

PCR-RFLP ANALYSIS OF THREE GENES ASSOCIATED WITH MEAT PRODUCTIVITY IN IL DE FRANCE SHEEP BREED

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Abstract

The present study was conducted in order to be investigated and identified the allelic variants of three genes associated with meat traits in sheep - myostatin (MSTN) gene, callipyge gene (CLPG) and calpastatin gene (CAST). Blood samples were collected from 30 adult sheep of Il de France sheep breed kept in Bulgaria. Genomic DNA was extracted and genotypes of the three genes were identified by means of PCR-RFLP analysis. The PCR products were digested with *HaeIII*, *BsmFI* and *MspI* restriction enzymes for MSTN, CLPG and CAST genes respectively. In the total population of sheep polymorphism was not found in any of the investigated regions. The three loci were found to be monomorphic – they were detected only the wild alleles and wild genotypes.

Keywords: Il de France sheep breed, CAST gene, MSTN gene, CLPG gene, PCR-RFLP method

Introduction

In recent years in Bulgaria are studied different genes associated with economically important traits in different domestic animals such as rabbits (Hristova et al., 2017), pigs (Stoikova-Grigorova et al, 2015; Stoikova-Grigorova et al, 2016) and sheep (Georgieva et al., 2015; Hristova et al., 2015).

Many of the meat qualities in sheep (such as tenderness) are heritable and depend on the genotype. There are clear effects of major genes on meat traits which worth more attention. (Hopkins et al., 2011).

Myostatin (MSTN) gene is considered as an inhibitor of skeletal muscle growth and therefore mutations in this locus could lead to increased muscling (McPherron et al., 1997; Shafey et al., 2014; Georgieva et al., 2015). This gene is part of mammalian growth transforming family (TGF-beta superfamily) (Sontegard et al., 1998). MSTN gene is located in chromosome 2, locus 2q32.2 from the sheep genome (*Ovis aries* L.). The gene contains 3 exons and 2 introns in all species studied (Bellingue et al., 2005; Nakev et al, 2013).

Other gene that could cause muscle hypertrophy is callipyge gene (CLPG). Callipyge phenotype in sheep is called “beautiful buttocks”. The hypertrophy is expressed only in paternal heterozygous animals when the mutant allele is inherited from father and wild allele in inherited from mother. This type of non-mendelian pattern of inheritance is named “polar overdominance”. CLPG locus is located in tellomeric region of ovine chromosome 18, within a cluster of imprinted genes (Cockett et al., 1996; Tellam et al., 2012).

Calpastatin gene (CAST) is used as candidate gene in marker-assisted selection for improving meat quality in sheep (Byon et al., 2009). It is located on the fifth chromosome of sheep genome (*Ovis aries* L.) and plays an important role in formation of muscles, degradation and tenderness of meat after slaughtering (Palmer et al, 2000; Sutikno et al, 2011).

Different polymorphisms have been detected in those three genes and they effect growth and carcass traits and could be used as efficient markers in genetic assisted selection in order to increase the quality and quantity of sheep meat (Gabor et al., 2009; Mirhoseini and Zare, 2012; Georgieva et al., 2014; Gorlov et al., 2016).

The aim of present study was investigation of genetic diversity in three genes associated with meat productivity in Il de France sheep breed using PCR-RFLP analysis.

Materials and methods

The present study was carried out in the DNA laboratory part of Faculty of Agronomy at the University of Forestry. The material included were 30 adult sheep from Il de France sheep breed kept in Bulgaria. Approximately 5 mL of peripheral blood was collected from *V. jugularis* in vacuum tubes, containing EDTA. DNA was extracted by manual commercial kit for DNA purification according to the manufacturer's instruction (QIAamp DNA Blood Mini Kit Qiagen). The DNA concentration of each sample was determined by spectrophotometer Biodrop and tested by agarose electrophoresis on 1 % agarose gel. .

PCR amplification reactions were 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) (Table 1) by using thermocycler QB-96 (Quanta Biotech). The primer pairs are presented in Table 2.

Table 1. Reaction mix for PCR amplification for the three genes

Item	Concentration	Total volume
ddH ₂ O	-	0.2 µl
Red Taq Master mix	2×	5.0 µl
<i>Forward</i> праймер	20 pM	0.4 µl
<i>Reverse</i> праймер	20 pM	0.4 µl
Genomic DNA	40 ng/µl	4.0 µl
Total volume	-	10µl

After PCR amplification were obtained PCR products with expected lengths of bp, 337 bp, 426 bp and 622 bp for MSTN, CLPG and CAST genes respectively. The PCR conditions are presented in Table 3.

Table 2. Locus, pairs and length of PCR product of each gene

Locus	Localization	Region	Primers	PCR product	References
MSTN	2	Exon 3	F: 5'- CCG GAG AGA CTT TGG GCT TGA - 3' R: 5'- TCA TGA GCA CCC ACA GCG GTC - 3'	337 bp	Dehnavi et al., 2012
CLPG	18	Telomeric region	F: 5'- TGA AAA CGT GAA CCC AGA AGC-3' R: 5'- GTC CTA AAT AGG TCC TCT CG-3'	426 bp	Gabor et al., 2009
CAST	5	Exon 1C/1D	F: 5'- TGG GGC CCA ATG ACG CCA TCG ATG - 3' R: 5'- GGT GGA GCA GCA CTT CTG ATC ACC - 3'	622 bp	Palmer et al., 1997

Table 3. PCR conditions for the three genes

Gene	Step	Primary denaturation	Cycles	Denaturation	Annealing	Elongation	Final elongation	Store
MSTN		95°C/5 min	30	95°C/30 s	54°C/45 s	72°C/1 min	72°C/10 min	10°C
CLPG		95°C/4 min	35	94°C/20 s	58°C/30 s	72°C/1 min	72°C/10 min	
CAST		95°C/5 min	30	95°C/30 s	62°C/45 s	72°C/1 min	72°C/10 min	

The digestion reactions were carried out in 10 µl final volume, containing 6 µl PCR product and 4 µl restriction enzymes. PCR products were incubated at 37°C for overnight (up to 15 h) (Table 4).

Table 4. Restriction conditions for the three genes

Locus	Enzyme	Time (h)	Temperature °C
MSTN	<i>HaeIII</i>	15	37
CLPG	<i>MspI</i>	15	37
CAST	<i>BsmFI</i>	16	37

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 in 1xTBE buffer, stained with GelRed (Biotium) and visualized under UV - transilluminator UVP.

Results and discussion

The quality of extracted genomic DNA samples was tested on 1% agarose gel and it was visualized under UV light (Figure 1).

After PCR amplification of exon 3 of MSTN sheep gene were obtained fragments with size 337 bp (Figure 2). After digestion with specific endonuclease *HaeIII* it was detected only wild

genotype *mm* (Figure 3) and only wild allele *m*. Mutant allele *M* and heterozygous genotypes *Mm* and mutant genotype *MM* were not detected in this herd of Il de France sheep breed. Exon 3 of MSTN locus was found to be monomorphic.

After digestion with *HaeIII* restriction enzyme in different Iranian sheep breeds other researchers detected two alleles – mutant allele *M* and wild allele *m* and three genotypes respectively: mutant genotype *MM* - one fragment with length 337 bp, heterozygous genotype *Mm* - four fragments: 337 bp, 131 bp, 123 bp and 83 bp and wild genotype *mm* – three fragments: 131 bp, 123 bp and 83 bp (Soufy et al., 2009; Dehnavi et al., 2012; Jamshidiet al., 2014).

The results in present study are in agreement with other studies. In our previous investigations on two Bulgarian sheep breeds – 121 adult sheep of Synthetic Population Bulgarian Milk and 32 rams of Northeast Bulgarian Merino we found similar results and all studied sheep were carried the genotype *mm* (Georgieva et al., 2015; Dimitrova et al., 2016). In Zel sheep Dehnavi et al., (2012) reported polymorphism in intron 2, but intron 1 and exon 3 were monomorphic. In Egypt Elkorshy et al. (2013) studied five Egyptian and Saudi sheep breeds and in all of them the exon 3 of MSTN locus was reported as monomorphic. In Iran Azzari et al. (2012) reported similar results in 110 native Dalagh sheep.

On the contrary, in Iranian Mehraban's sheep Jamshidi et al. (2014) detected two genotypes of exon 3 in MSTN gene – genotype *Mm* and genotype *mm* with frequencies 0,053 and 0,947, respectively. Soufy et al., (2009) observed polymorphism for exon 3 in Sanjabi sheep in Iran. They determined all three possible genotypes – *MM*, *Mm* and *mm* with frequencies 0,02, 0,01 and 0,97, respectively and the estimated allele frequencies were: for allele *M* 0,03 and for allele *m* 0,97.

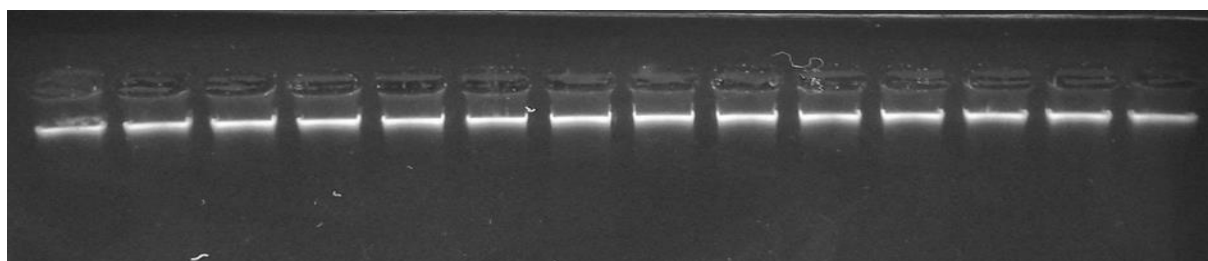


Figure 1. Testing of extracted DNA samples on 1% agarose gel with agarose electrophoresis

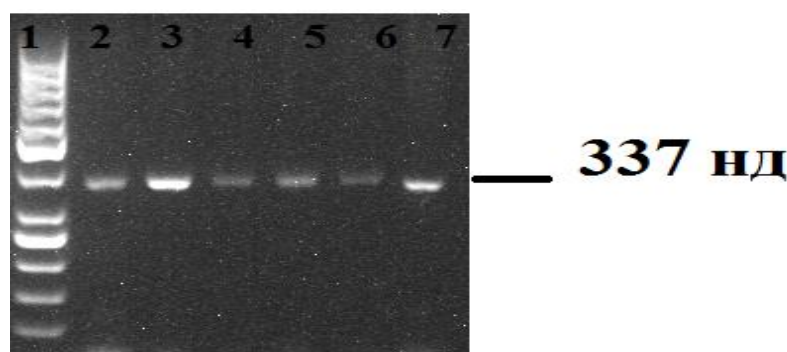


Figure 2. Testing of obtained PCR fragment after PCR amplification on 2% agarose gel for MSTN gene

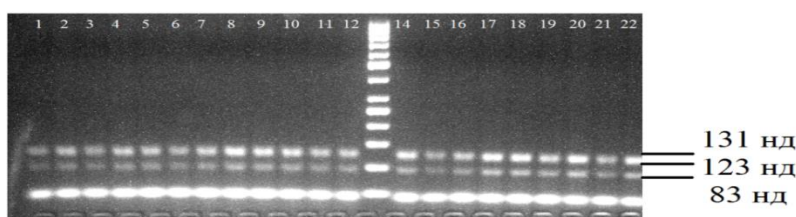


Figure 3. Restriction analysis of amplified product of MSTN gene with *HaeIII* restriction enzyme on 2% agarose gel electrophoresis

After PCR analysis of telomeric region of ovine CLPG gene of all 30 sheep were amplified 426 bp fragments (Figure 4).

After digestion process in present study was detected only wild homozygous genotype *AA* (Figure 5). Heterozygous genotypes *AG* and mutant genotype *GG* were not determined in this herd of Il de France sheep breed. CLPG locus was found to be monomorphic.

Results in this study are in agreement with other studies on CLPG sheep gene. Gabor et al. (2009) reported similar results for animals from several sheep breeds Lacaune, Tsigai, Improved valachian, East Friesian and crossbreed Tsigai and Lacaune. Alakilli (2015) analyzed the callipyge gene in two Saudi sheep breeds – Najdi and Harri, which were monomorphic for genotype *AA* of CLPG locus. In Iranian Lori sheep Nanekarani et al. (2014) also detected only wild homozygous genotype *AA*. This study disagrees with Jackson et al. (1997), which reported presence of the mutant allele *G* in sheep of breeds Dor-set, Rambouillet and Hampshire.

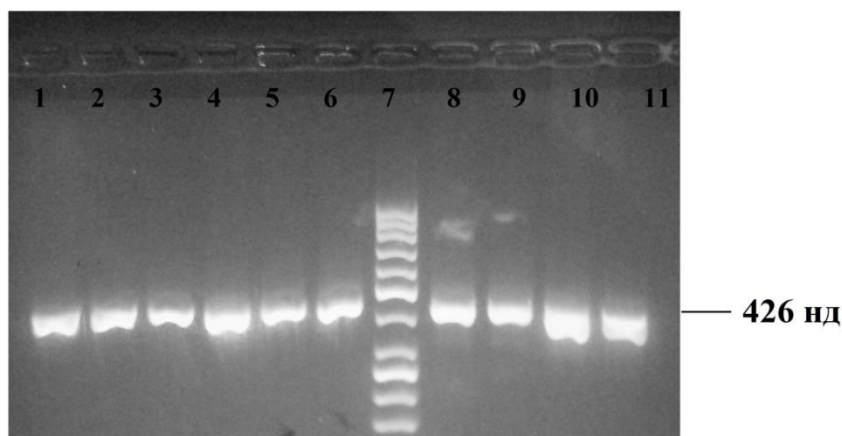


Figure 4. Testing of obtained PCR fragment after PCR amplification on 2% agarose gel for CLPG gene

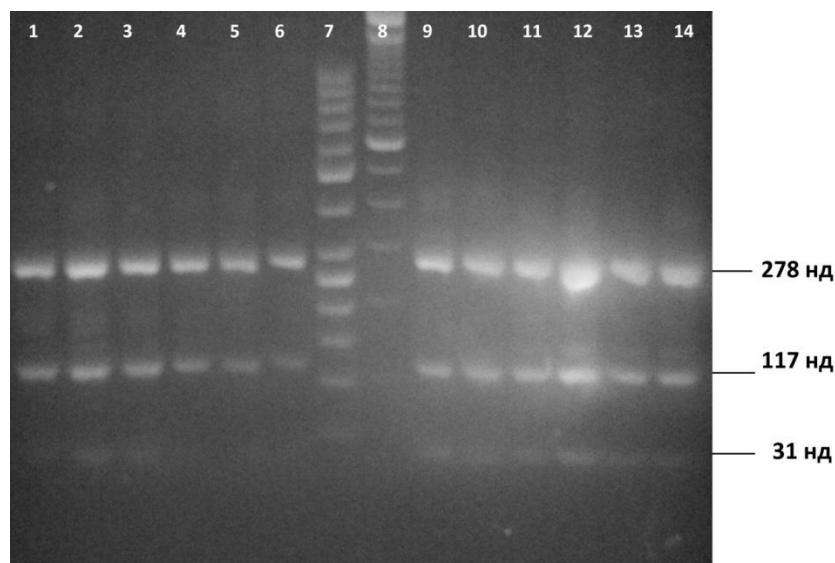


Figure 5. Restriction analysis of amplified product of CLPG gene with *BsmFI* restriction enzyme on 2% agarose gel electrophoresis

PCR fragments with size of 622 bp were amplified from exon 1C/1D of CAST sheep gene (Figure 6). In present study after digestion with restriction enzyme *MspI* was detected only wild genotype *MM* (Figure 6). Genotypes *MN* and *NN* were not determined in this herd. CAST locus was found to be monomorphic in this population (Figure 7).

Some authors reported for presence of two alleles of CAST gene and three genotypes respectively: genotype *MM* – two fragments with length 336 bp and 286 bp; genotype *MN* – three fragments – 622 bp, 336 bp and 286 bp; genotype *NN* – 622 bp (Palmer et al, 1998).

Results in this study are in agreement to other investigations on CAST sheep gene. Gabor et al. (2009) reported similar results for purebred Lacaune and East Friesian where only allele M was detected. Hristova et al., (2015) also reported for presence of allele M only in Bulgarian breed Local Karnobat.

In previous our study of another Bulgarian sheep breed - Synthetic Population Bulgarian Milk sheep it was found polymorphism in CAST locus - the allelic frequencies were 0,92 for allele M and 0,08 for allele N. Genotype frequencies were 0,84, 0,15 and 0,01 for *MM*, *MN* and *NN*, respectively (Georgieva et al, 2015). Hristova et al. (2015) in local Stara Zagora sheep have determined the frequency of homozygous genotype *MM* - 0.94 and heterozygous genotype *MN* - 0.06 (frequency of allele M – 0.97 and 0.03 of allele N).

High level of polymorphism was found in Kajli, Lohi and Thalli sheep breed in Pakistan, where Muhammad et al. (2012) detected all three possible genotypes in CAST gene. Szkudlarek-Kowalczyk et al. (2011) announced similar results in Il de France and Berishon de Cher sheep breed. In contrast Sumantri et al. (2008) found that the frequency of allele N is higher than the frequency of allele M in Indonesian Jonggol sheep breed. In Iran Tohidi et al.

(2013) also reported for high frequency of N allele, over 0,50 in Arch Merino and Mehraban sheep breed.

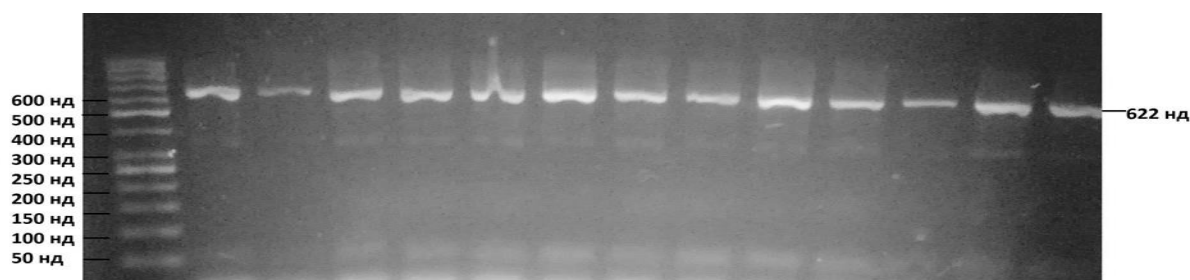


Figure 6. Testing of obtained PCR fragment after PCR amplification on 2% agarose gel for CAST gene

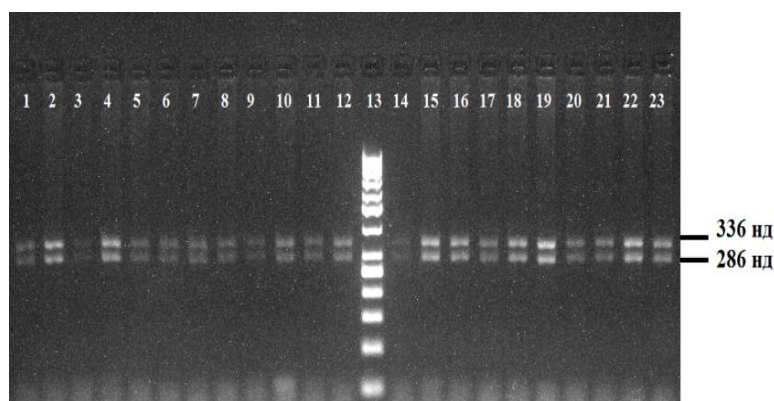


Figure 7. Restriction analysis of amplified product of CAST gene with *MspI* restriction enzyme on 2% agarose gel electrophoresis

Conclusion

In present investigation it may be concluded that exon 3 of MSTN sheep gene, telomeric region of CLPG gene and exon 1C/1D of CAST gene in Il de France sheep breed are found to be monomorphic in all studied animals. They were detected only the wild alleles of all three genes and only wild genotypes with frequencies 1.00.

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