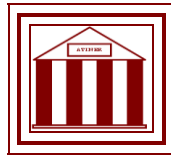


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**PCR-RFLP Analysis of CAST Gene in one
Bulgarian Sheep Breed**

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PCR-RFLP Analysis of CAST Gene in one Bulgarian Sheep Breed

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Abstract

The aim of the present study was investigation and identification of allelic variants of CAST gene associated with meat traits in sheep. The material involved one population of 25 animals of Bulgarian breed Karakachan sheep – 22 ewes and 3 rams. Genomic DNA was extracted so as calpastatin genotypes to be estimated by means of PCR amplification and PCR-RFLP method. The PCR products were digested with *MspI* restriction enzyme. In the total population of sheep, polymorphism was not found. The CAST locus was monomorphic, only genotype *MM* was observed.

Keywords: CAST gene, Karakachan sheep breed, PCR-RFLP method

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Introduction

CAST is an endogenous and specific inhibitor of calpains; it inhibits the calpain activity in postmortem tissue and thus regulates the rate and extent of postmortem meat tenderization (Kawasaki and Kawashima, 1996). Higher levels of calpastatin expression make meat less tender and increase its toughness. The calpastatin gene is considered as a candidate gene in marker-assisted selection for improving meat quality in sheep (Byon et al., 2009; Chung et al., 2001). The majority of studies showed that CAST may be a significant gene with a great effect on the carcass and the characteristics of meat quality (Palmer et al., 1998; Schenkel et al., 2006; Mohammadi et al., 2008).

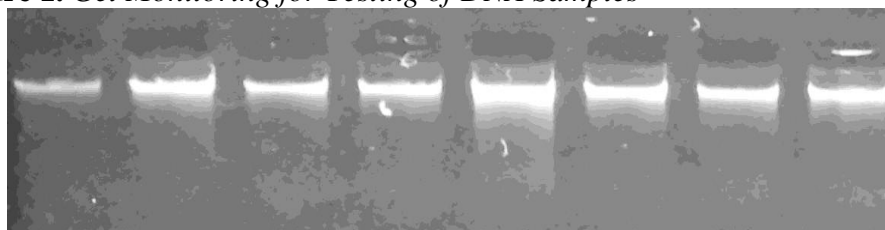
The CAST gene is located on the fifth chromosome of the sheep genome (*Ovis aries L.*) and plays an important role in formation of muscles, degradation and tenderness of meat after slaughtering (Palmer et al, 2000; Sutikno et al, 2011). Different investigations report that the calpastatin gene is found to be polymorphic in many sheep breeds. This gene is related with the increase of weight and improving of carcass quality. The CAST gene is a potential candidate gene for controlled selection programmes of farming animals (Byun et al., 2012).

The present study was conducted in order to be identified the allelic variants of calpastatin gene in one Bulgarian indigenous sheep breed - Karakachan.

Materials and Methods

The investigation was carried out in the DNA laboratory part of the University of Forestry, Department of Genetics and selection of agricultural crops. The material in the present study involved animals of a private herd in the town of Sapareva banya, in the Southwestern part of Bulgaria and included animals from the Bulgarian Karakachan sheep breed - 22 ewes and 3 rams. Approximately 5 mL of peripheral blood was collected from *V. jugularis* in vacuum tubes, containing EDTA. The DNA was extracted by a manual commercial kit for DNA purification according to the manufacturer's instruction (QIAamp DNA Blood Mini Kit Qiagen). The DNA quality and concentration of each sample were determined by gel monitoring (Figure 1) and spectrophotometer Biodrop.

Figure 1. Gel Monitoring for Testing of DNA Samples



PCR amplification reaction was carried out in total volume of 10 μ l containing 4 μ l DNA, 5 μ l Red Taq Polymerase Master Mix (VWR) and 0.4 μ l of each primer – forward and reverse (Bioneer) (Table 1). The primer sequences were (Palmer et al., 1998):

F: 5'- TGG GGC CCA ATG ACG CCA TCG ATG -3'

R: 5'- GGT GGA GCA GCA CTT CTG ATC ACC -3'

Table 1. *Reaction Mix for PCR Amplification*

Item	Volume
ddH ₂ O	0.2 μ l
Red Taq Master mix	5.0 μ l
Forward primer	0.4 μ l
Reverse primer	0.4 μ l
DNA	4.0 μ l
Total volume	10μl

After PCR amplification a PCR product, with a length of 622 bp, was obtained. The PCR conditions are presented in Table 2.

Table 2. *Conditions of PCR Amplification*

Stage	Temperature	Time
Primary denaturation	95 °C	5 min
30 cycles		
Denaturation	95 °C	30 s
Annealing	62 °C	45 s
Elongation	72 °C	1 min
Final extension	72 °C	10 min
Store	10 °C	

The digestion reaction was carried out in a 10 μ l final volume, containing a 6 μ l PCR product and 4 μ l *MspI* restriction enzyme with buffer (Bioneer). PCR products were incubated at 37°C for 15h. The fragment sizes were determined using the GeneRuler. Ladder, 50 bp (Sigma) supplied with 1 mL 6xDNA Loading dye. The obtained restriction products were tested on 2% agarose gel and visualized under UV light.

Results and Discussion

A 622 bp fragment was amplified from the CAST sheep gene. After digestion with the *MspI* restriction enzyme the authors reported for the presence of two alleles 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). In the present study only genotype *MM* was detected (Figures 2 and 3). Genotypes *MN* and genotype *NN* were not determined in the herd. The CAST locus was found to be monomorphic in this population (Table 3).

Table 3. Allele and Genotype Frequencies of CAST Locus

Locus	Allele number		Allele frequency		Genotype frequency			Heterozygosity	
	n_a	n_e	<i>M</i>	<i>N</i>	<i>MM</i>	<i>MN</i>	<i>NN</i>	H_0	H_e
CAST	1.00	1.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00

n_a – observed number of allele

n_e – effective number of allele

H_0 – observed heterozygosity

H_e – expected heterozygosity

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

Many authors studied the relation between different genotypes and some productive traits in various sheep breed.

Diyono et al. (2007) proved linkage between higher live body weight in male Indonesian local sheep and the presence of heterozygous genotype MN which was solid based for further studies associated with CAST locus and meat traits in sheep.

Putra et al. (2012) studied morphometric performances of nine thin tail sheep breeds from Jonggol with different calpastatin genotypes and established that sheep with a MM genotype had longer body length, heart girth, wither depth, and rump width than sheep with a MN genotype.

In the Kivircik lambs the association between the calpastatin (CAST) gene and carcass quality characteristics was investigated and it was found that the allele M of calpastatin locus was the most common allele. The authors found that the live weight, average daily gain, backfat thickness and skin+backfat thickness mean values were lower in animals with NN genotype compared to those with MM and MN genotypes. The results for Kivircik lambs showed that the calpastatin gene affected back fat and skin+back fat and animals with MM and MN genotypes had less fatty carcass than those with the NN genotype (Yilmaz et al., 2014a). That could be the reason why the allele M is the most common.

In our previous study of another Bulgarian sheep breed – in Synthetic Population Bulgarian Milk sheep was found polymorphism in CAST locus - the allelic frequencies were 92% for allele *M* and 8% for allele *N*. Genotype frequencies were 84%, 15% and 1% for *MM*, *MN* and *NN*, respectively (Georgieva et al., 2015). In another native Bulgarian sheep breed Stara Zagora, Hristova et al. (2015) determined the frequencies for the homozygous genotype *MM* – 93.7% and for the heterozygous genotype *MN* - 0.063 (frequencies of alleles *M* and *N* were 96.8% and 3.2%, respectively).

High levels of polymorphism were found in Kajli, Lohi and Thalli sheep breeds in Pakistan, where Muhammad et al. (2012) detected all three possible genotypes in the CAST gene and genotype frequencies of *MM*, *MN* and *NN* genotypes were found to be 77, 20 and 3% in the Lohi breed and 68, 26 and 6% in the Kajli breed respectively. In Thalli sheep only the *MM* (80%) and *MN* (20%) genotypes were detected. Szkudlarek-Kowalczyk et al. (2011) announced similar results in four sheep breeds: Polish Merino, Blackheaded Mutton, Il de France and Berishon de Cher sheep. They reported high level of the presence of the allele *N* in Polish Merino and the Blackheaded Mutton with a frequency of 23.8% and 18.6%, respectively. Mahrous et al. (2015) studied three Egyptian sheep breeds and determined genotypes *MM* and *MN* with frequencies: for Barki - 65% and 35%, for Rahmani - 40% and 60% and for Osseimi sheep breed - 30% and 70%, respectively. A high percentage of heterozygous genotype *MN* – 74%, was also observed in the Saudi sheep breed Harri (Saleha and Alakilli, 2015). Sumantri et al. (2008) found that the frequency of genotype *MN* was higher than the frequency of genotype *MM* in the Indonesian Garut fighting sheep from Ciomas (58 %). Similar results were identified by Yilmaz et al., (2014b) in the Sakis Turkish sheep breed where *N* allele and genotype *NN* were determined with frequencies of 65.52 % and 40.23 %, respectively. In Iran Tohidi et al. (2013) also reported a high frequency of *N* allele, over 50 % in Arch Merino and Mehraban sheep breed.

The genotyping of the animals could help classify them before slaughtering in order to improve selection programmes and meat productivity in sheep.

Figure 2. Restriction Analysis of PCR Product of CAST Gene with *MspI* Restriction Enzyme on 2% Agarose Gel Electrophoresis in Karakachan Breed

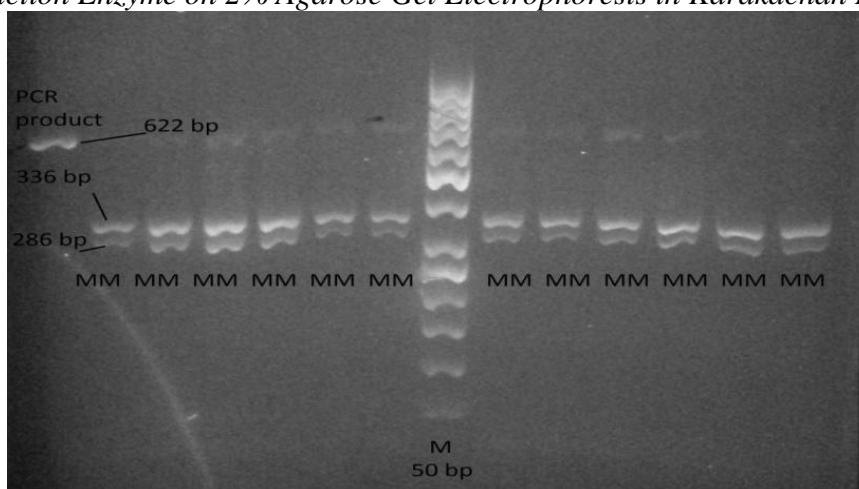
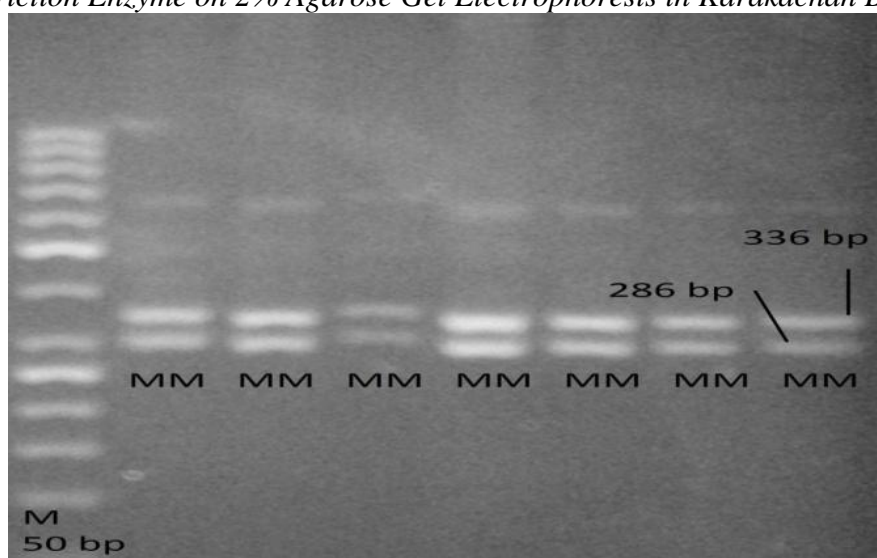


Figure 3. Restriction Analysis of PCR Product of CAST Gene with *MspI* Restriction Enzyme on 2% Agarose Gel Electrophoresis in Karakachan Breed



Conclusions

It may be concluded that the CAST gene is monomorphic for this herd of local Karakachan sheep raised in Bulgaria. Only the allele *M* and the genotype *MM* with a frequency of 1.00 were detected. The Karakachan sheep breed is an indigenous Bulgarian breed and it is considered that those breeds are a source of genetic diversity. In order to receive more accurate results, it is recommended for more animals, of the same breed from different herds and habitat, to be studied.

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