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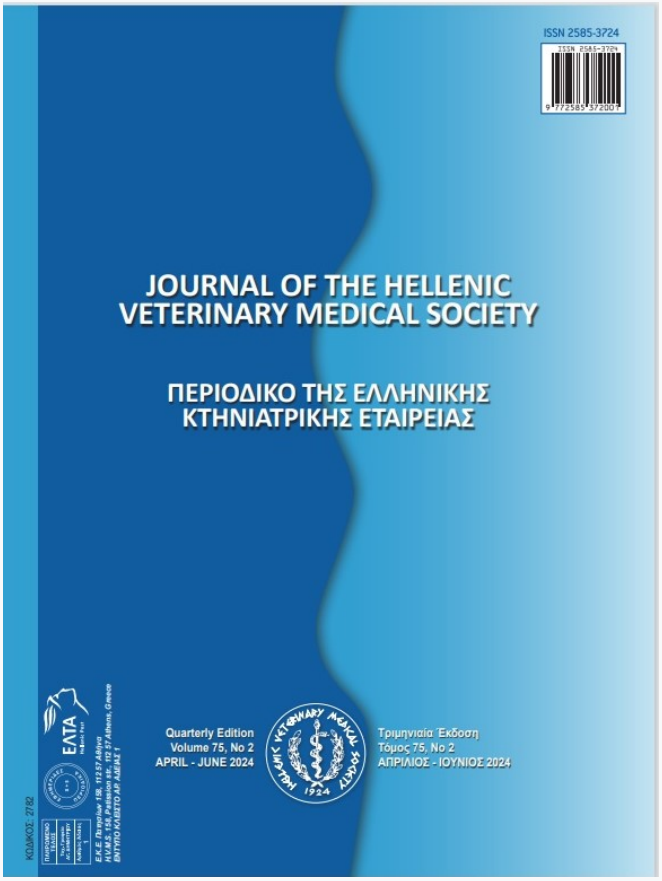

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Research article
Ερευνητικό άρθρο

Investigation of calpastatin *(CAST)* gene polymorphism in Norduz sheep by PCR-RFLP method

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ABSTRACT: The objective of this research was to determine polymorphism in the calpastatin (CAST) gene in a group of 102 Norduz sheep. Polymorphism was identified using the PCR-RFLP technique. The amplified PCR product with the length of 622 bp was digested with restriction enzymes MspI. It was found that the M and N alleles were present in CAST/MspI locus, their frequency being 78.43% and 21.57%, respectively. Homozygous for the M allele (MM) produced two bands of 336 and 286 bp. Three bands of sizes 622, 336 and 286 bp were seen in case of heterozygous genotype (MN), and homozygous for the N allele (NN) showed a 622 bp band only. The frequencies of homozygous MM, heterozygous MN and homozygous NN genotype were 64.7%, 27.5% and 7.8%, respectively. The genotype frequency distributions were in Hardy-Weinberg Equilibrium. In conclusion, the current study is the first study on genotyping of the CAST gene in Norduz sheep. The CAST gene was polymorphic in Norduz sheep. In the CAST gene, the predominant allele in the population was M, with a frequency of 78.43%. The MM and MN genotypes represented 92.2% of the genotype frequencies. The MM genotype was the most common, and NN genotype had the lowest genotype frequency. These results also indicate that in order to be used the polymorphism in the exon 1C/1D region of the CAST gene as a biomarker in Norduz sheep, primarily, association studies with economic traits should be performed.

***Keywords*:** Norduz sheep; calpastatin gene; polymorphism; PCR-RFLP

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INTRODUCTION

Sheep breeding has an important place in the livestock sector in Türkiye. Sheep breeding in Türkiye is done by traditional methods based on grazing in rural areas. The great majority of the sheep population of Türkiye is composed of multipurpose native breeds, producing meat, milk and wool. Norduz sheep is one of the most important endemic sheep of Türkiye. Norduz is a triple-purpose sheep (dairy, meat, and wool) and have a fat tail.

There are important relationships between the genetic structure of animals and their productivity. Recently, molecular genetics has been widely used to identify genes directly or indirectly related to livestock productivity. In studies conducted on farm animals at the molecular level, defining the effects of some genes on yields by associating them with the yields of animals allows genotype selection in breeding. One of the identified genes is the calpastatin (*CAST*) gene.

Calpains are intracellular calcium-activated cysteine proteases that have been implicated in a variety of physiological and pathological processes Calpains participate in the muscle development and in muscle fiber determination (Goll et al., 2003). The *CAST* gene an excellent candidate for controlling meat traits in livestock (Zhou and Hickford, 2008). Khan et al. (2012) reported that the *CAST* gene locus, was a potential molecular marker for MAS concerning growth rate in both Balkhi and Kajli sheep breeds. Koohmaraie (1992) indicated that the biological activity of the *CAST* gene played an important role in the tenderization in beef and lamb. Casas et al. (2006) reported that markers developed at the *CAST* gene are suitable for use in identifying animals with the genetic potential to produce meat that is more tender.

Studies to determine the genetic diversity of domestic sheep breeds in Türkiye are very limited. It is very important to determine genetic diversity in sheep and to reveal the relationships between determined genes and yield characteristics. The aim of this research was to determine polymorphism in the calpastatin (*CAST*) gene in Norduz sheep.

MATERIAL AND METHODS

Blood sample collection

The animal material of the study consisted of 102 Norduz sheep aged 1-4 years, which were raised in the Directorate of Animal Husbandry Application and Research Center of Van Yüzüncü Yıl University in Türkiye. Using sterile disposable needles, 9 ml blood sample was taken into vacuum EDTA tubes from the neck vein (Vena jugularis) of each sheep. The blood samples taken were brought to the laboratory via cold chain and kept in a deep freezer at 20 ºC until the day of DNA isolation.

DNA isolation from blood

Before starting the DNA isolation, the blood in the vacuum-EDTA tube was thawed at room temperature and mixed by vortex. Genomic DNA isolation was performed using a commercial DNA extraction kit (Invitrogen™ PureLink™ Genomic DNA Mini Kit, USA). Then, the amount of DNAs obtained was measured by spectrophotometer and the DNAs were adjusted to be at least 50 ng/µl for PCR application.

PCR

In the PCR process, primers located in the exon 1C/1D region of the sheep *CAST* gene were used (Palmer et al., 1998). The PCR reaction mix was made using PCR Master Mix (5xFIREPol Master Mix) according to the protocol reported by the manufacturer (Solis BioDYne, Estonia). DNA samples were first denatured completely by incubation at 95 °C for 5 min before the amplification cycle; then DNA was amplified by subjecting it to 30 cycles of (i) denaturation at 95 °C for 1 min, (ii) primer annealing at 60 °C for 1 min, and (iii) elongation at 72 °C for 2 min, using a Thermal cycler (Applied Biosystems^{TM}, Simpliamp^{TM}, Singapure). After the last amplification cycle, the samples were incubated further at 72 °C for 10 min for complete elongation of the final PCR products. After the PCR, the amplification results were visualized by performing 2.5% agarose gel electrophoresis and ethidium bromide staining.

Forward and reverse primer sequences used in the study are given in Table 1.

Table 1. Forward and reverse primer sequences used in the study.

Gene	Primers	PCR product	References
CAST	Forward: 5’ TGGGGCCCAATGACGCCATCGATG 3’ Reverse: 5’ GGTGGAGCAGCACTTCTGATCACC 3’	622 bp	Palmer et al. (1998)

RFLP

After successful amplification of the 622-bp PCR products was confirmed, the PCR products were subjected to *MspI* restriction enzyme (Thermo Scientific, Lithuania) digestion. For the RFLP process, PCR products were digested with *MspI* restriction enzyme for 1 hour at 37 °C. Enzyme-digested fragments were visualized under UV light (Vilber Lourmat, Super-Bright UV transilluminator, France) after ethidium bromide staining. As a result of the observation, the *CAST* gene genotypes of each sheep were determined according to the band patterns that appeared on 2.5% agarose gel.

Statistical analysis

The allele and genotype frequencies were analyzed by chi-square (X^2) for the Hardy-Weinberg equilibrium (SPSS, 2006).

RESULTS

The amplified calpastatin resulted in a DNA fragment with 622 bp including the sequences of exon and intron regions from a portion with PCR technique. From the analysis, two alleles (M and N) were observed, resulting in three genotypes (MM, MN and NN).

M and N allele frequencies were 0.7843 and 0.2157, respectively. The observed genotype frequencies were 0.647 for MM, 0.275 for NN and 0.078 for MN, respectively. The sheep population was in Hardy-Weinberg equilibrium (Table 2).

The animals with both alleles were assigned with MN genotype, whereas those possessing only M or N alleles were assigned with MM or NN genotypes, respectively. Genotype MM showed the two band pattern (336 and 286 bp). Genotype NN showed one band pattern (622 bp), while MN animals displayed a pattern with all three bands (622, 336 and 286 bp) (Figure 1).

Table 2. Allele and genotype frequencies of the calpastatin (*CAST*) gene.

Gene	Exon	Allele	Frequency	Genotype	Frekans	X^2	P
CAST	1C/1D	M	0.7843	MM	0.647	3.6295	0.056
		N	0.2157	MN	0.275		
				NN	0.078		

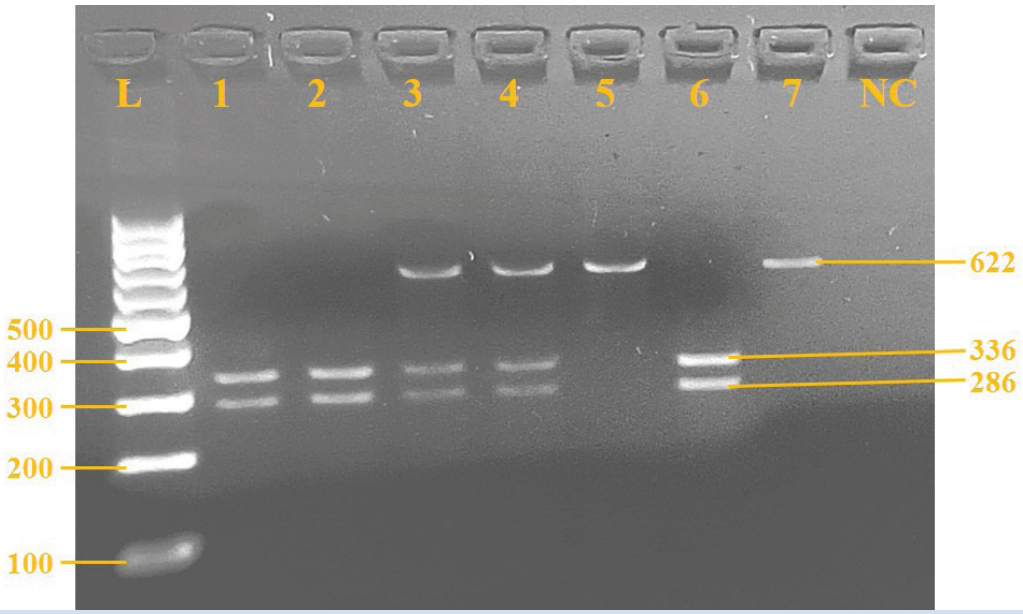

Figure 1. The PCR-RFLP image of the calpastatin (*CAST*) gene.

L: DNA size marker 100 bp DNA ladder
622 bp PCR fragment (7)
622 bp, 336 bp, and 286 bp for MN genotype (3, 4)
622 bp for NN genotype (5)
336 bp and 286 bp for MM genotype (1, 2, 6)
NC: Negative control

DISCUSSION

In the prsent study, 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 0.7843 and 0.2157 in Norduz sheep, respectively. Norduz sheep which shown 3 genotypes, MM, MN, and NN, with frequencies of 0.647, 0.275 and 0.078, respectively.

M allele frequency of *CAST* gene in Norduz sheep in the present study were higher than N allele frequency, which was similar to those in study conducted on Kangal, Awassi, Güney Karaman, Akkaraman, Morkaraman, Karayaka and Karakas sheep populations with respect to M and N allele frequencies of the *CAST* gene by Balcıoğlu et al. (2014).

Two alleles were observed allele M and allele N with frequencies 0.94 and 0.06 in Karnobat Merino breed, respectively (Dimitrova et al., 2017). Kowalczyk et al. (2011) reported that a frequency of 0.95 for M and 0.05 for N alleles in *CAST* locus of Ile de France sheep breed. Gorlov et al. (2016) determined a frequency of 0.88 for M and 0.12 for N alleles in Soviet Merino sheep. Frequencies of M and N alleless were found to be 0.90 and 0.10 in Thalli sheep, 0.82 and 0.18 in Lohi breed, 0.81 and 0.19 in Kajli breed, respectively (Suleman et al., 2012). When the M and N allele frequencies in the *CAST* locus were evaluated as a whole, both in our study and in the literature mentioned above, the allele M was the most frequent.

Suleman et al. (2012) reported frequencies of MM, MN and NN genotypes as 0.77, 0.20 and 0.03 in Lohi breed, respectively. Shahroudi et al. (2006) reported frequencies of 0.61, 0.36 and 0.03 for the MM, MN and NN genotypes in Iranian Karakul sheep, respectively. Khan et al. (2012) observed frequencies of 0.74, 0.24 and 0.02 for the MM, MN and NN genotypes in in Kajli sheep, respectively. The MM, MN and NN genotype frequencies reported by Suleman et al. (2012), Shahroudi et al. (2006) and Khan et al. (2012) are similar to the results of the present study.

Dimitrova et al. (2017) observed that two genotype MM and NN with frequencies 0.89 and 0.11 in Karnobat Merino breed, respectively. The absence of heterozygous MN genotype in Karnobat Merino sheep is inconsistent with the finding of our study. Azari et al. (2012) reported that a frequency of 0.36 for MM, 0.38 for MN and 0.26 NN genotype in Dalagh sheep. Also, Azari et al. (2012) reported that the calpastatin locus did not in Hardy-Weinberg equilibrium. This literature finding is inconsistent with the finding of our study. Because, in our study, the *CAST* locus was in Hardy-Weinberg equilibrium. Palmer et al. (1997) detected three genotypes (MM, MN and NN) in Corriedale rams for this locus which was in agreement with the present results.

Suleman et al. (2012) reported two genotype MM (0.80) and MN (0.20) genotypes in Thalli sheep. Khan et al. (2012) observed 2 genotypes (MM and MN) for the *CAST* gene in Balki sheep. Also, Khan et al. (2012) detected only the MM genotype in Beetal sheep. Sumantri et al. (2014) observed only the NN genotype in Rote sheep. These findings are inconsistent with the finding of our study. It is understood from these findings that different sheep breeds may have different genotypes related to the *CAST* gene. Therefore, gnotyping of sheep breeds is required in association studies related to the *CAST* gene.

Valencia et al. (2022) reported that in Colombian hair sheep, the *CAST* gene significantly infuenced birth weight. Armstrong et al. (2018) reported that in Texel sheep, single nucleotide polymorphisms in the *CAST* gene was associated with birth weight and growth rate. Byun et al. (2008) evaluated the *CAST* gene in Romney lambs; they observed a signifcant efect of the A allele on birth weight, and on pre-weaning daily gain only in animals from the simple calving type. Allele B had no infuence on weights at birth, weaning, and pre-weaning daily gain. They suggest that *CAST* gene is an important marker in the trait birth weight and growth performance.

CONCLUSION

The current study is the first study on genotyping of the *CAST* gene in Norduz sheep. The *CAST* gene was polymorphic in Norduz sheep. In the *CAST* gene, the predominant allele in the population was M, with a frequency of 78.43%. The MM and MN genotypes represented 92.2% of the genotype frequencies. The MM genotype was the most common, and NN genotype had the lowest genotype frequency. These results also indicate that in order to be used the polymorphism in the exon 1C/1D region of the *CAST* gene as a biomarker in Norduz sheep, primarily, association studies with economic traits should be performed.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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