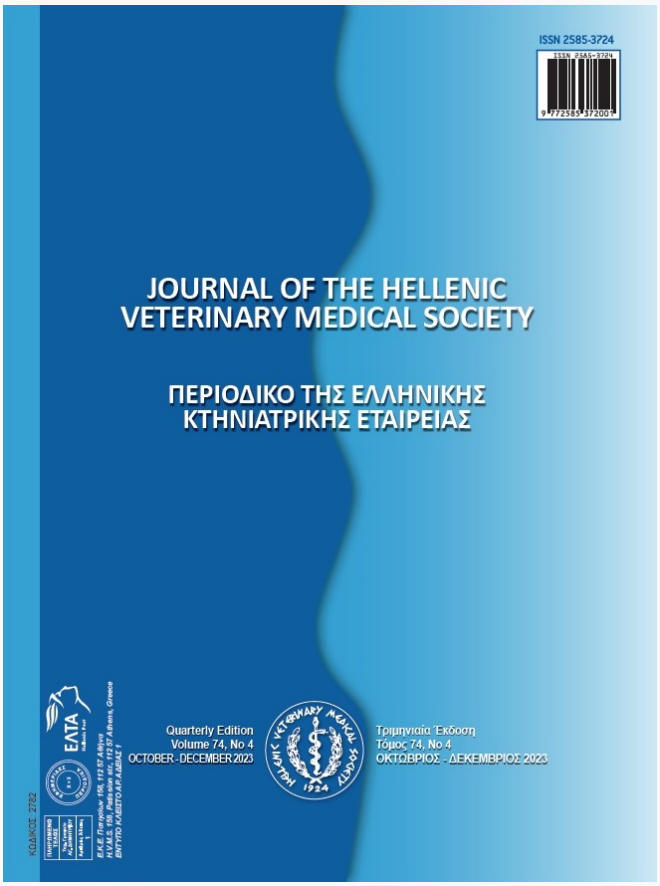
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Research article
Ερευνητικό άρθρο

Investigation of Genetic Polymorphisms of *CSN1S1* and *BLG* Genes in Norduz sheep by PCR-RFLP method

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ABSTRACT: This study was undertaken to examine genetic polymorphisms of Alpha-S1 casein (*CSN1S1*) and β-Lactoglobulin (*BLG*) genes in Norduz sheep by PCR-RFLP method. *CSN1S1* and *BLG* genes have known a significant effect on cheese making. Restriction fragment length polymorphism (RFLP) is a molecular biology tool that reveals the difference between samples of homologous DNA molecules from differing locations of restriction enzyme sites.10 ml blood samples from 102 heads of Norduz sheep was used in the study.The genotypes of *CSN1S1* and *BLG* genes was determination by PCR-RFLP method.

Statistical analysis, after calculating allele and genotype frequencies by direct gene counting method, the distribution of observed and expected genotypic frequencies was determined according to the Hardy-Weinberg Equilibrium, and whether it was compatible with the $\chi 2$ test.In the study conducted in Norduz sheep, polymorphism was observed in *CSN1S1* and *BLG* genes. AA, AB and BB genotype frequencies of *BLG* gene were found to be 17.6%, 69.6% and 12.7%, respectively. It was determined that the *BLG* gene was not in Hardy-Weinberg Equilibrium. AA, AC and CC genotype frequencies of *CSN1S1* gene were determined as 0.0%, 2.9% and 97.1%, respectively. The *CSN1S1* gene was determined to be in Hardy-Weinberg equilibrium. In conclusion, it was determined that the A allele and AB genotype of the *BLG* gene were more common, the C allele and the CC genotype of the *CSN1S1* gene were more common however, the AA genotype was not observed in Norduz sheep.

***Keywords*:** Alpha-S1 Casein; β-Lactoglobulin; PCR-RFLP; Norduz Sheep.

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INTRODUCTION

Sheep breeding is one of a very important sector of livestock production in Turkey. Many sheep breeds and varieties are bred in Turkey. According to Turkstatdata for 2021, the number of sheep in Turkey is 45.177. 690 heads, while according to Turkvetdata for 2022, the total number of sheep in Van is 2.800.327 heads. Production system of sheep in Turkey is mainly extensive, intensive and semi-extensive, and more focused on utilization of grasslands and pasture areas.

One of the sheep varieties bred in Turkey is Norduz sheep. Norduz sheep are fat-tailed, they have high endurance, survivability and adaptability in the region where they are raised (Tagem, 2009).Norduz sheeps are bred in the Norduz region of Gürpinar district of Van province in Turkey. Gürpinar province, located between 37°44′-28°29′ north latitudes and 43°07′-44°07′ east longitudes, is Turkey's biggest district, with a surface area of 4.063 km^2 inside the limits of Van province(Ezelhan et al., 2021).

One of the most important yields of farm animals is milk. Nowadays, the dairy sector is focused on producing an expanding number of various milk products, and the technical features of milk are attracting greater attention (Frajman and Dovc, 2004). This reveals the necessity of increasing milk production. The composition of the milk is very important in the production of high-quality milk. One of the most important components affecting the quality of milk is milk protein. The mammary gland epithelial cell produces and secretes proteins that are encoded by the milk protein genes (Aggeler et al., 1988). Animal breeding is very interested in milk protein genetic polymorphisms (Barillet et al., 2005). Additionally, their polymorphisms were utilized in phylogenetic research, genomic selection, conservation methods, and the study of genetic diversity (Rout et al., 2010).It has been suggested that a number of milk protein polymorphisms may be used to select dairy ruminants. Genotyping of milk protein genes regardless animals; age and sex is now achievable due to DNA-based molecular techniques, offering a potentially more effective and adaptable selection tool than protein electrophoretical genotyping of milk. However, the effectiveness of selection depends on breed-specific allelic frequencies and how these polymorphisms affect dairy characteristics and the technical aspects of milk (Barillet et al., 2005).Restriction fragment length polymorphisms nucleotide changes occur in all eukaryotic genomes (Beuzenet al.,2000).

Beta-lactoglobulin and alpha-lactalbumin are the two primary whey proteins (Morammazi et al., 2016). The *CSN1S1* and *BLG* loci are still the only ones being explored in dairy sheep investigations on casein and whey protein genetic polymorphisms, with less conclusive results than in goats(Barillet et al., 2005). The *CSN1S1* gene,according to Sanchez et al. (2005) may potentially enhances milk protein characteristics. There has also been evidence of a connection between milk composition and variations of the *BLG* gene. For instance, milks with the *BLG* AA genotype were shown to have greater total solid, fat, and protein levels when compared to milks with the AB and BB genotypes (Schmoll et al., 1999).

The genes for *CSN1S1* and *BLG* are crucial for the dairy industry and livestock breeding. However, investigation of these genes and their polymorphisms in sheep is limited. Thus, this study was undertaken to examine genetic polymorphisms of *CSN1S1* and *BLG* genes in Norduz ewes by PCR-RFLP method.

MATERIALS AND METHODS

Animal Material

In this study, 102 heads of Norduz sheep, which were raised in Van YüzüncüYıl University Livestock Application and Research Center Directorate, were used as animal material. This sheep breed is one of the fat-tailed native sheep breeds of Turkey.

DNA Isolation

In this study, 10 mLof blood samples were drawn from the vena jugularis (the neck veins) of Norduz sheep into tubes with K_3EDTA and stored at -20°C until use. Then, frozen blood samples were thawed and DNA was isolated using a DNA extraction kit according to the protocol determined by the manufacturer (PureLink™ Genomic DNA Mini Kit, Invitrogen™). The purity and quantity of the DNA's obtained were determined spectrophotometrically by using nanodrop.

PCR Reaction and RFLP

Restriction fragment length polymorphism (RFLP) is a molecular biology tool that reveals the difference between samples of homologous DNA molecules from differing locations of restriction enzyme sites. In this method, the DNA sample is digested by specific restriction enzymes, and the resulting restriction

fragments are resolved according to their size using gel electrophoresis. RFLP was discovered in 1984 by the English scientist "Alec Jeffreys" while working on area of hereditary diseases (Chaudharyand Kumar, 2019).

Firstly, the 236bp PCR product, in the exon 2 region, of the *BLG* gene was digested by the restriction enzyme *Rsa*I.

PCR reaction was carried out with 50-75 ng of the genomic DNA isolated from the blood samples, PCR master mix (5x FIREPol Master Mix Ready to Load, Solis BioDyne) and100-150 nM from each of the following primers, 5'- AGAGCATTCCCCAGGGTGCAGAG-3' and 5'-GTGGGCTTCAGCTCCTCCACGTA -3' (Schlee and Rottmann, 1992). The PCR reactions were performed on a SimpliAmp™ Thermal Cycler (Applied Biosystems™) with following conditions: an initial denaturation step at 95°C for 3 min followed by 35 cycles of denaturation for 95°C for 60 sec, an annealing for 65°C for 30 sec, an extension for 72°C for 45 sec and a final extension 72°C for 10 min.

The obtained PCR products were electrophorised for 45-60 minutes at 90-100 V on a 2% agarose gel containing ethidium bromide. Then 10 µL of the PCR products were digested at 37°C for 1 hour with 10 U *Rsa*I (Thermo Scientific™) restriction endonuclease enzyme in a total volume of 30 µL reactions. After digesting PCR product, the *BLG* variants were determined relative to the bands occurring on a 3% agarose gel.

The 372 bp PCR product, in the exon 3 region, of the Alpha-S1 casein gene was digested by the restriction enzyme of *Mbo*II.

PCR reaction was carried out with 50-75 ng of the genomic DNA isolated from the blood samples, PCR master mix (5x FIREPol Master Mix Ready to Load, Solis BioDyne) and 100-150 nM from each of the following primers: 5'- GGTGTCAAATTTAGCTGTTAAA -3' and 5'- GCCCTCTTCTCTAAAAAGGTTT -3'(Pilla et al., 1998).The PCR reactions were performed on a SimpliAmp™ Thermal Cycler (Applied Biosystems™) with the following conditions: an initial denaturation step at 95°C for 3 min followed by 35 cycles of denaturation for 95°C for 30 sec, an annealing for 60°C for 30 sec, an extension for 72°C for 45 sec and a final extension 72°C for 10 min.

The obtained PCR products were electrophorised for 45-60 minutes at 90-100 V ona 2% agarose gel electrophoresis containing ethidium bromide. Then 10 µL of the PCR product was digested at 37°C for 1 hour with 10 U *Mbo*II restriction endonuclease enzymes in a total volume of 20 µL reaction. After digesting PCR product, the Alpha-S1 casein variants were determined relative to the bands occurring on a 2% agarose gel under UV.

Statistical Analysis

Statistical analysis, after calculating allele and genotype frequencies by direct gene count method, the distribution of observed and expected genotypic frequencies was determined according to the Hardy-Weinberg Equation (HWE), and whether it was compatible with the $\chi 2$ test.

Ethical approval

Ethical approval for this study was obtained from Animal Experiments Local Ethics Committee of Van YuzuncuYil University (Turkey) (Decision No: 2022/07-04).

RESULTS

PCR-RFLP images of β-Lactoglobulin and alpha-S1 casein genes are presented in Figures 1 and 2, respectively.

Findings of the *BLG*Gene

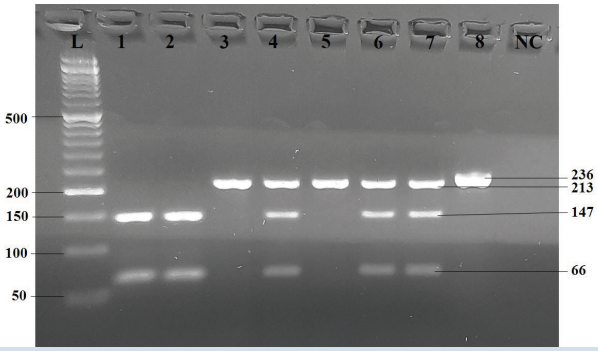


Figure 1. PCR-RFLP image of the β-lactoglobulin gene. (L: DNA Ladder; 1 and 2: AA genotype; 4, 6 and 7: AB genotype; 3 and 5: BB genotype; 8: PCR product; NC: Negative control).

The agarose gel image given in Figure 1;two bands of 66 and 147 bp in individuals with AA genotype (columns 1 to 2), three bands of 66, 147 and 213 bp in individuals with AB genotype, and a single band of 213bpwas observed in individuals with BB genotype (columns 3 and 5).

Findings of the *CSN1S1* gene

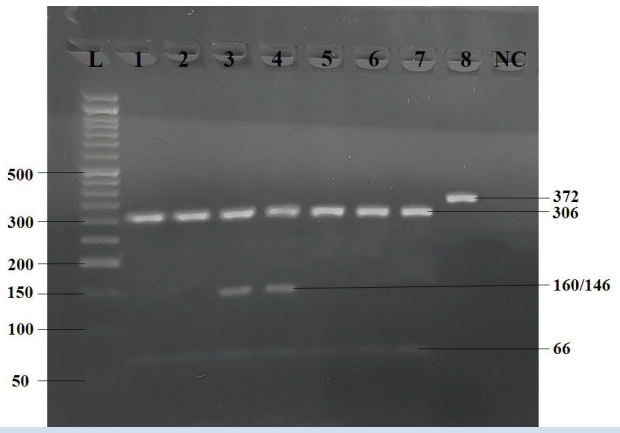


Figure 2. PCR-RFLP image of the alpha-S1 casein gene. (L: DNA Ladder; 1, 2, 5, 6, 7: CC genotype; 3 and 4: AC genotype; 8: PCR product; NC: Negative control).

The agarose gel image given in Figure 2 showed; Three bands of 66, 160/146 and 306 bp in individuals with AC genotype (columns 3 to 4) and two bands of 66 and 306bp of individuals with CC genotype, (columns 1, 2, 5, 6 and 7) were observed.

Allele and Genotype Frequencies

The description of the genes evaluated in this study is presented in Table 1.

In table 2 show that three genotypes in Norduz sheep were identified. Genotype frequencies of the *BLG* genes were found to be AA (17.6%), AB (69.6%) and BB (12.7%). A and B allele frequencies of the *BLG* gene were determined 0.52 and 0.48, respectively. In addition, it was observed that the allele frequencies distribution of *BLG* gene of Norduz breed was not in HWE.

In table 2 show that two genotypes of Alfa-S1 casein gene in Norduz sheep were found. The AA, AC and CC genotype frequencies of the *CSN1S1*gene were determined as 0.0, 2.9 and 97.1%, respectively. A and C allele frequencies of the *CSN1S1* gene were identified 0.01 and 0.99, respectively. In addition, it was observed that the allele distribution of the *CSN1S1* gene was determined to be in HWE.

DISCUSSION

β-lactoglobulin (*BLG*) is one of the milk proteins that is polymorphic in sheep and the gene encoding the protein is located on ovine chromosome 3. Variants of *BLG* loci was identified by PCR-RFLP technique on genomic DNA (Feligini et al., 1998; Anton et al., 1999).Caseins are the only coagulable milk proteins, the yield and the quality of cheese depends primarily on the amount of them. Casein genes are located on the chromosome two (Mercier et al., 1985).

Allele/genotype frequencies of the *CSN1S1* and *BLG* genes are summarized in Table 2. The frequencies of A and B alleles at the locus studied in the *BLG* gene were 0.52 and 0.48, respectively, being the AB genotype (0.70) as the most common genotype, followed by AA (0.17) and BB (0.13). The frequencies of A and C alleles at the locus studied in the *CSN1S1* gene were 0.01 and 0.99, respectively, being the CC genotype (0.97) as the most common genotype, followed by AC (0.03). In Norduz sheep, the distribution of allele frequencies of the *BLG* gene revealed that allele A was more common than allele B. Also, The *BLG* gene was not in HWE. However, the *CSN1S1* gene was in HWE.

Table 1. Description of genes evaluated in the study.

Gene name	Gene symbol	NCBI Gene ID	Chromosomal location	Number of exons	SNP location	SNP	Allele
β-Lactoglobulin	*BLG*	443385	3	7	Exon II	T>C	A/B
Alfa-S1 Casein	*CSN1S1*	443382	6	19	Exon III	T>C	A/C

Table 2. Allele and genotype frequencies of β-lactoglobulin and Alpha-S1 casein genes.

Gene	Exon	Allele	Frequency	Genotype	Frequency	χ2	P
β-Lactoglobulin	II	A	0.52	AA	0.17	15.955	*
		B	0.48	AB	0.70		
				BB	0.13		
Alfa-S1 Casein	III	A	0.01	AA	0.00	0.023	0.88
		C	0.99	AC	0.03		
				CC	0.97		

* $p<0.05$.

It has been reported that polymorphic *BLG*gene and A and B alleles of the *BLG* gene are common in many sheep breeds (Çelik and Özdemir, 2006; Elmacı et al., 2006; Mohammadi et al., 2006). However, it has been reported that the C allele of the *BLG* gene is rarely detected in Merinoland (Erhardt, 1989) and Merino (Recio et al., 1997) sheep breeds.

AB genotype (0.70) in the study was the most common genotype in the Norduz breed. Similar results for the genotype of ovine *BLG* in Awassi, Dağlıç, Akkaraman, Karakaş, Norduz, Güney Karaman, and Kangal sheep breeds were reported by Sahin et al (2011).On the other hand, this study was inconsistent with the results reported for HWE by Sahin et al (2011).

BLG A allele was high in Norduz sheep in this study. Similar findings were obtained in Kıvırcık (0.7759), Gökçeada (0.7632), and Sakız (0.9756) sheep breeds by Elmaci et al. (2006); Awassi (0.6316), Tuj (0.7188), Norduz (0.7059), and Güney Karaman (0.6552) sheep breeds by Sahin et al (2011).Similarly,Çelik and Özdemir (2006) reported that the A allele frequencies determined in Awassi and Morkaraman sheep were 0.63 and 0.56, respectively.

Sahin et al. (2011) showed that the *BLG* B allele was high in Dağlıç, Akkaraman, Karakaş, and Kangal sheep. This differs from the findings presented here in Norduz sheep.

The fact that the C allele of the *BLG* gene was not detected in Norduz sheep in this study is consistent with the finding reported for Chios, Gökçeada, and Kıvırcık domestic sheep breeds (Erhardt, 1989).

It has been observed that allele and genotype frequencies of the *BLG* gene vary in different sheep genotypes. This may be due to different breeding and combining methods applied in herds.

When the works of literature were evaluated as a whole, it was observed that allele C and CC genotypes of the *CSN1S1* gene were common for sheep herds. Alfa-S1 casein genetic variants have not been adequately studied for their potential role in highly adaptable local sheep genotypes.

Corral et al. (2010) reported that C allele (0.98) and CC genotype (0.98) frequencies in Merino sheep were the most common. Similar results for Sofia, Copper-Red Shumen, Local Karnobat, Pleven Blackhead, and Stara Zagora sheep breeds were reported by Gencheva et al (2020). *CSN1S1* gene findings of our study are consistent with that of Corral et al. (2010) and Gencheva et al. (2020).

Giambra et al. (2014) found that the *CSN1S1* A allele variant was not detected in the Lacaune sheep population, while the C allele variant was 100%. According to the A allele of *CSN1S1*, C allele in Serra da Estrela, White Merino, and Black Merino sheep breeds (Ramos et al., 2009), Black Faced Mutton Sheep (Giambra et al., 2014), as well as Pramenka sheep (Rustempasic et al., 2013) were found to be more frequent. In addition, Kevorkian et al. (2009) reported that no polymorphism was identified in the *CSN1S1* casein gene in sheep breeds bred in Romania.

CONCLUSIONS

In conclusion, it was determined that the most common allele and genotype of the *BLG* gene was the A allele and AB genotype, and the *CSN1S1* gene was the C allele and CC genotype in Norduz sheep. Also, the AA genotype of the *CSN1S1* gene was absent in Norduz sheep. It was concluded that it would be useful to investigate the economic effects of genetic polymorphisms of Alpha-S1 casein and β-Lactoglobulin genes on various yield traits in larger Norduz sheep populations.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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