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Genetic evaluation of Teleorman Black Head ewes using genes polymorphism for improving milk traits

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ABSTRACT

The purpose of the present study was to asses specific gene markers associated with the sheep milk production in Teleorman Black Head ewes. For the proper selection of the best candidates for milk production, the genetic markers such as *CSN3*, *BLG* and *PRL* were selected. To detect *CSN3*, *PRL* and *BLG* polymorphisms, genomic DNA was isolated from blood samples collected from 50 ewes. ASA-PCR and PCR-RFLP methods were used for genotyping of animals; the genotypes frequencies and Hardy-Weinberg equilibrium were estimated using R software. In our samples, for *CSN3* gene were identified two genotypes (CC and CT), the most frequent being CT genotype (94%). For *PRL* gene, were identified three genotypes (AA, AB and BB), AA and BB genotypes being the most frequent (36% and 40% respectively). Also, three genotypes were identified for *BLG* gene (AA, AB and BB), with an increased frequency of AB genotype (44%). These preliminary data will be correlated in the next experiments with phenotypic data, generating important tools for genetic selection of the Teleorman Black Head sheep population.

Keywords: sheep, kapa casein-3, prolactin, beta lactoglobulin, milk traits improvement, genetic polymorphism

INTRODUCTION

Identification of genetic markers specific for sheep milk production raised the research interest after the entire ovine genome was completely determined by DNA sequencing. The genetic markers are used as tools for the selection of the best individuals with superior phenotypes. Of genetic polymorphisms, those associated with milk production traits are of increasing interest, the genes coding for milk proteins being the most valuable candidate genes for marker-assisted selection (MAS).

Milk proteins include caseins and whey proteins (α-lactalbumin, β-lactoglobulin), Selvaggi et al (2014). Caseins are a milk proteins family, composed from four molecular fractions (αs1-, αs2-, β-, k- casein), Hristov et al (2016). Also, caseins represent the most abundant group of proteins found in milk, accounting 76-86 % from milk proteins, Ramunno et al., (2005).

A multitude of polymorphic forms of caseins were discovered in cows and goat with a significant impact on milk composition and technological properties, Pauciullo et al., (2015). In different sheep breeds were conducted molecular analyses of ovine *CSN3* gene, mainly of exon 4. In particular, Ceriotti et al. (2004) identified a C→T-SNP in exon 4 in Sopravissana sheep breed. Another genetic polymorphism, T→C, was reported in the mRNA sequence of ovine *CSN3* gene by Feligini et al. (2005). The *CSN3* gene is coding for k-casein and is the least of casein genes studied with respect to its effect on milk yield and composition.

The β-lactoglobulin is the main whey protein of ruminant milk, and is encoded by *BLG* gene, Selvaggi et al (2014). Genetic analysis showed that sheep breeds have polymorphic variants of *BLG* gene, being reported three co-dominant alleles, A, B and C, Selvaggi et al (2014). Alleles A and B are the most common variants in many sheep breeds: a high frequency of A allele was found in Bavarian sheep (Schlee et al, 1993) and Massese sheep (Rampilli et al, 1997), while B allele was frequent in Sarda (Pietrolà et al, 2000) and in Latxa, Manchega and Churra breeds (Barrilet et al, 2005).

Prolactin (PRL) is a lactogenic hormone essential for milk production, and the genetic polymorphism of *PRL* gene was less studied in ovine breed. Previous studies of Ramos et al., (2009) described a 2500 bp fragment of this gene correlated with milk production in sheep. Also, Ramos et al (2009) found two alleles, A and B, of PRL gene in Merino sheep breed. Also, our previous study identified the A and B alleles of PRL genes in Transylvanian Merino breed, Gras et al. (2017).

The identification of genetic markers in local sheep breed could be a important tool for their characterisation and also for the selection of animals with superior milk production traits. The aim of the present study is to identify the polymorphism of three important sheep milk genetic markers *CSN3, PRL* and *BLG* in Teleorman Black Head (TBH) local sheep breed.

MATERIALS AND METHODS

Animals. The study was conducted on 50 Teleorman Black Head ewes, reared in a farm from Teleorman County (Romania).

Ethical aspects. The handling of animals and collection of biological samples was performed in accordance with the UE legislation (206/2004), for the protection of farm animals in experiments. The study experimental design

was approved by the Ethical Committee of the National Research Development Institute for Biology and Animal Nutrition, Baloteşti, Romania.

Genomic DNA extraction. Genomic DNA was extracted from blood samples using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega Corp., USA), following the manufacturer recommendation.

CSN3 alleles identification

Allele specific polymerase chain reaction (ASA-PCR) method was used for identification of *CSN3* genotypes. Briefly, the two alleles of *CSN3* gene were amplified using one forward primer and two reverse primers, as described by Feligini et al (2005). Briefly, the ASA-PCR was performed using 75 ng of DNA template, 1.25U GoTaq DNA Polymerase, 200 µM dNTPs, 0.3 µM of primers and nuclease-free water (to a final volume of 25 µL). The primer sequences used are:

-Forward primer: 5′-CTTCGATGACAAAATAGCCAA-3′

T Revers primer: 5′-AATTGAGTCCATAACTAGGA-3′

C Revers primer: 5′-GGGGGGGGGGGGAATTGAGTCCATAACTAGGG-3′

The PCR cycling conditions were as follows: initial denaturation for 10 minutes at 95˚C, followed by 35 cycles of 30 seconds at 95˚C, 30 seconds at 56˚C, 30 seconds at 72˚C; the final elongation step was carried out at 72°C for 10 minutes.

The obtained *CSN-3* amplicons (of 97 bp and 87 bp respectively) were identified on 1 % (w/v) agarose gel.

PRL and BLG alleles identification:

The specific alleles of *PRL* and *BLG* were identified by PCR-RFLP method, as described by Gras et al (2016). The total volume of PCR reaction was 25 µL, with the same concentration of reagents as described for *CSN3* ASA-PCR. The primer sequences and characteristics are presented in Table 1. The thermal profile of PCR reaction was: initial denaturation step (10 minutes at 95°C), followed by 35 cycles of denaturation step (30 seconds at 95°C), annealing step (30 seconds at 60°C for *BLG* and at 56°C for *PRL* genes), extension step (30 seconds at 70°C) and final extension at 70°C for 10 minutes.

Table 1. Primer sequences used for amplification of PRL and BLG genes

Gene	Primer sequence 5' 3'	Orientation	Primer length (bp)	Tm (°C)	Amplicon length (bp)
Prolactin (*PRL*)	CAACTCAAGGTCCCTCTCCA	Fw	20	62	2500
	CTTCAGCTCCTCCACGTACA	Rv	20	62	
β-Lactoglobulin (*BLG*)	ACCTCTCTTCGGAAATGTTCA	Fw	21	45	120
	CTGTTGGGCTTGCTCTTTGTC	Rv	21	49	

For *PRL* gene, a 2500 bp fragment was subsequently digested with 10U *BsuRI (HaeIII)* enzyme (ThermoFisher Scientific, USA) for 3 hours at 37°C, and the products of digestion were visualized on a 2% agarose gel. The amplified PCR product of *BLG* gene (120 bp) was digested with 10U *Rsa*I endonuclease (ThermoFisher Scientific, USA), for 3 hours at 37°C and separated on ethidium bromide-stained 3% agarose gel.

Statistical analysis

Hardy-Weinberg equilibrium was tested using exact test, described by Graffelman and Moreno (2013). Statistical analysis was performed using statistical software environment R (https://www.r-project.org/).

RESULTS AND DISCUSSION

DNA integrity evaluation

In the present study were identified *CSN3, PRL and BLG* gene polymorphism. In order to detect these markers, *DNA* integrity was evaluated with a Bioanalyzer Tape Station 4150, Agilent and the *DIN* number was ranged between 9.8 and 8.8, (figure 1).

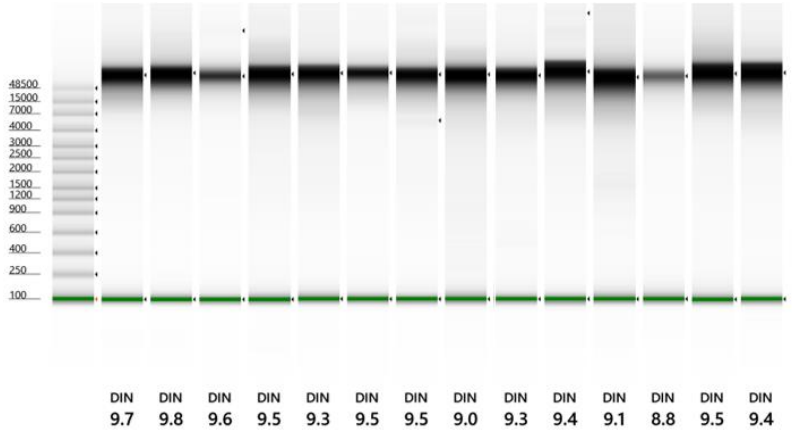

Figure 1. The evaluation of *DNA* integrity

CSN3 allele and genotypes identification in TBH ewes

The PCR pattern for *CSN3* gene was observed in Teleorman Black Head ewes on 2% (w/v) agarose gel electrophoresis by visualization of C and T alleles. A representative image of *CSN3* alleles identified in the present study is showed in Figure 2.

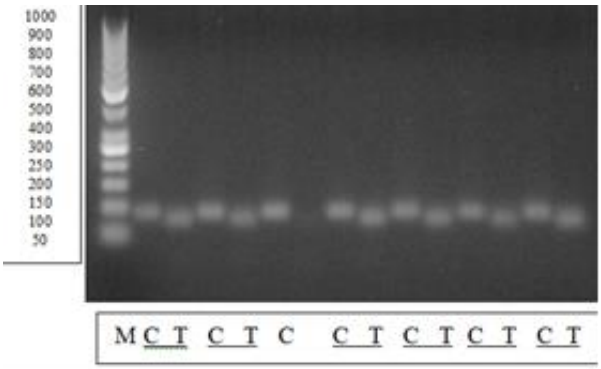

Figure 2. *CSN3* PCR pattern for allele C (97bp) and allele T (85bp) in Teleorman Black Head ewes

The genotypes for *CSN3* gene showed two genotypes, CC and CT. In our study, the allelic frequency, (figure 3) for casein gene was almost equally shared between C allele (0.53) and T allele (0.47), in a similar way with another research made for East Frisian breed by Staiger et al., (2010) (T with 0.49 and C with 0.51).

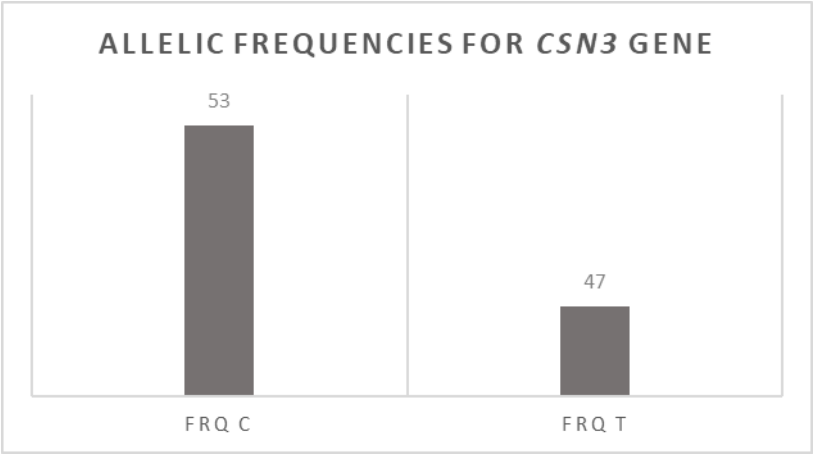

Figure 3. Allelic frequencies for *CSN3*-gene in Teleorman Black Head ewes

The CC homozygotes genotype frequency was 0.06, while for the heterozygotes CT was 0.94 in Teleorman Black Head ewes (figure 4). Different results for *CSN3* gene were discovered by Ceriotti et al., (2010) in Comisana and Sarda breeds, where the TT and CT genotypes are missing. Also, there was observed a correlation between CC genotype and milk quality in obtaining a high cheese productivity at Comisana and Sarda breeds, Ceriotti et al., (2010). Another research on East Frisian sheep pointed out the genotypic structure

regarding the *CSN3* polymorphism spread in three genetic variants CC (0.13), TT (0.11) and CT (0.76), different from the present study,

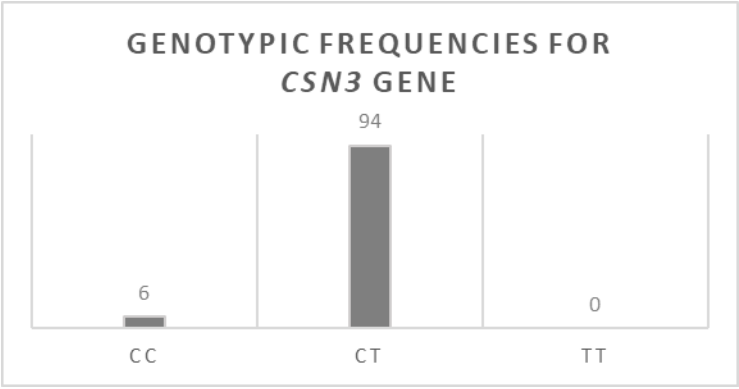

Figure 4. Genotypic frequencies for *CSN3* in Teleorman Black Head ewes

Hardy Weinberg (H-W) equilibrium was estimated for *CSN3* gene, and observed and expected genotypes were found in disequilibrium with the null hypothesis rejected. The lack of H-W equilibrium suggests an empirical selection of the individuals for the presence of C allele. The observed *CSN3* genotypes of the Teleorman Black Head ewes from the present study are explained by the prevalence of heterozygotes CT, followed by a small group of homozygotes CC. The state of H-W disequilibrium creates the opportunity to ~~s~~tudy the homozygosity of genotype CC of the *CSN3* gene in the future and its impact on milk yield (table 2). *CSN3* gene was observed in Hardy-Weinberg disequilibrium in this population data suggesting an empirical elimination of TT genotype form population. Taking into account the missing TT genotype in TBH ewes, in the future are needed more studies regarding effect of TT homozygosity on individuals.

Table 2. Genotypes and H-W equilibrium testing for *CSN3* gene in Teleorman Black Head ewes

Genotype	Observed	Expected
CC	3	14
CT	47	25
TT	0	11
$\chi 2$ at 2 df and 5% significance	3.85	3.85<39.32*
calculated $\chi 2$	39.32	

*Null hypothesis rejected

PRL allele and genotypes identification in TBH ewes

The amplicon of *PRL* gene, in this study was observed at 2500 bp (figure 5), and the amplification was followed by *RFLP* using HaeIII enzyme, resulting two alleles (A and B, figure 6).

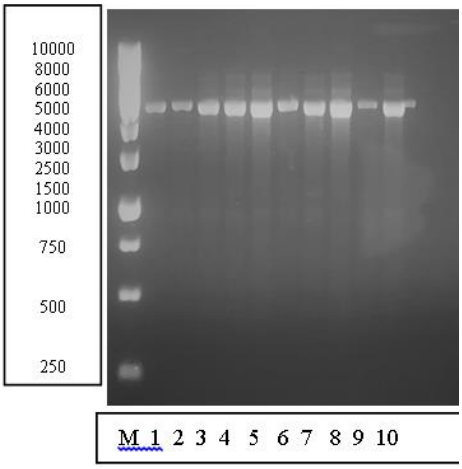

Figure 5. Amplicon of *PRL gene* in Teleorman Black Head ewes samples

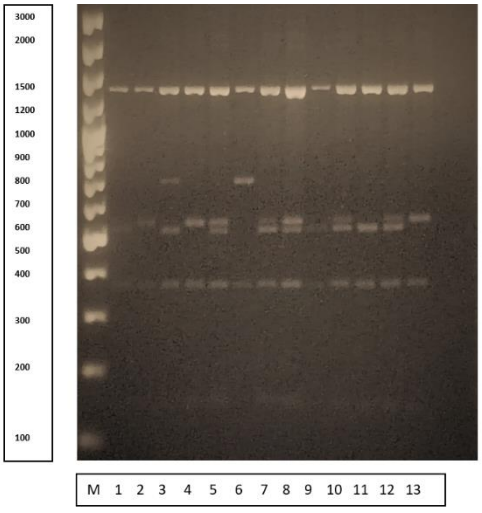

Figure 6. Restriction fragments polymorphism in Teleorman Black Head ewes for *PRL* gene

Allele A has 4 polymorphism fragments of 1400, 530, 360 and 150 bp; this allele has also an allelic frequency of 0.56 in TBH ewes. Allele B has five polymorphism fragments, at 1400, 530, 360 150 and 20 bp, its allelic frequency in THB population being 0.44 (figure 7).

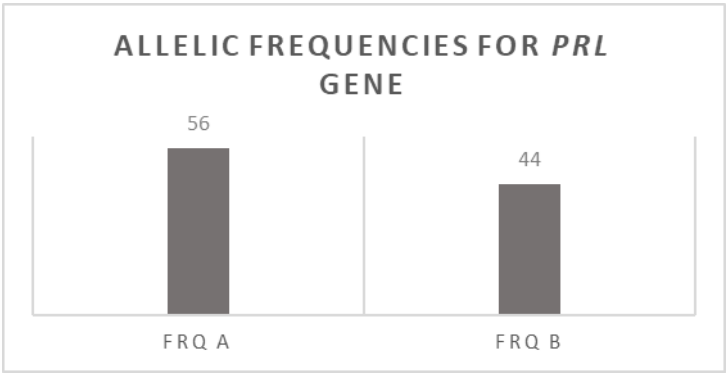

Figure 7. Allelic frequencies for *PRL* gene in Teleorman Black Head ewes

The three genotypes of the *PRL* gene were: heterozygotes AB (with a frequency of 0.40) and homozygotes AA and BB with a frequency of 0.36 and 0.24, respectively (figure 8).

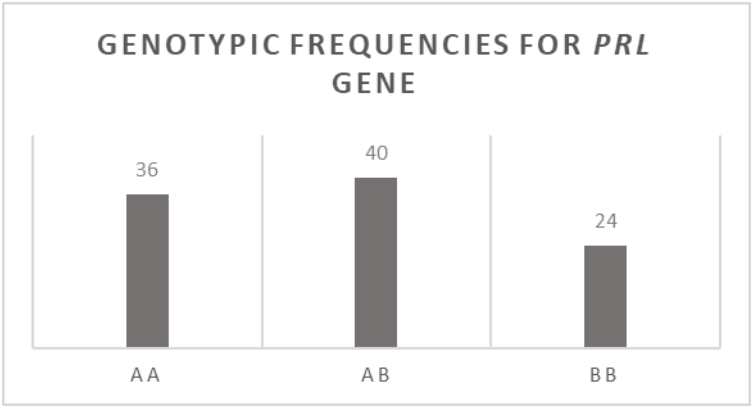

Figure 8. Genotypic frequencies for *PRL* in Teleorman Black Head ewes

Hardy-Weinberg equilibrium was estimated for *PRL* gene and genotypes were found in equilibrium, null hypothesis being accepted (table 3). Hardy-Weinberg equilibrium at this gene pointed out an absence of selection pressure on this locus and increased variability, with a highly potential for improvement.

Ozmen et al., (2016), observed in similar research for three milk breeds (Sakiz, Akkarman and Awassi) these two allele, A and B for *PRL* gene with a higher frequency for allele A in Sakiz ewes in comparison with the other two breeds.

Table 3. Genotypes and H-W equilibrium testing for *PRL* gene in Teleorman Black Head ewes

Genotype	Observed	Expected
AA	18	16
AB	20	25
BB	12	10
$\chi 2$ at 2 df and 5% significance	3.85	3.85>1.773**
calculated $\chi 2$	1.773	

**null hypothesis accepted

Akkarman and Awassi ewes had a higher frequency on allele B and distinctly from Teleorman Black Head ewes which for allele A and B of *PRL* gene were divided almost equally. In a study by Staiger et al., (2010) in East Frisian sheep the three genotypes of PRL gene were found, but with different frequencies (0.02 for AA, 0.24 for AB and 0.74 for BB).

There are studies demonstrating that, for the sheep breeds, the AA genotype of *PRL* is associated with a better milk production than AB and BB genotypes. The BB genotype of *PRL* resulted to be more appreciated with a higher milk fat percentage than AB and BB genotype. Ozmen et al., (2016) observed also, that AB heterozygotes had a higher protein percentage in Sakiz ewes than homozygotes AA and BB. A similarly research was done by Ramos et al., 2009 regarding the influence of *PRL* on milk production, protein and fat percentage on two breeds, Serra da Estrela and Black Merino, where allele A was more frequent than allele B (0.639 *vs* 0.361 and 0.722 vs 0.278 respectively.–Ramos et al., (2009) observed that animals with heterozygotes AB and homozygotes BB recorded a higher milk yield than those with homozygotes AA. Staiger et al., (2010) found on East Friesian sheep the same tendency of the genotype AA of *PRL* polymorphism associated with milk quantity, similar like that one found by Ozmen et al., (2016). Also, Staiger et al., (2010) supposed that the correlation between milk production and the *PRL* polymorphic forms resulted from HaeIII restriction were linked to the functional mutation of *PRL*.

BLG allele and genotypes identification in TBH ewes

In the present study we found an amplicon of *BLG* gene of 120 bp (figure 9). The digestion with RsaI enzyme leaded to the identification of three genotypes (AA, AB and BB, figure 10).

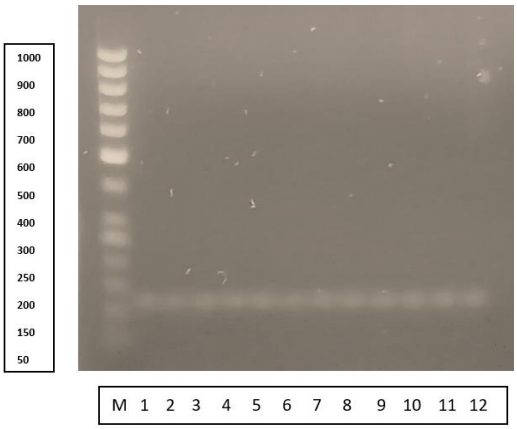

Figure 9. Amplicon of *BLG* gene in Teleorman Black Head ewes

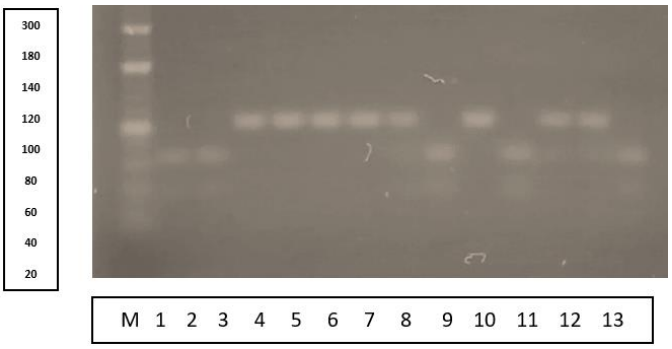

Figure 10. *BLG* allele identification in Teleorman Black Head ewes

BLG alleles A and B, identified in Teleorman Black Head ewes were found in equal amount among individuals taken in this study with allele frequency of 0.50 to 0.50 (figure 11).

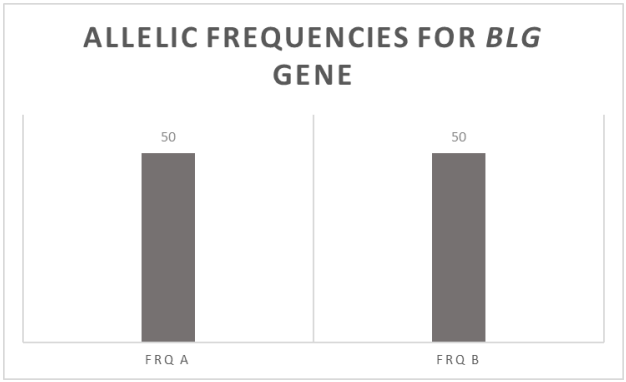

Figure 11. Allelic frequencies for *BLG* gene in Teleorman Black Head ewes

The structure of *BLG* genotypes is equally shared between homozygotes with a frequency of 0.28 (AA) to 0.28 (BB) and the heterozygotes AB had a frequency of 0.44 (figure 12).

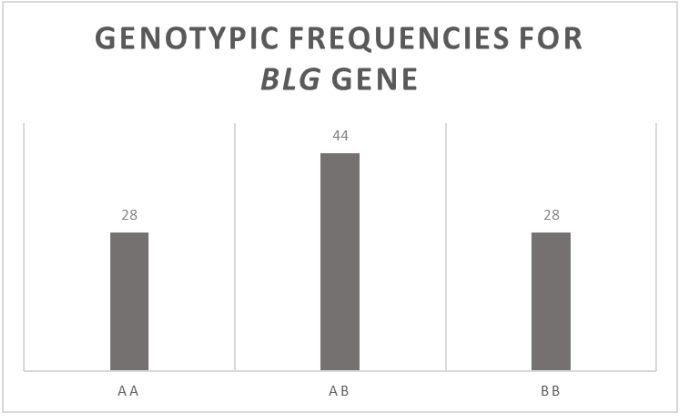

Figure 12. Genotypic frequencies for *BLG* in Teleorman Black Head ewes

BLG gene is in Hardy Weinberg equilibrium, similar with *PRL* gene. The state of H-W equilibrium, due to his increased variability, offer an excellent starting point in animal selection. (table 4).

Table 4. Genotypes and H-W equilibrium testing for *BLG* gene in Teleorman Black Head ewes

Genotype	Observed	Expected
AA	14	13
AB	22	25
BB	14	13
$\chi 2$ at 2 df and 5% significance	3.85	3.85>0.72**
calculated $\chi 2$	0.72	

**null hypothesis accepted

Similar studies were performed in India on the local sheep breed with three genetic polymorphisms for *BLG* A, B, and C, Arora et al., (2010). The same author identified the *BLG* polymorphic forms in Indian local sheep obtaining two alleles, A (0.37) and B (0.63).

Also, Arora et al., 2010 found three different *BLG* genotypes: homozygotes AA and BB with frequency of 0.175 and 0.436 respectively and heterozygote AB with frequency of 0.389. Arora et al., (2010) mentioned also that the obtained results (which demonstrated the dominance of B allele) were in contrasts to those obtained on sheep breeds from Eastern and Central Europe, Southwest Asia, and the Mediterranean area.

A similar study on local Turkish sheep breeds identified the polymorphism of *BLG* gene, discovering the same alleles A and B with frequencies between 0.763 and 0.975 for allele A and 0.024 to 0.236 for allele B, Elmaci et al., (2006). The authors concluded that in sheep breeds from European countries the most common allele encountered for *BLG* was the A allele, Elmaci et al., (2006).

CONCLUSION

In this research we studied the polymorphism of the most important candidate genes (*CSN3, PRL* and *BLG*) in the Teleorman Black Head sheep population.

The genotypes for *CSN3* gene showed two type of genotypes CC and CT, TT genotype missing. Allele frequency in the Teleorman Black Head sheep population is similar with those of other sheep populations from other genetic studies. *CSN3* gene was observed in Hardy-Weinberg disequilibrium in this population data suggesting an empirical elimination of TT genotype form population. Taking in account the missing TT genotype and the selection pressure at this gene, in the future are needed more studies regarding effect of TT homozygosity on individuals.

PRL and *BLG* genes exhibit three genotypes (AA, AB, BB) with both alleles evenly distributed in population. Hardy-Weinberg equilibrium pointing out an absence of selection pressure on those loci and increased variability, with a highly potential for genetic improvement of sheep population. High variability makes those genes excellent candidate genes for marker assisted selection programs. These preliminary data will be correlated in the next experiments with phenotypic data, generating important tools for genetic selection of the Teleorman Black Head sheep population.

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