Bulgarian Journal of Agricultural Science, 25 (Suppl. 3) 2019
Agricultural Academy

Genetic diversity and population structure of Bulgarian autochthonous sheep breeds based on nucleotide variation in Alpha S1- casein gene

Deyana Gencheva*, Svetlana Georgieva

Trakia University, Faculty of Agriculture, Department of Genetics, Breeding and Reproduction, Students town, Stara Zagora 6000, Bulgaria
**Corresponding author:* dhristova@uni-sz.bg

Abstract

Gencheva, D. & Georgieva, S. (2019). Genetic diversity and population structure of Bulgarian autochthonous sheep breeds based on nucleotide variation in Alpha S1- casein gene. *Bulg. J. Agric. Sci., 25* (Suppl. 3), 95–102

The genetic diversity, population structure and relationships of Bulgarian autochthonous sheep breeds were investigated based on nucleotide variation in exon III of the alpha S1- casein (CSN1S1) gene. Two genetic variants of the CSN1S1 gene (A and C) and two genotypes (AC and CC) have been identified using PCR-RFLP analysis on a total of 213 unrelated individuals representing five local sheep breeds: Breznik (BRNK), Copper-red Shumen (CRSH), Karakachan (KKCH), Pleven blackhead (PLBH) and Stara Zagora (STZG), reared in different private sheep-farms under the control of the Executive Agency on Selection and Animal Breeding in Bulgaria. As expected, a 306 bp fragment of the polymorphic locus in CSN1S1 gene was amplified and digested with endonuclease enzyme MboII. The allele frequencies determined a prevalence of the allele C (0.983) over the allele A (0.017) in the studied sheep populations. The obtained restriction fragments revealed two genotypes: AC and CC, observed in 3.4 and 96.6% respectively in the studied sheep populations. The homozygous genotype AA was not identified in the studied sheep breeds. All examined breeds indicated a low level of genetic diversity, with an average of 0.033. The CSN1S1 locus was monomorphic in the population STZG. The estimated negative values of the coefficient Fis (-0.017) showed a low level of inbreeding in all five sheep groups. The exact P-value ($P > 0.9$) for the separate breeds was established and all sheep breeds were in correspondence with HWE equilibrium at the degree of freedom df = 1. The obtained genetic differentiation between the examined sheep populations was not significant and genetic distances were relatively low. The greatest distance ($D_A = 0.004$) was found between STZG population and CRSH, also between STZG and KKCH, while the closest relationship was established ($D_A = 0.000$) between CRSH, KKCH and BRSK. The Nei's genetic distances calculated by UPGMA method produced a phylogenetic tree that separates the investigated sheep breeds into two main clusters: one including STZG and PLBH and the other one consisting of the three remaining sheep breeds – BRSK, CRSH and KKCH. The data obtained in the present study can contribute to developing an effective conservation strategy for traditional sheep breeds in Bulgaria.

Keywords: autochthonous sheep; *Ovis aries*, alpha S1; casein gene; *CSN1S1*; genetic diversity, PCR-RFLP

Introduction

In Bulgaria, sheep breeding and the use of local sheep genetic resources have a longtime tradition, despite its relatively small territory. The diverse ecological and economic conditions in the country and the various needs of the local people allowed raising a large number of sheep breeds, of which nineteen are autochthonous. At present, sheep breeding activities are realized in 13 breeding organizations with 179128 sheep in total. The dairy sheep have the biggest share, representing 67.3% and the autochthonous is about 25.8%. Stara Zagora sheep and Breznik sheep are on the edge of extinction while comparatively stable are the populations of Karakachan sheep and Copper-red Schumen sheep (EASAB, 2011).

The priority of dairy sheep breeding in Bulgaria is formed through the centuries because of the traditional use of dairy sheep products as well as the consumption of lamb meat (Dimitrov et al., 1993; Stankov et al., 2007). One of the principal goals of animal breeding specialists is selection of sheep in order to increase their milk production (Sotirov et al., 2006). Generally, the local sheep breeds have a high level of vitality and resistance to diseases, excellent adaptability to local ecological conditions and high suitability to breeding conditions. Also, they could be included in the system of organic farming (Danchev, 1994; Dimitrov & Dimitrova, 1994).

It is well known that the production of high-quality dairy products is closely linked with the preserved genetic diversity in local sheep breeds and strains. In this context, the genetic diversity within economically important loci, including S1- casein gene in European sheep breeds have been reviewed by Chessa et al. (2017), with insight into their development. In a review discussing the conservation of genetic resources of autochthonous domestic livestock breeds in Bulgaria, Tanchev (2015) pointed out that the preservation of specificity of a given breed would preserve the diversity among breeds in accordance with EC and global directives for genetic diversity protection at a worldwide scale. Barillet (2007) discussed the impact of milk genetic polymorphism for efficient breeding programmes in the dairy sheep industries. Therefore, the knowledge of the genetic polymorphism of ovine milk proteins is essential. In addition, production of typical dairy products is mainly based on the use of local populations in different breeding systems and therefore traditional sheep breeds play a direct role in biodiversity enhancement (Petrović et al., 2005; Scintu et al., 2007).

Caseins and their genetic polymorphisms are important due to their effects on quantitative and qualitative traits, also on technological properties of milk (Frajman & Dovč, 2004; Ceriotti et al., 2005; Park et al., 2007). Among the four milk fractions (αS1-CN, αS2-CN, β-CN and κ-CN), the alpha S1-CN constitutes 47.21% of individual fraction of whole ovine milk proteins and has a number of genetic polymorphs, which are due to a silent amino acid substitution or deletion in the triplet code (Clement et al., 2006; Chessa et al., 2010). The gene CSN1S1 encoding αS1-casein has been assigned on the sixth chromosome in the sheep genome and eight genetic variants CSN1S1 (A, B, C, D, E, F, H and I) have been described until now (Ferranty et al., 1995; Chianese et al., 1996; Ordas et al., 1997; Ferranty et al., 1998; Pilla et al., 1998; Amigo et al., 2000; Debeljak et al., 2000; Ceriotti et al., 2004; Ivanković & Dovć, 2004; Ceriotti et al., 2005, Chianese et al., 2007; Corral et al., 2010; Giambra et al., 2010). Considering the sequence for the caprine exon three of the ovine αS1-CN gene from GenBank, Kevorkian et al. (2009) have identified the sequence of the same exon in the ovine genome, which includes 30 nucleotides with base G in the position 15, instead T. Nevertheless available results did not allow a clear association between a specific genetic variant and ovine milk traits, several authors suggested that the genetic polymorphisms in CSN1S1 locus could be considered as a marker for milk and cheese quality and also for technological properties of milk (Chianese, 1997; Ramunno et al., 1997; Pirisi et al., 1999; Amigo, 2000; Banykó, 2007; Moioli et al., 2007; Martini et al., 2008).

The polymorphism of αS1-casein genes has been investigated in some Bulgarian sheep breeds, at the DNA level. Based on PCR-RFLP analysis, Hristova (2011) has established a single nucleotide polymorphism (SNP) in exon III of CSN1S1 gene in three sheep breeds – Karakachan, Local Karnobat and Copper-red Shumen. In order to evaluate the significance of the D allele (Welsh variant of the αS1-CN gene) for the technological properties of milk and the economic efficiency of breeding animals, Kalaydhziev et al. (2014) analyzed seven autochthonous sheep breeds.

The aim of the present research is focused on the study of genetic polymorphism in αS1-casein locus using PCR-RFLP assay in some of the most popular local sheep breeds in Bulgaria. This will give an opportunity to establish their population structure and define genetic diversity in order to facilitate and plan their sustainable development, utilization, and conservation.

Material and Methods

Sampling and DNA extraction

A total of 213 unrelated ewes belonging to the populations of five Bulgarian sheep breeds were analyzed: Breznik, Copper-red Shumen, Karakachan, Pleven blackhead, and Stara Zagora, reared in different private sheep-farms under the control of the Executive Agency on Selection and Animal Breeding in Bulgaria (EASAB). The abbreviations of the sheep breeds, their type, economic use, current size, degree of endangerment, geographical location, the number of individuals and sampled flocks per breed are presented in Table. 1.

Blood samples (3 ml) were collected from the *v. jugularis* into vacutainer tubes containing EDTA. The genomic DNA was extracted from the whole blood with Illustra Blood GenomicPrep DNA Purification Kit (GE Healthcare, UK) according to the manufacturer's instructions and stored at -20ºC until the analysis was performed. According to the manual, typically a 300 µl sample of whole blood yields about 10-50 ng DNA. The quality and quantity of the ob-

Table. 1. Sheep breeds under investigation

Sheep breed name	Code (Abbr.)	Examined number	Flocks (n)	Geographical location	Breed type, Tail type	Population size under breeding control*
Breznik	BRNK	50	1 (30) 2 (20)	Western Bulgaria, in Breznik, Pernik, Radomir and Kyustendil	Traditional selection, Long-thin-tailed	Total – 836 ♀ – 812 ♂ – 24 flocks – 9
Copper-red Shumen	CRSH	37	1	Northeastern Bulgaria	Traditional selection, Short-thin-tailed	Total – 4280 ♀ – 4138 ♂ – 142 Flocks – 40
Karakachan	KKCH	38	1 (31) 2 (7)	Higher mountainous regions of the country	Zackel Short-thin-tailed	Total – 3632 ♀ – 3529 ♂ – 103 Flocks – 20
Pleven blackhead	PLBH	48	1 (37) 2 (11)	Central and western parts of the Danubian plain	Tsigai Zackel Long-thin-tailed	Total – 13100 ♀ – 12836 ♂ – 264 Flocks – 52
Stara Zagora	STZG	40	1	Stara Zagora, Eastern part of the Tracian lowland	Tsigai Long-thin-tailed	Total – 694 ♀ – 680 ♂ – 14 Flocks – 7

* Data are obtained from Breeding Executive Agency on Selection and Animal Breeding (EASAB)
(n) – Number of individuals in each flock

tained genomic DNA were determined using NanoVue Plus Spectrophotometer (GE Healthcare) and through agarose gel electrophoresis.

PCR amplification and RFLP analysis

The alleles of the alpha S1-casein gene at exon III were established through PCR-RLFP analysis. PCR amplifications were carried out in a total volume of 20 µl, containing 100 ng DNA template, 2×Red Taq DNA Polymerase Master mix (VWR, Belgium) and 10 pM of each primer with sequence described by Corral et al. (2010). PCR reactions were performed in Gradient thermocycler (VWR) under the following conditions: an initial denaturation at 94°C/5 min, followed by 30 cycles at 94°C/30 sec, primer annealing at 53°C/45 sec, extension at 72°C/1 min and a final extension at 72°C/10 min. The digestion reactions were carried out in 25 µl final volume, containing 10 µl PCR product, incubated at 37ºC/15h using 1 U/µl of MboII restriction endonuclease (BioLabs). The obtained PCR products and restriction fragments were stained with fluorescent GelRed® dye (Biotium), separated on 2.5% agarose gel (TopVision agarose, Fermentas) in 1x TBE buffer and visualized under UV light using Electrophoresis Gel Imaging Analysis System (Bio-Imaging Systems). The genotypes of the analyzed individuals at the CSN1S1 gene were established using the restriction fragments observed in the agarose gel.

Statistical Analysis

For analysis of the genetic diversity and population structure were calculated: allele diversity, number of alleles per locus and their frequencies, observed and expected heterozygosity values (observed – Ho and expected – He) and coefficient of inbreeding within each population (Fis) through the software package ARLEQUIN, version 3.5.1.3 (Excoffier and Lischer, 2010). The same software was used to check deviation from Hardy-Weinberg equilibrium (HWE) by the method of Guo and Thompson (1992). POPGENE software, version 1.31 (Yeh and Yong, 1999; Labate, 2000) was used to estimate the genetic distances (D_A) between populations according to the method as per Nei (1978) based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm. PHYLIP ver. 3.69 software was used for phylogenetic tree reconstruction and depicting the dendrogram (Felsenstein, 2009).

Results

A 372 bp fragment of the target polymorphic region (exon III) of the CSN1S1 gene was successfully amplified

in the studied sheep populations. After enzymatic digestion with endonuclease MboII, two different genotypes were identified in the examined sheep populations –homozygous CC and heterozygous AC (Fig. 1).

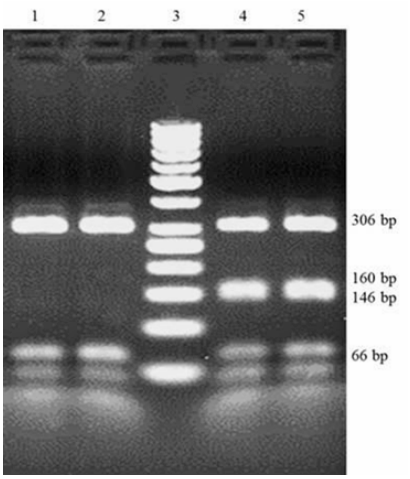

Fig. 1. Restriction fragments and PCR amplified products of exon III in CSN1S1 gene with Mbo II restriction enzyme in sheep populations on 2% agarose gel electrophoresis. Lanes: 1-2 – samples with CC homozygous genotype, lane 3: M – DNA ladder (50 bp), lanes: 4-5 – samples with AC heterozygous genotype

The homozygous CC genotype (presented on agarose gel with two fragments with size 306 bp and 66 bp) was detected in 48 Breznik ewes, 35 Copper-red Shumen ewes, 36 Karakachan ewes, 47 Pleven blackhead ewes and 40 Stara Zagora ewes. The heterozygous AC genotype (four fragments with size 306, 160, 146 and 66 bp) was found out in 2 Breznik ewes, 2 Copper-red Shumen ewes, 2 Karakachan ewes and only 1 Pleven blackhead ewe. The homozygous AA genotype was absent in all assessed sheep groups.

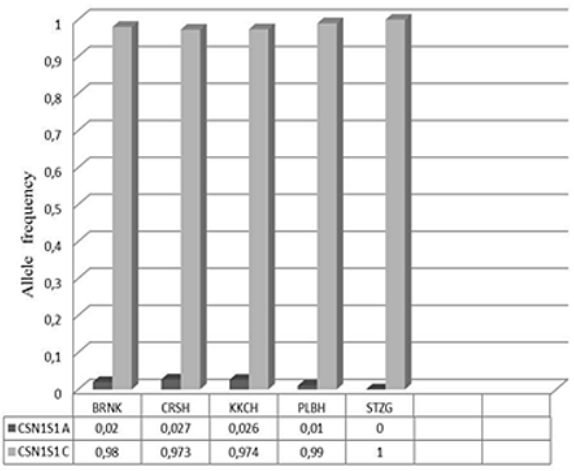

Fig. 2. Distribution of the allele frequencies for CSN1S1 gene in the examined sheep populations

Based on PCR- RFLP assay both allele (A and C) corresponding to the CSN1S1 gene were identified in the sheep populations under investigation. Allele frequencies distribution is presented on Fig. 2. The mean frequency value of the allele A was 0.017 and varied in a range from 0.000 in STZG to 0.027 in CRSH sheep. For allele C, the mean value of frequency was 0.983 and ranged from 0.973 in CRSH to 1.000 in STZG sheep.

Table. 2. Distribution of the genotype frequencies, expected heterozygosity (He), chi-square test of HWE (χ^2) and coefficient of inbreeding (Fis) for CSN1S1 gene in the examined sheep populations

Sheep breeds	Genotype frequencies			He*	χ^2 (P)	Fis
	AA	AC	CC			
BRNK	0.000	0.040	0.960	0.039	0.010	– 0.020
		(n = 2)	(n = 48)		(0.919)	
CRSH	0.000	0.054	0.946	0.053	0.014	– 0.028
		(n = 2)	(n = 35)		(0.905)	
KKCH	0.000	0.053	0.947	0.051	0.014	– 0.027
		(n = 2)	(n = 36)		(0.907)	
PLBH	0.000	0.021	0.979	0.021	0.000	– 0.011
		(n = 1)	(n = 47)		(1.000)	
STZG	0.000	0.000	1.000	0.000	–	–
		(–)	(n = 40)			
Mean	0.000	0.034	0.966	0.033		– 0.017

Legend: Breznik sheep (BRNK), Copper-red Shumen sheep (CRSH), Karakachan sheep (KKCH); Pleven blackhead (PLBH); Stara Zagora (STZG); He* – expected heterozygosity calculated as per Nei (1978)

The distribution of genotype frequencies in the different sheep populations is summarized in Table 2.

The heterozygous AC genotype was observed only in 3.4% of the studied ewes, while the homozygous CC was established in 96.6% of the individuals. The homozygous CC genotype was found with the highest frequency of 0.979 in PLBH, whereas for heterozygous AC it was 0.021. In the two other investigated sheep breeds, the distribution of genotype frequencies was similar as follows: 0.946 for CC and 0.054 for AC genotype in CRSH and 0.947 for CC and 0.053 for AC genotype in KKCH. The homozygous genotype AA was not identified in the studied sheep breeds. STZG sheep population was monomorphic in order to the studied CSN1S1 locus.

Observed heterozygosity (Ho) was higher than expected (He), resulting in a negative inbreeding coefficient with a mean value Fis = – 0.017, varied from – 0.011 in PLBH to – 0.028 in CRSH sheep (Table. 2). The chi-square test for Hardy-Weinberg equilibrium pointed values of χ^2 with a level of probability $P > 0.9$ at a degree of freedom df = 1 confirming the validity of the HWE for all studied sheep populations (Table 2).

The Nei's genetic distances (D_A) among the examined sheep breeds are present in Tab. III. The greatest distance (D_A=0.004) was found between STZG population and CRSH, also between STZG and KKCH. The closest relationship was established (D_A=0.000) between CRSH, KKCH, and BRSK. The Nei's genetic distances calculated by UPGMA method produced a phylogenetic tree which separates the investigated sheep breeds into two main clusters: one including STZG and PLBH and the other one consisting of the three remaining sheep breeds – BRSK, CRSH, and KKCH (Fig. 3).

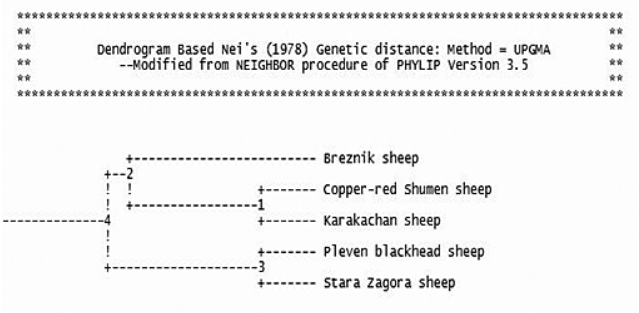

Fig. 3. UPGMA dendrogram generated from Nei's genetic distances (D_A) of the examined sheep breeds based on CSN1S1 genotypes

Discussion

Sheep breeding and the use of local sheep genetic resources has longtime traditions in Bulgaria. The priority of dairy sheep breeding is formed through the centuries due to the traditional consumption of dairy sheep products. The genetic polymorphisms of the αs1-CN fraction in milk proteins have been considered a potential tool for the selection of dairy sheep breeds. With the development of molecular genetics, PCR-RFLP method was already developed for establishment of the allele variants A, C, D in αS1-CN locus by Pilla et al. (1998), Ivankovic and Dovc (2004), Corral et al. (2010) etc. in different sheep breeds, strains and crossbred populations.

In the present investigation, single nucleotide polymorphism (SNP) in exon III of the alpha S1- casein (CSN1S1) gene was investigated on a total of 213 unrelated ewes from five sheep breeds reared in Bulgaria. The obtained experimental data showed that the frequencies of alleles A and C of CSN1S1 gene in the studied population are relatively close (Fig. 2). This indicates a small variation regarding CSN1S1 locus between the studied populations of sheep breeds. The distribution of allele frequency in all sheep breeds demonstrated a higher preponderance of allele C mean value 0.983) compared to allele A (mean value 0.017). Similar results have been reported by Ramos et al. (2009) in three sheep breeds (Serra da Esttrela, White Merino and Black Merino), with allele A frequencies of 0.023, 0.022 and 0.036, and for allele C – 0.923, 0.916 and 0.902, respectively, as well as by Giambra et al. (2010) in a total of 57 individuals of Black Faced Mutton sheep. The results corresponded with these reported by Rustempasic et al. (2013) for the total of 372 sheep collected from flocks of Pramenka breed located in Bosnia and Herzegovina, where the frequencies are 0.020 for allele A and 0.0980 for allele C (nonA). Based on PCR-RFLP analysis, Hristova (2011) has obtained frequencies similar to these from the present study – 0.020 for allele A and 0.980 for allele C, in a total of 90 individuals belonging to local sheep breeds, reared in Bulgaria – Karakachan, Local Karnobat and Copper-red Shumen. On the contrary, lower values of frequencies for allele C, ranging from 0.54 to 0.72 have been observed in different Italian and Spain sheep breeds by Amigo et al. (2000), but in general they were significantly higher than the frequencies of allele A (0.006-0,060).

It is important to point out that in the present study, a fixing of the allele C (frequency 1.000) in STZG sheep population is observed. This could be explaned with the highest milk yield by the long-term selection in this sheep population. Recently, in a study based on Isoelectric focusing (IEF) of the casein polymorphisms, Giambra et al. (2014) have been established genetic differences in East Friesian Dairy and Lacaune sheep breeds hold in three different flocks in Switzerland and Germany. The CSN1S1 C variant has been the most common in Lacaune sheep (100%), while the A

allele is missing. Contradictory results have been obtained by Kevorkian et al. (2009), who reported the absence of a polymorphism in the exon III in three sheep breeds reared in Romania.

At the present research, following SNP detection at position 13 of the third exon in CSN1S1 gene, two different genotypes homozygous – CC and heterozygous AC were established in assessed local sheep populations. It was observed that the CC genotype was a most common one in all breeds under the investigation and this might be due to the low frequency noticed for the allele A. In this study, the most frequently genotype CC at the αS1-CN locus was with a frequency of 0.979 in PLBH and 0.960 in BRNK sheep breeds. These results were agreed with experimental data reported by Corral et al. (2010) for the Spain Merino sheep breed (0.980).

The data obtained in this study for the mean value of expected heterozygosity (He = 0.033) calculated as per Nei (1978) indicate for a low level of genetic diversity in all examined sheep breeds at CSN1S1 locus (Table 2). The expected heterozygosity value was highest (0.053) in CRSH sheep population. The estimated values of χ^2 (Table 2) evidenced for a good relatedness between empirically obtained and theoretically expected genotypes in all studied sheep populations. This indicates for representativeness of the samples, despite their limited size as well as that current selection has not eliminated any of the alleles at the CSN1S1 locus. In contrast, a statistically significant deviation from HWE equilibrium has been reported by Rustempasic et al. (2013) for Pramenka sheep breed in Bosnia and Herzegovina. Nevertheless, the obtained values of observed heterozygosity (Ho) for different sheep population in this study were relatively low (varied from 0.021 to 0.054), a negative mean value for the coefficient of inbreeding (Fis = – 0.012) has been calculated (Table 2).

Study on genetic diversity by molecular genetic distances was in the focus of the researchers during the last decades. The obtained genetic differentiation between the examined sheep populations (Fig. 3.) was not significant and genetic distances based on UPGMA method were relatively low corresponding to CSN1S1 locus. As expected, two of the studied sheep breeds – STZG and PLBH are in the same cluster at the dendrogram. These populations are two of the widespread indigenous sheep breeds which have the highest milk yield compared to the other local sheep breeds reared in Bulgaria.

Conclusions

The present study revealed obvious genetic variation in autochthonous sheep breeds reared in Bulgarian with respect to the exon III of CSN1S1 gene, detected by PCR-RFLP analysis. The sheep population STZG was monomorphic in order to the CSN1S1 locus. The nucleotide variation in genes encoding ovine milk casein is important because the production of high-quality dairy products is linked with the preserved genetic diversity in local sheep breeds and strains.

Generally, the frequency of the allele C was significantly higher than that of allele A in all studied sheep population. The obtained experimental data showed that the frequencies of the two established genotypes – homozygous CC and heterozygous AC are relatively close in studied sheep populations. The most common genotype in all breeds under study was CC due to the low frequency of the allele A. The calculated mean value of expected heterozygosity indicates a low level of genetic diversity in all examined sheep breeds at CSN1S1 locus. The obtained genetic differentiation between the examined sheep populations was not significant and genetic distances were relatively low.

The data obtained in the present study can contribute towards developing an effective conservation strategy for traditional sheep breeds in Bulgaria. Analysis of more samples and longer part of the different polymorphic sites of the CSN1S1 gene are needed to reconstruct a correct phylogenetic tree. The genetic polymorphism of milk proteins can be studied and used further for assessment of the genetic structure of Bulgarian indigenous sheep populations and to establish the relationship with their dairy production traits.

References

Amigo, L., Recio, I. & Ramos, M. (2000). Genetic polymorphism of ovine milk proteins: its influence on technological properties of milk-a review. *International Dairy Journal*, *10*, 135-149.

Banykó, J. (2007). Distribution of αS1-casein "Welsh" variant in some Slovak and Czech sheep breeds. *Archive Tierzucht*, *50*, 381-387.

Barillet, F. (2007). Genetic improvement for dairy production in sheep and goats. *Small Ruminant Research*, *70*, 60-75.

Ceriotti, G., Chessa, S., Bolla, P., Budelli, E., Bianchi, L., Duranti, E. & Caroli, A. (2004). Single nucleotide polymorphisms in the ovine casein genes detected by Polymerase Chain Reaction-Single Strand Conformation Polymorphism. *Journal of Dairy Science*, *87*, 2606-2613.

Ceriotti, G., Chiatti, F., Bolla, P., Martini, M. & Caroli, A. (2005). Genetic variability of the ovine alpha S1-casein. *Italian Journal of Animal Science*, *4*, 64-66.

Chessa, S., Giambra, I., Brandt, H., Caroli, A., Gootwine, E. & Erhardt, G. (2017). Genetic diversity within economically important loci in European, Middle Eastern, and African sheep breeds: An insight into their development. *Small ruminant re-*

search, *155*, 72-80.

Chessa, S., Rignanese, D., Berbenni, M., Ceriotti, G., Martini, M., Pagnacco, G. & Caroli, A. (2010). New genetic polymorphisms within β- and αS2-caseins. *Small Ruminant Research*, *88*, 84-88.

Chianese, L., Caira, S., Garro, G. & Addeo, F. (2007). Primary structure of ovine deleted variant αs1-CN E. In: Proceedings of 5th international symposium on the challenge to sheep and goats milk sectors, Alghero/ Sardinia, Italy, pp. 70.

Chianese, L., Garro, G., Mauriello, R., Laezza, P., Ferranti, P. & Addeo, F. (1996). Occurrence of five α s1-casein variants in ovine milk. *Journal of Dairy Research*, *63*, 1, 49-59.

Chianese, L., Mauriello, R., Ferranti, P., Tripaldi, C., Taibi, L. & Dell'Aquila, S. (1997). Relationship between alphas1-casein variants and clotting capability of ovine milk. In Milk protein polymorphism, Palmerston North (New Zealand), Feb 1997. International Dairy Federation.

Clement, P., Agboola, S. & Bencini, R. (2006). A study of polymorphism in milk proteins from local and imported dairy sheep in Australia by capillary electrophoresis. *LWT-Food Science and Technology*, *39*, 63-69.

Corral, J., Padilla, J. & Izquierdo, **M.** (2010). Associations between milk protein genetic polymorphisms and milk production traits in Merino sheep breed. *Livestock Science*, *129*, 73-79.

Danchev, Y. (1994). The preservation of autochthonous breeds of domestic animals in Bulgaria, In: Bulgaria's Biological Diversity National Strategy: Conservation Status and Needs Assessment, 587-599, (Bg).

Debeljak, M., Susnik, S., Milosevic-Berlic, T., Medrano, J. & Dovc, P. (2000). *Gene Technology and Milk Production Food Technology and Biotechnology*, *38*,(2), 83-89.

Dimitrov, Ts. & Dimitrova, I. (1994). Preservation of Livestock genetic resources in Bulgaria, In: Animal Genetic Resources Information bulletin, Boyazoglu, J. & D. Chupin, (Eds.), 14, 41-59.

Dimitrov, Tz., Dimitrova, I. & Vassilev, D. (1993). Conservation of the Bulgarian Indigenous Livestock Resources, In Proceedings: International Animal Conservation Strategy, FAO Workshop, 27-28.

EASAB, 2011. Executive Agency on Selection and Animal Breeding, Livestock Breeds in the Republic of Bulgaria. Catalogue, 3rd Ed. ed V,. Nikolov, Sofia, Bulgaria, pp. 44-77 (Bg).

Excoffier, L., & Lischer, H. E. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular ecology resources*, *10*(3), 564-567.

Felsenstein, J. 2009. PHYLIP (Phylogeny Inference Package) Version 3.69. Department of Genome Sciences, University of Washington, Seattle, WA, USA.

Ferranti, P., Chianese, L., Malorni, A., Migliaccio, F., Stingo, V. & Addeo, **F.** (1998). Copresence of deleted protein species generates structural heterogeneity of ovine aS1-casein. *Journal of Agricultural and Food Chemistry*, *46*, 411-416.

Ferranti, P., A. Malorni, G. Nitti, P. Laezza, R. Pizzano, L. Chianese & F. Addeo, 1995. Primary structure of ovine alpha S1-caseins: localization of phosphorylation sites and characterization of genetic variants A, C and D. Journal of Dairy Research, *62*(2), 281-296.

Frajman, P. & Dovc, P. (2004). Milk production in the post-genomic era. *Acta Agriculturae Slovenica*, *84*(2), 109-119.

Giambra, I. J., Brandt, H. & Erhardt, G. (2014). Milk protein variants are highly associated with milk performance traits in East Friesian Dairy and Lacaune sheep. *Small Ruminant Research*, *121*(2-3), 382-394.

Giambra, I. J., Jäger, S. & Erhardt, G. (2010). Isoelectric focusing reveals additional casein variants in German sheep breeds. *Small Ruminant Research*, *90*(1-3), 11-17.

Guo, S. W. & Thompson, E. A. (1992). Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*, 361-372.

Hristova, D., 2011. Genetic polymorphism of alpha S1- casein gene in Bulgarian sheep breeds. *Agricultural Science and Technology*, *3*(1), 8-12.

Ivancovic, A. & Dovc, P. (2004). Polimorphism of β-Lactoglobulin and αS1-casein genes in the Pag Islad sheep. *Acta Agriculturae Slovenica*, *84*(2), 121-130.

Kalaydjiev, G., Angelova, T., Yordanova, D., Karabashev, V., Laleva, S., Cassandro, M., J. Krastanov, Oblakov, N. Dimov, D. & Popova, Y. (2014). „D-allele" frequencies in milk α-s1 casein from Bulgarian local sheep breeds. LXVIII Convegno Sisvet, XI Convegno Aipvet e XII Convegno Sira, 16-18 June, Pisa, Italy, pp. 142-145.

Kevorkian, S., Manea, M., Gavrila, M., Rebedea, M., Georgescu, S. & Costache, M. (2009). Sequencing of exon three of αs1- casein sheep and goad gene. *Archiva Zootechnica*, *12*(3), 87-92.

Labate, J. A. 2000. Software for population genetic analyses of molecular marker data. *Crop Science*, *40*(6), 1521-1528.

Martini, M., Salari, F., Scolozzi, C., Cecchi, F., Ceriotti, G. & Caroli, A. (2008). Relationship between milk genetic polymorphism and phisico-chemical and nutritional quality of sheep milk. *Small Ruminant Research*, *74*, 194-201.

Moioli, B., D'Andrea, M. & Pilla, F. (2007). Candidate genes affecting sheep and goat milk quality. *Small Ruminant Research*, *68*, 179-192.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, *89*, 3, 583-590.

Ordas, J., A. Rando, C. Senese & P. Masina, 1997. DNA polymorphisms of casein genes in Spanish dairy. *Small Ruminant Research*, *26*, 9-13.

Park, Y., Juarez, M., Ramos, M. & Haenlein, G. (2007). Physico-chemical characteristics of goat and sheep milk. *Small Ruminant Research*, *68*, 88-113.

Petrović, M. P., C. Mekić, D. Ružić & Žujović, M. (2005). Genetic principles relating to improvement of milk yield in sheep and goats. *Biotechnology in Animal Husbandry*, *21*, 5-6, 73-78.

Pilla, F., Bevilacqua, C., Leroux, C., Fraghi, A. & Martin, P. (1998). Genotyping of alpha-S1 casein in sheep. *Animal Genetics*, *29*, 472-473.

Pirisi, A., G. Piredda, C. Papoff, R. Di Salvo, S. Pintus, G. Garro, P. Ferranti &Chianese, L. (1999). Effects of sheep alpha S1-casein CC, CD and DD genotypes on milk composition and cheesemaking properties, *Journal of Dairy Research*, *66*, 409-419.

Ramos, A, Matos, C., Russo-Almeida, P., Bettencourt, C., Matos, J., Martins, A., Pinheirod, C. & Rangel-Figueiredo, T. (2009). Candidate genes for milk production traits in Portuguese dairy sheep. *Small Ruminant Research*, *82*, 117-121.

Ramunno, L., Cosenza, G., Rando, A., Macciotta, P., Pappalardo, M. & Masina, P. (1997). Identification of carriers of the Welsh CASA1 variant using an allele-specific PCR method. *Animal Genetics*, *28*, 154-155.

Rustempasic, A., Dokso, A., Zecevic, E., Hodzic, A., Bandzo, K., Miskoska-Milevska, E. & Brka, M. (2013). Polymorphism of CSN1S1 gene in Pramenka breed sheep in Bosnia and Herzegovina. In Proceedings of the 24th International Scientific-Expert-Conference of Agriculture and Food Industry, Sarajevo, Bosnia and Herzegovina, 25-28 September 2013 (pp. 91-94). Faculty of Agriculture and Food Sciences, University of Sarajevo.

Scintu, M. & Piredda, G. (2007). Typicity and biodiversity of goat and sheep milk products. *Small Ruminant Research*, *68*, 221-231.

Sotirov, L., Dimitrov, I., Djorbineva, M., & Jeliazkov, E. (2006). Effect of complement C3 genotypes on milk production in sheep. *Revue de médecine vétérinaire*, *157*(7), 391.

Stankov, I., Slavov, R., Panayotov, D. & Pamukova, D. (2007). State and perspective for development of sheep breeding in Republic of Bulgaria. Sheep breeding in Bulgaria and in Europe, In: Proceedings of scientific conferences, Kirilov A (Ed.), Sofia, 23-32, (Bg).

Tanchev, Sv. (2015). Conservation of genetic resources of autochthonous domestic livestock breeds in Bulgaria. A review. *Bulgarian Journal of Agricultural Science*, *21*(6), 1262-1271.

Yeh, F., & Yong, R. (1999). POPGENE version 1.31 (02.04. 2011). Microsoft based Freeware for Population Genetic Analysis. University of Alberta, Edmonton, Canada.