

Genetic polymorphism of alpha S1-casein in Bulgarian sheep breeds and its effect on milk composition

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Abstract: The genetic polymorphism of the alpha S1-casein (CSN1S1) was investigated in five sheep breeds reared in Bulgaria: Sofia (Elin-Pelin, SEPL), Copper-Red Shumen (CRSH), Local Karnobat (LKNB), Plevan Blackhead (PLBH), and Stara Zagora (STZG) sheep with an aim to establish the possible effect of a particular genotype on ovine milk composition. Based on nucleotide variation in exon III of the CSN1S1 gene, two genetic variants (A and C) and three genotypes (AA, AC, and CC) have been identified using PCR-RFLP analysis on a total of 217 unrelated ewes. The allele frequencies determined a prevalence of the allele C (0.886) over the allele A (0.114) across the studied sheep populations. The homozygous CC genotype was observed in nearly 80% of the studied ewes. The calculated values for observed ($H_o = 0.548$) and expected ($H_e = 0.468$) heterozygosity at CSN1S1 locus indicated a relatively high degree of genetic variability in the Sofia sheep population. The greatest Nei's genetic distance ($D_A = 0.080$) was found between the populations STZG and SEPL, while the closest relationship was established ($D_A = 0.000$) between PLBH and CRSH, also between STZG and CRSH. The results of the association analysis indicated that CSN1S1 AC genotype was significantly associated ($P < 0.05$) with the highest percentages of the fat, protein, casein, solids-nonfat and total solids in Sofia sheep ewes. The genotype CSN1S1 CC was associated with the highest noncasein protein percentage, while the genotype AA was linked with the highest lactose percentage. The CSN1S1 genotype did not show a significant effect ($P > 0.05$) in the Sofia sheep population in relation to the renneting time. In conclusion, the established single nucleotide polymorphism in the CSN1S1 locus could be used as a potential genetic marker for ovine milk composition traits, as well as for developing an effective conservation strategy towards traditional sheep breeds in the country.

Key words: Bulgarian sheep breeds, α S1-casein, CSN1S1 gene, single-nucleotide polymorphism, PCR-RFLP analysis, milk composition

1. Introduction

The primary goal of modern genomic selection is the association of all known sources of animal information (phenotype, origin, and genetic markers) in order to obtain the highest reliability of the estimated breeding value and ensure genetic improvement [1]. The authors pointed out that genomics offers extensive opportunities for genetic improvement of animals but can also be used as an appropriate tool for the assessment of genetic diversity of local sheep and goat breeds. Additionally, selection based on molecular techniques should involve the process of DNA molecular analysis in order to search for markers or single nucleotide polymorphisms (SNPs) to obtain information on the genetic potential of the product's characteristics of the individuals.

It is well known that genetic polymorphisms of ruminant milk proteins are important due to their effects on quantitative and qualitative traits and technological

properties of milk [2,3]. Moreover, clarification of the possible association between genetic variants and milk traits is connected to the fact that the variants of milk protein genes differ among themselves mostly in single nucleotide polymorphisms which could cause amino acid substitutions in mature proteins, with potential biological effect [4].

At present, the genetic polymorphism of milk proteins has been of considerable interest in animal breeding and in the dairy industry. Studies on milk protein polymorphisms and their effect on milk yields of sheep are mainly carried out in the Mediterranean countries, in which great importance is attached to milk performance [5]. In goat, the α S1-casein genotype had a significant effect on milk yield, fat content, and protein content [6]. On the other hand, sheep milk contains higher total solid and major nutrient content than that of goats and cows [7]. Some evidence indicated that genetic polymorphisms

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affect the physicochemical properties of ovine milk [8,9]. More in-depth knowledge of the genetic polymorphism of ovine milk proteins and their impact on ovine milk traits as well as technological properties is essential for the improvement of the quality of ewe's milk cheese [10,11]. Therefore, an important step for cheese curd formation is the presence of alpha S1-casein (α s1-CN) which is also a structural component of the casein [12]. The α s1-CN represent the main milk protein, constitutes 47.21% of the individual fraction of whole ovine milk proteins, and has a number of genetic polymorphisms, which are due to a silent amino acid substitution or deletion in the triplet code [13,14]. At present, eight genetic variants CSN1S1 (A, B, C, D, E, F, H, and I) have been described [15–17].

Compared to the cows and goats, the polymorphism of ewes milk is not yet well established. In many countries, milk production traits have a great extent, because the sheep milk is almost totally processed into cheese due to the influence of individual differences on milk proteins [18,19]. In addition, the impact of milk genetic polymorphism for efficient breeding programs in the dairy sheep industries has been pointed by Barillet [20]. Although the genetic polymorphisms of CSN1S1 have been widely proposed to affect milk composition traits in different sheep production systems, its potential role in the high-adaptive local sheep breeds has not been intensively investigated. Therefore, the aim of the present research was to identify the genetic polymorphism in sheep populations reared in Bulgaria with respect to CSN1S1 locus and establish the possible effect of a particular genotype on milk composition.

2. Materials and methods

2.1. Animals and sample collection

The present investigation was performed with a total of 217 unrelated ewes, representing five sheep breeds: Sofia (Elin–Pelin, SEPL, $n = 42$), Copper–Red Shumen (CRSH, $n = 34$), Local Karnobat (LKNB, $n = 46$), Pleven Blackhead (PLBH, $n = 51$), and Stara Zagora (STZG, $n = 44$). The animals were grown in private farms located in different regions of Bulgaria, under the control of the Executive Agency for Selection and Reproduction in Animal Breeding. Blood samples were obtained from vena jugularis into K2EDTA vacuum tubes (Becton Dickinson, UK) in a total volume of 3 mL.

2.2. DNA extraction and gene amplification

Total genomic DNA was extracted from the whole blood samples using Illustra Blood GenomicPrep DNA Purification Kit (GE Healthcare, UK) according to the manufacturer protocol and stored at -20°C until the analysis was performed. The quality and quantity (about 20–80 ng) of the obtained genomic DNA were determined using NanoVue Plus Spectrophotometer (GE Healthcare).

PCR amplifications were carried out in total volume of 20 μL , containing 80 ng DNA template, 10 pM of each primer and 2 \times Red Tag DNA Polymerase Master mix (VWR, Belgium). PCR reactions were performed with primers designed by Pilla et al. [21] and Corral et al. [22], and accomplished on Doppio (2 \times 48 well) Gradient thermal cycler (VWR, Germany) under the following cycling conditions: an initial denaturation step at $95^{\circ}\text{C} / 5 \text{ min}$, followed by 35 cycles of $95^{\circ}\text{C} / 30 \text{ s}$, primer annealing at $53^{\circ}\text{C} / 55 \text{ s}$, extension $72^{\circ}\text{C} / 1 \text{ min}$, final extension at $72^{\circ}\text{C} / 10 \text{ min}$, and stored at $4^{\circ}\text{C} / \infty$.

2.3. Genotyping through RFLP assay

The obtained amplicons with fragment size of 372 bp were digested using 1U / μL MboII restriction endonuclease (BioLabs) in a determined specific site at 5'...GAAGA(N8)↓...3'. The digestion reactions were carried out in a total volume of 25 μL , containing 10 μL PCR product and incubated at $37^{\circ}\text{C} / 15 \text{ h}$. The obtained restriction fragments were stained with GelRed[®] (Biotium, USA) fluorescent dye, separated on 2.5% agarose gel (TopVision agarose, Fermentas) dissolved in 1X TBE buffer and visualized under UV light using Electrophoresis Gel Imaging Analysis System (Bio-Imaging Systems, Israel). The exact genotype of the analyzed individuals at the CSN1S1 gene was established based on the number of the restriction fragments and their size identified in the agarose gel.

2.4. Milk composition analysis

The analysis of milk composition was performed with milk, obtained from 42 ewes representing purebred Sofia sheep, reared in the flock of the private farm in village Novi Han, municipality Elin–Pelin, district Sofia. Individual milk samples were obtained in the morning and the evening, proportionally to the milk yield, according to rules for milk sampling. The milk composition—fat, protein, casein, noncasein protein, lactose, solids-nonfat, and total solids (dry matter content)—was established by means of Milko-Skan 104 (A/S Foss Electric, Denmark), according to Barbano and Dellavalle [23]. The renneting time of the milk was measured visually in milk samples as the time from rennet addition until the formation of the first visible flocks according to the method suggested by Chomakov et al. [24].

2.5. Statistical analyses

The software package ARLEQUIN, version 3.5.1.3 [25] was used for calculation of the population genetic parameters: allele and genotype frequencies, observed (H_o) and expected (H_e) heterozygosity, as well as coefficient of inbreeding (F_{is}) within each group. The genetic distances (DA) between populations were estimated using Co-dominant Diploid data from the program POPGENE, version 1.31 [26,27], according to the method as per Nei [28] based on unweighted pair group method with

arithmetic mean (UPGMA) algorithm. The same software was used in order to check the validity of the Hardy-Weinberg equilibrium (HWE) by the method of Guo and Thommson [29]. POPGENE software, version 1.31 [26, 27] was used for phylogenetic tree reconstruction and depicting the dendrogram.

The association analysis includes obtaining the main statistics (mean values [\bar{x}] and the standard deviations [SD]) for the tested milk components (fat, protein, casein, noncasein protein, lactose, solids-nonfat, and total solids) and renneting time, a normality distribution verification of the data set with the tested milk components and univariate data analysis to establish the influence of the particular genotype of CSN1S1 gene on milk composition traits in Sofia sheep population. As a result, a general linear model was developed, given as $Y = \bar{x} + G + e$, where Y are the measurements of the milk traits, \bar{x} are the mean values, G are the factors of influence (the particular genotypes of CSN1S1 gene), and e are the random residual errors. Significant differences were tested using Tamhane test, and P-values < 0.05 were considered statistically significant. The IBM SPSS Statistics 17.0 WinWrap Basic, Copyright 1993 - 2007 statistical package was used to process the data [30].

Additionally, the genetic effects for dominance (D) and additivity (A) for CSN1S1 genotypes in the Sofia sheep population were estimated, according to the equations suggested by Russo et al. [31]: $D = CA - \frac{1}{2}(CC + AA)$ and $A = \frac{1}{2}(CC - AA)$, where CC and AA are homozygous groups and AC the heterozygous group. The ratio D/A was considered to indicate actual gene effects, as follows: $D/A < 0.2$, additive; $0.2 < D/A < 0.8$, partial dominance; $0.8 < D/A < 1.2$, dominance; $D/A > 1.2$, overdominance, despite any significance [32].

3. Results

3.1. Population genetic structure of examined sheep populations

The genetic structure and diversity of a total of five most widespread sheep populations in Bulgaria were established regarding the single nucleotide polymorphism (SNP) of the exon III at CSN1S1 locus. As expected, after the digestion of the PCR products of the exon III at ovine CSN1S1 gene with MboII enzyme, a total of four fragment sizes, 306, 160, 146, and 66 bp, were obtained representing three genotypes as follows: two homozygous ones, CC and AA; and a heterozygous one, AC (Figure 1).

The obtained results based on PCR-RFLP assay showed that 80% of the studied animals were homozygous (genotype CC) and their restriction profile revealed two electrophoretic bands (306 and 66 bp). Only 17% of the individuals were heterozygous (AC genotype), presented with four fragments (306, 160, 146, and 66 bp). The

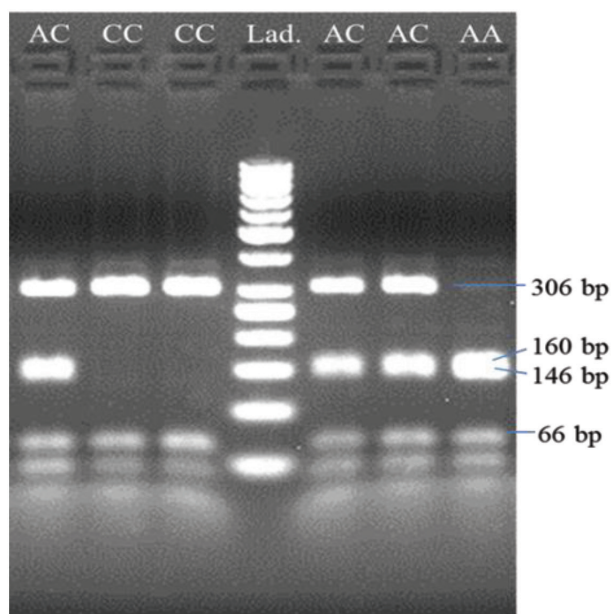


Figure 1. Electrophoregram with restriction fragments of amplified PCR products at the polymorphic locus (exon III) of the CSN1S1 gene obtained with MboII restriction enzyme in studied sheep. Identified genotypes: CC, homozygous (two bands of 306 and 66 bp); AC, heterozygous (four fragments of 306, 160, 146, and 66 bp); AA, homozygous (three bands of 160, 146 and 66 bp); Lad., DNA marker, 50 bp.

homozygous AA genotype was identified by the lowest frequency (about 3%) and was expressed with three bands (160, 146, and 66 bp) on the agarose gel image.

The allele and genotype frequencies at the polymorphic CSN1S1 locus were estimated for all studied breeds and summarized in Table 1.

The mean value of frequency for allele A at CSN1S1 locus was 0.096 across the studied sheep populations and varied from 0.009 in PLBH sheep to 0.393 in SEPL sheep. According to allele C, the mean value of frequency was 0.904 and ranged from 0.607 in SEPL sheep to 0.991 in BHPL. It is interesting to point out that allele A was absent in the population of STZG sheep and consequently the allele C showed the highest value of 1.000. Therefore, the STZG sheep population was monomorphic in regard to studied CSN1S1 locus.

The distribution of genotype frequencies in the different sheep populations is summarized in Table 1. The homozygous CC genotype was found with the highest frequency of 0.981 in the PLBH population, whereas for SEPL it was 0.333. The homozygous genotype AA was not identified in three studied sheep breeds: CRSH, PLBH, and STZG.

The observed (H_o) and expected (H_e) heterozygosities for the examined sheep populations are given in Table

Table 1. Allele and genotype frequencies, observed (Ho) and expected (He) heterozygosity, chi-square test for HWE (χ^2), and coefficient of inbreeding (Fis) at the polymorphic CSN1S1 locus in the studied sheep populations .

Sheep populations	n	Allele frequencies		Genotype frequencies			He Nei**	χ^2 (***)	Fis
		A	C	AA	AC*	CC			
SEPL	42	0.393	0.607	0.119 (5)	0.548 (23)	0.333 (14)	0.468	1.434 (0.231)	-0.196
CRSH	34	0.029	0.971	0.000 (0)	0.058 (2)	0.942 (32)	0.057	0.015 (0.901)	-0.030
LKNB	46	0.141	0.859	0.022 (1)	0.239 (11)	0.739 (34)	0.233	0.032 (0.858)	-0.015
PLBH	51	0.009	0.991	0.000 (0)	0.019 (1)	0.981 (50)	0.019	0.000 (1.000)	-0.009
STZG	44	0.000	1.000	0.000 (0)	0.000 (0)	1.000 (44)	0.000	-	-
Mean		0.114	0.886	0.028	0.173	0.799	0.157		

SEPL, Sofia (Elin-Pelin) sheep; CRSH, Copper-Red Shumen sheep; LKNB, Local Karnobat sheep; PLBH, Plevan Blackhead sheep; STZG, Stara Zagora sheep; *, Observed heterozygosity (Ho); **, Expected heterozygosity (He) calculated as per Nei (1978); ***, P-value (P); n, number of individuals.

1. Considering the whole sample containing a total of 217 individuals, Ho ranged from 0.019 in PLBH sheep population to 0.548 in SEPL sheep population with a mean of 0.173, while He varied from 0.019 to 0.468 with a mean 0.157. As a whole, the observed heterozygosity was higher than expected, resulting in a negative coefficient of inbreeding (Fis), varied from -0.009 in the PLBH sheep to -0.196 in the CRSH sheep population (Table 1). In regard to test possible deviation from Hardy-Weinberg equilibrium, exact P-values for the single breeds were obtained (Table 1). The chi-square test for genetic equilibrium pointed values of χ^2 with a level of probability $P > 0.2$ confirming the validity of the HWE for all studied sheep populations at a degree of freedom $df = 1$. The genetic distances among the studied sheep populations calculated using the UPGMA method are shown in Table 2. The closest Nei's genetic distance (DA = 0.001) was observed between the populations of PLBH, STZG, and CRSH sheep, while the distance estimated between SEPL and STZG sheep populations was the greatest (0.080).

Genetic relationships between the studied sheep breeds based on the polymorphism in CSN1S1 locus are presented in Figure 2. The calculated genetic distances produced a phylogenetic tree that separates the investigated sheep populations into two main clusters: one consisting of four groups: CRSH, PLBH, STZG, and LKNB; and the other including SEPL sheep population.

Table 2. Nei's genetic distances (DA) between studied sheep breeds.

Sheep populations	SEPL	CRSH	LKNB	PLBH	STZG
SEPL	****				
CRSH	0.068	****			
LKNB	0.026	0.008	****		
PLBH	0.076	0.001	0.011	****	
STZG	0.080	0.001	0.013	0.002	****

SEPL, Sofia (Elin-Pelin) sheep; CRSH, Copper-Red Shumen sheep; LKNB, Local Karnobat sheep; PLBH, Plevan Blackhead sheep; STZG, Stara Zagora sheep

3.2. Association analysis

In Figure 3 are presented the normal distributions of the investigated milk components of the Sofia sheep population (fat, protein, casein, noncasein protein, lactose, solids-nonfat, and total solids) and renneting time, grouped by the CSN1S1 genotypes. As can be seen from the graphs in Figure 3, there are some differences in skewness and kurtosis at the milk components distributions but still, it can be noticed that there is a similarity at the normal distributions of the data based on the genotypes. This

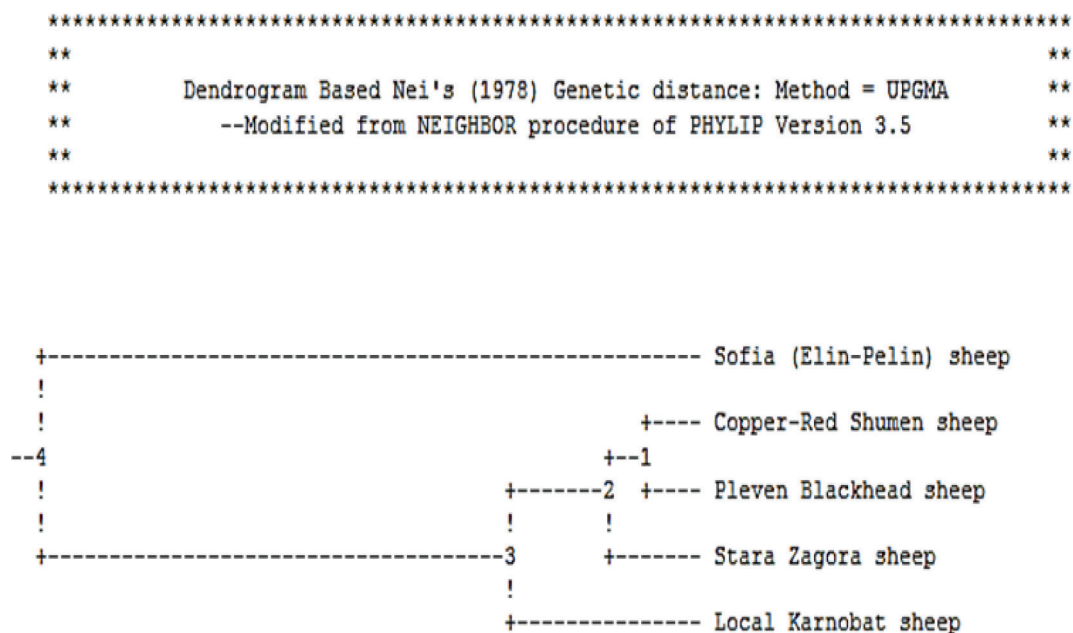


Figure 2. UPGMA dendrogram generated from Nei's genetic distances of the examined sheep breeds based on the polymorphism in CSN1S1 locus.

shows the normality of the data into the genotype groups and is a reason the multivariate data analysis by Tamhane tests to be applied.

In Table 3 are presented the mean values (\bar{x}) and the standard deviations (SD) for all the tested milk parameters for the Sofia sheep population, depending on the different genotypes of the studied polymorphic CSN1S1 locus.

The results presented in Table 3 indicating statistically significant differences between the mean values for the milk components fat, protein, casein, solids-nonfat, and total solids in individuals of the Sofia sheep breed with the homozygous AA genotype and heterozygous AC genotype. Regarding the noncasein protein and lactose components, statistically significant differences were observed between individuals of the two homozygous genotypes AA and CC, and between those with the AA and AC genotypes, respectively. Insignificant differences ($P > 0.05$) were established for CSN1S1 genotypes in terms of renneting time.

In Table 3 are shown the obtained coefficients of determination (R^2) for the observed milk components in the examined sheep population. The highest percentage of influence at the particular genotype was established compared to follow traits: lactose content ($R^2 = 12.8\%$), noncasein protein content ($R^2 = 12.4\%$), casein content ($R^2 = 11.5\%$), and protein content ($R^2 = 9.4\%$). In the examined sheep population, the lowest percent of influence for other milk traits was estimated for fat content ($R^2 = 6.1\%$), solids-nonfat content ($R^2 = 6.0\%$), and total solid content ($R^2 = 6.0\%$), respectively.

4. Discussion

Sheep breeding in Bulgaria is a thousand-year livelihood of the population and the use of local genetic resources has longtime traditions. The diverse ecological and economic conditions in the country and the different needs and interests of the local people led to development of a large number of sheep breeds [33]. The priority of dairy sheep breeding is formed through the centuries due to the use of traditional milk products [34,35]. Dimitrova et al. [36] pointed out that clarifying the genetic structure of sheep breeds is essential for their genetic improvement through genomic studies and precise analysis of quantitative traits.

One of the widespread local sheep breeds in Bulgaria is Sofia (Elin Pelin) sheep, located in the regions of Sofia and Pernik, but it is also found all over the south-western part of the county. The live weight of the ewes is 60–65 kg, and for rams is 90–100 kg. The animals possess a long body with strong bone system, narrow and deep chest, and wool-free head. The sheep have specific black spots around the eyes in the form of a domino. The average milk yield is 100–120 L per milking period and 130–160 L during lactation. The prolificacy is 120–140% with deviations up to 160–170% [37].

At present, the Sofia sheep breed is relatively stable with a total of 5698 animals (5474 ewes and 224 rams grown in 61 flocks) under selection control. This sheep breed has been used mainly for milk and wool production, but has never been selected for milk production traits. Nevertheless, the farmers have a great interest towards

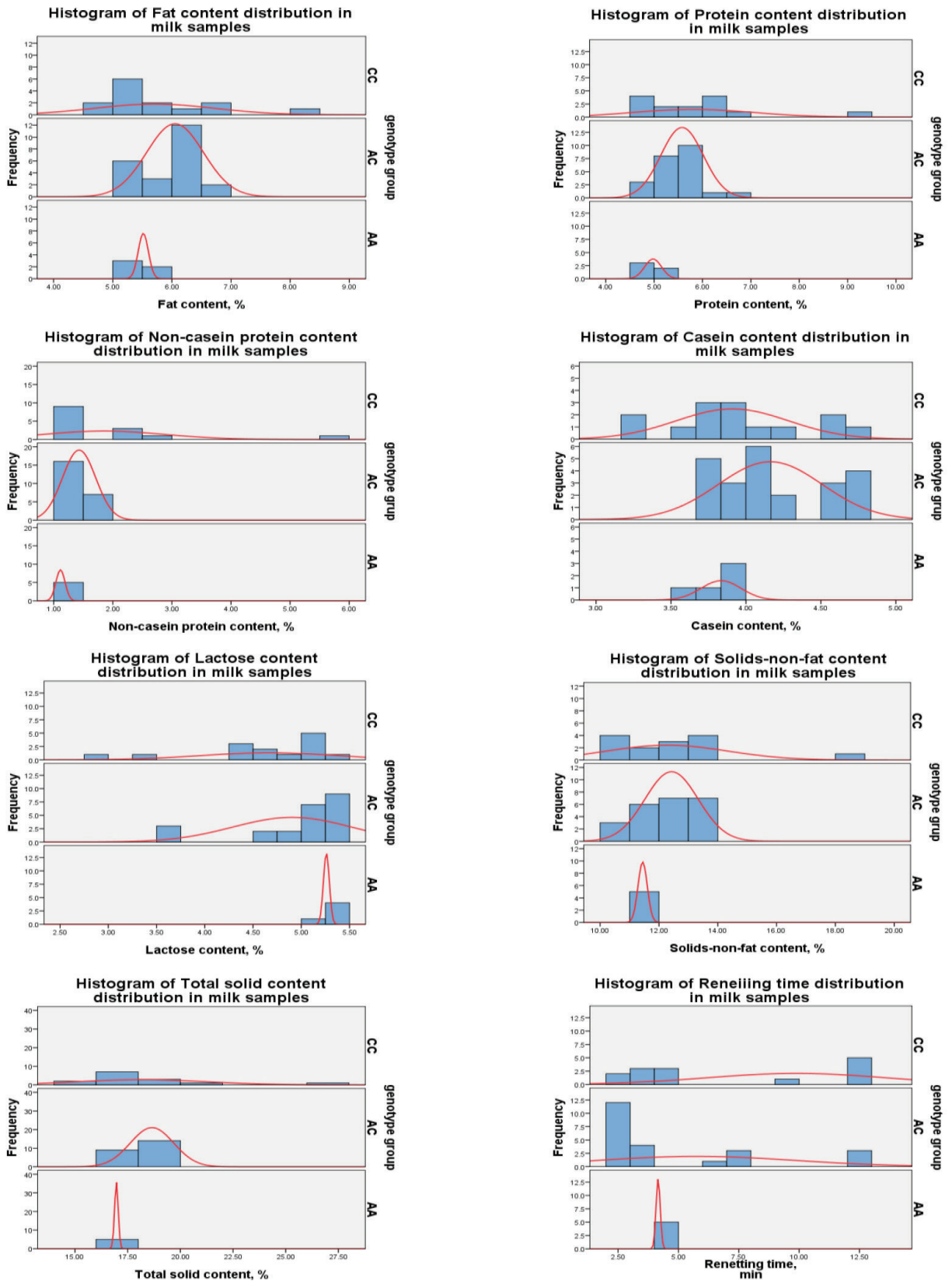


Figure 3. Histograms of all tested parameters of milk composition depending on different CSN1S1 genotypes in Sofia sheep population

Table 3. Effect of CSN1S1 genotypes on milk composition traits: fat, protein, noncasein protein (NCP), lactose, solids-nonfat (SNF), and total solids (TS), and renneting time (RT) in Sofia sheep population.

Milk traits (%)	R ²	CSN1S1 genotypes $\bar{x} \pm SD$			D	A	D/A
		AA (n = 5)	AC (n = 23)	CC (n = 14)			
Fat	0.061	5.516 \pm 0.086 ^a	5.981 \pm 0.507 ^b	5.704 \pm 1.017	0.371	0.094	3.945
Protein	0.094	4.978 \pm 0.174 ^a	5.591 \pm 0.434 ^b	5.807 \pm 1.242	0.198	0.415	0.477
NCP	0.124	1.114 \pm 0.078 ^{aa}	1.419 \pm 0.275 ^b	1.867 \pm 1.163 ^b	-0.072	-0.557	0.129
Casein	0.115	3.834 \pm 0.133 ^a	4.172 \pm 0.359 ^b	3.939 \pm 0.469	0.143	0.053	2.698
Lactose	0.128	5.260 \pm 0.037 ^{aa}	4.954 \pm 0.546 ^b	4.593 \pm 0.736 ^b	0.027	-0.334	-0.080
SNF	0.060	11.446 \pm 0.158 ^a	12.522 \pm 0.890 ^b	12.461 \pm 2.136	0.568	0.508	1.118
TS	0.060	16.964 \pm 0.087 ^a	18.503 \pm 1.155 ^b	18.165 \pm 3.083	0.938	0.601	1.560
RT (min)	0.097	4.160 \pm 0.086 ^{ns}	4.55 \pm 3.440 ^{ns}	6.87 \pm 4.288 ^{ns}	-	-	-

^{a,b} Different superscripts within the same row represent significant differences at the level of significance $P < 0.05$; ^{ns}, not significant differences ($P > 0.05$); R², coefficients of determination based on observed means through Tamhane test; A, additive genetic effect; D, dominance genetic effect; D/A ratio dominance effect/additive effect; n, number of the individuals.

increasing milk yield, as well as improving the quality of ewe's milk cheese [33].

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. Different DNA polymorphisms at the ovine alpha S1-casein locus, their inheritance and linkage relationships using the restriction fragment length polymorphism (RFLP) technique have been described by Levéziel et al. [38] based on endonuclease enzymes BglII, RsaI, and TaqI and by Di Gregorio et al. [39] and Ordás et al. [40] via EcoRI and TaqI digestion.

The polymorphism of whey milk proteins has been reported in some local Bulgarian breeds and strains, but only at the protein level [41,42]. At the DNA level, the polymorphism of the CSN1S1 gene has been previously investigated in Bulgarian sheep breeds by Hristova [43] in order to establish the genetic structure of three the most spread sheep populations: Karakachan, Local Karnobat, and Copper-Red Shumen. The most common genotype at this locus has been homozygous CC with a frequency of 0.970 in the Karakachan and Copper-Red Shumen sheep populations. In a recent study, the genetic diversity and population structure of five Bulgarian autochthonous sheep breeds (Breznik, Copper-red Shumen, Karakachan, Pleven blackhead, and Stara Zagora) have been investigated based on nucleotide variation at CSN1S1 locus by Gencheva and Georgieva [44]. The obtained experimental data in this study showed that the allele frequencies determined a prevalence of the allele C (0.983) over the allele A (0.017) and homozygous AA genotype was not identified in the studied sheep breeds. In conclusion, the authors point out that the genetic differentiation between the examined

sheep populations was not significant and genetic distances were relatively low.

In the present study, a comparison of the allele and genotype frequencies established for exon III in CSN1S1 gene revealed a difference between the studied sheep populations. In general, the obtained results indicated a prevalence of the CSN1S1 C allele in all studied sheep populations with a mean value of 0.886. Among the five examined sheep breeds, SEPL showed the highest frequency (0.548) of heterozygous AC genotype, which demonstrated the relatively high genetic diversity at CSN1S1 locus in this population. The obtained experimental data showed a fixing of the allele C (frequency 1.000) due to the absence of the allele A and in the STZG sheep population. Therefore, we considered the investigated CSN1S1 locus as a monomorphic site in this sheep population. This tendency has been reported by Giambra et al. [17] in the Lacaune sheep population where the variant CSN1S1 C was the most common (100%), while the allele A was missing. However, based on publications, allele CSN1S1 C seems to be more frequent compared to the allele A in Serra da Estrela, White Merino and Black Merino sheep breeds [45], in Black Faced Mutton sheep [17], also in Pramenka sheep [46]. On the contrary, relatively lower values (0.540–0.720) for the allele C frequencies were observed by Amogo et al. [10] in different Italian and Spanish sheep breeds, but in general, they were higher compared to the frequency of the allele A (0.006–0.060). On the other hand, Kevorkian et al. [47] did not identify any polymorphisms at the CSN1S1 gene in sheep breeds reared in Romania.

Because the coding SNPs have a considerable possibility to result in function involves, the present study

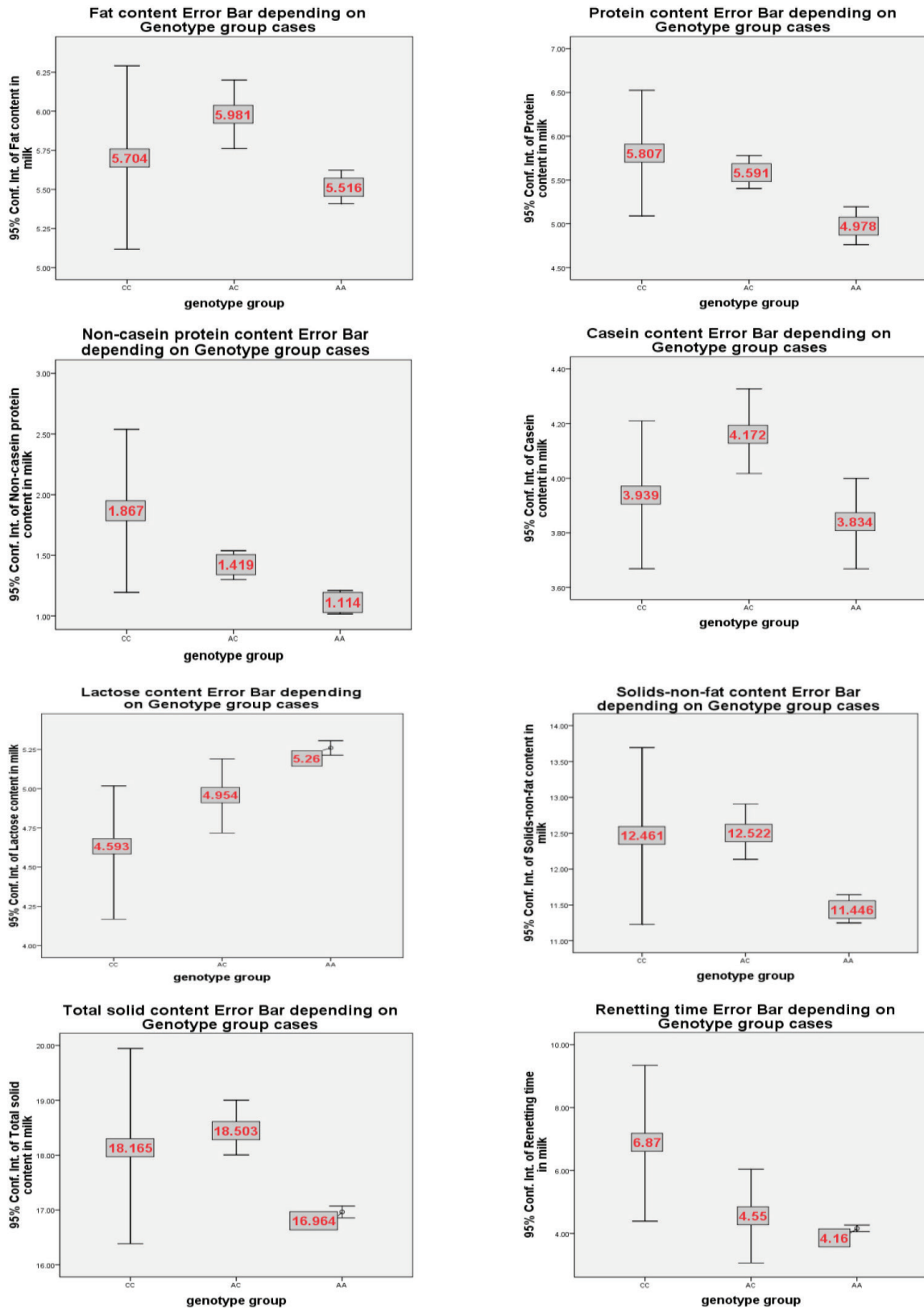


Figure 4. Significant differences between genotype groups for observed milk composition traits in Sofia sheep populatio

is focused on identifying the polymorphisms in CSN1S1 that are potentially associated with the composition traits of ovine milk. Therefore, of special interest in our study was the genetic variation of exon III at CSN1S1 gene and its effect on fat, protein, casein, noncasein protein, lactose, solids-nonfat, and total solids percentages and renneting time. Single marker-trait association analysis was performed on Sofia ewes because of the relatively high level of genetic diversity observed in this sheep population. In the other populations under study, it was not possible to conduct the influence of CSN1S1 genotypes on milk traits due to insignificant variability of the targeted locus, low frequency of the allele A, and lack of the animals carrying the homozygous AA genotype. Moreover, the CSN1S1 locus was monomorphic in the STZG sheep population.

The results obtained in this study showed that polymorphism of exon III at CSN1S1 locus has a significant genetic effect on the examined milk composition traits in Sofia sheep population. There were significant differences ($P < 0.05$) on fat, protein, casein, SNF, and TS between AA and AC genotypes. Thus, the highest fat (5.981 ± 0.507), protein (5.591 ± 0.434), casein (4.172 ± 0.359), SNF (12.522 ± 0.890), and TS (18.503 ± 1.155) percentages were associated with ewes carrying the CSN1S1 AC genotype, and detected individuals were significantly affected by the overdominance, partial dominance and dominance effects on the traits fat and casein ($D/A = 3.945$ and 2.698), on the protein ($D/A = 0.477$) and on the SNF ($D/A = 1.118$), respectively. A significant association was also established between NCP and lactose, and CSN1S1 genotypes ($P < 0.05$). The genotype CC was associated with the highest NCP percentage (1.867 ± 1.163), while the genotype AA was linked with the highest lactose percentage (5.260 ± 0.037), and the animals were affected by the additive effect on these traits ($D/A = 0.129$ and -0.080). However, it is important to emphasize that the homozygous genotype had a low frequency in the analyzed sheep population (0.119) compared to the other ones. In relation to the RT, CSN1S1 genotype did not show a significant effect ($P > 0.05$) in the Sofia sheep population. The results presented graphically in Figure 4 clearly show the mean values and standard deviations of the studied components of the milk from the Sofia sheep ewes according to the influence of the different genotypes, as well as the significant differences between them.

Although the studies of the genetic polymorphism in the ovine CSN1S1 gene are extensive, only a few of them have been dedicated to investigating the influence of the individual genotype on sheep's milk composition traits. Moreover, contradictory effects of the CSN1S1 variants on milk fat and protein contents were reported by Piredda et al. [48], Pirisi et al. [9], by Serrano Moyano et al. [49], etc. A suggestive effect of the CSN1S1 on milk yield has been detected in Serra da Estrela, but no

associations were found between the variants of this marker with milk fat and protein content. Concerning the ovine CSN1S1 polymorphism, Amigo et al. [10] reported that milk samples containing CSN1S1 CC show the best technological behavior, due to the higher amount of casein. Mroczkowski et al. [5] investigated a total of 207 Polish Merino sheep from central Poland within the α S1-CN and concluded that CC genotype exhibited a statistically significant advantage in terms of fat and solids percentage compared to the sheep carrying the AC α S1-CN genotype. Martini et al. [50,51] pointed out that the allele C of alpha S1-casein is the most favorable for cheese making. Despite the results available in the literature so far, they still do not allow associating specific variants of the ovine milk with the compositions traits.

In conclusion, the present study revealed genetic variation in autochthonous sheep breeds reared in Bulgarian with respect to the exon III of the CSN1S1 gene, detected by PCR-RFLP analysis. Generally, the frequency of allele C was significantly higher than that of allele A in all studied sheep populations. The obtained experimental data showed that the most common genotype in all breeds under the study was CC due to the low frequency of the allele A. The calculated values for observed ($H_o = 0.548$) and expected ($H_e = 0.468$) heterozygosity at CSN1S1 locus indicated a relatively high degree of genetic variability in the Sofia sheep population.

As a result of the multivariate analysis for the studied components of the sheep's milk composition (fat, protein, casein, noncasein protein, lactose, solids-nonfat, and total solids), the significance levels of $P < 0.05$ were determined in the Sofia sheep population. Therefore, we could confirm the statistically significant effect of genotypes on the studied milk composition traits. The obtained results obviously support that the polymorphism in exon III of the CSN1S1 gene could be used as a potential genetic marker for milk composition traits in Sofia sheep population, as well as in the other local sheep breeds in Bulgaria.

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. Moreover, the data obtained in the present study can contribute to developing an effective conservation strategy for traditional sheep breeds in Bulgaria.

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