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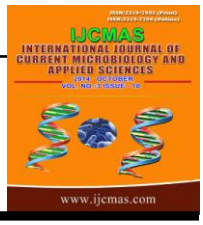

Original Research Article

Microsatellites based genetic diversity and population structure of seven Bulgarian indigenous sheep breeds

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A B S T R A C T

The diversity, population structure and genetic relationships of 7 Bulgarian indigenous sheep breeds were investigated using 6 polymorphic microsatellite markers. A total of 96 alleles were identified across the populations of 338 individuals. The mean number of alleles per locus varied from 6.43 (ILSTS11) to 19.00 (MAF70).All examined populations indicated a high level of genetic diversity with an average of 0.78. The exact P-values for the single breeds were obtained and only one breed (Breznishka) was out of HWE equilibrium. Estimates for inbreeding coefficient (Fis) were significant for all breeds studied, except for Starozagorska (0.10). The genetic differentiation between the examined populations was not significant and genetic distances were relatively low. The greatest distance (0.359) was found between the populations Local Karnobatska and Starozagorska, while the closest relationship (0.106) between Copper-Red Shumenska and Karakachanska. The unrooted neighbour-joining dendrogram obtained from the Reynold'sgenetic distances, and factorial correspondence analysis revealed a separation between Starozagorska and and the other sheep breeds. The data obtained in the present study can contribute towards development of effective conservation strategies for these traditional sheep breeds in Bulgaria.

Keywords

Indigenoussh eepbreeds (Ovisaries), microsatellite markers, diversity, genetic structure

Introduction

Recent studies in livestock genetic resources in Europe unambiguously emphasize the importance of local breeds for maintaining genetic diversity in order to respect different production, cultural and region needs. Local livestock genetic resources are also sources (origins) of genetic variation for providing genetic improvement, preventing diseases and loss of genetic diversity. Diversity of indigenous breeds contributes significantly to European food quality and variety. In this line conservation and sustainable utilization of farm animal genetic resources are of vital importance. Some issues with conservation and preservation of local breeds in Europe are also among the problems needed to be solved (Simianer *et al.,* 2003).

Genomic studies based on different molecular techniques became an obligatory tool for describing the diversity in livestock species. Microsatellites markers (simple sequence repeats-SSRs) have been increasingly used as the marker of choice because of their locus specificity, extensive genome coverage, and high degree of polymorphism, co-dominant inheritance and easy, automated scoring of genotypes. Microsatellites are widely used to characterize the genetic variability within and between populations and at present they allow the standard method to estimate genetic diversity (Chen *et al.,* 2009) and to assign individuals to a breed (Ozerov *et al.,* 2008; Glowatzki-Mullis *et al.,* 2009).

In recent years studies on sheep genetic diversity, population structure, genetic differentiation and phylogenetic reconstruction aiming at identifying endangered populations and developing genetic conservation strategies have been performed in several European countries such as Spain (Alvarez *et al.,* 2004; Rendo *et al.,* 2004; Legaz *et al.,* 2008), Greece (Altarayrah *et al.,* 2007; Ligda *et al.,* 2009), Italy (Bozzi *et al.,* 2009, Tolone *et al.,* 2012), Serbia (Cinkulov *et al.,* 2008b), Switzerland (Stahlberger-Saitbekova *et al.,* 2001), Austria (Baumung *et al.,* 2006), Albania and Kosovo (Hoda *et al.,* 2009).

In Bulgaria, sheep breeding and the use of local sheep genetic resources have a longtime tradition. Despite its relatively small territory, the country has a rich diversity of autochthonous domestic livestock breeds, of which nineteen are local sheep breeds (Dimitrov at al. 1993; EASRAB, 2011). The objective of this study is to assess the genetic diversity and to determine the genetic relationship within and between 7 indigenous Bulgarian sheep breeds using SSR markers. This will give an opportunity to define the genetic structure of the studied sheep breeds in order to facilitate and plan their sustainable development, utilization and conservation.

Materials and Methods

Sampling and DNA isolation

A total of 338 individuals were sampled (unrelated males and females), representing seven local Bulgarian sheep breeds: Breznishka (BRSK), Sofiiska (Elin-Pelinska, SEPL), Copper-Red (Mednochervena) Shumenska (CRSH), Karakachanska (KKCH), Local Karnobatska (LKNB), Blackhead (Chernoglava) Plevenska (BHPL) and Starozagorska (LSTZ). The abbreviations of the sheep breeds, their type, current size, geographical location, the number of sampled flocks per breed and the number of individuals per flock are given in Table 1. Blood samples were collected from jugular vein into vacutainer tubes containing EDTA. DNA was extracted from the whole blood with Illustra Blood GenomicPrep DNA Purification Kit (GE Healthcare, UK) according to the manufacturer's instructions.

Microsatellite markers

A set of six microsatellite markers (Table 2) was chosen based on their level of polymorphism, location on different chromosomes, preferably unlinked following the recommendation of the Food and Agriculture Organization (FAO) and the International Society for Animal Genetics (ISAG).

Polymerase chain reaction and fragment analyses

Primer sequences, size ranges and PCR protocols for the selected markers were

obtained from http://dad.fao.org/en/ Home.htm. PCR amplification was carried out in total 10µl volume, containing 50ng DNA template, 20pM primers and 1x AmpliTaq Gold PCR Master mix (Applied Biosystems, USA). Forward primers were Cy5 fluorescently labelled. The amplification was performed in a thermocycler GeneAmp 9700 (Applied Biosystems) using the conditions given in Table 2. PCR products were separated together with internal size standards on 6% denaturing polyacrylamide gel (ReproGel High Resolution) using automated laser sequencer (ALF Express II Amersham Biosciences).

Statistical analysis

The allele diversity, number of alleles per locus and their richness, observed and expected heterozygosity values (H_o and H_e) were calculated using POPGENE software, version 1.31, (Yeh and Yong, 1999; Labate, 2000). This program was used also to estimate 3 fixation indices: inbreeding coefficient within each population (F_{is}), coefficient of gene differentiation between populations (F_{st}) and inbreeding coefficient for all populations (F_{it}) (Wright, 1978). Polymorphism Information Content (PIC) which reflects the usefulness of the selected marker loci was calculated with Cervus ver. 3 (Kalinowski *et al.,* 2007).

The ARLEQUIN software, version 3.5.1.3 was used for population data analysis (Excoffier and Lischer, 2010). The number of alleles of each locus, heterozygosities values, F_{st} between all pairs of the tested breeds and gene diversity were calculated for each breed. The same software was used to check deviation from Hardy-Weinberg equilibrium (HWE) by the method of Guo and Thommson (1992). Reynold's distance (Reynolds *et al.,* 1983), recommended for populationswith short divergence time (Eding and Laval, 1999),were used to estimate pair-wise genetic relationships amongbreeds. The neighbor-joining tree was constructed using the estimated Reynold's genetic distances with the Neighbor procedure of PHYLIP ver. 3.69 (Felsenstein 2009). MEGA5 software (Tamura *et al.* 2011) was used for depicting of the dendrogram given on Figure 1.

The structure of the entire populations was examined with the software package STRUCTURE (Pritchard *et al.,* 2000) using the prior population information model.

Results and Discussion

Allele and gene diversity

In total of 96 alleles with an average 16 alleles per locus, ranging from 10 (ILSTS11) to 31 (MAF70) were observed for the 6 microsatellite loci surveyed (Table 3). The mean number of alleles (Mn) per locus varied between 6.43 (ILSTS11) and 19.00 (MAF70). The PIC considering all loci wasequal with an average of0.81, showing that the microsatellites panelused was highly informative. ILSTS11 was found to bethe least informative marker (0.75), whereas MAF70the most informative one (0.92).

The average observed (H_o) and expected (H_e) heterozigosities for the six microsatellite markers are given in Table 3. Considering the whole sample (338 individuals) H_o ranged from 0.38 to 0.83 with a mean 0.62 while H_e from 0.78 to 0.93 with a mean 0.83. Marker ILSTS11 showed lowest H_e (0.78) whereas MAF70 the highest one (0.93). H_o was always higher than 0.5 except for the marker MAF65 (0.38). At each locus the expected heterozygosity (H_e) was higher than the observed heterozigosity (H_o).

Genetic structure of the populations was analyzed by Wright's F statistics. Mean estimated values for F_{is}, F_{st} and F_{it} were 0.22, 0.06 and 0.26, respectively. The F_{is} index among the loci varied from 0.08 (MAF70) to 0.50 (MAF65). The mean F_{is} value was higher than F_{st} (0.22>0.06) and heterozigosity deficiency at all tested loci was detected (Table 3).

Population structure

The number of alleles per locus observed in each breed ranged from 6 to 21 (Table 4). Based on the mean number of alleles per population, the most diverse population was SEPL (11.5) and the least diverse was LKNB (9.17). Two populations (KKCH and LSTZ) have equal mean number of alleles (9.50).

In all examined populations the H_o levels were lower than the expected heterozigosities (H_e). The mean H_o and H_e per studied populations were between 0.55-0.69 (KKCH-LSTZ) and 0.76-0.82 (LSTZ-SEPL), respectively. Generally, a heterozygote deficit could be detected in all examined loci and across the populations it was the highest in SEPL and the lowest in LSTZ.

The coefficient of inbreeding (F_{is}) was positive in all populations, ranging from 0.10 (LSTZ) to 0.30 (SEPL) with a mean 0.23, indicating a risk of inbreeding. In order to test possible deviation from HWE, exact P-values for the single breeds were obtained (Table 4). The investigated breeds were in equilibrium, except BRSK.

Figure 2 shows the results of population structure evaluation using STRUCTURE with the prior population information model. With the gradual increase in the number of expected population groups, K, the different breeds separated from one another until the actual number of groups (7) was reached. Further increase in K did not change the results of the analysis.

Genetic relationships between populations

Pair-wisegenetic distances amongthe examined populations were determined using the Reynolds genetic distance estimates (Table 5). The lowest valueswere observed between KKCH and BHPL (0.100) and also between CRHS and KKCH (0.106), whereas thehighest one between LKNB and LSTZ breeds (0.359). The LSTZ breed showed the highest genetic distance in relation tothe other 6 breeds followed by LKNB breed. The Reynold's genetic distances were used to reconstruct the radial neighbor-joiningdendrogram (Figure 1), showing 2 clear clusters: one including KKCH, CRSH BHPL, SEPL and the other including BRSKand the LKNB, which emphasized that the LSTZ breed appears to be more distant from the other breeds.

In present study the allele diversity and genetic relationship of 7 indigenous sheep breeds in Bulgaria were examined using a set of 6 polymorphic microsatellites. The relatively high mean number of alleles per locus (10.07) and the high overall PIC value indicate the usefulness of the selected markers for studying the genetic diversity in Bulgarian sheep populations. The comparison of the N_e with the number of observed alleles per locus provides evidence for the predominance of certain alleles in each breed. In this sense, MAF70 could be considered as the most informative marker of our test panel. This observation is in accordance with other studies (Arranz *et al.*, 2001; Kusza *et al.*, 2008, 2010) and confirms the appropriateness of this marker in sheep genetic diversity.

The mean F_{is}, F_{st} and F_{it} (0.22, 0.06 and 0.26, respectively) indicated 26% heterozygote's deficit across populations and around 22% inbreeding within populations. The multilocus F_{st} showed that only 6% of the total genetic variation in Bulgarian sheep breeds is due to the population differences, while the remaining 94.4% corresponds to differences among individuals. The low value of F_{st} is an indication that the studied breeds are not differentiated enough. The lack of clear differentiation between Bulgarian sheep breeds could be due to geographic proximity, similarity in environment and breeding practices but most likely to the past and present gene flow among them. The estimates for genetic differentiation were similar to those reported in other genetic diversity studies, e.g. 4.9% for Sicilian sheep breeds (Tolone *et al.,* 2012), 5.7% for Alpine and European and Middle-Eastern breeds (Peter *et al.,* 2007; Dalvit *et al.,* 2008), 5.2% for West Balkan Pramenka sheep types (Cinkulov *et al.,* 2008a), but lower than in Slovak Tsigai sheep (13.3%, Kusza *et al.,* 2008), Bardhoka breed in Albania and Kosova (24%, Hoda *et al.,* 2009).

The overall F_{is} was higher than that of F_{st} (0.22 versus 0.06). The F_{is} values were positive in all examined populations, indicating medium to high rate of inbreeding, as for BHPL and LSTZ they were the least ones (0.17 and 0.10, respectively).Actually, the mostprobable reason of the high level of inbreeding as alreadystated by other authors (Dalvit *et al.,* 2008, Tolone *et al.,* 2012) is the management of the flocks and in particular the lack of definite breeding scheme to control inbreeding. In most cases the studied existing breeds originated from one common flock as a gene pool in the past generations from where later rams and ewes were distributed as founders of another flocks of the breed. Moreover,the reduced or absent exchange of rams betweendifferent flocks of the same breed may have also an impact. Estimatesforinbreedingcoefficientweresignificantalsoforthestudied Greek (Ligda *et al.,* 2009), and Tsigai and Zakel type of sheep breeds from the Central-Eastern- and Southern-European regions (Kusza *et al.,* 2008) but lower in Sicilian sheep breeds (Tolone *et al.,* 2012). As most of the breeds considered in this study have never been genetically characterized before it was not always possible to compare our data with that reported in the literature. Previous study (Kusza *et al.,* 2010) on the genetic relationship among five Bulgarian sheep breeds using 16 SSR markers also showed positive F_{is} values for the three indigenous Bulgarian sheep breeds - Patch-Faced Maritza (0.246), White Maritza (0.275) and BHPL (0.376) - which is the only common breed included also in our study. The lower inbreeding coefficient (0.17), the higher mean number of alleles (10.33) and genetic diversity (0.80) observed for BHPL population here could be explained with the sampling scheme and/or the panel of the polymorphic markers used of which five were common.

Regardless of significant heterozygote deficit the mean levels of H_o were relatively high in the investigated here sheep breeds (0.55-0.69), compared to these reported by Kusza *et al.* (2010) (0.458-0.577). Generally, all examined populations showed high level of genetic diversity with an average 0.79 which is close to that published by Oliveira *et al.* (2005) for Bordaleira de Entre Douro e Minho sheep (0.74), Alvarez *et al.* (2004) for Latxa sheep (0.77) and Kusza *et al.* (2010) for five Bulgarian sheep breeds (0.736). A significant deviation from HWE in two of the examined breeds BRSK and BHPL was observed, even though a

deficiency of heterozygocity was indicated in all studied sheep breeds. It could be explained with natural processes of mutation, migration, non-random mating, genetic drift and both artificial and natural selection (Diez-Tascon *et al.,* 2000).

In this study the genetic distances among the examined populations were relatively low, but higher than 0.05, which may indicate certain differences in their genetic structure. The closest genetic relatedness was found between CRSH and KKCH which is in accordance with their similar phenotypic traits. Both breeds are short thin-tailed type with black-brown color of the wool, predominantly coarse.

Similarly, KKCH and BHPL breeds are also genetically related. The geneticcloseness betweenboth breeds might be explainedconsidering that these breeds have partially overlapping geographical breeding area,which might have led to genetic exchange between them.In this study thegenetic differentiation of BHPL (Tsigai x Zackel) was not so evident as in the study of Kusza *et al.* (2010) who found one private allele at locus OarFCB20 even though this locus was also included in our test. This means that the number of defined population specific alleles depends not only on the markers used but also on the number of populations under the study and their genetic relatedness and even more specifically on the allele configuration at particular loci in each breed.

Among the examined sheep breeds, LSTZ is genetically unique and the most distinct from LKNB (0.626). The two breeds originated from different regions lying remote each other about 100 km and are phenotypically distinct. The animals of LKNB breed (Zakel type), historically originating from Karnobat region are relatively small (female body weight is 40kg) with short thin tail and red-brown colour of the wool. It is a dual-purpose breed with equal emphasis on meat and wool. LSTZ is a Tsigai type among the indigenous sheep breeds in lowlands and the regions of distribution are restricted around Stara Zagora. In contrast to LKNB, the female animals of LSTZ have a large body (up to 70-80 kg), long thin tail and white color of wool. The lower mean number of alleles and genetic diversity observed in the LSTZand LKNB breedsin comparison to other breeds isprobably due to a reduced effective population size.

The population structure was assessed by varying K from 1 to 14. The entire dataset showed that the samples had the highest probability to form 7 clusters. Based on the assignment of individuals without previous population information at K=2 the software program STRUCTURE (Pritchard *et al.,* 2000) placed them in 3 clusters one of which includes BRSK, the second one – SEPL, and the third cluster – all remaining breeds. Assuming K=2 the BRSK and the SEPL formed two distinct groups suggesting that admixture was nearly zero for these 2 breeds.

At K=4 CRSH separates from other breeds, while at K=5 KKCH and LKNB breeds appear isolated. At K=6 further splitting is occurred which leads to the separation of the BHPL breed. At K=7 LSTZ appears separated and each breed tends to has its own distinct cluster. K=7 was chosen as the best value to describe the genetic structure of the studied here breeds, since the increase of K from 7 to 14 was unchangeable in comparison to K1÷7. The high average percentage of assignment of individuals for BRSK and SEPL pointed out the existence of clear genetic differences compared to other breeds.

Table.1 Sheep breeds investigated in the study

Breed name	Code (Abbr.)	Examined number	Flocks (n)	Geographical location	Breed type, Tail type	Trends	Current population size*	Status (Degree of endangerment)
Breznishka	BRSK	50	1(30) 2(20)	Western Bulgaria, in the region of Pernik, Radomir, Breznik	Traditionally selection, Long thin-tailed	Milk, wool, meat	836 ♀-812 ♂-24	Threatened
Sofiiska (Elin-Pelinska)	SEPL	58	1(33) 2(22) 3(13)	Western Bulgaria, in the region of Sofia - Elin Pelin	Traditionally selection, Long thin-tailed	Milk, wool, meat and hide	1438 ♀-1380 ♂-58	Not at risk
Copper-Red (Mednochervena) Shumenska	CRSH	37	1	North-East part of Bulgaria	Traditionally selection, Short thin-tailed	Milk, meat, wool	4280 ♀-4138 ♂-142	Not at risk
Karakachanska	KKCH	38	1(31) 2(7)	In mountainous areas of the country	Zackel Short thin-tailed	Milk, meat, wool	3632 ♀-3529 ♂-103	Not at risk
Local Karnobatska	LKNB	48	1	South-Eastern Bulgaria in lowlands in the regions Burgas	Zackel Short thin-tailed	Meat, milk, wool and hide	265 ♀-255 ♂-10	Threatened
Blackhead (Chernoglava) Plevenska	BHPL	59	1(48) 2(11)	In the plains and foothill areas of the country	Tsigai × Zackel Long thin-tailed	Milk, meat and wool	13100 ♀-12836 ♂-264	Not at risk
Starozagorska	LSTZ	48	1	South part of the country, in Trakia lowland	Tsigai Long thin-tailed	Milk, meat and wool	694 ♀-680 ♂-14	Threatened

* Data are obtained from EASRAB , since November, 2011
(n) – Number of individuals in each flock

Table.2 SSR locus information

Marker name	Origin	Chromosome localization	NCBI accession number	Primer sequences F/R	T° annealing	Mg (mM)
OarFCB20	Ovine	2	L20004	F: AAATGTGTTTAAGATTCCATACAGTG R: GGAAAACCCCCATATATACCTATAC	55º	2,5mM
MAF70	Ovine	4	M77200	F: GCAGGACTCTACGGGCCTTTGC R:CACGGAGTCACAAAGAGTCAGACC	60°	1mM
ILSTS11	Bovine	9	L23485	F: GCTTGCTACATGGAAAGTGC R: CTAAAATGCAGAGCCCTACC	58°/55°	2mM
MAF65	Ovine	15	M67437	F: AAAGGCCAGAGTATGCAATTAGGAG R: CCACTCCTCCTGAGAATATAACATG	55°	2,5mM
OarCP20	Ovine	21	U15695	F: GATCCCCTGGAGGAGGAAACGG R: GGCATTTCATGGCTTTAGCAGG	62°/60°	2,5mM
OarJMP58	Ovine	26	U35058	F: GAAGTCATTGAGGGGTCGCTAACC R: CTTCATGTTCACAGGACTTTCTCTG	55º	1mM

Table.3 Allele range, number of identified alleles (Na), mean number of alleles per locus (M_n), heterozygosities: observed (H_o), expected (H_e), Polymorphism Information Content (PIC) and F-values in each locus

Locus	Allele range (bp)	Na	Mn	Ho	He	PIC	F_{is}	F_{st}	F_{it}
OarFCB20	88-114	13	9.71	0.59	0.84	0.82	0.25	0.05	0.29
MAF70	121-185	31	19.00	0.83	0.93	0.92	0.08	0.03	0.11
ILSTS11	268-286	10	6.43	0.65	0.78	0.75	0.12	0.05	0.17
MAF65	122-140	11	7.00	0.38	0.80	0.77	0.50	0.06	0.53
OarCP20	67-91	11	7.57	0.55	0.79	0.76	0.26	0.07	0.31
OarJMP58	133-173	20	10.71	0.70	0.86	0.85	0.12	0.07	0.19
Mean		**16**	**10.07**	**0.62**	**0.83**	**0.81**	**0.22**	**0.06**	**0.26**
Total		**96**							

Table.4 Number of alleles per locus, mean observed (Ho) and expected (He) heterozygosities, coefficient of inbreeding (Fis) and chi-square test for HWE in the examined populations

Population	Number of alleles/locus							Mean heterozygosity		Fis	χ^2 (df)*	P
	OarFCB20	MAF70	ILSTS11	MAF65	OarCP20	OarJMP58	Mean	Ho	He			
BRSK	9	18	6	7	8	12	10.00	0.60	0.81	0.26	27.87	0.02
SEPL	13	19	8	8	8	13	11.50	0.57	0.82	0.30	17.18	0.31
CRSH	8	18	7	8	10	12	10.50	0.63	0.82	0.24	11.80	0.70
KKCH	9	21	7	6	5	9	9.50	0.55	0.78	0.30	9.02	0.88
LKNB	9	19	6	6	7	8	9.17	0.61	0.77	0.21	9.48	0.85
BHPL	11	19	6	7	7	12	10.33	0.67	0.80	0.17	24.26	0.06
LSTZ	9	19	5	7	8	9	9.50	0.69	0.76	0.10	19.75	0.18
Mean								**0.62**	**0.78**	**0.23**		

*df - degree of freedom- all loci have one df-15

**P- value (degree of probability)

Table.5 Reynolds genetic distances between the examined breeds

Breed	BRSK	SEPL	CRSH	KKCH	LKNB	BHPL	LSTZ
BRSK	0.000	0.122	0.122	0.132	0.156	0.136	0.221
SEPL		0.000	0.139	0.135	0.219	0.107	0.244
CRSH			0.000	0.106	0.229	0.155	0.272
KKCH				0.000	0.255	**0.100**	0.217
LKNB					0.000	0.220	**0.359**
BHPL						0.000	0.236
LSTZ							0.000

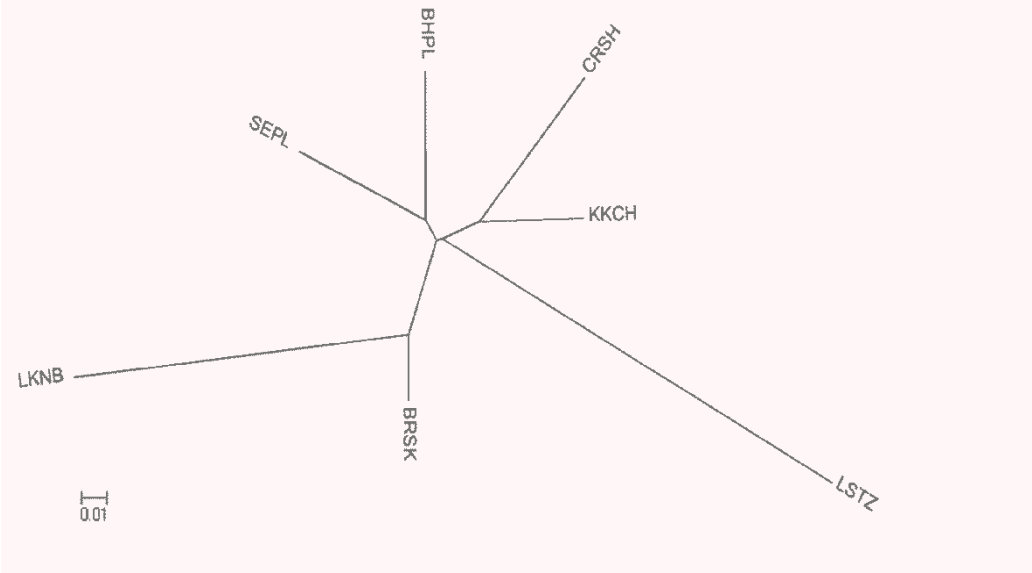

Figure.1 Radial neighbor-joining dendrogram generated from Reynolds genetic distances of the examined breeds

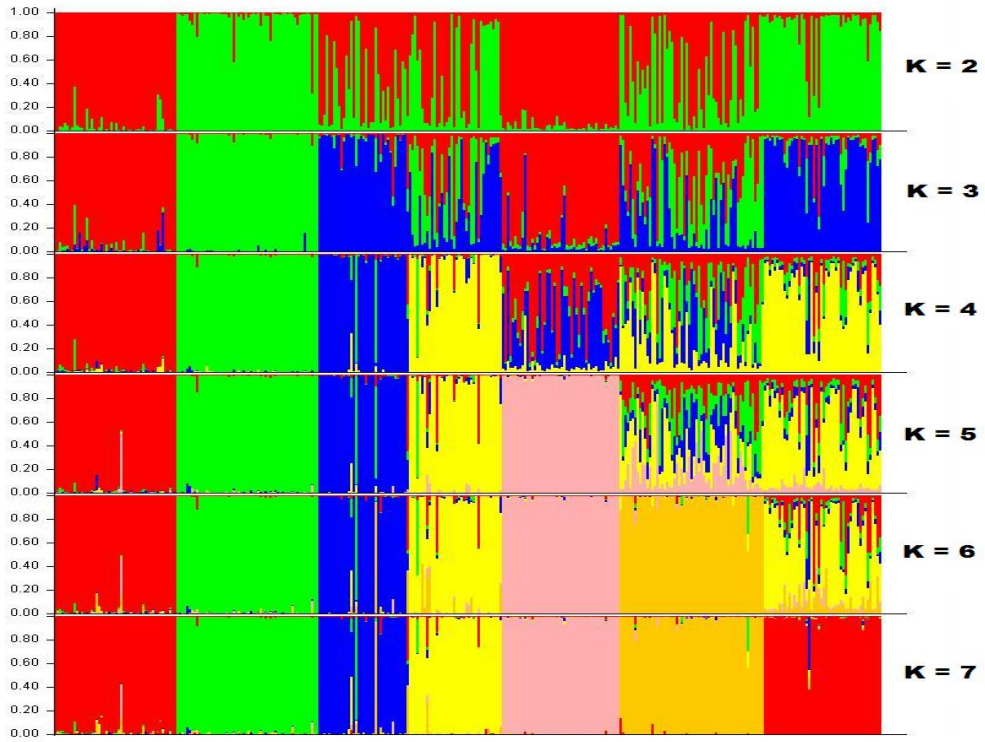

Figure.2 Analysis of the structure of the examined sheep breeds. In order from left to right: Breznishka, Sofiiska, Copper-Red Shumenska, Karakachanska, Local Karnobatska, Blackhead Plevenska, and Starozagorska. The parameter K specifies the assumed number of groups

This is also confirmed by the geographic distribution of both breeds in the Western regions of Bulgaria and different breeding system (mainly for milk production). The remaining populations exhibited the presence of admixture, in fact the CNSH, KKCH and LKNB clustered together up to K=4 and K=5. The highest level of admixture was observed for BHPL and LSTZ which appear separated at K=6 and 7. This result could be due to their phylogenetic relationship and/or migration of individuals among the several farms with location in the North-Eastern and South-Eastern Bulgaria as well as breeding towards the improvement of milk productivity traits.

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