1

Genetic Characterization of Albanian Sheep Breeds by Microsatellite Markers

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1. Introduction

Albania is a Mediterranean country, located in West of Balkan Peninsula. Albanian farmers have a long tradition in sheep breeding. Sheep comprise one of the most important domestic livestock species in Albania and play an important role in the livelihood of local community since they are a good source of meat, milk and coarse wool (Dobi et al., 2006; Porcu and Markovic, 2005). There are three important local sheep breed in Albania: Bardhoka, Ruda and Shkodrane, which are also the object of this study. The genetic characterization of a breed is very important for the evaluation of genetic variability, which is an important element in conservation of genetic resources and for breeding strategies. Genetic characterization can be done by different classes of molecular markers, such as Restriction Fragment Length Polymorphisms (RFLP) (Abdel-rahman et al., 2010), Single Stranded Conformation Polymorphisms (SSCP) (Bastos et al., 2001), Random Amplified Polymorphic DNA (RAPD) markers (Jawasreh et al., 2011; Kantanen et al., 1995; Paiva et al., 2005; Qasim et al., 2011), Amplified Fragment Length Polymorphisms (AFLP) (Xiao et al., 2009), Single Nucleotide Polymorphisms (SNP) (Pariset et al., 2006a,b), and microsatellites.

Microsatellites are short tandem nucleotide repeats that are randomly distributed throughout eukaryotic genomes. The repeat units can range from two to six base pairs motifs (Tautz and Schlotterer, 1994). They are called also as, simple sequence repeats (SSR) (Tautz, 1989), short tandem repeats (STRs), (Edwards et al., 1991) or variable number tandem repeats (VNTR). Alleles at a specific locus can differ in the number of repeats. They are generally found in nuclear genome, usually in non-coding parts of genome. Microsatellites are "junk" DNA, and are selectively neutral (Li et al., 2002).

Microsatellite loci are often hypervariable with high mutation rates and therefore are highly polymorphic in most mammalian species (Weber, 1990, Jeffreys et al., 1994). Mechanisms of mutation are believed to be unequal crossover during recombination (Smith, 1976), polymerase slippage and especially slipped-strand mispairing during replication (Levinson and Gutman, 1987) resulting in the addition or loss of one or a small number of repeats. They are inherited co dominantly in Mendelian fashion and are relatively easy to score directly.

There is several mutation models considered for microsatellites. The infinite allele model (IAM), (Kimura and Crow, 1964) assumes that all new alleles are unique. Stepwise mutation model (SMM), (Kimura and Ohta, 1978), involve addition or deletion of one repeat. Mutations are also described as or as a combination of single and multiple steps by the two-phase mutation model (TPM), (Di Rienzo et al., 1994).

Microsatellite markers are currently the markers of choice for molecular genetic studies such as reconstruction of phylogenetic and relationships among populations (Bowcock et al., 1994; Forbes et al., 1995; MacHugh et al., 1997), determination of paternity and kinship analyses (Glowatzki-Mullis et al., 1995; Heyen et al., 1997; Luikart et al., 1999; Schlotterer, 2004) forensic studies (Edwards et al., 1992), linkage analysis (Francisco et al., 1996; Kappes et al., 1997; Mellersh et al., 1997), population structures (Arora and Bhatia, 2004; Bruford and Wayne, 1993).

Microsatellites are used, in livestock species for estimating genetic variation within and among breeds (Buchanan et al., 1994; Cronin et al., 2009; Diez-Tascon et al., 2000a; Dowling et al., 2008; Schmid et al., 1999; Saitbekova et al., 1999), for admixture studies (Alvarez et al., 2004; Freeman et al., 2004, 2006; MacHugh et al., 1997; Vicente et al., 2008) and for assigning individuals to breeds (Baumung et al., 2006; Cornuet et al., 1999; Maudet et al., 2002; Meadows et al., 2006; Troy et al., 2001).

Microsatellites as DNA markers are advantageous over many other markers as they are highly polymorphic, highly abundant, co-dominant inheritance, simply to analyze and easy to score, but nevertheless this type of marker has disadvantages such as null alleles, or size homoplasy (Schlotterer, 2004).

Microsatellites are the commonest markers used for genetic characterization of sheep breeds. Diez-Tascon et al., (2000b) studied genetic variability among six Merino populations using 20 microsatellites. Stahlberger-Saitbekova et al., (2001) analyzed genetic diversity between seven breeds from Swiss Alps and Mufflon, using ovine, caprine and bovine microsatellite markers. Arranz et al., (2001) have analyzed genetic variability of five Spanish sheep breed and Awassi by 18 microsatellites markers. Grigaliunaite et al., (2003) studied variability, paternity and possible bottleneck in 7 Baltic sheep breeds, using 15 microsatellite markers. Pariset et al., (2003) have used 11 microsatellites for genetic variation and inbreeding analysis in 17 flocks from Sarda breed in Viterbo province. Rendo et al. (2004) analyzed genetic variability of six autochthonous Nordeuropean sheep breeds based on 11 microsatellite markers. Alvarez et al., (2004) have used 14 microsatellite markers to analyze the relationship between Nordeuropean sheep breeds. Tapio et al., (2005) have used 25 microsatellites in 20 native and 12 imported North European sheep breeds in order to evaluate the importance of each breed for gene diversity. Baumung et al., (2006) have used 25 microsatellite markers for genetic characterization and breed assignment in 11 Austrian sheep breeds. Peter et al., (2007) have examined in a comprehensive study the genetic diversity of 57 European and Middle Eastern sheep breeds, using 31 microsatellites markers. Part of this study has been also the three Albanian sheep breeds considered here. Cinkulov et al., (2008) have used 15 microsatellite markers and mtDNA to estimate genetic variation in seven Pramenka types from West Balkan. Ligda et al., (2009) have used 28 microsatellite markers to analyze genetic diversity and differentiation in 8 Greek sheep breeds. Dalvit et al., (2008) have used 19 microsatellite markers for genetic characterization of 8 sheep breeds from Italy, Germany and Slovenia. Dalvit et al., (2009) have used 19 microsatellite markers

for genetic variation and presence of breed substructure of four native sheep breeds from North Italy. Arora and Bhatia, (2004) have used 13 microsatellites to asses genetic effects of the population declines in Muzzafarnagri Sheep from India. Tapio et al., (2010) have used 20 microsatellite markers for genetic diversity and population structure of 52 sheep breed from three geographical regions Caucasus, Asia, and the eastern fringe of Europe.

The Food and Agriculture Organization (FAO) has proposed an integrated programme for the global management of genetic resources, Measurement of Domestic Animal Diversity (MoDAD) program, using panels of microsatellites for characterizing farm AnGR.

The genetic characterization of local sheep in Albania, for a long time has been very limited based mainly on blood or milk protein polymorphism and visible phenotypic profile (Zoraqi, 1991). Recently, in the frame of Econogene project (www.econogene.eu) these breeds are characterized at molecular level using several set of markers like microsatellite (Hoda et al., 2009; Peter et al., 2007), AFLP (Hoda et al., 2010), SNP (Hoda et al., 2011). The study was undertaken to characterize the genetic diversity, to evaluate the genetic relationship and structure of these local sheep breeds, using 31 microsatellite markers recommended by MoDAD/FAO.

2. Materials and methods

2.1 Sample collection and microsatellites

A total of 93 individuals representing 3 different Albanian sheep breeds were analyzed. The breeds were Bardhoka, Ruda and Shkodrane. For each breed, 31 unrelated individuals were selected. Sampling was carried out in mountainous area, where still have pure breed individuals, from ten to eleven flocks.

Bardhoka breed (Figure 1) is classified under the long tail group. Its origin is North/Northeast of Albania and Western part of Kosova as well. This is a sheep with triple productive profile, milk, lamb and wool. It has a good developed body and a strong skeleton. The head has strong mandibles, wide face and big ears. The legs are strong and with thick bones. Bardhoka has a totally white fleece/cover. A well developed udder is characteristic of the breed. It has good volume and well-developed teats, very appropriate for milking. Usually, ewes are polled while the rams are horned.

Fig. 1. Bardhoka sheep breed

Ruda (Figure 2) is triple purpose breed with half-fine wool belongs to the long tail group. This breed is part of Tsigaya family regarding to the wool quality and other zootechnic traits. It is originated and widespread in North-Eastern part of Albania. This breed is adapted to pastures in high altitude and for long distance transhumant. Animals have a well-developed body with long legs that is a characteristic for this breed. The animals are generally white but sometimes black ones can show up. Ewes are polled and rams have big horns. Animals are covered by a non-dense fleece; while their neck and abdomen is uncovered.

Fig. 2. Ruda sheep breed

Shkodrane sheep breed (Figure 3) belongs to the long tail group of a triple purpose use. Its origin is Northern Albania. The tendency is the reduction of population. Most of the crosses are made with Bardhoka breed aiming to increase the milk production and body weight features. Shkodrane is a small sheep, well adapted to poor and stony pastures of North Albania. It has low requirements for the feed and it is resistant towards cold and dry climate. The very long and coarse wool is typical for this breed. Characteristic of its exterior is the light brown pigmentation of the skin at legs and face. Ewes are polled while the rams are horned. This breed is estimated as "potentially endangered", and some efforts to establish conservation programs are in process.

Fig. 3. Shkodrane sheep breed

In Table 1 are shown some of phenotypic traits of Albanian sheep breeds (Dobi et al., 2006; Porcu and Markovic, 2006).

Breed	Color	Wool	Tail	Body weight (kg)		Withers Height (cm)		Milk Production (kg)	Use
				Male	Female	Male	Female		
Bardhoka	White,	Coarse	Long	60	40	70	60	150 - 200	Milk, wool, meat, fur
Ruda	White	Half-fine	Long	50	40	65	55	90	Milk, wool, meat, fur
Shkodrane	White, reddish face	Long, coarse	Long	42	36	55	52	130 - 150	Milk, meat, wool,

Table 1. Phenotypic traits of three Albanian sheep breeds.

2.2 DNA extraction and microsatellite analyses

Blood samples of 5 – 10 ml were collected in EDTA tubes and stored at –20^{0}C. DNA was isolated according to standard phenol-chloroform extraction method. All samples were genotyped for 31 microsatellite markers according to the methodology explained in detail, by (Peter et al., 2007).

2.3 Data analysis

Allele frequencies, observed heterozygosity (Ho), expected heterozygosity (He) were estimated for 31 microsatellite markers using Genalex 6 program (Peakall and Smouse, 2006). Polymorphic information content (PIC) was estimated for all markers in all breeds using the Cervus software (Marshall, 2001).

Tests of genotype frequencies for deviation from Hardy-Weinberg equilibrium (HWE) as well as for linkage disequilibrium were carried out using Markov Chain Monte Carlo simulation (100 batches, 5000 iterations and a dememorization number of 10 000) implemented in the Genepop V.1.2 program(Raymond and Rousset, 1995).

The program FSTAT, (Nei, 1987), and estimation of Wright's fixation index (Weir and Cockerham, 1984). A significance test on the estimates for each microsatellite locus were obtained by constructing 95% and 99% confidence intervals based on the standard deviations estimated by jackknifing across populations using FSTAT (Goudet, 2001). Estimates of genetic variability for each breed (He, Ho), mean number of alleles were computed using GENETIX program (Belkhir et al., 2001). Gene flow (Wright, 1931) was calculated using the same program (Belkhir et al., 2001).

Cavalli-Sforzas Chord Distance D_C (Cavalli-Sforza and Edwards, 1967), Reynolds' D_R distance (Reynolds et al., 1983), Nei's D_A distance (Nei et al., 1983) and Neis Standard Distance (Nei, 1972) among breeds were computed using Populations program (Langella, 2002). In order to test the presence of correlations between different distance matrices, a Mantel test, modified by (Manly, 2007) was carried out with Programm FSTAT (Goudet, 2001).

The genetic distance of Reynolds (D_R) among breeds was used for the construction of UPGMA consensus tree (Felsenstein, 1993). Bootstrap (1000 replicates) resampling was performed to test the robustness of the dendrogram topology.

Genetic distances among individuals were estimated as the proportion of shared alleles (D_{PS}) using Populations program (Langella, 2002). Individual distances were represented by a neighbor-joining tree and depicted using software package TreeView version 1.6.6 (Page, 1996).

The analysis of population's structure by a clustering analysis based in Bayesian model was carried out by the program STRUCTURE (Pritchard et al., 2000). The program uses Markov Chain Monte Carlo method based on the "admixture model", where allelic frequencies were correlated, with "burn in period" and "period of data collection" of 300000 iterations. The samples were analyzed with *K* from 1 to 4, applying 5 independent running. Evanno's method (Evanno et al., 2005) was used to identify the appropriate number of clusters using the *ad hoc* statistic Δk, which is based on the second order rate of change of the likelihood function with respect to successive values of K.

To test for evidence of a recent genetic bottleneck, the program BOTTLENECK (Piry et al., 1999) was used. The program tests for departure from mutation drift equilibrium based on heterozygosity excess or deficiency. It compares heterozygosity expected (*H*e) at Hardy–Weinberg equilibrium to the heterozygosity expected (*H*eq) at mutation drift equilibrium in the same sample, that has the same size and the same number of alleles. Wilcoxon signed rank test was used to test for heterozyosity excess under all three mutation models, infinite alleles (IAM), two-phase (TPM), and the step-wise mutation model (SMM).The method of graphical representation of mode-shift indicator, was also used for assessing distortion in allele frequency, indicative of possible bottleneck.

Nei genetic distance calculated from the allele data was plotted as PCA using GenAlEx program (Peakall and Smouse, 2006).

The Factorial Correspondence Analysis (FCA) is performed to visualize the relationships between individuals from different breeds and to test possible admixtures between the populations. FCA was computed using GENETIX program (Belkhir et al., 2001).

Geneclass2, (Paetkau et al., 1995), assuming a default allelic frequency of 0.001 and a threshold of 0.05. The assignment of individuals to the reference population was carried out using Bayesian approach (Rannala and Mountain, 1997). The "leave one out" procedure assignment was applied for the individuals. The confidence level was 99%.

The hierarchical analysis was carried out using analysis of molecular variance (AMOVA) implemented in the ARLEQUIN Ver. 3.0 package (Excoffier et al., 2005). AMOVA yields estimations of population structure at different levels of the specified hierarchy.

3. Results

3.1 Microsatellite markers

All markers were highly polymorphic. In table 2 are displayed the variability parameters of the investigated loci. A total number of 348 alleles were observed at 31 microsatellite loci. Except of SRCRSP4, all the markers displayed 5 or more alleles. The total number of alleles

varied from 4 (SRCRSP5) to 20 (INRA63) with an overall mean of 11.33 alleles/locus. Mean number of alleles per locus ranged from 4 (SRCRSP5) to 13.33 (INRA63) having a pooled mean of 8.54. The effective number of alleles (N_E) ranged between 2.17 (SRCRSP9) to 7.7 (OARFCB20), with an overall mean of 4.57. PIC ranged from 0.495 (SRCRSP9) to 0.856 (OARFCB20). The within-breed deficit in heterozygosity, as evaluated by the F_{IS} parameter, ranged between −0.081 (SRCRSP9) to 0.458 (SRCRSP5) having a total mean of 0.041 for all loci. A very high contribute displayed the markers Oarae129 (0.368), Srcrsp5 (0.458) and Mcm527 (0.237) with ($p<0.001$) high significant F_{IS} values. F_{IT} values ranged from -0.044 (BM8125) to 0.5 (Srcrsp5). The global heterozygosity deficit (F_{IT}) was estimated 0.052 and global breed differentiation evaluated by F_{ST}, was estimated 0.011. The contribution of the

Locus	Allelic range (bp)	TNA	MNA	NE	PIC	AR	F_{IS}	F_{IT}	F_{ST}
BM1329	162-180	6	5.00	2.82	0.584	5.309	0.02	0.027	0.007
BM8125	112-126	7	6.33	3.37	0.657	6.148	-0.047	-0.044	0.003
DYMS1	163-199	14	10.67	7.32	0.827	11.515	-0.029	-0.007	0.022***
HUJ616	114-162	15	10.00	5.03	0.762	10.939	-0.025	-0.019	0.006***
OARAE129	136-162	7	5.33	3.12	0.606	5.023	0.368***	0.367***	-0.001
OARCP34	118-130	7	6.33	5.02	0.754	6.237	-0.017	-0.006	0.01*
OARCP38	120-136	8	5.67	2.26	0.518	5.667	-0.04	-0.043	-0.003
OARFCB128	98-128	9	7.33	4.63	0.740	7.192	0.083	0.085	0.003
OARHH47	121-155	13	9.67	5.43	0.761	9.93	0.163***	0.193***	0.036
OARVH72	125-141	9	8.33	7.12	0.829	8.481	0.047	0.05	0.003
MAF65	122-138	9	7.33	3.99	0.704	7.069	0.021	0.013	-0.008
INRA63	158-206	20	13.33	5.41	0.781	12.909	-0.025	-0.023	0.002
OARFCB20	92-118	13	11.00	7.70	0.834	10.854	0.056	0.071*	0.016*
OARJMP58	141-175	15	11.67	5.48	0.779	11.734	0.034	0.05	0.016**
OARJMP29	115-157	15	11.00	5.82	0.777	10.653	-0.082	-0.048	0.031***
OAFCB193	95-133	15	9.67	2.87	0.620	9.557	0.001	-0.001	-0.002
BM1824	169-175	5	5.00	3.82	0.684	4.978	0.013	0.016	0.002
MAF70	128-160	16	12.33	5.65	0.784	12.5	0.065	0.084*	0.021**
MAF209	110-140	12	9.67	5.11	0.762	10.422	-0.023	-0.005	0.018**
OAFCB304	149-189	16	10.67	4.05	0.701	11.06	0.089	0.107*	0.02***
SRCRSP9	108-128	10	6.33	2.17	0.490	6.023	-0.089	-0.096	-0.007
ILSTS5	186-211	10	7.00	2.86	0.590	7.573	-0.026	0.008	0.033**
ILSTS11	271-286	9	6.67	4.61	0.736	7.163	0.017	0.02	0.003
ILSTS28	127-169	13	10.00	6.33	0.815	9.777	-0.05	-0.052	-0.002
MAF214	180-265	15	10.67	4.19	0.712	10.613	-0.08	-0.054	0.024*
SRCRSP5	146-154	4	4.00	2.56	0.510	3.916	0.458***	0.5***	0.077***
SRCRSP1	126-140	8	6.67	2.86	0.583	6.328	0.116	0.121*	0.006
MAF33	116-143	12	9.33	4.27	0.723	9.263	0.048	0.043	-0.005
MCM140	161-192	13	10.33	5.97	0.804	10.439	0.111*	0.107*	-0.005
OAFCB226	118-156	14	10.67	5.28	0.778	10.73	-0.064	-0.058	0.006
MCM527	159-179	9	6.67	4.66	0.736	6.93	0.237***	0.243***	0.007
Mean/breed		11.23	8.54	4.57	0.72		0.041***	0.052***	0.011***

Table 2. Fragment sizes, total number of alleles per locus (TNA), Mean number of alleles (MNA), effective number of alleles (NE), polymorphic information content (PIC), Allelic richness (AR), Wright's F-statistics (FIT, FIS, FST) for each locus and all loci in three Albanian sheep breeds.

microsatellite markers for breed differentiation was estimated by the significance of the F_{ST} statistics. Only twelve markers had significant F_{ST} values and therefore contributed to breed differentiation. F_{ST} values ranged from -0.008 (MAF65) to 0.077 (SRCRSP5). The overall estimates for F-statistics were significantly ($p < 0.05$) different from zero.

In table 3, is shown Nei genetic diversity for 31 markers H_T. The values for observed heterozygosity ranged from 0.315 (SRCRSP5) to 0.891 (ILSTS28), with an overall mean value of H_O of 0.72, while the values of expected heterozygosity ranged from 0.543 (SRCRSP9) to 0.865 (OARFCB20). The mean value of Nei gene diversity, H_T was 0.75. The diversity within

Loci	H_O	H_S	H_T	D_{ST}	G_{ST}
BM1329	0.632	0.645	0.648	0.003	0.008
BM8125	0.739	0.705	0.707	0.001	0.003
DYMS1	0.88	0.856	0.868	0.012	0.021
HUJ616	0.822	0.802	0.805	0.003	0.006
OARAE1	0.432	0.686	0.685	-0.001	-0.001
OARCP3	0.812	0.8	0.805	0.006	0.01
OARCP3	0.583	0.563	0.562	-0.001	-0.003
OARFCB	0.705	0.774	0.775	0.001	0.002
OARHH4	0.67	0.801	0.821	0.02	0.037
OARVH7	0.821	0.862	0.864	0.002	0.003
MAF65	0.742	0.758	0.754	-0.004	-0.008
INRA63	0.839	0.818	0.819	0.001	0.002
OARFCB	0.75	0.775	0.794	0.019	0.036
OARJMP	0.785	0.813	0.822	0.009	0.016
OARJMP	0.882	0.815	0.832	0.018	0.031
OAFCB1	0.628	0.635	0.634	-0.001	-0.002
BM1824	0.731	0.741	0.742	0.001	0.002
MAF70	0.763	0.816	0.828	0.011	0.021
MAF209	0.817	0.799	0.808	0.01	0.018
OAFCB3	0.677	0.747	0.757	0.01	0.02
SRCRSP	0.591	0.543	0.541	-0.002	-0.007
ILSTS5	0.656	0.641	0.655	0.014	0.033
ILSTS1	0.774	0.786	0.787	0.002	0.003
ILSTS2	0.891	0.848	0.847	-0.001	-0.002
MAF214	0.814	0.753	0.765	0.013	0.025
SRCRSP	0.315	0.582	0.614	0.032	0.076
SRCRSP	0.576	0.652	0.654	0.002	0.005
MAF33	0.733	0.771	0.769	-0.003	-0.005
MCM140	0.747	0.84	0.838	-0.003	-0.005
OAFCB2	0.864	0.811	0.814	0.004	0.006
MCM527	0.599	0.786	0.79	0.004	0.007
Overall	0.718	0.749	0.755	0.006	0.012

Table 3. Nei's genetic diversity for each loci across all populations.

breeds (H_S) was 0.749 and diversity between breeds (D_{ST}) of 0,006. The diversity within breeds relative to the diversity of the whole population, G_{ST} value was 0.011. This value is similar with F_{ST} value.

Except of BM1824, all the markers displayed private alleles. A total of 89 private alleles were found, but only 10 private alleles had a frequency higher than 5% (Table 4).

Breed	Locus	Allele	Freq
Bardhoka	BM1329	180	0.050
Bardhoka	DYMS1	175	0.100
Bardhoka	HUJ616	156	0.083
Ruda	OARJMP58	165	0.065
Ruda	OAFCB304	179	0.081
Ruda	MAF33	124	0.065
Ruda	OAFCB226	152	0.081
Shkodrane	DYMS1	177	0.065
Shkodrane	HUJ616	154	0.067
Shkodrane	MAF209	136	0.065

Table 4. List of private alleles with frequency higher than 5%.

3.2 Genetic variation

The genetic variability for each breed was studied, regarding mean number of alleles and allelic richness, (Table 5). All breeds have similar mean number of alleles. The lowest value was displayed by Shkodrane breed. Overall estimate of allelic richness was 8.61. Within breed mean expected heterozygosity varied from 0.74 in Shkodrane to 0.77 in Ruda having an overall mean value of 0.75. Mean estimate of observed heterozygosity overall breed and loci was 0.72. Bardhoka and Shkodrane showed a significant deficit of heterozygotes ($p<0.05$), while Ruda showed a nonsignificant excess of heterozygotes. Mean value of inbreeding coefficient (F_{IS}) was 0.04. Deviations from Hardy-Weinberg equilibrium were significant for 6 out 93 loci breed combinations ($p<0.01$). The microsatellites SRCRSP5 and OARAE129 showed deviations in Bardhoka and Shkodrane. OARJMP29 was deviating only in Bardhoka and MCM27 was deviating only in Shkodrane. Ruda showed deviations in none of the markers.

3.3 Genetic differentiation

Polymorphism information content (PIC) in three Albanian sheep breeds ranged from 0.690 (Shkodrane) to 0.722 (Ruda). The breeds showed poor genetic differentiation, where F_{ST} index was equal to 0.011. Also, the average G_{ST} values over all loci was 0.011 indicating that a 1.1% of total genetic variation corresponded to differences among populations, whereas 99% was explained by difference among individuals.

Breed	MNA	H_O(SD)	H_E(SD)	AR	F_{IS}	PIC	NPA	HWE
Badhoka	8.58	0.71(0.015)	0.76(0.014)	8.35	0.065***	0.712	24	3
Ruda	8.84	0.75(0.014)	0.77(0.019)	8.54	0.022	0.722	38	1
Shkodrane	8.19	0.71(0.015)	0.74(0.018)	7.97	0.037*	0.690	27	3
Total	8.54	0.72(0.13)	0.75(0.09)	8.61	0.041	0.708		

Table 5. Mean number of alleles (MNA), mean observed (H_O) and expected (H_E) heterozygosity, allelic richness (AR), within-breed heterozygote deficiency (F_{IS}), number of private alleles (NPA), polymorphism information content (PIC) and number of loci not in the Hardy- Weinberg equilibrium at $P < 0.05$, for each breed across 31 loci.

The correlations between different distance matrices, D_R, D_A, D_C and D_S were tested by Mantel-Test modified by (Manly, 2010). High significant correlations were obtained between different matrices. The highest correlations were observed between Nei's D_A, Distance and Cavalli-Sforza, D_C, Distance (0,999912, $p < 0,01$) and the lowest between Reynolds, D_R, Distance and Cavalli-Sforza, D_C, Distance (0.673549, $p < 0,01$) (Table 6).

	D_A	D_S	D_R
D_C	0.999912**	0.796600**	0.673549**
D_A		0.804546**	0.683290**
D_S			0.983366**

Table 6. Corelations between distance matrix, D_C, D_A, D_S and D_R and their significance (** $p < 0,01$).

D_R genetic distance between each pair of populations are shown in Table 7. Distance matrix was used to build NJ phylogenetic tree. The smallest distance was between Bardhoka and Shkodrane. In figure 4 is presented the UPGMA tree based on Reynolds distance. Bootstrapping tested the robustness of the tree. Bootstrap values ranged from 41 to 100 indicating rather strong topology of the phylogenetic tree. Pairwise F_{ST} values between each pair of three sheep breeds were very low, reflecting a poor genetic differentiation breeds.

	Bardhoka	Ruda	Shkodrane
Bardhoka		0.012 (21)	0.010 (24)
Ruda	0.032 29		0.011 (23)
Shkodrane	0.031 28	0.029 28	

Table 7. Reynold's D_R genetic distance matrix (below diagonal), pairwise F_{ST} distance between breeds (above diagonal) and gene flow (Nm) (in bracket)

The low degree of genetic differentiation found between Albanian sheep breeds is supported by high level of gene flow (Nm, number of migrants per generation) between breeds (Table 7). Similar values of gene flow between populations are observed, but the highest value is observed between Bardhoka and Shkodrane.

The program BOTTLENECK (Piry et al., 1999) was used to investigate the hypothesis of a recent bottleneck. Wilcoxon sign-rank test under three mutations models IAM, TPM and SMM and shift mode test were used to find out recent bottleneck (heterozygosity excess) in

the three Albanian sheep breeds. The heterozygosity excess obtained (Table 8) were nonsignificant ($P < 0.5$) under all the models in all sheep populations. These results were consistent with the normal L-shaped distribution of allele frequency in all populations (Figure 5). The results obtained here, demonstrate that the null hypothesis of mutation–drift equilibrium was fulfilled in these breeds.

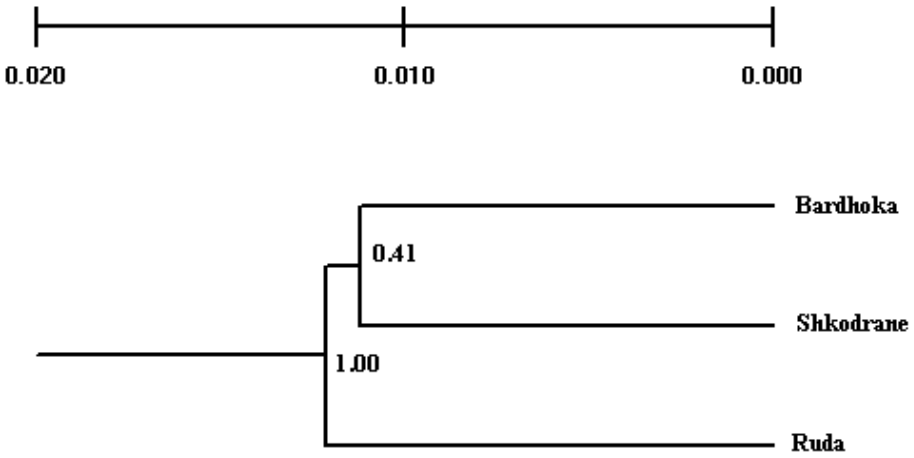

Fig. 4. Dendrogram of Reynolds genetic distance between Albanian sheep breeds by UPGMA algorithm

Breed	Mutation model		
	IAM	TPM	SMM
Bardhoka	0.995	0.795	0.999
Ruda	0.999	0.618	0.999
Shkodrane	0.951	0.909	0.999

Table 8. Bottleneck analysis for Albanian sheep breeds using Wilcoxon rank test under three mutation models

An AMOVA analysis was carried out to analyze the variation within and between breeds. The AMOVA revealed that percentage of variation among populations was 1.18% and within populations was 98.82% (Hoda et al., 2009). Variance components among population were highly significant ($p<0.001$). SRCRSP5 marker contributed the highest variability (8.42%) among populations. ILSTS11 and OARVH72 contributed the lowest variability (0.34% and 0.39% respectively).

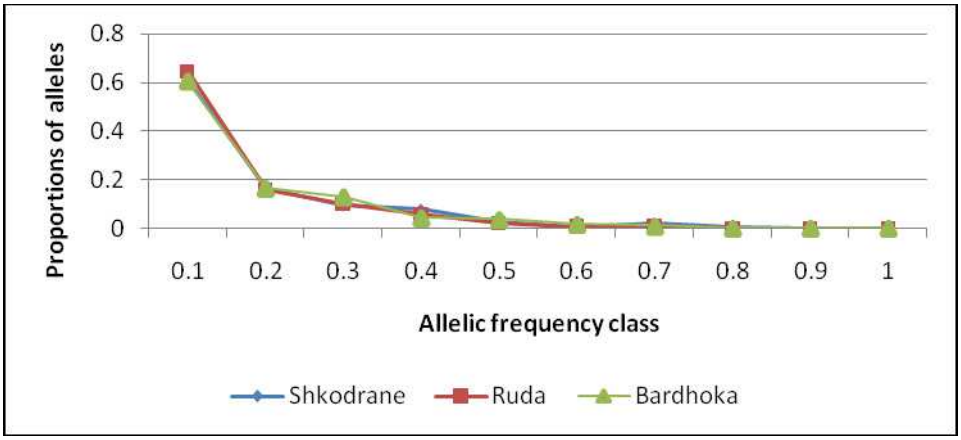

Fig. 5. Graphical representation of proportions of alleles and their distribution in three Albanian sheep breeds

3.4 Population structure

The program STRUCTURE (Falush et al., 2003; Pritchard et al., 2000) was run 5 times independently, with K ranging from 1 to 4, in order to choose the appropriate value of K. The results of the analyses with Structure are summarized in Table 9. We have used two methods to estimate the best *K* value: Pritchard and Evanno methods. According to Pritchard method, the average likelihood values Ln Pr(X | K) for each K, were plotted against K, in order to choose the optimal K value. The likelihood values reaches a maximum at K= 2 and afterwards decreased rapidly. Also the variance reaches the lowest value at K = 2. The results of this method are shown also previously (Hoda et al., 2009). Evanno method (Evanno et al., 2005) was applied and was calculated, an *ad hoc* statistic based on the second order rate of change of the likelihood function with respect to K. This statistic peaked at *K* = 2 (Table 9) (Figure 6) indicating strong support for 2 groups. Graphic representation of the estimated membership coefficients to the clusters for each individual, (*K*= 2), is shown previously (Hoda et al., 2009). The results of structure analysis show a high level of breed admixture.

K	Ln Pr(X | K)]	ΔK
1	-10080.2	
2	-10073.9	13.30326
3	-10198	0.702013
4	-10172.7	

Table 9. Estimated posterior probabilities [Ln Pr(X | K)] for different numbers of inferred clusters (K) and ΔK statistic.

The tree of individuals based on proportion of shared alleles D_{AS}, obtained through Neighbor-joining algoritm (figure 7) showed that animals from three different breeds are mixed together.

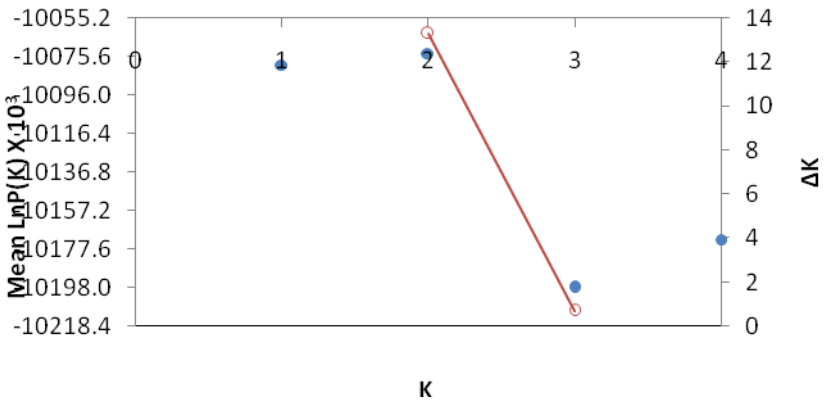

Fig. 6. Results of the STRUCTURE analysis showing mean Ln P(D) and ΔK values.

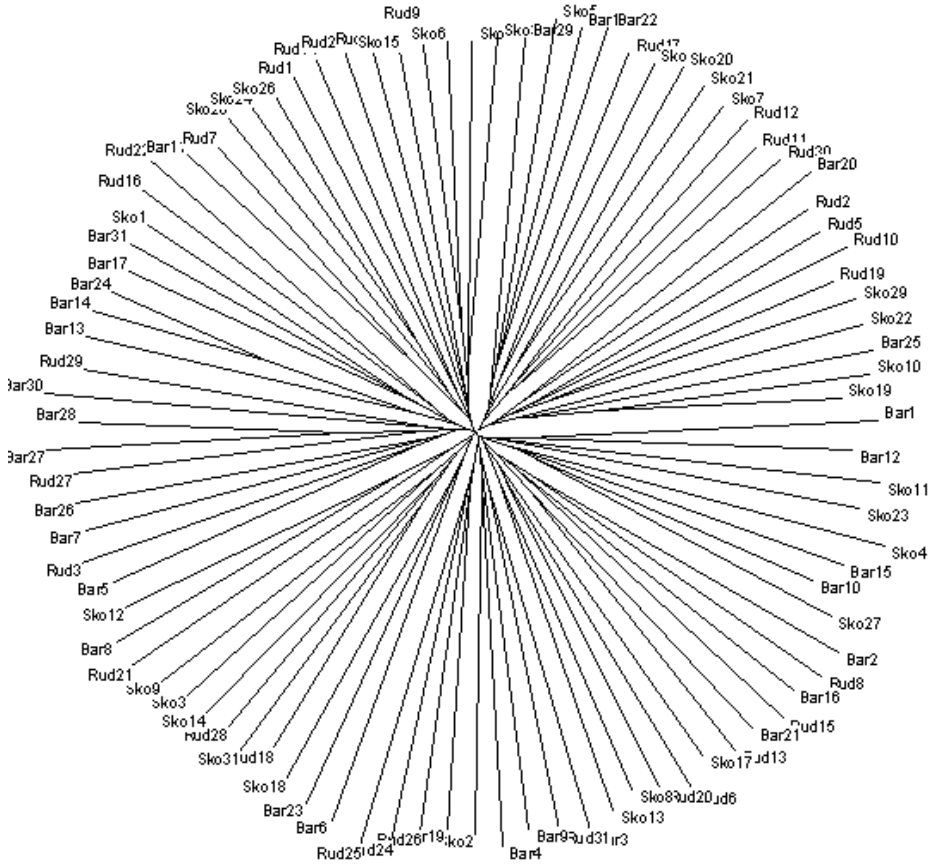

Fig. 7. Dendrogram of allele sharing distances between each individual of three sheep breeds

Relationship of populations based on Nei's genetic distance was performed by frequency Principle Component Analysis (PCA). The PCA was carried out with Genalex (Peakall and Smouse, 2006). In the Principal Component Analyses, the first and second axis accounted for 58.7, and 41.3% of the total inertia respectively. As is shown in figure 8, the first axis separate Ruda from two other breeds and the second axis separate Shkodrane from other breeds.

Fig. 8. Diagram of PCA based on Nei's genetic distance

The Factorial Correspondence Analysis (FCA) is performed to visualize the relationships between the individual using Genetix program (Belkhir et al., 2001), It is a multivariate method of analysis. Allele frequencies, of all populations and at all loci, are used as variables. The results of analysis are displayed in figure 9. Individuals of three breeds are grouped together indicating clear admixture among individuals.

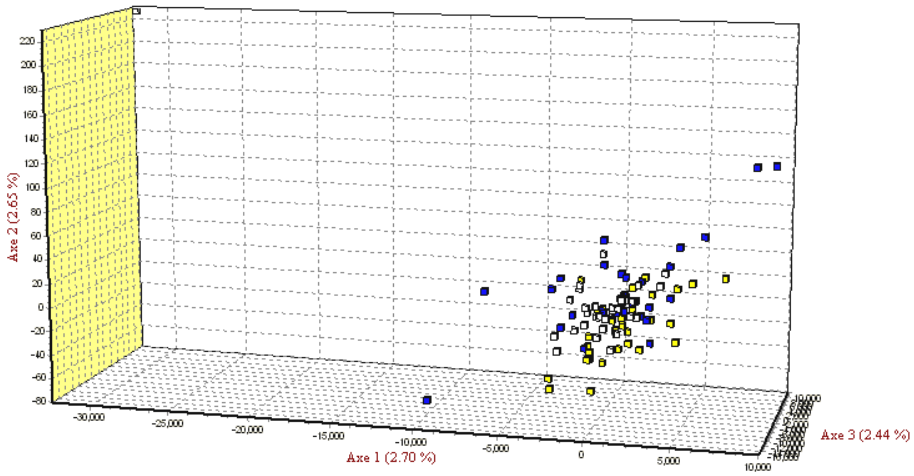

Fig. 9. Factorial Correspondence Analysis (FCA) results showing the relationship between all of the individuals analyzed in the study.

Breed	Direct					Simulation				
	D_C	D_S	D_A	Freq	Bayes	D_C	D_S	D_A	Freq	Bayes
Bardhoka	51.61	61.29	54.84	70.97	64.52	6.45	6.45	6.45	12.90	19.35
Ruda	58.06	64.52	54.84	64.52	67.74	19.35	12.90	25.81	22.58	29.03
Shkodrane	83.87	67.74	83.87	70.97	64.52	0.00	3.23	3.23	3.23	6.45
Total	64.52	64.52	64.52	68.82	65.59	8.60	7.53	11.83	12.90	18.28

Table 10. Percentage of individuals from each sheep breed correctly assigned to their reference population by likelihood and genetic distance methods.

Likelihood and genetic distance based methods were used for a direct assignment of and for an exclusion analysis of individuals to their reference population. The likelihood based methods are used also previously, (Hoda et al., 2009). Table 10 shows the results of the assignment test obtained through different methods. By direct assignment of individuals to their reference populations, the best scores were obtained with frequency method (68.82%). The highest rate of excluded animals out of the 10000 simulated individuals was obtained using Bayes theorem (18.28%). By direct assignment, using the genetic distance based methods, the highest percentage of correctly assigned individuals was obtained by Nei's genetic distances D_A and D_S. The highest number of correctly assigned animals, for all methods were from Shkodrane.

4. Discussions

The local breeds analyzed in this study are the most important Albanian sheep breeds that are reared on small familiar farms in extensive or semi-intensive systems. The aim of this study was to examine the genetic diversity within and between them using 31microsatellite analysis.

The number of alleles observed at a locus is an indication of genetic variability at that locus. FAO have recommended that microsatellite loci for genetic diversity studies should have more than four alleles. The total number of alleles per locus ranged from 4 (SRCRSP5) to 20 (INRA63), with a mean value of 11.23, indicating that all the microsatellite loci were sufficiently polymorphic and were appropriate to analyze diversity. This value was smaller than values found for four Romanian sheep breeds, by 11 microsatellite markers (17.9, Kevorkian et al., 2010)), Italian native sheep breeds, by 19 microsatellite markers (15.4, Dalvit et al., 2009)), or European sheep, by 23 microsatellite markers (19.9, Handley et al., 2007)), but were higher than values provided for Gentile di Puglia sheep breed, by 19 microsatellite markers (9.68, d'Angelo et al., 2009).

Takezaki and Nei, (1996) have determined that gene diversity should be in the range of 0.3 to 0.8 in the populations, in order that markers to be useful for measuring genetic variation. Gene diversity for each breed ranged from 0.74 to 0.77, with an average value of 0.75. This confirmed that these markers were appropriate for measuring genetic variation.

The polymorphic information content (PIC) is a parameter indicative of the degree of informativeness of a marker. All markers have PIC values higher than 0.5, indicating that are highly informative. Only SRCRSP9 had a PIC value close to 0.5 (0.490). This confirm

again that the set of microsatellite markers were effective for genetic diversity estimation in Albanian sheep breeds.

The mean allele number (allele diversity) provides a reasonable indicator of the levels of variability present within a breed. The range of allele diversity measure was 8.19 – 8.84 indicated high level of genetic variability of Albanian sheep breed. The allele diversity estimates obtained in this study are comparable with those reported for Italian native sheep breeds (7.1 – 9.6, Dalvit et al., 2009), for Baltic sheep (3.93 – 8.33, Grigaliunaite et al., 2003), for Brazilian sheep breeds (4.22 – 8.39, Paiva et al., 2005), for Austrian sheep (6.19 – 10.7, Baumung et al., 2006), in Romanian sheep (7.8-11.6, Kevorkian et al., 2010), for five Bulgarian sheep breeds (6.3 – 8.6, Kusza et al., 2010), in Six Indian Sheep Breeds, (7.72 – 9.56, Arora et al., 2010).

The allelic richness had an average value of 8.61 alleles per breed. That is higher than what is observed in Northern European sheep breeds (6.98, Tapio et al., 2005), in European sheep breeds (7.5,), in Romanian sheep (4.82, KEVORKIAN et al., 2010), in West Balkan pramenka sheep types (7.9, Cinkulov et al., 2008), and in native Italian sheep breeds (7.3, Dalvit et al., 2009).

The genetic diversity values found in Albanian sheep breeds ranged from 0.74 to 0.77, with a mean value of 0.75. The values of gene diversity estimates were comparable with values detected for Baltik (0.57 – 0.76, Grigaliunaite et al., 2003), Austrian (0.67 – 0.78, Baumung et al., 2006), Bulgarian (0.73 – 0.80, Kusza et al., 2010), Romanian (0.67 – 0.79, Kevorkian et al., 2010), Italian native breeds (0.70 – 0.80, Dalvit et al., 2009). These gene diversity estimates are smaller than those detected by for Egyptian sheep breeds (0.81 – 0.86, El Nahas et al., 2010), Iranian sheep breeds (0.83, Nanekarani et al., 2010), but higher than values found for Indian sheep breeds (0.59-0.65, Mukesh et al., 2006), Slovak Tsigai populations (0.46 – 0.61, Kusza et al., 2009), West Balkan Pramenka sheep types (0.739 – 0.830, Cinkulov et al., 2008).

Estimates of observed heterozygosity confirm the high level of diversity evidenced in the Albanian sheep breeds. The average observed heterozygosity was 0.72. Overall heterozygosity estimates are comparable with what found in Swiss sheep breeds (Stahlberger-Saitbekova et al., 2001), Austrian sheep breeds (Baumung et al., 2006), Spanish breeds (Alvarez et al., 2004) and Alpine sheep breeds (Dalvit et al., 2008), Sarda sheep flocks (Pariset et al., 2003).

The comparison of average observed and expected heterozygosity values did not show great differences between breeds. All breeds showed smaller observed than expected heterozygosities (Table 3). Bardhoka and Shkodrane showed on 3 loci out of 31, significant deviation from Hardy Weinberg proportion, but Ruda showed no deviations from Hardy Weinberg proportion. The observed heterozygosity (Ho) of microsatellite loci was always larger than 0.50 (Table 3), except of Oarae129 (0.432) and SRCRSCP5 (0.315). Most of the loci showed the heterozygote deficit as also depicted by positive F_{IS} value (Table 2).

Several factors can contribute to less than expected heterozygosity in a population. One reason might be inbreeding, i.e. mating between relatives. In case of inbreeding, the deficit affects all or most of the loci in a similar way. The number of loci, with a significant deficit of heterozygotes, is very small. The farmers do efforts to avoid as much as possible, the breeding between relatives, trying not to use the rams from their own flock. Other factor

that can also cause a deficit of heterozygotes in the population might be the presence of "null alleles" (non-amplifying alleles). This may cause a afalse observation of homozygotes excess. Peter et al., (2005) have indicated the presence of null alleles in locus OarAe 129. Finally, the presence of population substructure within the breed may lead to a Wahlund effect, since the animals were sampled from many small flocks. For all breeds sampling was carried out in 10 – 11 small flocks.

The high values of heterozygosities and allelic richness obtained in this study confirm that native breeds of sheep represent an important reservoir of genetic diversity, even though the level of differentiation among closely located breeds is small. This is in accordance with the prediction of (Handley et al., 2007) of a higher within-breed diversity and lower genetic differentiation in southern than in northern European breeds. Peter et al., (2007), observed a higher genetic diversity of Middle Eastern, Turkish, Greek, Albanian and Romanian sheep breeds compared with northwestern European breeds.

Grigaliunaite et al., (2003) showed that when unique allele has a frequency below 0.1 it might be an allele that is present in several populations at low frequency and could be found also in other breeds, if greater fraction of the total population would be screened. A high number of private alleles were observed in Albanian sheep breeds and none of them had frequency higher than 10%. Peter, (2005) in the study of 57 European and Middle-Eastern sheep breeds, including also the Albanian sheep breed considered here, showed the presence of 2 private alleles in Bardhoka, only one in Ruda and none to Shkodrane. Therefore all private allele data, obtained here are not informative

The studied breeds showed a poor, but significant genetic differentiation (0.011), which is very low compared to those from other genetic diversity studies, e.g. 18.3% for Indian sheep (Mukesh et al., 2006), 13.3% Slovak Tcigai populations (Kusza et al., 2009), 8.2% Romanian breeds (Kevorkian et al., 2010), 8.3% Bulgarian breeds (Kusza et al., 2009), 8% Austrian (Baumung et al., 2006), 6.1% Six Indian Sheep Breeds, (Arora et al., 2010), 5.7% for Alpine sheep (Dalvit et al., 2008) and European and Middle-Eastern breeds including also the Albanian sheep breeds (Peter et al., 2007), 5% for Pramenka types (Cinkulov et al., 2008), 4.6% for Ethiopian sheep (Gizaw et al., 2007), 3.7% in three Egyptian sheep (El Nahas et al., 2010) and Manchega sheep (Calvo et al., 2006). Our results are similar to those reported by (Nanekarani et al., 2010) for pelt sheep breeds of Iran (0.018). Low Fst value (0.29) have found also (Calvo et al., 2006) for Portugese native sheep, northern Spanish (F_{ST} = 0.029, (Rendo et al., 2004). The low genetic differentiation of Albanian sheep breed displayed here is in concordance with the results obtained with AFLP markers (Hoda et al., 2010) and SNP markers (Hoda et al., 2011).

An analysis of Nei genetic distance indicated that the three Albanian sheep breeds are closely related. The pairwise F_{ST} value of 0.05 implies moderate differentiation between breeds (Hartl, 1980). The pairwise F_{ST} values provided here between all pairs of the tested breeds are less than 0.05, indicating a low differentiation between Albanian sheep breeds. The degree of genetic differentiation was poor and had similar values between all pair of breeds. This is supported by the high level of gene flow (Nm) between breeds.

All the genetic distance measures employed to estimate inter-breed closeness showed low levels of distances between the sheep breeds. The smallest distance was observed between Shkodrane and Bardhoka, that is in concordance with results obtained previously using

AFLP markers (Hoda et al., 2010). The pattern of clustering observed with the allele-sharing distance measures (D_{AS}) among individual animals reflected the admixture of individuals coming from different breeds. This is in accordance also with the model of clustering of the same individuals, using Jaccard's similarity coefficients matrix based on AFLP data (Hoda et al., 2010). Results for the correct assignment of individuals to their reference origin, using different methods are shown in Table 10. Low percentage of correctly assigned individuals is found for all breeds. The results of assignment test can be used to identify pure breed individuals that might be used in the breeding programs in the near future. The low percentage of correctly assigned individuals to their reference population reflect also the high gene flow and intermixing of gene pool between the breeds and suggest that they are genetically very close. Low percentage of correctly assignment was obtained also by SNP markers (Hoda et al., 2011).The assignment tests, Factorial Correspondence Analysis (FCA) and Structure analysis showed a high degree of genetic similarity between individuals of three breeds and high level of breed admixture. The results of bottleneck analysis revealed that the sheep breeds have not undergone any recent bottleneck, i.e, any recent reduction in the effective population size and are at mutation drift equilibrium.

The results obtained here reflect sheep management in Albania. Sheep farming is an important activity for the farmers in Albania. Typically, the farms are small having 20 – 30 individuals with one ram. Management system is extensive or semi-extensive. The animals graze on natural grasses from morning till evening, without any supplement feed. They provide an important source of milk, meat and wool, mainly for family consumption. Product marketing and processing is limited and difficult due to the low rural socio economic level, poor infrastructure and investments. There is no breeding program for these sheep breeds. The mating is natural. In most of the cases there is only one ram per flock that breed all the ewes in the flock. The rams and ewes are housed and grazed together thereby no controlled mating is practiced at farmer's level. The rams are selected by the farmer, trying to avoid the use of males from their own flock. Usually the farmer buys the rams in the farm animal market, or from neighbor farms without any information or control of their origin, resulting in mating without parentage control. The lack of herd book, until nowadays, probably has facilitate the gene flow and the admixture of the breeds resulting to a low level of genetic differentiation.

Based on the results of this study, but also of previous studies by AFLP and SNPs markers we may conclude that Albanian sheep breed are important reservoir of genetic diversity, have a low level of differentiation and high level of admixture. All this results may be used and help in starting a breeding strategy and policy involving the decision on crossbreeding or pure breeding.

5. Conclusions

Traditionally, classifications of Albanian sheep breeds were based on visible phenotypic traits and productive traits. Characterization at molecular level using different set of markers was made possible in frame of Econogene project.

Molecular characterization using a huge set of microsatellite markers showed that Albanian sheep breed have more within breed variation than between breed variation.

All microsatellite markers have more than 4 alleles and a high level of gene diversity and high PIC values, indicating that were sufficiently polymorphic and were appropriate to analyze diversity.

Genetic distances between breed were small. The pairwise FST values were small and similar between all breeds. A high level of gene flow was detected between breeds. All these data show a poor level of genetic differentiation.

Factorial Correspondence Analysis (FCA) and Structure analysis showed a high degree of genetic similarity between individuals of three breeds and high level of breed admixture.

The results of bottleneck analysis revealed that the sheep breeds have not undergone any recent bottleneck, i.e, any recent reduction in the effective population size and are at mutation drift equilibrium.

Albanian sheep breed are important reservoir of genetic diversity, have a low level of differentiation and high level of admixture.

All this results may be used and help in starting a breeding strategy and policy involving the decision on crossbreeding or pure breeding.

6. Acknowledgements

This work has been supported by the EU Econogene contract QLK5-CT-2001-02461. The content of the publication does not represent necessarily the views of the Commission or its services.

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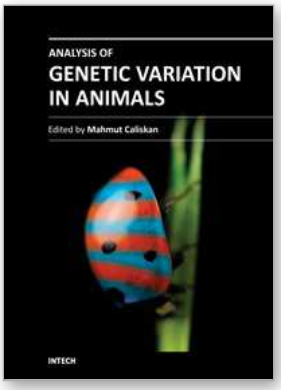

Analysis of Genetic Variation in Animals
Edited by Prof. Mahmut Caliskan

ISBN 978-953-51-0093-5
Hard cover, 360 pages
Publisher InTech
Published online 29, February, 2012
Published in print edition February, 2012

Analysis of Genetic Variation in Animals includes chapters revealing the magnitude of genetic variation existing in animal populations. The genetic diversity between and within populations displayed by molecular markers receive extensive interest due to the usefulness of this information in breeding and conservation programs. In this concept molecular markers give valuable information. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in animals and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation in animals by presenting the thoughts of scientists who are engaged in the generation of new idea and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

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