Genetic diversity and population structure in Albanian local sheep breeds analyzed by microsatellite markers

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SUMMARY

Sheep are considered as an important livestock species in Albania. The genetic structure and relationships among 3 autochthonous sheep breeds have been inferred from 31 DNA microsatellite loci using F-statistics. The study carried out in the frame of the ECONOGENE project, contributes to the investigation of genetic diversity estimate in three autochthonous Albanian sheep breeds "Bardhoka", "Ruda" and "Shkodrane". A total of 93 animals were investigated and 355 microsatellite alleles were found. The level of apparent breed differentiation is low and multilocus F_{ST} values indicate that only around 1.11% of the total genetic variation is explained by the between breeds component, while the remaining 98.89% by differences among individuals within breeds. This deficit is of 5.16% ($p<0.001$) in the whole population. It was carried out a direct assignment of individuals and an exclusion analysis based on 10 000 simulated individuals. The number of inferred populations was K = 2 for microsatellite markers. Population structure analysis revealed a high level of admixture between populations. Several reasons for the breed admixture are presented. A breeding strategy and policy has to be started in order to conserve valuable sheep breeds.

Key words: *genetic diversity, autochthones breeds, sheep, microsatellite, gene flow, breed admixture.*

INTRODUCTION

Sheep is one of the most important livestock species in Albania. There are four indigenous sheep breeds: Bardhoka, Ruda, Shkodrane and Recka. Bardhoka breed is classified in long tail group. Its origin is from north/northeast of Albania, as well as in Western part of Kosovo. Ruda is triple production breed with half-fine wool. This breed is part of Cigaia family regarding the wool quality. It is originated and widespread in north-eastern part of Albania. The Shkodrane sheep breed belongs to the long tail group. The area of origin is north Albania. Recka breed is part of Cakel group. It is located all over the Albanian territory, especially on mountain areas of central and south Albania. There were applied crossing between Bardhoka and Shkodrane, known as Baca, in order to improve the milk production. Selection programs in sheep are less advanced and systematic then in other livestock. Lack of parentage control and breed purity has facilitated a continuous gene flow. Because artificial insemination with frozen sperm is rarely practiced, this gene flow is limited by distance and geography. On the other hand, local management may have led to genetic isolation, which reduces the effective population size.

Polymorphic DNA markers are very useful in assessment of genetic diversity within and between breeds. Microsatellite is widely used as genetic markers for the analysis of genetic variability within and between breeds due to their high number, distribution throughout the genome and the efficiency of genotyping.

Recently, they are used for the study of diversity in European sheep breeds (Álvarez et al 2004, Arranz et al 1998, Arranz et al 2001, Diez-Tascón et al 2000, Farid et al 2000, Pariset et al 2003, Rendo et al 2004, Stahlberger-Saitbekova et al 2001, Tapio et al 2005, Tapio et al 2003, Peter et al 2007, Cinkulov et al 2008).

For long time the genetic diversity of indigenous sheep breed has been evaluated on the basis of biochemical methods. Zoraqi, 1991 has studied Bardhoka and Shkodrane breeds, based on visible traits, as well as milk and blood polymorphism. In this study we have characterized three local sheep breeds using 31

microsatellite markers. We intend to characterize the relationships among the breeds, by estimating genetic distances from microsatellite markers.

MATERIALS AND METHODS

Sample collection and microsatellite markers

A total of 93 randomly sampled animals representing 3 different Albanian sheep breeds were analyzed. The breeds were Bardhoka, Ruda and Shkodrane. For each breed were sampled 31 individuals and were selected maximum three unrelated individuals (two females and one male) per flock. Sampling was carried out from an average of 11 flocks per breed, based on the information provided by the farmer.

There are used 31 microsatellite markers: BM1329, BM1824, BM8125, DYMS1, HUJ616, ILSTS005, ILSTS011, ILSTS028, INRA63, MAF33, MAF65, MAF70, MAF209, MAF214, McM140, McM527, OarAE129, OarCP34, OarCP38, OarFCB20, OarFCB128, OarFCB193, OarFCB193, OarFCB304, OarHH47, OarJMP29, OarJMP58, OarVH72, SR-CRSP-1, SR-CRSP-5 and SR-CRSP-9.

Statistical analyses

The program FSTAT (http://www/2.until.ch.popgen.softwares/fstat.htm) was used for the calculation of corrected allele diversity (allelic richness). Also, FSTAT was used to compute F-statistic parameters, according to Weir and Cockerham (1984) (F_{IT} (W&C), F_{IS} (W&C and F_{ST} (W&C). The significance was tested by 1000 permutations (Goudet 1995). F_{IS} estimates were calculated across all populations and loci and for populations and loci individually. Also were performed F_{ST} calculations over all populations and all combinations formed by three populations (pairwise $_{FST}$). The polymorphic information content (PIC) index for each marker was calculated according to Botstein *et al*. (1980).

Exact tests of genotype frequencies for deviation from Hardy-Weinberg equilibrium (HWE) were performed using the GENEPOP V.1.2 package (Raymond and Rousset, 2001), performing a probability test using Markov chain Monte Carlo simulation (dememorization 10,000, batches 1000, iterations per batch 5,000).

Assignment of individual to their reference population was evaluated using GeneClass2 (Piry et al 2004). For all breeds was carried out a direct assignment of individuals and a exclusion analysis based on 10 000 simulated individuals. The methods based on allele frequencies frequencies (Paetkau et al 1995), as well as Bayesian approach (Rannala & Mountain 1997) were used.

The genetic structure of four sheep breeds is analyzed using the program STRUCTURE (Pritchard et al 2000). The program STRUCTURE uses the Markov Chain Monte Carlo method. The program was run 3 times independently, for K ranging from 2 to 4. All runs were carried out under "admixture model", with a burn-in period of 300,000 iterations and a period of data collection of 300,000 iterations.

RESULTS AND DISCUSSION

Microsatellite loci and genetic variability

The allele and genotype frequencies of 31 microsatellite loci were determined in 3 Albanian sheep breeds. All the markers were polymorphic. The total number of alleles and allele size for each locus are presented in Table 1. A total of 347 alleles were detected over all loci in 93 individuals. The average number of alleles was 8 and varied from 4 (SRCRSP9) to 20 (INRA063). 30 out of 31 markers showed 5 or more alleles. Observed heterosigosity (H_O) per locus ranged from 0.315 (SRCRSP5) to 0.882 (OARJMP5), with an average 0.891. In total of 10 from 93 locus-population comparisons revealed significant departures ($p<0.05$) from Hardy-Weinberg proportions. Loci Oarae129 deviated in Bardhoka and Shkodrane, locus Srcrsp5 deviated in all populations; therefore these loci are excluded from the genetic differentiation analysis. PIC values were higher than 0.6.

The different estimates of genetic differentiation (F_{ST} and G_{ST}) with F_{IS} and F_{IT} are shown in table 1 and table 2. On average breeds had a 4.1% deficit of heterozygotes, whereas the total population had a 5.16% deficit of heterozygotes. F_{ST} values of genetic differentiation and G_{ST} values of breed differentiation were similar. The average genetic differentiation between all breeds was poor, of 1.11% significantly different from zero ($p<0.001$). F_{ST} values indicated that about

1.1% ($p<0.001$) of the total genetic variation was explained by a population difference and 89% correspond to the differences among individuals. The contribution of the markers for breed differentiation was estimated by the significance of F_{ST}. All loci had a significant F_{ST} (table 1). The overall G_{ST} value was 0.011. In table 2 are shown the comparison of within population inbreeding. 1

Tab 1: Number of observed alleles (n), allelic richness (AR), heterozygosity and F-statistics for each of the 31 microsatellite markers and means estimates

Nr.	Loci	Chr.	n	AR	PIC	H_O	H_S	H_T	$F_{IS}\equiv f$	$F_{IT}\equiv F$	$F_{ST}\equiv\theta$	G_{ST}	$F_{IS}\equiv f$		
													Bardhoka	Ruda	Shkodrane
1	BM1329	6	6	5.309	0.842	0.632	0.645	0.648	0.0417*	0.0524*	0.0112*	0.008	-0.088	0.068	0.108
2	BM1824	1	5	4.978	0.841	0.731	0.741	0.742	0.0438*	0.0547*	0.0113*	0.002	-0.117	-0.057	0.028
3	BM8125	17	7	6.148	0.775	0.739	0.705	0.707	0.0438*	0.054*	0.0106***	0.003	0.049	-0.064	-0.074
4	DYMS1	20	14	11.515	0.864	0.880	0.856	0.868	0.0434*	0.0542*	0.0112***	0.021	0.03	-0.116	0.007
5	HUJ616	13	15	10.939	0.862	0.822	0.802	0.805	0.0312***	0.0423***	0.0114*	0.006	0.379**	0.263*	0.461**
6	ILSTS005	7	9	7.163	0.803	0.774	0.786	0.787	0.0432*	0.0537*	0.0111*	0.003	0.016	-0.148	0.083
7	ILSTS011	9	13	9.777	0.857	0.891	0.848	0.847	0.0431*	0.054*	0.0114*	-0.002	0.046	-0.259	0.081
8	ILSTS028	3	10	7.573	0.795	0.656	0.641	0.655	0.0395*	0.0505*	0.0114*	0.033	0.181*	0.009	0.063
9	INRA063	14	20	12.909	0.849	0.839	0.818	0.819	0.0368***	0.0465***	0.0101***	0.002	0.191*	0.154	0.144
10	MAF33	9	12	10.422	0.866	0.817	0.799	0.808	0.0409*	0.0518*	0.0114*	0.018	0.054	0.007	0.082
11	MAF65	15	15	10.613	0.821	0.814	0.753	0.765	0.0418*	0.053*	0.0117*	0.025	-0.107	0.056	0.119
12	MAF70	4	12	9.263	0.824	0.733	0.771	0.769	0.0435*	0.0544*	0.0114*	-0.005	0.079	-0.001	-0.171
13	MAF209	17	9	7.069	0.854	0.742	0.758	0.754	0.0405*	0.0509*	0.0108*	-0.008	0.003	0.021	0.143*
14	MAF214	16	16	12.500	0.828	0.763	0.816	0.828	0.0413*	0.0517*	0.0109*	0.021	0.041	0.081	-0.024
15	MCM140	6	13	10.439	0.870	0.747	0.840	0.838	0.0456*	0.0554*	0.0103***	-0.005	-0.046	-0.069	-0.132
16	MCM527	5	9	6.930	0.878	0.599	0.786	0.790	0.0422*	0.0531*	0.0114*	0.007	0.189*	-0.018	-0.192
17	OARAE129	5	7	9.557	0.834	0.656	0.656	0.655	0.042*	0.0529*	0.0113*	-0.002	-0.018	-0.071	0.121
18	OARCP34	3	15	10.730	0.834	0.864	0.811	0.814	0.0402*	0.0505*	0.0107*	0.006	0.031	0.162*	-0.013
19	OARCP38	10	14	11.060	0.855	0.677	0.747	0.757	0.0434*	0.0537*	0.0108*	0.02	0.062	-0.05	-0.087
20	OARFCB20	2	16	5.023	0.869	0.432	0.686	0.685	0.0395*	0.0499*	0.0108**	-0.001	0.322**	-0.09	0.06
21	OARFCB128	2	7	6.237	0.811	0.812	0.800	0.805	0.0442*	0.0551*	0.0115*	0.01	0.076	-0.142	-0.19
22	OAFCB193	11	7	5.667	0.641	0.583	0.563	0.562	0.043*	0.0529*	0.0104**	-0.003	-0.032	-0.138	0.078
23	OAFCB226	2	8	7.192	0.815	0.722	0.788	0.789	0.0419*	0.0528*	0.0113*	0.003	-0.013	0.133	-0.08
24	OAFCB304	19	9	10.854	0.912	0.817	0.865	0.875	0.0445*	0.0556*	0.0115*	0.016	-0.083	0.008	-0.077
25	OARHH47	18	13	9.930	0.879	0.670	0.801	0.821	0.0451*	0.0553*	0.0106*	0.037	-0.036	0.012	-0.224
26	OARJMP29	24	13	11.734	0.887	0.785	0.813	0.822	0.0305***	0.0394***	0.0092*	0.016	0.557***	0.4**	0.392**
27	OARJMP58	26	15	10.653	0.880	0.882	0.815	0.832	0.0389*	0.0497*	0.0112*	0.031	0.138	0.042	0.174
28	OARVH72	25	15	8.481	0.875	0.821	0.862	0.864	0.0409*	0.052*	0.0116*	0.003	0.085	-0.016	0.09
29	SRCRSP1	1	9	6.023	0.721	0.591	0.543	0.541	0.0385**	0.0497*	0.0116*	-0.007	0.118	0.125	0.088
30	SRCRSP5	18	10	3.916	0.856	0.315	0.582	0.614	0.0449*	0.0556*	0.0112*	0.076	-0.037	-0.037	-0.121
31	SRCRSP9	12	4	6.328	0.726	0.576	0.652	0.654	0.0343***	0.045***	0.0112*	0.005	0.07	0.295**	0.339***
	Average		8			0.722	0.753	0.759	0.041***	0.0516***	0.0111***	0.011			

Table 2: Correct assignment of individuals to their reference population by microsatellites markers

Breed	Nr. ind.	Assignment				Exclusion			
		Frequency		Bayes		Frequency		Bayes	
		Nr.	Percent.	Nr.	Percent.	Nr.	Percent.	Nr.	Percent.
Bardhoka	31	22	70.97	20	64.52	5	16.13	5	16.13
Ruda	31	20	64.52	21	67.74	9	29.03	10	32.26
Shkodrane	31	22	70.97	20	64.52	2	6.45	1	3.23
Total	93	64	68.82	61	65.59	16	51.61	16	51.61

Breed assignment

There are used two individual assignment/exclusion tests: assignment using a Bayesian-based approach (Rannala and Mountain, 1997), assignment based on allele frequencies (Paetkau *et al.*, 1995). The results of assignment of individuals to to their reference population, by both methods, under simulation and direct approach, are presented in Table 2. Both assignment tests give similar results. Ruda is the breed with the higher rate of correctly assigned animals, using Bayesian method of assignment. Ruda is also the breed with the higher rate of excluded animals (29.03% and 32.26% under frequency based method and Bayes theorem respectively).With the likelihood based method, based on allelic frequencies only 68.82% of the individuals were assigned to their reference population, but based on Bayes theorem, were assigned correctly 65.59% of the individuals. Exclusion tests based on both methods give the same results.

The program STRUCTURE (Pritchard et al 2000) infers the number of populations into which the analyzed genotypes can be divided. The program estimates the natural logarithm of the probability Ln P(D) of given genotype, being part of a given population *K*. By the Bayes Model-based Clustering Analysis, the highest likelihood value (Ln P(D)), correspond to the most probable number of subpopulations. In order to find the best value of K, the program was run 3 times, fitting *K* from 2 to 4, under "admixture model", for both type of markers. The best value of Ln P(D) (-10067.7) was obtained for *K* = 2, for microsatellite markers, and for K = 3 (-2338.3) for SNP markers.

Therefore we infer K= 2 as possible populations (Table 3). It is clear that the inferred populations, for both markers are formed by individuals of all three breed.

Table 3: Proportion of membership of Albanian sheep breeds when inferred clusters are 2

Breed	Inferred clusters	
	1	2
Bardhoka	0.513	0.487
Ruda	0.470	0.530
Shkodrane	0.533	0.467

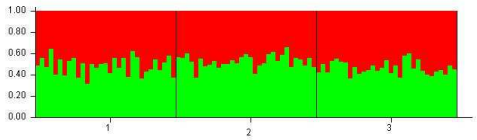

Figure 1: Clustering assignment of the three sheep breeds provided by STRUCTURE analyses.

Each of the 93 animals is represented by a thin vertical line that is divided into segments. Each line is broken into two colored segments (K=2), which represent the membership of each individual to the two clusters. Breeds are separated by thin black lines. With K = 2 inferred clusters, it can be seen breed admixture patterns.

The genetic analysis of 3 Albanian local sheep breeds with 31 microsatellite markers showed high gene diversity. The high number of alleles for each locus, as well as the high PIC values, suggested that all the markers used were appropriate to analyze diversity in Albanian local sheep breeds. The mean number of alleles per breed is a good indicator of genetic variation within populations. According to Takezaki and Nei (1996), a marker can be useful in measuring genetic variation if the average heterozygosity ranges from 0.3 to 0.8 in the population. This confirmed that these markers were appropriate for measuring genetic variation. These breeds are characterized by a local distribution, small population size, living in extensive condition, grazing in the poor pastures, linked to

traditional farming system. This may be the explanation for the high degree of genetic variability. Several markers displayed a significant deficit of heterozygotes due to within population inbreeding in each of the breed.

The genetic differentiation (F_{ST}) among the local sheep breeds in this study is quite low, 1.11%. The F_{ST} values obtained in this study suggested a poor genetic differentiation between Albanian sheep breeds. Gene flow between populations was quite high and could have likely played an important role in determining this poor differentiation. The values of genetic differentiation are lower than those reported by other authors, between 57 European and Middle Eastern sheep breeds (5.7%, Peter et al., 2007), between seven West Balkan pramenka sheep types (5.2%, Cinkulov et al 2008), between finish sheep breed, (8%, Tapio et al., 2003), between Baltic sheep breeds (8.8%, Tapio et al., 2005), or (8.5%, Forbes et al, 1995). In all these cases the number of markers was much higher than in our case. It is clear that 98.89% of genetic variation corresponds to differences among individuals and 1.11% is result of differences among breeds.

There is no herd book since 1990 and maybe there is some admixture of breeds. In Albania are applied crossing between Bardhoka and Shkodrane, known as Baca, in order to improve the milk production. This may be one explanation for the smallest distance between Bardhoka and Shkodrane and their grouping.

Therefore it is important the determination of population structure, in order to design and establish a proper conservation program for local sheep breeds. The microsatellites markers proved very useful for assessing admixture in Albanian sheep populations. The results revealed using program STRUCTURE and Geneclass suggested a high level of breed admixture.

Assignment methods have multiple application, like the identification of the source population of a given genotype, evaluation of population differentiation (Waser and Strobeck (1998) or in agriculture for the traceability of animals for breed confirmation (Maudet et al 2002. Two of these breeds, Bardhoka and Ruda are traditionally transhumant breeds. Their transhumance is oriented on the same region, which are western lowlands. This may bring to intercrosses that occur occasionally or voluntarily by farmers. Albanian farmers have never been organized in breeding associations that may apply breeding standards, identification and keep herd book. Nevertheless that several breeding stations were acting during the last 50 years, sheep herd book was not established and this has facilitated the breed admixture.

In Albania are applied crossing between Bardhoka and Shkodrane, known as Baca, in order to improve the milk production and body weight. All the sheep farms are private and the animal breeding is realized by the farmers himself, who buy the reproducing males in the farm animal market, having as a consequence lack of parentage control The results provided here, may be used to start a breeding strategy and a policy in order to conserve the important sheep breeds.

REFERENCES

ÁLVAREZ, I, ROYO, L.J, FERNÁNDEZ, I, GUTIÉRREZ, J.P, GÓMEZ, E AND GOYACHE, F (2004) Genetic relationships and admixture among sheep breeds from Northern Spain assessed using microsatellites. J. Anim. Sci. 82, 2246-2252.

ARRANZ J. J., BAYON Y., SAN PRIMITIOVO F. (2001) Differentiation among Spanish sheep breeds using microsatellites. Genet. Sel. Evol. 33. 529-542.

ARRANZ, J.J, BAYON, Y AND SAN PRIMITIVO, F (1998) Genetic relationships among Spanish sheep using microsatellites. Anim. Genet. 29, 435-440.

CINKULOV M, POPOVSKI Z, PORCU K, TANASKOVSKA B, HODZIC A, BYTYQI H, MEHMETI H, MARGETA V, DJEDOVIC R, HODA A, TRAILOVIC R, BRKA M, MARKOVIC B, VAZIC B, VEGARA M, OSLAKER I AND KANTANEN J (2008) Genetic diversity and structure of the West Balkan pramenka sheep types as revealed by microsatellite and mitochondrial DNA anaylsi, J. Anim. Breed. Genetic 125, 417-426.

CORNUET J M, PIRY S, LUIKART G, ESTOUP A AND SOLIGNAC M (1999) New methods employing multilocus genotypes to select or exclude populations as origins of individuals. *Genetics*, *153:* 1989-2000.

DIEZ-TASCO´N C, LITTLEJOHN R.P, ALMEIDA P.A AND CRAWFORD A.M (2000) Genetic variation within the Merino sheep breeds analysis of closely related populations

using microsatellites. Animal Genetics 31, 243–51.

FARID, A, O'REILLY, E, DOLLARD, C AND KELSEY, C.R (2000) Genetic analysis of ten sheep breeds using microsatellite markers. Can. J. Anim. Sci. 80, 9-17.

FORBES S.H, HOGG J.T, BUCHANAN, F.C, CRAWFORD, A.M AND ALLENDORF, F.W (1995) Microsatellite evolution in congeneric mammals domestic and bighorn sheep. Mol. Biol. Ecol. 12, 1106-1113.

GOUDET, J (1995) FSTAT V 2.9.3 a computer programme to calculate F-statistics http//www.unil.ch/izea/software/fstat.html. J. Hered. 8, 485-486.

MAUDET C, LUIKART G AND TABERLET P (2002) Genetic diversity and assignment tests among seven French cattle breeds based on microsatellite DNA analysis. J. Anim. Sci. 80: 942–950.

PAETKAU D, CALVERT W, STIRLING I AND STROBECK C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Mol. Ecol.*, *4:* 347-354.

PARISET, L, SAVARESE, M.C, CAPPUCIO, I AND VALENTINI, A (2003) Use of microsatellites for genetic variation and inbreeding analysis in Sarda sheep flocks of central Italy. J. Anim. Breed. Genet. 120, 425-432.

PETER, C, BRUFORD, M, PEREZ, T, DALAMITRA, S, HEWITT, G, ERHARDT, G AND THE ECONOGENE CONSORTIUM (2007) Genetic diversity and subdivision of 57 European and Middle Eastern sheep breeds. Anim, Genetics, 38, 37 – 44.

PIRY S, ALAPETITE A, CORNUET J M, PAETKAU D, BAUDOUIN L AND ESTOUP A (2004) GeneClass2: a software for genetic assignment and first generation migrants detection. *Journal of Heredity* 95,536- 39.

PRICHARD J K, STEPHENS M AND DONNELLY P (2000) Inference of population structure using multilocus genotype data. *Genetics*, *155*: 945–959.

RANNALA B AND MOUNTAIN J L (1997) Detecting immigration by using multilocus genotypes. *Proc. Natl Acad. Sci. USA*, *98:* 9197-9201.

RAYMOND, M. AND ROUSSET, F (2001) GENEPOP a population genetic software for exact test and ecunemism. http//www.cefe.cnrs-mop.fr. Stand 12.12.04.

RENDO, F, IRIONDO, M, JUGO, B.M, MAZÓN, L.I, AGUIRRE, A, VICARIO, A AND ESTONBA, A (2004) Tracking diversity and differentiation in six sheep breeds from the North Iberian Peninsula through DNA variation. Small Ruminant Res. 52, 196-202.

STAHLBERGER-SAITBEKOVA, N, SCHLÄPFER, J, DOLF, G. AND GAILLARD, C (2001) Genetic relationships in Swiss sheep breeds based on microsatellite analysis. J. Anim. Breed. Genet. 118, 379-387.

TAKEZAKI N, NEI, M (1996) Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. Genetics 144 389 – 3999.

TAPIO, I, TAPIO, M, GRISLIS, Z, HOLM, L.E, JEPPSSON, S, KANTANEN, J, MICEIKIENE, I, OLSAKER, I, VIINALASS, H. AND EYTHORSDOTTIR, E (2005) Unfolding of population structure in Baltic sheep breeds using microsatellite analysis. Heredity 94, 448-456.

TAPIO, M, MICEIKIENE, I, VILKKI, J AND KANTANEN, J (2003) Comparison of microsatellite and blood protein diversity in sheep inconsistencies in fragmented breeds. Mol. Ecol. 12, 2045-2056

WASER P, STROBECK C (1998) Genetic signatures of interpopulation dispersal, Trends Ecol. Evol. 13, 43_44.

WEIR, B.S AND COCKERHAM, C (1984) Estimating *F*-statistics for the analysis of population structure. Evolution 38, 1358-1369.

ZORAQI. G (1991) Studimi i strukturës gjenetike të racave të deleve autoktone "Bardhoka" e "Shkodrane" me anë të markerëve gjenetikë polimorfë dhe lidhjet gjenetike midis racave me anë të markerëve gjenetikë polimorfë dhe lidhjet gjenetike midis racave e me tiparet prodhuese. *Dizertacion për marrjen e gradës shkencore "Kandidat i Shkencave"*.