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Genetic diversity using microsatellite markers in four Romanian autochthonous sheep breeds

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Abstract

Investigation of genetic relationship among populations was traditionally based on the analysis of allele frequencies at different loci. The four Romanian sheep breeds we have analyzed in this study are very important from an economical point of view, as they are raised for milk, meat and pelts. The aim of this study was to analyze, the genetic diversity and variability of four autochthonous Romanian sheep breeds (Botosani Karakul, Karabash, Palas Milk Line and Palas Meat Line) through the use of 11 ovine-specific microsatellite markers,. The microsatellite analysis revealed high allelic and gene diversity in all four breeds. Botosani Karakul breed showed the highest mean number of alleles (11. 6), while the highest value for polymorphic information content was observed for Palas Milk Line breed (0.83). The genetic differentiation among breeds was low, but significant (F_{ST}=0.082) and highlighted breed differentiation. Genetic distance estimates and phylogeny analysis highlights genetic differentiation between Botosani Karakul and Karabash breeds and the two synthetic lines.

Keywords: sheep, microsatellites, polymorphism, diversity.

Introduction

The need to maintain and improve local genetic resources has been recognized as a priority, at the European level. The maintenance of genetic diversity in livestock species requires the adequate implementation of conservation priorities and sustainable management programs, which should be based on comprehensive information regarding the structure of the populations, including sources of genetic variability among and within breeds.

In Romania there are two indigenous sheep breeds: Tsurcana and Tsigai. Both breeds are supposed to descend from the wild *Ovis vignei arkar*. In this study we have analyzed four domestic Romanian sheep breeds (Botosani Karakul, Karabash, Palas Milk Line and Palas Meat Line), which are farmed for milk, meat and pelts. In the out bringing off these four breeds the two indigenous breeds were used.

Microsatellite markers have often been used for genetic diversity studies, because of their large number, distribution throughout the genome, high level of polymorphism, co-dominant inheritance, neutrality with respect to selection and easy automation of analytical procedures (CANON & al. 2001 [1]). Microsatellite markers have been shown to be useful tools for the analysis of genetic differentiation among sheep populations as well as aids in conservation decisions for genetic resources (ARRANZ & al, 2001 [2] RENDO & al., 2004

[3], PETER & al. 2007 [4]). Here, we apply recent methodologies to analyze multi-locus genotype information from four domestic Romanian sheep breeds which will allow determining genetic differentiation among populations, thereby contributing to the knowledge of the historical relationships among them.

Materials and methods

Sampling and DNA extraction

A total of 161 individual blood samples were collected in EDTA-treated plastic vacutainers from different sheep breeds: Botosani Karakul breed (55), Karabash breed (38), Palas Milk Line breed (30) and Palas Meat Line Breed (38). The isolation of genomic DNA from fresh blood was performed with Wizard Genomic DNA Extraction Kit (Promega).

PCR multiplex

Six of the 11 microsatellite markers (OarFCB 20, OarCP34, MAF70, MAF33, MAF214, MAF65) used for the molecular analysis belong to the panel of 30 markers of the Econogene Project (http://www.econogene.eu/list_of_msmarkers.html). The other five microsatellite markers used in this study were: OarFCB11, OarCP20, BM143, MCM42 and HSC and were selected for the polymorphism content and the efficiency of amplification (BAUMUNG &al., 2006 [5]). The primer sequences for all 11 microsatellites are shown in Table 1.

Table 1. Primers used in this study.

Primer	Sequence	Chromosome localization	Dye
OarFCB11 F	GCAAGCAGGTTCTTTACCACTAGCACC	2	**NED**
OarFCB11 R	GGCCTGAACTCACAAGTTGATATATCTATCAC		
OarFCB20 F	GGAAAACCCCCATATATACCTATAC	2	**NED**
OarFCB20 R	AAATGTGTTTAAGATTCCATACATGTG		
OarCP34 F	GCTGAACAATGTGATATGTTCAGG	3	**FAM**
OarCP34 R	GGGACAATACTGTCTTAGATGCTGC		
MAF70 F	CACGGAGTCACAAAGAGTCAGACC	4	**FAM**
MAF70 R	GCAGGACTCTACGGGGCCTTTGC		
OarCP20 F	GGCATTTCATGGCTTTAGCAGG	21	**FAM**
OarCP20 R	GTTTGATCCCCTGGAGGAGGAAACGG		
MAF214 F	AATGCAGGAGATCTGAGGCAGGGACG	16	**PET**
MAF214 R	GGGTGATCTTAGGGAGGTTTTGGAGG		
BM143 F	ACCTGGGAAGCCTCCATATC	6	**PET**
BM143 R	CTGCAGGCAGATTCTTTATCG		
MAF33 F	GATCTTTGTTTCAATCTATTCCAATTTC	9	**PET**
MAF33 R	GATCATCTGAGTGTGAGTATATACAG		
McM42 F	CATCTTTCAAAAGAACTCCGAAAGTG	9	**VIC**
McM42 R	CTTGGAATCCTTCCTAACTTTCGG		
MAF65 F	AAAGGCCAGAGTATGCAATTAGGAG	15	**VIC**
MAF65 R	CCACTCCTCCTGAGAATATAACATG		
HSC F	CTGCCAATGCAGAGACACAAGA	20	**VIC**
HSC R	GTCTGTCTCCTGTCTTGTCATC		

For each set of primers we optimized the PCR conditions by varying the annealing temperature between 52-62˚C on a gradient thermocycler IQCycler (BioRad). After determining the optimum temperature of 59˚C, the amplification reactions were carried out in

a 25 µL final volume containing 1X PCR Buffer, $MgCl_2$, 200µM dNTPs, DNA template (50ng), 0.5 units of AmpliTaq Gold DNA Polymerase and nuclease free water. The quantity for each primer was adjusted based on the amplitude of peaks obtained in monoplex reactions. PCR amplifications were performed in 0.2 ml tubes using a 34 cycles program. Denaturation was performed at 95˚C (30 seconds), annealing at 59˚C (30 seconds) and extension at 72˚C (1 minute and 15 seconds). The first denaturation step was of 10 minutes at 95˚C and the final extension was of 60 minutes at 72˚C.

In order to amplify the microsatellite loci we performed two multiplex PCR reactions as follows: 3-Plex reaction for microsatellite markers OarFCB11, OarFCB20 and MAF 33 and 8-Plex reaction for the microsatellite markers OarCP20, OarCP34, MAF70, MAF214, MAF65, BM143, McM42, HSC.

In order to enable analysis on automated sequencers, forward primers were labeled using one of the following fluorescent compounds PET, VIC, 6-FAM and NED according to their measures (Table 1). The PCR products were submitted to fragment analysis by capillary electrophoresis, in an automated sequencer ABI310 (Applied Biosystems), using the Gene Scan–500 LIZ Size Standard, according to manufacturer specifications. The sizes obtained for each microsatellite in each breed are shown in Table2. The results were examined with the GeneScan 3.1.2 and Genotyper 2.5.2 Softwares (AppliedBiosystems).

Table 2. Analyzed loci and sizes obtained for each breed (bp).

Marker	Milk Line Palas	Meat Line Palas	Karabash	Botosani Karakul
OarFCB11	120-135	120-143	119-136	120-145
OarFCB20	90-114	88-108	86-102	91-108
OarCP34	107-116	108-119	106-119	101-119
MAF70	132-157	128-157	124-154	125-154
OarCP20	72-83	72-79	62-72	72-99
MAF214	185-194	190-194	190-227	192-232
BM143	98-116	98-115	98-107	98-113
MAF33	120-137	136-139	121-133	124-143
McM42	87-99	81-95	73-101	81-103
MAF65	125-138	125-140	124-139	123-135
HSC	262-285	268-284	263-295	263-296

Data analysis

The allele frequencies, mean number of alleles per locus, and population mean observed (*H*o) and expected (*H*e) heterozygosities were calculated with the Genetix 4.0.4 software (BELKHIR & al., 1996 [6]). The polymorphic information content (PIC) was calculated per locus, using the Cervus 2.0 software (MARSHALL & al., 1998 [7]). Exact tests for deviation from Hardy–Weinberg equilibrium (HWE) were applied using the Markov Chain Monte Carlo simulation (200 batches, 5000 iterations per batch, and a dememorization number of 10000) as implemented in GENEPOP version 3.4 (RAYMOND & ROUSSET, 1995 [8]). The Fstat 2.9.3 software (GOUDET & al., 1995 [9]) was employed in calculations of allelic richness (an estimation of mean number of alleles per locus corrected by sample size), gene diversity (NEI & al., 1987 [10]), and estimation of Wright's fixation index (F_{IT}, F_{IS} and F_{ST}) (WEIR & COCKERHAM, 1984 [11]). Nei's standard genetic distances among breeds were obtained and phylogenetic trees constructed by neighbour-joining methodology with the Populations 1.2.28 software (LANGELLA, 2002 [12]). Robustness of the dendrograms was assessed by bootstrap re-sampling procedures, using 1000 replicates.

Visualization of individual (allele sharing tree) and populations (radial tree) dendrograms were performed with the tree drawing software TreeView (PAGE, 2001 [13]).

Results and discussions

Genetic variability at microsatellite loci

All 11 loci were successfully amplified and a total of 197 alleles were detected; MAF70 showed the highest number of alleles per locus (30) while OarCP20 the lowest (11) (Table 3) with a global mean of 17.9±5.87 allele.

Table3. Number of alleles per locus.

Marker	Milk Line Palas	Meat Line Palas	Karabash	Botosani Karakul	Total
OarFCB 11	8	12	6	13	18
OarFCB 20	10	9	7	13	20
OarCP34	10	8	9	11	14
MAF70	11	16	6	18	30
OarCP20	6	7	3	9	11
MAF214	7	4	7	7	12
BM143	12	11	6	9	16
MAF33	7	3	7	15	17
McM42	5	4	10	10	17
MAF65	9	7	10	9	15
HSC	15	12	15	17	27
Total					197

The majority of the markers were found to have high polymorphism, with PIC ranging from 0.621 (OarCP20) to 0.86 (HSC). The mean gene diversity across loci was 0.732 indicating high level of information of the chosen microsatellite set (Table 4). Overall, the mean *H*e across loci was approximately 0.733±0.09, while the mean *H*o was 0.611±0.17. The value for *H*e was highest for HSC (0.87) and lowest for OarCP20 (0.52).

Table 4. Mean number of alleles (MNA), polymorphic information content (PIC), gene diversity, observed heterozygosity (*Ho*), expected heterozygosity (*He*), in four Romanian sheep breeds.

Marker	MNA	PIC	Gene diversity	H_o	H_e
OarCP20	5.4	0.621	0.531	0.35*	0.52
OarCP34	8.0	0.689	0.83	0.81	0.82
MAF 70	10.4	0.771	0.782	0.66*	0.76
MCM 42	6.2	0.718	0.737	0.67	0.72
MAF 65	7.6	0.743	0.774	0.72	0.76
HSC	12.2	0.861	0.892	0.67	0.87
OarFCB 20	8.6	0.8	0.813	0.77	0.79
OarFCB 11	8.2	0.77	0.786	0.79**	0.77
BM 143	8.2	0.736	0.775	0.51	0.75
MAF 214	5.4	0.668	0.665	0.43*	0.65
MAF33	6.6	0.676	0.707	0.34*	0.66
Mean	7.89	0.732	0.754	0.61	0.73

*p<0.05
**p<0.001

In a comprehensive study involving 57 breeds from 15 European and Middle Eastern countries, Peter *et al.* (2007) report global means for the number of alleles/locus and expected heterozygosity of 6.4 and 0.72, respectively, which are comparable to the levels of genetic diversity found in our study.

In order to test possible deviations from Hardy-Weinberg equilibrium, exact p-values for single breeds were pooled and all investigated breeds were in Hardy-Weinberg equilibrium. When the Hardy-Weinberg testing was performed for the loci, deviations from the Hardy-Weinberg equilibrium were found to be significant ($p<0.05$) in OarCP20, MAF70, MAF214, MAF33 and highly significant ($p<0.001$) in OarFCB11.

Table 5. Mean number of alleles (MNA), mean observed heterozygosity (*Ho*), mean expected heterozygosity (*He*), allelic richness and F_{IS} estimates per breed across 11 loci.

Breed	MNA	H_o	H_e	Allelic richness	F_{IS}
Karabasch	7.8	0.58	0.67	5.14	0.227
Meat Line Palas	8.5	0.72	0.77	4.54	0.125
Milk Line Palas	9.2	0.59	0.74	4.88	0.239
Botosani Karakul	11.6	0.67	0.79	4.73	0.16
Mean	9.275	0.64	0.74	4.82	0.187

Mean estimates of observed and expected heterozygosity over all loci and breeds were 0.64±0.06 and 0.74±0.05, respectively. The allelic richness was very similar in all breeds, with an overall estimate of 4.82±0.25 (Table 5).

Genetic differentiation

Population differentiation examined by fixation indices F_{IT}, F_{IS} and F_{ST} for each of the 11 analyzed loci across four Romanian ovine breeds are given in Table 5. Mean estimates of F-statistics obtained over loci (Weir 1990) were: F (F_{IT}) = 0.247± 0.13, f (F_{IS}) = 0.182± 0.13, θ (F_{ST}) = 0.082 ±0.039. The genetic differentiation among the analyzed breeds (F_{ST}) was 8, 2%.

Table 5. Estimators of F statistics at each locus across the four sheep breeds.

Locus	F_{IS}	F_{ST}	F_{IT}
OarCP20	0.327	0.143	0.423
OarCP34	0.031	0.015	0.045
MAF 70	0.126	0.086	0.201
MCM 42	0.089	0.062	0.145
MAF 65	0.076	0.054	0.126
HSC	0.251	0.048	0.287
OarFCB 20	0.045	0.073	0.115
OarFCB 11	0.060	0.117	0.170
BM 143	0.241	0.115	0.328
MAF 214	0.377	0.049	0.407
MAF33	0.386	0.139	0.471
Mean:	0.182	0.082	0.247

The matrix of Nei's standard genetic distances (D_S) among breeds is presented in Table 6 and the correspondent phylogenetic tree is presented in Figure 1. The genetic

distances between breeds ranged from 0.263 for Milk Line Palas and Meat Line Palas to 0.606 for Karabash and Meat Line Palas. The phylogenetic tree (Figure 1) provides a method of visualizing the genetic relationship between populations, with the values in the nodes of the tree indicating the proportion of 1000 replicates of the 11 microsatellites.

Table 6. Estimates of pairwise F_{ST} distances between the analyzed breeds (above diagonal) and Nei's standard genetic distances (below diagonal).

	Botosani Karakul	**Karabash**	**Milk Line Palas**	**Meat Line Palas**
Botosani Karakul	-	0.0855	0.0803	0.0811
Karabash	0.381176	-	0.1063	0.1256
Milk Line Palas	0.412315	0.459566	-	0.0493
Meat Line Palas	0.448552	0.606219	0.263112	-

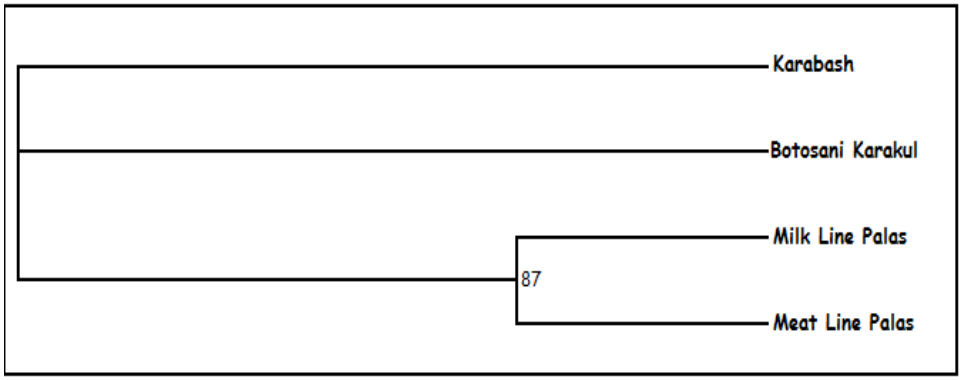

Figure 1. Neighbor-Joining phylogenetic tree based on Nei's standard genetic distances. Numbers indicate the proportion of bootstrap 1000 replicates.

The breeds included in this study are of big importance to the farmer community. The Palas Meat Line was formed after the inbreeding of Ile de France breed and Palas Merinos and Tsigai breeds, while the Palas Milk Line was formed after the inbreeding of Ostrize and Awassi breeds with Palas Merinos and Tsigai breeds. Both lines produced in 1973 are located in south-east part of Romania and are very important as they were formed in order to obtain a higher milk and meat productivity. Botosani Karakul was formed in 1944 after the inbreeding of Karakul breed from URSS and Germany with Romanian Tsurcana and recognized as breed in 1988. It is located in the north part of Romania and the main use of this breed is the production of pelts. The Romanian Blackhead Ruda, named by peasants Karabash, was first noticed in 1912. It is located in Teleorman county, on the left side of Danube. The two local sheep breeds (Tsigai and Tsurcana) and some foreigner breeds among which Berganmasca breed (TAFTA, 2008 [14]) contributed in its creation. It is a dual-purpose breed raised for milk and meat. The obtained tree revealed that the most closely related breeds were the two synthetic lines. Although the two local breeds (Tsurcana and Tsigai) participated in the creation of all analyzed breeds, clear differences can be noticed.

Conclusions

This study presents the first results regarding the characterization of genetic variability through molecular markers of four Romanian domestic sheep breeds. All the breeds revealed considerable genetic variation, based on their high mean number of alleles (average of 7.89

alleles/locus) and gene diversity (expected heterozygosity equal to or above 0.66, with exception of the OarCP20 locus).

From a genetic perspective, it is possible to support the contention that the Botosani Karakul and Karabash breeds are independent and lightly differentiated populations among themselves as well as from other sheep breeds.

In the future we intend to include the two indigenous sheep breeds (Tsurcana and Tsigai) in our studies in order to better evaluate the level of inbreeding and establish the appropriate conservation strategies aimed to avoid losses of genetic diversity. In addition, we want to evaluate the genetic relevance of Romanian sheep breeds at mtDNA level (cytochrome b and control region).

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