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Preliminary studies on genetic variability of chamois from Retezat massif

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Abstract The aim of the present work is to study of the genetic diversity among chamois eight populations in the central area of the Retezat massif. Laboratory analysis consisted of isolation and purification of total genomic DNA from dried feces, collected from eight different sites and identified as belonging to distinct populations (Aradeș, Secări, Zlata, Ștevia, Slăvei, Bucura, Cârjiț, Coșuri). The quantity and quality of isolated DNA were verified using the spectrophotometric method. The technique used is based on PCR (Polymerase Chain Reaction) and after obtaining the enzymatic reaction data, they were used to develop the matrix of genetic diversity and similarity matrices. A number of 14 polymorphic markers, eight ISSR markers and six DAMD markers were selected as to provide the best information but also a good amplification model as an indication of polymorphism. The result in establishing the relationship between populations was more accurate when data provided by the two markers are combined: In conclusion, it can be said that the analyzed chamois populations are distinct and only in exceptional conditions there is an exchange of genes between the physically isolated ones by long distance or habitat fragmentation.

Key words

chamois populations, molecular markers, genetic similarity, genetic divergence

The neutral genetic markers have become a very important tool for scoring populations genetic diversity of living species (Chambers et al, 2014). DNA sequences that may either be single copy (spacer DNA' between the coding regions of the genome) or exist in multiple copies, are being called repetitive DNA and can be found in the telomers of the chromosomes. The polymorphisms arising by this mechanism have been referred to as *mini* and *microsatellites* or variable number of tandem repeats (VNTRS) (Nakamura, 1987, Boldura et al, 2015a). VNTR markers are dispersed in the eukaryotic nuclear genome and their polymorphisms is resulting from the variations in the number of tandem repeats in a short core sequence (Bruford et al, 1998). Microsatellites are tandemly repeated motifs of 1–6 bases and can repeat from about 5–100 times at each locus. Minisatellites are tandemly repeating motifs of 8 to10 bases that can repeat from two to several hundred times at each locus being interspersed but often clustered in telomeric regions. Microsatellites are randomly spread throughout the genome and may frequently appear in transcription units. In this study two VNTR markers systems techniques were used: Inter simple sequence repeat markers (ISSR) (Zietkiwicz et al, 1994, Abdul-Muneer, 2014) - primers based on microsatellites are utilized to amplify inter-SSR DNA sequences, and Direct Amplification of Minisatellite DNA markers (DAMD-PCR), (Heath et al, 1993) order to detect the gene flow and the genetic relationship among the chamois populations.

Materials and Methods

Total genomic DNA was isolated and purified from 100 mg of dried feces collected from eight different locations and identified as belonging to distinct populations of chamois (*Rupicapra rupicapra L.*) using FastDNA SPIN Kit for Feces (MP Biomedicals, LLC QBioGene, Ohio,USA), (Table 1).

Table 1

Chamois samples analized in this study

No. crt.	Location
1	Rău Șes - Corciova
2	Rău Șes - Baicu
3	Rău Șes - Baicu
4	Rău Șes - Corciova
5	Tomeasa
6	Netiș
7	Lănciț
8	Coșuri

Isolated DNA quality and quantity of was assessed by specrophotometric method using a NanoDrop 8000, Spectrophotometer (Thermo Scientific). The final work DNA concentration was established at 50 ng/ul for each analyzed sample.

Two VNTR molecular markers systems: 16 - ISSR and 10 - DAMD, were used for assessing the potential genetic material exchange among populations (Table 2).

Table 2

Primers used in this study

No.crt.	Primer code	Sequence 5'…3'
ISSR molecular markers		
1	**UBC807**	**AGAGAGAGAGAGAGAGT**
2	UBC810	GAGAGAGAGAGAGAGAT
3	UBC814	CTCTCTCTCTCTCTCTA
4	UBC820	GTGTGTGTGTGTGTGTC
5	UBC824	TCTCTCTCTCTCTCTCG
6	**UBC827**	**ACACACACACACACACG**
7	UBC829	TGTGTGTGTGTGTGTGC
8	**UBC836**	**AGAGAGAGAGAGAGAGYA**
9	**UBC841**	**GAGAGAGAGAGAGAGAYC**
10	**UBC844**	**CTCTCTCTCTCTCTCTRC**
11	**UBC849**	**GTGTGTGTGTGTGTGTYA**
12	UBC852	TCTCTCTCTCTCTCTCRA
13	UBC829	TGTGTGTGTGTGTGTGC
14	**UBC857**	**ACACACACACACACACYG**
15	**UBC862**	**AGCAGCAGCAGCAGCAGC**
16	UBC867	GGCGGCGGCGGCGGCGGC
DAMD molecular markers		
11	**URP2F**	**GTGTGCGATCAGTTGCTGGG**
12	URP2R	CCCAGCAACTGATCGCACAC
13	URP6R	GGCAAGCTGGTGGGAGGTAC
14	**URP9F**	**ATGTGTGCGATCAGTTGCTG**
15	33.6	GGAGGTGGGCA
16	**14C2**	**GGCAGGATTGAAGC**
17	**M13**	**GAGGGTGGCGGCTCT**
18	**HBV 3**	**GGTGAAGCACAGGTG**
19	URP1F	ATCCAAGGTCCGAGACAACC
20	**YNZ22**	**CTCTGGGTGTGGTGC**

Single letter abbreviations for mixed-base positions: Y = (C, T), R = (A, G), B = (non A), H = (non G).

The primers listed in Table 2 were used in an initial screening study. The primers were selected from the collection of Molecular Genetics Laboratory of BUASVM from Timisoara, Romania. The primers that were selected for the actual genetic diversity study are highlighted in Table 2.

The final reaction volumes PCR was 25 µl and it contained 100 ng of DNA template. The composition of the master mix was carried out according the

producer instructions for GoTaqGreen PCR Master Mix (2X) commercial kit (Promega, USA). The reaction was performed on a DNA Engine Peltier Thermal Cycler (MJ Research, U.S.A.) and the PCR program consisted of a first denaturing step for 5 min at 94°C, followed by 45 cycles of denaturation at 94°C for 45 - 60 sec, annealing at 48°C- 55°C for 45 - 60 sec and extension at 72°C for 2 min, the final step of extension at 72°C for 5 min., according to literature (3, 4).

The PCR amplicons were run on 1.8 % agarose gels in TAE buffer at a constant voltage of 100 V for 100 minutes and were visualized and photographed under UV light (PhotoDocumentation System, *UVP, England*). The obtained data were analyzed with VisionWorksLC software (*UVP, England*) and based on identified alleles a binary matrix (a set of variables) was developed.

The Jaccard coefficient has been used to compare between this set of variables and to develop a similarity and a genetic diversity matrix. The program calculates and transforms similarity coefficients into distances and makes a clustering using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm.

Results and Discussions

Microsatellite molecular markers have been used in similar studies (Ichikawa et al, 2001, Boldura et al, 2015b) and also VNTR marker systems have been used successfully in studies on the genetic diversity of other mammals (Boldura et al., 2017). Due to the lack of information on the use of the two VNTR molecular marker systems, the first step of our study was initial screening with 26 primers designed to target each of the two molecular marker systems. The goal of this step was to select those markers that would provide the most informative data and a good amplification model as the indication of the polymorphism. The DNA matrix for this study consisted of a mixture made of each AND sample. For each set of primers the PCR amplification mix was performed separately. Based on the result obtained, a number of 14 primers, eight ISSR markers and six DAMD markers were considered as a valuable choice for the subsequent genetic diversity study (Table 2). Primers that gave a small number of amplicons were eliminated from the experiment. Therefore, the DNA fingerprint study was developed based on the 14 marker system. After selection of the primers, separate PCR amplifications were performed which target the selected molecular markers for each individual under study. The raw data obtained are summarized in Table 3.

In this experiment, using data provided from 14 polymorphic molecular markers, a binary matrix of 307 scored PCR amplicons, from which 299 were polymorphic, was constructed. ISSR primers yielded the highest number of amplicons, compared to DAMD primers, and overall they exceeded also in terms of polymorphic band percentage (Table 3). As revealed by the obtained data those markers can be successfully used in establishing genetic diversity among distinct population.

Analyzing the obtained matrixes it can be observed that the populations are very distinct from the genetical point of view, all the obtained values were around the medium value or above.

Nevertheless, when data provided from the two molecular markers are combined, the result is accurate and precise, in establishing the relationship among related populations. From the data resulted from the analyses of PCR results a binary matrix for each individual was developed. The binary matrix was used in assessing the similarity matrix and the genetic distance matrix using the Jaccard similarity coefficient (Table 4, 5).

Table 3

Data collected in DNA fingerprinting experiment

Primer	**Sequence 5'…3'**	Fragment size range (bp)	Fraction polymorphic fragments
UBC807	AGAGAGAGAGAGAGAGT	**1174 - 240**	**30 / 30**
UBC827	ACACACACACACACACG	**1054 - 265**	**23 / 23**
UBC836	AGAGAGAGAGAGAGAGYA	**1335 - 250**	**22 / 22**
UBC841	GAGAGAGAGAGAGAGAYC	**1225 - 117**	**20 / 20**
UBC844	CTCTCTCTCTCTCTCTRC	**1210 - 220**	**21 / 21**
UBC849	GTGTGTGTGTGTGTGTYA	**1436 - 126**	**24 /22**
UBC857	ACACACACACACACACYG	**1138 - 240**	**18 / 17**
UBC862	AGCAGCAGCAGCAGCAGC	**1230 - 185**	**20 /20**
URP2F	GTGTGCGATCAGTTGCTGGG	**1550 - 285**	**19 / 17**
URP9F	ATGTGTGCGATCAGTTGCTG	**1690 - 195**	**20 /19**
14C2	GGCAGGATTGAAGC	**1883 - 210**	**24 /24**
M13	GAGGGTGGCGGCTCT	**1270 - 170**	**22 /22**
HBV 3	GGTGAAGCACAGGTG	**1595 - 300**	**21 /20**
YNZ22	CTCTGGGTGTGGTGC	**1532 - 220**	**20 / 19**
Total			**307 / 299 (97.4%)**

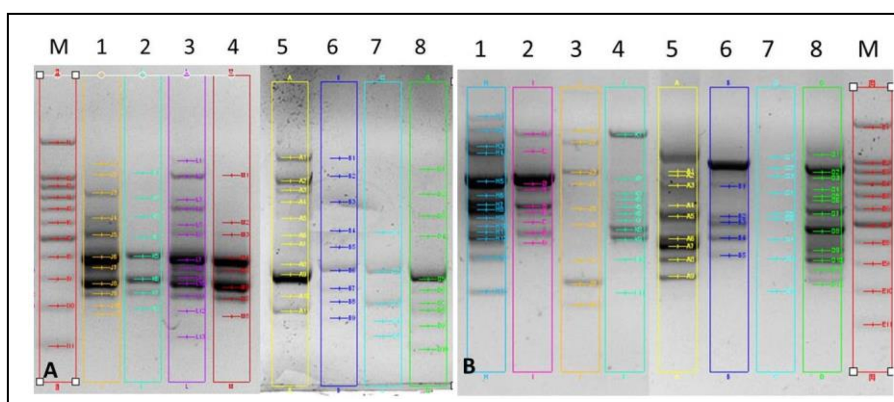

Fig.1: DNA fingerprint of chamois populations obtained using: panel A: ISSR primer UBC 857 and panel B: DAMD primer HBV 3; 1 - 8 chamois populations, M - Molecular weight marker - 100 bp DNA Ladder RTU, GeneDirex.

Table 4

Genetic distance matrix of the studied eight chamois populations

	1	2	3	4	5	6	7	8
1	0	0.599	0.656	0.435	0.667	0.696	0.717	0.63
2		0	0.411	0.633	0.624	0.704	0.763	0.696
3			0	0.646	0.581	0.684	0.73	0.64
4				0	0.656	0.709	0.709	0.683
5					0	0.633	0.701	0.588
6						0	0.657	0.655
7							0	0.447
8								0

The genetic distances are used to approximate the genetic divergence between analyzed populations. Analyzing the obtained matrix it can be observed that the chamois populations are poorly related, as all the obtained values were around or above the medium threshold. The minimum value of 0.411 was obtained in this case is between the populations from „ Rău Șes - Baicu" locations, meaning that the genes exchange is more likely to happen between the populations that are sharing the same territories. The higher value of 0.763 is recorded between populations „ Rău Șes - Baicu" and „Lănciț".

Table 5

Similarity matrix of the studied eight chamois populations

	1	2	3	4	5	6	7	8
1	1	0.401	0.344	0.565	0.333	0.304	0.283	0.37
2		1	0.589	0.367	0.376	0.296	0.237	0.304
3			1	0.354	0.419	0.316	0.27	0.36
4				1	0.344	0.291	0.291	0.317
5					1	0.367	0.299	0.412
6						1	0.343	0.345
7							1	**0.553**
8								1

The similarity coefficients represent the degree of relation among the studied individuals, and are expressed with values ranging from 0 to1. Considering a medium value of 0.5 the data are interpreted as it approaching to the lower extreme value, meaning that the degree of genetic similarity among analyzed population is very reduced. The highest value of 0.553 is recorded between population „ Lănciț" and „ Coşuri", and the lowest of 0.237is recorded between population „ Rău Șes - Baicu" and „ Lănciț".

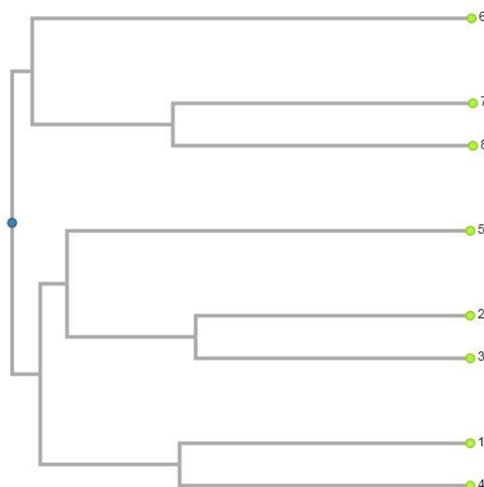

Fig. 3: UPGMA Dendrogram of *eight chamois populations* created by DendroUPGMA program using data from two molecular markers systems

Based on genetic similarity and genetic distance matrices, a UPGMA genetic dendrogram was developed (Figure 3). Similarity coefficients have been used to group populations in clusters. The size of clusters is dictated by genetic distance coefficients. The eight populations are grouped into two major groups, first consisting of three populations, namely: Netiș, Lănciț and Coșuri, those being also separated in two distinct sub-clusters. The second cluster contains populations from the locations: Rău Șes – Corciova, Rău Șes – Baicu and Tomeasa, those being also grouped in two sub-clusters. Various researches have been monitoring chamois population, as well as other mammals with genetic methods using non-invasive DNA samples (feces and hair) obtaining similar results (Ichikawa Y.et al 2001, Perez et al, 2002; Boldura et al, 2017).

Conclusions

The eight chamois analyzed populations from Retezat massif are very distinct from the genetic point of view, all values being around the average or higher. Based on the genetic similarity and genetic distance matrices, the UPGMA gene dendrogram reveals that the populations are grouped into two major but well isolated groups. The first group consists of three populations: Bucura, Cârjit and Coșuri and the second group of five populations Slăvei, Secari, Zlata, Aradeș and Ștevia, each being also separated into two subgroups in direct relation with their spatial distribution and habitat fragmentation. In conclusion, the present study shows the role of habitat spatial distribution and fragmentation in the genetic variability of chamois populations from Retezat massif.

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