POLIMORPHISM SCREENING USING A VNTR MOLECULAR MARKER SYSTEM IN ROMANIAN RESIDENT BISON INDIVIDUALS.

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Abstract:*Genomic DNA isolated from the blood sampled from 14 individuals of Bison bonasus was used in order to obtain a molecular fingerprint and based on that, to obtain a dendrogram exhibiting the phylogentic relationship among those individuals. By using the PCR technique and an VNTR (Variabile Number of Tandem Repeats molecular markers system: ISSR (Inter simple sequence repeats) was used in this study for establish set of DNA fingerprints. Based on molecular data a common binary matrix was developed. By using DendroUPGMA software, based on Jaccard coefficient the similarity indices and the genetic distances were calculated. Based on those data set a dendrogram containig the phylogenetic relationship among bison exemplars, was generated. By analyzing the molecular data it was observed that the degree of polymorphism was either absent or very low especially in this case, our recorded data suggest that among Romanian resident Bison exemplars, there is a high level of genetic similarity meaning that the individuals are most probably close blood relatives.*

Key words : *polimorphism screening, variabile number of tandem repeats*

Genomic DNA isolated from the blood sampled from 14 individuals of Bison bonasus was used in order to obtain a molecular fingerprint and based on that, to obtain a dendrogram exhibiting the phylogentic relationship among those individuals. By using the PCR technique and an VNTR (Variabile Number of Tandem Repeats molecular markers system: ISSR (Inter simple sequence repeats) was used in this study for establish set of DNA fingerprints. Based on molecular data a common binary matrix was developed. By using DendroUPGMA software, based on Jaccard coefficient the similarity indices and the genetic distances were calculated. Based on those data set a dendrogram containig the phylogenetic relationship among bison exemplars, was generated. By analyzing the molecular data it was observed that the degree of polymorphism was either absent or very low especially in this case, our recorded data suggest that among Romanian resident Bison exemplars, there is a high level of genetic similarity meaning that the individuals are most probably close blood relatives.

The wisent or European bison (Bison bonasus L.), was common in the Carpathian eco-region (about 210,000 km2 of mountain area) in Medieval Ages, it disappeared by late 18th century, due to overhunting and gradual habitat loss (Erlich and Erlich, 1981).). In the early twentieth century, only 54 individuals survived in the whole world population (Pucek, 1991). First attempts for its re-introduction are going back to the 1960s (Poland and Ukraine). In the late 1990s a restitution project was initiated and had involved five countries (Poland, Slovakia, Ukraine, Romania and Hungary) (Kajetan Perzanowski and Wanda Olech 2013). To date the specie accounts about 3600 exemplars with a high degree of inbreeding, as being

the descendants of only 12 individuals. Almost 40% live in small groups in captivity, and the rest live in a few, isolated, free-ranging and semi-free herds (Kajetan Perzanowski and Wanda Olech, 2007, Luenser, K). The genetic diversity among wisent populations is very limited making them vulnerable to diseases, affecting fertility and thus keeping the specie in the vulnerable estate (Małgorzata Tokarska, 2011, Radwan, J.,2010) . The loss of genetic variability can be prevented by allowing exchange of genes among sufficiently large numbers of animals (Kajetan Perzanowski and Wanda Olech, 2007, Tokarska, M 2009). All programs of re-establishing viable populations in the Carpathians are based on the genetic analysis of formerly released animals and of the ones to be released. (Wanda Olecha, Kajetan Perzanowski 2002).

The study presented in this paper is part of the genetic diversity scoring attempt of wisent individuals that were to be released in the in a protected area located Caras Severin county, Romania. Neutral genetic markers are an important tool for scoring genetic diversity and the similarity of living species. (Chambers GK, 2014) The molecular markers used in our DNA fingerprinting by PCR method experiments, in order to reveal the genetic diversity, were a type of VNTR (Variable Number of Tandem Repeats) molecular markers systems: namely ISSR (Inter simple sequence repeats). Inter simple sequence repeats (ISSRs) are dominant markers located between microsatellite sites in the genome, are often highly polymorphic at the species level and require no prior sequence information (Claes Ramel,1997; Heath DD,1993). Using VNTR markers, information about the population structure can be obtained and also spatial distribution, parentage, and movements of wisent small herd individuals. Those information are of high importance in re-constructing, preserving and protecting the European bison in natural environment. Obtained data were used to develop genetic diversity and similarity matrices that further allowed the construction of a genetic relationships dendrogram.

MATERIAL AND METHODS

Total genomic DNA was isolated and purified from 300 µl of total blood samples of 14 Bison bonasus L. individuals provided by World Wide Fund Romania the identity of individuals being their propriety. The quality and quantity of DNA was assessed by specrophotometric measurement (*NanoDrop 8000, Thermo Scientific*). ISSR molecular markers were chosen for the genetic diversity assessment. For the initial screening a set of 15

Table 1. ISSR primers sequences used in initial screening

primer code	**Sequence 5'...3'**
UBC810	GAGAGAGAGAGAGAGAT
UBC829	TGTGTGTGTGTGTGTGC
UBC834	AGAGAGAGAGAGAGAGYT
UBC836	**AGAGAGAGAGAGAGAGYA**
UBC840	**GAGAGAGAGAGAGAGAYT**
UBC 843	**CTCTCTCTCTCTCTCTRA**
UBC854	TCTCTCTCTCTCTCTCRG
UBC857	ACACACACACACACACYG
UBC880	**GGAGAGGAGAGGAGA**
UBC873	GACAGACAGACAGAC A

UBC885	**HBHAGAGAGAGAGAGAG**
UBC 886	VDVCTCTCTCTCTCTCT
A 12	**GAGAGAGAGAGACC**
A 21	**CACACACACACAAC**
A 17	**GACAGACAGACAGACA**

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

From those primers, based on the quality of amplification products, nine primers were chosen (marked in bold character, Table 1) to be used in scoring the genetic diversity.

PCR amplifications were carried out in volumes of 25 µl using 75 ng of DNA template. The composition of amplification mixtures were carried out following the producer instructions for GoTaq Green Master Mix (*Promega*, USA). The reactions were 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 95°C for 45 sec, annealing at 48°C- 55°C for 45 sec and extension at 72°C for 2 min, the final step of extension at 72°C for 5 min., according to literature data (Pharmawati Made et al., 2005).

PCR products were run on 1.8 % agarose gels in TAE buffer at room temperature at a constant voltage of 100 V for 90 minutes. The PCR products were visualized and photographed under UV light (PhotoDocumentation System, UVP, England). The obtained data were analyzed with VisionWorksLC software.

The genetic diversity and similarity matrices and the dendrogram were assessed from a set of variables by using DendroUPGMA (http://genomes.urv.cat/UPGMA/index.php), software. The program calculates a similarity matrix and transforms similarity coefficients into distances and makes a clustering using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm.

RESULTS AND DISCUSSIONS

Specific literature data does not contain information to state that VNTR markers systems were used in scoring genetic diversity and to molecular fingerprint the wisent population in our country. Considering this issue the first step of our study was a screening of ISSR molecular markers in order to choose those markers that will provide the most informative data. A set of 15 ISSR primers were chosen for an initial screening, in order to select polymorphic primers suitable to be used further in the experiment. A bulked sample consisting of 5 µl of each individual DNA was used as template for PCR amplification. For this screening experiment, a Gradient-PCR program, with annealing temperatures ranging from 47°C- 53°C was used. Based on the obtained screening result, 10 ISSR primers were selected to be used in the first step of the experiment (Figure 1), (Table 2).

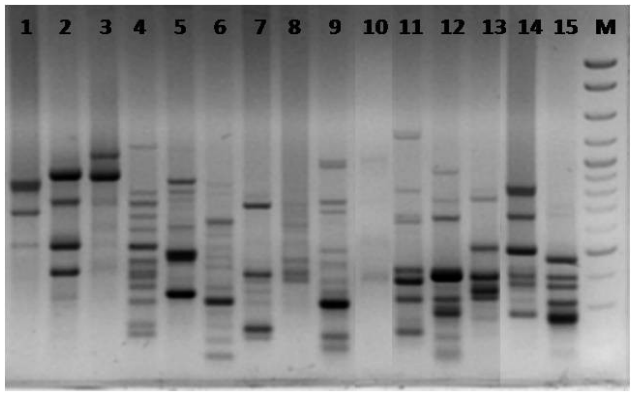

Fig. 1: PCR based screening with 15 ISSR primers:1 –UBC810, 2 –UBC829, 3 –UBC834, 4 - UBC836, 5 - UBC840, 6 - UBC843, 7 - UBC854, 8 - UBC857, 9 - UBC880, 10 - UBC873, 11 - UBC885, 12 - UBC886, 13 – A 12, 14 –A 21,15 – A 17, M- Molecular weight marker - GeneRuler Express DNA Ladder (*Fermentas*).

From the randomly selected ISSR primers only those that yielded a DNA fingerprint of good quality in terms of amplified sequences number and well defined PCR product (Figure 1), were considered as a valuable choice for further studies. Primers that yielded faint or low number of amplicons were eliminated from the experiment. Therefore, the DNA fingerprinting study was developed on the basis of 9 markers system (Figure 2, 3), (Table 2).

As o result of the genomic omogenity among studied individuals, four of used primers, namely A 17, UBC 880, UBC 843,UBC 886, did not reveal any polymorphism, in terms of amplified fragments, therefore their results were eliminated from the study (Figure 3). The others five primers: A 12, A 21, UBC 836, UBC 840 and UBC 885 provided enough data for the genetic diversity to be scored and a dendrogram to be developed (Table 3).

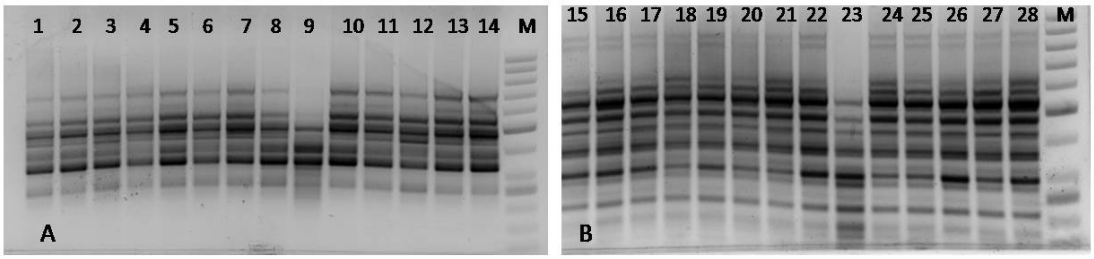

Fig. 3: Non- polymorphic DNA fingerprint obtained using ISSR primer: UBC 843 (panel A) and UBC 886 (panel B): 1 – 14; 15-28: wisent individuals; M - Molecular weight marker - GeneRuler Express DNA Ladder (Fermentas).

By using the primer A 12, a number of 16 amplicons were obtained with dimensions raging from 1800 to 210 base pairs, from which only two were polymorphic; both are situated in the area of low molecular weight fragments (Figure 2). Primer A 21 yielded 18 amplicons from which 11 were polymorphic, covering all areas of the molecular weights (Figure 3).

Table 3. Molecular markers and data collected in DNA fingerprinting experiment

Primer	**Sequence 5'…3'**	Fragment size range (bp)	Fraction polymorphic fragments
A 12	GAGAGAGAGAGACC	**1800 - 210**	**16 / 2**
A 17	GACAGACAGACAGAC A	-	-
A 21	CACACACACACAAC	**1810 - 205**	**18 - 11**
UBC836	AGAGAGAGAGAGAGAGYA	**1135 - 150**	**17 - 7**
UBC840	GAGAGAGAGAGAGAGAYT	**1450 - 160**	**19 / 13**
UBC880	GGAGAGGAGAGGAGA	-	-
UBC885	HBHAGAGAGAGAGAGAG	**1240 - 250**	**16 / 6**
total			**86 / 39 (45.3%)**

17 bands could be scored for primer UBC 836, with seven showing polymorphism. The polymorphic fragments are situated in the area of heavy and low molecular weight. For the primer UBC 840 19 amplicons were scored from which 13, most of them in the area of medium molecular weight fragments, are polymorphic. Primer UBC 885 yielded a total number of 16 amplified fragments. The six polymorphic bands were situated in all areas of the molecular weights.

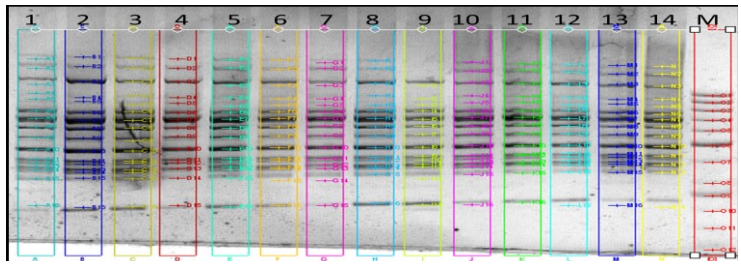

Fig. 2: DNA fingerprint obtained using ISSR primer: A 12: 1 – 14:wisent individuals; M - Molecular weight marker - GeneRuler Express DNA Ladder (Fermentas).

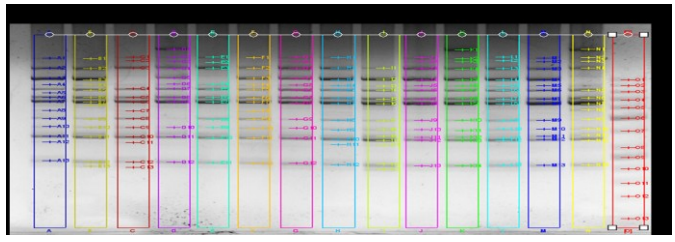

Fig. 3: DNA fingerprint obtained using ISSR primer A 21 : 1 – 14 wisent individuals; M - Molecular weight marker - GeneRuler Express DNA Ladder (Fermentas).

Data collected from all ISSR primers were used to develop a binary matrix of 86 scored PCR products, from which 39 were polymorphic. This matrix consisted of scores (1 for present and 0 for absent) for each of the 14 individuals DNA fingerprint obtained by using the 14 primers. The binary matrix was uploaded in the software as complete set of variables. The Jaccard coefficient has been used to compare among this set of variables and further, to construct the genetic distance and similarity matrices (Table 4, Table 5).

The genetic distances are used to approximate the genetic divergence between analyzed individuals. Thus, a value of 0 means the lack of genetic divergence, obtained only in the case when comparing an individual to itself, and the value of 1 which is a theoretical maximum threshold. The medium value is around 0.5. Analyzing the obtained matrix it can be observed that the wisent individuals are very closely related, as expected all the obtained values were beneath the medium threshold close to the lower value. The minimum value of 0.077 was obtained in this case is between individual „12" and „13". The higher value of 0.280 is recorded between individuals „14" and „9".

Table 4. Genetic distance matrix of the studied 14 wisent individuals

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0	0.154	0.165	0.192	0.167	0.120	0.190	0.169	0.145	0.184	0.217	0.195	0.198	0.244
2		0	0.141	0.215	0.212	0.192	0.190	0.169	0.192	0.208	0.195	0.195	0.175	0.200
3			0	0.203	0.154	0.156	0.130	0.156	0.203	0.218	0.183	0.160	0.207	0.210
4				0	0.133	0.184	0.133	0.184	0.253	0.224	0.165	0.188	0.212	0.259
5					0	0.108	0.107	0.133	0.205	0.173	0.115	0.091	0.141	0.212
6						0	0.108	0.135	0.184	0.176	0.165	0.165	0.167	0.192
7							0	0.108	0.228	0.197	0.115	0.139	0.165	0.190
8								0	0.160	0.200	0.141	0.165	0.190	0.237
9									0	0.224	0.232	0.210	0.235	**0.280**
10										0	0.132	0.156	0.158	0.184
11											0	0.076	0.077	0.127
12												0	**0.077**	0.173
13													0	0.104
14														0

Table 5. Similarity matrix of the studied 14 wisent individuals

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	1	0.846	0.835	0.808	0.833	0.880	0.810	0.831	0.855	0.816	0.783	0.805	0.802	0.756
2		1	0.859	0.785	0.787	0.808	0.810	0.831	0.808	0.792	0.805	0.805	0.825	0.800
3			1	0.797	0.846	0.844	0.870	0.844	0.797	0.782	0.817	0.840	0.793	0.790
4				1	0.867	0.816	0.867	0.816	0.747	0.776	0.835	0.812	0.787	0.741
5					1	0.892	0.893	0.867	0.795	0.827	0.885	0.909	0.859	0.787
6						1	0.892	0.865	0.816	0.824	0.835	0.835	0.833	0.808
7							1	0.892	0.772	0.803	0.885	0.861	0.835	0.810
8								1	0.840	0.800	0.859	0.835	0.810	0.762
9									1	0.776	0.768	0.790	0.765	**0.720**
10										1	0.868	0.844	0.842	0.816
11											1	**0.924**	0.923	0.873
12												1	0.923	0.827
13													1	0.896
14														1

The similarity coefficients represent the degree of relation among the studied individuals, and are expressed with values raging from 0 to1. The absolute similarity is represented by 1, the lack of it being represented with 0 values. Considering a medium value of 0.5 the data are interpreted as it approaching to the mentioned extremes.

As in the case of genetic distance, the obtained data quite resembled. All the coefficients are above the medium value. The highest value of 0.924 is recorded between individuals „11" and „12", and the lowest of 0.720 is recorded between individuals „9" and „14".

On the basis of resulted matrices an UPGMA dendrogram was designed (Figure 4). The similarity coefficients are used to group the individuals in clusters. The length of the clusters is being dictated by the genetic distance coefficients. The dendrogram illustrates the genetic relationship among studied wisent individuals. Dendrogram analyses revealed that the 14 individuals are grouped in two different major clusters. The first cluster contains the majority of individuals from „1" to „9", individual „4" being the most distinct among them as it is clustered separately. The most related individuals seem to be „5" and „7". In the second major cluster the most distinct individual is „10" and the more related are individuals „11" and „12". Individual „13" is also very close to the cluster of the previous two. Overall, the obtained data suggest, as expected, a high degree of similarity among the analyzed wisent individuals. As specific literature data state, this situation is of concern for all wisent herds that live in semi-wild environment all cross Europe protected areas. Genetic diversity studies are helpful for generating the population of a new small herd and also, can provide useful information in sustaining artificial exchange of individuals between those herds.

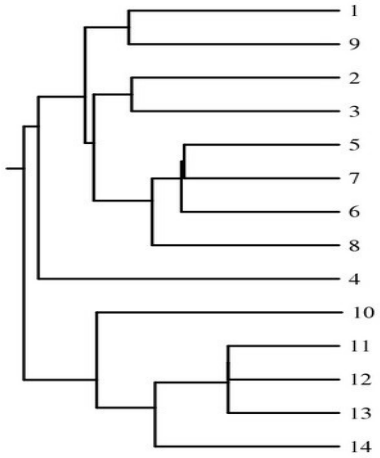

Fig. 4: Genetic distance Dendrogram of *Bison bonasus L.* individuals created by DendroUPGMA program using data from 5 ISSR molecular markers.

CONCLUSIONS

This paper presents a case study with the goal of developing a cheap molecular markers system that can become an important tool of fingerprint wisent individuals, thus combating severe inbreeding and, if applied on all individuals, can be used in scoring genetic diversity and phylogeny studies.

The proposed ISSR markers were proved to be reliable and provided accurate information in terms of establishing an individual molecular fingerprint but also in clustering individuals according to the degree of genetic similarity, despite the fact that there some non-informative molecular markers were anccounted. Even so, most accurate data will be obtained by similar studies with the help of an increased number of molecular markers systems. The developed similarity and genetic distance matrices differentiated among studied individuals according to their genetic inheritance. In order to develop the proposed method and further to create a genetic data base, higher number of VNTR markers systems must be analyzed in similar studies.

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