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GENETIC MONITORING OF POPULAR GAME SPECIES IN HUNTING AREAS OF VOJVODINA

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Summary: The aim of this paper is to present comparative data of genetic monitoring of two popular game species in Vojvodina, brown hares and wild boars. Determination and monitoring of genetic variability in game species is the basis for adequate ecological management and biological conservation. First determination of genetic diversity in brown hare populations from Vojvodina recommended optimal three-year monitoring period for these populations. Continual monitoring of brown hares from Vojvodina included analyses of three commonly used microsatellite markers. New alleles were determined in all three analyzed loci and there was no population substructuring revealed. The current status of genetic variability in wild boar populations from Vojvodina was analyzed using five microsatellite loci. All loci presented high degree of polymorphism and some level of genetic structure was found.

Key words: brown hare, wild boar, genetic monitoring

Introduction

One of the main aspects in continual monitoring of game species in hunting areas is genetic monitoring. This approach represents organized screening of game populations by pre-defined molecular markers and standardized protocols, in order to determine the level of genetic variability within population, genetic structure of population, level of inbreeding etc., and these data are necessary in order to predict possible events and to establish an adequate response to negative effects in the process of hunting and ecological management. The determination of appropriate molecular markers is essential in order to obtain data of practical interest such as degree of inbreeding, presence of population fragmentation, number of migrants, population site estimation, etc.

With the development of molecular markers and technology, different classes of molecular markers were used in characterization of game species in Vojvodina. The brown hare (*Lepus europaeus*) occurs throughout large parts of Europe and constitutes an important game species in agricultural areas, open woodland and grassland up to 1500m. At the end of 1950s, it was the most numerous game species in Vojvodina, with numbers between 400,000 and 500,000. Since then, environmental changes caused a rapid population decline to about 200,000 hares within only ten years [1]. In the face of the marked environmental changes for hares and their regional population declines in large parts of Europe, maintenance of genetic resources of locally adapted populations is considered important for the long-term development of this species. Hares do not only play an important role for the hunting economy of the Vojvodina, but they also represent a significant prey species particularly in the agrosystem of the region, and they contribute to the flow of organic matter and nutrients: for instance, assuming a density of 50 hares per 100 ha, we might expect over one ton of dry weight of dung per year. Brown hare populations from Vojvodina were the first that were analyzed and first markers employed were allozymes [2], [3]. These analyses revealed shallow gene pool divergence among populations, and low level of substructuring, which defined the Danube river as a main geographical barrier to gene flow. Due to technical limitations and low informativeness, allozymes were not employed in a continual monitoring of these populations, while microsatellites as the molecular markers of new generation were introduced. The first screening of brown hare populations from Vojvodina was done in 2006 [4], and the analysis revealed higher level of substructuring among populations. One of the recommendations after this research was determination of genetic variability in defined time period of three years. In the following screening of same populations after three years, additional microsatellites were analyzed together with previous ones [5], [6], and new alleles enabled additional improved definition of genetic structure of analyzed populations. Furthermore, mitochondrial DNA markers were also analyzed, primarily for phylogenetic analyses of named populations, but also as additional marker for population genetic study [7], [4], [8].

Furthermore, the genetic screening of game species in hunting areas of Vojvodina were extended to other popular game species, since the interest was pointed to wild boar populations. The wild boar is one of the most abundant terrestrial mammals in hunting areas in Vojvodina and it is an important wildlife species, in both economical and ecological terms. Across Europe, during the past, wild boar populations passed through local extinctions and translocations, but nowadays they are expanding throughout Europe at the fast pace and management is urgently required [9]. Understanding how past and recent events affected the genetic structure of this species represents the basis for future adequate management strategies. The first characterization of wild boars in Vojvodina included microsatellite analysis in the captive wild boar population from the Danube region [10],[11],[12]. Loci SW251 and

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SW2429 were successfully amplified in wild boars for the first time, showing adequate level of polymorphism. Obtained results showed that level of genetic variability in wild boar population from Vojvodina is at the level for the wild boar populations in European continent. Later research of wild boar population from the Podunavlje-Podravlje hunting area (located in the triangle between the Danube and Drava rivers) showed high level of gene diversity within analyzed population [13]. The description of genetic structure of this population was the first step towards characterization of wild boars in the West Balkan region, which is a major point in the development of conservation and management strategies. In order to get better insight into nuclear gene pool diversity, three wild boar populations (Vojvodina, Slavonia and Bosnia) from the extended region of the West Balkan was later analysed and the existence of some level of population structure was found [14].
From the conservation biology aspect, it is very important to define all present molecular variants in a population, since sometimes, the increase of the population number does not actually reflect good fitness of the population. In the large population with significantly increased homozygosity negative anthropogenic or any other effect may cause drastic changes such as in small populations.
The aim of this paper is to present comparative data of genetic monitoring of two popular game species in Vojvodina, brown hares and wild boars.

Material and methods

A total number of 41 brown hares was collected from three different regions in Vojvodina during hunting seasons in period 2004-2006, while 60 individuals were collected from the same region in hunting season 2009. Muscle tissue samples of total 67 wild boars were collected from the same three regions in hunting areas in Vojvodina. The following 3 regions, subsequently termed "populations", were included in genetic analyses: Bačka, Banat and Srem. Sampled tissue was frozen immediately after the death of animal.

Total DNA was extracted from liver and tongue tissue of hares and muscle tissue of wild boars using standard phenol chloroform isoamylalcohol extraction with proteinase K digestion [15]. In hares, eleven microsatellite loci with different levels of polymorphism were analyzed Sat2, Sat5, Sat12 [16], Sol03, Sol08, Sol28 [17], Sol33 [18], Lsa1, Lsa2, Lsa3 and Lsa8 [19] , in first data set, while in the second data set 3 most informative loci were employed: Sat2, Sat5 and Sat12. In wild boars, five microsatellite loci previously shown as high polymorphic were selected S0068, S0005 [20], SW251, SW857 [21], SW2429 [22].
The PCR conditions for primer pairs for loci Sat2, Sat5 and Sat12 were performed in 20µl volume with final concentrations 100ng of DNA template, 1xBuffer (with 15mM $MgCl_2$), 200µM dNTP, 10 pmol of each primer, 1 mM $MgCl_2$ and 1 unit of Taq DNA polymerase. Amplification reaction for primer pairs for loci S0068, S0005, SW251, SW2429 and SW857 was performed in total volume of 20µl, with 10pmol of each primer, 100µM dNTPs, 1x *Taq* buffer, 1 U *Taq* DNA polymerase, 2.5 mM $MgCl_2$ and 100ng of genomic DNA. Amplified PCR products were analysed on 6% denaturating polyacrylamide gel and detected by silver staining [15].
Comparison of genetic variability in brown hares in two hunting seasons was performed based on three mutual loci analyzed. Microsatellite loci in brown hares and wild boars were tested for deviation from Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium using the Markov chain method in GENEPOP version 3.4 [23]. Allele frequencies, mean number of alleles (A), observed (Ho) and expected (He) heterozygosity were calculated for each locus and for each population with GENETIX [24]. This program was also employed for calculating inbreeding coefficients (F_{IS}) in wild boar populations.

Results and discussion

Numbers of alleles per locus (A), numbers of unique alleles, and observed (Ho) locus-specific heterozygosity per population of brown hare for seasons 2006 and 2009 are given in Table 1.

Table 1. Basic parameters of genetic variability at 3 microsatellite loci in brown hare (*Lepus europaeus*) populations from Vojvodina

Year	2006			2009		
Locus	Sat2	Sat5	Sat12	Sat2	Sat5	Sat12
A	18	8	11	23	18*	8
Unique alleles	2	1	6	7	11*	3
Ho Bačka	0.692	0.280	0.708	0.941	0.556	0.737
Ho Banat	0.577	0.091	0.720	0.842	0.833**	0.824
Ho Srem	0.739	0.150	0.869	0.950	0.733**	0.945
Ho Vojvodina	0.680	0.146	0.744	0.911*	0.707*	0.835

* $p< 0.05$ ** $p< 0.01$; A – number of alleles per locus; Ho – observed heterozygosity

A total number of 37 alleles at three microsatellite loci were found in brown hare populations from Vojvodina analyzed in 2006, while higher number of 49 alleles was detected in populations from the same region analyzed in 2009. In analyses performed in 2009 new alleles were determined at all three loci. The higher numbers of alleles were detected at Sat2 and Sat5 loci, but the statistical significance was proven just for Sat5 locus. The number of unique alleles was also significantly higher for Sat5 locus (1 unique allele in 2006 and 11 unique alleles in 2009).Observed locus-specific heterozygosity per population varied from 0.091 (*Sat5* locus in Banat population) to 0.869 (*Sat12* locus in Srem population) in season 2006, while in season 2009 this values ranged from 0.556 (*Sat5* locus in Bačka population) to 0.95 (*Sat2* locus in Srem population). In season 2009, significantly higher Ho was detected at *Sat5* locus in populations Banat and Srem in comparison to obtained heterozygosity at this locus in the same populations analyzed in season 2006. In average, significantly higher Ho was found in season 2009 at loci *Sat2* and *Sat5* (Tab. 1).
No significant deviations from HWE for brown hare populations in Vojvodina were detected. Expected and observed heterozygosity per population in both analyzed seasons are presented in Table 2. Significantly higher obtained heterozygosity was determined in Banat population in season 2009 compared with season 2006. Average observed heterozygosity was also higher in season 2009, and this difference was very significant (Tab. 2).

Table 2. Expected (He) and observed (Ho) heterozygosity per population in brown hare from Vojvodina

Population	He	Ho	He	Ho
Year	2006		2009	
Bačka	0.760	0.567	0.864	0.745
Banat	0.821	0.477	0.903	0.850*
Srem	0.776	0.572	0.870	0.876
Vojvodina (average)	0.786	0.539	0.879	0.823**

* p< 0.05 ** p< 0.01

Average expected heterozygosity value for all brown hare populations from Vojvodina was 0.786 in 2006 and 0.879 in 2009. According to derived data, analyzed populations expressed medium level of genetic polymorphism as expected for large continuously dispersed populations of mammals [25].

Table 3. Genetic variability at 5 microsatellite loci in wild boar populations from Vojvodina

Population	Locus	S0068	S0005	SW251	SW2429	SW857	Average
Bačka	A	11	6	4	14	7	8.4
	Most frequent allele	210	220	130	160	148,150,156	
	Ho	0.42	0.67	0.9	0.68	0.99	0.73
	H_E	0.69	0.79	0.72	0.82	0.82	0.77
	F_{IS}	0.41	0.17	-0.23	0.19	-0.20	0.066
Banat	A	6	2	4	4	6	4.4
	Most frequent allele	192,250	234	130	160,164	150,156	
	Ho	0.50	0.50	0.50	0.99	0.99	0.69
	H_E	0.81	0.37	0.56	0.68	0.81	0.65
	F_{IS}	0.5	-0.20	0.25	-0.33	-0.09	0.067
Srem	A	12	7	3	14	8	8.8
	Most frequent allele	192	220	136	150	154	
	Ho	0.41	0.43	0.40	0.71	0.86	0.56
	H_E	0.88	0.70	0.64	0.88	0.76	0.77
	F_{IS}	0.55	0.39	0.39	0.21	-0.104	0.29
Vojvodina Average	A	17	8	4	15	9	10.6
	Allele range	150-260	210-240	126-140	100-170	144-160	
	Ho	0.44	0.53	0.60	0.79	0.95	0.67
	H_E	0.79	0.62	0.64	0.79	0.80	0.73
	F_{IS}	0.49	0.12	0.14	0.023	-0.13	0.13

A – number of alleles per locus; He – expected heterozygosity; Ho – observed heterozygosity;
F_{IS} – inbreeding coefficient

Numbers of alleles per locus (A), most frequent alleles, allelic size ranges (in bp), expected (He) and observed (Ho) locus-specific heterozygosity per population, and inbreeding coefficients in wild boar populations from Vojvodina are given in Table 3. Statistical analysis included all five analysed loci since linkage disequilibrium test indicated independent segregation of loci. A total of 53 alleles in three wild boar populations from Vojvodina were found, with an average of 10.6 alleles per locus. The highest number of alleles per locus was found at locus S0068 (A=17), while the lowest number was detected at locus SW251 (A=4). The highest number of alleles was found in Srem wild boar population (A=44, mean A=8.8). In the analysis of Portuguese wild boar genetic variability average number of alleles was the same as in our populations A=10.17 [26], and in Italian and Hungarian wild boars a higher number of alleles per locus was determined A=12.11 [27].

Observed locus-specific heterozygosity per population ranged between 0.40 (locus SW251 in Srem population) and 0.99 (locus SW857 in populations Bačka and Banat, and locus SW2429 in Banat population), with an average Ho=0.67. Similar value of average observed heterozygosity was found in Italian and Hungarian wild boar populations Ho=0.662 [27]. In Portuguese wild boars a lower average heterozygosity was detected Ho=0.627 [26], as well as in wild boar population from Podunavlje-Podravlje hunting area Ho=0.57 [13] and in wild boars from wide area of the European continent Ho=0.57 [9]. Expected locus-specific heterozygosity per population varied from 0.37 to 0.88, with an average He=0.73. Detected expected heterozygosity was lower then values found in other European populations of wild boars [13], [26], [9].
Deviation from Hardy-Weinberg equilibrium found due to significant heterozygote deficiency was detected for three (S0068, S0005 and SW2429) of the five analyzed loci and for populations Bačka and Srem, which might suggest the existence of some level of genetic structure [28]. Inbreeding coefficients varied from -0.33 (locus SW2429 in Banat population) to 0.55 (locus S0068 in Srem population), with a mean of 0.13. Overall positive inbreeding coefficients may indicate that inbreeding act as a main cause of detected HWE deviation.

Conclusion

This paper presented comparative data of genetic monitoring in popular game species, brown hare and wild boar, in Vojvodina. The continual genetic monitoring in brown hare populations from Vojvodina showed low level of substructuring among populations, while microsatellite analyses of wild boar populations from Vojvodina revealed some level of genetic structure among analyzed populations. In future research of continual monitoring in wild boar populations we will be able to confirm an exact level of substructuring among them.

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