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Comparison of allozyme and microsatellite variability in brown hare populations (*Lepus europaeus* Pallas) from Vojvodina (Serbia)

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Summary. The brown hare (*Lepus europaeus* Pallas) is a widely popular game species throughout Europe and a species of specific biological and economical interest, which plays an important role in the hunting economy of Vojvodina (Serbia). Awareness of the value of genetic resources has encouraged studies of the genetic diversity present in different game populations. The aim of the present study was to evaluate genetic diversity in brown hare populations from Vojvodina using two molecular marker systems, allozyme and microsatellite variability, and to compare the data obtained using these two methods. Standard protocols for allozyme and microsatellite analyses in brown hares were used to measure the degree of genetic variability within and among three brown hare populations from Vojvodina: Backa, Banat and Srem, defined using geographical criterion. Both marker systems revealed significant differences between observed and expected heterozygosity in the populations, as well as positive fixation indice values. Significant deviations from Hardy-Weinberg expectations were not detected for all analyzed loci, indicating that the detected deficit of heterozygotes was the consequence of slight gene pool substructuring, rather than inbreeding effects. Microsatellite analysis revealed negligible population differentiation, while allozyme analysis revealed slightly moderate differentiation, supporting previous data showing that there is a generally shallow gene pool divergence among brown hare populations in Europe. Graphical analysis of allozyme variability data suggests lower genetic differentiation between Banat and Srem brown hare populations, in contrast with phylogenetic trees derived from microsatellite variability data, where Backa and Banat brown hare populations were genetically less different. Considering previous analyses of the same populations using nuclear and mtDNA variability data, which suggest a closer gene pool affinity between Backa and Banat, we conclude that although fewer markers were employed, microsatellite-derived data still yields more reliable results than allozyme variability data.

Keywords: allozymes, brown hares, microsatellites.

INTRODUCTION

The brown hare (*Lepus europaeus* Pallas) is commonly found throughout large parts of Europe, where it represents one of the most widely hunted game species. It is the only *Lepus* species that can be found in the Balkan Peninsula (Suchentrunk et al. 2000; Mamuris et al. 2001; Kasapidis et al. 2005; Alves et al. 2008). The habitat of the brown hare includes agricultural areas, open woodland and grassland up to 1500 m (Mitchell-Jones et al. 1999). At the end of the 1950's, a sudden regression of brown hare populations in the whole of Europe began, most likely as a consequence of anthropogenic effects on habitat (Vapa and Selmic 1997; Fickel et al. 2005). The same phenomenon was registered in Serbia, especially in the northern province of Vojvodina, which is an area of Serbia with the largest number of hunting sites. Ecological research on brown hares from Vojvodina indicate that urbanization, agricultural techniques and traffic development, as well as the increasing number of hunters and the appearance of various infectious diseases, are likely responsible for the observed decline in brown hare populations (Vapa and Selmic 1997; Vapa et al. 2007).

Considering the continuous environmental changes impacting brown hares and their regional population decline in large parts of Europe, maintenance of genetic resources in locally adapted populations is considered important for

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the long-term development of this species (Vapa and Selmic 1997). A population's genetic structure has important ecological and evolutionary consequences and the disappearance of certain genes significantly reduces the chance of future genetic variability, as well as the chance of adaptations to changes in the environment. Awareness of the value of genetic resources has encouraged studies of the genetic diversity present in different game populations (Suchentrunk et al. 2000). Brown hares not only play an important role in the hunting economy of Vojvodina, but also represent a significant prey species, particularly in the agrosystem of the region, and contribute to the flow of organic matter and nutrients (Vapa et al. 2007).

Due to the above, continual monitoring of brown hares is crucial in order to obtain data concerning the genetic diversity of specific populations. Screening game populations via pre-defined molecular markers and standardized protocols is necessary in order to determine the level of genetic variability within a population, as well as the genetic structure of a population and the level of inbreeding, etc. These data are necessary in order to predict possible events and to establish adequate responses to negative effects during the course of hunting and ecological management. Determination of appropriate molecular markers is essential in order to obtain data of practical interest. Analyses of genetic diversity within and among *L. europaeus* populations from Serbia over the past 15 years have been based on different molecular markers: including allozyme and mtDNA variability studies, as well as studies of microsatellite variability. Allozymes have played a major role in evolutionary and conservation genetics for several decades, for determination of different levels of genetic variation: within individuals; among individuals of a population; and among populations, species and higher taxa (Hartl et al. 1994). Brown hare populations from Vojvodina were first characterized using allozymes (Vapa et al. 1999, 2002, 2007; Davidovic 2003; Djan et al. 2006). These analyses revealed shallow gene pool divergence among populations, and a low level of substructuring. In the following studies, microsatellites were introduced as a new generation of molecular markers (Djan 2008). During the last decade, microsatellite markers have proven useful in genetic studies of populations or species with low levels of allozyme polymorphism (Thulin et al. 2006). The high level of polymorphism in microsatellite loci make these markers particularly suitable in various analyses, such as individual characterization, population genetics and phylogenetic studies. One advantage of microsatellites over allozyme and mtDNA markers, is that they are in general phenotypically neutral, biparentally inherited and highly polymorphic. Thus, they are ideal for measuring the degree of variability in natural populations (Estonba et al. 2006). Large numbers of alleles segregating at hypervariable microsatellite loci can reveal differentiation between populations and individuals, especially if several loci are combined (Thulin et al. 2006). The first screening of brown hare populations from Serbia revealed a higher level of substructuring among populations. Furthermore, mitochondrial DNA markers were also analyzed, primarily for phylogenetic analyses of the above named populations, but also as an additional set of markers for population genetic studies (Djan et al. 2006).

The aim of the present study was to compare genetic diversity parameters among brown hare populations from Vojvodina, using two types of molecular markers, allozymes and microsatellites, in order to test the hypothesis that microsatellites are more precise and more informative for the detection of population structure.

MATERIAL AND METHODS

Material

Liver and muscle tissue samples of brown hare individuals were collected during regular hunting seasons from hunting grounds in Vojvodina (Northern Province of the Republic of Serbia). The following 3 geographical samples, subsequently termed "populations", were considered for genetic analysis: Backa, Banat and Srem (Fig. 1).

Allozyme diversity

Protein extractions from liver tissue samples of 63 individuals were used in allozyme variability analysis. Tissue preparation and protein specific staining were carried out following Hartl and Höger (1986) and Grillitsch et al. (1992). Polyacrilamide and starch gel electrophoresis were performed according to Munstermann (1979) and Hartl and Höger (1986). Genotypes for each individual were scored according to band patterns and enzyme structure.

The following 26 structural gene loci were analyzed: sorbitol dehydrogenase (SDH, 1.1.1.14, Sdh-1), lactate dehydrogenase (LDH, 1.1.1.27, Ldh-1,-2), malate dehydrogenase (MDH, 1.1.1.37, Mdh-1,-2), malic enzyme (MOD, 1.1.1.40, Mod-1,-2), isocitrate dehydrogenase (IDH, 1.1.1.42, Idh-1,-2), 6- hexokinase (HK, 2.7.1.1, Hk-1,-2, -3), esterases (ES, 3.1.1.1, Es-1; 4.2.1.1, Es-D), peptidases (PEP, 3.4.11, Pep-1,-2), mannose phosphate isomerase (MPI, 5.3.1.8, Mpi), aldolase (ALDO , 4.2.1.3, Aldo), creatine kinase (CK, 2.7.3.2, Ck-1, -2), fumarate hydratase (FH, 4.2.1.2, Fh), phosphoglucomutase (PGM, 2.7.5.1, Pgm-1, -2), superoxide dismutase (SOD, 1.15.1.1, Sod-1), xanthine dehydrogenase (XDH, 1.2.3.2, Xdh), 6-phosphpogluconate dehydrogenase (PGD, 1.1.1.44, Pgd).

The BIOSYS-1 PC package, release 1.7 (Swofford and Selander 1989) was used to calculate allele frequencies, average heterozygosity (H_o - observed, H_e- expected), proportion of polymorphic loci (P, 95% criterion), mean numbers of alleles per locus (A) and χ^2 tests of the deviation of observed genotype frequencies at polymorphic loci from Hardy-Weinberg equilibrium (P, 95% criterion).

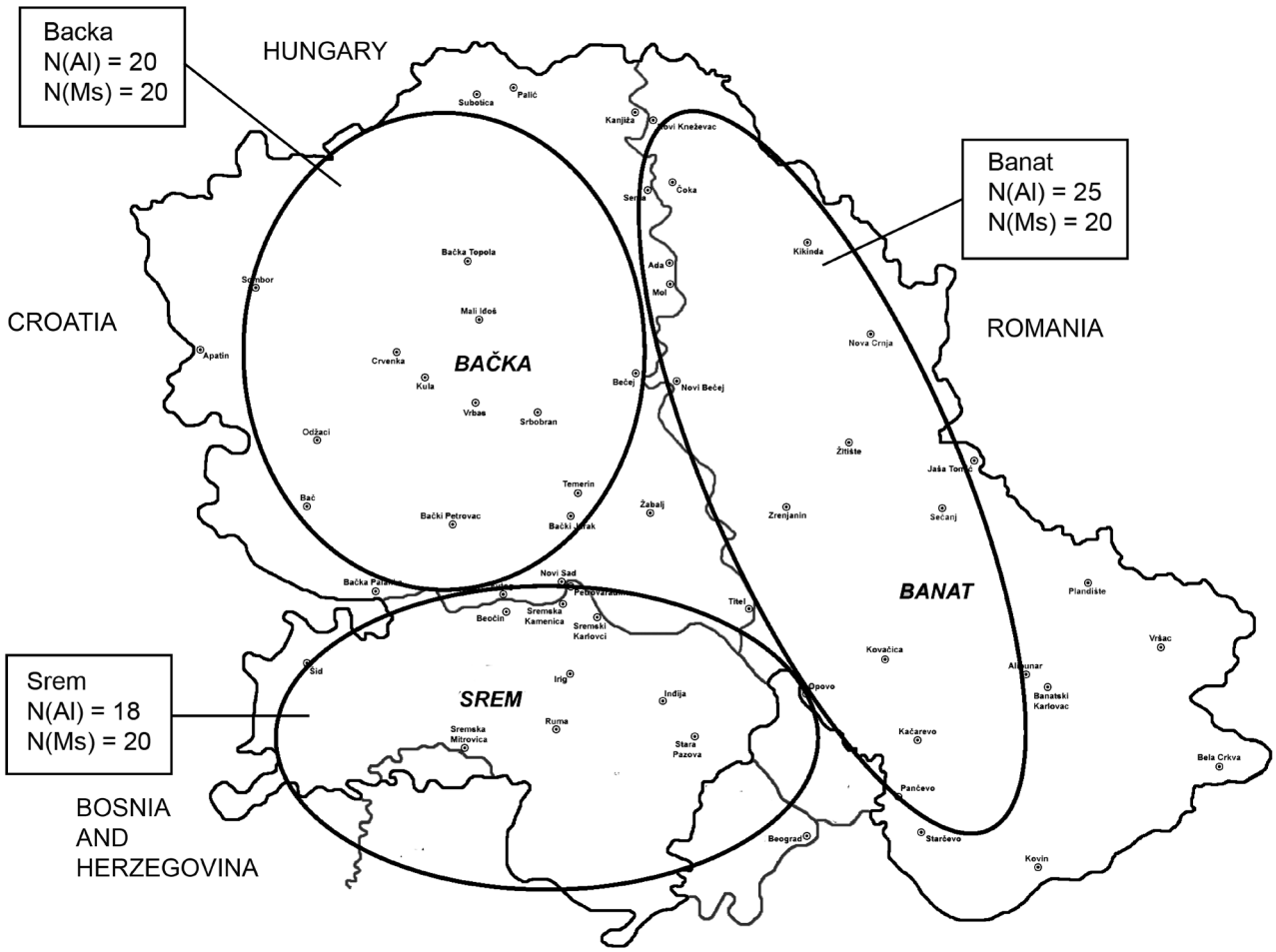

Figure 1. Geographical locations of sampling areas in Vojvodina (Serbia). Backa, Banat, Srem – geographical regions subsequently considered as populations. $N_{(Al)}$ – number of individuals included in allozyme diversity analysis. $N_{(Ms)}$ – number of individuals included in microsatellite diversity analysis.

Microsatellite diversity

For analysis of microsatellite variability, DNA was extracted from muscle tissues of 60 individuals using standard phenol chloroform isoamylalcohol extraction with proteinase K digestion (Sambrook and Russel 2001). Three microsatellites which previously showed a sufficient level of polymorphism were selected: Sat2, Sat5 and Sat12 (Mougel et al. 1997). PCR conditions for these loci were performed according to Andersson et al. (1999) using a modified procedure. Amplification reactions were performed in a volume of 20 µl, with 10 pmol of each primer, 200 µM dNTPs, 1x*Taq* buffer, 2U *Taq* DNA polymerase, 2.5 mM $MgCl_2$ and 100 ng of genomic DNA. The PCR program consisted of 95 °C for 2 min, followed by 25 cycles of 94 °C for 30s, 58 °C for 60 s, and 72 °C for 2 min, and a final extension of 72 °C for 10 min. Amplified PCR products were separated by 6% denaturating polyacrylamide gel electrophoresis, followed by silver staining (Sambrook and Russel 2001). A known DNA sequence was run with PCR products in order to determine the size of different alleles.

The Genepop 3.1d (Raymond and Rousett, 1995) PC package was used for the analysis of microsatellite data in order to calculate the number and frequencies of alleles per locus and population, average heterozygosity (H_o - observed and H_e- expected), and deviation of heterozygosity values from Hardy Weinberg equilibrium. Microsatellite loci were tested for deviation from Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium using the Markov chain method. Default parameter settings of 1000 dememorizations, 100 batches and 1000 iterations per batch were used for Markov estimations. Significance levels were adjusted using the sequential Bonferroni correction for multiple comparisons (Rice 1989).

Testing the difference between the level of polymorphism obtained by allozyme and microsatellite markers was conducted using χ^2 square test implemented in STATISTICA 10.0 MR1 (2011).

The degree of differentiation between and across all populations was quantified using Wright's F_{ST} (Wright 1978). Based on pairwise F_{ST} values obtained for both allozyme and microsatellite variability analyses, a UPGMA tree was constructed in MEGA version 5 (Tamura et al. 2011).

RESULTS

The selected 26 allozyme loci were successfully analyzed in all three brown hare populations. The genotype scoring was successful for all loci for 94.5% of individuals in Backa, 88.8% in Banat and 92.2% in the Srem population. The overall number of alleles per locus for allozyme analysis was 1.5, and polymorphism 42.3% (11 polymorphic loci out of 26 analysed). Measures of genetic variability based on allozyme variability were calculated for all three tested populations and for the total population of Vojvodina (Table 1). The χ^2-square test for deviations of allelic frequencies from Hardy-Weinberg equilibrium revealed the following deviations: at the Est-1 locus in the Backa population; the Idh-2 locus in the Banat population; and at Mod-2 and Pgd loci in the Srem population. Deviations at Mod-2, Pgd and Idh-2 loci were confirmed for the Vojvodina brown hare population. Heterozygote deficiency was detected in all three populations, as well as in the Vojvodina population, based on a global multi-locus U test with the implemented Markov chain method. The overall fixation index for the Vojvodina brown hare population was F_{IS}= 0.206.

The selected set of three microsatellite markers was successfully amplified in 93.33% of individuals at the Sat2 locus, 90% at Sat12 and 83.33% at the Sat5 locus. The highest number of alleles was found at the Sat2 locus, while the lowest number was found at the Sat12 locus. Based on determined genotypes for each individual at each locus, measures of genetic variability within the three test populations and the Vojvodina population in total were calculated (Table 2). Heterozygote deficiency was revealed in the Banat population at the Sat2 locus, while this deficiency was noted at the Sat5 locus in the Backa and Srem populations. In the Vojvodina brown hare population, heterozygote deficiency was confirmed for the Sat2 and Sat5 loci, while no heterozygote deficiency (or excess) was determined for the Sat12 locus. Negative F_{IS} values were found for Sat2 and Sat12 loci, while positive values were found for the Sat5 locus.

To obtain an overview of the genetic differentiation among the test populations, pairwise F_{ST} values were calculated according to allozyme variability data (Table 3) and microsatellite variability data (Table 4).

Graphical presentations of relative genetic differentiations were performed using the UPGMA method and resulting constructed trees revealed different branch topologies according to allozyme variability analysis (Fig. 2) versus microsatellite variability analysis (Fig. 3).

Table 1. Allele frequencies at polymorphic loci and indices of genetic variability in *Lepus europaeus* populations from Backa, Banat and Srem.

Locus	Alleles	Backa	Banat	Srem	Vojvodina
Mod-2	A	0.900	0.740	0.861*	0.825*
	B	0.100	0.260	0.139	0.175
Est-D	A	0.763	0.725	0.667	0.719
	B	0.237	0.250	0.333	0.272
	C	/	0.025	/	0.009
Est-1	A	0.071*	0.295	0.222	0.213
	B	0.643	0.568	0.417	0.537
	C	0.214	0.137	0.278	0.204
	D	0.072	/	0.083	0.046
Pgd	A	0.763	0.420	0.667*	0.597*
	B	0.237	0.580	0.333	0.403
Sdh	A	0.676	0.921	0.643	0.760
	B	0.324	0.029	0.357	0.240
Pep-2	A	0.938	0.676	0.821	0.809
	B	0.062	0.265	0.143	0.160
	C	/	0.059	0.356	0.031
Mpi	A	0.765	0.818	0.750	0.774
	B	0.235	0.182	0.250	0.226
Hk-3	A	0.500	0.818	0.964	0.738
	B	0.500	0.182	0.036	0.262
Idh-2	A	0.938	0.611*	1.000	0.833*
	B	0.031	0.389	/	0.156
	C	0.031	/	/	0.011
Ldh-1	A	0.147	0.409	0.750	0.283
	B	0.853	0.591	0.250	0.717
Mdh-1	A	0.789	0.652	0.583	0.675
	B	0.211	0.348	0.417	0.325
He		0.141	0.177	0.159	0.141
Ho		0.037*	0.148*	0.132*	0.125*
A		1.54	1.54	1.5	1.50
$P_{95\%}$		42.31%	42.31%	38.46%	42.30%

H_e – expected heterozygosity; H_o – observed heterozygosity; A – mean number of alleles per locus; $P_{95\%}$ – rate of polymorphism (95% criterion); asterisk (*) indicate level of significance $p<0.05$.

DISCUSSION

The first true molecular markers established in population genetic studies are allozymes, and because universal protocols for analyses have been developed, allozymes have since been used for a wide range of species (Schlötterer 2004). However, the development of new techniques that enable direct detection of variability at the DNA level, as well as the obvious disadvantages of allozyme markers (e.g. limited number of loci, use of fresh frozen material, and indirect detection of variability) has led to the increased use of DNA-based markers in population genetics (Schlötterer 2000; Selkoe and Toonen 2006). Among them, microsatellites

Table 2. Allelic variation at 3 microsatellite loci in *Lepus europaeus* populations from Backa, Banat and Srem.

Locus		Backa	Banat	Srem	Vojvodina
	n	13	17	14	23
	F	0.150	0.110	0.150	0.124
Sat2	He	0.932	0.952	0.918	0.879
	Ho	0.941	0.842*	0.950	0.850*
	F_{IS}	-0.010	0.118	-0.036	0.033
	n	11	13	11	18
	F	0.300	0.150	0.230	0.184
	He	0.865	0.922	0.883	0.753
Sat5	Ho	0.556*	0.833	0.733*	0.600*
	F_{IS}	0.364	0.043	0.174	0.205
	n	6	7	7	8
	F	0.340	0.240	0.330	0.303
	He	0.795	0.834	0.810	0.732
Sat12	Ho	0.737	0.824	0.945	0.750
	F_{IS}	0.075	0.013	-0.172	-0.024
Overall	He	0.864	0.903	0.870	0.879
	Ho	0.745*	0.850*	0.876	0.825*

n- number of alleles per locus, F – frequency of the most common allele; H_e – expected heterozygosity; H_o – observed heterozygosity; F_{IS} – inbreeding coefficient; asterisk (*) indicate level of significance $p<0.05$.

Table 3. Relative genetic differentiation based on pairwise F_{st} values according to allozyme variability data.

Population	1	2	3
1 Bačka	******		
2 Banat	0.0948	*****	
3 Srem	0.0691	0.0582	*****

Table 4. Relative genetic differentiation based on pairwise F_{st} values according to microsatellite variability data.

Population	1	2	3
1 Bačka	******		
2 Banat	0.0052	*****	
3 Srem	0.0133	0.0150	*****

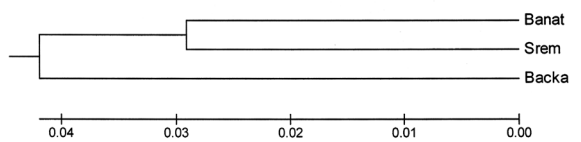

Figure 2. UPGMA dendogram based on pairwise F_{ST} values according to allozyme variability data.

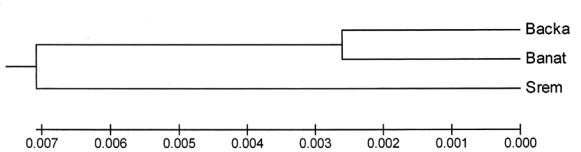

Figure 3. UPGMA dendogram based on pairwise F_{ST} values according to microsatellite variability data.

took over the dominant position in the population genetic analyses of many species, due to the high-level of information obtained, biparental inheritance, and conserved flanking sequences: which enable using previously developed microsatellite primers from closely related species, thus avoiding expensive and time-consuming development of new primers (Andersson et al. 1999). Our results presented here, from comparisons of genetic diversity in brown hare populations from Vojvodina using allozyme and microsatellite variability, partially support these facts.

Using basically the same sample of brown hares from Vojvodina, we analyzed 26 allozyme loci and a significantly lower number of microsatellite loci (3). Nevertheless, as was expected, polymorphism was significantly higher according to microsatellite analysis results (100% - all loci polymorphic) versus allozyme analysis (42.3%). The value of heterozygosity across all genes is often considered to be a general indicator of the amount of genetic variability of a population. The employment of allozyme markers revealed significantly lower values of observed versus expected heterozygosity in all test populations and in the Vojvodina brown hare population as a whole. The same phenomenon was recorded using microsatellite based data, except in the Srem population. Generally, if the observed heterozygosity is less than expected, it may indicate that inbreeding is present in a population (Frankham et al. 2004). However, in our analyzed population that is less likely, since a heterozygote deficiency was not detected for all polymorphic allozyme and microsatellite loci. Thus, the significant deviations from Hardy-Weinberg (HW) expectations detected for some loci might indicate slight gene pool substructuring, rather than inbreeding effects. Moreover, genetic variability in the total population of Vojvodina brown hares was within the range found for the many European populations (Hartl et al. 1994; Davidovic 2003; Suchentrunk et al. 2003; Thulin et al. 2006; Vapa et al. 2007; Ben Slimen et al. 2008; Djan 2008), and the average genetic diversity reported for a wide range of terrestrial mammal species (Tiedemann et al. 1996). Comparisons of heterozygosity values calculated from allozyme vs. microsatellite data revealed several fold higher heterozygosity values from microsatellite data (this difference was not statistically tested), as expected due to the direct analysis of DNA variability provided by microsatellites. Thus, even the lower number of microsatellite loci used appears to be sufficient for obtaining higher levels of heterozygosity compared to allozymes, since observed heterozygosity values depend on the number of individuals. In the present study, the number of individuals is not different, so the higher number of monomorphic loci decreases this value. The positive F_{IS} values obtained by allozyme and microsatellite methods, are in agreement with the reduced observed heterozygosity. Moreover, the positive F_{IS} values obtained for brown hares from Serbia were previously detected with an even larger number of individuals, using either allozyme (Davidovic 2003) or microsatellite (Djan 2008) loci.

Moreover, calculated F_{IS} values were within the range found for many European populations (Hartl et al. 1994; Davidovic 2003; Suchentrunk et al. 2003; Thulin et al. 2006; Vapa et al. 2007; Ben Slimen et al. 2008; Djan 2008). F_{IS} values obtained by microsatellite analysis were three fold lower, and closer to zero; thus supporting the conclusion that the observed significant deviations from HW expectations are a consequence of slight gene pool substructuring, rather than inbreeding or a possible bottleneck drift effect.

The major differences we observed during population genetic analyses of brown hares using two different marker systems is reflected in the analyses of genetic differentiation among the three test populations. F_{ST} values were calculated because it represents the most inclusive measure of population substructure and is most useful for examining the overall genetic divergence among subpopulations (Conner and Hartl 2004). F_{ST} values are calculated based on subpopulation average heterozygosity and total population expected heterozygosity: for this analysis higher values were calculated using allozymes compared to microsatellites. Considering that F_{ST} values up to 0.05 indicate negligible genetic differentiation, whereas >0.25 is associated with significant genetic differentiation within an analyzed population, it could be concluded that microsatellites revealed negligible genetic differentiation among the test populations, while allozymes revealed slightly moderate differentiation. Both results do not contradict previous results which show that there is in general shallow gene pool divergence among the brown hare populations in Europe; while relative genetic differentiation (F-statistics) suggests moderately reduced gene flow across longer geographic distances (Hartl et al. 1994; Mamuris et al. 2001; Vapa et al. 2002, 2007). Furthermore, the very low genetic differentiation revealed using microsatellites was confirmed for brown hares from Vojvodina in a large study of brown hare populations from Serbia (Djan 2008), even with a higher number of microsatellites used. The UPGMA dendrogram constructed according to allozyme variability data suggests a slightly closer nuclear gene pool affinity between the Banat and Srem brown hare populations, contrary to the phylogenetic tree derived from microsatellite variability data, where Backa and Banat brown hare populations were genetically less different. Regarding earlier studies of brown hare populations from the same region, the Backa and Banat brown hare populations were found to be genetically more similar according to data obtained by analysis of 40 allozyme loci in 77 individuals (Davidovic 2003), and six microsatellite loci in 78 individuals (Djan 2008). Moreover, the same branch position was obtained in UPGMA dendogram clustering of three brown hare populations from Vojvodina according to the nucleotide divergence of three mtDNA regions: cytochrome oxidase I (COI), a control region, and 12S/16S rRNA (Djan et al. 2006). Considering previously published data and the biogeography of the region, we may conclude that data derived using microsatellites in cluster analysis are more reliable than that obtained using allozymes. It might be possible that an increased number of allozyme loci would produce different genetic differentiation results. However, considering all of the above named disadvantages of allozyme-based molecular markers, as well as the results of this research, we recommend microsatellites for continuous monitoring of this game species, as was proposed for other hare populations (Andersson et al. 1999; Fickel et al. 2005; Estonba et al. 2006; Thulin et al. 2006; Alves et al. 2008; Ben Slimen et al. 2008), especially if the chosen number of microsatellites is increased. Nevertheless, we have shown that even a low number of microsatellites can produce valuable and reliable results.

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