

DOI: [10.15679/bjwr.v2i1.22](https://doi.org/10.15679/bjwr.v2i1.22)**GENETIC VARIATION WITHIN AND AMONG BROWN HARE (*LEPUS EUROPAEUS* PALLAS, 1778) POPULATIONS IN SERBIA AS INFERRED FROM MICROSATELLITES)**Djan M.¹, Veličković N.¹, Stefanović M.¹, Marković V.², Obreht Vidaković D.¹, Vapa Lj.¹

Summary: The European brown hare (*Lepus europaeus* Pallas, 1778) is the only *Lepus* species that can be found in Serbia and Balkan Peninsula, where it represents an important game species. Previous analyses of brown hares in Serbia based on allozyme and mtDNA sequence variability revealed moderate genetic diversity and indicated possible population structuring in the region. The aim of this study is to detect population genetic diversity and structure in brown hares from Serbia using microsatellites. **Material and Methods:** A total number of 157 brown hares were collected from six different regions in Serbia and polymorphism of six microsatellite loci was analyzed. Following computer packages were used for the data analyses: ARLEQUIN, STRUCTURE, GENETIX, BOTTLENECK and NEWHYBRIDS. **Results:** A total number of 81 alleles at 6 loci were found. The highest number of alleles per locus was found at Lsa2 microsatellite loci (20), while the lowest number of alleles was present at loci Sat5 and Lsa3 (9), with an average of 13.5 alleles/locus in total sample. Different statistical approaches revealed that brown hare population from Serbia is structured into two genetic subunits (i.e., subpopulations). First subpopulation, named Northern-Central-Eastern (NCE) Serbia consists of five initially defined groups (Bačka, Banat, Srem, Northern-Central Serbia and Northern-Eastern Serbia) while second subpopulation comprised of individuals from Northern-Western (NW) Serbia. Higher values of genetic diversity parameters were found in NCE Serbia subpopulation compared to NW Serbia subpopulation. **Conclusion:** Observed genetic differentiation should be considered in future brown hare management strategies and consider those subpopulations as separate management units, in order to preserve existing genetic diversity and to avoid further reduction of genetic variability in NW Serbia subpopulation or human mediated admixtures which can disturb present gene pools..

Key words: genetic diversity, *Lepus europaeus*, microsatellites, Serbia.

Introduction

The European brown hare (*Lepus europaeus* Pallas, 1778) is an important game species. It has a range that includes palearctic, nearctic, oriental and neotropical regions. In Europe, *L. europaeus* occurs throughout the continent and extends towards Central Asia (Thulin et al., 1997; Suchentrunk et al., 2003; Fickel et al., 2005). It is the only *Lepus* species that can be found in Serbia and in the Balkan peninsula (Suchentrunk et al., 2000; Mamuris et al., 2001; Kasapidis et al., 2005; Stamatis et al., 2009). The brown hare adapted successfully to the agricultural landscape and now can be found in open farmland all over its range.

Species of the lagomorph genus *Lepus* show no differentiation among each other in genetic characters such as chromosome number and karyotype banding (Robinson et al., 1983), and very little differentiation in allozyme markers (Hartl et al., 1993; Suchentrunk et al., 1999, 2000; Ben Slimen et al., 2006, 2007; Vapa et al., 2007). The employment of microsatellite markers provided better insight into population genetic diversity and structure in different *Lepus* species across Europe. Usually microsatellite markers developed and discovered in *Oryctolagus cuniculus*, are markers of choice in population genetic,

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studies of hares. Mougel et al. (1997) developed six new microsatellite markers in populations of wild rabbits and confirmed the usefulness of the three previously described by Rico et al. (1994) for genotyping hares. In the analysis of *L. europaeus* populations among these microsatellites, three proved to be particularly informative: Sat2, Sat5 and Sat12 (Mougel et al., 1997). Additional six new microsatellite markers detected in rabbits *Oryctolagus cuniculus* (SurrIDGE et al., 1997), proved to be applicable in 20 different taxa of Lagomorpha. The developed markers were employed for analysis of population diversity and structure and possible hybridization between *L. europaeus* and *L. timidus* in Scandinavian Peninsula (Andersson et al., 1999; Thulin et al., 2006a). Estimated genetic differentiation between species was low and authors suggested reticular evolution of these species, with a constant gene flow between them. Information provided by the microsatellites showed clear genetic differentiation and distinctiveness among *L. europaeus*, *L. castroviejoi*, and *L. granatensis* in the Iberian Peninsula (Estonba et al., 2006), and moderately high genetic diversity of *Lepus europaeus* populations. The comprehensive study by Fickel et al. (2005) of brown hare populations in Germany detected a high degree of genetic variability, matrilineally structured populations, male-biased gene flow, and lack of inbreeding. No geographical or anthropogenic barriers to gene flow were found. Ben Slimen et al. (2008) reported decrease of intraspecific genetic diversity in *L. capensis* and *L. europaeus* in south-north direction, from South Africa, North Africa, Israel, Anatolia, the Balkans, central Europe to north western Europe.

So far, only partial information about the population structure of brown hares (*Lepus europaeus*) from Serbia is available, despite the large amount of studies of brown hares populations structure from Europe. In previous studies, the allozyme composition of brown hares from Vojvodina (northern province in Serbia) were analyzed (Vapa et al., 2002, 2007; Djan et al., 2013), indicating very similar allele composition to brown hares from other parts of central Europe. Also, the analyses of mtDNA polymorphism in brown hares from Vojvodina province (Djan et al., 2006; Stamatis et al., 2009) adjoins the populations to the central European brown hare haplotype group. Different local hare populations from Europe have already been analyzed using microsatellites (Andersson et al., 1999; Fickel et al., 2005; Estonba et al., 2006; Ben Slimen et al., 2008), but from the Central Balkan region only brown hare population from Vojvodina was characterized using microsatellites (Djan et al., 2013).

Awareness of the value of genetic resources has encouraged studies of the genetic diversity present in different game populations. Game population genetic structure has important ecological and evolutionary consequences, but also precise knowledge on population structure is required for maintaining its economic value, because management plans could be designed for particular population. Usually, population genetic studies focus on fragmented populations, endangered species or on species with complex social systems. Lower number of investigations deals with the analyses of genetic structure in continuously distributed species, such as brown hares. Considering all available facts, in this study we aim to detect population genetic diversity and structure in brown hares from Serbia using microsatellites in order to reveal genetic diversity of previously unsampled population and define population genetic structure. Obtained data could be useful in future brown hare management plans and relevant for sustainability of brown hare populations in the Balkans.

Material and Methods

Sample collection

A total number of 157 brown hares were collected from six different regions in Serbia, during hunting seasons from 2004 to 2006. The following 6 regions, subsequently termed "groups", were included in genetic analyses: Bačka (26), Banat (26), Srem (26), Northern-Central (NC) Serbia (32), Northern-Eastern (NE) Serbia (13) and Northern-Western (NW) Serbia (34) (numbers of samples per group are given in parenthesis) (Fig. 1), not only due to geographic proximity, but also considering biogeographical features of the areas. Sampled tissue was frozen immediately after death of animal.

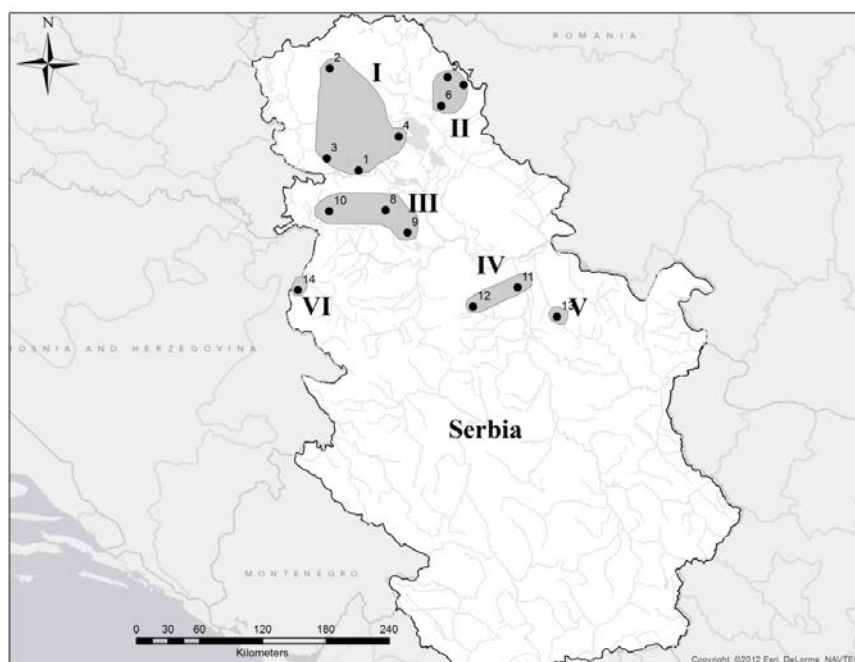


Figure 1. Map of sampling regions. I – Bačka (1–Futog; 2 – Stara Moravica; 3 – Nova Gajdobra; 4 – Čurug); II – Banat (5 – Kikinda; 6 – Bašaid; 7 – Novi Kozarci); III – Srem (8 – Žarkovac; 8 – Krnješevci; 10 – Martinci); IV – Northern-Central Serbia (11 – Osipaonica; 12 – Mladenovac); V – Northern-Eastern Serbia (13 – Petrovac na Mlavi); VI – Northern-Western Serbia (14 – Loznica)

Microsatellite analysis

Total DNA was extracted from liver, tongue and eye tissue using standard phenol chloroform isoamylalcohol extraction with proteinase K digestion (Sambrook and Russel, 2001).

Six microsatellite loci with different levels of polymorphism were analyzed: Sat2, Sat5, Sat12 (Mougel et al., 1997), Sol33 (SurrIDGE et al., 1997), and Lsa2 and Lsa3 (Kryger et al., 2002). Due to relatively small sample sizes of certain populations, microsatellite markers with varying levels of polymorphism were selected in order to outbalance the fact, as suggested by Shriver et al. (1995) and Takezaki and Nei (1996).

All reactions were performed in 20µl volume. The PCR reactions contained 100ng of DNA template, 1xBuffer (with 15mM MgCl₂), 200µM dNTPs, 10 pmol of each primer, 1 mM MgCl₂ and 1 unit of Taq DNA polymerase. The PCR conditions with optimized annealing temperatures for each locus are given in Table 1.

Table 1. PCR conditions with optimized annealing temperatures for amplification of microsatellites in brown hares

Locus	PCR conditions °C (s)	Reference
Sat2	95 (120), 25 x [94 (30), 55 (60), 72 (120)], 72 (600)	Mougel et al., 1997
Sat5	95 (120), 25 x [94 (30), 58(60), 72 (120)], 72 (600)	Mougel et al., 1997
Sat12	95 (120), 25 x [94 (30), 55 (60), 72 (120)], 72 (600)	Mougel et al., 1997
Sol33	94 (180), 7 x [94 (30), 52 (30), 72 (30)], 23 x [89 (30), 52 (30), 72 (30)]	SurrIDGE et al., 1997
Lsa2	94 (180), 35 x [94 (30), 57 (30), 72 (30)]	Kryger et al., 2002
Lsa3	94 (180), 35 x [94 (30), 56 (30), 72 (30)]	Kryger et al., 2002

The PCR products of loci Sat2, Sat5, Sat12, Sol33, Lsa2 and Lsa3 were applied to a 2% agarose gels, stained with ethidium bromide and fragments were visualized under UV light. Samples that were successfully amplified for the fragments of interest, were electrophoretically separated by applying 5µl of each to a 6% denaturing polyacrylamide gel with 1xTBE buffer, followed by silver staining in order to visualize the PCR products. A known DNA molecular weight-size marker was run with the PCR products to

measure the size of different alleles. The individuals that failed to produce signal were amplified a second time, under the same conditions.

Data analysis

All brown hare samples were divided into 6 groups based on their geographic proximity: (1) Backa, (2) Banat, (3) Srem, (4) Northern-Central Serbia, (5) Northern-Eastern Serbia, and (6) Northern-Western Serbia (Fig. 1). The analysis of molecular variance (AMOVA) and pairwise genetic distances among the presumed groups (F_{ST}) were calculated using ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010). Significance levels were adjusted using the sequential Bonferroni correction for multiple comparisons (Rice, 1989). In order to infer the existence of population structuring of brown hares in Serbia, Bayesian cluster analyses implemented in STRUCTURE 2.3.3 (Pritchard et al., 2000; Falush et al., 2003, 2007; Hubisz et al., 2009) were performed. STRUCTURE run was performed assuming admixture model with correlated allele frequencies with burn-in length of 100,000 followed by 1,000,000 MCMC (Markov Chain Monte Carlo) iterations. For the estimation of the most likely number of clusters and graphical interpretation of the results we used CLUMPAK (Kopelman et al., 2015). Genetic similarity among individual genotypes were also examined through factorial correspondence analysis using GENETIX (Belkhir et al., 2004).

Microsatellite loci were tested for deviation from Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium using ARLEQUIN in inferred genetic clusters (subpopulations). The same program was used to calculate mean number of alleles (A), observed (H_o) and expected (H_e) heterozygosities and inbreeding coefficients for each locus and for each defined subpopulation. The amount of partitioning of nuclear diversity among subpopulations was calculated by AMOVA model using the ARLEQUIN and pairwise genetic distances among the inferred subpopulations (F_{ST}) was also estimated.

Detection of migrants and hybrids between subpopulations was performed using computer package NEWHYBRIDS 1.0 (Anderson and Thompson, 2002). This analysis included all individuals that were analyzed for hybrid or parental origin. The analysis was performed in 3 replicas with burn in length of 50,000 followed by 100,000 MCMC iterations. In order to infer demographic history of brown hare populations from Serbia bottleneck analysis was carried out for each defined subpopulation using program BOTTLENECK (Cornuet and Luicart, 1997). We performed analysis considering two-phase model of microsatellite evolution set with 90% of mutations following the strict stepwise mutation model and 10% being multistep mutations. The results obtained for each locus were combined using the Wilcoxon test.

Results

Genotyping of all 157 brown hare specimens was successful for locus Sat2 (100%), while amplification success rate in the remaining loci ranged from 98.73% for the locus Sol33 to 87.9% for the locus Lsa2. Unsuccessful amplifications for certain samples might be consequence of null alleles that occur mostly due to mutations in primer binding region, but we cannot exclude another possible cause of inhibition of PCR reactions in individual samples.

For the initial analysis of the brown hare population structure from Serbia pairwise genetic distances among six presumed groups (F_{ST}) were calculated (Table 2.). The sampling group Northern-Western Serbia showed statistically significant genetic differentiation from all other sampling groups. Furthermore, AMOVA among six sampling groups showed 10.76% of variation among groups ($p < 0.0001$) and 89.24% within groups.

Table 2. Relative genetic differentiation between 6 presumed brown hares groups from Serbia based on pairwise F_{ST} (below diagonal) and number of shared alleles (above diagonal)

	Backa	Banat	Srem	NC Serbia	NE Serbia	NW Serbia
Backa		43	37	37	25	18
Banat	0.000		41	43	31	22
Srem	0.020	0.013		40	30	18
Central Serbia	0.027	0.021	0.022		25	21
Eastern Serbia	0.025	0.009	0.018	0.013		14
Western Serbia	0.191*	0.207*	0.261*	0.210*	0.282*	

* $p < 0.05$ (0.008 after Boniferroni correction)

Following the ΔK method, the most likely number of distinct genetic clusters was $K=2$ (Fig. 2). Bayesian approach implemented in the STRUCTURE revealed that the overall data set was most likely representing 2 genetic subunits (i.e., subpopulations).

First subpopulation, named Northern-Central-Eastern (NCE) Serbia consists of five initially defined groups (Backa, Banat, Srem, NC Serbia and NE Serbia) while second subpopulation comprised of individuals from Northern-Western (NW) Serbia. Observed population genetic structure was further supported by Factorial Correspondence Analysis (Fig. 3.) with clear separation of individuals from NW Serbia.

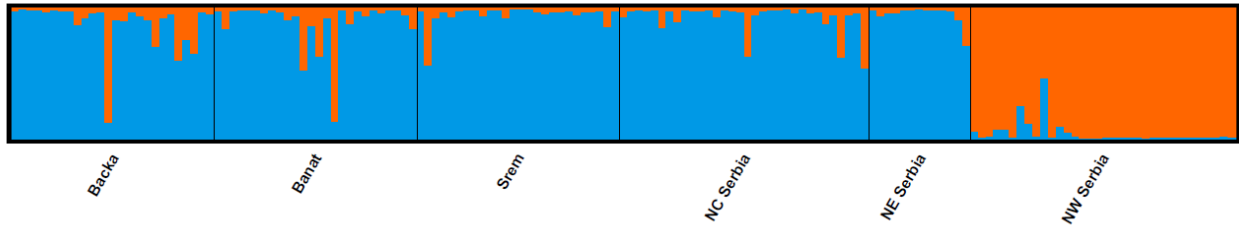


Figure 2. Estimated population structure of brown hare (*Lepus europaeus*) from Serbia. Each individual is represented by a vertical line, a thick black lines separate individuals from different sampling groups. Different colors represent different posterior probability of assignment of each individual to each of the inferred genetic clusters

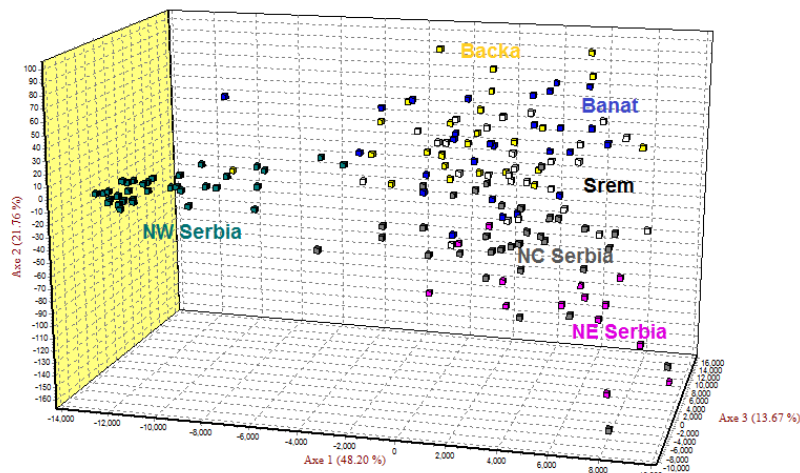


Figure 3. 3D Factorial Correspondence Analysis performed for 157 brown hares from Serbia.

Parameters of genetic variability were calculated for inferred genetic clusters (subpopulations). A total number of 81 alleles at 6 loci were found in brown hare populations from Serbia. The highest number of alleles per locus was found at Lsa2 microsatellite loci (20), while the lowest number of alleles was present at loci Sat5 and Lsa3 (9), with an average of 13.5 alleles/locus in total sample.

All six analyzed microsatellites were polymorphic in NCE Serbia subpopulation, and four were polymorphic in NW Serbia subpopulation (Table 3).

Observed heterozygosity (H_o) value was higher in NCE Serbia subpopulation (0.530) compared to NW Serbia subpopulation (0.470). The highest observed heterozygosity was found for locus Lsa2 in both subpopulations, and the lowest at locus Lsa3. Deviation from Hardy-Weinberg equilibrium was found in all loci for NCE Serbia subpopulation, and for three (Sat2, Lsa2, Lsa3) out of four polymorphic loci in NW Serbia subpopulation (Tab. 3). No genetic signature of recent bottleneck was detected for any subpopulation, but heterozygote deficiency was detected in NW subpopulation ($p=0.03125$).

Table 3. Allelic variation at 6 microsatellite loci in two brown hare (*Lepus europaeus*) subpopulations in Serbia

Locus	NCE Serbia subpopulation						NW Serbia subpopulation					
	2n	Na	AP	Ho	He	Fis	2n	Na	AP	Ho	He	Fis
Sat2	246	19	12	0.715	0.920	0.223	66	6	0	0.424	0.656	0.357
Sat5	216	9	8	0.167	0.657	0.743	/	1	0	/	/	/
Sat12	224	12	6	0.777	0.851	0.087	62	7	1	0.548	0.716	0.238
Sol33	242	11	10	0.430	0.641	0.331	/	1	0	/	/	/
Lsa2	210	20	15	0.847	0.913	0.072	64	5	0	0.687	0.528	-0.309
Lsa3	220	9	5	0.245	0.769	0.682	64	4	0	0.219	0.428	0.493
Mean	226.3	13.3	9.3	0.5	0.8	0.3	64	5.5	0.2	0.5	0.6	0.2

2n – total number of alleles/sample size; Na – numer of alleles per locus; AP – number of private alleles; Ho – observed heterozygosity; He – expected heterozygosity; Fis – inbreeding coefficient

Seven pairs of loci (out of 15 combinations) were in linkage disequilibrium in NCE Serbia subpopulation, and none pair of loci showed linkage disequilibrium in NW Serbia subpopulation. The pairwise genetic differentiation was high $F_{ST} = 0.168$ ($p < 0.0001$). Analysis of molecular variance showed higher intrapopulation variability (81.12%) comparing to interpopulation variability (18.88%) ($p < 0.001$), which was statistically significant and high (Table 4).

Table 4. Partitioning of nuclear diversity among brown hare subpopulations from Serbia by AMOVA model

Source of variation	Sum of squares	Variance components	% variation
Among subpopulations	51.388	0.48869	18.88
Within subpopulations	607.466	2.09975	81.12

The NEWHYBRIDS analyses matched genotype-based assignment of individuals and it was shown that two subpopulations defined in Bayesian cluster analysis represent two parental populations (Fig. 4). We didn't find migrants between these two subpopulations, while we found presence of F2 hybrids in each subpopulation. Higher number of F2 hybrids was observed in NCE Serbia subpopulation.

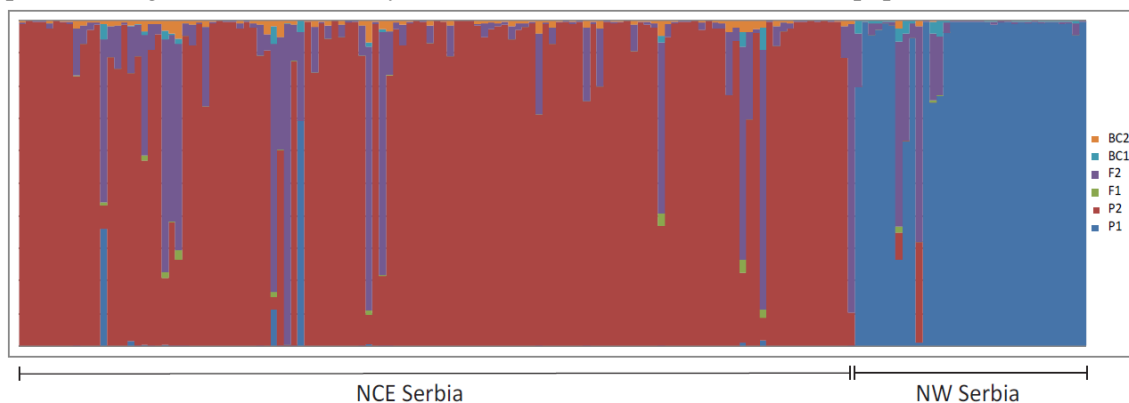


Figure 4. Results of the pairwise analysis for parental or hybrid origin of individuals from the two brown hare subpopulations in Serbia, performed with NEWHYBRIDS. Posterior probabilities of assignment to parental and hybrid classes are plotted in different colours.

Discussion

Advancements in molecular techniques constantly provide novel methods for rapid and reliable genotyping of animal natural populations. So far, genetic diversity of brown hare populations from Serbia was analyzed by detection of allozyme variability levels (Vapa et al., 2007). Moreover, previous studies were dealing with the analyses of genetic diversity of brown hare populations from Vojvodina (Vapa et al., 2002; Djan et al., 2006; Vapa et al., 2007; Djan et al., 2013), but no data were published on genetic variability of brown hare populations from other regions in Serbia using microsatellites or other nuclear

DNA markers. Previous studies based on analyses of CR-1 mtDNA sequence variability indicated possible structuring of brown hares from Serbia (Djan et al., 2006; Stamatis et al., 2009).

An average number of alleles per locus in our study was lower than detected in *L. europaeus* and *L. capensis* populations (14.7 alleles/locus) (Ben Slimen et al., 2008) and Scandinavian hares (16.3) (Thulin et al., 2006b), but higher than in Canadian snowshoe hares (13.4) (Burton et al., 2002) and brown hare populations from Germany (11.2) (Fickel et al., 2005). Observed differences might be due to number of individuals analysed, more restricted sampling area in our study or different set of microsatellite markers employed. Observed heterozygosity of the brown hare populations from Serbia was within the range found for the Central European populations (Fickel et al., 2005, Ben Slimen et al., 2008) and Scandinavian brown hares (Andersson et al., 1999).

A combined statistical analyses of the presently produced allele set suggested genetic differentiation of brown hare sample into two defined subpopulations: Northern-Central-Eastern Serbia (NCE) and Northern-Western (NW) Serbia. Heterozygosity values indicated that brown hares from this subpopulation are still sufficiently genetically diverse to avoid inbreeding depression and to exclude fixation of alleles. Observed significant deviations from the Hardy-Weinberg equilibrium (HWE) in all loci may suggest a high level of gene exchange among European brown hares from NCE and NW Serbia subpopulations and possibly unsampled neighbouring brown hare subpopulations. Moreover, NEWHYBRIDS results showed present but restricted gene flow between NCE Serbia and NW Serbia subpopulations. It was shown that higher number of F2 hybrids is present in NCE Serbia subpopulation and this indicates existence of gene flow between brown hares from NCE and NW Serbia, but also gene flow with a non sampled populations from neighbouring countries can not be excluded. In order to further speculate on reasons for the observed restricted gene flow, individuals from a broader sampling area should be analysed, specifically from Southern Serbia. Deviation from Hardy-Weinberg equilibrium was also found in majority of *L. europaeus* populations in Europe (Ben Slimen et al., 2008). Heterozygote deficiency detected in NW Serbia subpopulation may be caused by inbreeding or might be also explained by Wahlund effect, which is unlikely for our data. In Scandinavian hares heterozygote deficiency was detected in all populations (Andersson et al., 1999; Thulin et al., 2006b).

Genetic variation within subpopulations was much higher as among subpopulations, indicating probably successful reproductive activity by many females. According to Fickel et al. (2005) genetic variation within brown hare populations from Germany was twice higher as among populations. This ratio in our study was much higher, as well as in Ben Slimen et al. (2008).

It could be concluded that microsatellites revealed high genetic differentiation of brown hares from Serbia into two subpopulations, as it was previously slightly suggested by allozyme and mtDNA variability analyses (Vapa et al., 2002, 2007; Djan et al., 2006, 2013). Earlier studies show that there is in general moderate gene pool divergence among the brown hare populations in Europe and moderately reduced gene flow across longer geographic distances (Hartl et al., 1993; Mamuris et al., 2001; Vapa et al., 2002, 2007). Observed differentiation should be considered in future brown hare management strategies, in order to preserve existing genetic diversity and to avoid further reduction of genetic variability in Northern-Western Serbia subpopulation or human mediated admixtures which can disturb present gene pools. The detection of distinct gene pools (and in a sense, different random mating units) in Serbian brown hare populations can provide important insight for the management. As game species, hares are subjected to exploitation and management, not always following the best practices and integrated approaches. We believe that genetic population structure information should be integrated with other traditional methods for brown hare hunting management strategy (such as estimation of population size and age structure), in order to provide long-term sustainability of the population.

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