

MILOMIR STEFANOVIĆ, NEVENA VELIČKOVIĆ, DEJAN BEUKOVIĆ, MILOŠ BEUKOVIĆ,
MIHAJLA DJAN, Novi Sad/Serbia

Genetic diversity of brown hares (*Lepus europaeus*) from lowland hunting grounds in Serbia

1. Introduction

Brown hares (*Lepus europaeus*) are widely distributed throughout Europe, and occur in a variety of environments, from Mediterranean to subarctic regions, showing considerable ecological plasticity (STAMATIS et al. 2009). It is the only *Lepus* species present in Serbia, where it is one of the most important and most hunted game species (POPOVIĆ et al. 2012). Since 1960s, remarkable decline of brown hares populations has been detected throughout its range, as well as in Serbia, mainly as a consequence of agriculture intensification, together with increased predation, overhunting and changes in climate conditions (MODESTO et al. 2011). In Serbia, the most widespread hare populations are occurring in the agricultural regions in northern province of Vojvodina (POPOVIĆ et al. 2012). Intensification of agricultural practices and anthropogenic effects led to the changes in typical plain habitats for this species. The increase of areas under arable crops, together with intensive use of pesticides and reduction of areas under forage crops, affected the availability of high-quality diet for hares and reduced the area under favorable habitat types (BEUKOVIĆ et al. 2012). As a consequence of ever-increasing anthropogenic changes to natural habitats, together with continuous environmental and climatic changes, high inbreeding rates and a decrease of genetic

variability due to the reduced gene flow could be expected. Genetic monitoring and identification of management units through usage of different molecular markers is the best estimator of natural population sustainability and a long-term potential for survival and adaptations (SCHWARTZ et al. 2007). Previous genetic analyses of brown hares from Vojvodina indicated shallow to moderate genetic differentiation among populations, as well as local fixation of certain alleles and reduced gene flow (VAPA et al. 1999, VAPA et al. 2002, DJAN et al. 2006, DJAN 2008, DJAN et al. 2013). Apart from being important for the hunting economy of Vojvodina province, hares play an important role as a prey species and contribute to the flow of organic matter and nutrients (VAPA et al. 2007). Therefore, continuous genetic monitoring of hares in hunting grounds of Vojvodina province represent an important step in maintaining the sustainability of this species. In the present study, which is a part of continuous genetic monitoring of this species, genetic variability in seven hunting grounds in Vojvodina is estimated based on the mitochondrial and microsatellites molecular markers.

2. Material and methods

A total of 59 brown hares were collected from seven lowland hunting grounds in northern Ser-

bian province of Vojvodina (Fig. 1), during the regular hunting seasons.

Total DNA was extracted from muscle tissue samples following the standard phenol-chloroform procedure (SAMBROOK & RUSSEL 2001). Mitochondrial DNA was used to amplify hyper-variable d-loop region following the procedure described by KASAPIDIS et al. (2005). The PCR products were purified by ExoSAP protocol (Fermentas, Vilnius, Lithuania) and sequenced with an BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, Carlsbad, CA, USA) on ABI 3130xl Genetic Analyzer using the forward primer only. Obtained sequences were aligned using the Clustal W algorithm implemented in

BioEdit (HALL 1999), and final adjustments were done by visual inspection. After alignment dataset comprised of 59 sequences of total length of 409bp. Overall DNA polymorphism parameters (h-haplotype diversity, π – nucleotide diversity, k – mean number of pairwise differences) were calculated in DnaSP (LIBRADO & ROZAS 2009), while analyses of molecular variance (AMOVA) based on pairwise ϕ_{ST} differences between hunting grounds was performed in Arlequin (EXCOFFIER & LISCHER 2010). To asses for the existence of spatial genetic patterns, a multivariate method, the spatial principal component analysis (sPCA) was performed using the adegenet package (JOMBART et al. 2008) in R. Delaunay triangulation connection network was

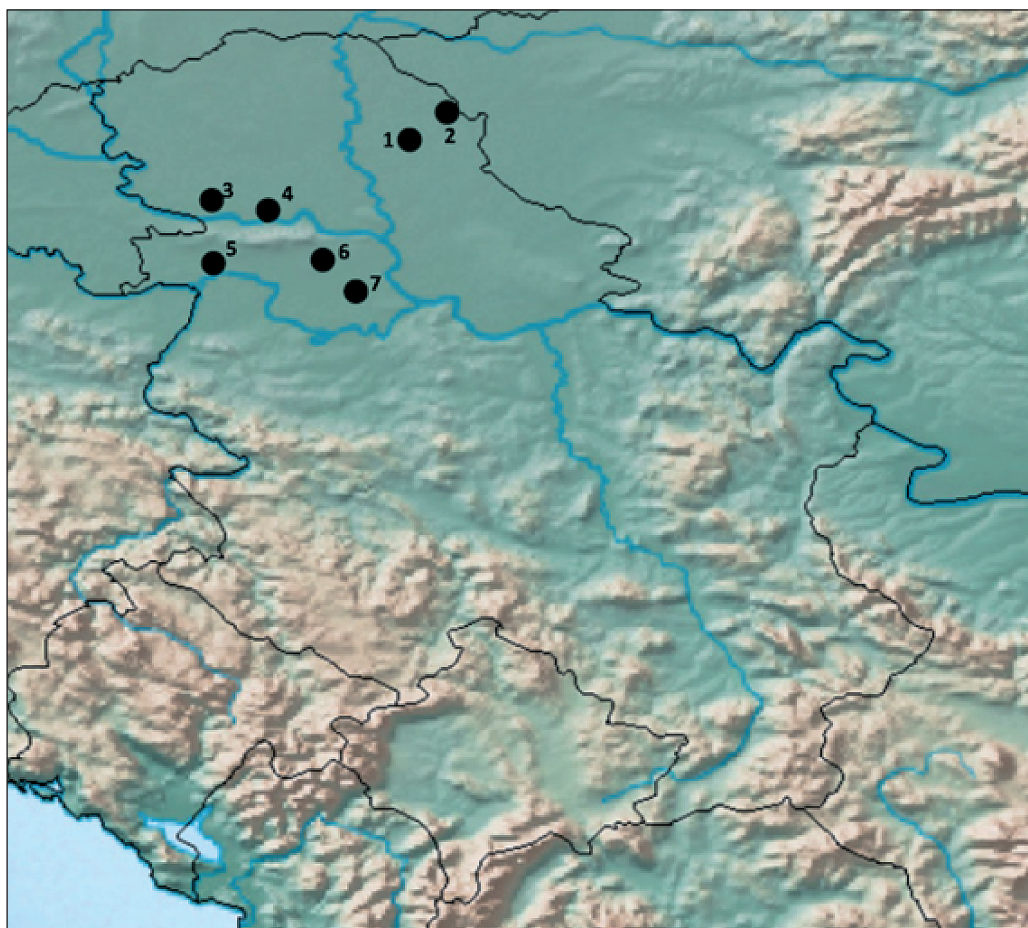


Fig. 1: Map of hunting grounds in Vojvodina province (1. "Varoški rit"; 2. "Melečin"; 3. "Palanački rit"; 4. "Podunavlje"; 5. "Granica"; 6. "Srem-sever"; 7. "Srem")

created between hunting grounds. Based on the genetic variance and spatial structure the largest eigenvalues were retained for the analysis.

Nuclear DNA was analysed based on five microsatellites loci: Sat2, Sat5 and Sat12 developed by MOUGEL et al. 1997, and, Lsa 1 and Lsa 3 developed by KRYGER et al. 2002. PCR amplification of selected loci and genotyping was done following the procedure described by DJAN (2008). Genetic diversity indices were calculated in GENETIX (BELKHIR et al. 1996–2004) and Arlequin. The latter was also used to test for the deviation from Hardy-Weinberg equilibrium, and to perform AMOVA based on F_{ST} pairwise differences between hunting grounds. The presence of spatial patterns was assessed by sPCA in adegenet as described above.

3. Results and discussion

Mitochondrial DNA diversity

In total, 15 different mtDNA d-loop haplotypes were detected based on 14 polymorphic sites, 13 of which were parsimony informative, and one singleton. One haplotype was common (30.5%), two were unique, while others were presented in frequencies ranging from 3.39% to 11.86%. Observed haplotype and nucleotide diversity in this study corresponds to previous data of genetic variability in brown hares from Vojvodina (DJAN et al. 2013) and to central European brown hare populations (FICKEL et al. 2005, 2008; KASAPIDIS et al. 2005; STAMATIS et al. 2009). Haplotype diversity value (H_d) was 0.875 ± 0.031 , while nucleotide diversity

(π) was 0.007 and the average number of nucleotide differences (k) was 2.957. Highest molecular diversity indices were detected within “Varoški rit” hunting ground, while the lowest values were detected within “Srem-sever” hunting ground (Table 1).

The analysis of molecular variance indicated moderate genetic differentiation between hunting grounds ($\phi_{ST} = 0.118$, $p < 0.01$), even though the most of the variation (88.12%) was due to the within hunting grounds variation. The private haplotypes were found only in the hunting ground “Srem”, while all other hunting grounds shared at least one haplotypes in common. This suggests the existence of gene flow and rather lower genetic differentiation in brown hares from studied hunting grounds in comparison to the previous study of brown hares from Vojvodina (DJAN et al. 2013), where ϕ_{ST} value was 0.201, and each population showed at least one unique haplotype. The results of sPCA analysis go in favor of shallow genetic differentiation, since neither global, nor local spatial patterns were statistically significant, even though a slight differentiation of hares from “Srem”, “Melečin” and “Varoški Rit” hunting grounds could be noticed (Fig. 2a).

Microsatellites diversity

Overall, the number of alleles per locus, for all 59 individuals, ranged from 6 to 18, with a mean value of 10 alleles over all loci. The highest number of alleles per locus was found at Sat2 locus, while the lowest number of alleles was found at Lsa 1 microsatellites locus. Expected heterozygosity was higher ($H_e = 0.787$)

Table 1: D-loop mtDNA molecular diversity indices in brown hares from hunting grounds in Vojvodina Province (Serbia)

	Podunavlje	Palanački Rit	Varoški Rit	Melečin	Srem-sever	Srem	Granica
n	7	10	6	10	5	11	10
h	4	5	5	4	2	7	4
H_d	0.810	0.756	0.933	0.778	0.401	0.927	0.733
π	0.004	0.006	0.009	0.008	0.004	0.008	0.005
k	2	2.531	3.730	3.781	1.6	3.273	2

n – sample size, h – number of haplotypes, H_d – haplotype diversity, π – nucleotide diversity, k – average number of nucleotide differences

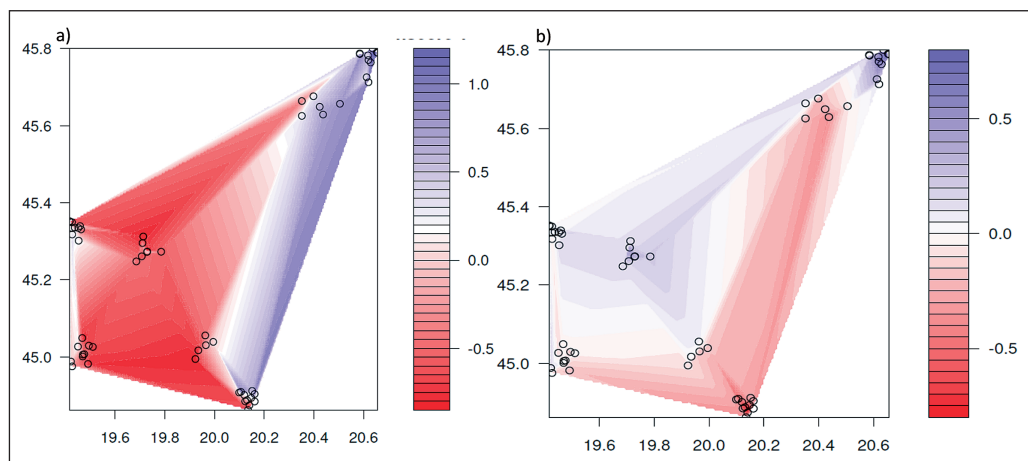


Fig. 2: Spatial principal components analysis (sPCA) showing the interpolated map of brown hares from hunting grounds in Vojvodina. Circles represent individuals and shade gradients quantify the degree of genetic differentiation based on a) d-loop mtDNA variability and b) microsatellites variability.

than the observed heterozygosity ($H_o = 0.506$). An overall F_{IS} value was 0.369, with four loci (Sat2, Sat5, Sat12 and Lsa3) found to be in significant deviation from Hardy-Weinberg equilibrium and five pairs of loci were in linkage disequilibrium after applying Boniferroni correction. Observed and expected heterozygosity values detected in this study are in range with the Central European hare populations (FICKEL et al. 2005, BEN SLIMEN et al. 2008).

When comparing between hunting grounds, the highest observed heterozygosity was detected in “Palanački rit” hunting ground, while the lowest observed heterozygosity was in “Srem-sever” hunting ground (Table 2). Mean number of different alleles over all loci ranged from 3.6 to 6.4. The highest number of alleles per locus was 11, observed in four loci (all except Sat5) in hunting ground “Srem”, while the lowest number of alleles per locus was 3, observed at locus Lsa3 in hunting ground “Srem-sever”. Significant deviation from Hardy-Weinberg equilibrium after Boniferroni correction was detected in two out of five loci in hunting grounds “Srem” and “Granica”; and one out of five in hunting grounds “Podunavlje” and Melečin”. The highest number of private alleles was 2 and it was found in locus Sat2 in hunting ground “Srem”, while the lowest number of private alleles was one, found in locus Lsa3 and Lsa1 in hunting

grounds “Melečin” and “Granica”, respectively. The observed deficit of heterozygosities, together with positive F_{IS} values could be attributed to the inbreeding effect due to the small number of individuals involved in reproduction, or to the fact that mating was occurring among the closely related individuals. It seems unlikely that positive F_{IS} values are caused by the Wahlund effect.

The sPCA analysis indicated no significant signal of local or global genetic structure, suggesting a pattern similar to the one observed by d-loop mtDNA analyses (Fig. 2b). Analysis of molecular variance further supported shallow genetic differentiation between hunting grounds with non-significant overall F_{ST} value of 0.099, and 90.05 % percentage of variation due to the within hunting grounds variation, which could be attributed to successful female reproductive activity.

4. Conclusions

Results of this study indicate that both mitochondrial and nuclear DNA markers reveal similar genetic diversity patterns. Multilocus estimates of genetic diversity and population structuring contribute to assessing the potential for population’s survival and adaptation, delineation of

Table 2: Allelic variation over all examined loci in brown hares from hunting grounds in Vojvodina Province (Serbia)

	Podunavlje	Palanački Rit	Varoški Rit	Melećin	Srem-sever	Srem	Granica
n	7	10	6	10	5	11	10
Na	4.8	5.6	4.2	6.4	3.6	5.2	5.6
Ne	3.89	3.75	3.17	4.99	2.89	3.5	3.83
LP	0	0	3	2	0	1	2
Ho	0.571	0.558	0.507	0.449	0.410	0.542	0.475
He	0.798	0.686	0.692	0.818	0.497	0.652	0.764
F _{IS}	0.226	0.187	0.298	0.427	0.155	0.241	0.339

n – sample size, Na – mean number of different alleles over all loci, Ne – mean effective number of alleles over all loci, LP – number of loci with private alleles, Ho – observed heterozygosity, He – expected heterozygosity, Fis – inbreeding coefficient

management units and development of sustainable management plans. Even though obtained results indicated moderate genetic diversity and the existence of gene flow, particular attention should be oriented toward further monitoring of hares from hunting grounds “Srem” and “Varoški rit” in order to avoid possible interruption in gene flow as well as further genetic isolation. In future, genetic monitoring should include more individuals and encompass more informative molecular markers, such as those linked to adaptive traits.

Summary

Brown hares (*Lepus europaeus*) are widely distributed throughout Europe and represent an important game species. Since 1960's, populations of this species have been experiencing remarkable decline throughout its range, raising concerns about the necessity of developing sustainable and conservation management measures. Understanding the patterns of genetic variability of this species and identifying possible obstacles to gene flow represent an important step in long-term population management. In order to assess genetic variability and structuring of brown hares from Vojvodina Province (Serbia), a total of 59 muscle tissue samples collected across the seven lowland hunting grounds were genotyped at five microsatellite loci and mtDNA control region. Genetic variability parameters were calculated in *Genetix*, *Arlequin* and *DnaSP*, while the pres-

ence of genetic structuring was assessed by spatial Principal Component Analysis (sPCA) in *adeget*. In total 15 d-loop mtDNA haplotypes were revealed, with haplotype and nucleotide diversity of 0.807 and 0.007, respectively. Expected heterozygosity (0.787) was higher than the observed heterozygosity (0.505), with an overall F_{IS} value of 0.359 and four loci in significant deviation from Hardy-Weinberg equilibrium. However, no signals of genetic structuring were observed. The observed deficit of heterozygosities could be attributed to the inbreeding effect due to the small number of individuals involved in reproduction, or to the fact that mating was occurring among the closely related individuals. Further genetic monitoring should encompass broader sampling and/or additional molecular markers.

Zusammenfassung

Der Feldhase (*Lepus europaeus*) ist als bedeutende Wildart in Europa weit verbreitet. Seit den 1960er Jahren ist ein starker Rückgang der Hasenpopulation zu verzeichnen, was Maßnahmen zum Management herausfordert. Genetische Untersuchungen sind ein wichtiger Schritt im langzeitlichen ökologischen Management. Aus 7 Gebieten der Vojvodina Provinz (Serbien) wurden von 59 Hasen Proben für genetische Analysen gewonnen. Es wurden 15d-loop mtDNA-Haplotypen gefunden. Es wird vermutet, dass ein Defizit an Heterozygobie zu einem „inbreeding effect“ beitragen könnte.

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Anschriften der Verfasser:

MSc MILOMIR STEFANOVIĆ
Department of Biology and Ecology
Faculty of Sciences, University of Novi Sad
Trg Dositeja Obradovića 2
21000 Novi Sad, Serbia
E-Mail: milomir.stefanovic@dbe.uns.ac.rs

Dr. NEVENA VELIČKOVIĆ
Department of Biology and Ecology
Faculty of Sciences, University of Novi Sad
Trg Dositeja Obradovića 2
21000 Novi Sad, Serbia
E-Mail: nevena.velickovic@dbe.uns.ac.rs

Dr. DEJAN BEUKOVIĆ
Department of Animal Science
Faculty of Agriculture, University of Novi Sad
Trg Dositeja Obradovića 8
21000 Novi Sad, Serbia
E-Mail: dejan.beukovic@stocarstvo.edu.rs

Dr. MILOŠ BEUKOVIĆ
Department of Animal Science
Faculty of Agriculture, University of Novi Sad

Trg Dositeja Obradovića 8
21000 Novi Sad, Serbia
E-Mail: milos.beukovic@stocarstvo.edu.rs

Dr. MIHAJLA DJAN
Department of Biology and Ecology
Faculty of Sciences, University of Novi Sad
Trg Dositeja Obradovića 2
21000 Novi Sad, Serbia
E-Mail: miihajla.djan@dbe.uns.ac.rs

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