Ministerul Educaţiei, Cercetării, Tineretului şi Sportului
Ministry of Education, Research, Youth and Sports
Autoritatea Naţională Pentru Cercetare Ştiinţifică
National Authority for Scientific Research

ACM-V

Asociaţia pentru Cercetare Multidisciplinară din Zona Vest a României

Association for Multidisciplinary Research of the West Zone of Romania

Universitatea Politehnica Timişoara

Politehnica University Timisoara

Universitatea de Vest „Vasile Goldiş" Arad

West University "Vasile Goldiş" of Arad

Lucrările celui de al XII-lea Simpozion Internaţional

„TINERII ŞI CERCETAREA MULTIDISCIPLINARĂ"

Proceedings of the XIIth International Symposium

"YOUNG PEOPLE AND MULTIDISCIPLINARY RESEARCH"

11 – 12 Noiembrie 2010
11 – 12 November 2010
Timişoara, România

Editura POLITEHNICA Timişoara

Authors (Autori): Veličković Nevena, Djan Mihajla, Obreht Dragana, Vapa Ljiljana

GENETIC DIVERSITY OF BROWN HARE (LEPUS EUROPAEUS) POPULATIONS FROM VOJVODINA

Keywords: brown hare, microsatellite, genetic diversity

Authors' address (Adresa autorilor):
Department of Biology and Ecology
Faculty of Sciences
University of Novi Sad
Trg Dositeja Obradovica 2
21000 Novi Sad Serbia

Summary

In the last decade several studies on nuclear gene pool diversity within and among brown hare populations from Vojvodina were performed (Vapa et al. 2002, 2007). First determination of genetic variability in brown hare populations from Vojvodina using microsatellites were done by Djan (2008). Optimal three-year monitoring period for brown hare populations was recommended, in order to perform adequate population management and conservation. The aim of this paper was microsatellite analysis of genetic diversity in brown hares from Vojvodina during hunting season 2008/09 and comparison of level of genetic diversity with the results obtained by Djan (2008). Total number of 60 individuals was analyzed from Backa, Banat and Srem populations. Three commonly used microsatellite markers: *Sat2*, *Sat5* and *Sat12* were included. New alleles were detected at all three loci. In Srem brown hare population, heterozygosity at loci *Sat2* and *Sat5* were significantly higher than in previous analyses, but some new sample sites from this particular region were included, so these findings as well as new alleles found might be consequence of this fact. Since the sample size in both researches was not statistically different, it could be concluded that heterozygosity remained at same level for these populations in the past three-year period.

Introduction

Awarness of the value of genetic resources has encouraged studies of the genetic diversity of different game populations. Population genetic structure has important ecological and evolutionary consequences. 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.

The European brown hare (*Lepus europaeus*) is an important game species, that occurs throughout the continent and extend 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 Balkan peninsula (Suchentrunk et al., 2000; Mamuris et al., 2001; Kasapidis et al., 2005).

In previous studies the allozyme composition of brown hares from Vojvodina (nothern province in Serbia) were analyzed (Vapa et al., 2002; Vapa et al., 2007; Davidovic, 2003) 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) adjoins the populations to the central European brown hare haplotypes group. Furthermore, analyzes of mtDNA

throughout Europe, including individuals from Vojvodina Province, showed that brown hares are not separated into discernable phyletic groups (Stamatis et al., 2009). Different local hare populations from Europe have already been analyzed using microsatellites (Fickel et al., 2005; Estonba et al., 2006; Andersson et al., 1999; Ben Slimen et al.,2008). First determination of genetic variability in brown hare populations from Vojvodina using microsatellites were done by Djan (2008). Optimal three-year monitoring period for brown hare populations was recommended, in order to perform adequate population management and conservation. The aim of this paper was microsatellite analysis of genetic diversity in brown hares from Vojvodina during hunting season 2008/09 and comparison of level of genetic diversity with the results obtained by Djan (2008).

Material and Methods

A total number of 60 brown hares were collected from three different regions at the Northern part of Serbia during hunting season 2008/2009. The following 3 regions, subsequently termed "populations", were included in genetic analyses: Backa (20), Banat (20) and Srem (20) (numbers of samples per region are given in parenthesis). Sampled tissue was frozen immediately after death of animal. Total DNA was extracted from eye and tongue tissue using standard phenol chloroform isoamylalcohol extraction with proteinase K digestion (Sambrook and Russel, 2001).

Three microsatellite loci with different levels of polymorphism were analyzed Sat2, Sat5, Sat12 (Mougel et al., 1997). The PCR conditions were performed in 20µl volume with final concentrations 100ng of DNA template, 1xBuffer, 200µM dNTP, 10 pmol of each primer, 2.5 mM $MgCl_2$ and 2 units of Taq DNA polymerase. The PCR program consisted of 92°C for 2 min, followed by 25 cycles of 94°C for 30s, 58°C for 60s, and 72°C for 120s, and final extension of 72°C for 10 min.

The PCR products 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 products. A known DNA sequence was run with the PCR products to measure the size of different alleles.

Microsatellite loci were tested for deviation from Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium using the Markov chain method in GENEPOP version 3.4 (Raymond and Rousett, 1995). 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). The same program was used to calculate allele frequencies, mean number of alleles (A), observed (Ho) and expected (He) heterozygosity. Difference tests in computer package STATISTICA 8.0 were done in order to compare heterozygosity values with the results obtained by Djan (2008).

Results and Discussion

A total number of 49 alleles at three loci were found in brown hare populations from Vojvodina. The highest number of alleles per locus was found at *Sat2* microsatellite (23), while the lowest number of alleles was present at locus *Sat12* (8), with an average of 16.33 alleles/locus. In total the highest number of alleles per population was detected in Banat brown hare population (37), and the lowest in Srem (30), with an average number of alleles per population 33. Microsatellite amplification

was successful in 83.30% (Sat5) to 93.30% (Sat2). Numbers of alleles per locus (A), allelic size ranges (in bp), size and frequency of the most common allele (S and F), expected (He) and observed (Ho) locus-specific heterozygosity per population for seasons before 2006. (Djan, 2008) and 2008/09 are given in Table 1.

Table 1. Allelic variation at 3 microsatellite loci in brown hare (*Lepus europaeus*) populations from Vojvodina

Population		Loci						Mean	
		Sat2		Sat5		Sat12			
		Djan (2008)	Season 2008/09	Djan (2008)	Season 2008/09	Djan (2008)	Season 2008/09	Djan (2008)	Season 2008/09
Bačka	A	13	13	7	11	8	6	9.33	10
	R	225-265	227-271	191-221	175-221	110-138	106-126		
	S	261	243	191	191	126	114		
	F	0.14	0.15	0.65	0.31	0.30	0.34		
	He	0.93	0.91	0.57	0.84	0.82	0.77		
	Ho	0.68	0.80	0.25	0.5	0.70	0.7		
Banat	A	14	17	8	13	9	7	10.33	12.33
	R	225-263	225-273	191-221	175-223	110-146	102-126		
	S	225	236,238,259	191	175,191,221	134	114,118,122		
	F	0.16	0.11	0.38	0.15	0.24	0.23		
	He	0.93	0.93	0.82	0.89	0.86	0.81		
	Ho	0.63	0.8	0.12	0.75*	0.79	0.7		
Srem	A	15	14	5	11	10	7	10	10.66
	R	225-265	225-275	191-215	177-223	110-154	100-122		
	S	247	238,241,265	191	191	134,138	114		
	F	0.23	0.15	0.58	0.23	0.22	0.33		
	He	0.89	0.89	0.63	0.85	0.86	0.79		
	Ho	0.65	0.95*	0.15	0.55*	0.87	0.85		

A – number of alleles per locus; R – allelic size range in bp; S – size in bp of most frequent allele; F – frequency of the most common allele; He – expected heterozygosity; Ho – observed heterozygosity; * - significant (Bonferroni correction $p<0.05$) difference between heteroyzgosities

In previous study (Djan, 2008), the highest number of alleles was also observed at *Sat2* locus, but the lowest number of alleles was found at *Sat5* microsatellite. In total highest number of alleles was observed in Banat brown hare population (31), same as nowadays.

New alleles were detected at all three loci. At *Sat2* locus 6 new alleles were determined in all three populations, while two of before found couldn't be detected in analyzed sample. At *Sat5* locus 10 new alleles were observed, while one of predefined were not found. At *Sat12* locus lower number of alleles was found, 6 of 13 previously detected alleles of this locus were confirmed and two new were found. At *Sat5* microsatellite locus one allele was shown as the most frequent in both studies in all populations.

Higher numbers of alleles were detected in German brown hare populations (57) and lower number in Scandinavian brown hare populations (33), but the higher number of microsatellites were analyzed as well, five in both studies (Fickel et al., 2005; Andersson et al., 1999). Higher number of alleles was found in Scandinavian hares *L. europeaus, L. timidus* and suspected hybrids (114), among seven microsatellite loci (Thulin et al, 2006a).

Ben Slimen et al. (2008) reported average value 14.7 alleles/locus, which is lower than in our study, and locus Sol03 had highest number of alleles (34), and at loci Lsa4 and Lsa6 lowest number (6) of alleles were detected. In Canadian

snowshoe hares average number of alleles was 16.3 (Thulin et al, 2006a), similar as in our study.

Observed heterozygosity (Ho) values for populations ranged from 0.66 (Bačka) to 0.78 (Srem), with an average 0.73. The highest observed heterozygosity was found for locus *Sat2* (0.85), and the lowest at locus *Sat5* (0.6), with an average 0.73. In previous analyses (Djan, 2008), observed heterozygosity values for populations were lower (0.51-0.56). The highest observed heterozygosity was at *Sat12* locus (0.79), and lowest was found for locus *Sat5* (0.17), with an average 0.54. In Srem brown hare population, heterozygosity at loci *Sat2* and *Sat5* were significantly higher than in previous analyses. Comparing to the previous analysis (Djan, 2008), some new sample sites from this particular region were included, so these findings as well as new alleles found, might be consequence of this fact. Average observed heterozygosity was also higher, but not significantly. Since the sample size in both researches was not statistically different, it could be concluded that heterozygosity remained at same level for these populations in the past three-year period.

In brown hare populations from Germany Ho varied in wider range between 0.527 – 0.782 (Fickel et al., 2005). In Scandinavian brown hares Ho varied form 0.333 to 1.000 (Andersson et al., 1999). In *L. europaeus* and *L. capensis* populations Ho values ranged from 0.45 to 0.63 (Ben Slimen et al., 2008). Heterozygosity values and microsatellite allele distribution indicated that brown hare populations from Serbia are still sufficiently genetically diverse to avoid inbreeding depression and to exclude fixation of alleles.

Average expected heterozygosity value for all brown hare populations from Vojvodina was 0.84, and 0.81 in previous study (Djan, 2008). In Canadian snowshoe hares (Burton et al., 2002), and L. europaeus and L. capensis populations (Ben Slimen et al., 2008) these values were lower, 0.67 and 0.624, respectively.

Deviation from Hardy-Weinberg equilibrium was found only for Bačka brown hare population due to heterozygote deficiency detected. Thulin et al. (2006b) detected observed heterozygote deficiency in one population for four loci Lsa2, Sat12, Sat5 and Sol08, and explained this fact as result of Wahlund effect because the samples were collected over large geographical area. We presume that Wahlund effect is not the cause of heterozygote deficiency in Backa brown hare population. In Scandinavian hares heterozygote deficiency was detected in all populations (Andersson et al., 1999; Thulin et al., 2006a). In Ben Slimen (2008) deviation from HWE was found in 11 populations due to overall heterozygosity deficits, while 7 populations were in HWE.

In order to determine overall difference in genetic diversity between two periods, it is necessary to analyze additional three loci (Sol33, Lsa2 and Lsa3) previously analysed, and after these broad spectrum analyses to compare again values of observed heterozygosity and genetic diversity within and among populations.

Conclusion

Determination and monitoring of genetic variability in natural game population is the basis for adequate ecological management and biological conservation of different species. Analyses of genetic variability in brown hare populations from Vojvodina using microsatellites for samples from hunting seasons from 2003 to 2006 recommended optimal three-year monitoring period for these populations. The aim of

this study was microsatellite analysis of genetic diversity in brown hares from Vojvodina during hunting season 2008/09 and comparison of those with the previous results. New alleles were detected at all three loci. In Srem brown hare population, heterozygosity at two loci were significantly higher than in previous analyses, but some new sample sites from this particular region were included, so these findings as well as new alleles found might be consequence of this fact. According to observed heterozygosity values, we concluded that heterozygosity remained at same level for these populations in the past three-year period.

References

1. Andersson AC, Thulin CG, Tegelstrom H. (1999) Applicability of rabbit microsatellite primers for studies of hybridization between an introduced and a native hare species. Hereditas 130: 309-315
2. Ben Slimen H, Suchentrunk F, Stamatis C, Mamuris Z, Sert H, Alves CP, Kryger U, Shanin AB, Elgaaied ABA. (2008) Population genetics of cape and brown hares (Lepus capensis and L. europaeus): A test of Petter 's hypothesis of conspecificity. Biochemical Systematics and Ecology 36: 22-29
3. Burton C, Krebs JC, Taylor BE. (2002) Population genetic structure of the cyclic snowshoe hare (Lepus americanus) in southwestern Yukon, Canada. Molecular Ecology 11: 1689-1701
4. Davidovic M. (2003) Molecular genetics analysis of brown hare populations (*Lepus europaeus*, Pallas) variability in Vojvodina. Master thesis, Faculty of Biology, Belgrade. (in Serbian)
5. Djan M, Obreht D, Vapa LJ. (2006) Polymorphism of mtDNA regions in brown hare (Lepus europaeus) populations from Vojvodina (Serbia and Montenegro). Eur J Wildl Res 52: 288-291
6. Djan M. (2008) Polymorphism of microsatellites and mtDNA in brown hare populations (*Lepus europaeus* Pallas). PhD thesis, Faculty of Sciences, University of Novi Sad, Serbia
7. Estonba A, Solis A, Iriondo M, Sanz-Martin JA, Perez-Suarez G, Markov G, Palacios F. (2006) The genetic distinctiveness of the Iberian hare species: Lepus europaeus, L. granatensis and L. castroviejoi. Mammalian Biology 71 (1): 52-59
8. Fickel J, Schmidt A, Putze M, Spittler H, Ludwig A, Streich JW, Pitra C. (2005) Genetic structure of populations of European brown hare: implications for management. Journal of Wildlife Management 69 (2): 760-770
9. Kasapidis P, Suchentrunk F, Magoulas A, Kotoulas G. (2005) The shaping of mitochondrial DNA phylogeographic patterns of the brown hare (Lepus europaeus) under the combined influence of Late Plaistocene climatic Fluctuations and anthropogenic translocations. Molecular Phylogenetics and Evolution 34: 55-66
10. Mamuris Z, Sfougaris AI, Stamatis C. (2001) Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDNA RFLP-PCR analysis implications for conserving genetic diversity. Biological Conservation 101: 187-196
11. Mougel F, Mounolou JC, Monnerot M. (1997) Nine polymorphic microsatellite loci in the rabbit, Oryctolagus cuniculus. Animal Genetics 28: 58-71

12. Raymond M, Rousett F. (1995) GENEPOP (version 3.1d): population genetic software for exact tests and ecumenism. J Hered 86: 248-249
13. Rice WR. (1989) Analyzing tables of statistical tests. Evolution 43: 223-225
14. Sambrook JF, Russel DW. (2001) Molecular Cloning: A laboratory manual, 3rd Edition. Cold Sprin Harbour Laboratory Press, USA
15. Stamatis C, Suchentrunk F, Moutou1 AK, Giacometti M, Haerer G, Djan M, Vapa Lj, Vukovic M, Tvrtkovic N, Sert H, Alves CP, Mamuris Z. (2009) Phylogeography of the brown hare (Lepus europaeus) in Europe: a legacy of south-eastern Mediterranean refugia? Journal of Biogeography 36: 515–528
16. Suchentrunk F, Michailov C, Markov G, Haiden A. (2000) Population genetics of Bulgarian brown hares *Lepus europaeus*: allozymic diversity at zoographical crossroads. Acta theriologica 45: 1-12
17. Suchentrunk F, Mamuris Z, Sfougaris A, Stamatis C. (2003) Biochemical genetic variability in brown hares (*Lepus europaeus*) from Greece. Biochemical Genetics 41 (5/6): 127-140
18. Thulin GC, Jaarola M, Tegelström H. (1997) The occurence of mountain hare mitochondrial DNA in wild brown hares. Molecular Ecology 6: 463-467
19. Thulin CG, Stone J, Tegelstrom H, Walker WC. (2006a) Species assignment and hybrid identification among Scandinavian hares Lepus europaeus and L. timidus. Wildl.Biol. 12: 29-38
20. Thulin CG, Fang M, Averianov AO. (2006b) Introgression from *Lepus europaeus* to *L. timidus* in Russia revealed by mitochondrial single nucleotide polymorphisms and nuclear microsatellites. Hereditas 143: 68-76
21. Vapa Lj, Obreht D, Vapa M, Selmic V. (2002) Genetic variability in brown hare (*Lepus europaeus*) populations in Yugoslavia. Z. Jagdwissenschaft, 48: 261–266
22. Vapa Lj, Djan M, Obreht D, Hammer S, Suchentrunk F. (2007) Allozyme variability of brown hares (Lepus europaeus) from the Vojvodina (Serbia), compared to central and south eastern European populations. Acta Zool. Acad. Sci. Hung. 53: 75-87