Genetic analysis for geographic isolation comparison of brown bears living in the periphery of the Western Carpathians Mountains with bears living in other areas

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ABSTRACT

Populations of the European brown bear (*Ursus arctos L.*) differ substantially in size, degree of geographic isolation and level of genetic diversity. Present patterns result from phylogeographic processes and profound human intervention. We assessed the genetic variability of a subpopulation of brown bears near the periphery of their range in the Western Carpathian Mountains and compared their genetic properties with those of bears in the core of the same population and elsewhere. Samples were collected non-invasively in 2007-2008 and 2010 in Strážovské vrchy Protected Landscape Area (PLA) in Slovakia (included to the NATURA 2000 networking programme). Seven polymorphic microsatellite loci (UaMU26, UaMU64, G10B, G1D, G10L, UaMU50 and UaMU51) were amplified using a nested PCR in order to assess the following parameters: variability, allelic combinations, heterozygosity, number of alleles and inbreeding coefficient. Sufficient brown bear DNA for analysis was obtained from 57 out of 140 samples (41%), among which 45 different genotypes were identified. Loci had a mean of 2.71 ± 0.76 alleles. Average observed heterozygosity was 0.59. The inbreeding coefficient was negative for all but one of the analysed loci (2007-2008). In the year 2010 was negative three of seven loci. These results imply that gene flow with other parts of the population has been maintained in the reduced level and the isolation level of bears in the study area was not so low. Nevertheless, the genetic variability of bears in Strážovské vrchy PLA was lower than that reported from other localities in the Carpathian Mountains. The results are discussed in the context of behavioural ecology and conservation genetics.

Keywords: Carpathian Mountains; European Brown Bear; *Ursus arctos L.;* Genetic Diversity; Microsatellite Markers; Non-Invasive Sampling

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MATERIAL AND METHODS

1. Sample Collection and DNA Isolation

A total of samples 57 (41.8%) out 140 from different sites of Strážovské vrchy Mts were collected. A 37 samples collected during the year 2010 (faeces and hair samples) and 20 samples from Strážovske vrchy territory (during the period 2007-2008) were examined. Sufficient brown bear DNA for analysis was obtained from 20 (2007-2008) out of 46 samples and 37 (2010) out of 94 samples collected in the field. A total of 45 different genotypes were identified among 28 samples from faeces and 29 from hair. DNA extraction from hairs was performed using 10% Chelex according to Kruckenhauser et al. [1], Depending on availability and quality hairs with visible roots were used, DNA extractions from non-invasive samples were performed with the QIAamp DNA Stool Kit (QIAGEN) with a final elution volume of 100 µl.

2. Microsatellites Analysis and Gender Identification

Seven microsatellite loci Mu26, Mu64, G10B, G1D, G10L, Mu50 and Mu51 were amplified using polymerase chain reaction [2] and fragment length (allele) analyses were carried out on eight-capillary sequencer (Genome Lab GeXP, BeckmanCoulter). Analyses were repeated in order to verify the reliability of individual allele length determination. DNA was extracted from hair using 10% Chelex solution [25] and from faeces with the Qiamp DNA Stool® kit (Qiagen). To test for individuals, seven microsatellite loci (UaMU26, UaMU64, G10B, G1D, G10L, UaMU50 and UaMU51) were amplified in a nested polymerase chain reaction (PCR) [2]: a longer fragment of each locus was amplified prior to amplifying a more specific area. Two-step PCR procedures improve genotyping success rate and limit genotyping errors [3]. Observed (H_O) and expected (H_E) heterozygosity were calculated using Cervus 3.0 software (Field Genetics). Results were compared with genetic data from brown bears in core ranges of the Carpathian Mountains in Slovakia [4,5] and Romania [4] as well as in central Austria [1].

Table 1. Genetic variability of brown bears in the Strážovské Vrchy Mountains, Slovakia. n_a- observed number of alleles, n_e- effective number of alleles. Pic – Polymorphic information content, H_O – observed heterozygosity, H_E – expected heterozygosity, Fis – inbreeding coefficient.

2007 - 2008

Locus	n_a	n_e	P_{IC}	H_O	H_E	F_{IS}
UaMU26	2	1.89	0.35	0.30	0.47	0.36
UaMU64	3	2.38	0.47	0.85	0.58	- 0.47
G10B	2	1.49	0.27	0.40	0.33	- 0.21
G1D	4	3.03	0.59	0.85	0.67	- 0.27
G10L	2	2.04	0.38	0.80	0.51	- 0.57
UaMU50	3	2.56	0.51	0.80	0.61	- 0.31
UaMU51	3	2.63	0.53	0.90	0.62	- 0.45
Mean	2.71	2.29	0.44	0.70	0.54	- 0.27
St. Dev	0.76	0.52	0.10	0.24	0.12	0.31

2010

Locus	n_a	n_e	P_{IC}	H_O	H_E	F_{IS}
UaMU26	2	1.89	0.35	0.07	0.47	0.85
UaMU64	3	3.13	0.59	0.54	0.68	0.21
G10B	2	1.96	0.37	0.32	0.49	0.35
G1D	4	3.33	0.64	0.79	0.70	-0.13
G10L	2	1.92	0.36	0.75	0.48	-0.56
UaMU50	3	2.50	0.51	0.50	0.60	0.17
UaMU51	3	2.27	0.48	0.75	0.56	-0.34
Mean	2.71	2.43	0.47	0.53	0.57	0.08
St. Dev	0,76	0.59	0.12	0.27	0.10	0.47

2007 - 2010

Locus	n_a	n_e	P_{IC}	H_O	H_E	F_{IS}
UaMU26	2	2	0.38	0.14	0.50	0.72
UaMU64	3	2.94	0.58	0.67	0.66	-0.02
G10B	2	1.96	0.37	0.35	0.49	0.29
G1D	4	3.33	0.64	0.81	0.70	-0.16
G10L	2	2	0.37	0.81	0.50	-0.62
UaMU50	3	2.44	0.50	0.61	0.59	-0.04
UaMU51	3	2.27	0.48	0.77	0.56	-0.38
Mean	2.71	2,42	0.47	0.59	0.57	-0.03
St. Dev	0,76	0.53	0.11	0.26	0.08	0.44

Table 2. Allelic combinations found at seven microsatellite loci in genomic DNA from brown bears in the Strážovské Vrchy Mountains. The most frequently occuring combination for each locus is shovn in bold. AC – allelic combinations detected.

2007 - 2008

AC	UaMU26	UaMU64	G10B	G1D	G10L	UaMU50	UaMU51
1	**182/182**	177/194	**114/114**	171/221	143/143	**118/121**	110/110
2	182/198	**184/194**	114/126	179/179	**143/171**	121/125	**110/136**
3	198/198	184/184		**179/208**	171/171	125/125	110/116

2010

AC	UaMU26	UaMU64	G10B	G1D	G10L	UaMU50	UaMU51
1	**182/182**	177/177	**114/114**	171/221	143/143	118/121	110/110
2	182/198	177/194	114/126	179/179	**143/171**	**121/121**	**110/136**
3	198/198	**184/194**	126/126	**179/208**		121/125	110/116
4		184/184				125/125	

3. Statistical Methods

Observed (H_O) and expected (H_E) heterozygosity were calculated with CERVUS software. Descriptive statistics for each locus (mean number of alleles per locus, heterozygosities and polymorphic information content (P_{IC}) were computed from allele frequencies. The Fisher's exact test was used to check for genotypic linkage disequilibrium for all pairs of loci by employing the Markov chain method, as implemented in GENEPOP [6]. Deviations from Hardy–Weinberg (HW) proportions were evaluated through the Weir and Cockerham's [7] and Robertson and Hill's [8] estimates of F_{IS} to test for heterozygote deficit with Levene's correction for small sample size, using the method described by Guo and Thompson [9].

Results

The mean observed heterozygosity (H_O) among the seven loci examined in the year 2007-2008 was 0.70 and 0.53 in the year 2010, the mean expected heterozygosity (H_E) 0.54 in the year 2007-2008 and 0.57 in the year 2010. Only one locus (UaMU26) had a H_O lower than HE in the year 2007-2008. In the year 2010 it was locus UaMU26, UaMU64, G10B and Ua MU50 (Table 1). Locus G1D had the most alleles (four), while UaMU26, G10B and G10L showed low allelic variability (two alleles per locus). With the exception of G10L in the year 2007-2008 and UaMU64 in the year 2010, the observed number of alleles at each locus (n_a) was greater than the effective number of alleles (n_e). The inbreeding coefficient was negative for six out of the seven loci (mean value F_{IS} = −0.27), the exception being UaMU26 (F_{IS} = +0.33) (2007-2008). In the year 2010 the inbreeding coefficient was negative only for three out seven loci (mean value F_{IS} = 0.08).

Locus UaMU26, UaMU64, G1D, G10L, UaMU50 andUaMU51 had the equal number of different allelic combinations (three) (Table 2). The least variability was found at loci G10B (2 alleles per locus in 2 different combinations) (2007-2008). Locus UaMU64 and UaMU50 had the equal number of different allelic combinations (four), followed by UaMU26, G1D, G10B and UaMU51 with three each. The least variability was found at loci G10L (2 alleles per locus in 2 different combinations) (2010).

DISCUSSION AND CONCLUSIONS

The brown bear is a wide-ranging species exhibiting malebiased dispersal [10]. Adult males commonly use hundreds of square kilometres in their search for food and mating opportunities and dispersing subadult male brown bears may roam over areas up to 12,000 km² [11]. Such movements facilitate gene flow and, in the case of dispersing young males, there is evidence that it operates as a mechanism to avoid inbreeding [10]. The relatively high level of heterozygosity and low degree of inbreeding we found in bears in Strážovské vrchy (this study) suggests that the subpopulation is not geographically isolated and gene exchange with other segments of the population has been maintained. The most obvious potential source of migration into the study area is the Malá Fatra mountain range, which lies immediately to the east (Figure 1) and has a high density of bears [12, 5]. The eastern edge of the Strážovské vrchy seems to present the least obstruction to wildlife movement in and out of the study area, as the unfenced primary road I/64 passes through a heavily forested landscape for the 20-km section between Rajecká Lesná and Kľačno, forest availability being the most important habitat constraint on bear distribution in the Western Carpathians [13, 14]. There are, however, several other nearby ranges from which bears could reasonably be expected to reach Strážovské vrchy, including Vtáčnik, Kremnické vrchy and Veľká Fatra. Knowledge of population size, distribution, social and sexual structure, home range and population trend on the local level as well as migration is crucial for the proper conservation and management of species within and between protected areas.

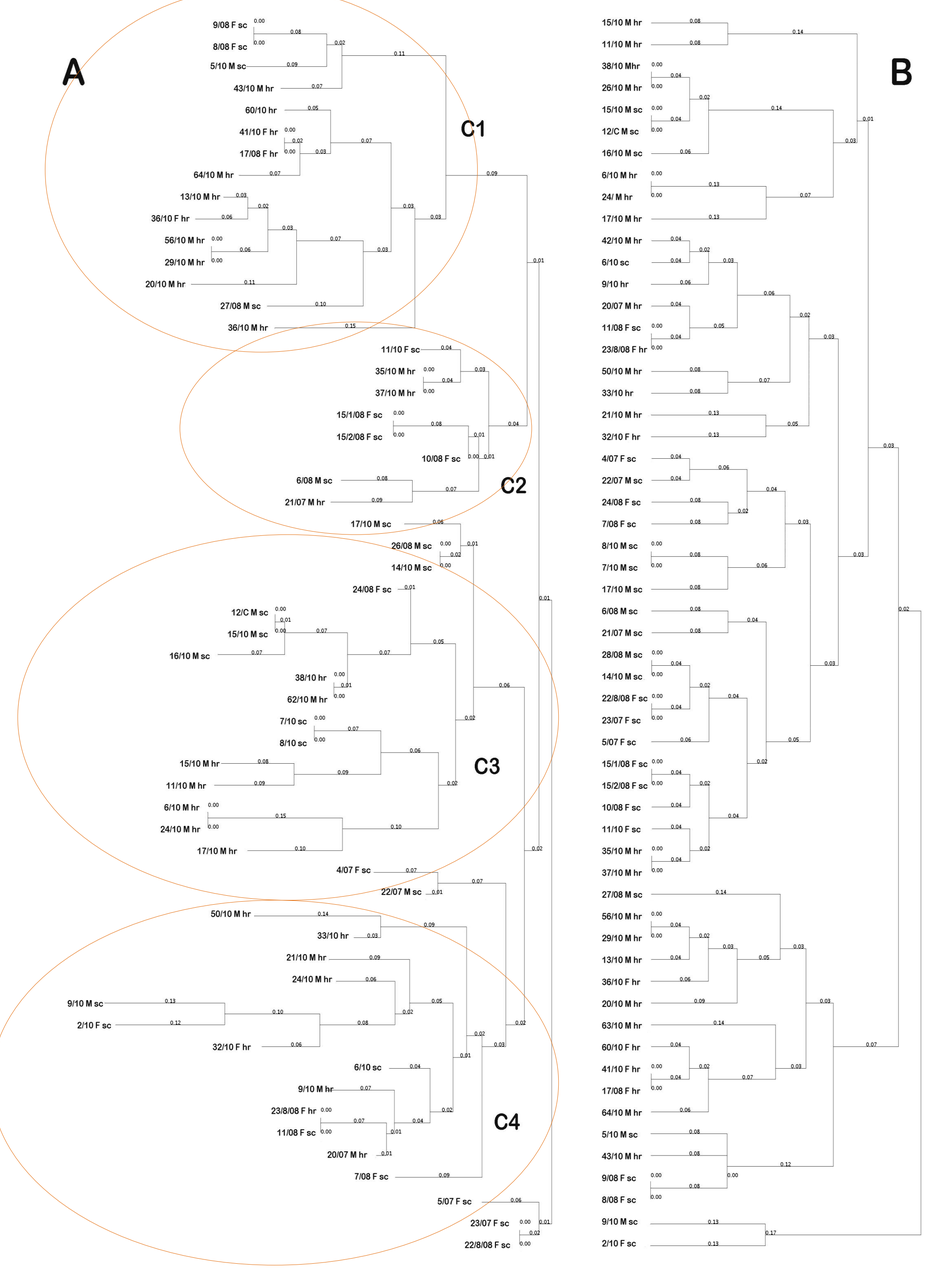

Figure 1. Scematic presentation of animals distribution into „family" clusters (C1 – C4) based on degree of reladness between individual microsatellite profiles. (a) present Neighbor-joining method and (b) the UPGMA method (hr – hair samples, sc – scat samples).

Division of Animals into Clusters

Processing of acquired data and in particular in finding answers to the question of individuals distribution on the site led them to propose procedures for evaluation of microsatellite data was processed using our original software (Java script). Two methods were used Neighbor-joining and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) for construction of clusters graphic presentation based on microsatellites data processing.

REFERENCES

[1] Kruckenhauser, L., Rauer, G., Däubl, B. and Haring, E. (2009) Genetic monitoring of a founder population of brown bears (Ursus arctos) in central Austria. Conservation Genetics, 10, 1223-1233. doi:10.1007/s10592-008-9654-6

[2] Taberlet, P., Camarra, J.J., Griffin, S., Uhrès, E., Hanotte, O., Waits, L.P., Dubois-Paganon, C., Burke, T. and Bouvet, J. (1997) Noninvasive genetic tracking of the endangered Pyrenean brown bear population. Molecular Ecology, 6, 869-876. doi:10.1111/j.1365-294X.1997.tb00141.x

[3] Bellemain, E. and Taberlet, P. (2004) Improved noninvasive genotyping method: application to brown bear (Ursus arctos) faeces. Molecular Ecology Notes, 4, 519-522. doi:10.1111/j.1471-8286.2004.00711.x

[4] Straka, M., Paule, L., Ionescu, O., Štofík, J. and Adamec, M. (2012) Microsatellite diversity and structure of Carpathian brown bears (Ursus arctos): Consequences of human caused fragmentation. Conservation Genetics, 13, 153-164. doi:10.1007/s10592-011-0271-4

[5] Janiga, M., Fečková, M., Korňan, J., Kalaš, M., Labudíková, I., Matiaško, K. and Londa, P. (2006) Preliminary results on genetic tracking of the Brown Bear (Ursus arctos) individuals in the Malá Fatra National Park
(Slovakia). Oecologia Montana, 15, 24-26.

[6] Bellemain, E. and Taberlet, P. (2004) Improved noninvasive genotyping method: application to brown bear (Ursus arctos) faeces. Molecular Ecology Notes, 4, 519-522. doi:10.1111/j.1471-8286.2004.00711.x

[7] Weir, B.S. and Cockerham, C.C. (1984) Estimating F- statistics for the analysis of population structure. Evolu- tion, 38, 1358-1370. doi:10.2307/2408641

[8] Robertson, A. and Hill, W.G. (1984) Deviations from Hardy-Weinberg proportions: Sampling variances and use in estimation of inbreeding coefficients. Genetics, 107, 703-718.

[9] Guo, S.W. and Thompson, E.A. (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics, 48, 361-372. doi:10.2307/2532296

[10] Zedrosser, A., Støen, O.-G., Sæbø, S. and Swenson, J.E. (2007) Should I stay or should I go? Natal dispersal in the brown bear. Animal Behaviour, 74, 369-376. doi:10.1016/j.anbehav.2006.09.015

[11] Swenson, J.E., Dahle, B., Gerstl, N. and Zedrosser, A. (2000) Action plan for the conservation of the brown bear in Europe (Ursus arctos)—Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). Nature and Environment No. 114, Council of Europe Publishing, Strasbourg.

[12] Rigg, R. and Adamec, M. (2007) Status, ecology and management of the brown bear (Ursus arctos) in Slovakia. Slovak Wildlife Society, Liptovský Hrádok, 128p.

[13] Fernández, N., Selva, N., Yuste, C., Okarma, H. and Jakubiec, Z. (2012) Brown bears at the edge: Modelling habitat constrains at the periphery of the Carpathian population. Biological Conservation, 153, 134-142. doi:10.1016/j.biocon.2012.04.013

[14] Finďo, S., Skuban, M. and Koreň, M. (2007) Brown bear corridors in Slovakia. Carpathian Wildlife Society, Zvolen.