Open Journal of Genetics, 2013, 3, 174-182 **OJGen**
http://dx.doi.org/10.4236/ojgen.2013.33020 Published Online September 2013 (http://www.scirp.org/journal/ojgen/)

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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Received 24 April 2013; revised 26 May 2013; accepted 10 June 2013

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 in 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

1. INTRODUCTION

The brown bear (*Ursus arctos*) re-colonised the entire European continent after the Last Glacial Maximum [1,2], and yet its current distribution shows a discontinuous pattern as a result of various human activities [3,4]. In contrast to the relatively large and contiguous populations with higher expected heterozygosity and allelic diversity in Eastern Europe, the Balkans and Scandinavia [5] population fragments in the western part of the continent are extremely small and isolated, with low levels of genetic variability and vulnerable to genetic drift and inbreeding [6-8].

An additional level of complexity is added by the existence of three mitochondrial subclades [6,9,10]. They may be the result of different founder populations having passed through bottlenecks prior to rapid recolonisation during the Holocene [11] or recent human-induced population fragmentation due to habitat loss and killing [12,13].

It has been suggested that one of the glacial refugia from which brown bears of the eastern lineage (subclade 3a) re-colonised most of continental Eurasia was in the Western Carpathian Mountains of present day Slovakia [2,11,14]. Alternatively, bears may have survived the glacial period in the cold tundra-steppe of central Europe [12]. Regardless of when and how they arrived, brown bears persisted in the Western Carpathians in a large, continuous population until the Middle Ages [15] and in most forested upland areas of present-day Slovakia

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through to the 19th century [16]. Deforestation, overhunting and eradication programmes then precipitated a catastrophic decline in range and numbers culminating in the 1930s, when it was estimated that only c.20 - 60 individuals remained in an isolated, relict population [16].

A 30-year moratorium on hunting facilitated population recovery [17]. The bears continued to increase in number and reoccupy parts of their former range despite the resumption of limited hunting from the 1960s [18]. The Western Carpathian bear population now numbers several hundred individuals and extends across all mountain ranges of central and northern Slovakia [19]. However, although it was thought that connection with the much larger Eastern Carpathian population was re-established in the 1980s [20], the two populations show a high degree of differentiation most likely resulting from genetic divergence during the c.100 years of their division and evidence of renewed gene flow is sparse [21].

Populations that have passed through a recent demographic bottleneck would generally be expected to have lost genetic diversity through stochastic drift [22]. However, medium-high levels of allelic variation have been found in the nuclear DNA of Western Carpathian bears and their level of genetic diversity seems to be within the range commonly observed in different populations of brown bears and other mammals [15,21,23], which suggests the population bottleneck may not have been quite so severe as feared by contemporary observers. The main aim of our study was to examine the degree of genetic diversity and geographic isolation of bears in a subpopulation near the periphery of their current distribution in the Western Carpathians and compare it with that of bears in core areas of the population and in select European subpopulations.

Fieldwork was conducted in the Strážovské vrchy mountain range (49˚23'702"N, 18˚73'46"E), which extends over an area of c. 900 km^2 in northwest Slovakia (**Figure 1**) and forms part of the Inner Western Carpathian Mountains. Brown bears were apparently absent until the mid 1960s [17,19] but re-colonised during the period 1967-1984, when the recovering Western Carpathian bear population expanded its range 40 km northwestwards [24].

Intense development in molecular technology, have moved bear ecology into a significant development in which genetic analyses can be performed with ease and with great informative value. Environmentalists can now routinely utilize genetic information obtained from DNA to formulate questions about the behavioural ecology and conservation genetics of bear populations. The highly variable microsatellites markers that have been analysed in this study offer an effective tool for individual identification. These DNA fingerprints can be used in an ecological context for the dendrogram construction based on the degree of microsatellite profiles similarity to linking individuals. Then we can get a view on the degree of genetic relatedness of individuals grouped into clusters. Implementation of population genetic features of the PLA Strážovské vrchy provides an overview of the migration rate between neighboring populations of the brown bears.

2. MATERIAL AND METHODS

2.1. Sample Collection and DNA Isolation

A total of samples 57 (41.8%) out 140 from different sites of Strazovske vrchy Mts were collected. A 37 samples collected during the year 2010 (faeces and hair samples) and 20 samples from Strazovske 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.* [25], 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.

Samples were collected in the Strážovské Vrchy Protected Landscape Area (**Figure 1**). The PLA covers c.300 km2 of which 78% is forest and 19% agricultural land. Altitude ranges from 315 to 1213 m a.s.l. On the basis of annual track surveys, opportunistic direct observations and camera trapping we estimated there to be approximately 20 different bears using the area, although some of them may have had home ranges extending beyond the study area.

2.2. Microsatellites Analysis and Gender Identification

Seven microsatellite loci Mu26, Mu64, G10B, G1D, G10L, Mu50 and Mu51 were amplified using poly-

Figure 1. Location of the study area in relation to the distribution of the Western Carpathian population of brown bears.

merase chain reaction [7] 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.

Molecular sexing of the bears identified by DNA fingerprinting was assessed by amplification of the Sry fragment on theY chromosome [26]. In each cell, the autosomal microsatellite locus is twice as concentrated as the *Sry* gene. PCR products were checked on 2.0% agarose gels. Males show two bands, the microsatellite and *Sry* fragment, while females show only the ZFY/ZFX band. The primer pair used for gender determination is not bear-specific, but amplifies the Sry fragment in a wide variety of mammals, including humans [26]. To avoid contamination PCR reactions were set up by a female investigator.

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) [7]: 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 [27].

Observed (HO) and expected (HE) 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 [21,28] and Romania [21] as well as in central Austria [25].

2.3. Statistical Methods

Observed (HO) and expected (HE) heterozygosity were calculated with CERVUS software. Descriptive statistics for each locus (mean number of alleles per locus, heterozygosities and polymorphic information content (PIC) 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 [29]. Deviations from Hardy-Weinberg (HW) proportions were evaluated through the Weir and Cockerham's [30] and Robertson and Hill's [31] estimates of FIS to test for heterozygote deficit with Levene's correction for small sample size, using the method described by Guo and Thompson [32].

The difference between Hardy-Weinberg heterozygosity (*HE*) and that expected from the observed number of alleles (*HEQ*) was tested under the assumption of mutation-drift equilibrium, given the sample size. Evidence for a recent reduction of population size is assumed when HE is significantly higher than *HEQ*. The patterns of microsatellite mutations appear to be extremely complex [33] and the evolution mode of bear microsatellite loci was not known. Therefore, calculations were made according to three models: the Infinite Alleles Mode [34], the strict Stepwise Mutation Model [34] and the Two Phase Model (TPM) with a 5% of multi-step changes: [35] Valdes, Slatkin and Freimer [36], an offshoot of the SMM, which accounts for addition or deletion of more than one repeat unit.

The loci screened in our study were evaluated for their reliability and resolving power when performing parentage tests. A simulation of parentage analysis was conducted using CERVUS version 2.0 [37]. That programme uses allele frequencies from the study population to run simulations of paternity inference when multiple males are non-excluded, allowing for user-defined inputs, such as the number of males that are candidates for paternity, the proportion of candidate males that are sampled and errors in genotyping. Success rates of parentage tests were derived assuming HW equilibrium in cases where one true parent was known and in cases where neither parent is known *a priori*, with 80 and 95% confidence levels. The simulations (10 000 repetitions) were conducted by changing parameters, e.g. the proportion of loci typed, the number of candidate parents, the fraction of candidates sampled and the level of potential laboratory mistyping.

2.4. 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) (will be published in Oecologia Montana). 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 (**Figure 2**).

Neighbor-joining method provides not only the topology but also the branch lengths of the final tree. A pair of "neighbors" is a pair of animals connected through a single interior node in an unrooted, bifurcating tree [38].

UPGMA is a simple agglomerative or hierarchical clustering method often used for the creation of phenetic trees (phenograms). UPGMA assumes a constant rate of similarity between animals. UPGMA was initially designed for use in protein electrophoresis studies, but is currently most often used to produce guide trees for more sophisticated phylogenetic reconstruction algorithms.

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

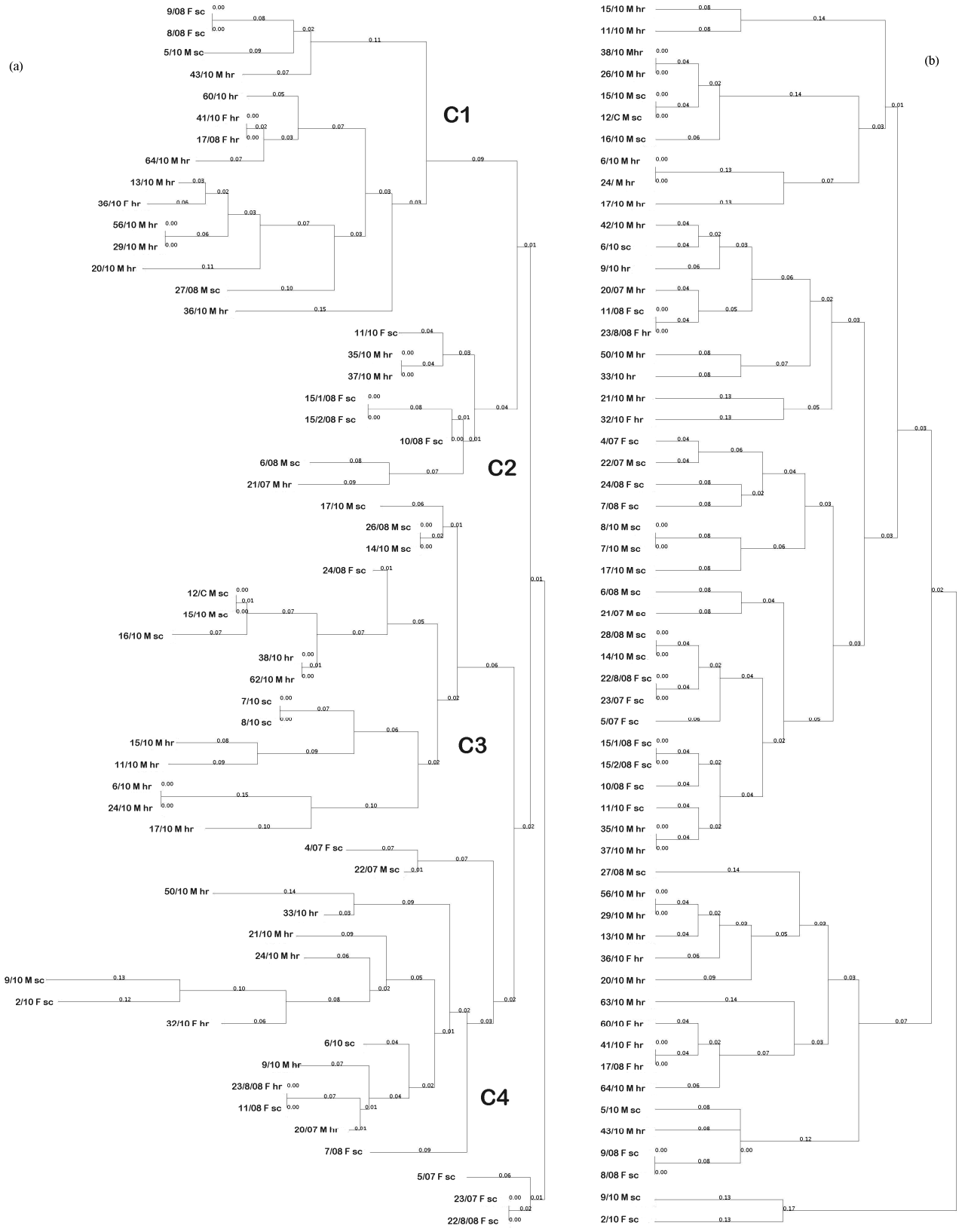

Figure 2. Schematic presentation of animals distribution into "family" clusters (C1 - C4) based on degree of relatedness between individual microsatellite profiles. (a) present Neighbor-joining method and (b) the UPGMA method (hr—hair samples, sc—scat samples).

2010. Only one locus (UaMU26) had a H_O lower than H_E 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 $F_{IS} = 0.08$).

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, P_{IC}—Polymorphic information content, H_O—observed heterozygosity, H_E—expected heterozygosity, F_{IS}—inbreeding coefficient.

(a)

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

(b)

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

(c)

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

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).

3. DISCUSSION AND CONCLUSIONS

The brown bear is a wide-ranging species exhibiting male-biased dispersal [39]. 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 km^2 [1]. 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 [39]. 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 [19,28]. 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 [40]. 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.

To elucidate their movements without the need to live-trap, immobilise and fit animals with telemetry equipment, DNA profiling or 'genetic fingerprinting' in combination with GPS localisation of sampling sites enables individuals to be identified and tracked non-invasively [7,28]. This may help to identify important biocorridors in need of protection or improvement [41,42]. Effective immigration can also be estimated from changes in observed and expected heterozygosity and heterozygote excess [13,43,44].

Although the results of the present study showed relatively high variability (increasing in the year 2010), microsatellite analysis of brown bears in Malá Fatra National Park [28] found higher numbers of alleles per locus (**Table 3**). Moreover, the difference between observed and expected heterozygosity was greater in Malá Fatra than in Strážovské vrchy and low values of FIS for each locus demonstrated a higher occurrence of heterozygotes. Higher variability has also been found in central Slovakia, northern Slovakia and Romania [21].

The leading edge of an expanding population might be expected to become less diverse as a result of a series of genetic bottlenecks [22]. However, two loci analysed in the small central Austrian bear population [25] showed a similar level of variability to that found in Malá Fatra and central Slovakia, even though all genotyped individuals were descended from just four founders. These four individuals arrived in the area 20 - 40 years ago, which is more recently than bears re-colonised the Strážovské vrchy, and there is no evidence of there having been any subsequent immigration.

The founders of the central Austrian bear population

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

(a)

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

(b)

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	

Table 3. The selected Carpatian Mts and central Austria Brown bear subpopulations genetic variability comparison. n_a—observed number of alleles, H_O—observed heterozygosity, H_E—expected heterozygosity, F_{IS}—inbreeding coefficient of Small Fatra [28]—SF, Northern Slovakia [21]—NS, Central Slovakia [21]—CS, Central Austria [25]—CA and Romania [21]—R.

Locality and locus	n_a	H_O	H_E	F_{IS}
SF-UaMU26	4	0.65	0.44	−0.48
CA-UaMU26	4	0.65	0.57	−0.13
SF-UaMU64	9	0.74	0.46	−0.61
NS-G10B	5	0.59	0.63	0.06
CS-G10B	4	0.60	0.63	0.05
CA-G10B	4	1	0.69	−0.45
R-G10B	8	0.76	0.75	−0.01
SF-G1D	4	0.63	0.43	−0.47
NS-G1D	6	0.68	0.79	0.14
CS-G1D	6	0.76	0.76	0.00
R-G1D	7	0.71	0.73	0.03
NS-G10L	6	0.41	0.48	0.16
CS-G10L	6	0.60	0.61	0.02
R-G10L	8	0.79	0.84	0.06
NS-UaMU50	6	0.76	0.75	−0.01
CS-UaMU50	6	0.72	0.65	−0.11
R-UaMU50	8	0.80	0.82	0.02
NS-UaMU51	6	0.52	0.74	0.30
CS-UaMU51	7	0.73	0.82	0.11
R-UaMU51	7	0.72	0.78	0.08

originated in Slovenia and are therefore part of the western lineage (subclade 1b), whereas Slovakia's bears belong to the eastern lineage known as subclade 3a [45]. There are bears of both mtDNA lineages in Romania [5] but the differentiation is not reflected in nuclear loci, perhaps due to male-mediated gene flow and female philopatry [21]. The genetic diversity of brown bears is highest in Romania [46], where the population has never fallen below 800 individuals, and is also high in Slovenia [46] as well as neighbouring Croatia [47].

Our results represent a comparative study of a subpopulation which has hitherto received little attention from researchers. However, the brown bear is one of the best-studied mammalian species [13]. In the last two decades there has been a proliferation of genetics studies mapping populations at different geographical scales from regional [7,8,48] to continental [49,50]. Standardised procedures have been developed for sampling and analysis in order to facilitate comparisons between studies [46,51,52]. Non-invasive genetic methods, especially appropriate for use with elusive species in small, endangered populations or over large areas, are now available to allow identification of individual animals, census populations and monitor migration and gene flow [13]. The potential for further work building on our study is therefore substantial. 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.

4. ACKNOWLEDGEMENTS

This study was supported by Structural Funds of EU Project of the Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic, Bratislava, ITMS No. 26110230078.

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