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Genetic Tracking of Brown Bear (*Ursus arctos*) in the Belianske Tatry Mountains

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Abstract. In the last decade, molecular tools have been often used in the field of ecology. This study describes the genetic tracking of brown bear population in the Belianske Tatry Mountains. We collected non-invasive samples such as hairs and faeces of brown bears which contain enough genetic material for the DNA isolation. The analysis of five microsatellites loci helped to determine 12 original genotypes in the study area. Besides the estimation of individuals living in our study area, we calculated the genetic variability of this population. Obtained data of genetic variability was helpful for the comparison with other brown bear populations. We find out a limited geneflow with the other Slovak subpopulation. However, a significantly verified bottleneck from the 20th century compared with the small isolated European brown bear population showed us a relatively big genetic diversity of brown bears living in the Belianske Tatry Mountains. This study shows the potential of using molecular technologies in the research of such mysterious animals as brown bears are.

Key words: The Belianske Tatry Mountains, genetic tracking, microsatellites, population size estimation, *Ursus arctos, non-invasive samples*

Introduction

The Slovak brown bear population is similar to other European brown bear populations. The same problems deal with the Slovak population as it is described by Swenson (2000) in the Action plan for the conservation of the brown bear in Europe. As a result of the increasingly intense human activity deforestation and the increase of agricultural land cause the loss and degradation of habitats suitable for bears. It results in fragmentation and isolation of particular brown bear populations. Hunting, poaching and missing research of brown bears living in Slovakia makes the proper management more complicated.

In many studies related to brown bears, the tools of molecular ecology are used. The genetic tracking of brown bear means the molecular identification of individuals by the means of residence signs containing the biological material, allowing the isolation of DNA. Samples of faeces and hairs are a suitable material for DNA analysis related to the studies designed to identify individuals, kinship analysis as well as an estimated number, sexual structure and genetic structure of the population. Without the need to capture, or observe, it is possible to obtain information about protected and hardly observable species (Straka *et al.* 2009). Therefore, such samples are called non-invasive.

The analysis of samples at several loci enables the evaluation of genetic diversity, population structure and the gene flow of brown bears. The additional information obtained in such research includes behavioural information. The habitat use, information about migration, movement through the area where a construction of highways, or other potential barriers that cause bear habitat fragmentation are planned (Karamanlidis *et al.* 2010). A long-term application of such research will enable to learn more about the use of bear habitat and the activities in the region. Such results recommend one direction, which should be continued. Non-fragmented habitats of northern Slovakia must be kept for large carnivores. It is the only way to preserve the genetic diversity of bears in the Western Carpathian Mountains (Janiga *et al.* 2006). The results can be used to assess the conservation measures needed to maintain the genetic diversity of the species (Větrovcová 2011).

The aim of our study was to describe the application of molecular tools in the ecology research. The non-invasive samples are the source for a finger printing method (Jeffreys *et al.* 1985), which allowed the determination of individuals whose home ranges engage the study area. We determined the length of five microsatellites, and with the help of different statistical programs, estimated the genetic diversity of the sample population. One statistical program helped us to verify the bottleneck in the population known from historical data. In the discussion part, we showed the importance of obtained data for the proper management and protection of large carnivores.

Material and Methods

Study area

We applied our research to the bio-tracking of brown bear in the Belianske Tatry Mountains (Fig. 1).

The Belianske Tatry Mountains consist of an area of 64 km^2. A part of these mountains is established as a protected area with a number of habitats that provide food, shelter and den options suitable for bears. The area is largely inaccessible for the public. Protecting nature is the reason for slowing down the changes in these biotopes. All of these make this protected area more suitable for large carnivores (Swenson 2000). Rigg (2007) points at the fact known from many research projects that suitable areas for bears are not located nearby cities and tourist centres. The area is also suitable for denning because of a low level of interference with man and his activities. Less accessible and distant places from civilization provide suitable shelters such as caves, holes and forests of primeval character.

The extended area of our research consists of a territory larger than 100 km^2. This is adequate to a home range area of a female bear, as shown by the latest telemetry research in Slovakia. Recent results from the years 2008 to 2013 report about 110 to 120 km^2 of the home range area of a female bear in the High Tatras national park, and about 400 km^2 of a male bear during a two year period (Lenko *et al.* 2014). We tried to determine the minimum number of brown bears whose home ranges are engaged in our study area. In the field, we collected GPS data of collected samples, which include the genetic material.

Collection, storage of samples and DNA isolation

In our research area we collected samples of the biological material (scat, hair) from April 9, 2013 till November 14, 2013. For collecting samples we used paved forest roads, hunting, hiking and animal trails. We collected 54 samples of scat and 56 samples of hair from bear trees. We stored the samples in closed plastic bags and sterile tubes with descriptions. Collected samples were stored in a deep freezer in the temperature of -20˚ C until we processed them further on. For the isolation of DNA from hair samples we used the DNeasy Blood & Tissue Kit (QIAGEN). Samples of scat were processed with the QIAamp DNA Stool Kit (QIAGEN). We used the protocol isolation of DNA from the Stool for Human DNA Analysis. The extracted DNA was stored in 1.5 ml microcentrifuge tubes at the temperature of -20˚ C in the freezer.

PCR amplification and sequencing

To identify individuals we chose the microsatellites DNA analysis. It is often used for estimating the genetic variability of brown bear populations (Paetkau *et al.* 1998). Five microsatellite loci UaMU64, UaMU50, UaMU51, G1D and G10L were amplified by a polymerase chain reaction. The primers were described originally by Paetkau and Strobeck (1994), Paetkau *et al.* (1995), Taberlet *et al.* (1997). Table 1 shows the overview of all used primers.

The PCR was performed in two steps to improve the genotyping success rate (Bellemain and Taberlet 2004). In the first step, external microsatellite primers set was used to reduce the formation of nonspecific artefacts. For the second step, internal primers were used to obtain the desired products. All processes of PCR amplification were optimized with respect to studies, which were made in our laboratory (Janiga *et al.* 2006, Graban *et al.* 2013). For every PCR per each of the loci, a special master mix was prepared. The master mix consisted of H_2O, buffer, MgCl2, dNTP, forward, reverse primers and the polymerase. We used all components from the same producer (Promega).

Then we pipetted 12.5 µl of the master mix and 1 µl DNA of the sample into 200 µl microcentrifuge tubes, and put that into the thermocycler. In the thermocycler PCR was repeated in 35 cycles, which started with the initial denaturation by the temperature of 95˚ C for 2 minutes, and ended with the final extension at the temperature of 72˚ C for 5 minutes. Every cycle consisted of the denaturation at temperature of 94˚ C for 30 seconds,

Fig. 1. Study area - Belianske Tatry Mountains, Slovak republic.

	Msat loci	Forward primer (5'-3')	Internal primer (5'-3'), (F: forward, R: reverse)	Reverse primer (5'-3')	T (°C)	References
1	UaMU64	ACTCAACACAACCAT-TAAATA	AGGACCCAAATGACAC-TACA (R)	GGTATC-TACTCCCCAAAGGA	56	(Taberlet *et al.* 1997)
2	UaMU51	CCAGAATCCTA-AGAGACCT	AAGAGAAGGGACAGGAG-GTA (R)	GAAAGGTTAGATG-GAAGAGATG	58	(Taberlet *et al.* 1997)
3	UaMU50	TCTCTGT-CATTTCCCCATC	GAGCAGGAAACATGTAA-GATG (R)	AAAGGCAATGCA-GATATTGT	56	(Taberlet *et al.* 1997)
4	G10L	GGACAGGATATTGA-CATTGA	ACTGATTTTATTCA-CATTTCCC (F)	CAGAAACCTACCCAT-GCG	56	(Paetkau and Strobeck 1994)
5	G1D	ATCTGTGGGTTTATAG-GTTACA	CTACTCTTCCTACTCTTTA-AGAG (R)	CTAGCACCCAGCAAGG-TA	58	(Paetkau *et al.* 1995)

Table 1. Overview of all ordered primers. Annealing temperature (T)

annealing at the temperature of 56° C, or 58° C (according to primers sets, Table 1: annealing temperature) for 30 seconds and the extension at the temperature of 72° C for 1 minute. The second step of the PCR was the same as the first one, only external primers were replaced by the internal, and instead of 1 µl DNA of the particular sample, 1 µl of the PCR product from the step one was used. After the second amplifying in the thermocycler the final product was ready for the fragment length analysis by a sequencer. The length of microsatellites was measured by an eight-capillary sequencer (GenomeLab GeXP, Beckman Coulter). For the preparation of the fragment analysis master mix, a sample loading solution (SLS), marker and a final product from the above described PCR are necessary. We pipetted the master mix and the PCR product into a 96-well plate, and dropped the mineral oil at the top instead of closing it by a lid. For the automatic sequencer it is necessary to prepare another plate for washing, where we added a separation buffer. The last steps before we started with sequencing were changing water in the reservoir, replenishing of gel and capillaries.

Genetic variability and statistical methods

With the help of GIMLET software v1.3.3 we calculated the number of alleles with their frequencies (Valière 2002). The genetic variability was measured as an observed number of alleles per locus (n_a) and effective number of alleles (n_e), observed heterozygosity (Ho), and expected heterozygosity (He). For the calculation of the observed, expected heterozygotes and effective number of alleles, we used the CERVUS 3.0.3 software. This software calculated the polymorphic information content (PIC), too. The polymorphism information content (PIC) value is a measure of polymorphism to describe the genetic marker's usefulness. For calculating the effective number of alleles (n_e), we used the formula ne=1/1-He. The "effective number of alleles (n_e) is the number of breeding individuals in an ideal population that would show the same amount of dispersion of allele frequencies under random genetic drift or the same amount of inbreeding as the population under consideration" (Wright 1931). The Fisher's exact test was used to check the genotypic linkage dis-equilibrium for all pairs of loci (FIS) calculated with the formula FIS = (He-Ho)/He. It shows the inbreeding coefficient. If the value of this inbreeding coefficient is between minus one and zero, it describes a closed population, but if the value is above zero, it does not come to inbreeded crossing. Afterwards we calculated the probability of identity using the GIMLET software v1.3.3 (Valière 2002). This software calculated a biased probability of identity (PI), and probability of identity between siblings (PISIBS). The software sorted the loci from the most informative to less informative. By ourselves we calculated the general probability of identity, and also the probability of identity between siblings that two individuals would have the same genotype on all five loci.

The BOTTLENECK software computes the heterozygosity (Heq) distribution for each locus, expected from the observed number of alleles (k), given the sample size (n) under the assumption of mutation-drift equilibrium. The number of iterations influences the precision of the Heq estimates. We based the estimation on 1000 replications. The distribution of the heterozygosity is obtained through simulating the coalescent process of n genes under each of two possible mutation models: single-step mutations and multiple-step mutations. This distribution enables the computation of an average expected equilibrium heterozygosity (Heq) for each locus, which is compared to the Hardy–Weinberg heterozygosity (He) in order to establish whether there is a heterozygosity excess, or deficit at each locus. In addition, the standard deviation (SD) of the mutation ndrift equilibrium distribution of the heterozygosity is used to compute the standardized difference for each locus. The distribution obtained through simulations also enables the computation of a P-value for the expected heterozygosity (He). The P-value is the probability of obtaining the measured He in a sample (n) from an equilibrium population that has the observed number of alleles (k). The way in which the coalescent process is simulated is unconventional due to conditioning by the observed number of alleles.

For most microsatellites, the TPM (two-phase model) is apparently even more appropriate than the only single step mutation (Di Rienzo *et al.* 1994). The TPM is an option in the BOTTLENECK software. We used the TPM with 95% single-step mutations and 5% multiple-step mutations, how it is recommended

by microsatellites. Once all loci in a population sample have been processed, the three statistical tests are performed for each mutation model, as explained in (Cornuet and Luikart 1996). The Wilcoxon test was successful.

Division of the brown bear population into clusters

The first method is the Neighbor-Joining method, which provides not only the topology, but also branch lengths of the final tree. A pair of "neighbours" is a pair of animals connected through a single interior node in an unrooted, bifurcating tree (Saitou and Nei 1987). The second one is the Unweighted Pair Group Method with the Arithmetic Mean (UPGMA). UPGMA is a simple agglomerative or hierarchical clustering method, often used for the creation of dendrograms. UPGMA assumes a constant rate of similarity between animals. UPGMA was initially designed for use in protein electrophoresis studies, but is currently more frequently used to create guide trees for more sophisticated phylogenetic reconstruction algorithms (Saitou and Nei 1987).

Population size estimation

We estimated it from the count of discovered unique genotypes. For the estimation size of population by genetic research, the capture-mark-recapture (CMR) method is usually used (Schwarz and Seber 1999, Buckland *et al.* 2000, Williams *et al.* 2002, Amstrup *et al.* 2005). For the analysis of capture and recapture rates of individuals identified in each of the DNA-analyses, it is possible to use different softwares to calculate the size of population. We used the software CAPWIRE (Miller *et al.* 2005), which is developed for genetic tracking. This software works on the base of data accommodation with multiple marking of an individual within a single mark.

Results

Results of measured microsatellites

After automating the sequencing, we received final results. The results of measured alleles with their calculated frequencies and proportion of all loci are shown in Tables 2-4. From analysed samples we identified 12 individuals with a unique genotype (Fig. 2). One individual was identified from both sources. In 10 individuals, the genotype was identified repeatedly. In the Table 5 the allelic combinations are detected.

Genetic variability of brown bear population in the Belianske Tatry Mountains

We used the measured microsatellites as input data for calculating the main values of genetic variability. The results are shown in the Table 6.

cc					**UaMU51**				
Allele	f	prop.	Hetero-zygotes	Homo-zygotes	Allele	f	prop.	Hetero-zygotes	Homo-zygotes
171	0.481	25/52	10	8	120	0.135	7/52	7	0
150	0.308	16/52	0	8	112	0.212	11/52	11	0
143	0.212	11/52	10	0	108	0.654	34/52	12	11

Table 2. Detected alleles, allelic frequencies (f) with the proportion of all alleles per each locus (prop.), and with the count of homo- and heterozygotes per G10L and UaMU51 locus.

UaMU64					**UaMU50**				
Allele	f	prop.	Hetero-zygotes	Homo-zygotes	Allele	f	prop.	Hetero-zygotes	Homo-zygotes
199	0.096	5/52	5	0	139	0.096	5/52	5	0
197	0.077	4/52	4	0	134	0.250	13/52	5	4
195	0.058	3/52	3	0	132	0.115	6/52	6	0
192	0.192	10/52	10	0	130	0.212	11/52	5	3
188	0.231	12/52	6	3	128	0.096	5/52	5	0
184	0.135	7/52	7	0	121	0.154	8/52	0	4
181	0.096	5/52	5	0	118	0.077	4/52	0	2
179	0.058	3/52	3	0					
177	0.038	2/52	2	0					
175	0.019	1/52	1	0					

Table 3. Detected alleles, allelic frequencies (f) with the proportion of all alleles per each locus (prop.), and with the count of homo- and heterozygotes per UaMU64 and UaMU50.

G1D				
Allele	f	prop.	Heterozygotes	Homozygotes
224	0.038	2/52	2	0
210	0.096	5/52	5	0
208	0.019	1/52	1	0
200	0.096	5/52	5	0
188	0.385	20/52	10	5
180	0.135	7/52	7	0
177	0.192	10/52	10	2
172	0.038	2/52	2	0

Table 4. Detected alleles, allelic frequencies (f) with the proportion of all alleles per each locus (prop.), and with the count of homo- and heterozygotes per G1D locus.

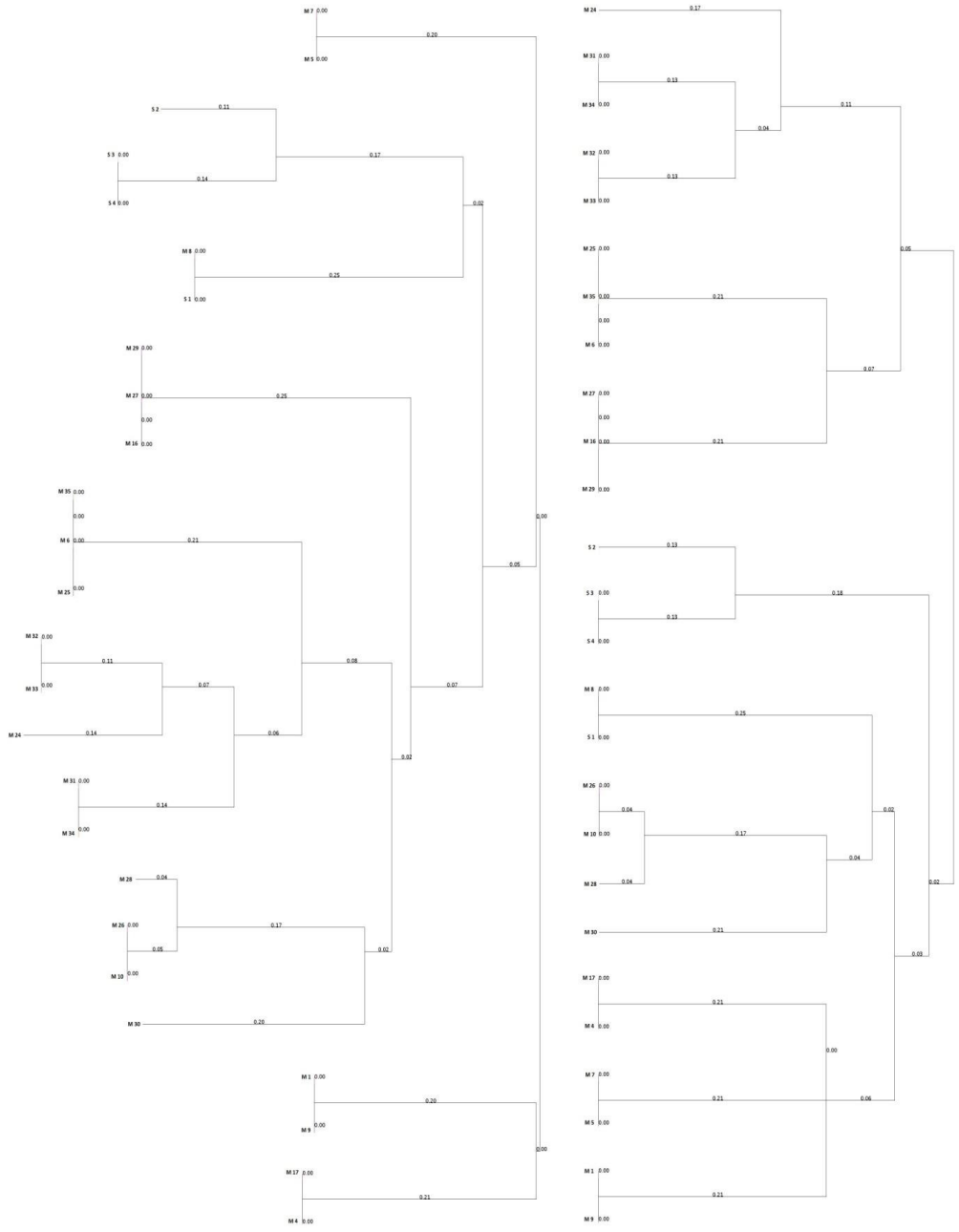

Fig. 2. Cluster of the brown bear family in the Belianske Tatry Mountains, on the left the Neighbour-Joining method, and the UPGMA method on the right.

No.	UaMU64	G1D	G10L	UaMU50	UaMU51
1	181/199	177/180	150/150	134/134	112/120
2	179/192	188/200	143/171	118/118	108/112
3	177/192	188/210	171/171	130/132	108/108
4	188/192	188/188		128/134	108/120
5	184/195	177/177		134/139	
6	181/188	172/224		128/139	
7	188/188	180/208		130/130	
8	195/199			121/121	
9	175/179			132/134	
10	184/192				

Table 5. Detected allelic combinations. Boldfaced are the combinations known from research (Graban *et al.* 2013), which was made in our laboratory, and underlined are the alleles with the known length from the same study.

Locus	na	ne	HO	HE	PIC	FIS
UaMU64	10	8.064516	0.885	0.876	0.844	-0.01027
G1D	8	4.761905	0.731	0.790	0.748	0.074684
G10L	3	2.702703	0.385	0.630	0.546	0.388889
UaMU50	7	6.578947	0.500	0.848	0.810	0.410377
UaMU51	3	2.083333	0.577	0.520	0.454	-0.10962
Mean	6,2	4.838281	0.6156	0.7328	0.6803	0.150812

Table 6. Genetic variability of brown bears in the Belianske Tatry Mountains (na -observed number of alleles, ne -effective number of alleles, PIC -Polymorphic information content, HO -observed heterozygosity, HE -expected heterozygosity, FIS -inbreeding coefficient).

Locus	PI	PISIBS	Number of alleles	PIC	rank
UaMU64	4.74x10-1	1.21	10	0.844	1
G1D	8.76x10-2	4.20x10-1	8	0.748	3
G10L	6.77x10-2	5.90x10-1	3	0.546	4
UaMU50	1.93x10-2	2.88x10-1	7	0.810	2
UaMU51	2.09x10-2	4.46x10-1	3	0.454	5
Mean	1.34x10-1	5.90x10-1	6.2	0.6803	

Table 7. The sorting of locus by the PI value (probability of identity): PI-biased probability of identity, PISIBS-probability of identity between siblings. Ranking, locus with the rank 1 is the most informative one.

	observed		under the two phase model				
locus	n	k	He	HEQ	SD	DH/sd	P
UaMU64	52	10	0.876	0.829	0.045	1.038	0.1170
G1D	52	8	0.790	0.773	0.066	0.263	0.4920
G10L	52	3	0.642	0.420	0.159	1.394	0.0620
UaMU50	52	7	0.848	0.738	0.075	1.459	0.0090
UaMU51	52	3	0.520	0.424	0.154	0.623	0.3400

Table 8. Results of bottleneck verified by the BOTTLENECK software. Sample size (n), observed number of alleles (k), expected heterozygosity (He), average expected equilibrium heterozygosity (Heq) , standard deviation (SD), DH/sd =(He-HEQ)/SD, probability (P)

The GIMLET software calculated the probability of the identity of two individuals and the probability of the identity of two siblings separately for each locus (Table 7). We counted probabilities for all five loci. The probability that two individuals have the same genotype in all of five loci is 1: 6581433. The probability that two siblings could have the same genotype in all five loci is 1:40. We estimated the ranking of loci according to informativeness. For the ranking (Table 7), we used the PIC of each locus. A higher number of alleles mean a higher number of the PIC, but it depends on frequencies of alleles, too. A locus with a higher number of the PIC is more informative.

Bottleneck testing

For the testing of the recent reduction of population size we used the BOTTLENECK software (Piry *et al.* 1999), and its two phase model. For the testing of heterozygosity excess the model needs different values shown in the Table 8. The heterozygosity excess was detected by the Wilcoxon test. Results of the Wilcoxon test are shown in the Table 9.

The UPGMA method shows a genetic distance in the form of phylogenetic tree based on the constant mutation rate of microsatellites. A similar principle has the neighbour-joining method, but in this method pairs of closely related individuals are created. The length of branches is shorter in every stage of the tree. Both clusters are shown in the Fig. 2, and can help the interpretation of relationships between individuals and the so-called families.

Estimation of population size

We created a table (Table 10) with an overview of estimated population sizes according to two different methods. The minimum population size is estimated according to identified individuals with a unique genotype. The second method is the so-called CMR (Capture-Mark-Recapture) method, which is calculated with the software CAPWIRE.

Map of analysed samples

We used the computer software ArcMap 10 for creating the GIS layer of analysed samples (Fig. 3).

Wilcoxon test	
Assumptions:	all loci fit T.P.M., mutation-drift equilibrium
Probability (one tail for He deficiency):	1.00000
Probability (one tail for He excess):	0.01563
Probability (two tails for He excess or deficiency):	0.03125

Table 9. Results of the Wilcoxon test

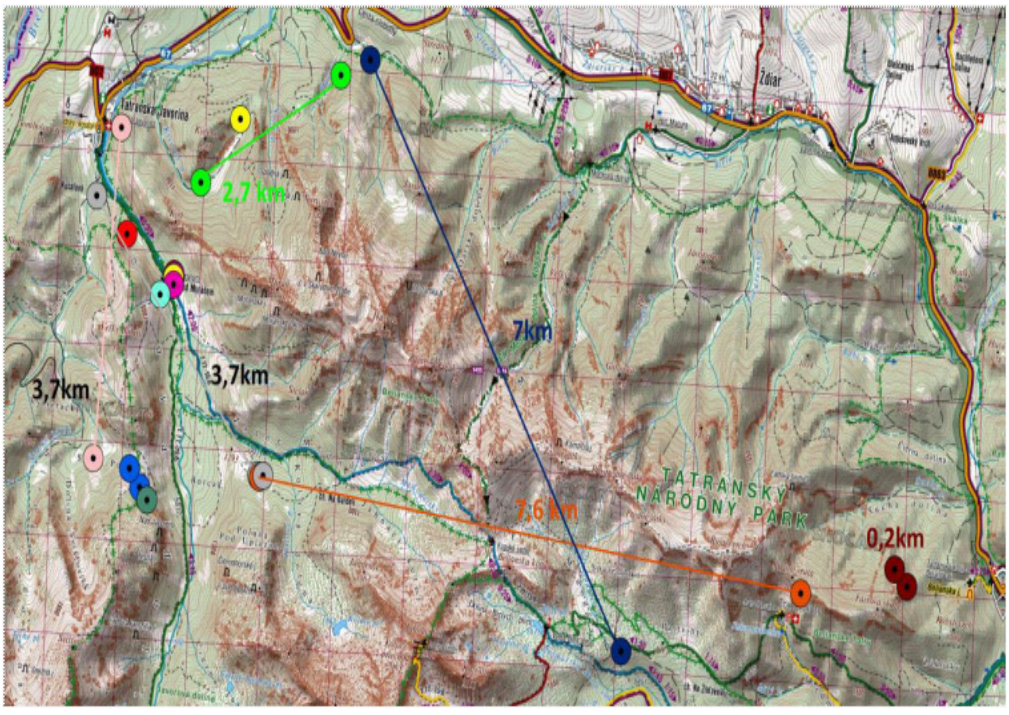

Fig. 3. Identification of individuals based on the same genotype of the samples marked with the same colour for each particular individual.

Method	Size of population (No. of individuals)
Minimum population size	12
CMR	15

Table 10. Overview of estimated population size. The left column shows the type of method used for the estimation, and the right column shows the number of individuals in an estimated population.

Discussion

For the interpretation of our results of the genetic structure of bear population, which lives in the Belianske Tatry Mountains we especially created the comparison table (Table 11). From collected data we calculated the Fisher's exact test to obtain the inbreeding coefficient of particular populations. Our research shows that the highest value of the inbreeding coefficient is in the Belianske Tatry Mountains population. That means that there should be the lowest level of inbreeding. The value might not be exact because of the low number of loci and samples, but it is enough to see that the population, which lives in the Belianske Tatry Mountains, shows a relatively high genetic variability. Our results confirm the results of other studies which were made in Slovakia (Straka *et al.* 2009; Straka 2011; Straka *et al.* 2012).

From the comparison table (Table 11) we can see that in European bear populations there are different numbers of genetic diversity. It is known that populations with different genetic diversity are divided into more subpopulations. The reason can be, for example, in any geographic barriers to the gene flow followed by different genetic drift in every subpopulation. That overlaps with the Swenson's (Swenson *et al.* 2011) characteristic of European bear populations, which have been reduced and fragmented. For example, in Romania and Scandinavia the diversity is higher than in our study population, but in some other populations like in Spain or Italy the diversity is considerably lower.

The comparison with other studied brown bear subpopulations in Slovakia shows differences in the genetic diversity observable, which only confirms their limited gene flow. The expected heterozygosity from the Strážovské Vrchy Hills (Graban *et al.* 2013) is smaller than in the Belianske Tatry Mountains. We think that it might be due to a different position of both populations. The Belianske Tatry Mountains are closer to the eastern subpopulation of brown bear, and the probability for the discontinuous gene flow between them is higher.

Straka (2011) found differences in genotypes of Slovak bears, not only between the West and East, but also between central and northern Slovakia, too. He estimated that the main barrier for the gene flow could be the Váh River Valley, especially the construction of water reservoirs and the highway parallel to railroads. According to the study by Straka (2011), bears, which we compared, should belong to different genotype groups. Bears from the Strážovské Vrchy Hills should belong to the central Slovak population, and bears

Population	N	L	A	Ho	He	Fis	Reference
Romania	109	13	8.46	0.76	0.8	0.05	(Straka 2011)
Romania	16	9	7.80	0.72	0.81	0.11	(Zachos *et al.* 2008)
Dinaric Mountains, Croatia	156	12	7.58	0.74	0.75	0.01	(Kocijan *et al.* 2011)
Southern Balkans	49	6	6.33	0.76	0.77	0.01	(Karamanlidis *et al.* 2010)
Central Pindos	128	12	5.6	0.65	0.69	0.06	(Straka 2011)
Scandinavia NS	108	19	6.20	0.66	0.66	0.00	(Waits *et al.* 2000)
Northern Slovakia	71	13	6.08	0.69	0.71	0.03	(Straka 2011)
Central Slovakia	96	13	6.00	0.69	0.7	0.02	(Straka 2011)
Scandinavia M	88	19	5.80	0.65	0.66	0.02	(Waits *et al.* 2000)
Scandinavia NN	29	19	5.50	0.66	0.66	0.00	(Waits *et al.* 2000)
Scandinavia S	155	19	5.40	0.76	0.66	-0.15	(Waits *et al.* 2000)
Eastern Slovakia	16	13	5.23	0.66	0.65	-0.02	(Straka 2011)
Slovakia MF	23	4	5.5	0.67	0.45	-0.49	(Janiga *et al.* 2006)
Slovakia SV	57	7	2.42	0.59	0.57	-0.03	(Graban *et al.* 2013)
Slovakia BT	26	5	6.20	0.62	0.73	0.15	**This study**
Central Austria	379	9	2.86	0.76	0.61	-0.25	(Kruckenhauser *et al.* 2009)
Western Cantabrian, Spain	30	12	2.2	0.40	0.44	0.09	(Pérez *et al.* 2009)
Apennines, Italy	34	8	2.13	0.29	0.26	-0.12	(Lorenzini *et al.* 2004)

Table 11. Comparison of the genetic structure of brown bear populations from different studies in Europe. Scandinavia NN, NS, M,S - different study areas in Scandinavia; Slovakia MF- Malá Fatra Mountains; Slovakia SV- the Strážovské Vrchy Hills; Slovakia BT- the Belianske Tatry Mountains. Number of samples (N), number of used loci (L), mean number of alleles per locus (A), observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficient (Fis).

from the Belianske Tatry Mountains to the northern Slovak population. In contrast, Straka (2011) found different genotypes in each subpopulation, which means that migration sometimes occurs.

In the cluster (Fig. 4), there are the bears from our study population together with the bears from the Strážovské Vrchy Hills (Graban *et al.* 2013). Helped by the UPGMA method, it is possible to see relationships in the cluster. Boldfaced squares mark the bears from the Belianske Tatry Mountains. This indicates the fragmentation into two subpopulations with some individuals from the population living in the Strážovské Vrchy Hills, which are more closely related to the population living in the Belianske Tatry Mountains.

We consider poaching as one of other reasons for the indicated fragmentation. Poaching disrupts the social organization of brown bears (Baláž 2003). Big male bears could represent some tool for the gene flow between populations, because of their large home ranges. However, big males are often the main object of interest for poachers.

In case of males the probability of the dispersion is higher than in case of females (Zedrosser *et al.* 2007). Based on Swedish research, the dispersion in males is 94%, while in females only 41%. Females are generally more philopatric and prioritize home ranges near their mother districts (Swenson 2000, Baláž 2002, Støen *et al.* 2005, Zedrosser *et al.* 2007, Baláž and D'Amicis 2010).

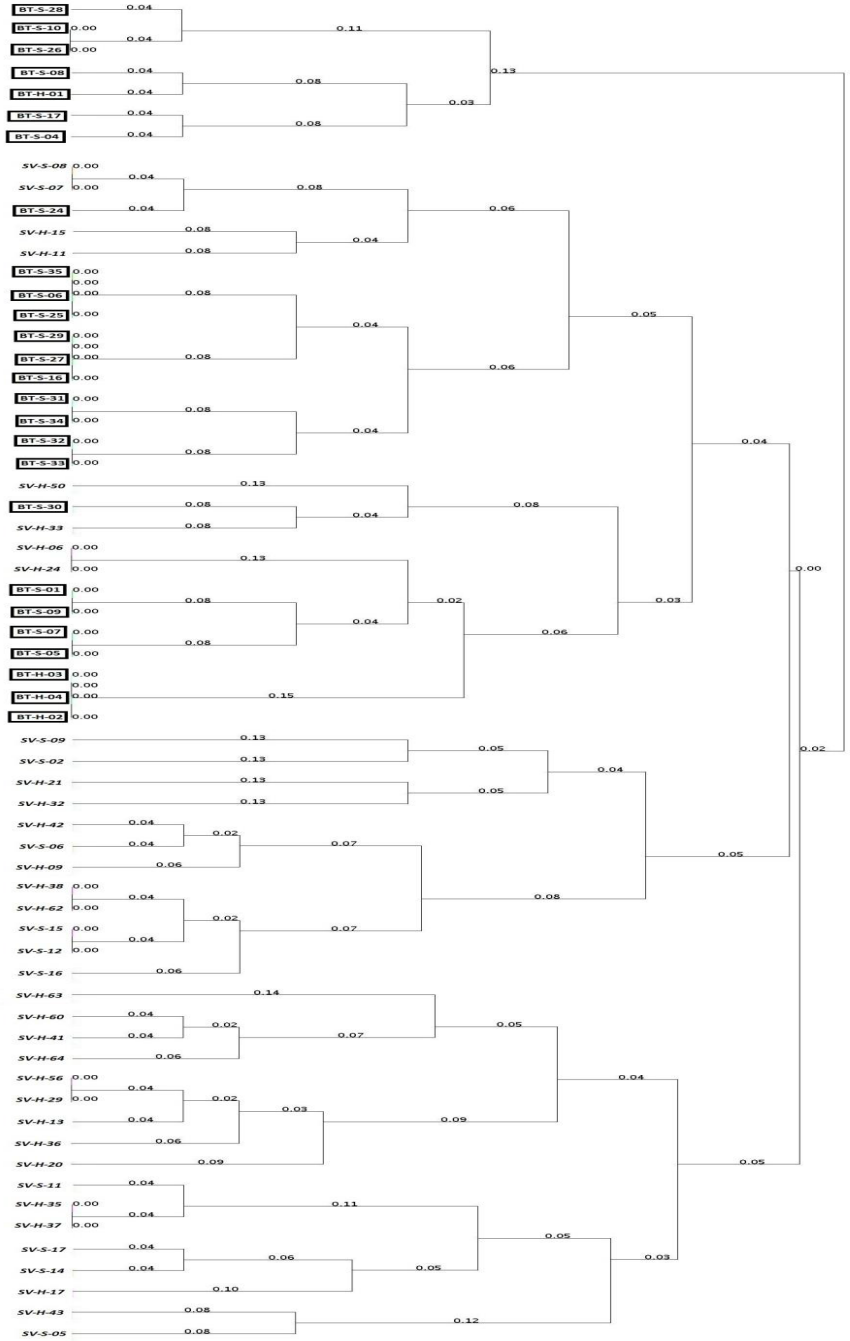

Fig. 4. Family cluster made according to the UPGMA method of the brown bear population in the Belianske Tatry Mountains (this study), and the population in the Strážovské Vrchy Hills (Graban *et al.* 2013). In boldfaced squares bears from the Belianske Tatry Mountains are displayed.

Males monitored in Sweden dispersed in average 119 km between reproductive pairs (Støen *et al.* 2006, Zedrosser *et al.* 2007). In the context of social organization there may be a suppression of reproduction of younger subordinate females and their late first birth giving. Research in Sweden and Norway found out that in philopatric females, which remained in the districts of their mothers, the age of first reproduction was higher than in dispersed females. Because females in the family relations, whose home ranges are overlapping could develop the hierarchy of dominance and suppression of reproduction towards younger females. This can reduce the competition for resources (Støen *et al.* 2006). A delayed birth of cubs is considered by Baláž (2002), and Baláž and D'Amicis (2010) as one of self-regulatory mechanisms of the population, because thanks to the presence of an old female in the territory, and the pressure on the young females, the number of females with cubs is limited to a number of the carrying capacity of the environment. Støen (2006) indicates, based on long period of research in Scandinavia, that probability of dispersion and distance of dispersion by juvenile individuals is inversely dependent on the population density, which is regulated by social interactions. Mentioned observations suggest that brown bears distinguish between relatives and unrelated individuals (Støen *et al.* 2006, Zedrosser *et al.* 2007), and also when it comes to solitary living animals, they have a social structure based on familiar relationships (Støen *et al.* 2005).

Knowledge about social structure and a relatively high genetic variability of the both mentioned subpopulations support the hypothesis of Hell and Sabadoš (Hell and Sabadoš 1993), and Janík (Janík 1997), who state that the absence of large territorial bears killed for trophies could be a possible reason for the rapid growth of population in the 20th century. Janík (1997) states that of 366 bears killed by hunters in the period of 1958-1980, 79% were males and 21% females. According to Jamnický (1988), large males in the period from the mid-70s to the mid-80s of the 20th century almost disappeared from some areas.

Hunting and poaching disrupt the natural social structure of brown bears. This phenomenon could reduce self-regulation mechanisms, known as infanticide. This could be the reason for a rapid increase of abundance since the half of the 20th century. Because of the mating of younger and weaker individuals than in a strict social structure, the genetic diversity of the population could grow faster. The consequence of the bottleneck effect in the Slovak brown bear population in the first half of the 20th century is unknown. We can observe an increase of the so-called synanthropic individuals, and the following problems. For the verification of the bottleneck, we used our microsatellites data and the analysis proved the bottleneck as significant (Table 9). To find out the consequences, the further molecular research is needed.

We tested a recent reduction of the effective population size with the BOTTLENECK software (Piry *et al.* 1999). Results of the Wilcoxon test, which detected the heterozygosity excess across loci, confirmed the genetic signature of the recent population bottleneck. The Table 12 is a comparison of our signature results with the genetic study of brown bear in the Carpathians (Straka 2011). The comparison of results shows that our results correspond with the results provided by Straka (2011).

The estimation of population size is important for the conservation of species and right management of wildlife species. The estimation should be repeated to figure out a trend in the population, if the population is relatively stable, or in a substantial increase or decline phase (Witmer 2005). It can be used for the estimation of hunting limits and damage prevention (Halley and Rosell 2002).

Population	p (one tail for heterozygosity excess)	Reference
Northern Slovakia	0.035	Straka 2011
Central Slovakia	0.029	Straka 2011
Romania	0.002	Straka 2011
Belianske Tatry Mts	0.015	This study

Table 12. Result comparison of the Wilcoxon test for the bottleneck.

Results for the abundance of the whole Slovak brown bear population estimated by a reliable scientific method are still not known. The most reliable source is the so-called expert estimates. Expert estimates of the population count in the last decade state that the maximum number of individuals is 868 because of the maximum capacity of the environment (Adamec *et al.* 2005, Hell *et al.* 2005, Rigg *et al.* 2007). Assuming that the maximum number of bear individuals in Slovakia is currently 868 in the total area of 13 000 km^2, the average density is 6 individuals per 100 km^2. The highest population density is in protected areas with very high quality habitats, and relatively low interference, the density is estimated there to about 11 individuals per 100 km^2 (Rigg *et al.* 2007). Our study area has the size of about 100 km^2, therefore we used this count as the so-called "expert estimate ". This corresponds with our results. As we estimated in the results (Table 10), 12 original genotypes were detected in our study area during the season 2013. It is probable that during the season some bears migrate in and out of the study area because of a relatively small study area, and big territories of some brown bear individuals. That is the reason why we can just estimate a number of individuals with home ranges interfered into our study area. Our results roughly correspond to the number of individuals estimated in the study by Lenko (2014) for the Belianske Tatry Mountains -South and the Belianske Tatry Mountains - North. For this area they count 11 individuals, but according to their method, they deduct the duplicate individuals from neighbouring areas. We calculated a theoretical count of individuals according to the CMR method, which uses the frequency of multiple registered individuals. According to this method, it should be possible to calculate an approximate count of individuals

in the area. Because of large home ranges, it is possible that for some reason (as good feeding possibilities in the area) more individuals than in calculated expert estimates can occur. In the capacity of environment, some factors can artificially enlarge the capacity to a normal condition. Feeding stations of hunters can draw more individuals, which in natural conditions would not come into the area.

During our research, we found out a higher count of brown bears, whose home ranges extended to our study area, as far as the capacity of the environment is calculated. The unnatural increase of home ranges could be caused by feeding stations of hunters. That is why we would suggest managing the feeding of only ungulates.

The estimation of population size is one of the most important tasks for proper management. The use of non-invasive samples for the analysis of microsatellites is a very useful method for that. It eliminates the disturbance of animals, and opens many different possibilities for further research. The indicated fragmentation of population can be helpful for building bio-corridors. Properly built bio-corridors can help the gene flow not only for bears, but also for other large carnivores, which are most threatened by the population fragmentation. Their relatively small count increases inbreeding in the fragmented population. This can result in a higher number of genetic diseases, and lower adaptation abilities of the whole population. The genetic study of the whole Slovak brown bear population could help manage the proper links between different fragmented populations by conservation and creating suitable habitats or biocorridors. It helps increase the genetic variability, and makes the population more resistant to different changes in the environment.

We suggest supporting more the research oriented towards the social structure and population dynamic of brown bears with the focus on self-regulation and the function of big males. As to the research of the natural social structure, it is extremely important to prevent poaching. One of the possibilities for genetic research is to identify bear individuals from trophies. Other possibility is to control the protective shooting of individuals damaging and threatening people. A more effective control could also help reduce poaching.

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