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Genetic differentiation of Carpathian brown bear (*Ursus arctos*) populations reflects the human caused isolation

Key words: *Ursus arctos*, genetic differentiation, population fragmentation, Carpathians

1. Introduction

Original distribution of brown bear (*Ursus arctos*) in Europe has been dramatically reduced during the last two centuries (Servheen 1990, Swenson et al. 2000). In Carpathian Mountains, formerly large and continuous population was split early in 20th century into two isolated stocks: the smaller Western Carpathian population located in central part of Slovakia and southern Poland and the larger Eastern Carpathian population including Eastern Slovakia, South-Eastern Poland, Ukraine and Romania (Hartl & Hell 1994, Finďo et al. 2007). In Western Carpathians, the excessive hunting pressure and habitat loss were the main causes of population decline (Feriancová 1955, Hell & Slamečka 1999). As a result, the population size was estimated to be only 15–75 individuals in the beginning of the 1930's (Žuffa 1932, Tobiáš 1933). Thanks to protection from 1932, the population started to recover and today's population size estimate is up to 800 individuals (Finďo et al. 2007).

On the other side, the population in Eastern Carpathians in Romania never reached such low numbers and minimum size was estimated to be about 860 individuals in the beginning of 1950's (Ionescu 1999), therefore more genetic variation could have been preserved in comparison to Western Carpathians. The maximum population size of almost 8,000 bears was reached in 1988 (Ionescu 1999).

The bear population in Ukraine experienced significant reduction in the 20th century and after the 2nd World War only few individuals were left (Tatarinov 1973). Although bear numbers increased to 1,000 individuals in late 1960's thanks to protection, the population size estimate in the beginning of 1990's dropped to 594 individuals and in 2001, only 240 individuals were recorded therefore the future of the population remains uncertain (Delehan et al. 2002). Data above indicate that populations on both sides of Carpathians were isolated for almost 100 years.

Low population density in Ukraine could have affected the gene flow within Eastern Carpathian range of distribution. Furthermore, bears in Western Carpathians were on the verge of extinction.

It has been documented that such isolation together with population bottleneck could significantly affect the distribution and the amount of genetic variation in wildlife populations (Pérez et al. 2009, Hellborg et al. 2002, Waits et al. 2000, Paetkau et al. 1998; Kyle & Strobeck 2001).

In this study we applied nuclear microsatellite markers in order to assess the genetic structure of Carpathian brown bear populations and genetic diversity due to human caused habitat fragmentation. Moreover, we evaluated the affect of recent population bottleneck on the level of genetic diversity within study populations.

2. Materials and Methods

2.1. Samples

Samples for genetic analysis were collected throughout the Carpathian range in three different areas: (1) core distribution area in central part of Slovakia, (2) Eastern Slovak Carpathians and (3) along Carpathian range in Romania (Fig. 1).

The most of the samples were soft tissues from legally culled animals as well as blood and bones from hunter trophies or museum specimen. In the territory of Eastern Slovakia where hunting is not allowed samples were collected non-invasively. The number of samples from each sampling area is given in Table 1.

2.2. DNA isolation

DNA was extracted using several methods depending on the type of the sample. Tissue samples were extracted either by modified method of (Sambrook et al. 1989) involving overnight digestion with proteinase K followed by phenol-chloroform extraction or by Chelex 100 Resin (Biorad) with 20 minutes at 99 °C in 10 % Chelex solution. For bone samples, the decalcification in EDTA was performed in first step and after that DNA was isolated by NucleoSpin® Tissue kit (Macherey-Nagel). Non-invasive samples were extracted in laboratory designated for this kind of samples. DNA from faeces was isolated using QIAamp DNA Stool Mini Kit (Qiagen) according producer's manual. Hair samples were extracted by Chelex

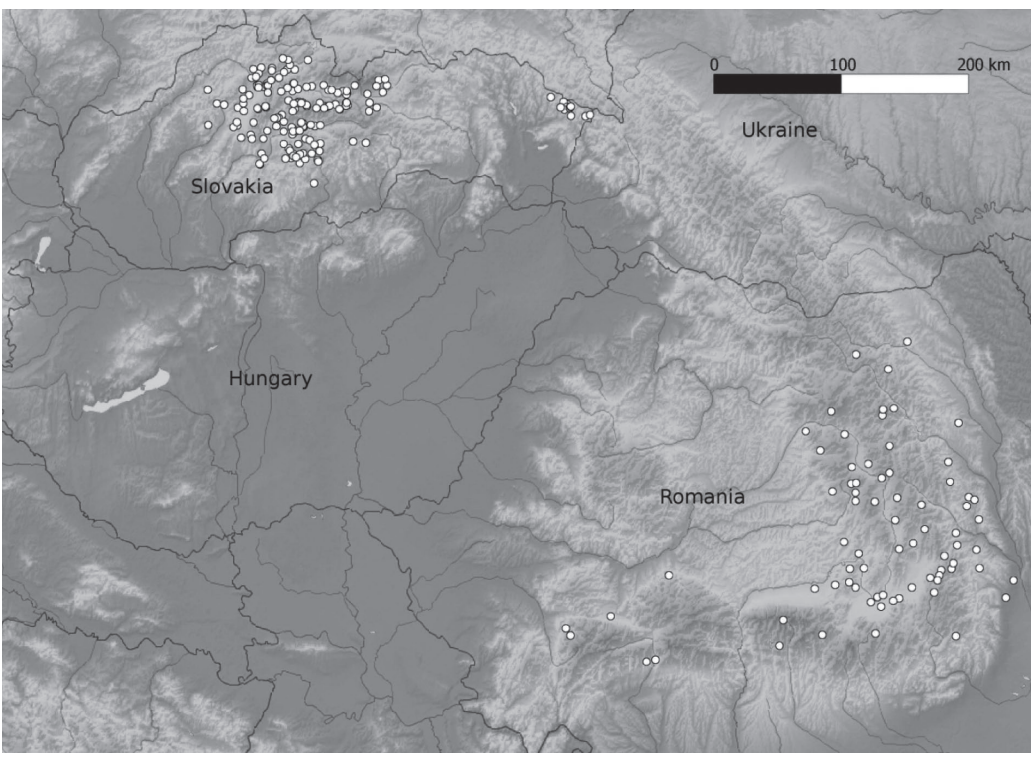

Fig. 1 Localization of samples used in this study

using the same protocol as for tissue samples. To detect contamination, one or two negative controls were used for each batch of samples.

2.3. Genetic typing

Genotypes were obtained using 13 microsatellite loci originally published by Paetkau and Strobeck 1994, Paetkau et al. 1995, Taberlet et al. 1997, and Bellemain and Taberlet 2004. Gender of individuals was determined using SRY locus (Taberlet et al. 1997). For tissues and blood, PCR reactions were performed in 15 µl mixtures containing 7.5 µl of Qiagen Multiplex PCR Kit, 2 µl of DNA extract and fluorescently labelled primers (concentration depending on marker). For bones, faeces and hair, the total volume of PCR was increased to 20 µl with 3 µl of DNA extract. Temperature conditions were as follows: initial denaturation at 95 °C for 15 min followed by 30 cycles of 1min denaturation at 94 °C, 90s annealing (temperature depending on marker) and 1 min elongation at 72 °C. Final elongation step was performed at 60 °C for 30 min. One from each pair of primers was fluorescently labelled to enable detection on capillary sequencer ABI 3130 (Applied Biosystems). Primer concentrations and annealing temperatures are shown in table 2. Primers were amplified in three multiplexes: (i) Mu10, Mu23, Mu50, Mu51, Mu59, G10L, SRY; (ii) G10B + G10C + G1D + G10P

Table 1 Samples used in this study

Sample type	Sampling location		
	Slovakia – core distribution	Slovakia – Eastern Carpathians	Romania
Soft tissues	146	–	102
Blood	12	–	–
Bones	4	1	3
Faeces	6	27	–
Hair	–	2	4

Table 2 Genetic markers employed in this study

Marker	Annealing temperature (°C)	Concentration (µl)	Range (bp)	Reference
Mu10	60	0.50	108–132	(Bellemain & Taberlet 2004)
Mu23	60	0.60	136–156	(Bellemain & Taberlet 2004)
Mu50	60	0.40	76–102	(Bellemain & Taberlet 2004)
Mu51	60	0.60	105–129	(Bellemain & Taberlet 2004)
Mu59	60	0.50	90–122	(Bellemain & Taberlet 2004)
G10L	60	0.50	141–161	(Bellemain & Taberlet 2004)
G10B	58	0.22	130–152	(Paetkau et al. 1995)
G10C	58	0.13	87–109	(Paetkau et al. 1995)
G1D	58	0.25	167–181	(Paetkau et al. 1995)
G10J	52	0.18	73–103	(Paetkau & Strobeck 1994)
G10M	52	0.35	202–218	(Paetkau et al. 1995)
G10P	58	0.15	141–173	(Paetkau et al. 1995)
G10X	58	0.12	132–156	(Paetkau et al. 1995)
SRY	60	0.50	75	(Taberlet et al. 1997)

+ G10X; (iii) G10J + G10M. Products from multiplexes 2 and 3 were mixed (5:2) to get 7 marker systems. These 7 marker systems (multiplex 1 and mix 2/3) were loaded on automated sequencer in mixture: 0.7 µl of PCR product; 9.3 µl of formamide and 0.2 µl of size standard. Electrophoregrams were analyzed by GeneMapper 4.0 software.

2.4. Reliability of typing of non-invasive samples

Non-invasive samples are prone to genotyping errors (Taberlet et al. 1996, Gagneux et al. 1997). Therefore special care must be taken in order to estimate the error rate and minimize it. We employed RELIOTYPE software (Miller et al. 2002) which uses a maximum-likelihood approach that minimizes errors by estimating genotype reliability and strategically directing replication at loci most likely to harbor errors. We used allele counts from tissue samples as input data. Samples from Core area of distribution in Slovakia and Romania were used to perform the allele counts as allelic frequencies from Eastern Slovak Carpathians were unknown. Genotyping error rate was assessed as number of allelic differences between genotypes obtained in each replicate to the consensus genotype (Bonin et al. 2004). In order to identify non-identical genotypes among analyzed non-invasive samples we applied GIMLET software (Valière 2002). To be conservative, samples were treated as identical if they were consistent in at least 12 out of 13 loci. Probability of identity (*PI*, Paetkau & Strobeck, 1994) and probability of identity between siblings (PI_{SIBS}, (Evett & Weir 1998, Taberlet & Luikart 1999) were calculated in GIMLET in order to assess the power of loci to discriminate between individuals.

2.5. Data analysis

We employed STRUCTURE software (Pritchard et al. 2000) in order to assess the genetic structure of studied populations. Program performs model-based clustering to estimate the likelihood for different number of clusters (*K*) defined by user. Analysis was set up to run 10 times for each *K* with 200,000 burn-in and 1 million Markov chain Monte Carlo (MCMC) replicates the admixture and correlated allele frequencies models. The most probable value of *K* was calculated in series of *R*-functions implemented in STRUCTURE-SUM (Ehrich et al. 2007) based on three criteria: (i) as recommended in STRUCTURE manual (Pritchard et al. 2007), *K* with the highest likelihood and consistency between runs was chosen as the most appropriate; (ii) similarity among runs was calculated according to (Rosenberg et al. 2002) with a slightly modified formula (Ehrich et al. 2007); (iii) ΔK statistics based on the rate of change in the log probability of data between the successive *K* values (Evanno et al. 2005) was used.
Population genetic measures were calculated in ARLEQUIN (Excoffier et al. 2005). Genetic diversity was expressed as number of alleles per locus (*A*), observed (H_O) and expected heterozygosity (H_E).
In order to assess if the studied populations were affected by bottleneck we performed *M* ratio test implemented in ARLEQUIN. The test is based on the assumption that the total number of alleles (*k*) is reduced faster than overall range in allele size (*r*) when the population is reduced in size. Thus, the ratio $M = k/r$ is expected to be smaller in recently reduced populations than in equilibrium populations (Garza & Williamson 2001).

3. Results

3.1. Genetic structure

Bayesian clustering analysis performed in STRUCTURE revealed that the most probable number of clusters (*K*) for our data set is two. This was confirmed by consistency between runs, similarity coefficient as well as ΔK statistics (Fig. 2). Two clusters were consisting of individuals from Core distribution area in Slovakia in Western Carpathians (therefore we called the cluster as WC) and individuals from Eastern part of Carpathians in Slovakia and Romania formed second cluster (Eastern Carpathians – EC).
Both clusters from the first part of analysis were analyzed separately in order to detect further

sub-division. Cluster EC from previous step was separated according to sampling locations into Eastern Slovak Carpathian (ES) and Romanian (RO). Western Carpathian cluster was divided into two separate clusters (Fig. 3).

The division of Western Carpathian samples into two genetic clusters was unexpected. Moreover, this division follows interesting geographical pattern. First cluster was consisting of individuals from northern part of Slovakia (NS) and second one included individuals from central Slovakia (CS). The border between these two areas is formed mainly by the Váh valley (Fig. 4).

The Váh river itself does not create an obstacle for gene flow; a dam on Váh river and highway (both built in late 1960s and in addition two industrial agglomerations created a barrier for effective gene flow between the northern and central part of Slovakia.

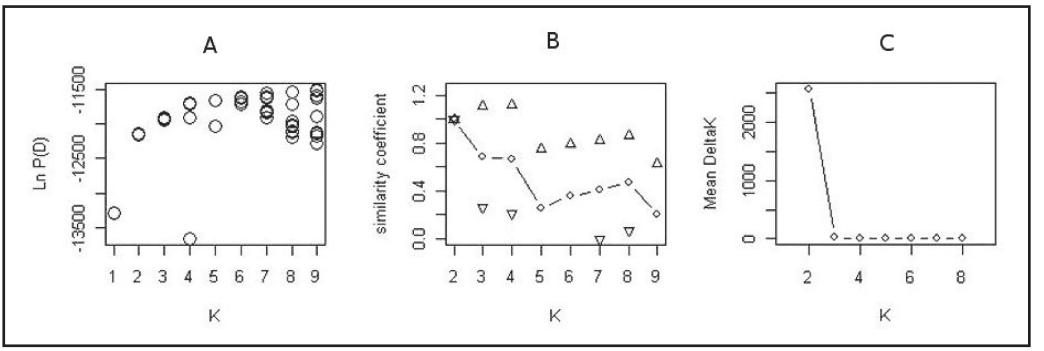

Fig. 2 Calculation of most probable value of K. The highest likelihood was for K=2. [A] – Plot of ln P(Data) in function of K. [B] - Plot showing the average similarity coefficient for each K with standard deviations. [C] – Plot of ΔK – the second order rate of change of ln P(Data) with respect to K.

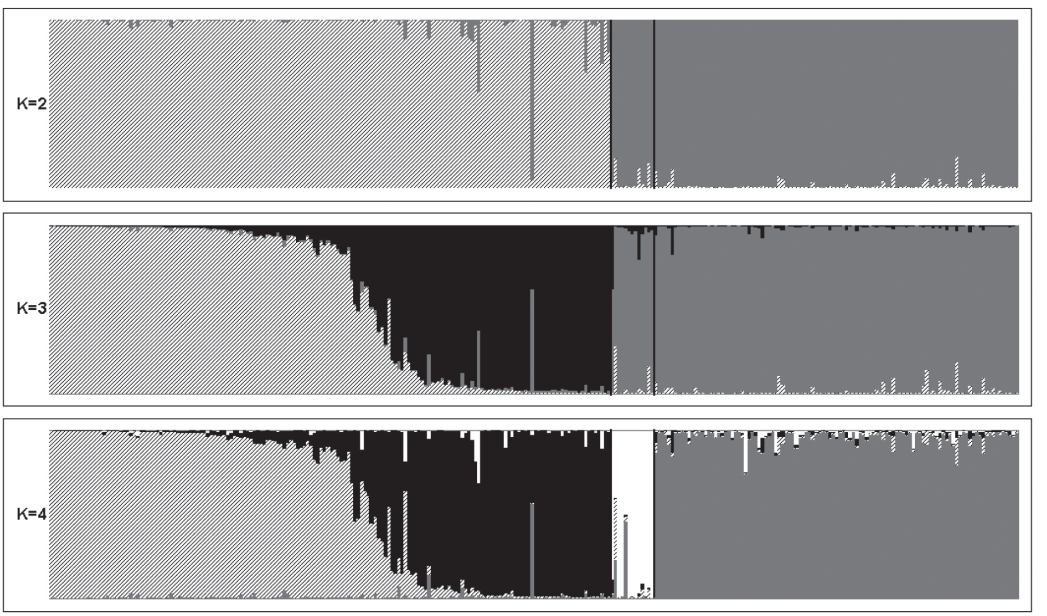

Fig. 3 Estimated population structure for different values of K. Individuals are represented by vertical lines which are partitioned into K segments that represent individual's membership fractions in K clusters.

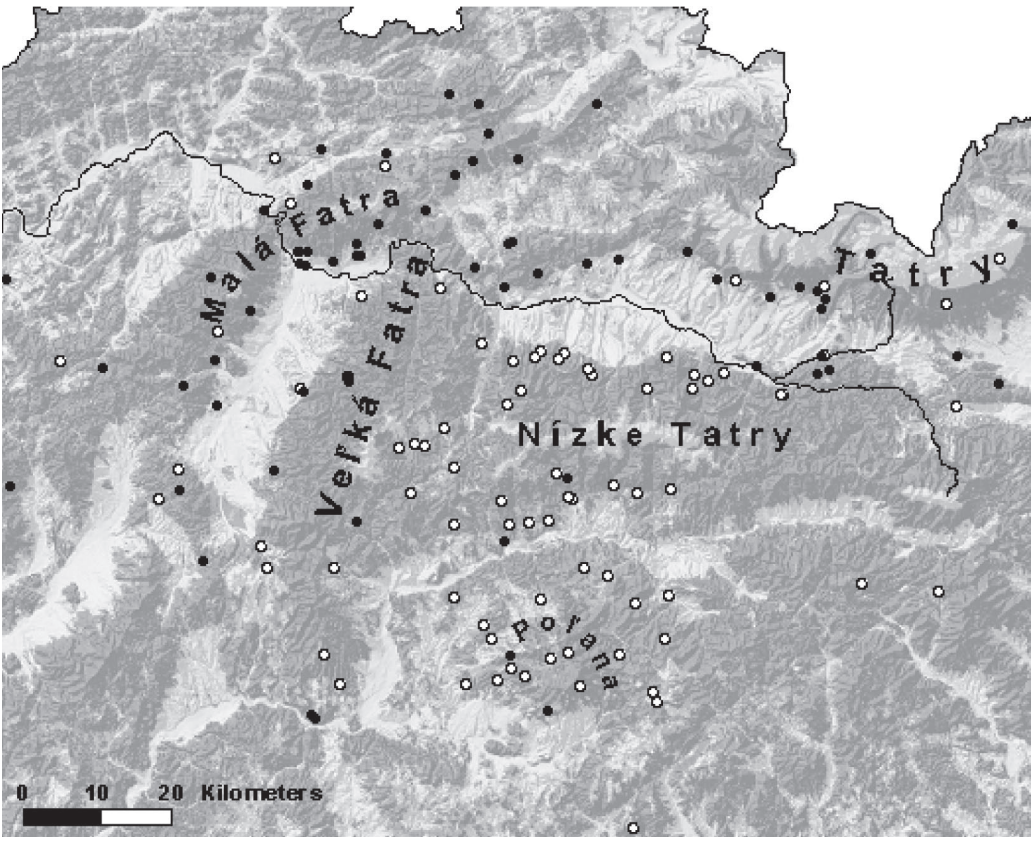

Fig. 4 Localization of two genetic clusters in the territory of Western Carpathians in Slovakia: Northern Slovakia (NS – black dots) and Central Slovakia (CS – white dots). The Váh river valley with agriculture and human settlements forms a border between the genetic clusters.

3.2. Genetic diversity

The highest number of alleles per locus (8.5) as well as the highest expected (0.796) and observed heterozygosity (0.757) was found in Romanian population. On the other side, the lowest level of genetic diversity was found in population from Eastern Slovakia with 5.2 alleles per locus and H_e and H_o 0.662 and 0.647 respectively. However, the results for eastern Slovakian subpopulation should be interpreted with caution due to low sample size. The values of genetic diversity measures are given in table 3.

3.3. Population bottleneck

The M ratio test revealed that genetic bottleneck occurred within the populations Central Slovakia (0.650), Northern Slovakia (0.647) and Eastern Slovakia (0.588). The value for Romanian population is slightly higher (0.746) indicating that the reduction of population size was less severe in the Eastern Carpathian populations.

4. Discussion

Originally large Carpathian brown bear population was reduced due to intensive hunting at the end of the 19th and the beginning of the 20th century. At that period brown bear was still con-

Table 3 Genetic diversity within studied populations. A – number of alleles per locus, H_o – observed heterozygosity, H_e – expected heterozygosity.

Locus	Northern Slovakia			Central Slovakia			Eastern Slovakia			Romania		
	A	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e
Mu10	6	0.807	0.753	8	0.779	0.755	5	0.667	0.582	7	0.757	0.831
Mu23	6	0.809	0.785	7	0.779	0.770	6	0.500	0.752	8	0.778	0.811
Mu50	6	0.711	0.646	6	0.773	0.743	4	0.688	0.579	8	0.798	0.816
Mu51	7	0.708	0.822	6	0.539	0.745	6	0.688	0.720	7	0.718	0.775
Mu59	7	0.727	0.714	7	0.718	0.738	7	1.000	0.766	15	0.832	0.894
G10L	5	0.622	0.630	6	0.390	0.415	5	0.500	0.476	8	0.787	0.843
G10B	4	0.589	0.633	4	0.597	0.604	5	0.625	0.653	8	0.761	0.753
G10C	6	0.789	0.750	7	0.675	0.698	5	0.375	0.579	9	0.807	0.820
G1D	6	0.761	0.759	6	0.688	0.783	5	0.813	0.772	7	0.706	0.732
G10J	5	0.744	0.751	6	0.795	0.790	5	0.688	0.605	8	0.780	0.789
G10M	4	0.530	0.562	6	0.732	0.747	5	0.667	0.638	7	0.615	0.674
G10P	6	0.727	0.772	5	0.592	0.626	4	0.400	0.487	8	0.736	0.803
G10X	5	0.460	0.415	4	0.757	0.691	6	1.000	0.802	10	0.757	0.796
Mean	5.615	0.691	0.692	6.000	0.678	0.700	5.231	0.662	0.647	8.462	0.756	0.795
s.d.	0.923	0.105	0.108	1.109	0.114	0.099	0.799	0.188	0.104	2.061	0.053	0.052

sidered as damage-causing game making significant impact on herds of cattle and ships grazing the pastures along the Carpathians. Except that the brown bear was considered to be the valuable hunting trophy. In the between-war period the brown bear population was almost extinct in Western Carpathians and the population size estimates were far below 100 (estimates ranged between 15 and 75). Since the range of the brown bear populations in the between-war period covered about 300,000 ha it can be assumed that in fact there were several nuclei composed of few individuals.

Thanks to the species protection which started in 1932 the brown bear population started to increase in size and the natural range was shifted towards west and south. At present the range covers about 700,000 ha. Development of industrial activities and building of transport and energetic infrastructure is causing the habitat fragmentation. Detailed analysis of habitat fragmentation and corridors in Slovakia described Finďo et al. (2007).

The particular case of the habitat fragmentation of brown bear range in the Western Carpathian was caused by human activities. Building the dam, highway and enlargement of the industrial zone around the cities Ružomberok and Liptovský Mikuláš caused about 30 km long barrier in effective gene flow. Eastwards and westwards of this barrier are effective corridors used also by brown bears in which annually some fatal accidents occur. These are between the Little and the Great Fatras (vicinity of villages Ľubochňa and Kraľovany) and the Low and the High Tatras (Važec to Lučivná). While in the first case no technical equipment is built to enable wildlife migration, in the second case there are several passages over and under the recently built highway enabling the migration between fragmented habitats.

Similar danger is expected in the Eastern Slovakia due to building the motorway connecting Prešov and Rzeszów in Poland. Geographically and genetically fragmented brown bear occurrence in Eastern Slovakia (Poloniny and sporadic occurrences in Slovak Ore Mountains and Levočské vrchy) will persist as fragmented also after finishing the motorway where no wildlife passages were designed and built.

The consequence of habitat fragmentation has been shown also in genetic differentiation within both parts of fragmented population in Western Carpathians. It is, however, difficult

to ascribe all genetic differentiation to human caused migration barriers in last 40 years. We do not have any genetic proofs about the genetic differentiation of the populations in West Tatras and Low Tatras from the period prior building the dam and highway in late sixties. Due to small population sizes of these fragments the genetic drift might have caused the genetic differentiation preceeding the human caused fragmentation.

The consequences of habitat fragmentation of brown bear populations within its European range have been a hot issue at building the highway infrastructure in Slovenia, Croatia and Greece. Pressure of the scientific community in Slovenia and Croatia helped to incorporate building of numerous passages on the highways crossing the brown bear habitats (Adamić, Huber). On the other hand, Karamalidis et al. (2011) run a project aimed at estimation the levels of the genetic diversity and differentiation in Pindos and Olympos mountains prior building the Egnatia highway and study the consequences of the human caused fragmentation.

The diversity estimates based on isozyme study were published for West Carpathian brown bear population by Hartl and Hell (1994). They stated that regardless the very small population size of the Slovak brown bear population in the between-war period there are no signs of any bottleneck effect and the results are comparable with results from the other European regions.

Our results, based on 13 microsatellite loci, showed that the heterozygosities found in west Carpathian populations are comparable with Scandinavia, although they do not reach the values obtained in Alaska, British Columbia and Romania (Paetkau et al. 1998, Waits et al. 2000). The tests of the bottleneck effect were significant for some population from West Carpathians.

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Summary

The present distribution of brown bear (*Ursus arctos*) in Europe is only fragment of its original range. In Carpathians, formerly continuous range was split into the Western and the Eastern Carpathian population as a consequence of human activities in the early 20th century. Due to excessive hunting pressure brown bear was in the verge of extinction in the Western Carpathians. On the other side, the bear population in the Eastern Carpathians in Romania never reached less than 800 individuals. Human caused isolation and population bottleneck of the Western Carpathian population might have affected the genetic variation. In this study we employed thirteen microsatellite markers to assess the genetic structure and the level of genetic diversity within the Carpathian brown bear range. High level of genetic differentiation was found. Although populations showed some degree of bottleneck, the level of genetic diversity was in medium range. We discuss the possible role of recent human caused changes of species distribution and population size in forming of present genetic structure of studied populations.

Zusammenfassung

Die genetische Differenzierung der Populationen des Karpathen-Braunbären (*Ursus arctos*) als Folge anthropogen verursachter Isolation

Die heutige Verbreitung des Braunbären (*Ursus arctos*) in Europa ist nur ein Fragment seines ursprünglichen Areals. Innerhalb der Karpaten wurde sein früheres Verbreitungsgebiet als Folge der menschlichen Aktivitäten anfangs 20. Jahrhunderts in die west- und ostkapratische Population zersplittert. Wegen des hohen Jagddruckes befand sich der Braunbär in den Westkarpaten an der Grenze der Ausrottung. Auf

der anderen Seite fiel die Braunbärpopulation in den Ostkarpaten (Rumänien) nie unter 800 Individuen. Durch die von Menschen verursachte Isolation und den Populationsbottleneck der westkarpatischen Population wurde die genetische Diversität beeinflusst. In unserer Studie verwendeten wir 13 Mikroisatellitenmarker um die genetische Struktur und die genetische Diversität innerhalb des westkarpatischen Verbreitungsgebietes zu analysieren. Wir haben eine hohe genetische Differenzierung festgestellt. Obwohl die Populationen einen gewissen Grad des Bottlenecks zeigten, gab es nur mittlere Diversitätswerte. Es wird die mögliche Rolle der rezenten durch menschlichen Einfluss verursachten Änderungen im Verbreitungsgebiet und in der Populationsgröße auf die Formation der heutigen genetischen Struktur der untersuchten Populationen diskutiert.

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