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Population genetic parameters of brown bears in western Serbia: implications for research and conservation

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Abstract: The Alps–Dinaric–Pindos (ADP) bear population is considered to be one of the largest populations remaining in Europe. Despite its international importance for large-scale bear conservation, detailed and accurate information about the genetic and conservation status of some of its sub-populations is lacking. Serbia is located in the geographic center of the ADP bear population, and is of special importance because it connects this population to bear populations in southeastern Europe. Our aim was to establish a research protocol for genetic monitoring and provide information on genetic parameters of brown bears in western Serbia. From hair samples collected non-invasively from hair traps and 2 live-captures, we identified 10 individual bears; a comparison to other bear populations in Europe suggests a favorable genetic status (i.e., increased genetic diversity) of bears in this part of the country. The close geographic proximity of bears in western Serbia to bear populations in adjacent countries, and our results, suggest that the ADP population is interconnected in this region. We recommend a coordinated, multi-national approach for the monitoring and conservation of bears in southeastern Europe, for example, through the establishment of a common genetic database.

Key words: Alps–Dinaric–Pindos population, microsatellite analysis, non-invasive genetic monitoring, Ursidae, *Ursus arctos*

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Introduction

Non-invasive genetic monitoring has emerged as a reliable, as well as time- and cost-efficient tool for studying rare, elusive, and endangered animals, such as large carnivores (Schwartz et al. 2007), and is therefore of prime importance in conservation biology (Taberlet et al. 1999). Non-invasive genetic monitoring has been successfully applied to study several large carnivores, including wolves (*Canis lupus;* Valière et al. 2003), tigers (*Panthera tigris;* Mondol et al. 2009), and wolverines (*Gulo gulo;* Hedmark et al. 2004). It has also become an integral part of research and conservation efforts regarding the brown bear (*Ursus arctos*), especially in Europe (Swenson et al. 2011), where several of the remaining populations are small and endangered (Zedrosser et al. 2001).

The Alps–Dinaric–Pindos (ADP) brown bear population recently has been estimated to number >3,000 individuals (Kaczensky et al. 2013) and is one of the largest bear populations remaining in Europe (Zedrosser et al. 2001). It ranges from Austria, Italy, and Slovenia in the north over the Dinaric mountain range into Greece in the south. The ADP bear population has been the focus of numerous studies, especially genetic-based studies (e.g., Italy [de Barba

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et al. 2010], Austria [Kruckenhauser et al. 2009], Slovenia [Skrbinšek et al. 2012b], Croatia [Kocijan et al. 2011], Former Yugoslav Republic [FYR] of Macedonia [Karamanlidis et al. 2014], and Greece [Karamanlidis et al. 2012]). However, despite its international importance for large-scale bear conservation in Europe (Zedrosser et al. 2001), detailed and accurate information about the genetic and conservation status of some ADP sub-populations, such as those in Bosnia and Herzegovina, Serbia, and Montenegro, are lacking or incomplete (Kaczensky et al. 2013).

Serbia is located in the geographic center of the ADP population range, and its bears belong phylogenetically to the western European lineage (Randi et al. 1994, Taberlet and Bouvet 1994). Two bear populations remain in geographically separated regions in the eastern and western parts of the country (Fig. 1). Bears in eastern Serbia are connected to the Stara Planina bear population in Bulgaria and the Carpathian population in Romania and are currently thought to be declining (Kaczensky et al. 2013). The bear population in western Serbia belongs to the ADP (Zedrosser et al. 2001) and is now considered stable (Kaczensky et al. 2013). Overall, bear populations in Serbia are thought to be declining since about 1995. Major threats are poaching, habitat loss and fragmentation, and the illegal capture of wild animals for exhibition (Huber 1999; Zedrosser et al. 2001; Paunović et al. 2005a, b). According to the latest population estimate in 2010, only 62 ± 10 bears are thought to exist in Serbia (Kaczensky et al. 2013). However, this estimate does not include bears in the Kosovo and Metohija regions in southern Serbia, for which no data have been available since 1998. Bears are a protected species in the Serbian legislation, and studying and monitoring their populations has been identified as a conservation priority (Paunović and Ćirović 2006). Our goal was to establish a research protocol for genetic monitoring and provide initial information on genetic parameters of brown bears in Serbia, using non-invasive sampling.

Materials and methods

Study areas

Non-invasive genetic sampling of brown bears was conducted in 2 study areas in western Serbia, in the Tara National Park and at Mount Čemerno (Fig. 1). Bears in these 2 study areas are considered to belong to the western Serbian bear sub-population (Kaczensky et al. 2013). Tara National Park is geographically located around Mount Tara in western Serbia (43°54′24″N, 19°18′18″E), and is part of the Dinaric mountain range. The slopes of the mountains are covered with dense forests and include numerous high-altitude clearings and meadows, steep cliffs, and deep ravines. Tara National Park was established in 1981, and comprises approximately 200 km^2. Mount Čemerno (450 km^2) is located approximately 70 km to the southeast of Tara National Park (43°34′57″N, 20°25′33″E), and is of similar topography.

Sampling, DNA extraction, and microsatellite analysis

Because of logistic and financial limitations, we conducted non-invasive hair sampling only in areas where previous knowledge indicated presence of bears. We collected hair samples from hair traps made of barbed-wire placed around feeders used for supplemental feeding of bears with corn, or from hair traps placed on rub trees (Kendall and McKelvey 2008). We placed 11 hair traps at 3 sites in Tara National Park (i.e., Gorušice: 4 hair traps; Makaze: 4 hair traps; and Račanska Šljivovica: 3 hair traps) and 3 hair traps at the site Gornji Dubac at Mount Čemerno.

We collected hair samples once per month from April to November 2009. We considered all hair on an individual trap barb a unique sample. In addition, we analyzed 2 hair samples collected from 2 live-captured bears that were part of a telemetry study in Tara National Park in 2007 (D. Ćirović and M. Paunović, University of Belgrade and Natural History Museum of Belgrade, unpublished data). We placed samples in paper envelopes and stored them at room temperature in sealable plastic bags with silica gel (Roon et al. 2003) until samples were analyzed by Wildlife Genetics International (Nelson, British Columbia, Canada).

Sample DNA was extracted using DNeasy Blood and Tissue kits (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Samples were genotyped at microsatellite loci G1A, G1D, G10H, G10J, G10L, G10U (Paetkau and Strobeck 1994, Paetkau et al. 1998); G10C, G10M, G10P (Paetkau et al. 1995); MU23, MU26, MU50, MU51, MU59 (Taberlet et al. 1997); CXX110 (Proctor et al. 2002); Msut-2 (Kitahara et al. 2000); REN144A06, and REN145P07 (Breen et al. 2001). Sex identification was established through analysis of the amelogenin

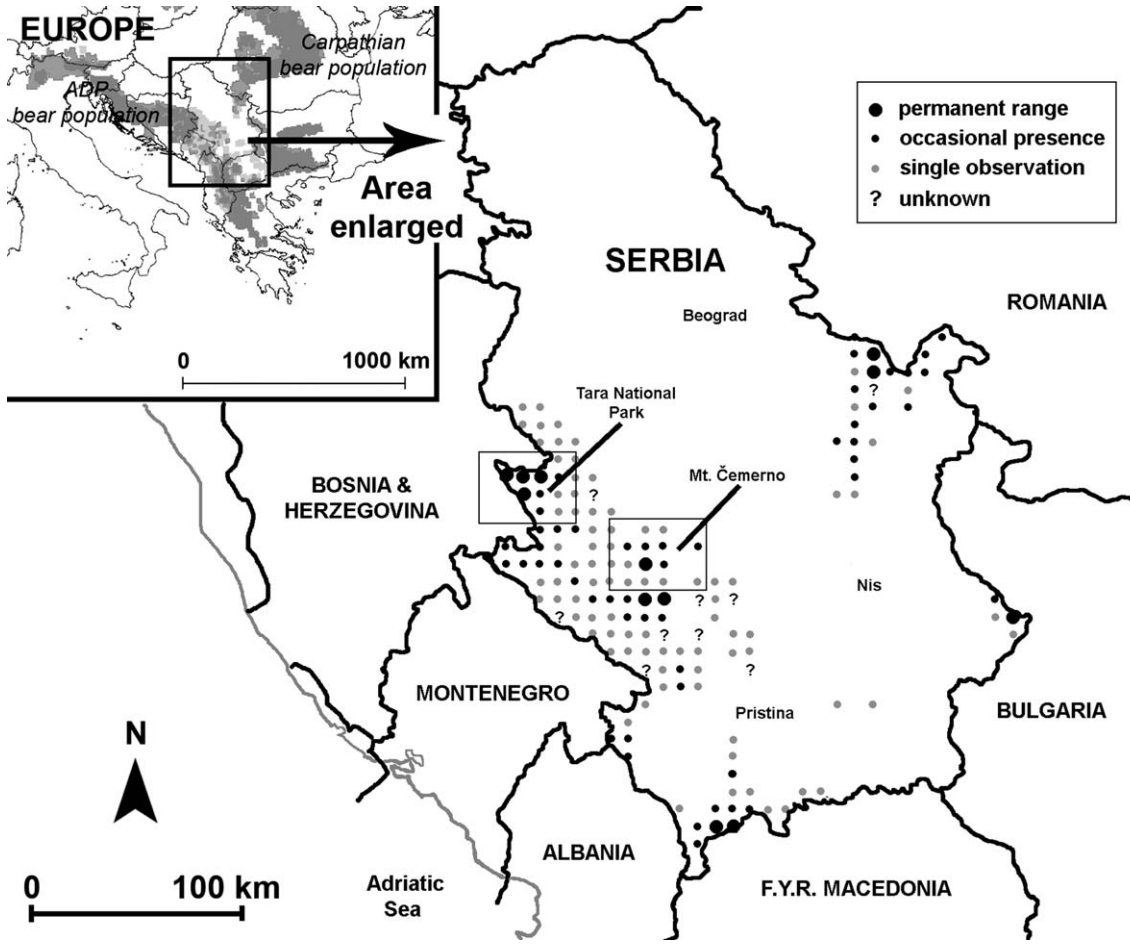

Fig. 1. Map of Serbia indicating the study areas in Tara National Park and Mount Čemerno in relation to the distribution of brown bears in Serbia (distribution of brown bears redrawn from Paunović et al. 2005b). The inset map indicates the location of Serbia in southeastern Europe in relation to the Alps–Dinaric–Pindos (ADP) and Carpathian bear populations.

gene (Ennis and Gallagher 1994). Thermal cycling was performed using a MJ Research PTC100 thermocycler with 96 well 'Gold' blocks (MJ Research Inc., St. Bruno, Quebec, Canada). Polymerase chain reaction (PCR) buffers and conditions were used according to Paetkau et al. (1998), except that markers were not co-amplified, because co-amplification may reduce the success rates for hair samples (D. Paetkau, Wildlife Genetics International, personal communication). Two mM MgCl2 was used for all markers except G10J (1.8 mM). An automated sequencer (ABI 310) was used, and genotypes were determined using ABI Genescan and Genotyper version 2.1 software (Perkin Elmer-Applied Biosystems, Foster City, California, USA). The sizing of the PCR products was performed using capillary electrophoresis. To minimize genotyping errors in the final data set, low-quality and putatively mixed samples were excluded from further analyses (Paetkau 2003). Genotypes that were closely related, matching at all but 1 or 2 markers, were reanalyzed at the markers where they differed to check for genotyping errors that could lead to false individual identification (Paetkau 2003, Kendall et al. 2009). Test for allelic dropout, presence of null alleles, and scoring errors caused by stutter peaks were performed with Micro-Checker version 2.2.3 (van Oosterhout et al. 2004).

Statistical methods

We assessed the utility of loci used for evaluating genetic diversity by calculating the Polymorphism Information Content (PIC; Botstein et al. 1980) using the program PowerMarker, version 3.25 (Liu

and Muse 2005). To evaluate the suitability of the marker set for identifying individuals, we calculated the probability of identity among siblings ($P_{ID\text{-}Sib}$; Waits et al. 2001) using the software GIMLET version 1.3.2 (Valière 2002). To allow for the possibility of mismatches caused by genotyping error, we also looked for the pairs of genotypes that were matched at all but 1, 2, and 3 loci (1-MM, 2-MM, and 3-MM pairs) using the program GenAlEx 6 (Peakall and Smouse 2006).

We measured nuclear DNA diversity using number of alleles per locus (A), observed heterozygosity (H_o), and Nei's unbiased expected heterozygosity (H_e; Nei 1978) using the program PowerMarker version 3.25 (Liu and Muse 2005). We tested deviations from Hardy–Weinberg equilibrium (HWE) for each locus in the population using the exact probability test implemented in the software GENEPOP version 4.0.10 (Raymond and Rousset 1995) using the method proposed by Guo and Thompson (1992). We used a Markov chain set to 100 batches, with 5,000 iterations/batch and 10,000 steps of dememorization to obtain an unbiased estimate of the exact probability. We performed pairwise tests for linkage disequilibrium using the Fisher's method (Sokal and Rohlf 1994), with 1,000 batches and 10,000 iterations/batch and adjusted P-values for multiple comparisons using the sequential Bonferroni correction (Holm 1979). We conducted global HWE tests across all loci for heterozygote deficiency using the Fisher's method.

To compare genetic diversity of bears in Serbia to other bear populations, we used the reference population approach (Skrbinšek et al. 2012b). In this approach, the locus sets of the reference and the studied populations are reduced to the loci they have in common. To correct for unequal sample size, genotypes from the reference population are then resampled with replacement multiple times to the same sample size as that of the studied population, and average allelic richness, expected heterozygosity and their standard deviations are calculated over all subsamples. Heterozygosity ratio (H_{er}) and allelic richness ratio (A_{rt}) indices are then calculated as a ratio between these summary statistics in the studied population and their sub-sampling–corrected values in the reference population. This provides a comparison of genetic diversity of the studied population with the reference population and all other populations that have had these indices calculated using the same reference population (Skrbinšek et al. 2012b). We used the data by Skrbinšek et al. (2012b) on brown bears in Slovenia as the reference population, because this large data set has previously been used as a reference for comparison with other brown bear populations. We used the 11 loci that our study has in common with the reference data set (i.e., G1A, G10C, G10D, G10J, G10L, G10M, G10P, MU23, MU50, MU51, MU59). We applied R-scripts provided in Skrbinšek et al. (2012c) to run the subsampling with 1,000 random subsamples. All analyses were run in R version 2.14.2 (R Development Core Team 2011). We used the heterozygosity ratio (H_{er}) and allelic richness ratio (A_{rt}) to compare genetic diversity of bears in Serbia with some of the other populations in the ADP range, as well as with other bear populations of known conservation status in other parts of Europe. Because errors of H_{er} and A_{rt} are normally distributed, we used the Z-test (Sokal and Rohlf 1994) to test for statistical significance of the difference between these indices in bears in Serbia and in Slovenia.

Results

We collected 145 hair samples of which 65 samples were removed before initial analysis because they did not contain any follicles. Of the remaining 80 samples, a complete 18-loci genotype was obtained for 43 samples and 10 unique genotypes were identified, including the 2 individuals live-captured in 2007. In Tara National Park, we identified 3 female and 4 male bears, while at Mount Čemerno only 1 male bear was identified. Five individuals were identified on several occasions, while movements of bears between the sampling sites in Tara National Park were recorded (max. travel distance between sampling sites: 16.7 km), but not between the 2 sampling areas (Table 1).

All loci used were polymorphic, with the number of alleles per locus ranging from 4 to 8, with a mean of 5.4 (Table 2). The mean observed heterozygosity was 0.78, and the unbiased expected heterozygosity was 0.69 (Table 2). Global tests across loci showed no deviation from HWE for heterozygote deficiency ($P = 0.555$), while loci REN145P07 ($P = 0.001$) and Msut2 ($P = 0.040$) had a greater number of homozygotes.

Results from analysis in Micro-checker indicated the presence of null-alleles at locus REN145P07. The low number of individuals identified in our study makes it difficult to confirm the reason for this and whether the excess homozygotes at locus Msut-2

Table 1. Monthly captures and recaptures of 3 female (F) and 5 male (M) brown bears identified during a non-invasive genetics study in western Serbia in 2009. Numbers indicate the code numbers of the animals identified.

	Month							
Sampling site	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**
			Mount Čemerno					
Gornji Dubac		M1	M1					
			Tara National Park					
Gorušice		M2	M3, F1			F2		
Makaze		M4	M2	M2, F3				M4
Račanska Šljivovica	M2	M5	M5, M2		F2	F2		

were null alleles, but as a precaution both loci were excluded from all downstream analyses. Eighty-one percent of the selected markers and the overall mean of all markers used in the study had a PIC value >0.600, and the cumulative probability of identity among siblings ($P_{ID\text{-}Sib}$) was <0.010, which is sufficient for identifying individual bears in our study area (Table 2); there were no samples matching at all but 1, 2, or 3 loci. Statistical tests for linkage disequilibrium were computed for all pairs of loci; and after adjustment of *P*-values with the sequential Bonferroni correction, none of the 152 tests were significant ($P \geq 0.050$).

There was no difference between the allelic richness of bears in Serbia and the reference population in Slovenia (A_{rt} difference = 0.1, P = 0.550), while the expected heterozygosity was practically identical between Serbia and Slovenia (H_{er} = 1.000). In relation to the allelic and heterozygosity ratios, the diversity of bears in Serbia was similar to that of other bears in the ADP population, lower than that of the bears in the Carpathian Mountains in Romania, but considerably higher than that of the small and threatened populations in the Cantabrian Mountains in Spain and the Apennine Mountains in Italy (Table 3).

Discussion

We collected genetic data from a poorly studied population of brown bears in western Serbia and conducted a preliminary evaluation of the genetic status of the species in that area. This was the first genetic study of brown bears in Serbia; therefore, we

Table 2. Descriptive statistics at 16 polymorphic loci of genetic samples of 10 brown bears from western Serbia, including number of alleles (*A*), unbiased expected (H_e) and observed (H_o) heterozygosity, Polymorphism Informative Content (PIC), probability of identity among siblings ($P_{ID\text{-}Sib}$) and multi-locus probability of identity among siblings (*Prod.* $P_{ID\text{-}Sib}$).

Locus	*A*	H_e	H_o	PIC	$P_{ID\text{-}Sib}$	*Prod.* $P_{ID\text{-}Sib}$
MU59	8	0.86	0.9	0.84	3.32E-01	3.32E-01
G10P	7	0.82	1.0	0.80	3.54E-01	1.17E-01
G10H	8	0.80	0.9	0.77	3.69E-01	4.34E-02
G10C	7	0.79	1.0	0.77	3.69E-01	1.60E-02
CXX110	6	0.77	0.8	0.74	3.87E-01	6.20E-03
MU50	6	0.75	0.8	0.71	4.03E-01	2.49E-03
G10U	6	0.75	0.8	0.71	4.03E-01	1.00E-03
G10M	4	0.74	0.8	0.69	4.09E-01	4.11E-04
G10J	5	0.72	0.9	0.67	4.22E-01	1.73E-04
MU23	5	0.68	0.7	0.63	4.49E-01	7.79E-05
MU51	4	0.68	0.8	0.61	4.55E-01	3.54E-05
G1A	4	0.65	0.8	0.58	4.72E-01	1.67E-05
G10L	5	0.64	0.8	0.60	4.72E-01	7.88E-06
REN144A06	4	0.64	0.9	0.59	4.74E-01	3.73E-06
G1D	4	0.47	0.4	0.42	6.00E-01	2.24E-06
MU26	4	0.27	0.2	0.26	7.51E-01	1.68E-06
Mean	**5.44**	**0.69**	**0.78**	**0.65**		

Table 3. Descriptive statistics of genetic diversity of brown bears in Serbia and other parts of Europe, including N = number of samples, A = allelic richness, H_e = expected heterozygosity, SE = standard error, H_{er} = expected heterozygosity ratio, and A_{rt} = allelic richness ratio between the compared population and re-sampling–corrected, marker-set specific values in the reference population (Table modified after Skrbinšek et al. [2012b]).

		Compared population		Reference population (re-sampled)		Ratio		
Population[a]	N	A (SE)	He (SE)	A (SE)	He (SE)	A_{rt} (SE)	H_{er} (SE)	Reference
Carpathian—Romania	16	7.78 (0.81)	0.81 (0.010)	5.15 (0.56)	0.70 (0.030)	1.51 (0.23)**	1.16 (0.05)**	(Zachos et al. 2008)
Carpathian—Romania	109	8.46 (0.57)	0.80 (0.014)	6.33 (0.54)	0.73 (0.023)	1.34 (0.15)*	1.09 (0.04)*	(Straka et al. 2012)
ADP—Croatia	156	7.58 (0.54)	0.74 (0.028)	6.48 (0.60)	0.73 (0.025)	1.17 (0.14)	1.01 (0.05)	(Kocijan et al. 2011)
ADP—Serbia	**10**	**5.36 (0.43)**	**0.71 (0.032)**	**4.88 (0.45)**	**0.70 (0.028)**	**1.10 (0.14)**	**1.10 (0.06)**	**This study**
ADP—Slovenia	513	6.68 (0.41)	0.73 (0.020)			1.00 (0.06)	1.00 (0.03)	(Skrbinšek et al. 2012b)
Carpathian—N. Slovakia	71	6.08 (0.29)	0.71 (0.025)	6.20 (0.54)	0.73 (0.023)	0.98 (0.10)	0.97 (0.05)	(Straka et al. 2012)
ADP—Greece	49	6.33 (0.42)	0.76 (0.02)	6.55 (0.52)	0.77 (0.02)	0.97 (0.10)	0.99 (0.04)	(Karamanlidis et al. 2012)
Carpathian—E. Slovakia	16	5.23 (0.22)	0.65 (0.028)	5.47 (0.49)	0.72 (0.025)	0.96 (0.09)	0.91 (0.05)	(Straka et al. 2012)
Carpathian—Central Slovakia	96	6.00 (0.25)	0.70 (0.031)	6.30 (0.54)	0.73 (0.023)	0.95 (0.09)	0.95 (0.05)	(Straka et al. 2012)
Cantabrian—W. Spain	39	3.44 (0.30)	0.48 (0.050)	5.73 (0.49)	0.71 (0.022)	0.60 (0.07)***	0.67 (0.07)***	(Pérez et al. 2009)
Apennines	17	2.44 (0.24)	0.44 (0.069)	5.19 (0.56)	0.70 (0.030)	0.47 (0.07)***	0.63 (0.10)***	(Zachos et al. 2008)
Cantabrian—E. Spain	8	1.75 (0.17)	0.28 (0.062)	4.56 (0.38)	0.68 (0.026)	0.38 (0.05)***	0.41 (0.09)***	(Pérez et al. 2009)

[a]ADP = Alps–Dinaric–Pindos.
*P = 0.10, ** P = 0.05, *** P = 0.01 (statistical significance of A_{rt} and H_{er} compared with bears in Serbia).

initially assessed the suitability of our genetic marker system to evaluate the reliability of our results. All markers used had a PIC value greater than the recommended value of 0.6 (Buchanan et al. 1993), indicating a high degree of informativeness. No pairs of samples matching at all but 1, 2, or 3 loci were identified and the cumulative probability of identity among siblings ($P_{ID\text{-}Sib}$) was considerably lower than 0.050, so the marker set we used is suitable for individual identification and population size estimation (Waits et al. 2001).

Measures of genetic diversity of populations with known recent demographic history and conservation status provide a useful approach for comparing diversity levels to that of populations of unknown history and status, and can provide valuable insights into the consequences of rarity that are critical for conservation planning (Johnson et al. 2009). Genetic diversity of brown bears in western Serbia was high and similar to that of populations considered to have a favorable conservation status, such as the bear populations in Croatia and Romania (Table 3). In general, bears in western Serbia had levels of genetic diversity similar to other bears from the ADP population, indicating they are part of this larger population and not an isolated fragment. This is supported also by recent telemetry data that show bears moving from Serbia to Bosnia and Herzegovina (D. Ćirović and M. Paunović, unpublished data). It has been suggested that the ADP population is discontinuous, with the population possibly being divided into 2 demes (Swenson et al. 2000). The bears in our study probably belong to the northern deme, which has a relatively large effective population size (Skrbinšek et al. 2012a), providing for the high genetic diversity observed in this study. Our results indicate increased levels of genetic diversity (i.e., moderate levels of allelic richness and increased levels of expected heterozygosity), which in turn suggest a favorable genetic status of brown bears in western Serbia. However, because of the small sample size, these results should be interpreted with caution.

Research and conservation implications

Our identification of 8 different bears via use of non-invasive genetic sampling represents approximately 12% of the entire bear population estimated in the country (Kaczensky et al. 2013), and is higher than the 10% total population identification limit proposed for studies assessing the genetic status of

bear populations in the region (Karamanlidis et al. 2010a). The non-invasive genetic sampling approach we used appears suitable for monitoring brown bears in western Serbia, and could provide information on genetic diversity, population status, and individual movements. Considering the success of similar non-invasive monitoring efforts in the southern Balkans (e.g., Greece [Karamanlidis et al. 2012], FYR Macedonia [Karamanlidis et al. 2014]), a non-invasive (genetic) monitoring scheme would seem appropriate for detecting presence, evaluating habitat use, and monitoring population trends of bears in that country.

A successful strategy for the genetic monitoring of bears in Serbia should focus on the creation of an extensive network of sampling stations for the non-invasive collection of hair samples (Karamanlidis et al. 2010b). This sampling network should cover a larger portion of bear range in Serbia than the present study, with more sampling stations, and greater sampling frequency (i.e., the time between sampling sessions should be shorter, e.g., approx. 14 days), because this appears to improve DNA yield extracted from hair samples collected in the field (Foran et al. 1997). The research protocol should use the 5 most informative loci identified in this study (i.e., MU59, G10P, G10H, G10C, CXX110) and should consider using the ''multi-tube'' approach (Taberlet et al. 1996) when analyzing samples. The $P_{ID\text{-}Sib}$ of 0.050 suggested by Waits et al. (2001) as sufficient to recognize individual animals was achieved with 3 of these loci (Table 2). To provide some redundancy, while at the same time minimizing genotyping error (Paetkau 2005) and costs, we suggest the use of these 5 loci, yielding a cumulative $P_{ID\text{-}Sib}$ of 0.006, for larger mark–recapture studies. To obtain more information on the genetic status of brown bears in Serbia, the use of multiple sources of genetic samples (e.g., scats [Bellemain 2004, Bellemain and Taberlet 2004]) should be considered (Boulanger et al. 2008).

The results of our study, in combination with the data from a telemetry study in the same area (D. Ćirović and M. Paunović, unpublished data), as well as the close geographic proximity of the western Serbian bears to the bears in neighboring Croatia, Bosnia and Herzegovina, and Montenegro, support the existence of a single, interconnected population. We suggest the need for a coordinated, international approach to monitor and conserve this species in southeastern Europe. The establishment of a multi-national genetic bear register (Karamanlidis et al. 2010a) could be a first step in this direction. The register could consist of a genetic database including genotypes of bears in the region and would ensure comparability of genetic data among countries and populations and further improve cross-border monitoring efforts.

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