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Original Investigation

Noninvasive genetic assessment of brown bear population structure in Bulgarian mountain regions

Christiane Frosch[a,b,*], Aleksandar Dutsov[c], Diana Zlatanova[c,d], Kostadin Valchev[c], Tobias E. Reiners[a], Katharina Steyer[a], Markus Pfenninger[e], Carsten Nowak[a]

[a] Conservation Genetics Group, Senckenberg Research Institute and Natural History Museum Frankfurt, Clamecystrasse 12, 63571 Gelnhausen, Germany
[b] Frankfurt Zoological Society, Bernhard-Grzimek-Allee 1, 60316 Frankfurt am Main, Germany
[c] Balkani Wildlife Society, 67 Tsanko Tserkovski Str, 1421 Sofia, Bulgaria
[d] Faculty of Biology, Sofia University, 8 Dragan Tsankov, 1164 Sofia, Bulgaria
[e] Molecular Ecology Group, Biodiversity and Climate Research Centre (BiK-F) by Senckenberg Gesellschaft für Naturforschung and Goethe-University, 60325 Frankfurt am Main, Germany

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ABSTRACT

The Balkans are one of the last large refugia for brown bear (*Ursus arctos*) populations in Europe, and Bulgaria, in particular, contains relatively large areas of suitable brown bear habitat and a potential population of more than 600 individuals. Despite this, the majority of brown bear research remains focused on bear populations in Central and Western Europe. We provide the first assessment of genetic population structure of brown bears in Bulgaria by analysing tissue samples ($n = 16$) as well as samples collected with noninvasive genetic methods, including hair and faecal samples ($n = 189$ and $n = 163$, respectively). Sequence analysis of a 248 base pair fragment of the mitochondrial control region showed that two highly divergent mitochondrial European brown bear lineages form a contact zone in central Bulgaria. Furthermore, the analysis of 13 polymorphic microsatellite markers identified 136 individuals and found substantial genetic variability ($H_e = 0.74$; $N_A = 8.9$). The combination of both genetic markers revealed the presence of weak genetic substructure in the study area with considerable degrees of genetic admixture and the likely presence of migration corridors between the two subpopulation in the Rhodope Mountains and Stara Planina as evidenced from the genetic detection of two male long-distance dispersers. A detailed assessment from densely collected samples in the Rhodope Mountains resulted in a population size estimate of 315 (95% CI = 206–334) individuals, indicating that not all available habitat is presently occupied by bears in this region. Efficient management plans should focus on preserving connectivity of suitable habitats in order to maintain gene flow between the two Bulgarian brown bear subpopulations.

Introduction

As a flagship species for European nature conservation, the genetic population structure of the brown bear (*Ursus arctos*) has been intensively studied throughout its western and central European range (Swenson et al. 2011). In Italy, for example, extensive long-term genetic monitoring has been conducted since 2002 in order to monitor population status and provide data for optimised bear management (DeBarba et al. 2010). In contrast, and only relatively recently, has research begun to focus on the genetic composition and structure of Eastern Europe bear populations (e.g. Romania, Straka et al. 2012; Croatia, Kocijan et al. 2011; Macedonia, Karamanlidis et al. 2013; Greece Karamanlidis et al. 2012; Caucasus, Murtskhvaladze et al. 2010; Slovakia, Graban et al. 2013; Serbia, Karamanlidis et al. 2014). This delay in rigorous scientific assessments of eastern European populations is somewhat surprising, considering that some of these bear populations number still above estimations for minimum viable population sizes and thus are of primary conservation relevance for the long-term preservation of the species in the remainder Europe. Given this and because genetic diversity is generally higher in eastern than in western bear populations (Swenson et al. 2011), the eastern populations of this species serve as valuable source populations for potential active future recolonisation (e.g., see Gutleb 1998) as well as donor regions for anthropogenic translocations (Kruckenhauser et al. 2009) of the largely bear-free western part of the continent.

* Corresponding author at: Conservation Genetics Group, Senckenberg Research Institute and Natural History Museum Frankfurt, Clamecystrasse 12, 63571 Gelnhausen, Germany. Tel.: +49 6051 61954 3138; fax: +49 6051 6195 43118.
E-mail address: cfrosch@senckenberg.de (C. Frosch).

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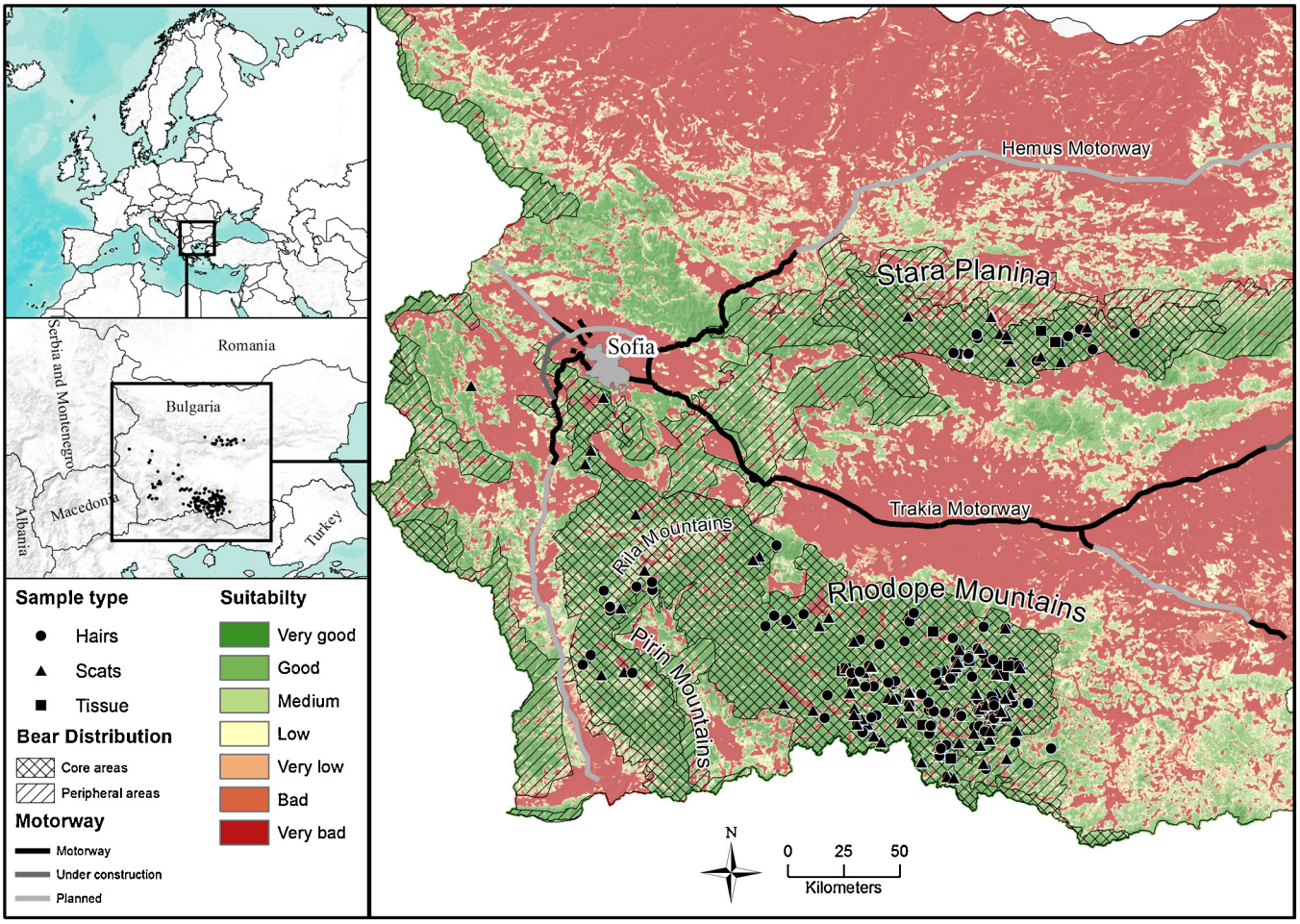

Fig. 1. Overview of sampling. A total of 368 samples were collected. Dots indicate hair samples ($n = 189$), triangles indicate scat samples ($n = 163$) and squares indicate tissue samples ($n = 16$). Colours from green to red indicate the degree of habitat suitability for brown bears (see Zlatanova et al. 2009). Lines in the main map show motorways (Kaphegyi et al. 2013), raster grid displays the current bear distribution (Action Plan for the Brown Bear in Bulgaria, 2008). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Bulgaria is located centrally within the south-eastern European bear range and still comprises large areas of near-natural montane habitat, including non-fragmented areas suitable for species such as the brown bear (Fig. 1). Additionally, anthropengic impacts in Bulgaria are relatively low due to the small populations size (66 inhabitants/km^2) and low level of infrastructure (road density of 0.293 km/km^2) (Kaphegyi et al. 2013). Perhaps due to these reasons, Bulgaria has one of the largest brown bear populations in the Balkan region, estimated at between 600 and 800 individuals (Kaphegyi et al. 2013). The population is restricted to mountainous areas, such as the Central Balkan Mountains (Stara Planina) and the Rilo-Rhodopean massif (Fig. 1). The amount of gene flow between the two Bulgarian mountain ranges and other adjacent bear regions in Greece, Serbia or Romania is virtually unknown, but at least the connection between the Bulgarian populations is under immediate threat due to increasing human pressure in the area with only few potential migration corridors (Zlatanova et al. 2009).

In this study we present the first genetic assessment of brown bear populations from the Bulgarian region of the Balkan Peninsula. We assessed population structure, genetic diversity, census size and migration routes within Bulgarian brown bear populations. As the presence of two highly divergent mitochondrial brown bear lineages is known to occur in Europe, with contact zones in nearby regions such as Romania (Zachos et al. 2008; Straka et al. 2012), we aimed to assess if this phylogeographic dichotomy also extends to Bulgaria. For this, we sampled hair, scat and tissue material from different Bulgarian regions and genotyped them with microsatellites and mitochondrial control region haplotype analysis. In particular we aimed to answer the following questions:

1. Are brown bears in Bulgaria regionally isolated or does gene flow occur among different mountain ranges, suggesting the existence of effective dispersal corridors?
2. Do genetic data support the official census population size estimation of brown bears in Bulgaria?
3. Is the degree of genetic diversity in Bulgarian brown bears comparable to other bear populations in Eastern Europe or is there evidence for genetic erosion?
4. Do both European brown bear mitochondrial lineages occur in Bulgaria and is there a geographic separation between these lineages?

Materials and methods

Sampling and DNA extraction

A total of 355 samples were collected between 2009 and 2012, and 13 samples were collected between 2004 and 2008 (Fig. 1). We analysed 16 tissue samples obtained from dead animals, legally killed following the derogation rules of EU directive 92/43 and animals trapped for GPS collaring, 189 non-invasively collected hair samples from barbed wire traps, scratch trees and fences, and

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163 scat samples. While usually bear scats and hair samples are quite well distinguishable from other species, there might be misidentifications, e.g., if only few hairs from the underfur were sampled from a hair trap. In order to proof the potential occurrence of bears in corridor regions sample material of unclear source was occasionally collected as well. Tissue and scat samples were stored in 96% ethanol. Hair samples were stored dark in filter paper with silica gel packs at room temperature. For DNA extraction from tissue we used the Qiagen Blood and Tissue Kit following the manufacturer's instructions, measured DNA concentration using a spectrometer (NanoDrop1000; Thermo Scientific) and diluted DNA to 6.5 ng/μl for further analyses. For DNA extraction from hair samples we cut the roots from 5 to 15 hairs and processed these with the Qiagen Investigator Kit as per manufacturer's instructions. The QIAamp DNA Stool Kit was used to extract DNA from faecal samples. We included negative controls in extraction process to check for cross-contamination during this step. The analyses of hair and scat samples were performed in a laboratory dedicated to the handling of noninvasive and forensic DNA samples following standard routines for avoidance of contamination (Taberlet et al. 1999).

Analysis of mitochondrial haplotypes

We amplified and sequenced a ~370–800 base pair (bp; depending on the respective species) part of the hypervariable left domain of the mitochondrial control region using the oligonucleotides L15995 (5′-CTCCACTATCAGCACCCAAAG-3′; Taberlet and Bouvet 1994) and H16498 (5′-CCTGAAGTAAGAACCAGATG-3′; Fumagalli et al. 1997) as described in Nowak et al. (2014a,b) for species determination and brown bear haplotype assignment. Sequences were aligned with ClustalW 1.83 (Thompson et al. 1994) in MEGA 5.10 (Kumar et al. 2004). Basic sequence analyses and calculation of pairwise genetic distances were also performed in MEGA 5.10. For further analyses we optimised the alignment manually, cut the fragment to a widely used 248 bp long fragment and excluded a ~20 base pair TC-stretch starting at site 75 (e.g., Taberlet and Bouvet 1994). Haplotype and nucleotide diversity were analysed using the DnaSP 5.10 software package (Librado and Rozas 2009). Further, a phylogenetic network was constructed in TCS 1.21 (Clement et al. 2000) by statistical parsimony with default settings. Sequences of new haplotypes were deposited in GenBank (KJ638591–KJ638597).

Microsatellite genotyping

For genetic profiling we used 13 microsatellite loci (Msut2, Kitahara et al. 2000; G10C, G10P, G10D, G10L, Paetkau et al. 1995; G10H, G10J, G10U, Paetkau and Strobeck 1994; UarMU26, G1A Taberlet et al. 1997; Mu10, Mu23, Mu51, Bellemain and Taberlet 2004) in three multiplexes and four PCR replicates for hair and scat samples. We also tested the microsatellites Mu51, Mu59, Mu23 (Bellemain and Taberlet 2004); CXX110 (Ostrander et al. 1995) and G10M (Paetkau et al. 1995) but did not use them in the final analyses because of general unreliable amplification or poor amplification in PCR multiplex reactions. PCR conditions were similar as described in Frosch et al. (2011). Microsatellite analyses were conducted on a 3730 DNA Analyzer (Applied Biosystems) with subsequent determination of fragment lengths using Genemarker 1.6 software (SoftGenetics). After replicated genotyping the consensus genotype was constructed by hand if at least two of the four replicates matched. Error rates were estimated using Gimlet 1.3.3 (Valière 2002) including the consensus genotypes in the input file.

Sex identification

For sex identification from noninvasively collected material we used the Y chromosome-specific fragments SMCY and 318.2 as well as the X chromosome-specific fragment ZFX as described in Bidon et al. (2013). Reactions were performed in a single multiplex with three replicates per sample.

Individual assignment and relatedness estimation

The individualisation of brown bears from the complete data set was performed using the program Coancestry 1.0.1.1 (Wang 2011). To determine which relatedness estimator is the best for our type of data, we used the observed allele frequencies of our microsatellite data and simulated 500 dyads of brown bears of varying relatedness coefficients (unrelated $r = 0$, monozygotic twins $r = 1$, parent–offspring $r = 0.5$, fullsibs $r = 0.5$, halfsibs $r = 0.25$ and first cousins $r = 0.125$). Based on this simulation we found the triadic likelihood estimator (TioML) described in Wang (2007) and the dyadic likelihood estimator (DyadML; Milligan 2003) to perform best. These produced a strong correlation with expected values of r ($R^2 = 0.91$ and 0.9). All pairwise relatedness values of the analysed bear samples were obtained from Coancestry with standard settings to construct a genetic relatedness matrix. We checked all sample combinations with $r > 0.6$ manually and involved sex and mitochondrial haplotype information for individualisation. The unbiased (PID_{unb}) and the expected (PID_{sibs}) probability-of-identity among full sib dyads (Mills et al. 2000) was calculated using the software GenAlex 6.41(Peakall and Smouse 2006; http://www.anu.edu.au/BoZo/GenAlEx).

To analyse kinship we calculated the pairwise relatedness between individual bears using the software ML-Relate (Kalinowski et al. 2006a, http://www.montana.edu/kalinowski/Software/MLRelate.htm) with 50,000 randomisations. Expected and observed mismatch (MM) values were computed using MMdist (Kalinowski et al. 2006b).

Genetic diversity

GenAlex 6.41 was used to estimate allele frequencies by locus and population, mean number of alleles per locus (N_A) and the probability of identity (PID_{unb} and PID_{sib}) for the complete data set. Wright's inbreeding estimator F_{IS} (Weir and Cockerham 1984), observed (H_O) and expected unbiased (H_e) heterozygosity and departures from Hardy–Weinberg Equilibrium (HWE) were computed for the complete population using Genetix4.05 (Belkhir et al. 1996; http://www.genetix.univmontp2.fr/genetix/genetix.htm).

Population admixture analysis

For inferring the population structure and assigning individuals to populations, we used a bayesian model-based clustering method implemented in Structure version 2.3.3 (Falush et al. 2007). We used the admixture model with correlated allele frequencies and ran the software for 1,000,000 steps of which the first 200,000 were discarded as burn-in. We tested a range of K from 1 to 15 (10 replicates for each K) for the complete data set. The presence of null alleles was estimated simultaneously, using the option 'RECESSIVE ALLELES = 1′ as described in Senn and Pemberton (2009). The most likely number of clusters was then inferred using Evanno et al.'s (2005) method in Structure Harvester(Earl and vonHoldt 2012; http://taylor0.biology.ucla.edu/structureHarvester/).

Population size estimation

We used Capwire (Miller et al. 2005) for estimation of total population size (N_c) based on the identified genotypes. The estimation is based on the number of resampling the genetically distinct individuals at multiple sampling occasions. Due to unequal capture probability of different individuals determined by the test in our

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Table 1
Number of collected samples, number of analysed samples with mtDNA of *Ursus* including percentage of all samples, number of successful genotyped samples and percentage of all samples as well as percentage of all *Ursus* samples. Number of failed samples including percentage of all analysed samples and number of non-bear samples and percentage of all analysed samples.

	n tissue	*n* hair	*n* scat	*n* all
Collected samples	16	189	163	368
Ursus (mtDNA)	16	145	138	299
Successfully genotyped	16	126	108	250
Failed	–	14	12	26
Other species	–	30	13	43
Bos taurus	–	9	1	10
Canis lupus	–	4	2	6
Capreolus capreolus	–	1	–	1
Equus caballus	–	6	1	7
Homo sapiens	–	2	2	4
Meles meles	–	1	–	1
Ovis aries	–	1	–	1
Rupicapra rupicapra	–	1	–	1
Sus scrofa	–	3	3	6
Vulpes vulpes	–	1	4	5
Cervus elaphus	–	1	0	1

sample ($\alpha = 7.8$), the TIRM Model was selected. Calculation of N_c was done only for samples collected in 2009 and 2010 to ensure that they represent the current population size.

Long distance dispersal

A least cost analysis was performed in ArcGis 10.1 (Cost Path Function of Spatial Analyst) for each of the two bears with proven use of the corridor through Sredna Gora Mountain (Zlatanova et al. 2009). The least cost path was developed based on cost matrix built with the Weighted Sum Function from the habitat suitability model (Zlatanova et al. 2009), with weight 0.6 and slope usage by the bears in Bulgaria (derived from telemetry data), with weight 0.4 (Kaphegyi et al. 2013).

Results

Success of analyses and error rates

All 16 tissue samples yielded reliable results for mitochondrial DNA (mtDNA) analysis and microsatellites. In 80.4% of the analysed noninvasive samples we found bear DNA (based on mitochondrial haplotyping). We were able to obtain bear DNA from 76.2% of all hair samples whereas from 7.4% of all hair samples we did not gain evaluable sequence data (due to low DNA quality or contamination). For 15.9% of the samples, mtDNA analyses clearly revealed that hairs did not originate from brown bears but from a variety of wild or domestic mammals (Table 1). For scat samples, 84.7% were brown bear and 7.4% failed, showed ambiguous bases or signs of contamination. In 7.4% DNA from scat samples resulted in the detection of other species (Table 1).

Microsatellite analyses were performed with all 299 samples showing bear DNA in the mitochondrial sequence analyses. We accepted genetic fingerprints when at least eight out of thirteen microsatellites could be scored in the consensus genotype. The majority of all samples provided microsatellite genotypes for all 13 loci and 94% of all samples resulted in ten or more successfully amplified microsatellites (13 = 62%; 12 = 19.6%; 11 = 8.8%; 10 = 4%; 9 = 2.4%; 8 = 3.2%). We detected an overall PCR success of 87.7%, with 11% allelic dropout errors and 1.1% of false alleles.

Distribution of mitochondrial lineages

For all successfully identified individuals ($n = 136$) the cut 248 bp fragment of the mitochondrial control region was available. We observed five haplotypes in 88 individuals, belonging to the western lineage and six haplotypes from 48 individuals from the eastern lineage as defined in Taberlet and Bouvet (1994; Fig. 2). Two individuals from the western lineage and one individual from the eastern lineage showed ambiguous bases and were thus excluded from further analyses of mitochondrial population structure. Both lineages could be easily differentiated with an average pairwise genetic distance of 7.1% calculated in MEGA 5.10 (Kumar et al. 2004). The genetic pairwise distance within the western and eastern lineages was ~1.6% and 0.7%, respectively. Haplotype diversity for the complete data set was 0.769 ± 0.026 (Hd ± s.d.), for the western lineage 0.6 ± 0.054 and 0.488 ± 0.00614 for the eastern lineage. Statistical analyses of the nucleotide diversity resulted in 0.03289 ± 0.00133 ($\pi \pm$ s.d.) for all successfully sequenced samples, 0.01077 ± 0.00228 within in the western lineage and 0.00219 ± 0.00042 in the eastern lineage. The minimum spanning network of the 11 haplotypes yielded two distinct groups that could not be connected under the 95% probability criterion used by TCS (e.g., Zachos et al. 2008).

Individual assignment, kinship and genetic diversity

All microsatellite loci were polymorphic in the entire data set with number of alleles per locus ranging from 6 (Msut-2) to 14 (G10U) (mean = 8.9 alleles per locus) (Table 2). Mean observed heterozygosity (H_O) and expected heterozygosity (H_e) across all samples was 0.64 and 0.74, respectively. Nine markers showed significant deviations from HWE calculated in Genetix4.05 (Belkhir et al. 1996). All F_{IS} values were positive, indicating a deficit of heterozygous individuals in the region. Markers Uar-Mu-26 and G10U showed high values of F_{IS} (0.45 and 0.36). For these markers relatively high proportions of null alleles were estimated (both 0.16) using Structure version 2.3.3. However, removing these two loci from all performed analyses did not alter our results or conclusions. The cumulative probability-of-identity with 13 loci was $PID_{unb} = 2.4 \times 10^{-14}$, and $PID_{sib} = 7.4 \times 10^{-6}$. The estimated expected fraction of individuals sharing an identical genotype (calculated as the PID x population size) was 3.2×10^{-12} (PID_{unb}) and 10^{-3} for a population composed of full-sibs (PID_{sib}). Based on the pairwise relatedness values calculated in Coancestry, we manually checked all samples and identified136 brown bear individuals in our data set. Sex identification resulted in 58 females (42.7%), 73 males (53.7%) and five unclear assignments (3.7%). The maximal straight distance between samples of the same individual was calculated for 14 recaptured males ranging from 4.6 to 146 km (mean 35.7 km, median 19.2 km) and for 12 recaptured females ranging from 1.4 to 76 km (mean 17.9 km, median 6.1 km).

Results of kinship analysis implemented in ML-Relate and MM-Dist suggested that the sample set is composed of related individuals. For all possible 9180 combinations of individuals ($136 \times 135/2$) we found 85.6% as not related, but 12.6% off all combinations showed possible halfsibs, 1.2% fullsibs and 0.7% parent offspring. However, mismatch distributions among family classes overlapped considerably, suggesting a high level of uncertainty in assignments of relatedness in our dataset (Fig. 3).

Population differentiation and estimation of population size

The most likely number of genetic clusters calculated with Structure Harvester was $K = 7$, but Delta K was also increased for $K = 2$ and $K = 5$ (Fig. 4). Most of the individuals could be clearly assigned with high posterior probability (>0.8) to one of the

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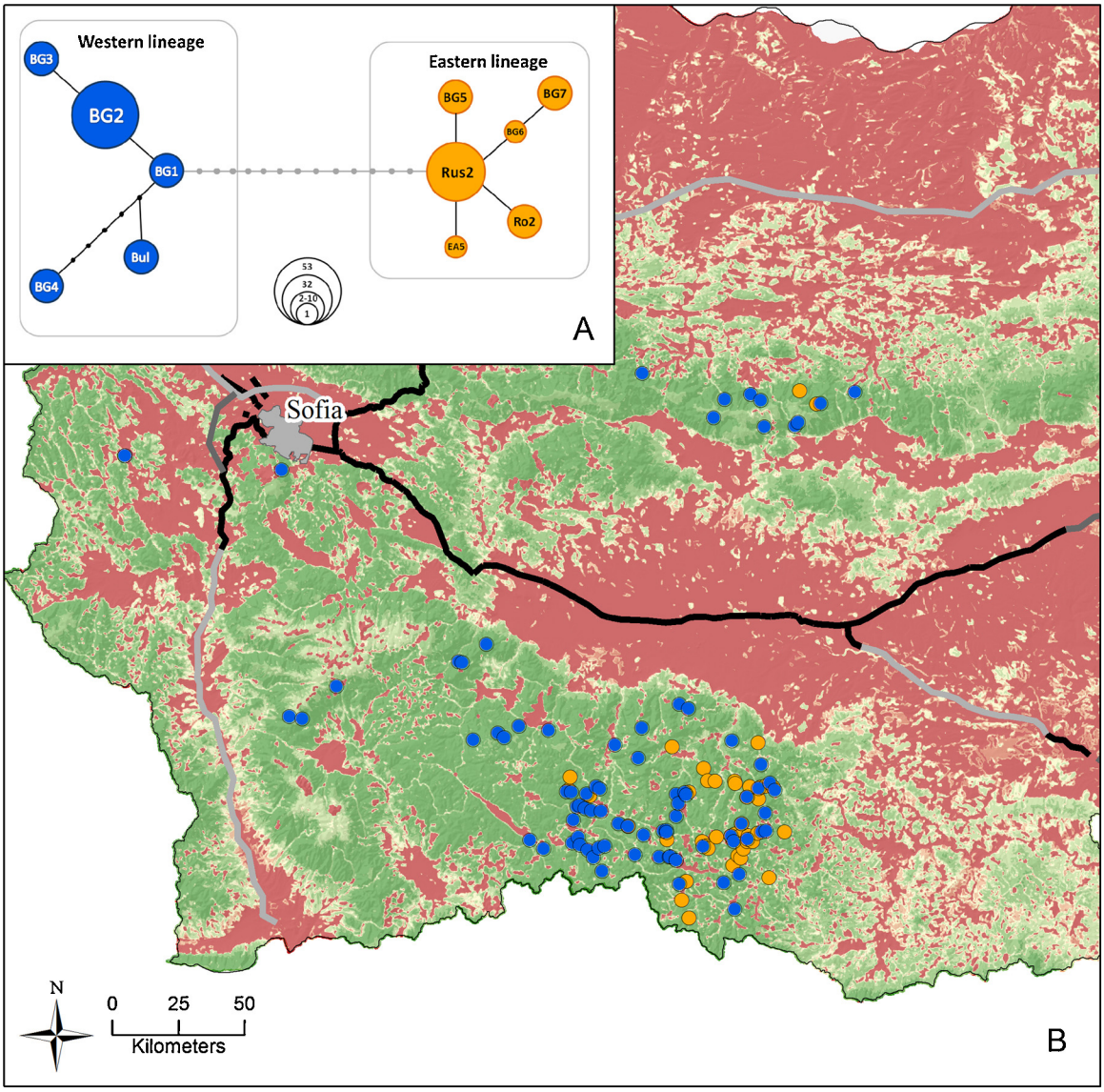

Fig. 2. (A) TCS network based on a 248 bp fragment of the mitochondrial control region, representing five haplotypes from the western lineage and six haplotypes from the eastern lineage. Four haplotypes have been described before: Bul from Bulgaria (Genbank accession number X75864), Ro2 from Romania (X75873), Rus2 from Russia (X75876) and EA5 from Europe (EU526769). Haplotypes BG1-BG7 are firstly described in this study (KJ638591–KJ638597). Grey lines show connection that could not be calculated under the 95% probability criterion in TCS. Size of circles indicate sample size. (B) Map of Bulgaria. Dots show individuals with a western (blue) or eastern (orange) mitochondrial haplotype. Map colouration indicates the habitat suitability for brown bears in Bulgaria, as described in Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Table 2
Name of locus, multiplex PCR reaction (MP), number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_e), deviations from Hardy–Weinberg equilibrium (P; ns, not significant; *significant, $P < 0.05$), inbreeding coefficient (F_{IS}), values of null alleles ($Null_S$), cumulative values of unbiased (PID_{unb}) and sibling (PID_{sib}) probability-of-identity, and percentage values of allelic dropout (D) and false alleles (fA).

Locus	MP	N_A	H_O	H_e	P	F_{IS}	$Null_S$	PID_{unb}	PID_{sib}	D	fA
Mu23	A	8	0.63	0.69	ns	0.10	0.05	0.12	0.44	10.73	1.07
Msut-2	A	6	0.71	0.73	ns	0.03	0.02	0.11	0.41	5.37	0.93
G10C	A	9	0.80	0.84	ns	0.05	0.04	0.04	0.34	7.47	0.00
UarMu-26	A	7	0.25	0.46	*	0.45	0.16	0.32	0.60	13.97	0.73
G1A	A	7	0.60	0.64	*	0.06	0.04	0.19	0.48	7.70	0.77
Mu51	B	7	0.67	0.78	*	0.14	0.06	0.09	0.38	8.47	1.83
G10P	B	9	0.65	0.76	*	0.15	0.08	0.08	0.39	13.73	1.50
G1D	B	8	0.78	0.82	*	0.06	0.04	0.05	0.35	13.90	0.00
Mu10	C	9	0.66	0.66	ns	0.01	0.02	0.14	0.45	11.10	0.77
G10U	C	14	0.57	0.87	*	0.36	0.16	0.03	0.32	16.93	1.10
G10J	C	10	0.65	0.77	*	0.15	0.06	0.08	0.38	8.50	3.37
G10H	C	13	0.69	0.82	*	0.16	0.07	0.05	0.35	14.70	0.20
G10L	C	9	0.65	0.73	*	0.11	0.06	0.11	0.41	10.97	2.57
Mean		8.92	0.64	0.74		0.14	0.07	0.11	0.41	11.04	1.14

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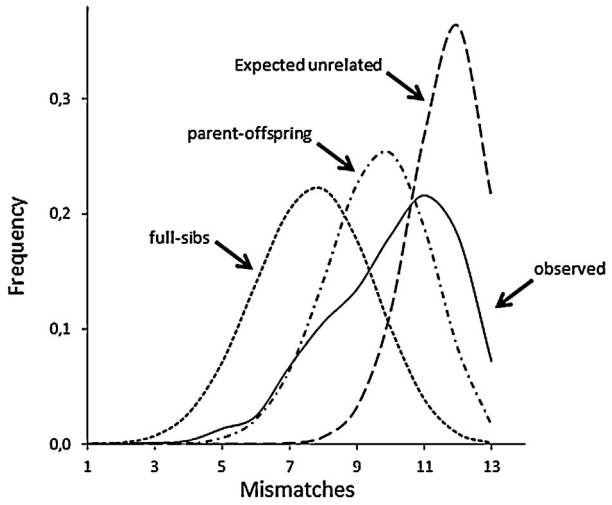

Fig. 3. Distribution of mismatches among genotypes of Bulgarian brown bears. Plotting of the proportions of expected and observed mismatch values in bear genotypes computed using the software MM-Dist (Kalinowski et al. 2006b).

clusters. For $K = 2$, 77.9% of all individuals were assigned to one of two clusters (106/136), 72.8% (106/136) to one of five clusters, and 51.5% (10/136) to one of seven clusters. Interestingly, all individuals from Stara Planina were assigned to one cluster for $K = 2$ and 9 of 11 individuals were assigned to one cluster for $K = 5$ and $K = 7$, respectively (Fig. 4). Individuals from Stara Planina never formed a separate cluster even in higher K models. The association between the two mtDNA lineages and nuclear clusters inferred from STRUCTURE ($K = 2$) analysis were calculated using a chi-square test. A significant association between mitochondrial and nuclear markers was observed ($p = 0.002$).

Samples of two male brown bears were found both in the Bulgarian part of the Rhodope Mountains and Stara Planina. From Male I, one scat sample was found in 06/2009 in the Rhodope Mountains and one scat sample was found in 09/2009 in Stara Planina. From Male II, four samples (hair = 1, scat = 3) were found between May and July in the Rhodope Mountains and two scat samples were found in September in Stara Planina. Both males bear the H1 haplotype of the western mitochondrial lineage (Fig. 2A) and were assigned to the Rhodopean population in STRUCTURE (purple; Fig. 4). The straight-line distance is 144 km and 146 km, respectively. Under consideration of the bear habitat suitability model the two long dispersal males covered a distance of 248 km and 270 km when using suitable habitat only.

The total population size (N_c) in the Bulgarian Rhodope Mountains analysed with the TIRM model in CAPWIRE was estimated to be 315 individuals (95% CI = 206–334).

Discussion

The occurrence of two major mitochondrial lineages in Europe – western and eastern – is well known for brown bears (Taberlet and Bouvet 1994). Nevertheless the origins of these lineages and how they formed are still under discussion (Swenson et al. 2011). The initial separation was primarily thought to correspond with different glacial refugia during the earlier Quaternary (Taberlet and Bouvet 1994), however recent analyses suggest that the timing of the split is inconsistent with this theory and may be much younger (70,000–250,000 years ago; Saarma et al, 2007). Analyses of ancient samples did not match the contemporary phylogeographical structure (Hofreiter et al. 2004), implying that population fragmentation may have been caused by human impact rather than glacial refugia (Valdiosera et al. 2007). Contact zones between eastern and western lineages are known from Scandinavia (Taberlet et al. 1995) and Romania (Zachos et al. 2008; Straka et al. 2012). Here, we detected this contact zone between the eastern and western mtDNA brown bear lineages in Bulgaria as well. Both lineages occur in the two major Bulgarian mountain ranges, Stara Planina and the Rhodope Mountains. We found numerous individuals bearing an eastern mitochondrial haplotype in the eastern part of the country, in the Smolyan region and in the south eastern Plovdiv region, and individuals carrying a western haplotype also often occurred in western regions (e.g. in the national parks of Rila and Pirin we observed only individuals bearing a western haplotype). In Stara Planina a larger number of samples are needed to clarify the spatial distribution patterns, but our restricted sampling indicates a similar pattern of a potential east–west separation. The observed nonrandom distribution of mtDNA lineages is likely due to philopatry of female bears which is known for stable or declining populations (Støn et al. 2006). This female philopatry might as well explain the significant correlation between the two mitochondrial lineages and the nuclear genetic differentiation. Within this study, the maximal straight line distance between samples of female individuals was on average 17.9 km ($N_{individuals} = 12$), compared to an average of 35.7 km in males ($N_{individuals} = 14$) including two male bears with long-distance dispersal from the Rhodope Mountains to Stara Planina. This considerable level of male-dominated dispersal does explain the overall weak spatial structuring of genetic variation in the study region based on microsatellite data.

We further observed a relatively consistent genetic clustering among different Ks and some degree of separation of samples from Stara Planina (Fig. 4A, green) as well as Rila and Pirin Mountains (red), whereas individual assignments from these clusters were also found in the Rhodope Mountains. This pattern may suggest there are few suitable corridors for bear migration between distant mountain regions. In our study we relied on an opportunistic sampling with unequal sample sizes in different areas, including heavy sampling in the Rhodope Mountains. All cluster assignments in the Rhodope Mountains were detected with only slight tendencies for geographic substructuring. The overall degree of relatedness between analysed bears using ML-RELATE and MM-DISTwas moderate. However, predominately mating with neighbours and the subsequent close relatedness of bears in densely sampled regions such as the Rhodope Mountains can result in our findings of spurious genetic clusters, especially in fine scale analyses (Schwartz and McKelvey, 2009). Although most Bulgarian bear individuals (51.5%) were clearly assigned to one cluster in STRUCTURE ($K = 7$; Fig. 4), we assume that these divisions are not exclusively due to biologically meaningful units. In general the interpretation of genetic cluster results can be challenging. Schwartz and McKelvey (2009), for example, tested different sample schemes using one continuous simulated population and found that when using a mixed sample approach, false, not meaningful, clusters were formed in their STRUCTURE analysis. Therefore, we conclude that the clustering in our study may be due to a combination of spatially unequal sampling, relatedness between individuals and isolation through landscape barriers (Rhodope Mountains – Stara Platina). We thus urge not to overinterpret the separations found in this study, for example by treating them as separate management units or using them for specific conservation decisions. Our study found substantial evidence for gene flow between regions and even between the two largely separated major mountain ranges of Bulgaria, which, in combination with the apparent mitochondrial lineage separation, provides evidence for a pattern of male triggered gene flow and female philopatry as observed previously for other bear populations (Straka et al. 2012; Taberlet et al. 1995; Støn et al. 2006). Swenson et al. (2000) defined a bear subpopulation as populations in nearby, but geographically isolated areas with male-mediated interchange, but without interchange of females. Based on our

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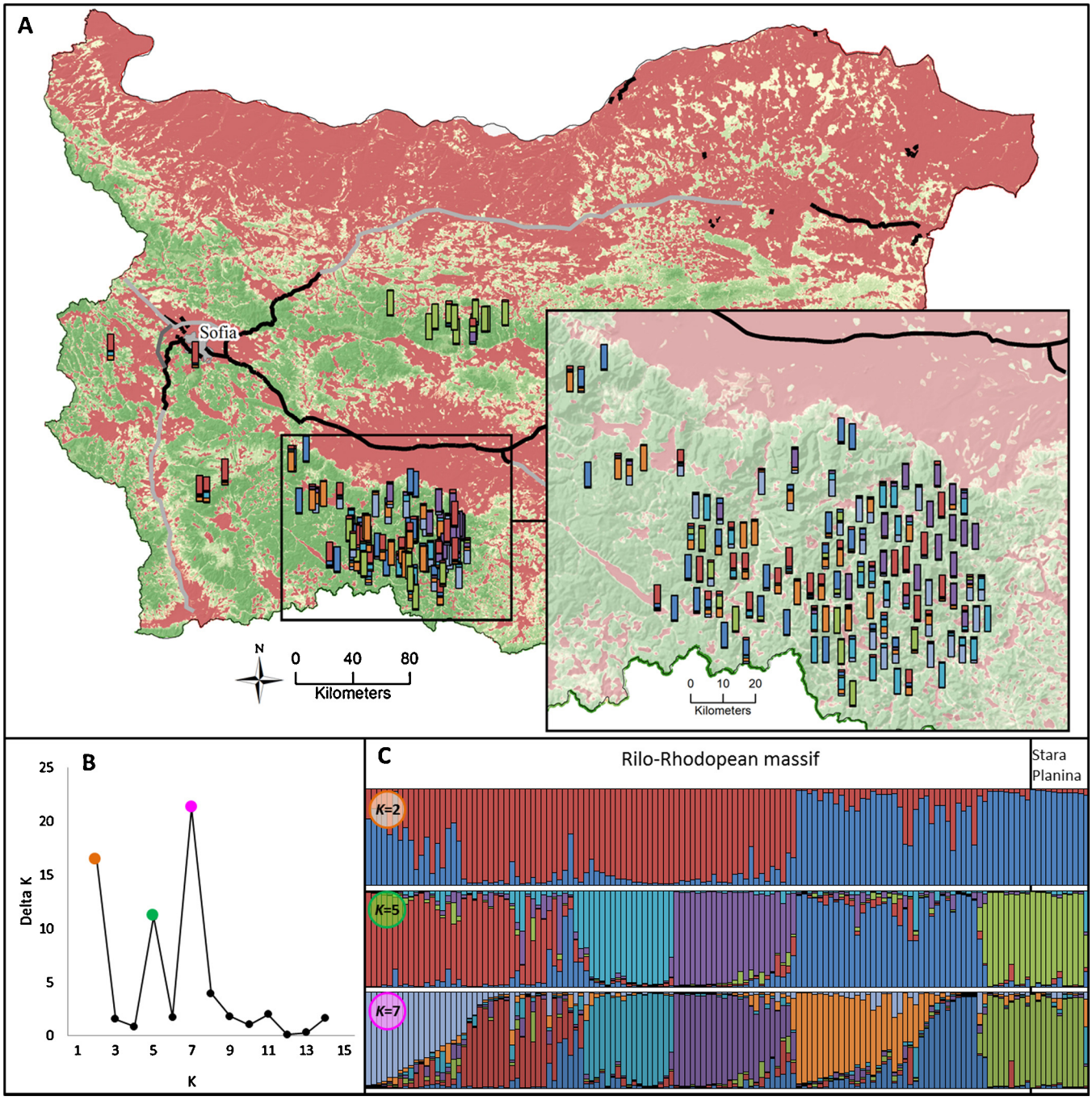

Fig. 4. Genetic population structure in Bulgarian brown bears. (A) Map of Bulgaria; every bar symbolises one individual with Structure model $K = 7$. (B) Evanno et al. (2005) plot obtained from Structure Harvester ΔK calculated as $\Delta K = m|L''(K)|/s[L(K)]$. (C) Population structure inferred by Structure using the 'admixture' model for $K = 2$, $K = 5$ and $K = 7$. Vertical lines represent brown bear individuals, colours represent individual assignment probabilities to genetic clusters revealed from Structure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

observation we thus conclude that Bulgaria harbours two subpopulations, the Stara Planina subpopulation and the subpopulation in the Rhodope Mountains. Further monitoring can reveal if the corridors were however sporadically used by females. Although we observed only a low degree of population substructure, we found significant differences between observed and the expected heterozygosity ($H_O = 0.64$; $H_e = 0.74$), most probably due to relatedness of bears in some regions and the weak spatial substructure among regions. We observed similar deviations from HWE if we tested individuals from the Rhodope Mountains and the Stara Planina Mountains separately. Interestingly, we observed a higher number of alleles (mean $N_A = 8.9$) than recorded for most other European studies. An overlapping marker set of eight microsatellites was analysed in our study and in the Romanian Carpathian Mountains (Straka et al. 2012), showing mean $N_A = 8.6$ in our study and $N_A = 7.45$ in Romania. Documented translocation of Romanian bears to different locations in Bulgaria (Nowak et al., 2014b) may have additional significant impact on genetic diversity measures. When excluding genotypes with clear Romanian introgression ($n = 7$, see (Nowak et al., 2014b) the mean number of alleles slightly decreases to $N_A = 8.77$ and the expected heterozygosity decreases to $H_e = 0.71$ while the observed heterozygosity of $H_e = 0.64$ does not change,

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indicating that potentially occurring admixture of Bulgarian bears with Romanian genotypes did have no major effect on genetic diversity in the region.

Generally, the nuclear genetic diversity of European brown bears is positively correlated with population size (Swenson et al. 2011). In Europe, high expected heterozygosity levels are found in Romania, Northern Finland and Russia (0.79–0.82; Straka et al. 2012; Zachos et al. 2008; Tammeleht et al. 2010). In areas with more fragmented habitats, such as Southeastern and Central Europe, slightly lower values of 0.61–0.77 were reported (Alps-Dinara-Pindos population (Croatia: Kocijan et al. 2011; Serbia: Karamanlidis et al. 2014; Slovenia: Skrbinšek et al. 2012; Greece: Karamanlidis et al., 2010); Austria (Kruckenhauser et al. 2009), Estonia (Waits et al. 2000) and Slovakia (Straka et al. 2012). In contrast, low values of H_e ranging from 0.25 to 0.45 were found in southwestern Europe (Spain, Italy, France; Pérez et al. 2009, 2010; Lorenzini et al. 2004; Taberlet et al. 1997). In comparison, H_e in this study is comparable to other large populations from the Balkan peninsula.

Approximately one-fourth of Bulgaria is known to be suitable brown bear territory (28,268 km^2) whereas less than half that (11,000 km^2) is currently occupied by brown bears (Action Plan for the Brown Bear in Bulgaria, 2008). However, the reported population size estimation for Bulgaria of 600–800 individuals is only a rough estimation and our finding of 315 (95% CI = 206–334) individuals based on noninvasively collected sample material in the Bulgarian part of the Rhodope Mountains is not a reliable basis for management decisions. According to the Bulgarian habitat suitability model, the carrying capacity of Rhodope Mountains alone is between 430 and 530 individuals (Zlatanova 2010). Sampling in this study was mainly opportunistic and geographically biased. In addition, non-resident individuals like migrants from Greece or long-distance dispersers from other mountain ranges, as well as cubs, were included in the calculations. In future, area-wide sampling of genetic material as well as sampling across state borders and a standardised capture-recapture design may help to improve the data set and provide a more precise population size estimation. In general Bulgaria offers large areas of suitable bear habitat with the potential for increasing bear population sizes. However, the ongoing expansion of humans and the increase of anthropogenic factors like road systems (Kaphegyi et al. 2013) influences the coexistence of humans and bears. Noninvasively collected sample material of two male brown bear individuals was found in both large regions, namely the Stara Planina and Rilo-Rhodopean massif. This provided, for the first time, direct evidence that narrow corridors exist (Zlatanova et al. 2009), are currently used by bears, and connect the two main population segments in Bulgaria. These potential migration corridors between Central Balkan and the Rilo-Rhodopean massif are rather narrow and most likely result in a low exchange rate. Moreover, the existing Trakia motorway is poorly equipped with animal crossing infrastructures and forms a significant barrier which additionally prevents the exchange between the two population segments (Kaphegyi et al. 2013). Thus, there is a need of multiple animal crossing structures over the motorway to preserve the existing genetic exchange between the two areas. The proper management of this stepping-stone area should play a significant role in further land use management. Transnational network and a combination of all recent genetic studies of the Balkan region is crucial to optimise the management and therefore ensure long term survival of species such as the brown bear.

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