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ORIGINAL PAPER

Genetic diversity, structure, and size of an endangered brown bear population threatened by highway construction in the Pindos Mountains, Greece

Alexandros A. Karamanlidis · Martin Straka · Elena Drosopoulou · Miguel de Gabriel Hernando · Ivna Kocijan · Ladislav Paule · Zacharias Scouras

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Abstract One of the major negative effects of roads is the creation of barriers to the movement of wildlife, ultimately disconnecting populations and increasing extinction risk. We collected genetic data from a threatened brown bear population in the central part of the Pindos mountain range in northwestern Greece to provide information about this, as yet genetically undescribed, population and to evaluate its status prior to the construction of a major highway. We used noninvasive genetic sampling methods and microsatellite analysis to investigate nuclear genetic diversity, population genetic structure, demographic history, relatedness within the population and estimated effective and total population size. Brown bears in the study area were found to possess a relatively high level of nuclear genetic diversity and low levels of inbreeding; the population did not show any signs of substructuring but seems to have gone through a genetic bottleneck in the recent past. The estimated effective population size was 29, and the total population size estimate obtained by two different methods was 33 and 51 individuals, respectively. Our results indicate a good conservation status of this bear population and provide baseline genetic data for the future evaluation of the effects on bears from the construction of a major highway, for monitoring the genetic status of this and other bear populations in Greece and for assessing gene flow in bear populations in southern Europe.

Keywords Conservation · Genetic status · Greece · Highway construction · Noninvasive genetic monitoring · *Ursus arctos*

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A. A. Karamanlidis (✉) · M. de Gabriel Hernando
ARCTUROS, Civil Society for the Protection and Management of Wildlife and the Natural Environment,
Roggoti Str. 3,
GR-54624 Thessaloniki, Greece
e-mail: akaramanlidis@gmail.com

A. A. Karamanlidis
Department of Ecology and Natural Resource Management,
Norwegian University of Life Sciences,
NO-1432 Ås, Norway

M. Straka · L. Paule
Department of Phytology, Faculty of Forestry, Technical University in Zvolen,
SK-96053 Zvolen, Slovakia

E. Drosopoulou · Z. Scouras
Department of Genetics, Development and Molecular Biology, School of Biology, Faculty of Sciences,
Aristotle University of Thessaloniki,
GR-54124 Thessaloniki, Greece

I. Kocijan
Division of Biology, Faculty of Science, University of Zagreb,
Rooseveltov Trg 6,
HR-10000 Zagreb, Croatia

Introduction

Current rates of species extinction have exceeded by far the normal background rates (Pimm et al. 1995; Barnosky et al. 2011) and therefore conservationists are constantly in search of ways to monitor the effects of human activity and identify the threats to global biodiversity in order to take effective protection measures. In an increasingly developing world, there have been growing concerns over the ecological effects of transportation infrastructures (e.g., roads,

railroads, canals) on wildlife (Forman et al. 2003). To thoroughly understand these effects, one should obtain and compare information from before and after the onset of a specific threat (Wandeler et al. 2007). While collecting samples from after the onset is relatively easy, it is not always so straightforward to obtain historic samples and where they do exist, the ages of the specimens may postdate the time period of interest (Matocq and Villablanca 2001). Few studies have managed to collect data on levels of genetic diversity prior to the construction of a major highway as a reference point for comparison with levels of genetic diversity in the future, after the construction of the highway (Balkenhol and Waits 2009). Difficulties in understanding the ecological processes associated to the operation of major highways are compounded when trying to study the effects on species, such as large carnivores, that have low population densities, large home ranges, and are often in direct conflict with humans and are therefore elusive (Karanth and Chellam 2009).

The brown bear (*Ursus arctos*) is one of only three large terrestrial carnivores surviving in highly industrialized Europe (Linnell et al. 2001), where human persecution and habitat loss have led to the disappearance of the species from large parts of its original range (Zedrosser et al. 2001). Despite increasing threats to the survival of bears on the European continent, basic biological and population parameters still remain largely unknown for some European bear populations (Swenson et al. 2011). This is particularly true for bear populations in the southern Balkans, including the bear populations in the Pindos mountain range in Greece, which are considered to be endangered, not only because of their small sizes but also due to the lack of systematic and coordinated efforts to study them (Zedrosser et al. 2001).

Brown bears in the Pindos mountain range belong to the large Alps-Dinaric-Pindos bear population, estimated at 2,800 individuals (Zedrosser et al. 2001). The species in Greece is considered threatened, and the total Pindos population is currently estimated to number 160–230 individuals (Mertzanis et al. 2009). The distribution of the species in this part of the country is continuous. Despite research and conservation efforts dating back to the 1990s, the Pindos bear population is characterized by a paucity of information regarding basic biological parameters, while available population estimates should be considered mere educated guesses. Bears in Greece are fully protected but illegal killing, habitat loss, and fragmentation threaten the survival of the species (Mertzanis et al. 2009). In addition to habitat loss and fragmentation, a new threat to the species has emerged in recent years in Greece through the rapid expansion of the national transportation network and the construction and operation of high-volume, high-speed motorways (Karamanlidis and Georgiadis 2009). One of the most imminent threats is considered to be the construction and operation of the "Egnatia" highway, which dissects the core area of brown bear distribution in the central part of the Pindos mountain range in northwestern Greece (Karamanlidis and Mertzanis 2003).

High-volume, high-speed motorways can have numerous negative effects on wildlife (Coffin 2007; Shepard et al. 2007; Balkenhol and Waits 2009; Holderegger and Di Giulio 2010) and bear populations, in particular, including increased mortality and habitat loss (Wooding and Maddrey 1994), changes in behaviour and activity patterns (Brody and Pelton 1989; Kasworm and Manley 1990; Brandenburg 1996; Proctor et al. 2002; Waller and Servheen 2005), and population fragmentation (Proctor et al. 2005). Because bears reproduce slowly, occur in low densities and have large home ranges, highways that are impermeable to bear movements can reduce genetic interchange and compromise population persistence (McCown et al. 2009).

The aim of the study was to assess the status of a brown bear population which inhabits the central part of the Pindos mountain range in Greece, prior to the construction of the "Egnatia" highway. We investigated nuclear genetic diversity, population genetic structure, demographic history, relatedness within the population, and effective and total population size using noninvasive genetic sampling methods and microsatellite analysis. Noninvasive genetic monitoring has been recognized as a sensitive, reliable, and time- and cost-efficient tool for studying rare, elusive, and often endangered animals, such as bears (Beja-Pereira et al. 2009; Pérez et al. 2009; de Barba et al. 2010; Karamanlidis et al. 2010b; Straka et al. 2011; Swenson et al. 2011), and has been used recently to evaluate the effects of habitat fragmentation and transportation infrastructure (Simmons et al. 2010), also on ursid populations (Proctor et al. 2005; Dixon et al. 2006, 2007). Considering that bear habitat in the central part of the Pindos mountain range has remained uninterrupted until recently, we expected to find preserved nuclear genetic diversity and no evident population structure. At the same time, we hypothesized that the population may have gone through a genetic bottleneck, as did many other brown bear populations in Europe that share a common history of habitat loss and hunting campaigns (Swenson et al. 2011). This study used for the first time genetic tools in the monitoring of a bear population in Greece; hence, the results of the study will have direct implications for the management and conservation of the species in Greece, because they will be used as baseline data for the future evaluation of the effects of the "Egnatia" highway on the Pindos bear population and for monitoring other populations of the species in the country. Moreover, the results of the study will substantiate the genetic information necessary for the effective monitoring and conservation of brown bears in Europe (reviewed in Swenson et al. 2011).

Materials and methods

Study area

The study area encompassed 850 km^2 in the Prefecture of Grevena, in the central part of the Pindos mountain range in northwestern Greece (Fig. 1). Major forest vegetation types consist of oak (*Quercus* sp.) and black pine (*Pinus nigra*); the study area is located at the centre of the western nucleus of the distribution of brown bears in Greece (Mertzanis 1994) and is currently crossed by the "Egnatia" highway, a four-lane, fenced motorway, from northeast to southwest (Fig. 1). To meet environmental requirements and mitigate potential negative impacts on brown bear habitat, the construction company Egnatia Odos, in cooperation with the nongovernmental organization ARCTUROS and foreign experts, designed and implemented a special road alignment that includes dual-carriage way bridges, twin-tube tunnels and wildlife underpasses (Egnatia Odos 2010). During the present study (2003–2005) construction efforts of the highway had not yet begun; works started in 2006 and now the "Egnatia" highway is fully operational.

Sampling, DNA extraction, and microsatellite analysis

In 2003–2005, scat and hair were collected opportunistically during regular field surveys. In order to obtain adequate capture probabilities for population estimation (Woods et al. 1999; Mowat and Strobeck 2000), an intensive 4-month sampling session was carried out in April–July 2005. In this time, only hair left behind when bears marked and/or rubbed on power poles were collected monthly, according to a protocol developed during a pilot study in the area (Karamanlidis et al. 2007; 2010b). From 2003 to 2005, 444 samples were collected (27 scat and 417 hair); we culled many of the hair samples before the first stage of analysis based on inadequate number of follicles (82%) and subsampling criteria (18%) (Karamanlidis et al. 2010b). We analyzed 131 samples (27 scat and 104 hair). In addition, six blood and hair samples were collected from live-captured bears within the framework of a concurrent telemetry study.

Hair samples were placed in paper envelopes without contacting human skin and then stored at room temperature in zip lock bags with silica gel (Roon et al. 2003). Hair and blood DNA extractions were performed using the DNeasy Blood & Tissue kits (Qiagen, Germany) following the

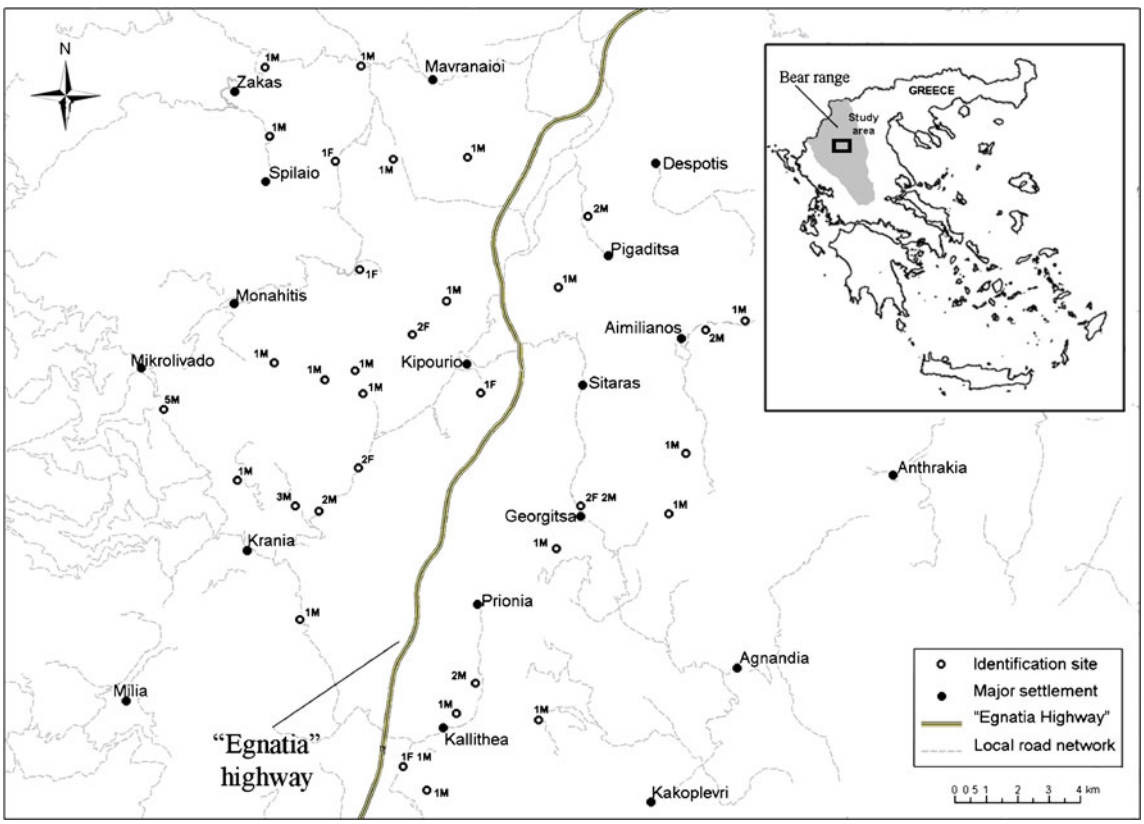

Fig. 1 Map of the study area at the Prefecture of Grevena in northwestern Greece, indicating the approximate distribution of the Pindos brown bear population and the location of the study area, the alignment of the "Egnatia" highway and the sampling locations and numbers of individual female (*F*) and male (*M*) bears identified by noninvasive genotyping

manufacturer's instructions. We aimed at using ten guard hairs where available. Scat samples were placed in a freezer until DNA extraction, which was performed using the DNA Stool kit (Qiagen, Germany), following the manufacturer's instructions. All extractions from noninvasive samples took place in a separate facility and extraction and PCR-negative controls were used. Each sample was genotyped at the microsatellite loci G1D, G10C, G10L, G10M, G10P (Paetkau et al. 1995), G10J (Paetkau et al. 1998), MU23, MU50, MU51, and MU59 (Taberlet et al. 1997). Sex identification was established through the analysis of the amelogenin gene (Ennis and Gallagher 1994). Thermal cycling was performed using a PTC100 thermocycler (MJ Research, USA) with 96-well 'Gold' blocks. PCR buffers and conditions were according to Paetkau et al. (1998), except that microsatellite markers were not co-amplified. We used 3 μl of DNA extract per PCR reaction, except during error-checking, when 5 μl was used. The concentration of $MgCl_2$ was 2.0 mM for all markers except G10J where it was 1.8 mM. Microsatellite analysis used ABI's four-color detection system; we used an ABI 310 automated sequencer (Applied Biosystems, USA) and genotypes were determined using ABI Genescan and Genotyper software. Error-checking and general quality assurance followed the recommendations of Paetkau (2003) and Roon et al. (2003).

Statistical methods

Software Microchecker v. 2.2.3 (van Oosterhout et al. 2004) was used to detect potential genotyping errors caused by large allele drop-out, scoring of stutter peaks, and null alleles. The informativeness of the loci for evaluating genetic diversity was assessed by calculating the polymorphism information content (PIC; Botstein et al. 1980), using the program PowerMarker v. 3.25 (Liu and Muse 2005). To evaluate the suitability of the marker set for identifying individuals, the probability of identity (P_{ID}; Paetkau and Strobeck 1994) and the more conservative probability of identity among siblings ($P_{ID\text{-}Sib}$; Waits et al. 2001) was calculated using the software Gimlet v. 1.3.2 (Valiere 2002). In addition, the pairs of genotypes that matched all, but one, two, or three loci (1-mm, 2-mm, and 3-mm pairs) were identified using the program GenAlEx 6 (Peakall and Smouse 2006).

Nuclear DNA diversity was measured as the number of alleles per locus (A), the observed heterozygosity (H_o) and Nei's unbiased expected heterozygosity (H_e; Nei 1978) using the program PowerMarker v. 3.25 (Liu and Muse 2005). Deviations from Hardy–Weinberg equilibrium (HWE) were tested using the exact probability test implemented in the software Genepop v. 4.0.10 (Raymond and Rousset 1995); a Markov chain set to 100 batches, with 5,000 iterations per batch, and 10,000 steps of dememorization was used to obtain an unbiased estimate of the exact probability. Pairwise tests for linkage disequilibrium were performed using Fisher's method (Sokal and Rohlf 1994) with 1,000 batches and 10,000 iterations per batch and P-values were adjusted for multiple comparisons using the Bonferroni sequential correction (Rice 1989).

The population assignment test implemented in the program Structure v. 2.3.3 was used to assign bears to a cluster or population based on their genotypes regardless of where the samples were collected (Pritchard et al. 2000). The admixture model was used, allele frequencies were assumed independent and analyses were conducted with a burn-in period of 50,000 followed by 20,000 Markov chain Monte Carlo repetitions. We ran Structure ten times with the number of clusters (K) set from 1 to 4 to determine the likely number of clusters representative of the data. The most probable value of K was inferred from the mean log-likelihood values [Ln$P(D)$] according to the criteria by Pritchard et al. (2000); K with the highest likelihood and consistency between runs was chosen as the most appropriate. A factorial correspondence analysis (FCA) implemented in the program Genetix v. 4.05.2 (Belkhir et al. 1996–2004) was performed to graphically visualize the genetic relationship between individuals and inferred groups. In addition, an exclusion test (Cornuet and Luikart 1996) for detecting potential migrants in the population was performed using the software GeneClass v. 2.0 (Piry et al. 2004), applying the frequency-based method (Paetkau et al. 1995) and the simulation algorithm of Paetkau et al. (2004).

Two different tests were used to detect genetic evidence of a recent bottleneck. The principle of the first test, which is implemented in the software Bottleneck (Piry et al. 1999), is based on the fact that in populations affected by a recent reduction in effective population size, allelic diversity is reduced faster than heterozygosity. Thus, heterozygosity becomes larger than the heterozygosity expected at mutation-drift equilibrium calculated from the number of alleles (Cornuet and Luikart 1996; Luikart et al. 1998). Heterozygosity excess was detected with a Wilcoxon test (Maudet et al. 2002), using the two-phase mutation (TPM) model with 90% single-step mutations and 10% multi-step mutations, as recommended previously (Piry et al. 1999; Garza and Williamson 2001). The second test used for the detection of a bottleneck, the M-ratio test, is based on the ratio of the number of alleles to the range in allele size. This ratio is expected to decrease in bottlenecked populations. The test was performed using the Critical-M and M-P-Val programs from Garza and Williamson (2001), with two sets of parameter values. In the first case, the proportion of one-step mutations (ps) was 0.9 and the average size of non-one-step mutations (Δg) was 3.5, and in the second case, the parameters were ps=0.88 and Δg=2.8 (Garza and Williamson 2001). In both cases, the parameter $\theta=4Ne\mu$ (Ne=*is*

effective population size, and μ is mutation rate) was varied over seven values (0.002, 0.01, 0.1, 1, 2, 5, and 10) to account for a range of mutation rates and possible effective population sizes prior to the bottleneck, as the true effective population size was unknown. With this conservative approach, the range of possible effective population sizes extends from 25 to 250,000, if we assume a mutation rate for microsatellite loci between 10^{-4} and 10^{-5} (Jarne and Lagoda 1996).

The overall within population inbreeding estimate (F_{IS}) was calculated using the program PowerMarker v. 3.25 (Liu and Muse 2005) and pairwise genetic relatedness between pairs of individuals was calculated using the estimators of Wang (2002), Queller and Goodnight (1989), and Lynch and Ritland (1999) as implemented in the software Coancestry (Wang 2011). Relatedness values range from 1 to −1, indicating the percentage of alleles shared among individuals. Theoretically, a value of 1 means that genotypes are identical and a value of 0.5 indicates that 50% of the alleles are shared; unrelated individuals have relatedness values ranging from 0 to −1 with the more negative values indicating greater differences in the genotypes of the individuals (Bellemain et al. 2007).

To meet assumptions of a closed population, demographic estimations were made using only data collected during the 4-month systematic sampling session in 2005. Effective population size (N_e) was calculated based on summary statistics and the approximate Bayesian computation implemented in the program ONeSAMP (Tallmon et al. 2008). This method assumes that all loci are neutral and unlinked and is based on simulations of a single, closed population. Based on our assumptions of the size of the local bear population, lower and upper bounds of the prior for N_e were used of two and 200; moreover, N_e was calculated also using the upper bound of 400 to help further substantiate our results. Total population size was estimated using the estimator implemented in the capture–mark–recapture-based program for noninvasive genetic sampling, Capwire (Miller et al. 2005). This software accommodates data with multiple observations of an individual within a single session and appears to work well for small populations, such as the one expected in our study area (Miller et al. 2005). Because of suspected capture heterogeneity in our data, due to the collection of genetic samples from power poles (Karamanlidis et al. 2007, 2010b), we calculated population size only using the two innate rates model (TIRM). Total population size was calculated also as the asymptote of the function between the cumulative number of unique genotypes and number of samples typed. The asymptote was calculated using two different rarefaction curve methods. The first method was described by Eggert et al. (2003) as the equation $y=a(1-e^{bx})$. The second method was suggested by D. Chessel in the GIMLET software manual (Valiere 2002) and is defined by the equation $y=a-a[1-(1/a)]^x$. The samples were regrouped in GIMLET and the output file was analyzed using R software (Ihaka and Gentleman 1996). The order in which the samples were analyzed can have an effect on population size estimates (Kohn et al. 1999), and therefore the input was randomized 10^4 times to prevent the bias.

Results

We obtained a complete ten-locus genotype for 70% of the scat and 61% of the hair samples analyzed and identified 49 unique genotypes (i.e., individual bears, 10 females and 39 males). No mismatches were recorded when analyzing blood and hair samples from the same individual. Unique genotypes from both sexes were identified on both sides of the highway alignment (Fig. 1); 16 unique genotypes were identified more than once, ten of which at different locations (Fig. 2). These ten individuals, both females and males, crossed the future highway alignment in both directions a total of 11 times.

None of the loci used in the study showed evidence of frequency distortion through large allele drop-outs or stutter bands; however, Micro-Checker indicated the possible existence of null alleles at loci G10M, MU23, and MU51. Nevertheless, these loci were used in the data analyses, except the bottleneck tests and when estimating relatedness and probability of identity. Sixty percent of the selected markers and the overall mean of all markers used in the study had a PIC value higher than the recommended value of 0.6 (Buchanan et al. 1993), pointing to a high degree of informativeness of these markers in evaluating genetic diversity. The accumulated, more conservative probability of identity among siblings ($P_{ID\text{-}Sib}$) of the seven most informative loci was lower than 0.01, the value recommended if the data are to be used for population size estimation (Waits et al. 2001; Table 1). Finally, in our sample set, there were no genotypes matching at all but one, two, or three loci.

All loci in the study were polymorphic, with the number of alleles per locus ranging between 3 and 8 and a mean of 5.6 (Table 2). The mean observed heterozygosity was 0.653, and the unbiased expected heterozygosity was 0.686. The level of nuclear genetic diversity of brown bears in northwestern Greece compared to some other bear populations in Europe was relatively high (Swenson et al. 2011). Global tests showed that the population was in HWE ($P=0.302$), although loci MU23 and G10M had a significant deficiency in heterozygotes at the $P<0.05$ level (Table 2). Statistical tests for linkage disequilibrium were computed for all pairs of loci, and only four pairs (i.e., Mu50 and G10M, G10P and G10M, Mu59 and G10L, and Mu23 and Mu51) were in linkage disequilibrium. However, after adjustment of P values using the Bonferroni sequential correction, none of the

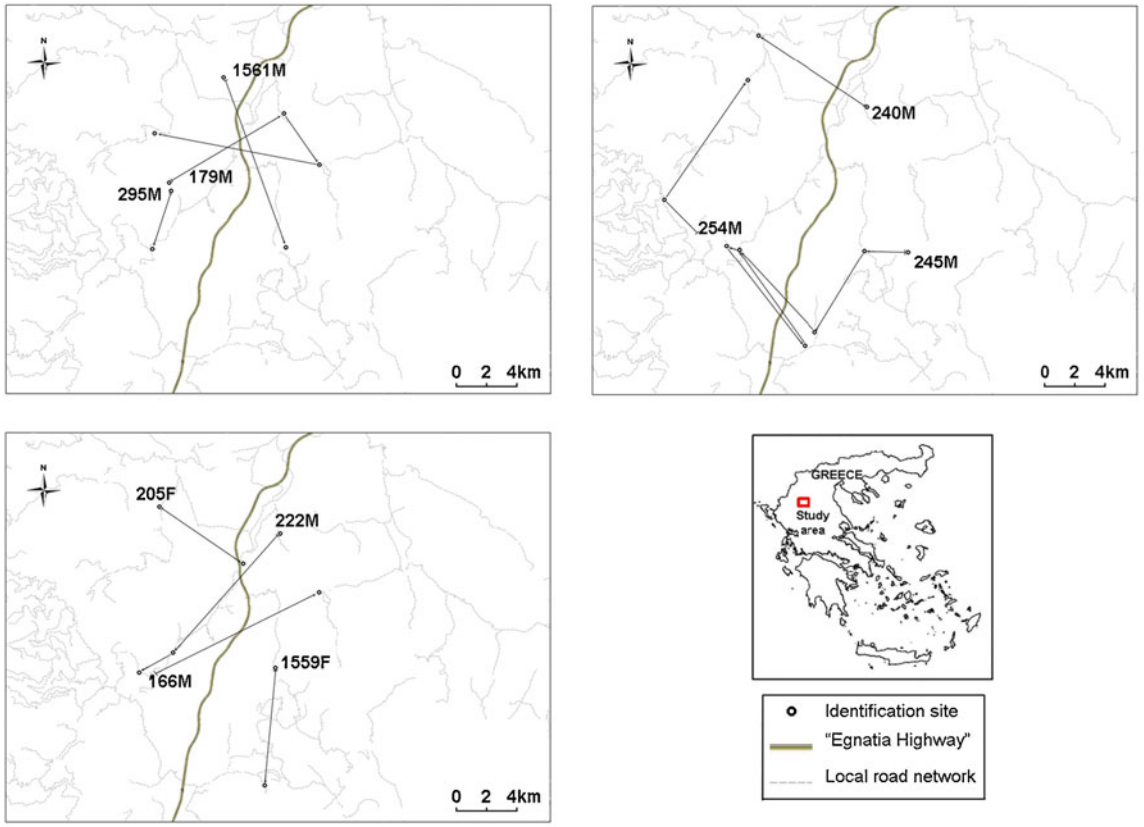

Fig. 2 Identification sites of ten individual bears and direct distances travelled between recaptures, prior to the construction of the "Egnatia" highway in the Prefecture of Grevena, Greece. Identification number and sex (*F* Female, *M* Male) is shown for each bear. The alignment of the "Egnatia" highway is indicated

45 test revealed significant results. The overall within-population inbreeding estimate (F_{IS}) was 0.059 (Table 2) and the average pairwise relatedness was −0.0221 for Wang's estimator and −0.0232 for Queller and Goodnight's and −0.0222 for Lynch and Ritland's estimators, respectively;

Table 1 Descriptive statistics at ten polymorphic loci in 49 brown bears in the central part of the Pindos mountain range in northwestern Greece, including the polymorphism information content (*PIC*), the probability of identity (P_{ID}), and the probability of identity among siblings ($P_{ID\text{-}Sib}$)

Locus	PIC	P_{ID}	$P_{ID\text{-}Sib}$	Prod. $P_{ID\text{-}Sib}$
G1D	0.768	6.090×10^{-2}	3.685×10^{-1}	3.685×10^{-1}
G10C	0.747	7.236×10^{-2}	3.796×10^{-1}	1.398×10^{-1}
MU59	0.741	7.354×10^{-2}	3.838×10^{-1}	5.638×10^{-2}
G10P	0.733	8.067×10^{-2}	3.872×10^{-1}	2.079×10^{-2}
MU50	0.678	1.130×10^{-1}	4.176×10^{-1}	8.681×10^{-3}
G10J	0.633	1.394×10^{-1}	4.447×10^{-1}	3.861×10^{-3}
G10L	0.574	1.816×10^{-1}	4.805×10^{-1}	0.001855
MU23	0.549	2.037×10^{-1}	4.942×10^{-1}	-
G10M	0.449	2.840×10^{-1}	5.741×10^{-1}	-
MU51	0.524	2.116×10^{-1}	5.256×10^{-1}	-
Mean	0.640			

The multilocus product of $P_{ID\text{-}Sib}$ is calculated sequentially in increasing order of single-locus values, the first locus being the most informative

Table 2 Nuclear genetic diversity of a brown bear population (N=49) in the central part of the Pindos mountain range in northwestern Greece, including the number of alleles (A), unbiased expected (H_e) and observed (H_o) heterozygosity, deviation from HWE by locus (P_{HWE}) and within-population inbreeding estimate (F_{IS})

Locus	A	H_e	H_o	P_{HWE}	F_{IS}
G10J	6	0.686	0.694		−0.001
G10C	6	0.782	0.771		0.025
G1D	6	0.798	0.735		0.090
MU23	6	0.619	0.468	0.006	0.254
MU50	5	0.727	0.755		−0.029
MU59	7	0.775	0.854		−0.092
G10P	8	0.771	0.729		0.065
G10M	3	0.502	0.372	0.046	0.270
G10L	5	0.636	0.636		0.011
MU51	4	0.564	0.511		0.104
Mean	5.6	0.686	0.653		0.059
SE	0.45	0.032	0.049		0.037

these values indicate low levels of population inbreeding and relatedness.

The clustering method implemented in program Structure indicated that the value of clusters (K) with the highest likelihood and consistency between runs for the whole data set was 1 {Ln$P(D)$=−1,316.08; Var[Ln$P(D)$]=22.41}. At K >1, mean log-likelihood was more negative and variance was higher {K=2, Ln$P(D)$=−1,333.28 Var[Ln$P(D)$]=65.19; K=3, Ln$P(D)$=−1,331.91 Var[Ln$P(D)$]=64.13; K=4, Ln$P(D)$=−1,320.90 Var[Ln$P(D)$]=39.3}. This result was supported by the results of the FCA analysis (Fig. 3), as only one cluster of individuals was identified and the first and second axes represented 9.68% and 6.85% of the variation, respectively. The exclusion test performed in GeneClass indicated that one individual most likely did not originate from the sampled population and was a migrant (P<0.001; Fig. 3).

A bottleneck signature was detected with the heterozygosity excess test under the TPM mutation model when proportion of single-step mutations was 90% (one-tailed Wilcoxon's test, P=0.008). Depending on the parameters used, the values of the mean M ratio ranged between 0.792 and 0.951 and that of the critical M ratio between 0.685 and 0.843, which were higher than the average sample M ratio of 0.625 (P value <0.01 in all simulated models). Thus, the M ratio test also indicated a recent bottleneck.

Out of 49 individuals, 26 (6 female and 20 male) were identified during the intensive sampling period in 2005 and were used in the estimation of effective and total population size. The number of recaptures per individual ranged from 1 to 8 (mean 1.807), with more than half of the bears (N=16) identified from a single sample. Using OneSamp, the median estimate of N_e was 29 with 95% credible limits (CL) of 24.4–39.2 breeding individuals; this estimate did not change markedly when using upper priors of 400 (i.e., N_e=28; 95% CL=23.6–39.9). For our study area, the Capwire point estimate was 51, which is considerably higher than the 26 genotypes actually identified. The 95% confidence interval (CI) was 29–68 individuals; ten individuals were classified as easier to capture (type A), and the remaining 41 as harder to capture (type B). The rarefaction analysis produced the following results: Chessel's method produced a point estimate of 33 (CI: 22.29–47.76; SD: 3.16) and Eggert's method produced a point estimate of 46 (CI: 25.57–2211.42; SD: 46.03). Due to the large variance and standard deviation, Eggert's method does not appear appropriate for the analysis and interpretation of our data.

Discussion

Nuclear genetic diversity of brown bears in the central part of the Pindos mountain range was high compared to other bear populations in the Cantabrian, Pyrenees, and the Apennine mountains, which have experienced population bottlenecks in the past and are currently considered critically endangered. In fact, the observed heterozygosity value in the central Pindos population is just slightly lower than that of bear populations in Scandinavia and Romania, which are considered to have a good conservation status (Swenson et al. 2011). Obtaining 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 are therefore critical for conservation planning (Johnson et al. 2009). Considering the paucity of information on brown bears in Greece, the relatively high levels of nuclear genetic diversity recorded in the present study indicate that the conservation status of this population might be better than previously assumed; intensive, noninvasive genetic monitoring efforts throughout the entire range of the species in the country are urgently required to determine the conservation status of brown bears in Greece and promote effective conservation and management measures.

We did not detect any evidence of substructure in our study population; there were no deviations from HWE and no linkage disequilibrium which, if present, would have indicated population substructuring. No evidence of substructure in our sampling area was detected also using the Structure and FCA analysis. The movements documented through the genetic recaptures during the present study also

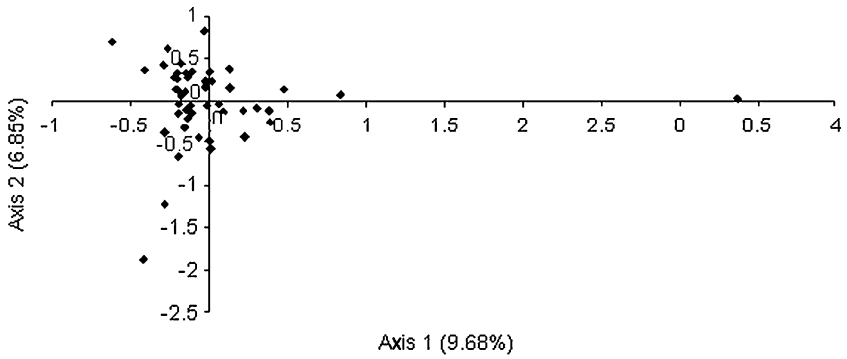

Fig. 3 Projection of samples on the plane defined by the two first factorial axes of the factorial correspondence analysis. The individual located at the far right side of the x-axis (Sample ID 1545) was probably a migrant

suggest a panmictic population. This result was largely expected, since brown bear habitat in the central part of the Pindos mountain range prior to the construction of the "Egnatia" highway had been mainly uninterrupted. Recent studies have demonstrated that anthropogenic barriers, such as major roads, can contribute to genetic structuring of bear populations even more than the linear distance between them (Dixon et al. 2007; Pérez et al. 2009; Simmons et al. 2010). In order to decrease extinction risk and ensure the persistence of large carnivore populations, it is therefore important to maintain opportunities for movements (Ernest et al. 2000). The results of the present study showed that the "Egnatia" highway dissects home ranges of several individual bears from our study population (Fig. 2). This finding, in conjunction with current monitoring efforts that have recorded fatal bear–vehicle collisions in the area (Karamanlidis 2007), suggest that this highway could indeed become a barrier to gene flow. Although the current alignment of the highway is designed to ensure sufficient movements, there have been no efforts to confirm this assumption, and it remains unknown whether the recommended gene flow of 1–10 migrants/generation (Mills and Allendorf 1996) is being achieved. Thus, a study is needed that would collect data from various sources (e.g., genetic and telemetry data) to evaluate the extent of bear movement over the highway, and even more so because the wider study area is now the planned construction site of another highway (Karamanlidis and Georgiadis 2009).

Demographic bottlenecks and the resulting increased extinction chances from declines in genetic variation are of great concern to conservation biologists (Hedrick and Miller 1992; Lacy 1997; Matocq and Villablanca 2001). The two tests that were applied to investigate the genetic evidence of a bottleneck, the heterozygosity excess test under the TPM and the *M* ratio test, gave concordant results. Both methods are considered appropriate for microsatellite data (Di Rienzo et al. 1994; Garza and Williamson 2001; Williamson-Natesan 2005), and in both cases, there was a strong indication of a past reduction in population size. Similarly, genetic studies have revealed bottlenecks in other brown bear populations in Europe (Lorenzini et al. 2004; Tallmon et al. 2004; Kocijan et al. 2011). Bearing in mind that many brown bear populations in Europe share a very similar demographic history of extinctions through habitat loss and hunting, it could be that a genetic trace of past bottlenecks is a common feature. Although no information on the historical population size of bears in Greece is available, the recent increased number of extra-limital sightings near our study area (Karamanlidis et al. 2008) are in accordance with the assumption of a depleted population that is gradually recovering. The low values of the within-population inbreeding estimate and average pairwise relatedness indicate furthermore that brown bears in our study area in the central part of the Pindos mountain range are currently not at risk of inbreeding, despite their relatively small size.

Using noninvasive genetic monitoring methods, we estimated an effective population size during a 4-month intensive sampling session for the brown bear population in the Prefecture of Grevena of 29 individuals and a total population size of 33 and 51 individuals, depending on methodology used. Estimating population size is important in identifying populations with a high extinction risk and predicting their long-term persistence (Creel et al. 2003; Prugh and Ritland 2005). The validity of estimates from closed population estimators, such as the ones used in the study, relies on demographic and geographic closure during sampling. Considering information on the reproductive and activity patterns of the species (Mertzanis et al. 2005; Kaczensky et al. 2006) and data from intensive field monitoring in the area that did not record any deaths during the intensive sampling period (Karamanlidis 2008), we believe that the assumption of demographic closure was not grossly violated in our study. Telemetry data from 13 bears monitored (ARCTUROS, unpublished data) and the fact that the study area is surrounded by human development, which limits bear movements, suggest that geographic closure also was reasonably given (Arandjelovic et al. 2010). However, considering that we cannot exclude the possibility that in an area of continuous bear distribution migration has occurred and that despite intensive sampling efforts, we did not manage to obtain the recommended recapture rates of 2.5–3.0 observations per individual (Miller et al. 2005), the confidence intervals of our demographic estimations are relatively large and therefore our demographic estimations should be treated with caution.

Populations with recently reduced effective population size may be particularly prone to extinction (Newman and Pilson 1997) and a minimum *Ne* of 50 individuals has been suggested for avoiding inbreeding depression (Frankham et al. 2002). Considering that the effective population size in our study area was lower than the minimum recommended threshold of 50 individuals and the fact that potentially new anthropogenic stressors might be operating in the area (i.e., operation of a highway, construction of a new one), the low effective population size in our study is a matter for concern and should be closely monitored in the future (see also Whiteman et al. 2006; Hale and Briskie 2007; Reed et al. 2007). Census size was higher than effective population size, as, depending on methodology used, 33–51 individuals were estimated to frequent the study area during the 4-month intensive sampling period. The high proportion of type B (i.e., harder to capture) individuals detected in Capwire reflects the fact that a high number of the bears were identified on the basis of a single genetic sample; future DNA-based monitoring efforts in Greece should increase

sampling frequency and the number of loci analyzed and collect data from multiple sources in order to increase the accuracy of population estimates (Boulanger et al. 2008). The fact that census size in our study area corresponds to approximately 25% of the minimum bear population estimate in Greece (Mertzanis et al. 2009) signifies the importance of this population for the long-term survival of the species in the country and underlines the urgency for taking appropriate management measures for mitigating the potential negative impacts from the construction of the "Egnatia" highway.

Conclusions and management recommendations

The results of this study indicate relatively high genetic diversity and lack of substructuring in a panmictic brown bear population in the central part of the Pindos mountain range in northwestern Greece that despite having experienced a population bottleneck recently is currently in low risk of inbreeding. Effective population size was small, but total population size was, considering the total population estimate for the species in Greece, relatively large. We consider these results as evidence for a good conservation status of this subpopulation.

The genetic data presented here provide baseline information that can be used in future studies that should evaluate the effects of the construction and operation of the "Egnatia" highway on the Pindos brown bear population. Maintaining connectivity should be a priority for the conservation of the species not only on a local but also on a national and international level. Considering that the bear population in our study area is connected to other bear populations in Greece and constitutes a part of the larger Alps-Dinaric-Pindos brown bear population, maintaining sufficient levels of gene flow should be a management priority to ensure survival of bears in Europe.

Considering the paucity of information regarding the species in Greece and the fact that noninvasive genetic monitoring techniques have proven to be efficient in assessing the status of the species, we recommend the wide-scale application of this method throughout the entire range of the species in the country. Similar studies should be carried out in the newly established National Parks of Northern Pindos and Rodopi, which have important bear populations.

The Alps-Dinaric-Pindos brown bear population is one of the largest populations of the species in Europe. At the same time, it is, however, also one of the most difficult to manage and protect since it is distributed over ten different countries; effective protection will require the collection and analysis of data in a comparable manner. The present study managed to collect more than 10% of the estimated number of individuals living in the country, which has been proposed as a common guideline for the genetic study of bears in the Alps-Dinaric-Pindos population (Karamanlidis et al. 2010a). The results of the study will contribute in the better understanding of gene flow within the Alps-Dinaric-Pindos population, and the evaluation of its long-term survival prospects while promoting the overall conservation planning for large carnivores in the increasingly urbanized landscape of Europe.

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