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Genetic tracking of the brown bear in northern Pakistan and implications for conservation

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ARTICLE INFO

Article history:
Received 4 April 2006
Received in revised form
23 August 2006
Accepted 3 September 2006
Available online 30 October 2006

Keywords:
Bottleneck
Feces
Individual identification
Pakistan
Ursus arctos

ABSTRACT

Asian bears face major threats due to the impact of human activities as well as a critical lack of knowledge about their status, distribution and needs for survival. Once abundant in northern Pakistan, the Himalayan brown bear (*Ursus arctos isabellinus*) has been exterminated in most of its former distribution range. It presently occurs sparsely, in small populations, the Deosai National Park supporting the largest isolate. This decline might imply a reduction in genetic diversity, compromising the survival of the population. Using a combination of fecal DNA analysis and field data, our study aimed at assessing the size and genetic status of the Deosai population and give guidelines for its conservation and management. Using fecal genetic analysis, we estimated the population to be 40–50 bears, which compares well with the field census of 38 bears. The northern Pakistani brown bear population may have undergone an approximate 200–300-fold decrease during the last thousand years, probably due to glaciations and the influence of growing human population. However, in spite of the presence of a bottleneck genetic signature, the Deosai population has a moderate level of genetic diversity and is not at immediate risk of inbreeding depression. Gene flow might exist with adjacent populations. We recommend careful monitoring of this population in the future both with field observations and genetic analyses, including sampling of adjacent populations to assess incoming gene flow. The connectivity with adjacent populations in Pakistan and India will be of prime importance for the long-term survival of Deosai bears.

1. Introduction

Brown bears (*Ursus arctos*) are the most endangered and least studied in Asia, where populations have declined by more than half in the past century (Servheen, 1990; Servheen et al., 1999). Asian bears face threats due to the impact of human activities and there is a critical lack of knowledge concerning their status, distribution and requirements for

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0006-3207/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biocon.2006.09.004

survival (Servheen et al., 1999). The Himalayan brown bear (*U.a.isabellinus*), a highly threatened subspecies, is distributed in small isolated populations over the Himalaya, Karakoram, Hindu Kush, Pamir, western Kun Lun Shan, and Tian Shan ranges in southern Asia.

This bear has been exterminated in most of its former distribution range in Pakistan, and occurs very sparsely in small populations with limited connectivity in northern mountainous areas. Deosai National Park is the main stronghold of the brown bear population in Pakistan (Schaller, 1977; Roberts, 1997). Once abundant in Deosai, bear numbers declined drastically to as low as 19 in 1993 (Himalayan Wildlife Project, 1994). Although the population in Deosai has been recovering gradually since 1993 due to strict protection and conservation efforts, the decline could have reduced the genetic variability considerably. As a consequence, this population might suffer from inbreeding, and its survival might be compromised. Small population size is a great concern in conservation biology because small populations are more vulnerable to genetic factors, demographic and environmental stochasticity, genetic drift and inbreeding and have increased probability of extinction (Soulé, 1987). Evolutionary processes such as mutations, migration, selection and stochasticity are also fundamentally different than those in large populations. In small populations the role of stochasticity increases and the impact of selection is limited (Frankham et al., 2002). The loss of genetic diversity as a result of a bottleneck or continued small populations has been documented in many endangered species such as the northern elephant seal (*Mirounga angustirostris*) (Bonnell and Selander, 1974), Mauritius kestrel (*Falco punctatus*) (Groombridge et al., 2000), Indian rhinoceros (*Rhinoceros unicornis*) and Siberian tiger (*Panthera tigris*) (Hedrick, 1992). Fragmented populations are prone to many subtle threats, such as limited dispersal and colonization and restricted access to food and mates (Primack, 2002).

Documenting the status and distribution of Asian bears has been identified as a priority action for conservation by the IUCN/SSC Bear Specialist Group (Servheen et al., 1999). A comprehensive action plan is required for the long-term management of Himalayan brown bears. In order to be effective, an action plan should be based on reliable biological data, such as trustworthy estimates of population size, population genetic status and connectivity with other populations. Population size estimates are difficult to obtain for rare and elusive animals like brown bears (Bellemain et al., 2005). Field methods based on observations of recognizable individual bears have been used to estimate the size of the Deosai population, but these methods have not been compared with censuses using independent methods in order to evaluate their consistency.

To assess the genetic status and size of the Deosai population and give guidelines for the conservation and management of this population, we used the increasingly popular non-invasive genetic technique (Taberlet et al., 1996, 1999), in combination with field data. Using DNA analyses of fecal sampling, we aimed to answer the following questions: (i) Is the population size estimated from field data consistent with genetic censuses? (ii) Did the population suffer from a bottleneck at the genetic level and how long ago did it begin to decline? (iii) Are Deosai bears at risk of inbreeding depression? (iv) Is the population genetically isolated?

2. Material and methods

2.1. Study area and studied populations

The study was conducted in the Deosai National Park, Northern Areas, Pakistan. Deosai National Park is a plateau in the alpine ecological zone encompassing about 20,000 km^2, situated 30 km south of Skardu and 80 km east of the Nanga Parbat Peak. Elevations range from 3500 to 5200 m and about 60% of the area lies between 4000 and 4500 m. Recorded mean daily temperatures range from −20 °C to 12 °C. The annual precipitation in Deosai is 510–750 mm, and falls mostly as snow (Himalayan Wildlife Foundation, 1999a). The Deosai plains are covered by snow during winter months between November and May, and life on the plateau is confined to a window of five months.

The Deosai Plateau is situated between two of the world's major mountain ranges, the Karakoram and Himalaya. The biota includes plants and animals from Karakoram, Himalaya and Indus Valley. As a result Deosai is a center of unique biota in northern Pakistan. The documented biota of Deosai National Park includes 342 species of plants, 18 of mammals, 208 of birds, three of fishes, one of amphibian, and two of reptiles (Woods et al., 1997). Most of the plant species are herbaceous perennials, and cushions forming and tufted plants are common growth forms. Plains present a mosaic of plant communities according to the availability of water. The low lying areas usually consist of bogs and pools with associated flora consisting predominantly of grasses and sedges and plants such as *Saxifraga hircus*, *Swetia perfoliata* and *Aconitum violaceum*.

Deosai National Park supports the largest population of brown bears in Pakistan (unpublished data). The brown bear population in this park has been protected and closely monitored since 1993, and primary data on population size, behavior and ecology have been gathered (Himalayan Wildlife Foundation, 1999b). Field personnel were able to recognize dominant bears from their physical characteristics, coloration and well defined home ranges on this open plateau (Himalayan Wildlife Foundation, 1999a,b; Nawaz et al., 2006). Based on this, they estimated the number of bears annually, the approximate age of some males and females, as well as their reproductive behavior and, in some cases, relatedness (mothers and their young).

2.2. Fecal sampling

The study area was searched for bear feces from July to early October 2004. We divided the study area into five blocks, and each block was searched for bear feces in order to cover most of Deosai National Park (Fig. 1). Transects of 40–60 km length were placed in each block, and walked by a team of 2–3 people. The transect routes were planned in a way that these covered the maximum extent of the block and passed through areas known for frequent bear sightings. Transect routes usually resembled a loop, starting from the central road, progressing towards periphery of the park, and ended at the starting point. The team walked along opposite borders of a block while going towards the periphery of the park and coming back to the road. Each transect was completed in 2–3 days,

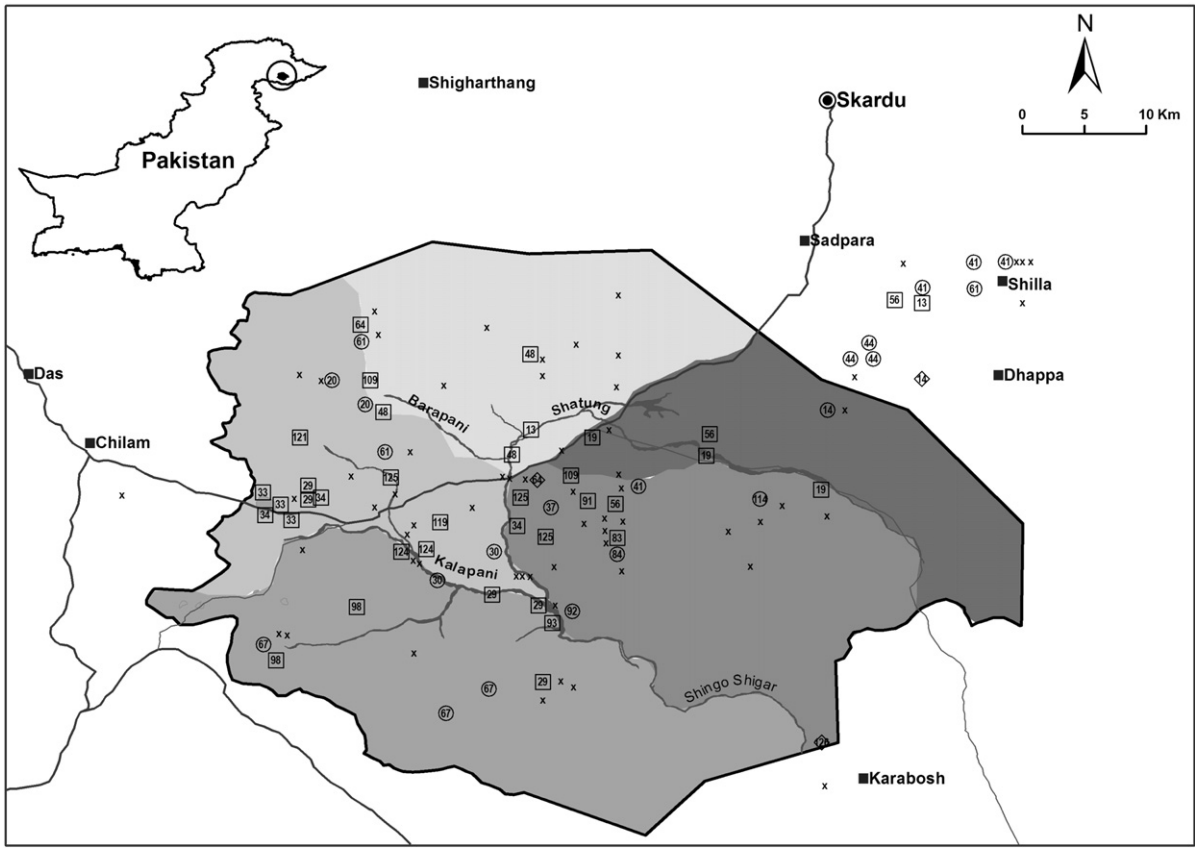

Fig. 1 – Map of the study area in the Deosai National Park, Northern Areas, Pakistan. Spatial distribution of brown bear genotypes is represented with squares for males, circles for females, and diamonds for unknown sex. Numbers within squares or circles represent individuals' identification numbers. Samples with negative/poor amplification are shown as "x". Five survey blocks are represented by different shades of grey.

with night stays made in portable tents. Apart from this planned collection, the field staff of Deosai National Park collected samples during their normal patrolling of the park.

Brown bears exhibit altitudinal migration in Deosai, and spend part of their life in surrounding valleys. We therefore collected feces from valleys connected to the park. When we found many feces together, usually at a bedding site, we collected one sample from the freshest feces. However if several feces were found at a food source (e.g. carcass) or we could differentiate different sizes, we took multiple samples. We picked up each fecal sample with a stick of wood and put 1 cm^3 of it in a 20-ml bottle. For each fecal sample, a sampling date, a geographical location and coordinates (latitude/longitude) were recorded using a GPS receiver (Garmin 12XL). Bottles were then filled with 95% alcohol to preserve the samples until DNA extraction.

Approximate ages of fecal samples were evaluated on the field and categorized into five classes; (1) fresh feces of the same day, (2) two–three days old, (3) one week old, (4) feces of the same month, and (5) feces older than one month.

2.3. *DNA extractions and typing*

2.3.1. *Extraction*

For every collected fecal sample, DNA extractions were performed using the Qiamp DNA Stool Kit (Qiagen, Hilden, Germany), developed especially for this type of material and following the manufacturer's instructions. All extractions occurred in a room dedicated to processing hairs and feces. Tubes containing samples and tubes without feces were treated identically to check for exogenous DNA contaminations.

2.3.2. *Genotyping for individual identification*

The extracted DNA was amplified using the six microsatellite primers described in Bellemain and Taberlet (2004) on a set of 16 feces to test for their polymorphism. The number of alleles per locus ranged from one to eight. The two primers showing only one or two alleles (Mu10 and G10L) were discarded for this analysis (but included later, see below) and the four others (Mu23, Mu50, Mu51, and Mu59) were kept. In order to obtain a probability of identity low enough to differentiate among all individuals, we redesigned two other microsatellite primer pairs, namely G10J and G10H (from Paetkau and Strobeck, 1994; Paetkau et al., 1995):

G10HFIpak: GGAGGAAGAAAGATGGAAAAC
G10HRpak: AAAAGGCCTAAGCTACATCG
G10JFpak: GCTTTTGTGTGTGTTTTTGC
G10JRIpak: GGTATAACCCCTCACACTCC

For sex identification, we used the SRY-primers described in Bellemain and Taberlet (2004).

We simultaneously amplified the following loci: Mu23 with Mu50; SRY with Mu51 and Mu59; G10Jpak with G10Hpak, using the internal fluorescent primers together with the appropriate external primers. We repeated each amplification eight times following the multi-tube approach (Taberlet et al., 1996). The fluorescent PCR products were loaded together on the single electrophoresis (ABI Prism 3100 DNA sequencer; Applied Biosystems, Foster City, California). The gels were analyzed using Genemapper (version 3.0) software package (Applied Biosystems, Foster City, California). We typed samples as heterozygous at one locus if both alleles appeared at least twice among the eight replicates and as homozygous if all the replicates showed identical homozygous profiles. If neither of those cases occurred, the alleles were treated as missing data.

We calculated a quality index for each sample following the rules defined in Miquel et al. (2006). To be conservative, we discarded the samples that had a quality index below 0.5.

2.3.3. *Genotyping for population genetics analyses*

To estimate population genetics parameters and relatedness, we increased the number of loci for each genetically identified individual. The highest quality sample per individual was selected, based on quality indices when the individual was represented by several samples. We amplified the following 12 additional microsatellites: G1A, G1D, G10B, G10C, G10L, G10P, G10X, G10O (Paetkau and Strobeck, 1994; Paetkau et al., 1995) and Mu05, Mu10, Mu15, Mu61 (Taberlet et al., 1997), using a modified protocol from Waits et al. (2000). The amplifications were performed using five combinations of loci: (1) G10B, G10C (2) G10X, G10P; (3) Mu61, Mu05; (4) G10O, G10L (5) G1D, Mu15; loci Mu10 and G1A were amplified separately. PCR reactions of 12.5 μL containing 2 μL template DNA, 0.1 mM each dNTP, 0.5 μM of each primer, 3 mM MgCl 2, 0.5 U AmpliTaq Gold Polymerase (Applied Biosystems) and 1 × Taq buffer (containing 100 mm Tris–HCl, pH 8.3, 500 mm KCl, according to the manufacturer's specifications; Applied Biosystems). Amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems) with the following conditions: 10 min at 95 °C, 35 cycles composed of 30 s denaturing at 95 °C, 30 s annealing at 57 °C for combination 1, 45 °C for combination 2, 48 °C for combination 3, 52 °C for combination 4, 55 °C for combination 5, 52 °C for Mu10 and 55 °C for G1A, 1-min extension at 72 °C, and as a final extension step, 7 min at 72 °C. We repeated each amplification four times. The PCR products were mixed in three multiplexes (1st: 2 μL G1A, 3 μL G10B/G10C, 5 μL Mu61/Mu05; 2nd: 3 μL G1D/Mu15, 7 μL G10P/G10X; 3rd: 5 μL Mu10, 5 μL G10O/G10L). One μL of this multiplex was added to a 10 μL mix of formamide and ROX 350 (10:0.2), and then loaded on an automatic sequencer ABI3100 (Applied Biosystems, Foster City, California). The gels were analyzed using Genemapper (version 3.0) software package (Applied Biosystems, Foster City, California). The same rules as described above were applied for defining homozygous and heterozygous loci.

A new quality index Miquel et al. (2006) was calculated for each sample and locus. The loci G10P, Mu05 and Mu61 were discarded from the analysis because of their low quality indices (below 0.6). Finally, genotypes were obtained based on 15 loci.

2.3.4. *Calculating the probability of identity*

Using the software GIMLET version 1.3.1 (Valière, 2002), and both datasets (6 and 15 loci), we computed the probability of identity, i.e. the overall probability that two individuals drawn at random from a given population share identical genotypes at all typed loci (Paetkau and Strobeck, 1994). We also computed the probability of identity among siblings (Waits et al., 2001).

2.3.5. *Estimating current population size using rarefaction indices*

Following the method described in Kohn et al. (1999), we compared the 6-loci genotype of each sample with all those drawn previously and calculated the population size as the asymptote of the relationship between the cumulative number of unique genotypes and the number of samples typed. This curve is defined by the equation $y = (ax)/(b + x)$, where a is the asymptote, x the number of feces sampled, y the number of unique genotypes, and b the rate of decline in the value of slope. Eggert et al. (2003) derived another estimator with a similar equation; $y = a(1 - e^{bx})$. These are referred to as the Kohn and Eggert methods, respectively. We analyzed data with the software package GIMLET version 1.3.1 (Valière, 2002), with 1000 random iterations of the genotype sampling order. Rarefaction equations were run using R software (version 1.7.1; available at http://www.r-project.org). Confidence intervals were calculated using the iterative approach, which is usually employed for rarefaction curves. However, this gives an indication of only the sampling variance and not the estimator variance.

2.3.6. *Investigating the genetic signature of the bottleneck*

At selectively neutral loci, populations that have experienced a recent reduction of their effective population size exhibit a characteristic mode-shift distortion in the distribution of allele frequencies (alleles at low frequency (<0.1) becoming less abundant; Luikart et al., 1998) and develop heterozygosity excess (Cornuet and Luikart, 1996). We used a Bayesian approach to detect and date a potential bottleneck in the Deosai bear population. This method is implemented in the MSVAR program (Beaumont, 1999) available at http://www.rubic.rdg.ac.uk/~mab. MSVAR calculates the Bayesian posterior distribution of demographic and mutational parameters, using a Markov Chain Monte Carlo approach. Mutations are assumed to occur under a stepwise mutation model with a rate $\theta = 2N_0\mu$, where μ is the locus mutation rate; the change in population size is assumed linear or exponential. The model assumes demographic history in a single stable population that was of size N_1 t_a generations ago and subsequently changed gradually in size to N_0 over the period from t to the current time. The program estimates two demographic parameters $t_f = t_a/N_0$ and $r = N_0/N_1$, where r indicates the population trend (population expansion if $r > 1$; population decline if $r < 1$).

For calculations we used the exponential growth models with the default parameters, as it is more suitable than the linear growth model for modeling population changes over a shorter time scale (Beaumont, 1999). For each population, 2×10^8 updates were calculated and only the last 90% of the chains were used. The model was run twice to test the general

stability of the solution from the Markov chain. In addition, we estimated the time since the population had started to decline (t_a) with $t_a = t_f * N_0$ and N_0 corresponding to the estimated population size, as well as the ancestral population size (before the decline), with $N_1 = N_0/r$.

2.3.7. Estimating nuclear DNA diversity, Hardy Weinberg equilibrium and linkage disequilibrium

Based on the 15 loci genotypes, we ran population genetic analyses using the softwares GENEPOP version 3.4 (Raymond and Rousset, 1995) and GENETIX version 4.02 (Belkhir et al., 1996–2004). Nuclear genetic diversity was measured as the number of alleles per locus (A), the observed heterozygosity (Ho), as well as Nei's unbiased expected heterozygosity (He) (Nei, 1978). Deviations from Hardy–Weinberg equilibrium were tested using an exact test. For loci with more than four alleles, a Markov chain was used to obtain an unbiased estimate of the exact probability. The Markov chain was set to 100 batches, with 5000 iterations per batch and 10 000 steps of dememorization. Global tests across loci for heterozygote deficiency and heterozygote excess and pairwise tests for linkage disequilibrium were performed using Fisher's method (Sokal and Rohlf, 1994) with 10,000 batches and 10,000 iterations per batch.

2.3.8. Comparing genetic diversity with other brown bear populations

We compared the genetic diversity of the Deosai population with the one from other documented bear populations in Europe and North America (A, Ho and He when available). However the values given in the literature cannot be compared directly with our data as they do not represent the same number of individuals and the same set of loci. Consequently, we took the opportunity of having the whole dataset from the Scandinavian brown bear population (Bellemain, 2004) for a comparison based on the same number of individuals and the same loci. A random selection of 28 bears, in each of the 3 subpopulations of the Scandinavian genetic dataset (M, N and S; Waits et al., 2000), was repeated 1000 times to estimate genetic diversity (A, He, Ho) and compare it with the corresponding values in the Deosai population.

2.3.9. Assessing relatedness

Based on the 15 loci genotypes of the different individuals identified in the population, we calculated pairwise genetic relatedness between pairs of individuals using Wang's estimator (Wang, 2002) and the software SPAGeDi version 1.0 (Hardy and Vekemans, 2002). This estimator includes (1) low sensitivity to the sampling error that results from estimating population allele frequencies; and (2) a low sampling variance that decreases asymptotically to the theoretical minimum with increasing numbers of loci and alleles per locus (Blouin, 2003). 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; a value of 0.5 indicates that 50% of the alleles are shared (e.g. parent/offspring or siblings relationship). Unrelated individuals have relatedness values ranging from 0 to −1 with the more negative values indicating greater differences in the genotypes of the individuals. We also used the genetic dataset for the Scandinavian subpopulations (M, N and S) to compare the level of pairwise relatedness between the Deosai population and those 3 subpopulations (using the same loci).

3. Results

3.1. Individual identification, probability of identity and reliability of the data

Totally, 136 samples were collected and 63 (~46%) of those samples were successfully amplified for 4–7 loci (including the sex locus). Twenty-three samples were from females, 37 from males and the sex could not be determined for three samples.

The data were judged to be reliable due to a high global quality index among successfully amplified samples (Fig. 2). Nine samples were discarded for further analysis due to their low quality index (below 0.5; Fig. 2). Finally, 54 samples typed for 6–7 loci were considered. Among those 54 samples, 28 individual genotypes were obtained (16 males, 10 females and 2 individuals of unknown sex). Each multilocus genotype was found from 1 to 5 times, with a mean of 2.22 ± 1.08 (SE) times. One sample for each of the 28 genetically identified individuals was further typed with 9 more microsatellites. The mean quality index per sample was 0.85 ± 0.13 for the 54 samples typed using 6 microsatellite loci and 0.91 ± 0.10 for the samples typed using 15 microsatellite loci.

Age of the feces was estimated for all but 11 samples. There was a significant negative correlation between the age of fecal samples and the proportion of positive amplification (Spearman's $\rho = -0.279$; $p = 0.01$) (Fig. 3) as well as between the age of fecal samples and the quality index (Spearman's $\rho = -0.271$; $p = 0.02$).

The probability of identity among the six amplified microsatellite loci for unrelated individuals was 1.881e−05 and 1.206e−02 for related individuals (sibs), thus we could identify each individual reliably. The probability of identity among the 15 amplified microsatellite loci unrelated individuals was 5.827e−10 and 1.329e−04 for related individuals. This allowed us to perform reliable parentage and relatedness analyses.

3.2. Estimating current population size

The population size estimates varied depending on the rarefaction equation used. The Kohn's estimate yielded a population size of 47 bears (95% CI: 33–102), whereas the Eggert's estimate gave a size of 32 bears (95% CI: 28–58).

3.3. Investigating the signature and age of the bottleneck

The analyses of the population's expansion and decline using MSVAR, based on the exponential growth model (Beaumont, 1999) gave the following values: $\log_{10}(r) = -2.423$, $\log_{10}(t_f) = 0.297$, $\log_{10}(\theta) = -1.410$. The low r value ($r < 1$) implies that the original population size declined to current population size. Considering the mean population size estimates for each rarefaction equation (see above), the number of generations

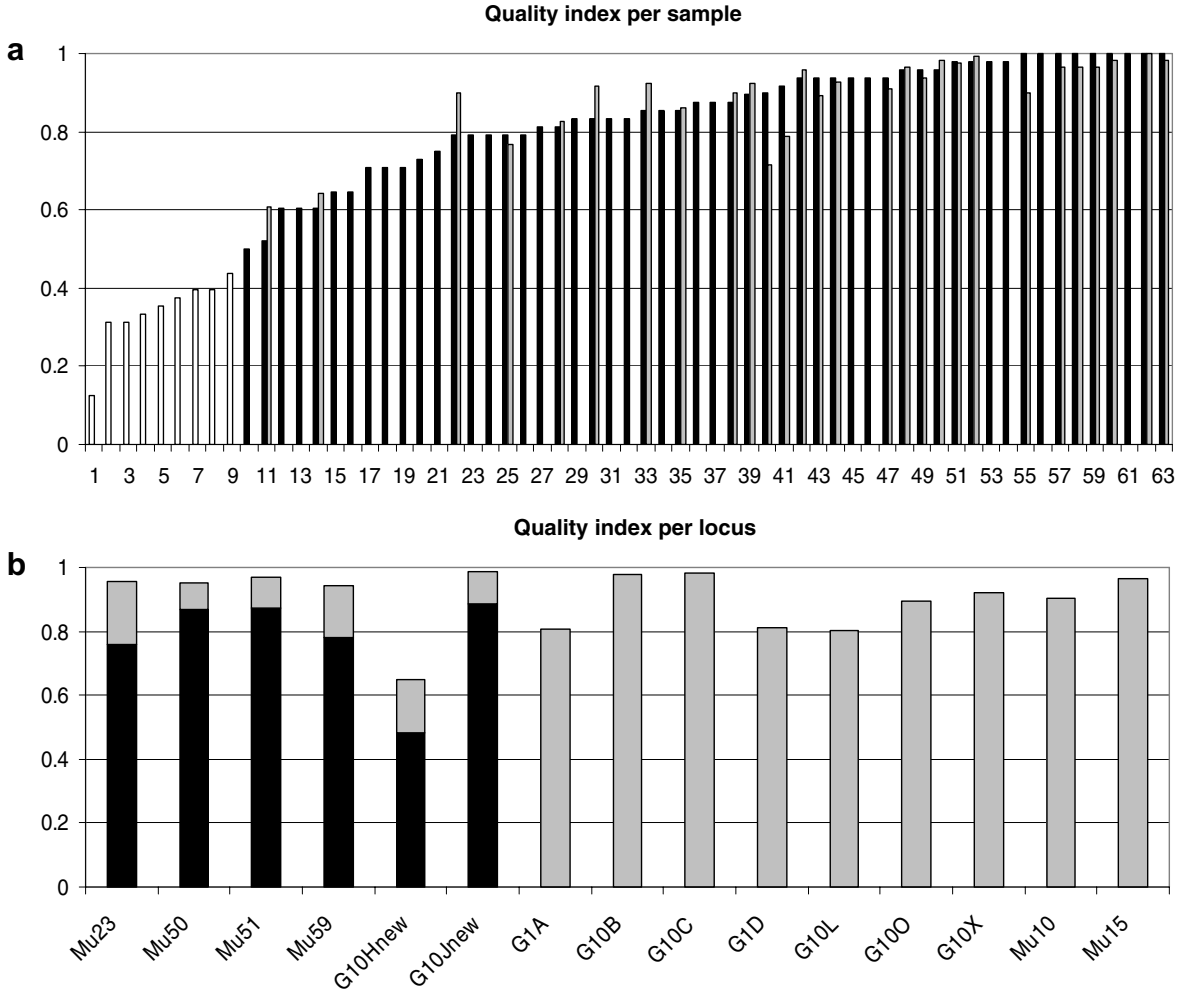

Fig. 2 – Quality indices (QI) per sample (a) and per locus (b) for successfully amplified genetic samples from brown bears in Deosai National Park, Pakistan. Black bars indicate QI for samples typed with 6 loci (for individual identification), grey bars indicate QI for samples typed with 15 loci (further analysis) and white bars indicate samples discarded from the analysis (because of their low QI).

since the population started to decline (t_a) was estimated to be between 63 and 93 and the ancestral population size ($N1$) ranged from 8000 to 11,750 individuals.

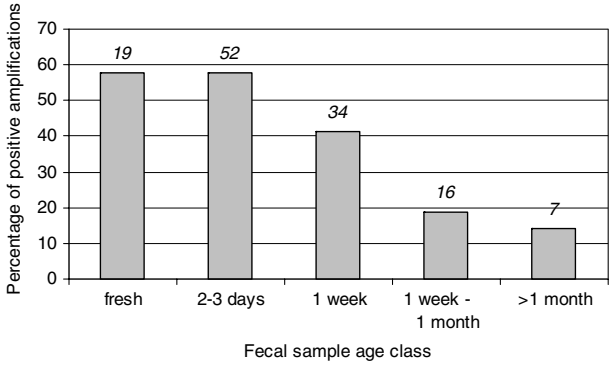

Fig. 3 – Success of brown bear fecal DNA amplifications from Deosai National Park, Pakistan, according to the age class of the fecal samples. Numbers above the bars, represent the sample size of each age class.

3.4. *Nuclear DNA diversity, Hardy–Weinberg equilibrium and linkage disequilibrium*

The number of alleles per locus among the 28 individual genotypes ranged from 2 to 7, with an average of 3.87 ± 1.36 (Table 1). The mean observed heterozygosity was 0.557, a value not significantly different from the unbiased expected heterozygosity (0.548). Global tests showed that the population is in Hardy–Weinberg equilibrium, although three loci (G10L, G10O, Mu10) had a significant deficiency in heterozygotes at the $p < 0.05$ level (Table 1). The overall multilocus Fis value was −0.016. Statistical tests for linkage disequilibrium were computed for all pairs of loci, and 15 of 105 tests revealed significant results ($p < 0.05$).

3.5. *Comparing genetic diversity with other bear populations*

The level of heterozygosity in the Deosai bear population ($Ho = 0.557$) was lower than in other bear populations in North America that are considered to have a good conservation sta-

Table 1 – Nei's unbiased expected (He) and observed (Ho) heterozygosities, and deviation from Hardy Weinberg equilibrium by locus from fecal samples of brown bears from Deosai National Park, Pakistan

Locus	Alleles	Allelic frequencies	He	Ho	P
Mu23	136	0.232	0.770	0.893	
	140	0.339			
	144	0.161			
	146	0.054			
	150	0.214			
Mu50	92	0.643	0.541	0.571	
	94	0.125			
	96	0.036			
	100	0.196			
G10B	136	0.382	0.466	0.518	
	150	0.618			
Mu59	95	0.25	0.830	0.857	
	109	0.196			
	111	0.054			
	113	0.089			
	115	0.036			
	117	0.214			
	119	0.161			
G10Jpak	80	0.518	0.656	0.678	
	84	0.089			
	86	0.232			
	88	0.161			
G1D	171	0.17	0.642	0.679	
	175	0.038			
	177	0.302			
	179	0.491			
Mu51	119	0.714	0.425	0.50	
	121	0.268			
	127	0.018			
G10Hpak	241	0.442	0.602	0.76	
	243	0.115			
	245	0.423			
	249	0.019			
G1A	189	0.593	0.496	0.5	
	191	0.019			
	193	0.389			
G10C	104	0.4	0.492	0.518	
	108	0.6			
G10L	143	0.204	0.773	0.583	0.009
	155	0.224			
	157	0.286			
	159	0.265			
	163	0.02			
G10O	193	0.019	0.037	0.037	
	195	0.981			
G10X	142	0.849	0.281	0.115	0.023
	154	0.057			
	156	0.057			
	158	0.038			
Mu10	140	0.094	0.656	0.5	0.0002
	142	0.057			
	150	0.019			
	152	0.434			
	154	0.396			

(*continued on next page*)

Table 1 – *continued*

Locus	Alleles	Allelic frequencies	He	Ho	P
Mu15	137	0.018	0.527	0.556	
	139	0.473			
	141	0.509			
Average			0.548	0.557	

Only significant *P*-values are shown ($P < 0.05$).

tus (*Ho* = 0.78 in North America; Paetkau et al., 1998 and *Ho* = 0.66–0.76 in different regions of Canada and USA; Waits et al., 1998). However, it is comparable to the level of heterozygosity in the Yellowstone area (*Ho* = 0.55; Paetkau et al., 1998) and higher than the level observed in some isolated populations such as the Kodiak Islands in Alaska (*Ho* = 0.26; Paetkau et al., 1998) or the Pyrenees in France (*Ho* = 0.39; Taberlet et al., 1997).

In comparison with each of the three subpopulations in Scandinavian bears, Deosai bears had a significantly lower number of alleles and observed and unbiased expected heterozygosity (for the same number of individuals and loci subsampled; Table 2). When compared to the mean genetic characteristics in the entire Scandinavia, the expected heterozygosity in the Deosai population is reduced by 17.5% and the number of alleles per locus by 44%.

3.6. *Assessing relatedness*

The average pairwise relatedness in the Deosai bear population was 0.0265 ± 0.292 (SE). This was not significantly different from the average pairwise relatedness in the subpopulations of the Scandinavian bears for the same loci (paired *t*-tests for each subpopulation: N: $r = -0.0232 \pm 0.044$; $p = 0.231$; S: $r = 0.015 \pm 0.044$; $p = 0.206$; M: $r = -0.001 \pm 0.032$; $p = 0.052$).

4. Discussion

4.1. *Quality of the genetic data*

We ensured a high reliability of the genetic data by repeating amplifications (multi-tubes approach) and selecting samples with high quality indices. The probability of misidentification was low, allowing us to identify unambiguously each individual. Therefore, we are confident that we have not overestimated the number of individuals in the fecal sampling.

The amplification success was correlated negatively with the age of fecal samples. Amplification success was relatively good (~58%) for fresh feces or feces that were only 2–3 days old and dropped to 41% for 1 week old samples, but this rate might still be acceptable. However, samples older than one week had a poor amplification success. We recommend, for future studies in Deosai, that fecal samples older than one

Table 2 – Comparison of the genetic diversity of brown bears between the Deosai population in Pakistan and the three subpopulations in the Scandinavian genetic dataset (mean over 28 randomly and repeatedly chosen individual bears)

	Pakistan			Scandinavia South			Scandinavia Middle			Scandinavia North		
	A	He	Ho	A	He	Ho	A	He	Ho	A	He	Ho
Mu23	5	0.77	0.89	7	0.70	0.73	7	0.82	0.83	6	0.72	0.70
Mu50	4	0.54	0.57	7	0.74	0.72	7	0.79	0.76	9	0.71	0.69
Mu51	3	0.43	0.50	7	0.78	0.80	8	0.77	0.75	8	0.76	0.74
Mu59	7	0.83	0.86	10	0.76	0.77	11	0.83	0.86	11	0.83	0.83
G10Jnew	4	0.66	0.68	6	0.57	0.58	6	0.66	0.66	7	0.75	0.75
G10Hnew	4	0.61	0.76	8	0.59	0.58	8	0.53	0.47	11	0.74	0.74
G1A	3	0.51	0.50	6	0.63	0.69	5	0.71	0.70	7	0.67	0.63
G1D	4	0.64	0.77	7	0.61	0.59	5	0.66	0.65	8	0.74	0.79
G10B	2	0.48	0.52	5	0.69	0.68	8	0.64	0.69	8	0.74	0.70
G10C	2	0.49	0.52	5	0.69	0.66	5	0.67	0.69	6	0.68	0.68
G10L	5	0.77	0.58	7	0.77	0.79	7	0.69	0.63	8	0.81	0.74
G10O	2	0.04	0.04	3	0.38	0.38	3	0.36	0.36	3	0.12	0.12
G10X	4	0.28	0.12	4	0.54	0.56	5	0.65	0.62	7	0.54	0.53
Mu10	5	0.66	0.50	8	0.80	0.79	8	0.74	0.75	8	0.78	0.75
Mu15	3	0.53	0.56	4	0.66	0.66	4	0.53	0.50	5	0.51	0.52
Mean	3.80	0.55	0.56	6.27	0.66	0.67	6.47	0.67	0.66	7.47	0.67	0.66
SD	1.37	0.20	0.24	2.07	0.11	0.11	2.07	0.13	0.13	2.07	0.18	0.17
P-values				6.82e−07	0.0121	0.059	1.02e−05	0.008	0.065	6.98e−07	0.0006	0.0161

P-values represent the significance of paired *t*-tests performed between the Pakistan population and each of the three Scandinavian subpopulations.

week not be collected in order to optimize the cost and benefit of the genetic analyses.

Brown bears in Deosai are mainly vegetarians (Schaller, 1977; unpublished data of fecal analysis). Previous studies have suggested that plant secondary compounds can inhibit PCRs (Huber et al., 2002). However, this study demonstrated that reasonable brown bear DNA amplification can be obtained from fecal samples composed mainly of plants (Murphy et al., 2003).

4.2. *The genetic status of the brown bear population in Deosai*

The analyses performed from the fecal DNA dataset allowed us to answer important questions regarding the management and conservation of bears in the Deosai population. First, the population size estimates provided by the two rarefaction indices are in the same order of magnitude as the numbers derived from the field censuses, which gives us confidence that those results are realistic. The census carried out during summer 2004 recorded 38 bears from the Deosai National Park, with a density of 19 bears per 1000 km^2 area (Nawaz et al., 2006). Based on this, the Eggert method seemed to underestimate the population size, whereas Kohn's method seemed to be more realistic, although the upper limit of the confidence intervals seems to be an overestimate. Unfortunately, the small sample size and small number of recaptures prevented us from using the MARK method, which is thought to give better estimates of population sizes (Bellemain et al., 2005). Considering the minimum number of individuals captured from the fecal samples (28) and the rarefaction method estimates, the field estimates appear to be conservative, though they fall within the range of the other estimates. Field methods usually give underestimates of wild populations, particularly for elusive animals (Solberg et al., 2006). The open terrain of the Deosai plateau, which allows bears to be observed, the small population size, distinctive marks on many bears, and the expertise that the field staff had gained over a period of 12 years from observing bears, probably contributed to the realistic observation-based estimates in Deosai National Park. We conclude that approximately 40–50 bears were present in the park in 2004.

The results from the analysis using the program MSVAR suggested that a decline in the Deosai population occurred approximately 63–93 generations ago using the mean estimates given by the rarefaction analysis and 80–100 generations ago, using a more realistic population size of 40–50 individuals. This period approximately corresponds to 800–1000 years ago, with a generation time of 10 years (calculated using the software RAMAS, Ferson and Akçakaya, 1990 and considering an age of first reproduction of 6 years old). The ancestral population (before the decline; N1) was estimated to contain 8000–11,750 individuals using rarefaction estimates or 10,000–12,500 individuals using a more realistic population size of 40–50 individuals. This estimate seems realistic considering an approximate area of 200,000 km^2 of bear distribution range in northern Pakistan and Kashmir, which gives a density of about 55 bears per 1000 km^2. These results suggest that the brown bear population in northern Pakistan might have undergone an approximate 200–300-fold decrease during the last thousand years. This decline cannot be linked to a single event or phenomenon. It was probably affected by both natural (climatic and geological) and socio-political factors. In the medieval warm period (1000–1200 AD), the bears certainly formed a single, large population, with a contiguous habitat in Hindu Kush, Karakoram and Western Himalaya ranges. The historic phase of glaciations in High Asia identified as a "little ice age" (1180–1840 AD; Kuhle, 1997; Esper et al., 2002; Mackay et al., 2005) is considered to have been similar in extent to the Neogeological stages (Meiners, 1997) and may have acted as a proximal cause of decline, destroying part of the population and fragmenting the rest. The influence of a growing human population, including large deforestation in the Middle Ages (Bertrand et al., 2002), political unrest due to presence of the Tibetan army in the area and its clashes with local people and China (Sheikh, 1998; Rashid S, personal communication) and the spread of firearms in the late 19th century, probably contributed further to the population decline and did not allow bears to colonize in a natural way.

Third, we assessed whether the Deosai population is currently at risk of inbreeding depression. The population genetics analyses revealed that the level of nuclear genetic diversity of the Deosai population is globally lower than brown bear populations considered to have a good conservation status, such as in Scandinavia or North America. In addition, and for the first time, we made an unbiased comparison of nuclear diversity between two populations, based on the same loci and same number of individuals. This analysis supports the conclusion that the Deosai population harbors significantly less heterozygosity and a smaller number of alleles per locus than any of the three subpopulations in Scandinavia. However, this population is in Hardy Weinberg equilibrium and its level of relatedness is similar to that in the Scandinavian brown bear population. Therefore, the Deosai bear population does not appear to be at immediate risk of inbreeding depression. Its level of genetic diversity is comparable to the brown bear population in the Yellowstone area, USA, which has become an isolated remnant, separated from other brown bears for nearly a century (Paetkau et al., 1998). A similar scenario could be envisaged for the Deosai brown bear, which probably lost genetic diversity due to isolation and genetic drift in the last centuries and due to the currently small population size.

Our final goal was to examine the degree of isolation of the Deosai population. Four individuals in our genetic dataset showed private alleles at two different loci, suggesting that they could be migrants (or descendants from migrants) from outside of the study area. Field observations support this hypothesis. Brown bears also exist in the Minimerg and Astore valleys, which are adjacent to Deosai National Park. Movements of bears have been observed between these areas during recent surveys, and the Deosai population may have interchanged not only with bears in these valleys, but also with the bear populations in the Neelam Valley and in Indian Kashmir through these valleys (unpublished data). When we began our studies of the Deosai brown bear population, we had expected to find genetic loss due to isolation and a small population; however, we documented a moderate level of genetic diversity. This strongly suggests that connectivity exists

between the Deosai population and the neighboring populations through movements of individuals.

4.3. *Conclusions and recommendations*

We have documented that the Deosai brown bear population shows moderate levels of diversity and is not at immediate risk of inbreeding. The population probably began to lose genetic diversity about 1000 years ago, when it began to decline from a single large population throughout northern Pakistan. This resulted in fragmentation of the population into smaller units that lost connectivity during the course of time. The population decline stopped in Deosai about 15 years, ago when the population received increased protection. Under a scenario of an isolated population, the population would probably suffer from inbreeding today. Therefore, we believe that the moderate level of genetic diversity observed has been maintained by gene flow with adjacent populations in Pakistan and India. Nevertheless, this level of genetic diversity is lower than in healthy populations in Europe or North America. Maintaining and improving the connectivity with adjacent populations in Pakistan and India will be of paramount importance for the long-term survival of this small population in future.

We suggest that future studies continue to monitor the population carefully, both with field observations and genetic analyses. Concrete management actions should aim at maintaining and improving connectivity with other populations to maintain or improve levels of genetic diversity. Otherwise, the population will continue to lose genetic diversity over time. Increasing the size and range of fecal sampling would not only allow a more precise estimate of the population size, but also give a better estimate of incoming gene flow.

Acknowledgements

The fieldwork for this study was supported by the Himalayan Wildlife Foundation (HWF), Islamabad. All field staff of the HWF, particularly M. Yunus, G. Murtaza and A. Khan, helped in collection of fecal samples. The cost of lab analysis was funded by the International Bear Association (John Sheldon Bevins Memorial Foundation), Norwegian University of Life Sciences, and Laboratoire d'Ecologie Alpine (LECA), University Joseph Fourier, France. We are grateful to L. Gielly, D. Rioux, C. Miquel and C. Poillot for their help in the laboratory work. Special thanks to Vaqar Zakaria and Dr. Anis ur Rahman, coordinators of HWF, for their support and suggestions during the study.

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