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The power of genetic monitoring for studying demography, ecology and genetics of a reintroduced brown bear population

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Abstract

Genetic monitoring has rarely been used for wildlife translocations despite the potential benefits this approach offers, compared to traditional field-based methods. We applied genetic monitoring to the reintroduced brown bear population in northern Italy. From 2002 to 2008, 2781 hair and faecal samples collected noninvasively plus 12 samples obtained from captured or dead bears were used to follow the demographic and geographical expansion and changes in genetic composition. Individual genotypes were used to reconstruct the wild pedigree and revealed that the population increased rapidly, from nine founders to >27 individuals in 2008 (λ = 1.17–1.19). Spatial mapping of bear samples indicated that most bears were distributed in the region surrounding the translocation site; however, individual bears were found up to 163 km away. Genetic diversity in the population was high, with expected heterozygosity of 0.74–0.79 and allelic richness of 4.55–5.41. However, multi-year genetic monitoring data showed that mortality rates were elevated, immigration did not occur, one dominant male sired all cubs born from 2002 to 2005, genetic diversity declined, relatedness increased, inbreeding occurred, and the effective population size was extremely small (Ne = 3.03, ecological method). The comprehensive information collected through genetic monitoring is critical for implementing future conservation plans for the brown bear population in the Italian Alps. This study provides a model for other reintroduction programmes by demonstrating how genetic monitoring can be implemented to uncover aspects of the demography, ecology and genetics of small and reintroduced populations that will advance our understanding of the processes influencing their viability, evolution, and successful restoration.

Keywords: effective population size, noninvasive genetic sampling, pedigree, spatial patterns, translocation, Ursus arctos

Introduction

Population monitoring is critical for effective management and conservation of wildlife (Nichols & Williams 2006). Information gathered through monitoring at different points in time can be used to understand population dynamics as well as detect system-level responses to management actions (Martin et al. 2007). Our ability to monitor wildlife populations, including elusive species and small endangered populations, has expanded with the use of DNA and population genetic data, which allow researchers to investigate demographic as well as genetic parameters and to quantify their

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temporal changes (i.e. genetic monitoring, sensu Schwartz et al. 2007). The utility of DNA-based monitoring programmes through noninvasive genetic sampling (NGS) is becoming increasingly recognized in the wildlife community (Waits & Paetkau 2005; Long et al. 2008). However, only a small number of studies have used NGS for comprehensive genetic monitoring over multiple years.

Long-term monitoring is particularly important following reintroductions or translocations to assess the success of such programmes and to ensure prompt actions for improving the status and probability of population persistence (Miller et al. 1999). Demographic information such as reproduction, recruitment, mortality, age structure and sex ratio need to be monitored at biologically relevant time intervals to evaluate vital rates and their variances, population trends, as well as the impact of demographic and environmental stochasticity (Sarrazin & Barbault 1996; Miller et al. 1999; Seldon 1999). Understanding the ecology of the reintroduced species, including patterns of geographical expansion from the reintroduction area, dispersal behaviour, connectivity with neighbouring populations, habitat use and identification of potential sink habitats, is critical for conservation and management (Miller et al. 1999; Frankham et al. 2002). In small populations, mating behaviour is also influenced by the small number of available mates, thus knowledge of mating and social structure is important for monitoring genetic structure, effective sizes and rate of inbreeding accumulation (Chesser 1991; Sugg et al. 1996; Sigg et al. 2005; Vonholdt et al. 2008).

Often reintroduction programmes release a small number of animals; therefore, effective population sizes are initially small and monitoring changes in genetic parameters is of primary concern for population viability (Lande & Barrowclough 1987; Frankham 2005). In the absence of gene flow, levels of coancestry are expected to rise; genetic diversity is lost as a result of drift and mating between relatives; adaptive evolutionary potential is limited; and accumulation of deleterious alleles is accelerated (Ralls et al. 1988; Hedrick 2000). Despite their recognized importance in the phases following the reintroduction, comprehensive monitoring programmes are often overlooked or their results are not reported (Sarrazin & Barbault 1996; Ostermann et al. 2001), and genetic monitoring is currently underused (Schwartz et al. 2007).

The recent translocation of brown bears (Ursus arctos) in northern Italy (Trentino) offers a unique case study for the application of genetic monitoring for small and reintroduced populations. Brown bears were originally present throughout most of the European continent, but during the 20th century, they were almost eradicated from southwestern Europe (Swenson et al. 2000). The decline of the bear population in northern Italy started during the 18th century and continued in the 20th century because of human persecution and habitat loss and fragmentation (Mustoni et al. 2003). Bears in the Italian Alps became isolated from the larger population that once extended from the Balkans to the Alps, and by the 1990s only a few relict individuals (~3) survived in Trentino; therefore, the population was considered functionally extinct (Mustoni et al. 2003). In 1999, a translocation plan was initiated for re-establishing a viable bear population in the central Alps (50–90 bears) (Mustoni et al. 2003). Between 1999 and 2002, nine bears captured in Slovenia were released in Trentino and were initially radiomonitored. After loss of radio-collars, genetic monitoring was preferred over traditional field-based methods for tracking the relict and reintroduced bears.

In this study, we focus on genetic monitoring of the small brown bear population in the Italian central Alps to follow its demographic and geographical expansion, plus changes in genetic composition since the translocation. Over 7 years (2002–2008), we used a combination of NGS of hair and faeces and collection of other tissue, blood, teeth and hair samples to (i) determine the wild pedigree, (ii) evaluate demographic and spatial expansion and (iii) monitor genetic diversity, inbreeding and effective population size. Our results provide important information for planning future monitoring and conservation actions for the bear population in the Italian Alps as well as a model for other reintroduction programmes.

Methods

Study area

The study area is ~26 000 km² mainly in the central Alps of northern Italy but also in trans-border areas of Germany, Switzerland and Austria (Fig. 1), encompassing a mosaic of natural and human impacted habitats. The elevation ranges from below 100 m to above 3900 m, comprising a variety of vegetation belts such as submediterranean, submontane, montane, subalpine and alpine (Mustoni et al. 2003). Towns in the valley bottoms and roads fragment the natural landscape. A major highway and railway run N–S through the study area along the urbanized Adige river valley. Human density is high (i.e. 81 inhabitants/km² in the Trento province) and mostly concentrated in the valleys, where the economy is dominated by tourism and agriculture. At higher elevation (1000–2000 m), diffuse farming and livestock are typical, and the overall density of residents is lower and concentrated around the main villages.

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The bear translocation was carried out in Trentino (Fig. 1). Translocated bears prefer deciduous and mixed forest, but areas with bushes and conifer trees are also used regularly (Preatoni et al. 2005).

**Sample collection and sampling design**

In 2002, scat and hair samples were collected opportunistically during a preliminary field data collection (Table 1) and founders’ genotypes were determined from known samples (De Barba et al. 2010). From 2003 to 2008, we sampled brown bears through a formalized noninvasive genetic monitoring programme. Hair and faecal samples were collected through hair trapping and opportunistic sampling, but in 2004 transect sampling was also applied (De Barba et al. 2010) (Table 1). Field work was carried out by personnel of various agencies following standardized procedures for sample collection and storage (De Barba et al. 2010). In 2008, we collected from the outside surface of the scat (Stenglein et al. 2010) rather than homogenizing the sample as in previous years. Tissue, blood, teeth and hair samples were collected when bear carcasses were found and during capture of problem bears.

Hair trap sampling design varied each year reflecting the distribution patterns of the bear population, experience gained in previous years, weather conditions and resources available (Table 1). Detailed description of aspects considered for establishing the sampling grid and hair trap methods can be found in De Barba et al. (2010). Grid extent varied from a minimum of 272 km² in 2007 to a maximum of 976 km² in 2008. Sites were visited for sample collection and lure replacement 21 days after initial setting in 2003–2004 and after 14 days in 2005–2008, for 5–8 sampling sessions from May to August/October. Midway through the entire sampling season, a variable proportion (34–93%) of traps was moved to a new location at least 0.5 km away.

**Table 1** Sampling design used for genetic monitoring in 2002–2008

<table>
<thead>
<tr>
<th>Year</th>
<th>Hair traps</th>
<th>Opportunistic sampling</th>
<th>Transect sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. grid cells</td>
<td>Grid extent</td>
<td>No. sessions</td>
</tr>
<tr>
<td>2002</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>2003</td>
<td>39</td>
<td>624 km²</td>
<td>8</td>
</tr>
<tr>
<td>2004</td>
<td>41</td>
<td>656 km²</td>
<td>7</td>
</tr>
<tr>
<td>2005</td>
<td>41</td>
<td>656 km²</td>
<td>7</td>
</tr>
<tr>
<td>2006</td>
<td>47</td>
<td>752 km²</td>
<td>7</td>
</tr>
<tr>
<td>2007</td>
<td>17</td>
<td>272 km²</td>
<td>7</td>
</tr>
<tr>
<td>2008</td>
<td>61</td>
<td>976 km²</td>
<td>5</td>
</tr>
</tbody>
</table>

na, not applied.
No., number.
Opportunistic sampling of hair and faeces was carried out throughout the bear range during the entire year (Table 1). Samples were collected during normal activities of the agencies in the field or following notification by third parties including cases of putative bear damages (De Barba et al. 2010). Transect sampling of hair and faeces was conducted in 2004 as part of a pilot study for evaluating the feasibility of this method compared to hair trapping and opportunistic sampling (De Barba et al. 2010). Seventeen transects (total length 145 km; mean transect length 8.5 km) were surveyed monthly May–October (Table 1).

Genetic methods

Genetic analyses were performed at the Italian Institute for Environmental Protection and Research, Italy, and the University of Idaho upon obtaining CITES permits (MA066574-0). Extraction methods, PCR protocols, protocols for individual identification, molecular sexing and for screening brown bear samples (Table 2) are described in detail in Appendix S1 and in De Barba et al. (2010). Eight loci were used for individual identification, and 10 loci were used for the population genetic analyses described later.

Wild pedigree

Parentage assignment based on the exclusion method was performed using the program GIMLET (Valière 2002) to determine whether new genotypes were descendents of the founders and to construct the pedigree since the translocation. The exclusion probabilities of the marker set \( P_{\text{ID(sib)}} \) for parentage assignment were computed. Field and observational data were used to refine the pedigree.

Demographic and spatial data

Demographic data were directly derived or estimated from the pedigree as well as from the complete monitoring history of each bear inferred from unique genotypes detected every year. For example, an adult bear not detected in 2003, but sampled in 2004, was considered alive in 2003. Mortality for different life stages (i.e. cubs, subadults, etc.) was estimated from age-specific composite life tables using presence/absence information from radiotelemetry during 1999–2002 and pedigree data from 2002 to 2008.

Cubs were defined as bears of age 0, and the transition to adulthood was established by the onset of sexual maturity for both sexes. For females, this was determined using reproductive data for the population, considering that brown bears in this region mate during May–July and that cubs are born the following year in January–February because of delayed implantation (Mustoni 2004). Because a smaller number of males reproduced, we used data reported in the literature to define life stages subsequent to age 0 for males. In other European bear populations, males are sexually mature as early as 3 years (Bellemain et al. 2006), but mating success is usually lower for 3- to 4- year-old males (Zedrosser et al. 2007). Thus, we considered 1- to 2- year-olds to be subadults and ≥3- year-olds as adults, but we distinguished younger males (3- to 4- year-old), from older males (≥5- year-old).

For estimating mortality rates, we considered individuals not detected with genetic sampling or observed for ≥2 consecutive years as mortalities because from our data, bears not detected for two subsequent years were never detected afterwards. In contrast, we observed two cases in which bears were not sampled in one year but were detected in following years. For this reason, 2008 was excluded from mortality estimates. Further, because it was not possible to know whether individuals not detected in 2008 only were dead or simply not sampled, we reported two population sizes for 2008 and two mortality rates considering those individuals as dead or alive. Generation time was estimated using deterministic projections in the program VORTEX v. 9.72 (Lacy et al. 2005) (Table S1).

Spatial patterns of the population and individual movements were evaluated by entering geographical

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Summary of the laboratory protocols used in 2002–2008 for individual and sex determination, and species ID screening (protocols are described in detail in Appendix S1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of loci analyzed for individual ID</td>
<td>8 (2002–2008)</td>
</tr>
<tr>
<td>( P_{\text{ID(sib)}} ) threshold</td>
<td>0.048 (2002)–0.0028 (2008)</td>
</tr>
<tr>
<td>Individual ID reliability protocol</td>
<td>Reference genotype approach (De Barba et al. 2010)</td>
</tr>
<tr>
<td>Sex ID</td>
<td>Amelogenin locus (Ennis &amp; Gallagher 1994)</td>
</tr>
<tr>
<td>Species ID screening for brown bear samples</td>
<td>150 bp segment of mtDNA control region (Murphy et al. 2000)</td>
</tr>
</tbody>
</table>

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coordinates of bear locations for each year in a geographic information system (GIS) database and visualizing them with ArcView v.3.3 (ESRI) software.

Genetic diversity, inbreeding and effective population size

Data analyses were conducted using 10-locus genotypes of the bears in the population in each year. Annual observed heterozygosity (H_E) was computed using the program GIMLET, and annual unbiased expected heterozygosity (H_E) and allelic richness (A) were calculated using FSTAT v. 2.9.3.2 (Goudet 2001). A paired t-test in SYSTAT v. 11 (SYSTAT Software Inc.) was used to evaluate whether a significant reduction in H_E and A occurred between the translocation and 2008. We used GENEPOL v. 3.4 (Raymond & Rousset 1995) to test for Hardy Weinberg equilibrium (HWE) and linkage disequilibrium (LD) for each locus in each population per year using probability exact tests, to obtain an overall (across loci) estimate of F_IS for the bear population in each year and test specifically for heterozygote excess and deficiency, and to examine changes in allele frequencies to detect the effect of genetic drift using \( \alpha = 0.05 \) and Bonferroni corrections for multiple comparisons.

We assessed inbreeding accumulation in the population by deriving pedigree estimates of inbreeding coefficients (Wright 1922) for each bear and average inbreeding (F). To monitor changes in relatedness levels in the population, program PEDITREE (Van Berloo & Hutten 2005) was used for obtaining kinship coefficients for single individuals in the pedigree and mean kinship values for the population in each year were derived. Effective population size (\( N_E \)) was estimated using demographic and genetic approaches. Our goal was to compare the performance of different genetic-based \( N_E \) estimators to the demographic approach, for evaluating accuracy of the genetic methods for estimating \( N_E \) in our study system in the future when we are unlikely to have detailed demographic data. For the demographic method, we estimated \( N_E \) in every year using the minimal model developed by Nunney & Elam (1994) based on ecological data for organisms with overlapping generations:

\[
N_E = 4(r(1 - r)TN)/[rA_f(1 + I_{af}) + (1 - r)A_m(1 + I_{am}) + (1 - r)I_{bm} + I_{bsd}].
\]

\( N \) is the number of adults in the population; \( T \) is the mean generation time of males and females; \( r \) is the adult sex ratio; \( A_f \) and \( A_m \) are the mean adult lifespan of females and males; \( I_{af}, I_{am} \) are their respective standardized variance; and \( I_{bf}, I_{bm} \) are the standardized variance in reproductive success of females and males. Demographic parameters entered in the equation were estimated from the yearly pedigree data (Table S2). The number of genotypes corresponding to adult bears identified in each year was entered for \( N \). We used the mean generation time of the two sexes from VORTEX for \( T \). Values for \( A_f, A_m, I_{af} \) and \( I_{am} \) were computed according to Nunney & Elam (1994) for type 2 survivorship because mortality of adult bears is independent of age (Saether et al. 1998). \( A_f = 1/(1-\nu_f) \), \( \nu_f \) is the adult survival rate of sex i from the bear life table, and \( I_{af} = \nu_f \), \( I_{bf} \) and \( I_{bm} \) were calculated as standardized variances in the number of offspring that survived to 1 year.

We applied several methods to estimate \( N_E \) from genetic data as variance effective size. We used the program NEESTIMATOR v. 1.3 (Peel et al. 2004) to implement two single sample estimators, one based on LD (Bartley et al. 1992) and the other on heterozygote excess (Pudovkin et al. 1996), and three temporal estimators: the moment based approach (Waples 1989), a Bayesian likelihood approach in program TM3 (Berthier et al. 2002) and a pseudo-likelihood approach in program MLNE (Wang 2001). We set \( N_{E \ max} = 100 \) in TM3 and MLNE and used 1000 iterations in TM3.

Results

Sample collection

A total of 2781 samples were collected noninvasively, 2712 using hair trapping and opportunistic sampling during 2002–2008 (Table 3), and 69 using transect sampling in 2004 only. In addition, 12 samples (eight hair, two tissue, one blood, one tooth) were obtained from dead individuals (8) or captured bears (4).

Microsatellite genotyping and sex determination

From 2002 to 2008, 2296 samples were analyzed. Microsatellite genotyping success rate was 14–82% for different sample types and was highest for hair samples collected at hair traps (Table 3). Transect sampling in 2004 was less efficient than other methods for detecting individual bears (De Barba et al. 2010) and was not applied afterwards. The number of individual bears identified increased each year (Table 3), and genotype detection was used to construct the monitoring history for bears in the population (Fig. 2). Each unique genotype was detected in 1–327 (mean 31.2) samples.

The \( P_{(D_{sib})} \) for the eight microsatellite loci used for individual ID ranged from 0.00065 to 0.0008 providing high discriminatory power. In addition, \( P_{(D_{sib})} \) using five (2002–2004), six (2005) and seven (2006–2008) loci was below the threshold set for each year (see
Appendix S1) (Table 2). All 2002–2007 eight-locus reference genotypes differed at ≥3 loci [there were no 1, 2 mismatch-pairs (Paetkau 2003)]. In 2008, two genotypes for new cubs differed only at one locus out of eight; however, they differed at three loci when the panel of ten markers was used.

Genotyping error rate per locus because of allelic dropout was 2.8–9.4% for hair trap samples, 5.3–23.3% for hair collected opportunistically and 17.3–44% for scat samples. Per locus error rates because of false alleles were 0–1.6% for hair trap samples, 1–3.4% for opportunistic hair and 2.4–9.9% for scat. Every year during 2003–2008, we replicated 15% of hair samples that were genotyped from only one PCR amplification to check for human errors caused by sample mix-up or mislabelling, and all matched the original genotype.

Table 3 Samples collected, individual identified and genotyping success over 7 years of genetic monitoring using hair trapping and opportunistic sampling

<table>
<thead>
<tr>
<th>Year</th>
<th>Hair trapping</th>
<th>Opportunistic sampling</th>
<th>Individual identified</th>
<th>Genotyping success</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hair</td>
<td>Hair</td>
<td>Scat</td>
<td>Hair (%)</td>
</tr>
<tr>
<td>2002</td>
<td>NA</td>
<td>15</td>
<td>16</td>
<td>31</td>
</tr>
<tr>
<td>2003</td>
<td>227</td>
<td>73</td>
<td>63</td>
<td>136</td>
</tr>
<tr>
<td>2004</td>
<td>480</td>
<td>85</td>
<td>167</td>
<td>252</td>
</tr>
<tr>
<td>2005</td>
<td>153</td>
<td>121</td>
<td>94</td>
<td>215</td>
</tr>
<tr>
<td>2006</td>
<td>255</td>
<td>137</td>
<td>84</td>
<td>221</td>
</tr>
<tr>
<td>2007</td>
<td>135</td>
<td>88</td>
<td>111</td>
<td>199</td>
</tr>
<tr>
<td>2008</td>
<td>83</td>
<td>128</td>
<td>197</td>
<td>325</td>
</tr>
</tbody>
</table>

NA, not attempted.

Fig. 2 Monitoring history of the translocated brown bear population in the Italian central Alps showing how single individuals were monitored through radiotelemetry, genetic sampling and observations since the reintroduction, and the level of certainty in the data. Founders (in bold) were initially radiocollared and problem bears were also captured and radiomonitored at different times during the study; death (†) was reported only when bear carcasses were found or bears were legally killed; some bears were genetically sampled only when found dead (†g); one problem bear was removed (R) from the population and placed into captivity during the study; observational data refers to bear cubs not detected genetically but observed with their mother and siblings, (m = males, f = females).
A total of 79 samples from 37 individuals were processed for molecular sexing. Error rate for X and Y PCR fragment dropout was 8.3%. Three hundred ninety-three of 827 samples that failed microsatellite genotyping were identified as bear samples after mtDNA fragment analysis.

**Wild pedigree**

The exclusion power of the marker set used was high (P1 0.995–0.999, P2 0.950–0.979). All new individuals were identified as progeny of introduced bears or their descendents (Fig. 3). Field observational data were used to refine the pedigree in three cases: bears BJ1, MJ2G1 and M6 (Fig. 3) were genetically sampled for the first time in 2006, 2007 and 2009 (after the completion of this study), respectively, but their mothers were sampled and observed with cubs in the previous year (BJ1, MJ2G1) and two years before (M6). Therefore, BJ1 and MJ2G1 were assumed to be born 1 year and M6 2 years prior to their detection with genetic sampling (Fig. 2). If this assumption is correct, the litter MJ2J1/MJ2G1 would have been produced by two different fathers providing evidence for multiple paternity.

**Demographic and spatial data**

DNA analyses of a tooth sample recovered from the remains of an old bear found in spring 2002 revealed the genotype of one of the autochthonous remnant bears. All other genotypes identified in following years were assigned to founders or their progeny (Fig. 3); therefore, indigenous bears were assumed to be dead without genetic exchange with the translocated bears, and no immigration was detected. The bear population increased from the initial nine founders to a population size of 27–31 in 2008 with a growth rate $\lambda = 1.17–1.19$ (Fig. 2, Table 4a). Since 2002, 10 females (five founders, five born after translocation) reproduced, producing 35 cubs. Three females born after the translocation had their first litter when they were 3 years old; therefore, we classified 1- year-old females as subadults and ≥2- year-olds as adults. For females that had multiple litters, the interbirth interval was 2 years (Fig. 3). In contrast, no males born in the study area have reproduced yet, and the two male founders that reproduced were ≥7 years old when their first offspring was born. The older founder sired all cubs from 2002 to 2005, and a second male founder started reproducing in 2006 (Fig. 3, Table 4a).

Nine females (possibly 10) born after the translocation survived to adulthood, while only two males did in 2008 (Fig. 4). As a consequence, adult sex ratio was skewed towards females (Table 4a). Average fecundity of females was 0.42; however, females that had offspring when they were 3–4 years old had lower fecundity rate (0.17) than older females (0.54).

Since the translocation programme was completed, death was documented for seven bears (including one founder), one founder was moved into captivity, and eight (four founders) were not observed or detected with genetic sampling for ≥2 years (Figs 2 and 3, Table 4a). Mortality rates for cubs and subadults were higher for males than females, but female adults had higher mortality rates than male adults, and young adult males had higher mortality rates than older males (Table 4b). Mean generation time was 11.5 years (8.3 years females, 14.6 years males).

Most bears were sampled in the surroundings of the translocation site (Fig. 1a). The greatest movement distance from the translocation site or natal area was...
42 km for a female and 163 km for a male. All females and the two male founders that reproduced were sampled within a 34 km radius of the translocation site (Fig. 1b). Subadult and young adult males were sampled at the greatest distances and were responsible for all of the movements detected outside of the reintroduction area. The greatest distance between any two locations for the same individual was 54 km for females and 215 km for males.

Genetic diversity, inbreeding and effective population size

Genetic diversity was relatively high; however, $H_e$ and $A$ showed a declining trend over the study period and were significantly lower in the 2008 population than in the founders ($t = 2.487, P = 0.035$ for $H_e$; $t = 4.776, P = 0.001$ for $A$) (Table 5). Single locus tests revealed deviation from HWE in three cases: locus G10M in 2006 ($P = 0.03$) and in 2008 ($P = 0.0068$), and locus G10X in 2008 ($P = 0.0062$). The bear population was in HWE (global test) each year ($P > 0.05$) except in 2006–2008 ($P \leq 0.01$) (Table 5). We found no significant changes in allele frequencies across years at the population and single locus levels ($P > 0.05$).

We detected one inbred cub in 2006 and two in 2008 resulting from two father–daughter mating events ($F = 0.25$) (Fig. 3), which were reflected in the pedigree inbreeding levels (Table 5). Mean kinship increased from 0.075 to 0.134 between 2002 and 2008 (Table 5).

Demographic estimates of $N_e$ using the ecological approach were extremely low ranging from 2.75 in 2002 to 3.08 in 2005 (Table 5). Genetic estimates of $N_e$ and performance of different estimators varied considerably. The LD method provided $N_e$ of 8.1 (6.9–9.5 95% CI), and the heterozygote excess method gave a point estimate of 4.9 (95% CI not available). Among the temporal methods, the Bayesian likelihood approach gave $N_e$ of 5.3 (3.8–9.9 95% CI), the moment estimator $N_e$ of infinity (12.5–infinity 95% CI) and the pseudo-likelihood estimator $N_e$ of 99.7 (0.8–99.7 95% CI).

---

**Table 4** Demographics of the bear population derived from seven years of genetic monitoring: (a) population growth, reproduction and sex ratio; (b) mortality rates; (No. = number)

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of unique genotypes sampled</th>
<th>No. of bears present</th>
<th>Lambda</th>
<th>Reproductive events</th>
<th>Number of cubs</th>
<th>Males reproducing</th>
<th>Adult sex ratio (m:f)</th>
<th>Cubs sex ratio at birth (m:f)</th>
<th>Documented deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>10</td>
<td>10</td>
<td>1.11</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2.6</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>2003</td>
<td>10</td>
<td>11</td>
<td>1.10</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2.5</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>2004</td>
<td>15</td>
<td>15</td>
<td>1.36</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>2.7</td>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>2005</td>
<td>17</td>
<td>18</td>
<td>1.20</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>2.7</td>
<td>3.1</td>
<td>0</td>
</tr>
<tr>
<td>2006</td>
<td>24</td>
<td>24</td>
<td>1.33</td>
<td>5</td>
<td>11</td>
<td>2</td>
<td>2.7</td>
<td>6.5</td>
<td>3</td>
</tr>
<tr>
<td>2007</td>
<td>23</td>
<td>24</td>
<td>1.00*</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2.8</td>
<td>2.1</td>
<td>0</td>
</tr>
<tr>
<td>2008</td>
<td>27</td>
<td>27–31</td>
<td>1.17–1.3†</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>4:10 (11)‡</td>
<td>4:4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><strong>Tot</strong></td>
<td><strong>16</strong></td>
<td><strong>34</strong></td>
<td><strong>2</strong></td>
<td><strong>4.9</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>18:16</strong></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td><strong>1.17–1.19</strong></td>
<td><strong>2.3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>1:0.8</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stage-specific mortality rates§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females (%)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Cubs</td>
</tr>
<tr>
<td>Subadults</td>
</tr>
<tr>
<td>Adults</td>
</tr>
</tbody>
</table>

*Lambda for 2007 was calculated including one bear (M6) that was only observed but not detected using genetic sampling in 2007.
†Lambda for 2008 was calculated including one radiocollared bear (JJ3) not detected using genetic sampling in 2008 (lower estimate) and four bears (DG3, DJ1G1, DJ3G1, M6) that were alive in 2007 but not detected in 2008 (higher estimate) (see methods).
‡Including one female alive in 2007 not detected in 2008.
§The range refers to mortality rates computed considering individuals alive in 2007 but not detected in 2008 (subadult DG3, cubs DJ1G1, DJ3G1, M6) as alive (lower value) or dead (higher value) (see methods).
Discussion

A number of recent long-term (>3 years) projects have demonstrated the utility of genetic monitoring for gathering diverse information on the study species (Rudnick et al. 2005; Fabbri et al. 2007; Hedmark & Ellegren 2007; Vonholdt et al. 2008; Kruckenhauser et al. 2009), but the value of comprehensive genetic monitoring through NGS and its application for wildlife reintroductions remains relatively unexplored, and only very few studies exist (Koelewijn et al. 2010). Our study provides a good example of the comprehensive knowledge that can be gained through multi-year noninvasive genetic monitoring for this and other small and reintroduced populations.

Wild pedigree

Studies of pedigrees of wild animals are essential for understanding processes driving population dynamics under natural ecological conditions (Haig & Ballou 2002; Pemberton 2008). Further, pedigree estimates of population genetic metrics, such as coancestry and inbreeding coefficients, are more accurate than marker-based estimates (Keller & Waller 2002). However, wild pedigree reconstruction is often constrained by the difficulty of sampling sufficiently large numbers of individuals (Kruuk & Hill 2008). Our study demonstrates that the ability to derive wild pedigrees is one of the most important benefits of genetic monitoring for small and reintroduced populations. Reconstructing the wild pedigree each year after the translocation allowed us to track demographic and genetic changes while also providing insight into the social structure and mating system of the population. Pedigree information obtained through genetic monitoring, such as the age of first reproduction for both sexes, the reproductive output of single individuals, inbreeding events and variance in reproductive success among founder males, could not have been gained by traditional field-based methods.

These data are also critical for understanding behavioural and biological factors influencing reintroduction outcomes and are useful for guiding subsequent reintroduction efforts. For example, the fact that only the older male founder sired all cubs during the initial 4 years and that the second male had his first litter at 7 years points to the importance of the age structure of the males released. In a promiscuous mammal such as the brown bear, males compete for mating and older and larger males have higher reproductive success (Craighead et al. 1995; Bellemain et al. 2006; Zedrosser et al. 2007). Our results, together with previous results from Sigg et al.

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Table 5 Estimates of genetic parameters for founders and the population from 2002 to 2008 using 10 microsatellite loci: $H_e$, expected unbiased heterozygosity; $H_o$, observed heterozygosity; $A$, allelic richness; $F$, mean inbreeding coefficient from pedigree; $K$, mean kinship from pedigree; $F_{IS}$, average individual inbreeding coefficient within each population; $N_{e demo}$, effective population size computed using the demographic method. Levels of significance (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$) are shown for the paired t-test comparison of $H_e$ and $A$ of the 2008 population to the founders, and for $F_{IS}$.

<table>
<thead>
<tr>
<th>Year</th>
<th>$H_e$</th>
<th>$H_o$</th>
<th>$A$</th>
<th>$F$</th>
<th>$K$</th>
<th>$F_{IS}$</th>
<th>$N_{e demo}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Founders</td>
<td>0.79</td>
<td>0.76</td>
<td>5.41</td>
<td>0.039</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>0.78</td>
<td>0.79</td>
<td>4.99</td>
<td>0.075 –0.014</td>
<td>2.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>0.76</td>
<td>0.79</td>
<td>4.72</td>
<td>0.095 –0.038</td>
<td>2.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2004</td>
<td>0.76</td>
<td>0.81</td>
<td>4.48</td>
<td>0.106 –0.079</td>
<td>2.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>0.74</td>
<td>0.83</td>
<td>4.48</td>
<td>0.121 –0.116</td>
<td>3.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2006</td>
<td>0.75</td>
<td>0.83</td>
<td>4.49</td>
<td>0.100 –0.115***</td>
<td>2.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>0.75</td>
<td>0.86</td>
<td>4.38</td>
<td>0.121 –0.155***</td>
<td>3.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>0.74*</td>
<td>0.85</td>
<td>4.55***</td>
<td>0.017</td>
<td>0.134 –0.152***</td>
<td>NC</td>
<td></td>
</tr>
</tbody>
</table>

NC: Reproductive success could not be estimated for 2008; therefore, $N_e$ was not computed.
Demographic expansion and geographical patterns

Monitoring survival and reproductive output of released animals is among the first steps for documenting the establishment of a self-sustaining population and hence the success of a reintroduction (Griffith et al. 1989). Studies undertaking this task are uncommon (Goossens et al. 2002; Sigg et al. 2005; Koolewijn et al. 2010) perhaps because of the significant effort necessary to acquire the desired information. In our study, we were able to decrease monitoring costs and efforts by using NGS and by implementing the sampling as part of the regular activities of field personnel (De Barba et al. 2010).

Over the seven years after reintroduction, the bear population approximately tripled in size with a growth rate (λ = 1.17–1.19) similar to an expanding Scandinavian brown bear population (Sæther et al. 1998) and higher than interior brown bear populations in North America (Pease & Mattson 1999; Wakkinen & Kasworm 2004; Garshelis et al. 2005). This potential to grow is reflected in a mean litter size that was comparable to other brown bear populations (Frković et al. 2001; Swenson et al. 2001; Schwartz et al. 2003) even though seven of 16 litters were produced by young females with lower fecundity. The early age (3 years) of female sexual maturity and the inter-birth interval of 2 years have been reported for other European populations (Dahle & Swenson 2003b; Zedrosser et al. 2004).

Although survival rates are higher than reported for other brown bear reintroductions in Europe (i.e. Kruckenhauser et al. 2009), a large number of animals are dead or suspected dead. We could not verify mortality for animals that were not detected for ≥2 years, but it is unlikely that a bear could go undetected for that time period because of the high density of the human population in the Alps, and the high degree of forest patrolling and management. Among the seven documented deaths, four were caused by human–bear interactions (i.e. car accidents), but it is suspected that a number of the nonverified mortalities could also be human induced. Only one female founder remains, and high mortality rates of subadult and young adult males resulted in the failure of recruitment among these age classes and skewed adult sex ratios. In addition, only two of the three male founders reproduced creating additional variance in founders’ reproductive contribution. Given its small size, the population is highly vulnerable to the stochastic and deterministic variation in these demographic parameters. Further, such aspects of the population dynamics and their interaction during this initial stage of the population establishment have important consequences for the evolution of population structure and genetic composition (Rhodes & Latch 2010).

Patterns of population expansion will also affect the demography and genetics of translocated populations. For example, natal dispersal may be the result of inbreeding avoidance or resource/mate competition mechanisms (Wolff 1993) with consequences for gene flow and postrelease mortality (Rhodes & Latch 2010). Similarly, social structure can influence the reproduction and survival of populations (Stoen et al. 2005). The spatial data collected through genetic monitoring in the postrelease phases will allow investigating these processes for the bear population in northern Italy.

The distribution of bear samples suggested that the immediate surroundings of the translocation site have been progressively occupied and are still used by most bears. In our study, spatial inferences can potentially be biased by unequal sampling intensity. However, all individuals were either released in the translocation site or born in its surroundings, and we sampled a high proportion of the population every year, allowing us to make general conclusions about bear spatial distribution and movements patterns.

The detection of all females within a limited distance from the translocation site during the study period suggests that this area comprises the core area of the population (Swenson et al. 1998). A feasibility study conducted prior to the reintroduction on ~6500 km² around the translocation site estimated sufficient suitable bear habitat to support a minimum viable population of ~40–60 bears (Dupré et al. 2000). Bear detections outside the core area and beyond the area considered in the feasibility study confirmed movements and settlements into new regions, suggesting the availability of a larger space and resources for population expansion. The map of bear samples (Fig. 1) depicts a high degree of range fidelity and natal philopatry of females and a greater level of roaming behaviour by males consistent with other bear studies (Dahle & Swenson 2003a; Schwartz et al. 2003; Proctor et al. 2004). As documented in other expanding brown bear populations (Swenson et al. 1998; Kojola et al. 2003), young males were found more often in peripheral areas than older males. In the Italian Alps, males were active dispersers at the age of 1.5 years.

Pedigree analysis and the spatial data confirmed the isolation of the reintroduced population from the brown bear population in the Balkans, particularly from Slovenia, the source of the founders (~300 km to the Slovenian border). The Adige valley and the highway are major barriers to dispersal in the East and only two...
bears are known to have crossed them. Another impediment to connectivity is the general fragmentation of the remaining Alpine habitat. Although bears are able to move between habitat patches, human activity and roads can limit long-distance dispersal (Proctor et al. 2004).

Genetic diversity, inbreeding and effective population size

For ensuring the success of reintroductions, it is necessary not only to quantify changes in the genetic composition of reintroduced populations, but to identify factors and processes that caused the observed changes so that proper conservation strategies for alleviating further losses of genetic variation can be implemented (Rhodes & Latch 2010). The significant decline in expected heterozygosity and allelic richness indicates that the reintroduced brown bear population in the Italian Alps has lost genetic diversity because of drift in less than a generation. Deviations from HW and linkage equilibrium conditions in 2008 are most likely reflective of the founding event and the small population size. Similarly, heterozygote excess could have been caused by differences in allele frequencies among individuals of the two sexes that can occur in small populations even with random mating (Allendorf & Luikart 2007). Further, the detection of two mating events between close relatives and the progressive increase in mean kinship indicate that, under persistent demographic isolation, inbreeding will be an immediate risk (Keller & Waller 2002). Our pedigree estimates of inbreeding and kinship assume that founders were unrelated and not inbred. However, high relatedness or past inbreeding among founding individuals will underestimate loss of genetic diversity and inbreeding derived from the pedigree (Ewing et al. 2008).

The demographic-ecological method provided the most conservative \( N_e \) estimates. The major advantage of this method is that it incorporates data from current population parameters such as sex ratio and variance in reproductive success. However, implementation of this approach for long-term monitoring is unlikely because it requires detailed demographic information. Thus, we compared the performance of genetic-based \( N_e \) estimators. The heterozygote excess and the Bayesian likelihood approaches were superior as they provided estimates closer to the ecological equation and had narrower confidence intervals (Bayesian method only). However, future application of the heterozygote excess method might be limited by the attenuation of heterozygote excess, the assumption of random mating and large sample size requirement (Leberg 2005). More importantly, our results illustrate that relatively accurate estimates of the variance effective size can be achieved using temporal methods when less than a generation passed between samples. The performance of the tested methods differed from other studies using a combination of demographic and genetic approaches (Kaeuffer et al. 2004; Rowe & Beebee 2004) underlining the importance of using multiple estimators and considering whether the underlying assumptions are appropriate for the study system.

Loss of genetic diversity because of founder effects and genetic drift has been documented for a variety of species (Ewing et al. 2008; Wisely et al. 2008). The rate of loss of genetic diversity and inbreeding accumulation is determined by the effective population size (Waples 2002). The estimated ratio of \( N_e/N \) for this bear population is \(~0.13–0.17\) (ecological in 2007 – Bayesian), and within the range estimated (0.037–0.27) for other brown bear populations worldwide (Paetkau et al. 1998; Miller & Waits 2003; Tallmon et al. 2004). \( N_e \) of the translocated bear population in northern Italy has remained extremely small and is far below the minimum of 50 recommended for short-term conservation (Allendorf & Ryman 2002). Therefore, the ability to counteract losses of genetic variation and the negative effects of inbreeding will depend on how long the population remains at such a small \( N_e \) and on the degree of demographic isolation.

Concluding remarks

The translocated brown bear population in the Italian central Alps has grown rapidly since the release of the initial nine founders, has high genetic diversity and is starting to expand beyond the translocation area. This information attests the success of the important early stages of the translocation. However, mortality rates of female founders, subadult males and young adult males are elevated; no immigration has occurred; genetic diversity is declining; inbreeding is an immediate risk; and the effective population size has remained extremely low. Further, because of its small size, the population is still vulnerable to stochastic processes that can lead to extinction. Recent studies on the genetic consequence of reintroduction on other species (Ewing et al. 2008; Grueber & Jamieson 2008; Wisely et al. 2008) have highlighted the importance of favouring conservation and management actions that allow \( N_e \) to increase rapidly after reintroduction through increasing population size and low rates of immigration. For the bear population in the Italian central Alps, prompt measures are needed to limit causes of non-natural mortality and facilitate gene flow from the Balkans before genetic variation deteriorates and inbreeding depression occurs.
Our study demonstrates how genetic monitoring can be implemented as a powerful tool for the conservation of small and reintroduced populations of free ranging animals through the efficient collection of comprehensive long-term data. The implementation of genetic sampling at biologically relevant time intervals allows managers to simultaneously collect pedigree, demographic, spatial, genetic and ecological data. All this information is critical for evaluating status and trend, enabling accurate assessment of viability and suitable conservation actions, and improving success of reintroduction programs.

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Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices.

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success of released male bridled nailtail wallabies. Biological Conservation, 125, 289–300.


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Supporting information
Additional supporting information may be found in the online version of this article.

Table S1 Parameters used in VORTEX for estimating generation time

Table S2 Parameters used for estimating $N_e$ with the ecological method

Appendix S1 Protocols for DNA extraction and amplification, screening of bear samples, and individual and sex determination.

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Table S1. Parameters used in VORTEX for estimating generation time for the translocated brown bear population in northern Italy. We report two mortality rates (lower and higher) considering individuals not detected in 2008 only as dead and alive respectively.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial population</td>
<td>21</td>
</tr>
<tr>
<td>Age of first offspring for females</td>
<td>3</td>
</tr>
<tr>
<td>Age of first offspring for males</td>
<td>7</td>
</tr>
<tr>
<td>Maximum age of reproduction</td>
<td>27</td>
</tr>
<tr>
<td>Maximum number of progeny per year</td>
<td>3</td>
</tr>
<tr>
<td>Sex ratio at birth (% males)</td>
<td>55.5%</td>
</tr>
<tr>
<td>% adult females breeding</td>
<td>41.2% (30.9% SD)</td>
</tr>
<tr>
<td>% female per yr producing 1, 2 and 3 offspring</td>
<td>18.75%, 43.75%, 37.5%</td>
</tr>
<tr>
<td>% adult males siring offspring</td>
<td>64.3%</td>
</tr>
<tr>
<td><strong>Male mean mortality rates</strong></td>
<td></td>
</tr>
<tr>
<td>age 0</td>
<td>0.30 (0.43SD)</td>
</tr>
<tr>
<td>age 1-4</td>
<td>0.304 (0.175 SD)</td>
</tr>
<tr>
<td>age = 5</td>
<td>0</td>
</tr>
<tr>
<td>Females mean mortality rates</td>
<td></td>
</tr>
<tr>
<td>age 0</td>
<td>0.033 (0.07 SD)</td>
</tr>
<tr>
<td>age 1-2</td>
<td>0.040 (0.08 SD)</td>
</tr>
<tr>
<td>age = 3</td>
<td>0.092 (0.11 SD)</td>
</tr>
</tbody>
</table>
Table S2. Parameters used for estimating $N_e$ with the ecological method of Nunney & Elam (1994) for the translocated brown bear population in Northern Italy. N. adults is the number of genotypes corresponding to adults detected each year; $r$ is the adult sex ratio; $A_f$, $A_m$ are the adult life span of females and males; $I_a f$, $I_a m$ are their respective standardized variance; $I_b f$ and $I_b m$ are the standardized variance in reproductive success of females and males; $T$ is the generation time from VORTEX.

<table>
<thead>
<tr>
<th>Year</th>
<th>N. adults</th>
<th>$r$</th>
<th>$1-r$</th>
<th>$A_f$</th>
<th>$I_a f$</th>
<th>$A_m$</th>
<th>$I_a m$</th>
<th>$I_b m$</th>
<th>$I_b f$</th>
<th>$T$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>8</td>
<td>0.33</td>
<td>0.67</td>
<td>9.4</td>
<td>0.89</td>
<td>16</td>
<td>0.94</td>
<td>2</td>
<td>5</td>
<td>11.46</td>
</tr>
<tr>
<td>2003</td>
<td>7</td>
<td>0.40</td>
<td>0.60</td>
<td>9.4</td>
<td>0.89</td>
<td>16</td>
<td>0.94</td>
<td>2</td>
<td>2</td>
<td>11.46</td>
</tr>
<tr>
<td>2004</td>
<td>9</td>
<td>0.29</td>
<td>0.71</td>
<td>9.4</td>
<td>0.89</td>
<td>16</td>
<td>0.94</td>
<td>0.08</td>
<td>0.13</td>
<td>11.46</td>
</tr>
<tr>
<td>2005</td>
<td>9</td>
<td>0.29</td>
<td>0.71</td>
<td>9.4</td>
<td>0.89</td>
<td>16</td>
<td>0.94</td>
<td>2</td>
<td>3.3</td>
<td>11.46</td>
</tr>
<tr>
<td>2006</td>
<td>9</td>
<td>0.29</td>
<td>0.71</td>
<td>9.4</td>
<td>0.89</td>
<td>16</td>
<td>0.94</td>
<td>0.37</td>
<td>1.67</td>
<td>11.46</td>
</tr>
<tr>
<td>2007</td>
<td>10</td>
<td>0.25</td>
<td>0.75</td>
<td>9.4</td>
<td>0.89</td>
<td>16</td>
<td>0.94</td>
<td>0.37</td>
<td>1.67</td>
<td>11.46</td>
</tr>
</tbody>
</table>

Literature cited

Appendix S1. Protocols for DNA extraction and amplification, screening of bear samples, and individual and sex determination used for genetic monitoring of the translocated brown bear population in the Italian Alps in 2002-2008.

DNA extraction and PCR

Genetic analyses were performed at the Conservation Genetics Laboratory of the Italian Institute for Environmental Protection and Research (ISPRA) and the Laboratory for Conservation and Ecological Genetics of the University of Idaho upon obtaining CITES permits (MA066574-0). Hair and scat samples were extracted as described in De Barba et al. (2010) in a room dedicated to low quantity DNA samples. The Qiagen tissue kit protocol (Qiagen Inc., Valencia, CA) was used for tissue and blood extraction using 25 mg of tissue and 200 µl of blood. Tooth extractions were conducted following protocols in Miller & Waits (2003) in a room dedicated to low quantity DNA samples.

Different protocols and microsatellite loci were used to genotype DNA samples across years. In 2002-2006, eight microsatellite loci were used for individual identification (De Barba et al. 2010). In 2002-2005, DNA extracts were amplified using standard PCR protocols for scat and hair samples described in De Barba et al. (2010). In 2004 only, scat DNA extracts were amplified using the multiplex pre-amplification method (De Barba & Waits 2010). In 2006, the Qiagen Multiplex PCR kit was used for combining the loci into two multiplex PCR reactions. The 7 µL PCR reaction consisted of 1x concentrated Qiagen Master Mix, 0.5x concentrated Qiagen Q Solution, 0.1 µM G10C, 0.09 G1D, 0.1 µM G10M, 0.15 µM G10P in multiplex 1, and 0.07 µM Mu15, 0.07 µM Mu23, 0.04 µM Mu50, 0.06 µM Mu59 in multiplex 2, and 1 µL DNA
extract for hair and 2 µL DNA extract for scat. The PCR profile had an initial denaturation step of 15 minutes at 94°C followed by a touchdown with 12 cycles of 30 seconds at 94°C, 90 seconds at 57.3°C with a decrease in annealing temperature by 0.4°C each cycle, and 1 minute at 72°C followed by 27 cycles of 30 seconds at 94°C, 90 seconds at 52.5°C, 1 minute at 72°C and a final elongation at 60°C for 30 min. A PCR negative was included in each group of reactions to test for contamination. PCR products from each multiplex were added to 10 µl mix of formamide and ROX350 size standard (Applied Biosystems) (10:0.26 µl) and run in two separate capillaries on the Applied Biosystems 3130xl ABI capillary machine. PCR products were scored using Genemapper3.7 software (Applied Biosystems). In 2007, G10C and G10M were replaced with CXX20 (Ostrander et al. 1993) and Mu11 (Taberlet et al. 1997) and in 2008, Mu23 was replaced with G10M. PCR protocols and profiles were the same as in 2006 except adjusting the multiplexes for 0.15 µM CXX20 and 0.06 µM Mu11. In addition, a panel of ten loci (all of the above except that G10C was substituted with 0.08 µM G10X – Taberlet et al. 1997) was used for confirming all unique genotypes identified since the beginning of the project using two samples per genotype when available.

Molecular sexing was performed using the Amelogenin locus (Ennis & Gallagher 1994) as described in Robinson et al. (2007). To screen for brown bear samples among samples that failed microsatellite genotyping, a 150 bp segment of mitochondrial DNA (mtDNA) control region was amplified as described in Murphy et al. (2000) and separated by size on an ABI3130xl.
Protocol for individual identification and sex determination

In 2002-2004 we established a standardized data analysis protocol for individual ID assuring reliable genotyping and assigning genotypes to samples based on a reference genotype approach (De Barba et al. 2010). This protocol allowed us to accept a genotype after one PCR for hair samples when the genotype matched a known genotype, and after observing an allele in at least two PCRs for scat samples. The data analysis protocol used in the following years was the same, except that i) in 2006-2008 the initial screening of sample quality was done by performing 2 multiplexes (8 loci); ii) in 2006-2008 samples were kept for further analyses if they had scorable results at = 50% loci; iii) six loci were sufficient to unambiguously identify a genotype in 2005, and seven in 2006-2008; iv) in 2007-2008 a ten locus genotype was generated for each unique genotype sampled; v) the location of genotyped samples was checked against field data and knowledge of bears’ distribution gained in previous years of sampling. In each year we required that the $P_{ID(sib)}$ (Waits et al. 2001) was lower than the threshold established considering the number of first-order relatives in the population (De Barba et al. 2010). The threshold ranged from 0.048 (2002) to 0.0028 (2008). Error rate due to allelic dropout and false alleles was estimated following the approach of Broquet & Petit (2004). In 2002 error rate was not calculated due to the low number (< 10) of samples genotyped from scat and faecal samples. In 2003-2004 all genotyped samples and PCRs performed were used for calculations. In 2005-2008 the first PCR and either all (when < 40) genotyped samples or 40 randomly selected from each sampling method were used. Only newly identified genotypes were processed for sex ID and were replicated a minimum of three times. Known samples were used as positive controls in each sex ID PCR reaction, and when possible, more than one sample from the same individual
was included. Error rate of molecular sexing was estimated on all samples using the first PCR performed.

**Literature cited**


