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Noninvasive genetic tracking of the endangered Pyrenean brown bear population

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Abstract

Pyrenean brown bears *Ursus arctos* are threatened with extinction. Management efforts to preserve this population require a comprehensive knowledge of the number and sex of the remaining individuals and their respective home ranges. This goal has been achieved using a combination of noninvasive genetic sampling of hair and faeces collected in the field and corresponding track size data. Genotypic data were collected at 24 microsatellite loci using a rigorous multiple-tubes approach to avoid genotyping errors associated with low quantities of DNA. Based on field and genetic data, the Pyrenean population was shown to be composed at least of one yearling, three adult males, and one adult female. These data indicate that extinction of the Pyrenean brown bear population is imminent without population augmentation. To preserve the remaining Pyrenean gene pool and increase genetic diversity, we suggest that managers consider population augmentation using only females. This study demonstrates that comprehensive knowledge of endangered small populations of mammals can be obtained using noninvasive genetic sampling.

Keywords: conservation genetics, faeces, genetic typing, hair, microsatellites, multiple-tubes approach, polymerase chain reaction, *Ursus arctos*

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Introduction

The brown bear *Ursus arctos* has a Holarctic distribution, stretching from Spain east across Asia to North America. The species formerly occupied most of the European continent, but its present range has been dramatically reduced since the mid-1800s (Servheen 1990) by habitat destruction and excessive hunting. In western Europe, the brown bear now exhibits a patchy geographical distribution with no possibility of re-establishment of continuous habitat (Sørensen 1990) or migration corridors for gene flow. The remnant brown bear population in the Pyrenees mountains on the border of France and Spain is among the most threatened in Europe.

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To obtain critical information for the management of the brown bear population in the Pyrenees, the French Ministry of the Environment initiated a research programme in 1991 with three major goals: (i) identify the potential conservation units at the European level; (ii) develop a method to determine the sex of free-ranging bears, (iii) establish unique genetic identification of all remaining individuals using noninvasive sampling techniques. The identification of potential conservation units was previously described (Taberlet & Bouvet 1994), as well as a sex-identification technique based on the presence/absence of the *SRY* gene (Taberlet *et al*. 1993). This manuscript presents the results and conclusions of the sex identification and individual genetic identification studies.

Before the beginning of this genetic study, field data based mainly on the recording of track sizes suggested the presence of 8–10 bears in the Aspe and Ossau valleys (Camarra 1992; Camarra & Dubarry 1992). Among these 8–10 bears, the sex of only one individual (a male) was

identified with good confidence based on very large track sizes when compared with the other individuals. The absence of cub tracks or of direct observations during the period from 1990 to 1995 suggested that no cubs were present, and it raised doubts concerning the presence of any female in the population. One possible method for avoiding extinction is to reinforce this small isolated group with bears from larger, nonendangered populations (Taberlet & Bouvet 1994). To make management decisions for this population, a precise knowledge of the number of individuals and of the sex-ratio is critical. However, obtaining this information by capturing and marking the last Pyrenean bears entails too many risks for the remaining individuals, and therefore cannot be considered.

Previous studies have demonstrated the potential for hair or faeces collected in the field to provide a suitable source of DNA for genotyping and sexing free-ranging mammals (Höss *et al*. 1992; Taberlet & Bouvet 1992; Taberlet *et al.* 1993; Morin *et al*. 1994a; Constable *et al*. 1995; Gerloff *et al*. 1995; Kohn *et al*. 1995; Taylor *et al*. 1997). Thus, we chose this noninvasive approach to obtain a comprehensive genetic profile of the Pyrenean bear population.

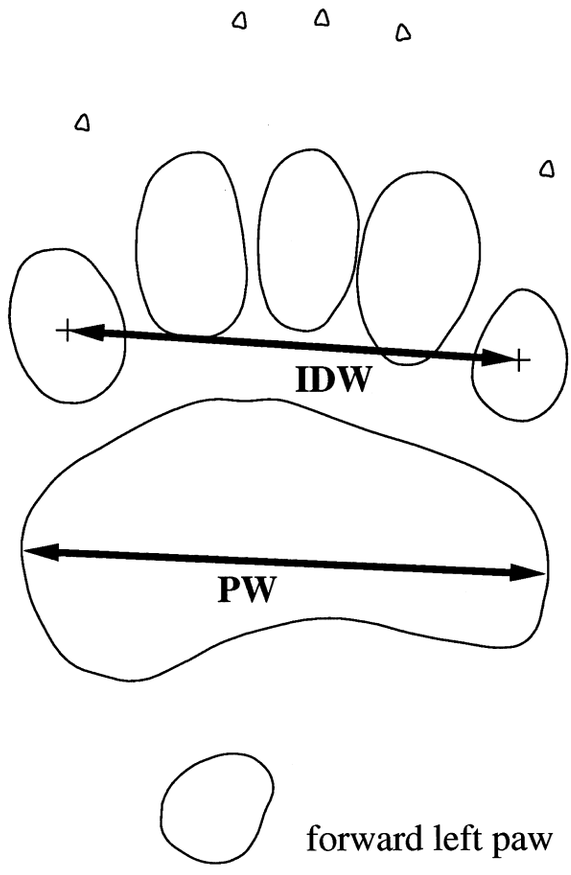

Fig. 1 Method used to record track sizes. Two measurements were taken: the pad width (PW), and the inter digital width between the middle of the two external toes (IDW). The measurement between the middle of the two external toes and not between the external edge of these toes increases the precision of IDW estimates among different tracks of the same bear, even if the consistency of the substrate is different.

Despite recent advances, noninvasive genetic sampling represents a difficult challenge as hair and faeces provide only picogram quantities of degraded template DNA. Under these limiting conditions, there are two major genotyping errors that may lead to inaccurate results: (i) an allele of a heterozygous individual may not be detected (Gerloff *et al*. 1995; Navidi *et al.* 1992; Taberlet *et al*. 1996), (ii) PCR-generated alleles or 'false alleles' may arise (Taberlet *et al*. 1996). To overcome these difficulties, a multiple-tubes approach has been recently developed (Taberlet *et al*. 1996). Using this method, the DNA extract is distributed among several tubes and amplified separately to determine the genotype with a 99% accuracy [see Taberlet *et al*. (1996) for further explanations concerning the confidence level].

In this paper, we present the results of the first large-scale application of this multiple-tubes approach to noninvasive genetic sampling of free-ranging animals. By combining field data with genetic data, we obtain the sex-ratio, minimum population size, and home range estimates for the endangered Pyrenean bear population.

Materials and methods

Sampling

The sampling of bear hairs and faeces in the Pyrenees was co-ordinated by J.-J. Camarra, and achieved by the brown bear network ('Groupe Ours'). Because the sex-identification method is not bear-specific, our field collection procedure was designed to avoid possible contamination by human DNA. All samples collected in the field were placed in paper envelopes without contacting human skin, and when possible, the associated tracks were measured and recorded as described in Fig. 1. All the samples were preserved dry until the DNA extraction.

DNA extractions

DNA extractions from bear hairs were carried out using the Chelex method as described by Walsh *et al*. (1991). A hair sample usually included many hairs. Generally, one to five extractions were carried out per sample. Only one hair was used per extraction. The suitable hairs were chosen by microscopy according to the presence of dry cells. The root part (2–3 mm) of the hair was cut and added to 500 μL of a 5% Chelex 100 (Bio-Rad) solution (w/v in H_2O). The tube was incubated with constant shaking at 56 °C for 6–8 h, thoroughly vortexed, left in boiling water for 8 min, and then centrifuged at 12 000 *g*. Five microlitres

of the supernatant, corresponding to about 1/100 of the extract, were used as template in each PCR.

DNA extractions from bear faeces were performed using the silica method (Boom *et al*. 1990; Höss *et al*. 1992; Höss & Pääbo 1993). Generally, two to five extractions were carried out for each faeces. For each extraction, about 50 mg of dry bear faeces were added to 1 mL of L6 extraction buffer (10 M GuSCN, 0.1 M Tris-HCl pH 6.4, 0.02 M EDTA pH8.0, 1.3% Triton X-100). After incubation overnight at 60 °C with constant agitation, 500 µL of the liquid phase were added to 500 µL of fresh L6 extraction buffer and 40 µL of silica suspension prepared as described in Boom *et al.* (1990). The mixture was incubated at room temperature for 10 min with constant agitation. After centrifugation (1 min, 7000 ***g***), the silica pellet was washed three times with 500 µL of L2 buffer (10 M GuSCN, 0.1 M Tris-HCl pH 6.4), once with 1 mL of 100% ethanol, and once with 1 mL of acetone. The pellet was then dried at 60 °C for 10 min, and nucleic acids were eluted at 60 °C for 5 min in 200 µL of TE (10 mM Tris-HCl, 10 mM EDTA, pH 8.3). The tube was centrifuged (3 min, 10 000 ***g***), and 160 µL of supernatant were carefully removed to avoid pipetting silica particles and transferred to a new Eppendorf tube. The tube was centrifuged again (3 min, 10 000 ***g***), and only 120 µL were removed to ensure that no silica particles remained in the extract. Five microlitres of the supernatant, corresponding to 1/24 of the extract, were used as template in each PCR.

Both extraction procedures were performed wearing a face mask in a room dedicated to processing ancient samples, hair and faeces. To detect whether contamination with exogenous DNA had occurred during extractions, tubes without bear faeces or without hair root (extraction negative control) were treated identically through both the extraction procedure and the subsequent amplifications.

Genetic typing

The genetic typing was performed via the multiple-tubes approach according to Taberlet *et al. (*1996). Three positive PCRs were first analysed, and then, depending on the results, a genotype was assigned or four additional experiments were carried out. This two-step procedure was designed to avoid analysing too many positive PCRs if the amount of template DNA is compatible with a reliable genotyping using only three experiments. Our first rule was to record an allele only if it was observed in two separate PCRs. To identify homozygous samples, our second rule was to score an individual as a homozygote only if seven independent experiments detected the same allele (see the justification of this procedure in Taberlet *et al*. 1996).

The DNA amplifications were performed in a two-step PCR. The first step used diluted external microsatellite primers to reduce the formation of primer–dimer artefacts (Ruano *et al*. 1989, 1990). In the second step, a nested primer was introduced, so three primers were designed for each of the 24 microsatellite loci tested (see Table 1). The first step was performed in a total volume of 25 µL (750 mM Tris-HCl (pH 9.0), 200 mM $(NH_4)_2SO_4$, 50 µM of each dNTP, 1.5 mM $MgCl_2$, 5 ng of BSA, 0.1 U of Red GoldStar DNA polymerase (Eurogentec), 0.01 µM of each of the external primers, 5 µL of the extract) and a PCR amplification of 20–25 cycles was carried out (93 °C for 30 s, 50–55 °C for 30 s, 72 °C for 1 min using a Perkin Elmer Gene Amp PCR System 9600). Between the first and second steps, a volume of 25 µL (750 mM Tris-HCl (pH 9.0), 200 mM $(NH_4)_2SO_4$, 50 µM of each dNTP, 1.5 mM $MgCl_2$, 5 ng of BSA, 0.1 U of Red GoldStar DNA polymerase, 1 µM of the nested primer and 1 µM of the appropriate external primer) was added to the same tube. The second step consisted of 35–40 cycles of amplification (93 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min). The PCR products were purified on a low-melting agarose gel, diluted in 200 µL ddH_2O, and 10 µL were used as the template for an additional amplification of 2 cycles (93 °C 10 s, 55 °C 30 s, 72 °C 1 min) performed in a volume of 25 µL (750 mM Tris-HCl (pH 9.0), 200 mM $(NH_4)_2SO_4$, 50 µM of each dNTP, 1.5 mM $MgCl_2$, 0.1 U of Red GoldStar DNA polymerase, 0.2 µM of the appropriate external primer and 0.02 µM of γ-^{33}P-labelled internal primer). Amplification products were separated by electrophoresis on a 6% polyacrylamide gel (sequencing gel) for 2 h. This gel was dried, and exposed to autoradiography film. This procedure allows the detection of a single target molecule in the template DNA (Taberlet *et al*. 1996).

An alternate protocol was used for the samples collected in 1996, except when field data suggested that the sample came from a bear that may not have been characterized previously. The genotype at two discriminant microsatellite loci (G10B, G10L) was determined in order to identify the bear, and to determine home ranges. The method used was as described for the radioactive detection, except that the two loci have been multiplexed (with one fluorescent primer per locus), for 60 cycles using DNA polymerase Amplitaq Gold (Perkin Elmer).The PCR products were resolved on polyacrylamide gels using an ABI 377 prism automated sequencer, and analysed using the Genescan and Genotyper software. A small number of samples were also analysed for each polymorphic locus on the ABI automated sequencer in order to obtain precise sizing of the PCR products using Genescan 350 Tamara (Perkin Elmer) as a standard.

The genetic analysis was carried out in two stages. In the first stage, 24 microsatellite loci were screened for polymorphism using 10–15 samples believed to represent different individuals based on field data and preliminary genetic results. It was not possible to use exactly the same

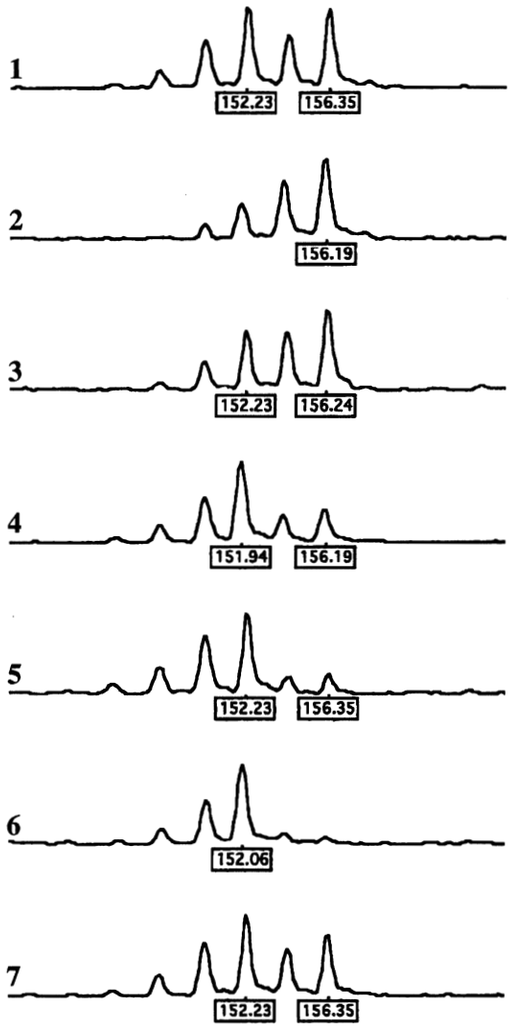

Fig. 2 Results of seven independent genotyping experiments (multiple-tubes approach) at microsatellite locus G10L (Paetkau *et al.* 1995). A single DNA extract of a shed hair collected in the field was used as a template. Each PCR consisted of 60 cycles using Amplitaq Gold (Perkin Elmer) in the presence of a fluorescent primer. The PCR products were detected and analysed on a ABI 377 prism automated sequencer. The bear (Cannelle) is a heterozygote with alleles 152 and 156 (bp). PCRs 1 and 7 detect both alleles. PCRs 2 and 6 detect only one of the two alleles. PCRs 3, 4 and 5 exhibit ambiguous results as one of the two alleles is much weaker than the other.

samples for the different loci, as DNA extractions from hairs and faeces do not provide enough template DNA for analysing 24 loci using the multiple-tubes approach. In the second stage all samples with adequate amounts of extracted DNA were genotyped for the polymorphic loci.

Sexing

The sex-identification was carried out using a two-step PCR as described by Taberlet *et al.* (1993), with two modifications: the internal positive control was the microsatellite locus UarMU64 (primers added at the first and the second steps as for the genetic typing), and the primer SRY121R was replaced by the primer RG7 (Griffiths & Tiwari, 1993). But, as the internal positive control is two times more concentrated (two autosomal copies per cell) than the SRY target sequence (one copy per cell), we adjusted the multiple-tubes procedure for this particular case using the model described in Taberlet *et al.* (1996). Nine independent positive experiments were carried out for each sample. The PCR products were run on a 4% agarose gel. The sample was identified as originating from a male if at least two experiments showed the SRY band, and as originating from a female with a confidence of 99% if the nine experiments showed only the internal positive control [see Taberlet *et al.* (1996) for the justification of the confidence level].

Results

Between 1993 and 1996, 352 samples (247 hair, 105 faeces) were collected in the Pyrenees. Only 57 of these samples (36 hair, 21 faeces) provided enough DNA for a complete genetic typing at all polymorphic loci using the multiple-tubes approach. Fig. 2 displays the different results that can be obtained from a single extract and emphasizes the importance of performing multiple genotyping experiments to obtain reliable results. Genetic variation was assessed using the 24 (CA/TG)*n* microsatellite loci (Table 1), but only six loci were found to be polymorphic, with only two alleles each (Table 2). Five unique genotypes were identified from the six polymorphic microsatellite loci using the multiple-tubes approach. The genotypic data for each sample were compared with the corresponding track size recorded when the hair or faeces samples were collected in the field in order to obtain a minimum estimate of the population size (Table 2). Because one genotype was found with two nonoverlapping track sizes (Papillon and Chocolat), we were able to detect six individual bears. The genotype of Pestoune was not found in the samples collected after 1993. Therefore, the Pyrenean bear population currently contains at least three adult males (Papillon, Chocolat and Camille), one adult female (Cannelle), and one yearling male (Pyren).

The observation of bear scat, hair and tracks far (15–25 km) from the core habitat zone previously suggested that additional bears were present in the Pyrenees. We were able to test this hypothesis. Using both field and genetic data, we detected the same genotypes associated with the same track sizes as in the core area.

Table 1 Sequences of the primers and accession numbers of the microsatellite loci used in this study. Loci UarMU05, UarMU09, UarMU10, UarMU11, UarMU15, UarMU23, UarMU26, UarMU50, UarMU51, UarMU59, UarMU61, UarMU63, and UarMU64 were cloned and sequenced for this study, the other loci are from Craighead *et al.* (1995) and Paetkau *et al.* (1995). No internal primer was used for UarMU26

Locus	Forward primer	Internal primer (F: forward; R: reverse)	Reverse primer	Acc. number
UarMU05	5'-GTGATTTTTCTTGTAGCCTAGG-3'	(F) 5'-AATCTTTTCACTTATGCCCA-3'	5'-GAAACTTGTTATGGGAACCA-3'	Y09640
UarMU09	5'-AGCCACTTTGTAAGGAGTAGT-3'	(F) 5'-TTGAAGTTCAGGGTAAATGC-3'	5'-ATATAGCAGCATATTTTTGGCT-3'	Y09641
UarMU10	5'-TTCAGATTTCATCAGTTTGAC-3'	(R) 5'-CAGCATAGTTACACAAATCTCC-3'	5'-TTTGTATCTTGGTTGTCAGC-3'	Y09642
UarMU11	5'-AATGTGAAAAAGAAAAGGTAGG-3'	(R) 5'-AAGTAATTGGTGAAATGACAGG-3'	5'-GAACCCTTCACCGAAAATC-3'	Y09643
UarMU15	5'-GCCTGACCATCCAACATC-3'	(F) 5'-CTGAATTATGCAATTAAACAGC-3'	5'-AAATAAGGGAGGCTTGGGT-3'	Y09644
UarMU23	5'-GCCTGTGTGCTATTTTATCC-3'	(R) 5'-AATGGGTTTCTTGTTTAATTAC-3'	5'-TTGCTTGCCTAGACCACC-3'	Y09645
UarMU26	5'-GCCTCAAATGACAAGATTTC-3'		5'-TCAATTAAAATAGGAAGCAGC-3'	Y09646
UarMU50	5'-TCTCTGTCATTTCCCCATC-3'	(R) 5'-GAGCAGGAAACATGTAAGATG-3'	5'-AAAGGCAATGCAGATATTGT-3'	Y09647
UarMU51	5'-GCCAGAATCCTAAGAGACCT-3'	(R) 5'-AAGAGAAGGGACAGGAGGTA-3'	5'-GAAAGGTTAGATGGAAGAGATG-3'	Y09648
UarMU59	5'-GCTCCTTTGGGACATTGTAA-3'	(R) 5'-GACTGTCACCAGCAGGAG-3'	5'-TGGATAGCATTCAGGCAT-3'	Y09649
UarMU61	5'-ACCCAGAGAAGTCCGATTAC-3'	(R) 5'-TCCACTGGAGGGAAAATC-3'	5'-CTGCTACCTTTCATCAGCAT-3'	Y09650
UarMU63	5'-AACCATTCACTGAAAATCAATT-3'	(R) 5'-CCTTGAATGGTTAAGTAATTGG-3'	5'-CAGGGAAGTTCCATTTTGT-3'	Y09651
UarMU64	5'-ACTCAACACAACCATTAAATCA-3'	(R) 5'-AGGACCCAAATGACACTACA-3'	5'-GGTATCTACTCCCCAAAGGA-3'	Y09652
G1A	5'-TCCAGTGTCCTCCCTTTCT-3'	(F) 5'-GCATACTCTCCTCTGATGGG-3'	5'-AGATTAGTGAAAAAGAAGCAGG-3'	U22095
G1D	5'-ATCTGTGGGTTTATAGGTTACA-3'	(R) 5'-CTACTCTTCCTACTCTTTAAGAG-3'	5'-CTAGCACCCAGCAAGGTA-3'	U22094
G10B	5'-AAGCCTTTTAATGTTCTGTTG-3'	(F) 5'-TGCTAATATTTTCTTGAGGACT-3'	5'-AGGACAAATCACAGAAACCT-3'	U22084
G10C	5'-CAACAAAAGGTTGAAGGGAG-3'	(F) 5'-GTCTGCAAAAGCAGAAGG-3'	5'-AAACACCGAGACAGCAGG-3'	U22085
G10H	5'-CTCTTGCCTTACTTACATGG-3'	(F) 5'-CCCACTCCAGCTCTCTAAAG-3'	5'-ATCAGAGACCACCAAGTAGG-3'	U22086
G10J	5'-GCTTTTGTGTGTGTTTTTGC-3'	(R) 5'-GGATAACCCCTCACACTCC-3'	5'-TACTGGGAAAATCACTCACC-3'	U22087
G10L	5'-GGACAGGATATTGACATTGA-3'	(F) 5'-ACTGATTTTATTCACATTTCCC-3'	5'-CAGAAACCTACCCATGCG-3'	U22088
G10M	5'-ATATTTCCCCTCATCGTAGG-3'	(F) 5'-GTTTGCCTCTTTGCTACTGG-3'	5'-TTTAAATGCATCCCAGGG-3'	U22089
G10O	5'-CTTTGGCTACCTCAGATGG-3'	(R) 5'-AATCCAAAGATGCATAAAGG-3'	5'-TGCCTACTGCACCAACAG-3'	U22090
G10P	5'-CCAGGGCAAGAAATAATGAG-3'	(F) 5'-TACATAGGAGGAAGAAAGATGG-3'	5'-AAAAGGCCTAAGCTACATCG-3'	U22091
G10X	5'-TTCCAATTCTCCCAGTAGC-3'	(F) 5'-CCCTGGTAACCACAAATCTC-3'	5'-ATCTGTGAAATCAAAACAAACA-3'	U22093

The sampling location of each genotype was plotted on a map to estimate the home range of each bear (Fig. 3). The results demonstrated that the female is confined to a small core bear area. However, the three adult males have much larger home ranges, and were sometimes located up to 25 km from this core area.

Discussion

The minimum population size and the sex-ratio of the Pyrenean brown bear population have been assayed via a noninvasive molecular approach using hair and faeces collected in the field as a source of DNA. Genetic data and corresponding track size data indicate that this population currently includes at least five bears, four males and only one female. Remote sensing photography utilized from 1994 to 1996 suggested the presence of at least four bears, including Papillon, Chocolat, Cannelle, and Pyren. The first evidence of the presence of Pyren, the cub, was obtained by the observation of very small tracks, and by remote sensing photography (in 1995). Later, two faeces from Pyren were found and analysed to provide genetic typing of this yearling.

The very good agreement between field data (track sizes, remote sensing photographies) and the genetic typing suggests that all individuals have been detected. The home range data for the Pyrenean bears obtained via our genetic approach are consistent with results from

Table 2 The six Pyrenean brown bears identified using genetic and field data. The genotype of Pestoune was not observed after 1993. Pyren was born in 1995. IDW, inter digital width between the middle of the two external toes; PW, pad width (see Fig. 1)

			Microsatellite loci (size in bp)						Track sizes (forward paw)	
Name	Sex	Age	G1D	UarMU64	G10X	G10B	UarMU26	G10L	IDW (mm)	PW (mm)
Papillon	M	Adult	173/173	208/208	133/133	115/123	193/195	152/152	122-127	135-140
Chocolat	M	Adult	173/173	208/208	133/133	115/123	193/195	152/152	96–105	110-115
Camille	M	Adult	173/173	206/208	133/143	115/115	193/193	152/156	112-116	123-128
Cannelle	F	Adult	171/173	208/208	133/133	115/123	193/193	152/156	92-97	103-108
Pyren	M	Yearling	173/173	208/208	133/133	123/123	193/195	152/156	75-79	88-93
Pestoune	F	Adult	173/173	208/208	133/133	115/115	193/195	152/156	95-100	106-111

Table 3 Genetic polymorphism of the Pyrenean brown bear population compared with three other North American populations. The eight microsatellite loci published in Paetkau *et al.* (1995) were compared. For the Pyrenean population, the five genotypes observed have been used to estimate the mean number of alleles per locus. For the three remaining populations, the mean number of alleles per locus was calculated for five randomly chosen genotypes (10 000 replicates)

	Pyrenees	Kodiak Island	Yellowstone	NCDE
Estimated population size	5	2600	300	600
Total no. of alleles observed (no. of individuals analysed)	12[6]	17[32]	35[53]	54[49]
Mean no. of alleles for five genotypes	1.50	1.69	3.08	3.93

radio-tracking studies in Europe and in North America, which have demonstrated that females have smaller home ranges than males, and the males often travel substantial distances outside the core area (Roth 1983; Blanchard & Knight 1991).

For future studies, it is important to recognize that the achievement of our goal was much more difficult than expected. We faced four main difficulties: (i) the very low amount of DNA available by using hair and faeces collected in the field; (ii) the characterization of polymorphic loci; (iii) the problems of obtaining a reliable genotype using picogram amounts of template DNA; (iv) and the very low genetic polymorphism of the remaining individuals of the Pyrenean population.

Only about 15% of hair samples and 20% of the faeces samples collected in the field provided enough DNA for the genetic analysis. This does not mean that we were not able to get some PCR products with the remaining 80–85%, but that the proportion of positive PCRs was too low to repeat the experiments and to obtain a reliable genotyping at six loci. The percentage of usable samples could be significantly increased by analysing less loci, and by multiplexing the loci during the amplification. The analysis of two loci (G10B and G10L) is sufficient for unambiguously characterizing the four genotypes, and preliminary results concerning the multiplexing of these two loci are encouraging. Therefore, in these conditions (two loci multiplexed), the template DNA could be sixfold concentrated in regard to the experiments carried out in this study, and the proportion of usable samples might reach 50%. Indeed, we observed that more than 50% of the DNA extracts yielded enough template DNA for obtaining some PCR products, but not enough to analyse six loci using the multiple-tubes approach.

The second difficulty was identifying the polymorphic loci without knowing the individuals, and in a context where it was technically impossible to analyse all the 24 loci for each sample due to low quantities of template DNA. Thus, we used preliminary genetic results and field data (track sizes, geographical distribution of the samples) to test the different primer sets on samples originating from four adult bears known to currently inhabit the Pyrenees. We believe that this strategy allowed us to identify all polymorphic loci, but we cannot exclude the possibility that a polymorphic locus was missed in the unanalysed samples.

The third difficulty concerns the reliability of nuclear DNA genotyping under conditions of low DNA quantity. This difficulty required the development of a new methodology, the multiple-tubes approach (Taberlet *et al.* 1996), that is much more expensive and time consuming than classical assays of microsatellite polymorphism.

The last problem was the very low genetic polymorphism in the population: only six loci were polymorphic (with only two alleles each) out of 24 tested and known to be polymorphic in other bear populations (Craighead *et al.* 1995; Paetkau *et al.* 1995; L. P. Waits, P. Taberlet, unpublished data). The fact that we were unable to distinguish the genotypes of Papillon and Chocolat suggests that the Pyrenean population has low levels of genetic diversity and increased levels of inbreeding, but estimates of heterozygosity are uninformative due to the small sample size. Thus, we compared genetic diversity in the Pyrenean population with other brown bear populations by estimating the mean numbers of alleles per locus for the five

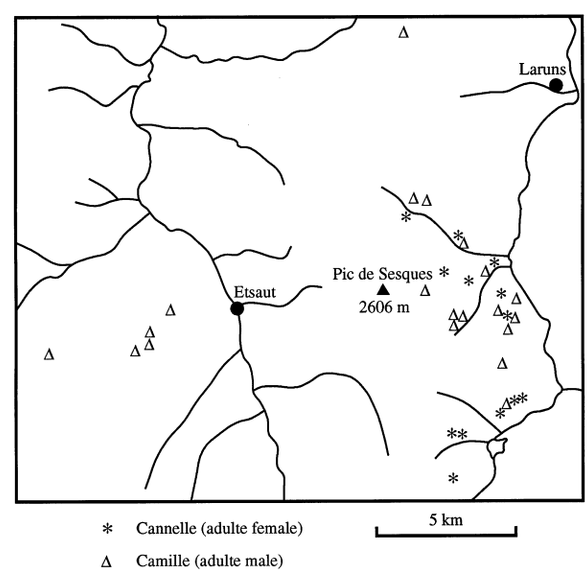

Fig. 3 Home range of two Pyrenean brown bears obtained by noninvasive genetic sampling and genotyping.

Pyrenean genotypes, and for five randomly chosen genotypes for the other populations. The comparison of microsatellite variation at eight identical loci (Table 3) clearly confirms that the Pyrenean population exhibits very low genetic polymorphism. The Kodiak Island population also has individuals that cannot be discriminated using eight polymorphic loci (Paetkau *et al.* in press). Therefore, it is not surprising that Papillon and Chocolat had the same genotype at all the six polymorphic loci analysed.

The genetic approach contributed significantly to the knowledge of this population. First, the population size was estimated more precisely as the tracks or faeces found 15–25 km from the core habitat zone were clearly attributed to one of the genotypes already present in the core zone, suggesting long distance movements of males. This may explain the differences between earlier field data which suggested more individuals, and the current estimate of the population size. Second, it was possible to identify unambiguously the sex of all characterized individuals, including the yearling Pyren. Third, information was provided about the level of genetic diversity and inbreeding in the population. Fourth, knowledge of the genotypes will allow researchers to trace pedigrees in the future (such as after population reinforcement) using noninvasive techniques. Finally, the genetic approach showed that the bears currently in the Pyrenees have no overlapping track sizes; as a consequence, before the paws of Pyren grow, it will be possible to assess home ranges of all individuals using only track sizes.

Management guidelines can be deduced from the population size and the sex-ratio detected in this study. It is clear that without population reinforcement, the Pyrenean bears are heading towards extinction. To preserve the population, managers should consider population augmentation using only females. This decision would partially preserve the remaining Pyrenean gene pool while adding genetic diversity to decrease the currently high risk of inbreeding depression.

This work extends the application of molecular methods in conservation biology by demonstrating that not only population size is accessible, but also sex-ratios and individual home ranges. However, caution is warranted as the very low quantities of DNA obtained from noninvasive sampling make accurate genetic typing difficult. The authenticity of the genotypes obtained must be verified by an appropriate methodology, based on the multiple-tubes approach (Navidi *et al.* 1992; Taberlet *et al*. 1996). Previous studies involving noninvasive genetic sampling (Morin & Woodruff 1992; Morin *et al*. 1994a, b) have not incorporated a multiple-tubes approach, and thus are at risk of misinterpreting results. To attain the full potential of noninvasive sampling in the field of conservation biology, researchers should strive for the experimental rigour required in forensic and ancient DNA studies.

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