Mitochondrial DNA polymorphism, phylogeography, and conservation genetics of the brown bear *Ursus arctos* in Europe

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SUMMARY

Some small European populations of the brown bear (*Ursus arctos*) are threatened by the risk of extinction in the near future. The reinforcement of these populations with bears from other regions might provide a solution to their future survival. However, before any population transfer, the different conservation units must be identified. The phylogeographic approach has been advocated for this purpose. The different European populations were assayed for mitochondrial (mt) DNA polymorphism. A remarkable degree of concordance was found between the geographic distribution and the mtDNA haplotypes. Two clearly distinct lineages differing by more than 7% in mtDNA control region sequences were found and, furthermore, the western lineage appears to be organized into two clades which correspond to two different ancestral refugia. The potential conservation units can be deduced from these results, and a management policy can consequently be inferred. This study clearly demonstrates the relevance of the molecular phylogeographic approach to the identification of conservation units.

1. INTRODUCTION

To manage the endangered population of brown bear in the Pyrenees, the French Ministry of the Environment initiated a research programme in 1991 including the identification of the potential conservation units at the European level, and, by using hairs found in the field, the sexing (Taberlet *et al.* 1993) and genetic typing of all the individuals of this population. The results concerning the identification of the potential conservation units are presented here.

The brown bear is a widespread species found in Europe, Asia and North America. It formerly occupied most of the European continent. Its present range has been dramatically reduced since the mid-1800s (Servheen 1990) by habitat loss and excessive hunting. In western Europe, this species now exhibits a patchy geographic distribution (figure 1) with no possibility of re-establishment of continuous habitat (Sørensen 1990). The Pyrenean and the Trentino populations (figure 1, A and E, respectively) may each number less than ten individuals today (Camarra & Parde 1990; Osti 1990; Höss *et al.* 1992; J.-J. Camarra, personal communication) and face the threat of extinction in the near future. One possible solution to avoid extinction is to reinforce these small isolates with bears from larger, non-endangered populations.

However, as conspecific populations may be geographically structured (Avise 1992), any management programme must begin by the identification of the potential units of conservation (or evolutionary significant units) (Ryder 1986; Woodruff 1989). Such units correspond to sets of populations which have a common recent history, and among which population transfer could be performed. Usually, management programmes are founded on the current morphologically based taxonomic assignments, but in some cases morphological traits lead to either recognition of groups showing little evolutionary differentiation (Laerm *et al.* 1982), or lack of recognition of distinct forms (Avise & Nelson 1989). We propose here to make use of the phylogeographic approach (Avise *et al.* 1987; Dizon *et al.* 1992) to try to understand the recent history of the European brown bear and then to identify the potential conservation units. The phylogeography of this species could be revealed by comparing the geographic distribution with the intraspecific phylogeny based on variation in mtDNA sequences. Indeed, the analysis of mtDNA sequences is particularly well adapted for recovery of intraspecific phylogenies (see, for example, Avise *et al.* 1979; Ferris *et al.* 1983*a*; Lansman *et al.* 1983; Cann *et al.* 1987), owing to an evolutionary rate which is 5–10 times higher than that of the single copy genes in mammals (Brown *et al.* 1979, 1982), and owing to the presence of highly variable zones in the control region (Brown *et al.* 1986; Southern *et al.* 1988; Saccone *et al.* 1991).

2. MATERIALS AND METHODS

Preliminary results (Taberlet & Bouvet 1992) have shown that the sequencing of 269 base pairs (b.p.) of the mitochondrial (mt) DNA control region exhibits enough variations to reveal the relationships among the different European populations of the brown bear. Therefore, this region, flanking the Pro tRNA gene, has been sequenced for 60 brown bears representative of the different European

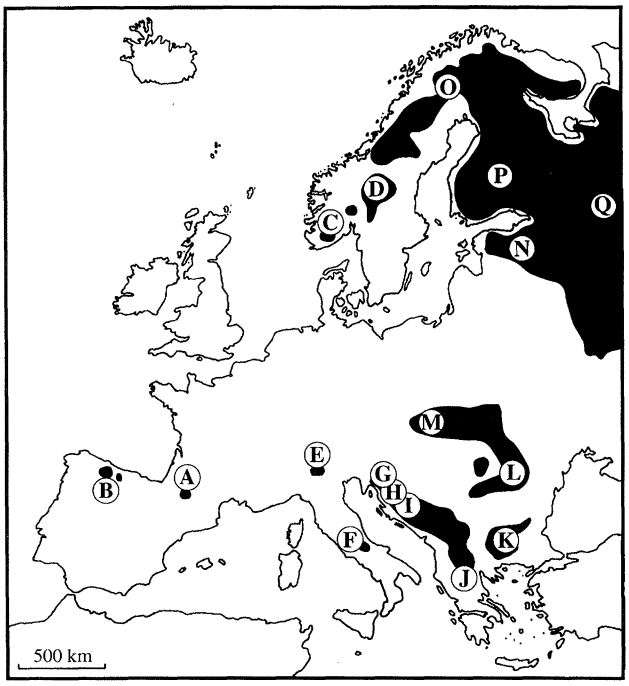

Figure 1. Geographic range (modified from Servheen (1990) and Sørensen (1990)) and sampling localities of brown bear in Europe. For each locality, the number of individuals analysed and the collector name(s) are indicated in brackets. A, Pyrenees, France ($n = 4$; J.-J. Camarra, C. Plisson, G. Caussimont, H. Laborde, M. Clemente, J. Herrero); B, Cantabrian mountains, Spain ($n = 2$; G. Palomero, A. Fernandez); C, Norway ($n = 1$; K. Elgmork); D, Dalarna, Sweden ($n = 7$; R. Franzén); E, Trentino, Italy ($n = 1$; F. Osti); F, Abruzzo, Italy ($n = 4$; G. Boscagli, H. Roth, L. Gentile); G, Slovenia ($n = 13$; M. Adamic, D. Huber); H, Croatia ($n = 2$; D. Huber, A. Frkovic); I, Bosnia ($n = 2$; D. Huber); J, Greece ($n = 1$; D. Bousbouras); K, Bulgaria ($n = 1$; N. Spassov); L, Romania ($n = 4$); L. Kalabér, N. Lestienne); M, Slovakia ($n = 2$; P. Hell); N, Estonia ($n = 4$; A. Allos, M. Kaal); O, Lapland, Sweden ($n = 7$; R. Franzén); P, Finland ($n = 2$; E. S. Nyholm); Q, Karelia, Russia ($n = 3$; P. Danilov).

Table 1. *Distribution of the 16 mtDNA genotypes found in European brown bear populations*

(The locality codes are the same as figure 1.)

	locality	mtDNA genotype code	number of individuals
A	Pyrenees, France	*Pyr*	4
B	Cantabrian mountains,	*Can*	1
	Spain	*Can′*	1
C	Norway	*Nor*	1
D	Dalarna, Sweden	*Dal*	7
E	Trentino, Italy	*Slo*	1
F	Abruzzo, Italy	*Abr*	4
G	Slovenia	*Slo*	13
H	Croatia	*Slo*	1
		Cro	1
I	Bosnia	*Slo*	2
J	Greece	*Gre*	1
K	Bulgaria	*Bul*	1
L	Romania	*Ro1*	1
		Ro2	3
M	Slovakia	*Rus′*	1
		Rus″	1
N	Estonia	*Rus*	2
		Est	2
O	Lapland, Sweden	*Rus*	7
P	Finland	*Rus*	2
Q	Karelia, Russia	*Rus*	3

populations (figure 1). Only hairs from wild, captive or killed animals were used as a source of tissue for the genetic analysis, except the locality M and P (skin samples). Hairs were preserved either in 70% alcohol or dry in paper envelopes. Three individuals in locality A, identified by their track size (J.-J. Camarra, personal communication) and their sex (Taberlet *et al.* 1993), and one in each of the localities C, E, and J, were assayed by using hairs found in the field (Taberlet & Bouvet 1992). One American black bear individual (*Ursus americanus*) was also analysed to provide an outgroup for the phylogenetic analysis.

Total DNA was extracted from the root part of hairs as described for humans by Walsh *et al.* (1991). This method, now widely used in forensic laboratories, involves the use of Chelex 100 resin (Biorad) at 50 g l^{-1} for the preparation of DNA before amplification. The hair root is incubated in the mixture of Chelex 100 and water (200 µl) at 56 °C for 6–8 h, and then left in boiling water for 8 min; a portion which represents 2.5–5.0% of the total extract was used as a template for the polymerase chain reaction (PCR). DNA extractions from skin samples were as described by Kocher *et al.* (1989). The primers used to amplify a portion of the mtDNA control region were 5′-CTCCACTATCAGCACCCAAAG-3′ (forward), and 5′-GGAGCGAGAAGAGGTACACGT-3′ (reverse). Each cycle, of which 40–45 were performed, consisted of denaturation at 93 °C for 60 s, annealing at 50 °C for 60 s, and extension at 72 °C for 60 s. The PCR product was then purified on low-melting agarose gel, as described by Kocher *et al.* (1989), and used as template for an asymmetric PCR (Gyllensten & Erlich 1988) of 30–35 cycles (denaturation at 93 °C for 60 s, annealing at 50 °C for 60 s, extension at 72 °C for 60–90 s) to generate single-stranded DNA suitable for direct sequencing after the elimination of excess nucleotides and primers by using either Ultrafree-MC 30000 (Millipore) or Microcon 100 (Amicon) columns. The sequencing of single-stranded DNA was done following a standard protocol (Sambrook *et al.* 1989; Palumbi 1991) by using the Sequenase 2.0 kit (United States Biochemicals) and either the primers used for the amplification or other internal primers.

The sequences were aligned by eye, and a matrix of transition–transversion was deduced. The genetic distances among the different genotypes were estimated by using the two-parameter model of Kimura (1981), and these were used to construct an unweighted pair-group method with arithmetic mean (UPGMA) phenogram (Sneath & Sokal 1973). The sequence data were also analysed via a parsimony approach by using PAUP (Swofford 1991*a*).

3. RESULTS

Sequencing of 272 base pairs of the control region enabled 16 different haplotypes to be distinguished out of the 60 individuals analysed. The first base of these sequences corresponds to position 16028 of the standard numbering (Anderson *et al.* 1981). The most striking result is that these haplotypes exhibit a precise geographic pattern, with almost no local variation (table 1). The four Pyrenean bears have exactly the same sequence, as do the seven individuals from

Table 2. *Estimates of mtDNA control region sequence divergence between some haplotypes of European brown bear*

(This uses the two-parameter model of Kimura (1980) (estimate above diagonal; standard deviation below diagonal). Insertions–deletions were not taken into account. The letters after the haplotype names indicate the localities (figure 1) where the haplotype was found.)

	Pyr (A)	Can (B)	Slo (E, G, H, I)	Bul (K)	Rus (N, O, P, Q)	Ro2 (L)
Pyr (A)	—	1.52	3.09	3.49	7.24	7.68
Can (B)	±0.77	—	2.30	2.69	6.38	6.81
Slo (E, G, H, I)	±1.11	±0.95	—	0.75	7.24	7.68
Bul (K)	±1.18	±1.03	±0.54	—	6.81	7.24
Rus (N, O, P, Q)	±1.77	±1.65	±1.77	±1.71	—	0.38
Ros2 (L)	±1.83	±1.71	±1.83	±1.77	±0.38	—

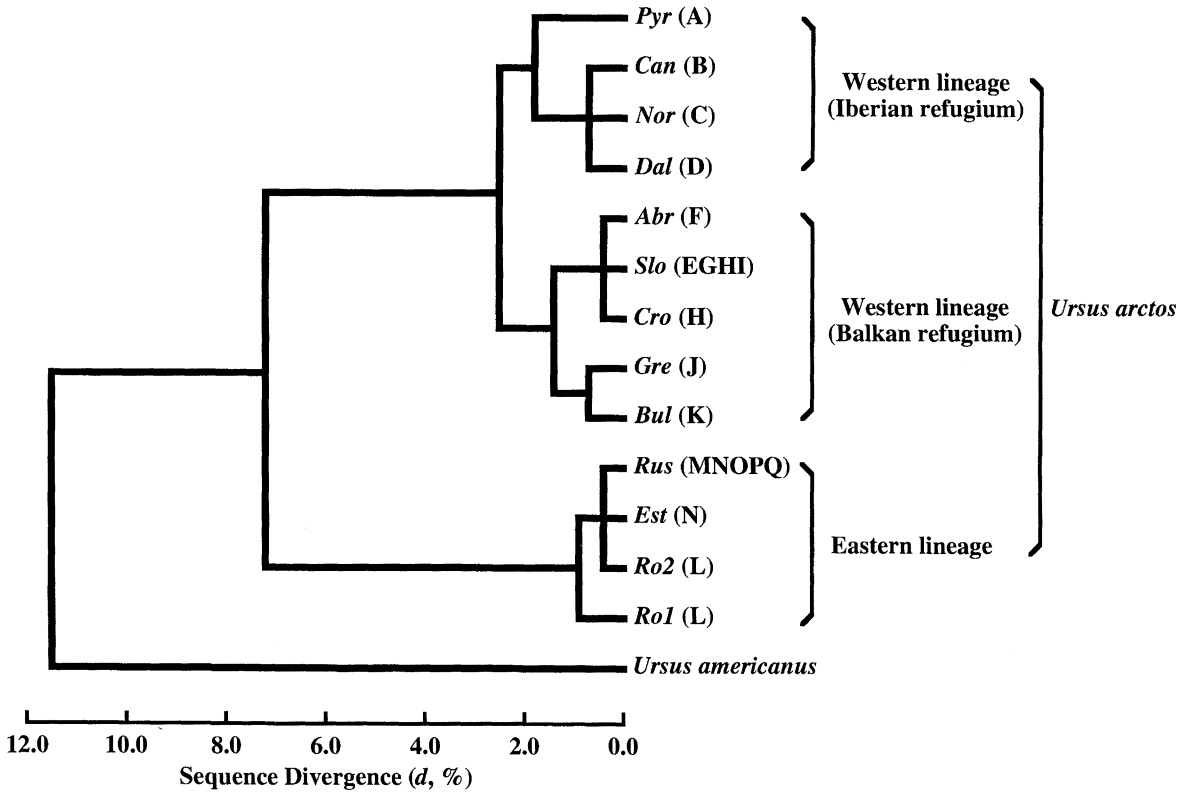

Figure 2. UPGMA phenogram (Sneath & Sokal 1973) summarizing mtDNA relationships among the 13 haplotypes of European brown bear and one haplotype of American black bear (outgroup). The letters after the haplotype names indicate the localities (figure 1) where the haplotype was found. The phenogram was deduced from a matrix of sequence divergence estimated by using the two-parameter model of Kimura (1980) and the sequence data. The cophenetic correlation is 0.985.

Dalarna (Sweden), the seven from Lapland (Sweden), and the 13 from Slovenia. The haplotypes *Can′*, *Rus′* and *Rus″* (table 1) correspond to length variants in a T stretch of haplotypes *Can* and *Rus*, and were not taken into consideration for the phylogenetic analysis. Sequences have been deposited in EMBL database under the accession numbers X75862–X75878. A multiple alignment is also available on request from the authors.

Both the genetic distances (table 2) and the phylogenetic tree (figure 2) deduced from these sequences show the presence in Europe of two main lineages which differ from each other by a mean pairwise genetic distance of 7.13%. The eastern lineage is mainly represented by the large populations of Russia and Romania, whereas the western lineage includes several isolates which contain less than 100 individuals (Cantabrian mountains (Del Campo *et al.* 1990); Pyrenees (Camarra & Parde 1990); Trentino (Osti 1990); Abruzzo (Boscagli 1990; Zunino 1990): localities B, A, E and F, respectively, in figure 1). Furthermore, the western lineage itself appears to be organized into two clades (figure 2).

By using the branch-and-bound option of the PAUP program (Swofford 1991*a*), a parsimony analysis was also done: the 12 shortest trees were found (tree length: 54), which confirmed the clade of the eastern lineage and one of the two clades of the western lineage (localities E, F, G, H, I, J and K). However, the other clade of the western lineage (localities A, B, C and D) found in the UPGMA tree is not supported by the shortest trees of the parsimony analysis, where all the haplotypes of the localities A, B, C and D exhibited abnormally short branch lengths due to homoplasy with the outgroup (figure 3): this constitutes one of the

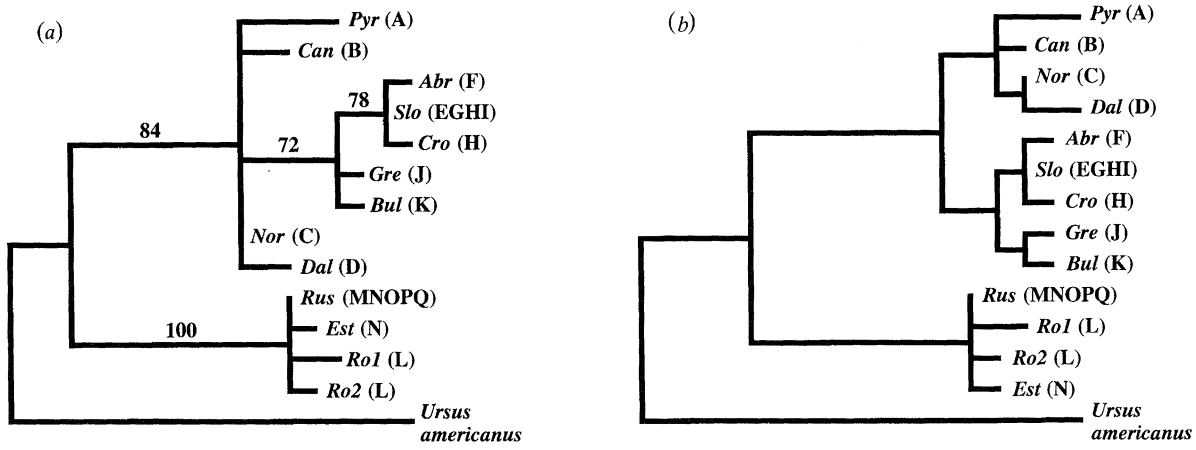

Figure 3. Parsimony analysis of the sequence data. The letters after the haplotype names indicate the localities (figure 1) where the haplotype was found. (*a*) Bootstrap 50% majority-rule consensus tree (tree length: 54). Bootstrap values are indicated on the branches concerned. (*b*) Example of a suboptimal tree (tree length: 56) showing the same branching pattern as the UPGMA tree (figure 2) for the three main lineages.

pitfalls of parsimony analysis (Stewart 1993). Nevertheless, a bootstrap analysis (Felsenstein 1985) was done (2000 replications): the clade of the eastern lineage (localities L, M, N, O, P and Q) and one of the two clades of the western lineage (localities E, F, G, H, I, J and K) are supported by bootstrap value of 100% and 72%, respectively. Some suboptimal trees of the parsimony analysis (Swofford 1991*b*), one or two steps longer, gave the same branching pattern as the UPGMA tree for the three main lineages (figure 3).

4. DISCUSSION

In Europe, a remarkable degree of concordance was found between the geographic distribution of the brown bears and their mtDNA haplotypes. This could be explained by very low dispersal habits of females (mtDNA is maternally inherited), and by the stochastic extinction of the primitive haplotypes in small populations, as was suggested for the grey wolf (*Canis lupus*) in Europe by Wayne *et al.* (1992). The quasi-absence of local variation in the European brown bear contrasts with the results obtained in North America for the coyote (*Canis latrans*), where the main genotypes occur throughout the geographic distribution, suggesting that there is high gene flow among subpopulations (Lehman & Wayne 1991).

Two main mtDNA lineages were found in European brown bears (figure 2): the eastern lineage and the western lineage. They differ from each other by a mean pairwise genetic distance of 7.13%. Based on the evolutionary rate of the homologous human sequence (Vigilant *et al.* 1989), we can assume these two main mtDNA lineages would have diverged 0.85 Ma ago. This crude estimate of absolute date of divergence corresponds to the time since the common maternal ancestor, which must precede the time of the population divergence. It has to remain qualified due to uncertainties both in the genetic distance estimates and in the molecular clock calibration, which is based on humans. The first occurrence of the brown bear in the fossil record has been estimated at 0.5 Ma (Kurten 1968). Therefore the split between these two lineages occurred at an early stage in the history of the brown bear species, and was probably due to geographic separation during the earlier Quaternary cold periods.

The western lineage itself appears to be organized into two clades (figure 2). The first includes bears from Abruzzo, Trentino, Slovenia, Bosnia, Croatia, Greece and Bulgaria (E, F, G, H, I and J in figures 1 and 2), and may correspond to populations which may have been isolated in a Balkan refugium during recent cold periods. The second clade is composed of bears from the Cantabrian and Pyrenean mountains, and from the south of Sweden and Norway (A, B, C and D in figure 1). We can assume that these last populations may have originated from an Iberian refugium, and would have been able to reach the south of Scandinavia via land of low altitude during the last post-glacial warming. During the same period, for the bears of the Balkan refugium, the ices of the Alps would have constituted a barrier towards the north and the west, as would the presence of the eastern lineage towards the east. These two barriers could explain the fact that bears of the Balkan refugium were unable to spread towards the north: when the ice sheets of the Alps melted, the north of Europe was already occupied by bears of another lineage.

This scenario assumes that the mtDNA polymorphism observed exactly reflects the phylogeography, and that no mtDNA introgression had occurred among the different bear populations. Indeed, for mammals, mtDNA transfers are possible and have been documented several times between closely related species (Ferris *et al.* 1983*b*; Carr *et al.* 1986; Tegelström 1987; Lehman *et al.* 1991). However, such a mtDNA introgression seems to be unlikely in the European brown bear, because the expansion pattern from the different refugia corresponds to that observed in other mammalian species: the expansion from an Iberian refugium towards the western European plains is the case for *Sorex coronatus* (Taberlet *et al.* 1994); the ice sheets of the Alps also constituted a barrier to the spread towards the north and the west for the Valais race of *Sorex araneus* (Taberlet *et al.* 1994); and the colonization of Scandinavia both by the north and by

the south is well documented for *Sorex araneus* (Fredga & Nawrin 1977), for *Microtus agrestis* (Fredga & Jaarola 1989), and for *Clethrionomys glareolus* (Tegelström 1987). These examples concerning other species tend to confirm the interpretation proposed for bears which could be tested by studying historical and fossil samples of brown bears throughout the historical range, and by comparing the mtDNA polymorphism with the variations of several nuclear markers such as microsatellites.

The identification of the potential conservation units can be deduced from the phylogeographic data. The first possibility is to include all European populations of brown bear in the same conservation unit, but this choice must be rejected due to the clear genetic break which separates the eastern and the western lineages. The second possibility is to consider each of these two main lineages as two separate conservation units. However, in this case, the geographic structuring corresponding to the Iberian and the Balkan refugia is not taken into account. Because some transplantations can lead to the irretrievable loss of the historical genetic record (Avise 1992), we prefer a third possibility: we suggest that populations originating from the Balkan and the Iberian refugia (figure 2) should be considered as two distinct potential conservation units.

This suggestion has clear consequences for management policy: (i) bears from Romania, Russia and Lapland are not suitable for reinforcement of the threatened isolates of the western lineage, and further studies will be necessary to delimit the exact border between the eastern and the western lineages, both in central Europe and in Scandinavia; (ii) in the short term, the management efforts must focus on the most endangered populations, i.e. those originating from the Iberian refugium.

In practice, the reinforcement of the Pyrenean population is difficult to achieve. Indeed, the other populations which originated from the Iberian refugium cannot provide a ready source of bears for transplantation: the populations from the Cantabrian mountains themselves are endangered, and, of the southern Scandinavia populations, those originating from the Iberian refugium are not clearly delimited at this time. Therefore, if it becomes obvious that the Pyrenean population has no chance of independent survival, and if further studies suggest that the populations from southern Scandinavia cannot provide bears, then the two potential conservation units of the western lineage could be pragmatically pooled into a single operational conservation unit, and animals from the Balkan refugium (Slovenia–Croatia–Bosnia and Bulgaria) might ultimately be used for the reinforcement.

Following these guidelines, the French Ministry of the Environment has already decided to assess two populations, one in southern Scandinavia and one in Bulgaria, to provide a ready source of bears for transplantation to the Pyrenees.

One of the main fundamental questions in conservation biology is to identify what has to be preserved at the intraspecific level. This study clearly demonstrates the relevance of the non-invasive molecular phylogeographic approach to reveal cryptic conservation units.

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