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Conservation genetics of the European brown bear – a study using excremental PCR of nuclear and mitochondrial sequences

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Abstract

In the Brenta area of northern Italy, a brown bear *Ursus arctos* population is rapidly going extinct. Restocking of the population is planned. In order to study the genetics of this highly vulnerable population with a minimum of stress to the animals we have developed a PCR-based method that allows the study of mitochondrial and nuclear gene sequences from droppings collected in the field. This method is generally applicable to animals in the wild. Using excremental as well as hair samples, we show that the Brenta population is monomorphic for one mitochondrial lineage and that female as well as male bears exist in the area. In addition, 70 samples from other parts of Europe were studied. As others have previously reported, the mitochondrial gene pool of European bears is divided into two major clades, one with a western and the other with an eastern distribution. Whereas populations generally belong to either one or the other mitochondrial clade, the Romanian population contains both clades. The bears in the Brenta belong to the western clade. The implications for the management of brown bears in the Brenta and elsewhere in Europe are discussed.

Keywords: brown bear (*Ursus arctos*), control region, excremental PCR, mitochondrial DNA, sex determination

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Introduction

The Adamello-Brenta Nature Park, Trentino, Northern Italy, is the last area in the Alps where brown bears have existed continuously as far back as recorded history goes and up to the present. This population is currently dwindling to extinction, the current number of individuals being perhaps as few as two or three. In an effort to ensure the future presence of bears in this area, the restocking or augmentation of the population is being considered. The success of such an effort is contingent on several factors, one of which may be the question of whether genetic substructure exists among European bears. A previous study of mitochondrial DNA polymorphisms in European brown bears (Taberlet and Bouvet 1994) has indicated that such substructure exists. In particular, two clades of related mitochondrial lineages exist in European bears, one predominantly in the west and one in the east. In order to elucidate the phylogeographic relationships of the Brenta population, we have extensively sampled that population, as well as other populations that could possibly be used as sources for its augmentation.

A general problem when studying the genetics of endangered species is to obtain samples without disturbing animals that are often shy and sensitive to stress. For bears, hair can serve as a convenient source because of the tendency of the animals to rub themselves against trees (Taberlet *et al.* 1992). However, a more general type of organic remains that animals leave behind is their faeces. We have previously shown that mitochondrial DNA as well as DNA stemming from plants consumed by bears can be amplified from nucleic acids extracted from droppings (Höss *et al.* 1992). Here, we demonstrate that nuclear genes can also be retrieved from droppings and show that the number of samples from which sequences can be retrieved increases if multiple extracts from one and the same faecal sample are performed.

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Materials and methods

Sample collection

From the Brenta population, excremental samples were collected and stored in 70% ethanol or dried and refrigerated at 4 °C. In addition, single hairs were collected from trees against which the bears rub themselves as well as other trees in the area. Liver samples were collected from bears killed during legal hunting and stored in ethanol. Table 1 summarizes the samples used.

DNA extractions

DNA was extracted from liver samples by proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation (Sambrook *et al.* 1989). From hair and droppings, DNA was extracted as described (Höss *et al.* 1992; Höss & Pääbo 1993). Briefly, approximately 50 mg of the dry excrement samples was crushed, 1 mL extraction buffer L6 (5 M guanidinium thiocyanate (GuSCN), 0.1 M Tris-HCl pH 6.4, 0.02 M EDTA pH 8.0 and 1.3% Triton X-100) was added and the sample suspended by vigorous vortexing before incubation overnight at room temperature under constant agitation. Droppings stored in alcohol were dried before extraction. Extracts were centrifuged at 5000 rpm for 10 min and the supernatants (~ 700 µL) were transferred to a tube containing 300 µL of fresh L6 buffer and 50 µL of silica matrix [for the preparation of the silica matrix (Sigma, St. Louis, MO), see Höss (1994)]. After vigorous vortexing, the mixture was incubated for 10 min at room temperature under agitation. The silica was washed twice with 1 mL of L2 washing buffer (5 M GuSCN, 0.1 M Tris-HCl pH 6.4, 0.02 M EDTA pH 8.0) and once with 1 mL of 70% ethanol. The pelleted beads were dried in a heating block at 56 °C and the DNA eluted by incubation at 56 °C with 120 µL water. Extraction blanks were included in each extraction. The extraction procedure was carried out in a room exclusively dedicated to DNA extraction from low copy number samples.

PCR and sequencing

Using the primers L15774 and H16498 (Shields & Kocher 1991), and L15997 (Kocher *et al.* 1989), we amplified and sequenced a part of the mitochondrial control region, which has been shown to be variable at the species level in *Ursus arctos* (Shields & Kocher 1991), from a Romanian bear. Based on this sequence, we designed three bear-specific primers:

(L636) 5′-AGCCAAAAACGGAGAATATCTA-3′,
(L573) 5′-TCCACTATCAGCACCCAAAGC-3′,
(H623) 5′-TAATGCACGATATACATAGG-3′.

L and H refer to the light and heavy strand, respectively. In addition, previously described primers (Höss *et al.* 1992; Taberlet & Bouvet 1994) were also used.

Enzymatic amplification from 5 µL of the excremental and hair extracts was carried out using wax-mediated hot starts (Chou *et al.* 1992; Bloch 1992) and 40 cycles in a 30-µL volume containing 67 mM Tris-HCl (pH 8.8), 2 mM MgCl2, 1.3 mg/mL bovine serum albumine (BSA), 1 mM of each nucleotide, 10 µM of each primer and 0.75 units of *Taq*. DNA polymerase (Perkin Elmer Cetus, Emeryville, CA). The cycles consisted of a denaturation step for 1 minute at 94 °C, an extension step for 1 min at 72 °C, and an annealing step of 1 minute at a temperature adjusted for the G/C content of the primers used. Ten microlitres of the amplification products were separated on a 4% low-melting agarose gel and visualized by ethidium bromide staining. Bands of expected size were cut out with a sterile razor blade, transferred to 100 µL of water and melted at 65 °C. Five microlitres was subjected to 30 cycles of a second amplification in a volume of 50 µL of 67 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 2 mM of each nucleotide, 20 µM of each primer and 0.75 units of *Taq* polymerase. PCR blanks were always amplified alongside with the samples and extraction blanks to check for contamination of the PCR reagents. The first amplifications were set up in a laboratory dedicated to procedures involving low copy number DNA whereas the second amplifications were set up in a regular laboratory.

Amplification from DNA extracted from tissue samples was carried out for 30 cycles in 25-µL volumes containing the buffer used for the first PCR (see above). Alternatively, the liver samples were pierced with a yellow pipette tip and the PCR mixture was directly inoculated with the 1–3 µL of tissue fragments and blood in the tip.

PCR products smaller than 150 bp were purified with Ultrafree-MC 30 000 (Millipore, Bedford, MA). Products longer than 150 bp were purified using the Prep-a-Gen kit (BioRad, Hercules, CA) following the supplier's instructions. Direct sequencing reactions were performed using Sequenase™ (United States Biochemical, Cleveland, OH) with incorporation labelling in a protocol modified according to Bachman *et al.* (1990) in order to circumvent fast strand renaturation of the short PCR products. The sequence reactions were separated in 6% denaturing polyacrylamide gels and visualized by autoradiography.

Sexing

The sex identification of the hair and excrement samples was carried out by a hot start PCR using the primers for the testis-determining factor gene (*SRY*) designed by Taberlet *et al.* 1993. The 131-bp fragment of the *SRY* gene was coamplified with a 295-bp mitochondrial fragment generated by the primers L573 and H-primer from Höss

et al. 1992 using 40 cycles and 50 °C annealing. The mitochondrial fragment was included to demonstrate that the PCR was successful. Aliquots of 10 µL from each PCR reaction were run on a 3% agarose gel and visualized by ethidium bromide staining.

Sequence analysis and phylogenetic estimation

Sequences were aligned by eye using the program PAUP, version 3.1.1 (Swofford 1993). Positions where deletions or insertions occurred as well as positions where the alignment could not be unambiguously determined were excluded from the phylogenetic analysis. Phylogenetic relationships were estimated with parsimony, neighbour-joining and maximum likelihood methods, and tested by bootstrap resampling (Felsenstein 1985) as implemented in PAUP 3.1.1 (Swofford 1993) and PHYLIP 3.4 (Felsenstein 1991). Pairwise distances were calculated using PAUP as well as an unpublished program (A. von Haeseler, unpublished).

Results

Excremental PCR

Droppings that were assumed from their morphology to be of bear origin were collected in the Adamello-Brenta Nature Park, Trentino, Italy and nucleic acid extracts were prepared using a standard phenol/chloroform protocol. When enzymatic amplifications of mitochondrial gene segments were attempted, no amplification products were obtained. It was observed that no primer artefacts appeared after 40 cycles of PCR and that addition of human DNA failed to result in an amplification. We concluded the *Taq* polymerase was inhibited by some component in the extracts. The inhibition could not be overcome by the addition of albumin (Pääbo *et al.* 1988). However, extractions prepared by adsorption of nucleic acids onto silica beads (Höss *et al.* 1992; Höss & Pääbo 1993) did not inhibit the polymerase (Fig. 1). Presumably, the extensive washing of the beads to which nucleic acids are adsorbed allows inhibiting components to be removed from the extract.

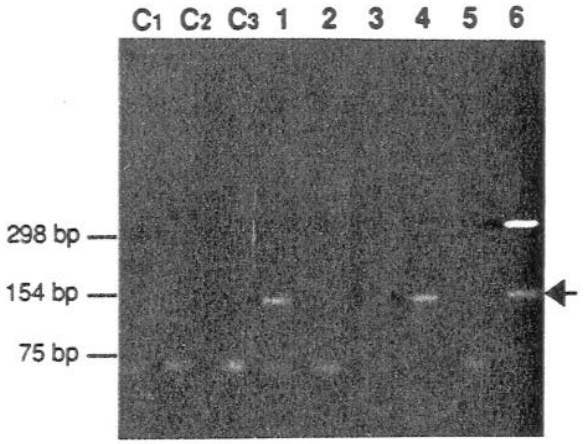


Fig. 1 Amplification of a mitochondrial DNA segment from six extracts prepared from one dropping. C_{1-3} denote amplifications from one PCR control and two extraction controls, respectively. The arrow indicates the amplification product and the numbers on the left the migration positions of molecular size markers.

However, not all samples yielded amplifiable DNA. Figure 1 shows that out of six extracts performed in parallel from one and the same excremental sample, one extract yielded no specific amplification and another only an extremely weak product. Inhibition of the reaction is not the cause of the failure to amplify specific sequences as primer artefacts appear in the reactions. Four other extracts from the same dropping yielded bands that upon sequence determination were shown to be derived from bear mitochondrial DNA. In addition, nonspecific amplification products of varying sizes can be seen in three of the amplifications. Since individual extracts from the same dropping can differ in their content of amplifiable DNA, six parallel extracts were performed from every dropping analysed. Out of the twelve samples analysed in this fashion, eight yielded specific amplification products in at least one of the extracts.

In order to evaluate the length of amplifiable DNA that can be extracted from excremental samples, amplifications of the mitochondrial control region of 141 bp, 295 bp and 398 bp (including primers) were attempted from DNA extracted from three excremental samples, a hair sample and one liver sample. Figure 2 illustrates that

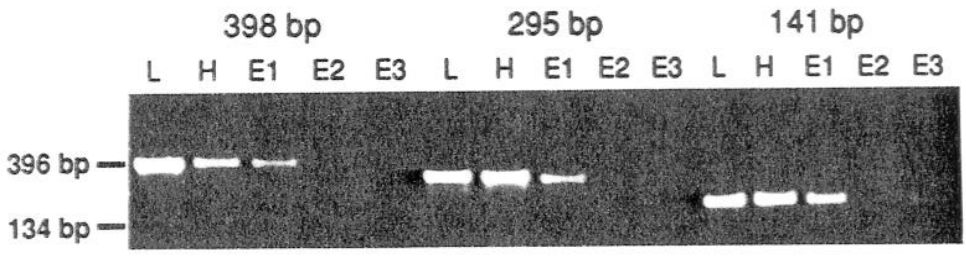


Fig. 2 Amplifications of various lengths from extracts of the excremental samples (E1–3), hair (H) and liver (L). Numbers indicate the migration positions of molecular size markers. The sizes of the amplification products are indicated above the lanes.

whereas the liver as well as the hair sample allowed amplifications of up to 398 bp, only one excremental sample contained DNA fragments allowing an amplification of this length. The other two samples allowed products of maximally 295 bp and 141 bp, respectively, to be amplified. In total, out of the 12 excremental samples analysed, one allowed the 398 bp product as well as the two shorter products to be amplified whereas five produced the 295 bp and the 141 bp products, and the 141 bp product could be amplified in only two. In the remaining four cases not even the shortest product could be amplified.

In addition, 16 single hairs from the Brenta were extracted and analysed as above. Fifteen samples yielded amplifiable DNA, all of which allowed the amplification of the 398-bp fragment. From a total of two excremental samples and ten hair samples, 213 bp of the mitochondrial control region were sequenced.

Sexing

In order to determine the sex of the bears in the Brenta as well as to find out if single copy nuclear gene sequences can be determined from droppings, we made use of the recently described primers for the *SRY* gene (Taberlet *et al.* 1993). In addition to the primers for a 131-bp-long *SRY* fragment, a mitochondrial bear-specific amplification of 295 bp was coamplified in the same tube as the *SRY* amplification in order to ascertain that the amplifications where the *SRY* band was not observed contained amplifiable bear DNA.

Figure 3 shows the amplifications from extracts of two bear droppings from the Brenta. One shows an *SRY* band as well as a mitochondrial band and is therefore interpreted as stemming from a male bear. The other dropping shows a mitochondrial band of the same intensity as the first extract but no *SRY* band and is therefore inferred to come from a female bear. The identity of the *SRY* band was sequenced and shown to be identical to the bear sequence (Taberlet *et al.* 1993) in a region that showed seven differences from the human sequence (data not shown). Out of six additional hair and excremental samples, three were of male and three of female origin.

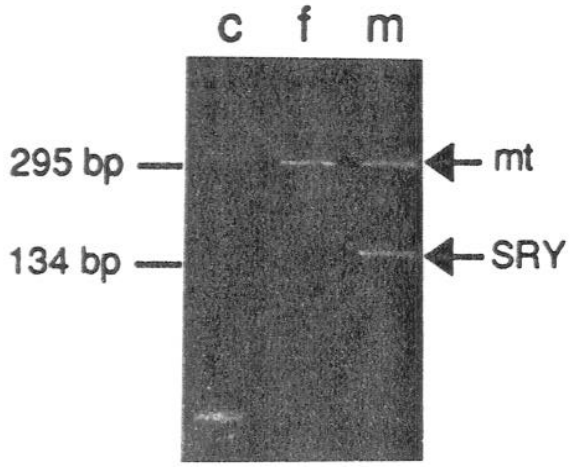


Fig. 3 Sexing of excremental samples. The arrows on the right indicate the migration positions of the amplification products of the SRY gene and a mitochondrial control amplification, respectively. Amplifications from a control extract (c) as well as two excremental samples determined to be female (f) and male (m) in origin are shown. The numbers to the right indicate the migration positions of molecular size markers.

Mitochondrial sequence variation in Europe

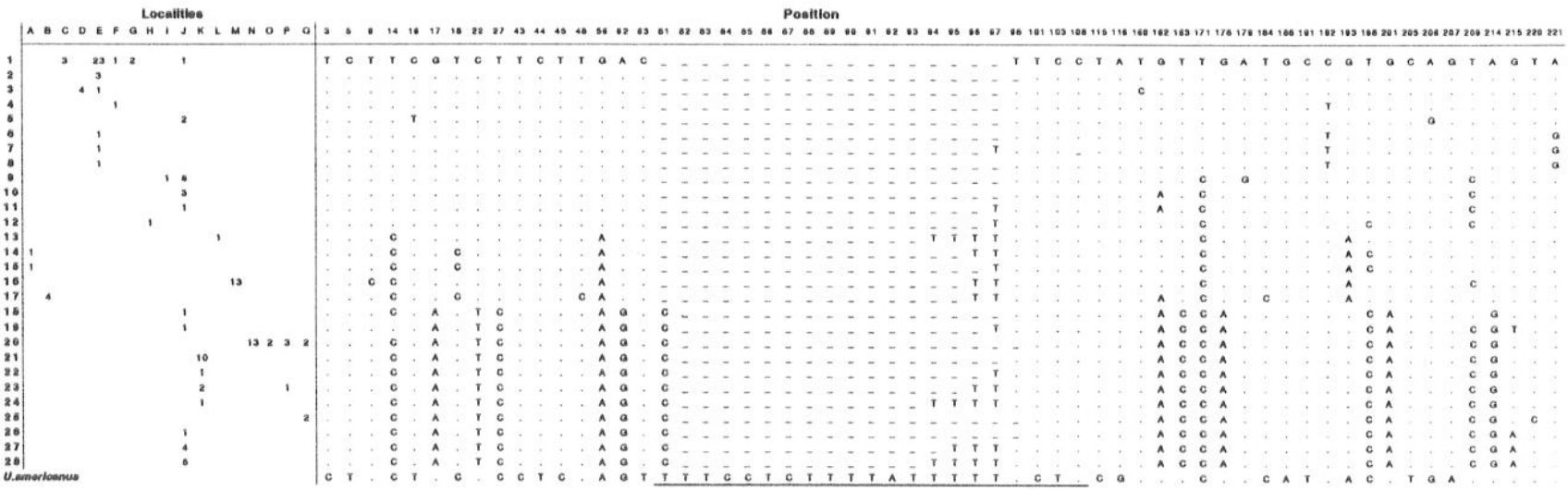
The homologous mitochondrial sequences were determined from 70 individuals from various European localities (Table 1). Of the 218 bp determined, 30 positions carried substitutions and at one position a 1–6-bp insertion/deletion occurred. These variable positions defined 20 different sequences. When they were compared to the 16 lineages previously found in European brown bears (Taberlet & Bouvet 1994), 8 were found to be identical. Thus, a total of 28 mitochondrial lineages has been identified. In Table 2, the aligned variable positions are shown along with an American black bear sequence (*Ursus americanus*).

Of the 28 lineages found in Europe, five exist in more than one population whereas the remaining 23 have been found in only one population (Table 2). In the alignable parts of the sequences, the number of substitutions among the brown bear lineages vary between 1 and 17 whereas the differences between brown bears and the American black bear vary between 17 and 27 substitutions (Table 2). A single transversion was observed among the sequences. This is in agreement with the observation that transitions predominate over transversions by a factor of at least ten in mitochondrial DNA (Brown

Table 1 Samples analysed in this study. The numbers and types of sample are indicated for each region. All samples from Brenta-Adamello were collected in 1991 except two excremental samples which were collected in 1993. Liver samples stem from bears legally shot in 1992 and 1993. The Swedish bear samples were provided in the form of DNA by Dr Leif Andersson

	Samples			
Population	total	liver	hair	faeces
Brenta	28		16	12
Slovenia	17	17		
Romania	23	23		
Slovakia	12	12		
Southern Sweden	6	6		
Northern Sweden	12	12		
Total	98	70	16	12

Table 2 Variable positions in mitochondrial control region sequences in European brown bears. The position numbers correspond to the numbers given in Taberlet and Bouvet 1994. The localities where the lineages 1–28 (given on the right) were found are: A = Spain (Cantabrian mountains), B = French Pyrenees, C = Brenta, D = Abruzzia, E = Slovenia, F = Croatia, G = Bosnia, H = Greece, I = Bulgaria, J = Romania, K = Slovakia, L = Norway, M = Southern Sweden (Dalarna), N = Northern Sweden (Lapland), O = Finland, P = Russia, Q = Estonia. The underlined positions (81–108) indicate an area which was excluded from the analysis since an insertion/deletion with respect to the black bear (Taberlet & Bouvet 1994) as well as among the brown bears makes the alignment ambiguous. The sequences are deposited in the EMBL Database under accession numbers L38261–L38280 at Genome Sequence Database (Genebank) 1800 Old Pecos Trail, Santa Fe, CA, USA.



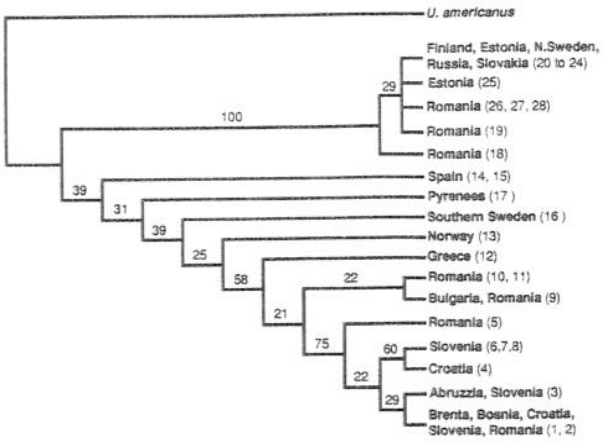


Fig. 4 Phylogeny of mitochondrial lineages in European brown bears. The parsimony tree shown is a consensus of 1000 bootstrap replications. Numbers indicate the percentages that the internal edges were retrieved. Numbers within parentheses denote the lineages given in Table 2. Neighbour-joining and maximum likelihood trees agreed in all major respects with the topology shown.

et al. 1982). The fact that the amount of intraspecific variation observed is smaller than the interspecific variation as well that very few transversions occur among the sequences indicates that multiple substitutions are not numerous enough to compromise greatly a phylogenetic analysis of these sequences.

The phylogenetic relationship between the lineages was estimated using maximum parsimony, neighbour-joining and maximum likelihood tree reconstructing methods. The American black bear sequence was used to root the tree. Trees estimated by the neighbour-joining and maximum likelihood methods yielded tree topologies that agreed in all major respects with the parsimony tree shown in Fig. 4. As has been noted by others (Taberlet and Bouvet 1993), the tree topology indicates the existence of two distinct clusters of mitochondrial DNA types in the European brown bear. One of these clusters of related lineages is found in northern Sweden, Finland, Russia, Estonia, Slovakia and some individuals in Romania whereas the other cluster contains some other lineages from Romania as well as all lineages at the other localities sampled. Thus, one of the two clusters of related mitochondrial lineages is found in populations in the western part of Europe whereas the other cluster is found in the east. An exception to this pattern is Romania, where both clusters exist in the same population.

The sequences from areas in which bears exist as continuous populations and from which thirteen individuals or more have been studied were used to calculate the mean pairwise differences between members of the same population (Table 3). It was found that the populations in southern Sweden and in Slovakia display no intrapopulation variation. However, in the latter, a homopolymer of thymidine residues varies in length, defining four different lineages. In contrast, the Romanian population is by far the most diverse, having 9 lineages among 23 samples analysed which differ by an average of 7.9 substitutions in the alignable part of the sequence. The Slovenian population has seven lineages among 34 samples. However, the lineages differ by an average of only 0.5 substitutions. Similarly, the population in northern Sweden, Finland, western Russia and Estonia exhibits little diversity, the 24 individuals studied having a mean pairwise sequence difference of 0.3. The Brenta population, finally, displays only one lineage among the 12 samples sequenced, which are estimated to stem from three individuals (see below).

When the mean pairwise sequence differences between populations are calculated (Table 3), it is found that the populations in Slovenia and neighbouring countries differ from the bears in Brenta by only 0.3 substitutions. Similarly, the populations in Slovakia and northern Scandinavia and western Russia differ by only 0.1 substitutions. All other interpopulation differences vary between 6 and 16 substitutions.

Discussion

Excremental PCR

The main problem encountered when amplifying DNA from excremental samples is inhibition of the *Taq* polymerase by unknown components in the extracts. When a silica-based extraction method (Höss & Pääbo 1993) is used for the direct isolation of nucleic acids from the droppings rather than as a means to purify extracts prepared by conventional methods as used in our previous work (Höss *et al.* 1992), these components can be

Population	*n*	1	2	3	4	5	6
Brenta	3	0.0	0.3	6.0	8.2	14.0	14.1
Bosnia, Croatia, Slovenia	34		0.5	6.2	8.4	14.2	14.3
Southern Sweden	13			0.0	11.6	16.0	16.1
Romania	27				7.9	7.5	7.6
Slovakia	14					0.0	0.1
Estonia, Finland, N. Sweden, Russia	23						0.3

Table 3 Mean pairwise sequence differences within and between some populations of European brown bears. Samples from areas between which direct evidence of gene flow exists were pooled. Thus, Slovenia, Croatia and Bosnia are treated as one population, as are northern Sweden, Finland, Russia and Estonia. The population in southern Sweden is generally considered to be separate from the one in northern Sweden

eliminated. This allows not only mitochondrial DNA, which occurs in several hundred copies per cell, to be amplified from droppings but also nuclear single copy genes such as the *SRY* gene on the Y chromosome. It is therefore possible to perform sexing and to study biparentally inherited genetic polymorphisms such as microsatellite loci using excremental PCR. In other experiments, excremental extracts from bison, elephant and polar bear have yielded sequences that by sequencing have been shown to be derived from the animal in question (M.K., unpublished). Thus, excremental PCR is a generally applicable tool for the noninvasive study of animals in the wild.

However, amplification of DNA sequences from faecal samples is not always experimentally straightforward. A peculiar feature is that when several extracts are performed from one and the same dropping, some extracts yield amplifiable DNA whereas others do not (Fig. 1). This presumably reflects an uneven distribution of cells shed from the intestinal lining in the faeces. It is therefore useful to perform multiple extracts from each dropping collected. Furthermore, the DNA extracted from droppings is clearly very degraded. This is evident from the fact that only eight out of twelve samples yielded amplifications of mitochondrial sequences and from the variation in the length of the amplifications that can be performed from the excremental samples (Fig. 2).

It was sometimes observed that sequencing of an amplification product yielded a mixture of sequences or even only a sequence of different origin than bear. These sequences were derived from a vertebrate control region and may stem from animals consumed by the bear. We have previously shown (Höss *et al.* 1992) that DNA from plants consumed by the bears can be retrieved from droppings. Thus, it may be possible to study also the feeding habits of carnivores as well as herbivores by excremental PCR. It is worth noting that sequences from pathogenic bacteria in patient stools (Frankel *et al.* 1989), *ras* oncogene mutations in cells shed from colorectal cancers (Sidransky *et al.* 1992) and tapeworm snRNA genes in fox faeces (Bretagne *et al.* 1993) have also been reported. Thus, molecular scatology promises to establish itself as a tool to study the genetics, feeding habits and pathology of wild animals as well as a diagnostic tool in medicine.

The Brenta population

The bear population in Adamello-Brenta is rapidly dwindling to extinction. At a feeding place monitored by an automatically activated infrared camera two individuals were observed in 1991 and tracks suggested the existence of one other individual. In 1992 only one individual frequented the feeding place. Thus, in 1991, when the samples were collected, the population was estimated to consist of three individuals. Assuming that the samples are randomly collected within the home range of all the bears, a total of 12 samples are needed in order to sample all three bears with 95% probability. Therefore, 12 samples were sequenced for 213 bp of a hypervariable part of the mitochondrial control region. All samples proved to contain the same mitochondrial lineage (Table 2).

In order to further investigate whether these samples stemmed from different individuals, a total of eight samples were sexed using primers for the bear SRY gene and an internal mitochondrial control amplification. It was shown that the samples stemmed from at least one female and one male bear (Fig. 3). Thus, the mitochondrial lineages found stem from at least two different bears and the disappearing brown bear population in Adamello-Brenta seems to contain a single mitochondrial lineage.

The intraspecific diversity of European brown bears

The Adamello-Brenta population is not unique in containing only one mitochondrial lineage. A previous study (Taberlet & Bouvet 1992) that investigated four samples from Pyrenean bears found only one lineage in this population which may currently number less than ten individuals. Thus, although the sampling in that case was not extensive, the Pyrenean population may also contain only one lineage. Furthermore, the samples from the population in southern Sweden also contains only one lineage. Further work is necessary to clarify if this could be due to sampling artefacts. That sampling may play a significant role is shown by the fact that the previous study found only one lineage among 13 samples from Slovenia (Taberlet & Bouvet 1994) whereas 17 samples in this study found six lineages, one of which was identical to the previously identified lineage. Even more strikingly, four hair samples from Romania yielded two lineages that differed by 2 substitutions, whereas 23 samples in this study revealed 9 lineages that differed by an average of 8 substitutions.

It has earlier been shown (Taberlet & Bouvet 1994), that the European lineages fall into two clades, one occurring in the populations in the western parts of Europe and one in the eastern parts. When the 73 individuals studied here are added to the analysis, this conclusion is largely upheld. The two clades are supported by eight substitutions conserved among all lineages in the respective clades. Thus, eight out of a total of 19 informative positions in the sequences support the dichotomy of the mitochondrial gene pool. When the geographic occurrence of the lineages are added to the tree (Fig. 4), the overall pattern that one clade occurs in western populations and one in eastern ones is also confirmed. However, the suggestion (Taberlet & Bouvet 1994) that the western clade can be divided into two subclades, one containing lineages

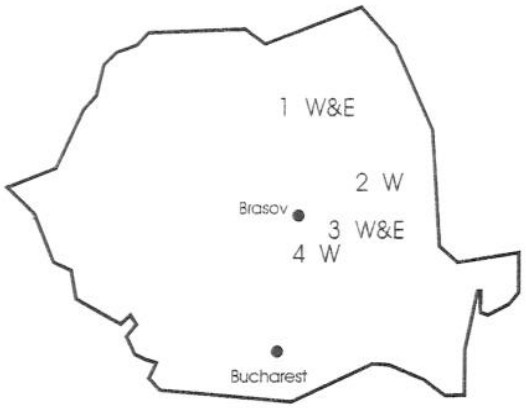


Fig. 5 Map of the localities sampled in Romania. Localities are denoted by numbers and the occurrence of mitochondrial lineages of the 'western' (W) and 'eastern' (E) clades (Fig. 4), respectively, are indicated. Locality 1 (Bistrita area) yielded 11 individuals with 'western' mitochondrial lineages (7 with lineage 9, 3 with lineage 10, 1 with lineage 11) and 7 with 'eastern' lineages (1 with lineage 26, 4 with lineage 27, 2 with lineage 28). Locality 2 (Tirgut Ocna area) yielded one bear with the 'western' lineage 5, locality 3 (Brasov area) yielded two individuals with 'western' lineages (1 with lineage 1, 1 with lineage 9) and one with an 'eastern' lineage (no. 10) whereas locality 4 (Sinaia) yielded one individual with a 'western' lineage (no. 5).

from the extreme west of Europe and one from the south-east is supported by only one informative substitution unique to these lineages and is thus tenuous.

In Romania, the previous study found two lineages among four samples, both belonging to the 'eastern clade'. Among the 9 lineages found in this study, four belong to the 'eastern clade' whereas five belong to the 'western clade'. In all cases where several samples have been collected from one locality in Romania, they contain bears carrying mitochondrial lineages of both clades (Fig. 5). This is in contrast to Sweden where a recent survey (Taberlet *et al.* submitted) shows that the 'eastern' clade in northern Sweden overlap only to a very limited extent with the 'western' clade in southern Sweden. Thus, Romania is the only population so far found where the two clades exist side by side.

The fact that the lineages show a dichotomy between eastern and western types indicates that the history of bears in Europe has involved a separate history of two groups of bears. It is reminiscent of the division between several eastern and western subspecies and species in Europe, e.g. house mice, frogs, and crows. This presumably goes back to the existence of separate refugia during quaternary glaciations, probably located in the south-west on the Iberian Peninsula or North Africa, and in the south-east of Europe or in Asia, respectively. It is interesting that the dichotomy in the bear gene pool was not suggested by morphological or behavioural traits prior to its discovery through the study of mitochondrial DNA sequences.

Management implications

The plans to augment the bear population in Adamello-Brenta and the dichotomy in the phylogeny of European bear mitochondrial lineages raises some interesting issues in conservation genetics and the biology of brown bears. The phylogeographic pattern (Fig. 4) shows that brown bears in the west and the east have different histories. If the main aim of population augmentation is to restore the situation that existed before the decimation of bears, then obviously the most suitable population for restocking the Brenta population is the Slovenian population, where the lineage now surviving in the Brenta exists at a high frequency and the other lineages are closely related (Tables 2 and 3). However, even more relevant for this approach would be an assessment of the situation before the decimation of the Brenta population when it was presumably more diverse. Such information could be provided by the study of skins from the beginning of the this century.

The only other population where the Brenta lineage is found is Romania. However, here not only western lineages, differing from the Brenta lineage by 0–3 substitutions, occur but also a substantial proportion of eastern lineages which differ from the Brenta lineage by 11–12 substitutions. It is presently unclear if this deep dichotomy in the maternally transmitted mitochondrial gene pool is reflected also in the nuclear gene pool, which is biparentally transmitted. If it exists predominantly or exclusively in the mitochondrial gene pool, it is probably maintained by a lower dispersal rate of female than of male bears. However, if it exists also in the nuclear gene pool, it probably reflects hitherto unrecognized physiological or behavioural traits that restrict the successful mating between 'western' and 'eastern' brown bears. This, obviously, would have profound consequences for the management of the European bear in the wild as well as in zoological gardens. Further work on the genetics of European brown bears is therefore of great importance.

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Felix Knauer and Wolfgang Schröder as well as Petra Kaczenfky work at the Munich Wildlife Society on the protection, reintroduction and management of bears in the Alps. Together with Alberto Stoffela of the Italian Forestry Service, and with the Adamello-Brenta Nature Park and the Provincial Government of Trentino they are developing a plan for the long-term management of the Brenta bears. Stimulated by the problems encountered in the sampling of the animals in Brenta, Michael Kohn and Matthias Höss developed the excremental PCR in the laboratory of Svante Pääbo.