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Evolution

Tracking the origins of the cave bear (*Ursus spelaeus*) by mitochondrial DNA sequencing

(ancient DNA/phylogeny)

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ABSTRACT **The different European populations of *Ursus arctos*, the brown bear, were recently studied for mitochondrial DNA polymorphism. Two clearly distinct lineages (eastern and western) were found, which may have diverged ≈850,000 years ago. In this context, it was interesting to study the cave bear, *Ursus spelaeus*, a species which became extinct 20,000 years ago. In this study, we have amplified and sequenced a fragment of 139-bp in the mitochondrial DNA control region of a 40,000-year-old specimen of *U. spelaeus*. Phylogenetic reconstructions using this sequence and the European brown bear sequences already published suggest that *U. spelaeus* diverged from an early offshoot of *U. arctos*—i.e., approximately at the same time as the divergence of the two main lineages of *U. arctos*. This divergence probably took place at the earliest glaciation, likely due to geographic separation during the earlier Quaternary cold periods. This result is in agreement with the paleontological data available and suggests a good correspondence between molecular and morphological data.**

Rare are extinct mammals that are known from such a wealth of fossilized bones as the cave bear, *Ursus spelaeus*. The majority of remains date from the Late Pleistocene, yet this bear lived much earlier, about 300,000 years ago. Most of the populations disappeared about 20,000 years ago; however, some of the remains found are slightly more recent. In the Late Pleistocene, the cave bear ranged northward from southern England, to southern Poland, and southward into the northern parts of Spain, Italy, and Greece. It spread eastward to Odessa and the Caucasus but did not occupy the greater part of Russia (1, 2). Therefore, the cave bear is an endemic European species. Despite this limited distribution area, a number of local forms have been described, which suggests limited migration movements between populations (1). From a general point of view, the cave bear was larger and more powerfully built than the brown bear. The most striking characteristics seen in cave bear skulls compared with those of the brown bear are a domed forehead, a convex lower border of the jaw, and a clearer adaptation of the teeth toward vegetarianism by the absence of premolars and by grinding molars with broad masticatory surfaces (2, 3).

Based on the fossil record, two different hypotheses have been proposed concerning the evolutionary history of Eurasian bears. Both suggest an unbroken lineage *Ursus deningeri* to *U. spelaeus*: in several caves, remains from the Middle Pleistocene correspond to intermediate forms between these two species (e.g., refs. 4 and 5). However, the two hypotheses differ on the common ancestor of *Ursus arctos* and *U. spelaeus* proposed and by the number of ancient lineages of *U. arctos* identified. Kurten (2) suggested that *Ursus etruscus* would be the common ancestor, that only one lineage of *U. arctos* evolved in Asia, and that this lineage recently colonized Europe. Based on a complete revision of the fossils of Eurasian bears, Mazza and Rustioni (6) proposed the *U. minimus–thibetanus* group as the common ancestor, two ancient lineages of *U. arctos* (one in Asia and one in Europe), and *U. spelaeus* originating from the European lineage of *U. arctos*.

For conservation purposes, the different European populations of the brown bear were recently assayed for mitochondrial DNA (mtDNA) polymorphism (7, 8). The control region flanking the $tRNA^{Pro}$ gene of 60 representative individuals was sequenced. One of the most striking results was the identification of two very distinct lineages in Europe: the eastern lineage is mainly represented by the large populations of Russia and Romania, whereas the western lineage includes animals from the Cantabrian mountains (Spain), the Pyrenees (France), Trentino and Abruzzo (Italy), Slovenia, Croatia, Bosnia, Greece, and Bulgaria. These two lineages differ in mtDNA control-region sequences by a mean pairwise genetic distance of 7.13%, may have diverged about 0.85 million years ago, and probably correspond to the Asiatic and European lineages proposed by Mazza and Rustioni (6).

The analysis of DNA present in ancient bones or teeth has become a powerful way to analyze the genetic material of extinct species (9–11). For example, such analysis has been successfully applied to the study of the extinct moa (reviewed in ref. 12), to 14,000-year-old bones of the saber-toothed cat *Smilodon* (13), and to a 25,000-year-old bone of *Equus hemionus* (14). In this context, it was tempting to use PCR amplification to sequence the mtDNA control region in the cave bear, in order (*i*) to determine the relationships between this extinct species and the two recent lineages of the brown bear in Europe and (*ii*) to test the phylogeny based on paleontological data. Furthermore, our study emphasizes the difficulties in obtaining reliable results when very few ancient molecules are present in fossil bones.¶

MATERIALS AND METHODS

Samples. The bone samples of *U. spelaeus* were as follows: four bones from the cave of "Prélétang," Isère, France, 40,000 years old—TAB1 (femur), TAB2 (metatarsus), TAB3 (femur), and TAB4 (mandible of young)—and one femur (TAB5) from the "Grotte de l'Ours," Saint Pierre d'Entremont, Savoie, France, 25,000–30,000 years old. Two rib bones from the cave of "La Balme à Collomb," France, gave no positive result (data not shown). For comparison, we

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¶The cave bear mtDNA sequence has been deposited in the GenBank database (accession no. X80259).

added to the study a subfossil bone (TAB7, 300–2000 years old) of *U. arctos* from the Vercors Mountains, France.

DNA Extraction. Extractions were carried out in a laboratory where no bear DNA is handled. The bone surfaces were removed by scraping with a sterile scalpel, ground in a frozen mill apparatus, and extracted by our "classical" method (11, 15). Two grams of powder was dissolved in 9 ml of 0.5 M EDTA, pH 8.0/0.1% *N*-lauroylsarcosine and containing proteinase K (125 μg/ml; Appligene, Strasbourg, France) for ≈18 hr at 50°C with constant shaking. Two extractions using a mixture of phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol), were performed. The samples were dialyzed (dialysis tubing from Polylabo, Strasbourg, France) against 10 liters of 1 mM EDTA for 4 hr. The solutions were then concentrated by 2-propanol precipitation and the pellets were dissolved in 30 μl of water.

Amplification and Sequencing. The precise knowledge of the mtDNA variation in the brown bear gave us the opportunity to design sets of primers which amplify the most variable and informative part of the control region, where 18 variable sites have been described for the brown bear. Thus two bear-specific primers were designed to amplify a 139-bp fragment of the mtDNA control region: L16164, 5′-GCCCCATGCATATAAGCATG-3′, and H16299, 5′-GGAGCGAGAAGAGGTACACGT-3′ (8). Numbering and letters L and H refer to the light and heavy strands of human mtDNA. One run of 40 cycles of PCR was performed in a programmable heating block (Perkin–Elmer/Cetus DNA thermal cycler) as follows: 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C plus 7 min at 72°C for the last cycle. The preparation of the reagents for PCR amplifications was carried out in a DNA-free room where DNA is never handled. PCR products were cloned into the PCR vector from Invitrogen and sequenced from both sides using "dye-deoxy" dNTPs on a 370A automated sequencer (Applied Biosystems) with the reverse and forward universal primers.

Phylogenetic Reconstructions. Sequences were aligned by eye and treated by parsimony using the PAUP package (16) and by distance methods with Kimura correction (17), using the Fitch (18) and neighbor-joining (NJ) (19) algorithms available on the French CITI-II/Bisance network (20). Bootstrap analysis was carried out on the PAUP (2000 replicates) or NJ (1000 replicates) results (21).

RESULTS

DNA Extraction and Sequencing. We have extracted DNA from five samples of *U. spelaeus* bones ranging from 25,000 to 40,000 years old and from one subfossil bone of *U. arctos*. Fig. 1*A* shows the visualization of the extracted DNA on a 1% agarose minigel. We noticed the presence of DNA in three of the *U. spelaeus* samples (TAB1, -4, and -5). This DNA was of high molecular weight for TAB1 and TAB4, but the presence of smaller molecules which migrated at 200–400 bp in the TAB1 and TAB5 samples was also observed. We have not noticed the presence of a blurring of blue fluorescence which is frequently observed after extraction of ancient bones. This could be due to the extraction method, which used 2-propanol precipitation, as it greatly decreases the amount of blue fluorescence in the ancient extracts (15).

The mtDNA control region was amplified with PCR using synthetic primers. This region offers the unique advantage of being bear-specific and allowing population identification of bears, because it accumulates mutations at a high rate (8). In addition, by working in a laboratory where no bear DNA is handled, it is possible to amplify ancient bear extracts without high risk of contamination. Using two specific primers we were able to amplify the expected 139-bp sequence in the TAB2 and TAB7 samples (Fig. 1*B*). It is interesting that the successful amplifications were obtained in two samples

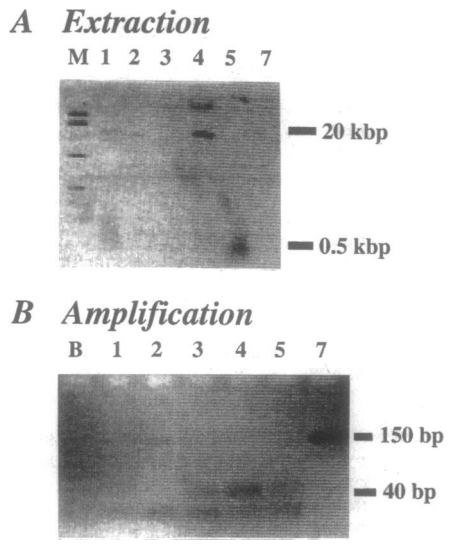

FIG. 1. (*A*) Visualization on a 1% agarose gel stained with ethidium bromide of the extracts realized from bear bones. Lane M, molecular size marker (the 20-kbp and 0.5-kbp positions are indicated at right). (*B*) Result of the PCR amplification of a 139-bp fragment in the mtDNA control region, loaded on a 3% Nusieve (FMC) agarose gel. The positions of 150-bp and 40-bp (oligonucleotide dimers) are indicated. Lane B, blank (PCR without DNA sample). In both *A* and *B* lanes 1–5 correspond to the TAB1–5 *U. spelaeus* bones; lanes 7 correspond to the TAB7 sample, which is from a subfossil bone of *U. arctos*.

where no DNA could be visualized after the extraction procedure. In all the samples where DNA was visible (TAB1, TAB4, and TAB5) several amplifications never yielded amplification products. Therefore, there is no correlation between the presence of visible DNA and amplification, and the visible DNA may not be of bear origin but may rather represent DNA from soil microorganisms. This sharp contrast between visualization of extracted ancient DNA and PCR amplification was noticed also by other authors (22). It is noteworthy that the cave bear sample which yielded a positive amplification signal is a metatarsus bone, as several authors have observed a better preservation of ancient DNA in bones or soft tissues coming from the extremities (12, 23). It is hypothesized that these parts are more rapidly desiccated after death. Such a relatively dry condition could protect the DNA from hydrolytic degradation.

The PCR fragments obtained from TAB2 and TAB7 were cloned and several independent clones were sequenced. From Fig. 2 it is clear that the two sequences are different and thus do not result from cross contamination of the samples (see *Discussion*). The TAB7 sequence, coming from a subfossil *U. arctos* bone from the Alps, is identical to the mtDNA sequences of individuals coming from Slovenia and Trentino (Italy) and contains four mutations when compared with the Pyrenean sequences (8). This observation is a strong argument in favor of the validity of the TAB7 sequence, because the now-extinct French Alps populations were strongly related, if not identical, to the living bears from Slovenia and Trentino.

Clones representing the TAB2 fragment from a 40,000-year-old *U. spelaeus* metatarsus differ at two positions only, which may be due to errors of the *Taq* polymerase acting on the chemically modified ancient DNA template (data not shown; see *Discussion*). When these two differences are excluded, the *U. spelaeus* control-region sequence comprises seven mutations compared with the Pyrenean bear mtDNA sequence (7). Interestingly, this sequence is different from other *U. arctos* sequences of this region determined by Taberlet and Bouvet (8), as well as from the mtDNA control-

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                10        20        30        40        50        60        70
PYR    TAAGCATGTA CATATTATGC TTGGTCTTAC ATGAGGACCT ACGTTCCAAA AGTTTGTTTC AGGTGTATAG
CAN    .......... ......G... .......... ........T. .......... ..C....... ..........
NOR    .......... ......G... .......... ........T. .......... .......... ..........
DAL    .......... ......G... .......... ........T. .......... .......... ...C......
ABR    .......... ....C.G... .....T.... ........T. .......G.. .......... ..........
SLO    .......... ......G... .....T.... ........T. .......G.. .......... ..........
TAB7   .......... ......G... .....T.... ........T. .......G.. .......... ..........
CRO    .......... ......G... .....T.... ........T. ......TG.. .......... ..........
GRE    .......... ......G... .......... ........T. .......G.. ..C....... ...C......
BUL    .......... ......G... .......... ...G....T. .......G.. .......... ...C......
RUS    .......... .......C.. .......... ..A.....T. .......G.. ..C..A.... ...C....G.
EST    .......... .......C.. .......... ..A.....T. .......G.. ..C..A.... ...C....G.
RO1    .......... .......C.. .......... ..A.....T. .......G.. ..C..A.... ...C....GT
RO2    .......... .......C.. .......... ..A.....T. .......G.. ..C..A.... ...C....GA
TAB2   .......... .......... .......... .......... ..A..T.G.. .....A.... .A........
BLACK  .......... ......G... .......... .......... ..A..T..... .C......T GA......G.

                80        90        100       110       120       130       140
PYR    TCTGTAAGCA TGTATTTCAC TTAGTCCGGG AGCTTAGTCA CCAGGCCTCG AGAAACCAGC AATCCTTGC
CAN    .......... .......... .......... .......... .......... .......... .........
NOR    .......... .......... .......... .......... .......... .......... .........
DAL    .......... .......... .......... .......... .......... .......... .........
ABR    .......... .......... .......... .......... .......... .......... .........
SLO    .......... .......... .......... .......... .......... .......... .........
TAB7   .......... .......... .......... .......... .......... .......... .........
CRO    .......... .......... .......... .......... .......... .......... .........
GRE    .......... .......... .......... .......... .......... .......... .........
BUL    .......... .......... .......... .......... .......... .......... .........
RUS    .......... .......... .......... .....GA... .......... .......... ..C......
EST    ....C..... .......... .......... .....GA... .......... .......... ..C......
RO1    .......... .......... .......... .....GA... .......... .......... ..C......
RO2    .......... .......... .......... .....GA... .......... .......... ..C......
TAB2   ..........  .......... .......... ......A... .......... .......... ..C......
BLACK  ....C..... .......... .......... .....GA... .......... .......... ..C......
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FIG. 2. Sequences of the TAB2 (*U. spelaeus*) and TAB7 (*U. arctos*) PCR fragments compared with the sequences of a representative panel of brown bear populations (8) as well as *U. americanus* (BLACK) as an outgroup. The abbreviations of brown bear lineages are identical to those of Fig. 3. Brown bear and cave bear sequences have been deposited in the GenBank/EMBL data library under the accession numbers X75862–X75878 and X80259, respectively.

region sequences from the bank of M. Höss and S. Pääbo (personal communication).

Phylogenetic Analysis. The TAB2 sequence, which contains no insertions or deletions when compared with other known *U. arctos* sequences, was aligned by the CLUSTAL V algorithm with a panel of European brown bear sequences representing the various known populations. A sequence of the mtDNA control region of the American black bear *U. americanus* (8) was used as an outgroup. The bear sequences have 91.4–98.6% homology among themselves. The compared bear sequences do not contain transversions, with the sole exception of the RO1 sample, which contains a G-to-T transversion at position 70 of the alignment (see Fig. 2). The cave bear sequence carries only transitions. This strong bias for transitions over transversions is in agreement with the known high transition/transversion ratio found for mtDNA of other closely related species of mammals.

Table 1 shows the number of substitutions among the different brown bear lineages, the cave bear, and the American black bear. Both distance-matrix analyses by NJ and Fitch and parsimony analysis (PAUP) were performed, and the robustness of the various trees obtained was determined by the bootstrap resampling procedure. Fig. 3*A* presents a simplified version of the various topologies obtained with the three methods used. A representative topology containing all the sequences used in this study and obtained with the NJ algorithm is presented in Fig. 3*B*.

The topology found by both parsimony (PAUP) and distance (Fitch, NJ) analysis shows the two previously described lineages (supported by high bootstrap values both in PAUP—

Table 1. Number of substitutions among the various brown bear, the cave bear (TAB2), and the American black bear (BLACK) lineages

	CAN	NOR	DAL	ABR	SLO	TAB7	CRO	GRE	BUL	RUS	EST	RO1	RO2	TAB2	BLACK
PYR	3	2	3	5	4	4	5	5	5	11	12	12	12	7	12
CAN	—	1	2	4	3	3	4	2	4	10	11	11	11	10	11
NOR		—	1	3	2	2	3	3	3	11	12	12	12	9	12
DAL			—	4	3	3	4	2	2	10	11	11	11	10	13
ABR				—	1	1	2	4	4	12	13	13	13	10	15
SLO					—	—	1	3	3	11	12	12	12	9	14
TAB7						—	1	3	3	11	12	12	12	9	14
CRO							—	4	4	12	13	13	13	10	15
GRE								—	2	8	9	9	9	10	13
BUL									—	10	11	11	11	10	15
RUS										—	1	1	1	10	13
EST											—	2	2	11	12
RO1												—	1	11	14
RO2													—	11	14
TAB2														—	9

Abbreviations of brown bear lineages are identical to those of Fig. 3.

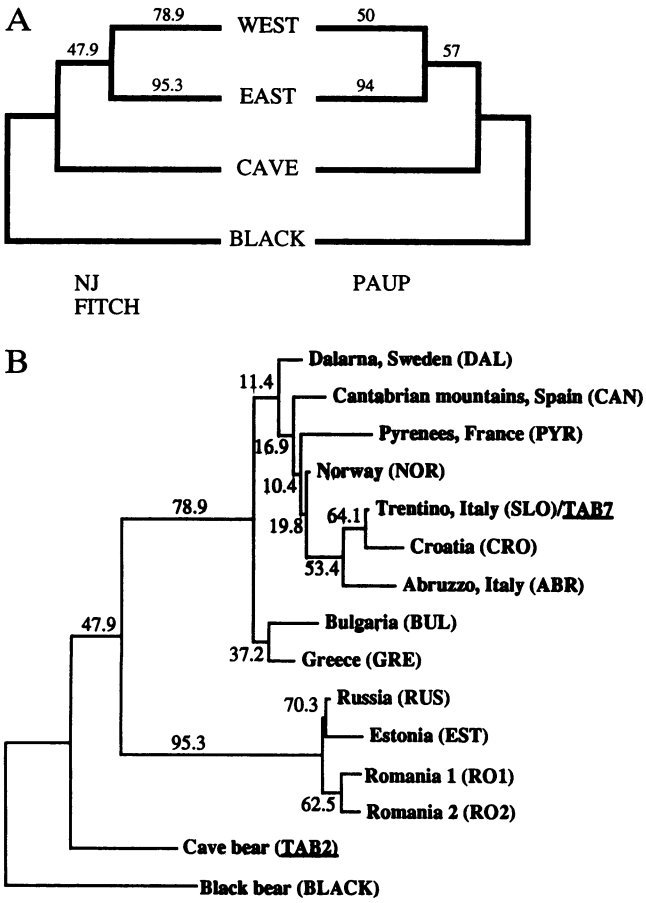

FIG. 3. (*A*) (*Left*) Consensus distance-matrix trees calculated with NJ or Fitch algorithms and rooted with the *U. americanus* sequence. The distance matrix was estimated from data of Fig. 2 by using the two-parameter model of Kimura (17). (*Right*) Consensus parsimony tree obtained with PAUP. The various brown bear sequences are regrouped on east and west lineages for clarity. Bootstrap estimates are indicated for each branch. (*B*) An example of a complete NJ tree with the corresponding bootstrap values.

94% for east, 50% for west—and in NJ—78.9% for east, 95.3% for west) clustering together in 57% and 47.9% of bootstrap replicates in PAUP and NJ, respectively. Then this monophyletic brown bear group is joined by the cave bear sequence. The parsimony analysis (branch-and-bound search option) gives 24 equally parsimonious trees which vary only in the branching order of the individual brown bear sequences within each of the two lineages (data not shown). These most parsimonious trees require 36 steps and have consistency and retention indexes of 0.667 and 0.789, respectively. The bootstrap values obtained with the parsimony analysis are on average weaker than those obtained with NJ, confirming the weaker resolving power of parsimony analysis for these sequences. Our phylogenetic analysis gives much the same results as a previous analysis (8), although ours is based on shorter sequences. The short sequence used may explain why the various brown bear populations are poorly resolved within each lineage when compared to Taberlet and Bouvet's results (8).

DISCUSSION

Authenticity of the Cave Bear Sequence. In this study, we amplified and sequenced DNA from a 40,000-year-old bone of *U. spelaeus*. To our knowledge, this represents the oldest DNA fragment yet sequenced from ancient bones (24). Contamination represents the most crucial problem when working on such ancient DNA. Because ancient DNA is very often cut into small fragments and is chemically modified, it is a very bad substrate for the *Taq* polymerase. For that reason, in cases of contamination with even a minute amount of modern DNA, this contaminant DNA will always be preferentially amplified. Thus, special care against accidental introduction of contaminants into the templates must be taken when working with ancient samples. Because molecules of ancient DNA are highly damaged, the *Taq* polymerase sometimes does make errors and creates artifactual mutations (25). For these two reasons (contamination and artifactual mutations) it is particularly important to assess carefully the authenticity of the amplified ancient sequence. In our case we have several reasons to be confident of the authenticity of our cave bear sequence.

(*i*) The study of an animal species which has never previously been used in the laboratory where extraction and amplifications are carried out can reduce the risk of contamination. For that reason, the cave bear bones were studied in the Lille laboratory, where modern bear DNA was never handled and where no bear ever came!

(*ii*) The cave bear sequence we have isolated is different from all the sequences of bears known to date (refs. 19 and 26; P.T., unpublished data; M. Höss and S. Pääbo, personal communication).

(*iii*) The subfossil bone of *U. arctos* (TAB7) gave a typical *U. arctos* sequence which corresponds to its geographical origin (Alps). This shows that there was no cross contamination of the samples during extraction and/or PCR amplification. That several samples gave negative PCR amplification results also confirms this observation.

(*iv*) The presence of two artifactual mutations between the sequenced clones argues for the ancient origin of the PCR template. For comparison, using the same reagents and conditions, we amplified a 1-kb fragment containing the entire 12S rRNA gene of modern rodents without observing mutations between sequenced individual clones (27).

(*v*) The mitochondrial genome accumulates many more transitions than transversions. The mutations observed in our cave bear sequence are all transitions. If mutations were created at random by the *Taq* polymerase, we should have observed an equal number of transitions and transversions.

(*vi*) As previously observed, the control region is variable between different bear populations. The mutations are clustered in some highly variable sites. The variable positions in the cave bear sequence all correspond to one of these variable sites, again stressing the fact that the mutations were not created at random by PCR but indeed represent the result of an evolutionary process.

Taken together, all of these observations provide strong evidence confirming that the extracted and sequenced DNA issued from extinct specimens rather than from contaminations. The phylogenetic analysis also gives a good argument for the validity of the sequence, as the position of *U. spelaeus* observed in the molecular phylogeny is consistent with paleontological information.

Knowledge of the polymorphism of a living species (here *U. arctos*) closely related to the cave bear is very important for several reasons. First, it permits us to trace back the origin of a possible contamination. Because we know the various types of *U. arctos* control regions, we can be confident of the fact that the cave bear sequence is clearly different from all the geographical variations yet described for *U. arctos*. If the cave bear sequence were due to a contamination of the sample, we would have observed a sequence corresponding to the present-day Italian population. Indeed, this type of sequence was observed in the subfossil TAB7 *U. arctos*, which came from the Vercors mountains and which was extracted during the same round of experiments as the cave bear DNA. Second, knowledge of

the polymorphism of *U. arctos* control region allows us to check that the 139-bp fragment contains consistent phylogenetic information which permits retracing the cave bear origin (see below).

The cave bear sequence presented in this paper was found in only one bone (TAB2) and only in the Lille laboratory. The extraction and PCR experiments were conducted in two labs (in Lille and in Grenoble) with two different extraction methods. In Lille we used the method described in *Materials and Methods*, whereas in Grenoble all the extractions were conducted in a small scale with only 400 μl of extraction buffer and 0.1 g of bone powder. So in Lille the extraction scale was 20 times higher than in Grenoble. This may explain the failure to amplify bone DNA in Grenoble, because we can easily imagine that there is only a very small number of ancient DNA molecules in a 40,000-year-old bone. During a large-scale extraction, we have many more chances to pick up one ancient molecule, whereas a small extraction procedure will miss it. This hypothesis is consistent with the fact that the much more recent *U. arctos* subfossil bone (TAB7) gave positive amplification signals and the same sequence both in Lille and Grenoble. The bone powder of the sample TAB2 was sent to M. Höss and S. Pääbo in Munich. They were unable to amplify any cave bear DNA (M. Höss and S. Pääbo, personal communication). The same hypothesis may also explain the failure in Munich, since a small-scale silica-based purification method (14) was used in this laboratory. It is important to note that the conditions of bone conservation in Prélétang (the cave where the TAB2 bone was found) seem not especially favorable to DNA conservation.

Phylogeny of the Cave Bear. In the main, the molecular phylogeny based on the analysis of a 139-bp fragment of the mtDNA control region (Fig. 3) is congruent with the paleontological data. The sequence divergence observed (Table 1) confirms that the cave bear diverged during the earlier Quaternary cold periods, and is compatible with the affiliation *U. deningeri–U. spelaeus* observed in the fossil record. The molecular phylogeny (Fig. 3) suggests that the cave bear diverged before the split of the eastern and western lineages of the brown bear. This branching pattern exactly corresponds to the hypothesis of Kurten (2). However, we cannot exclude the views of Mazza and Rustioni (6), where *U. spelaeus* diverged about at the same time as the two lineages of *U. arctos*, because (*i*) the genetic distances observed between the cave bear and each lineage of brown bear are comparable to the distance obtained between the two lineages of brown bear (Table 1) and (*ii*) the node connecting the cave bear to the brown bear lineage is weakly supported despite the occurrence of 17 informative sites in the sequence analyzed. Kurten (2) hypothesized that the brown bear colonized Europe relatively recently. The presence of two distinct brown bear lineages invalidates this hypothesis. Between the split of these two lineages 850,000 years ago and the extinction of the cave bear about 20,000 years ago, the cave bear could have been responsible for the isolation of the two brown bear populations, the first in Asia, which corresponds to the eastern lineage, and the second in Europe, which corresponds to the western lineage in an area where the cave bear fossils have never been found (i.e., in the south of Spain). It seems reasonable to hypothesize that the two brown bear lineages became neighbors when the cave bear disappeared 20,000 years ago. Such a hypothesis may explain the relatively high degree of divergence existing between the two brown bear populations, which seems surprising given their present geographical proximity (8).

It will be important to study the population genetics of the cave bear in order to further delineate its relationships with the European brown bears. This aim will be reached if we find new samples coming from deposits where the conditions of conservation are better. Such well-preserved samples could enable the study of a higher number of individuals in order to obtain information about the genetic variability existing within the cave bear lineage.

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