Ancient DNA analysis reveals divergence of the cave bear, *Ursus spelaeus*, and brown bear, *Ursus arctos*, lineages

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The cave bear, *Ursus spelaeus*, represents one of the most frequently found paleontological remains from the Pleistocene in Europe. The species has always been confined to Europe and was contemporary with the brown bear, *Ursus arctos*. Relationships between the cave bear and the two lineages of brown bears defined in Europe, as well as the origins of the two species, remain controversial, mainly due to the wide morphological diversity of the fossil remains, which makes interpretation difficult [1, 2]. Sequence analysis of ancient DNA is a useful tool for resolving such problems because it provides an independent source of data [3]. We previously amplified a short DNA fragment of the mitochondrial DNA control region (mt control region) of a 40,000-year-old *Ursus spelaeus* sample [4]. In this paper, we describe the DNA analysis of two mtDNA regions, the control region and the cytochrome b gene. Control region sequences were obtained from ten samples of cave bears ranging from 130,000 to 20,000 years BP, and one particularly well-conserved sample gave a complete *cyt b* sequence. Our data demonstrate that cave bears split largely before the lineages of brown bears around 1.2 million years ago. Given its abundance, its wide distribution in space and time, and its large morphological diversity, the cave bear is a promising model for direct observation of the evolution of sequences throughout time, extinction periods, and the differentiation of populations shaped by climatic fluctuations during the Pleistocene.

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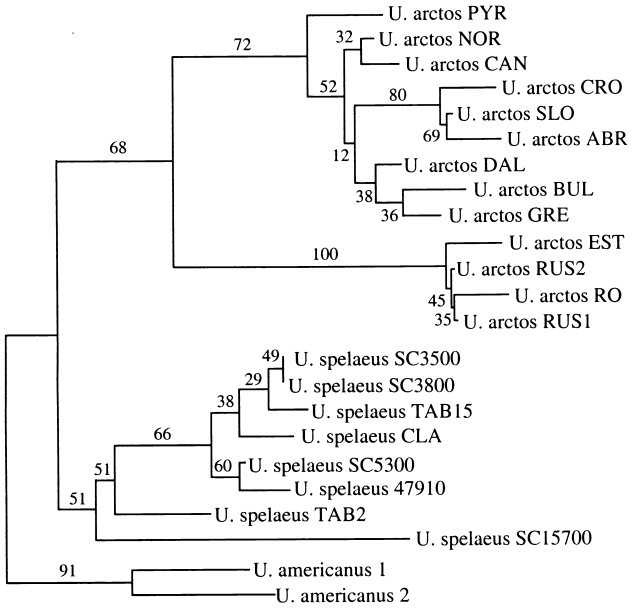
Results and discussion

We extracted DNA from 22 samples of *U. spelaeus* bones, ranging from 20,000 to 130,000 years old and coming from 8 different European deposits. The potential presence of PCR inhibitors in the DNA extracts was tested by the inclusion of increasing amounts of bear extract in a PCR assay amplifying modern duck DNA. We used several concentrations of extracts in PCR amplification reactions, for which we used bear-specific primers targeting the mitochondrial DNA control region. First, we amplified a 139 bp fragment of the mt DNA control region, which offers the unique advantage of being bear specific. It also allows population identification of bears because it accumulates mutations at a relatively high rate [4, 7]. From the 22 samples used in PCR amplification, 10 gave a positive signal at the expected size, whereas the various negative controls remained blank. We compiled several sequences per sample to generate the final sequence of the 139 bp fragment (see Supplementary material, including Table S1, available with this article on the internet). For one sample (SC11700), the use of a set of internal primers allowed us to obtain only a single positive amplification and resulted in an 88 bp product.

The 10 samples for which PCR was succesful gave different sequences that exclude cross-contamination between the samples. The only exceptions are the two samples from Cova Linares (CLA and CLB), which come from the same deposit, and the samples of the Sclayn deposit (SC3500 and SC3800), which came from the same layer [14] (40,000–45,000 years BP, layer 1a). The ancient sequences were aligned with *U. arctos* and *U. americanus* (American black bear) sequences. The *U. spelaeus* sequences do not contain any transversions when they are compared to other bear sequences, and this finding is in accordance with the known bias toward transitions in mt DNA. In a phylogenetic tree based on distance analysis, all *U. spelaeus* sequences cluster together, and this finding provides a strong argument in favor of their authenticity (Figure 1). The tree derived from these sequences (Figure 1) is similar to that obtained previously with a unique cave bear sequence [4] even though it is supported by low bootstrap scores and few informative sites.

We amplified longer PCR fragments to obtain more sequence information. For 6 samples, we obtained a complete 282 bp fragment encompassing the previously sequenced 139 bp fragment. The alignment of these sequences with the homologous region of brown and black bears as well as of *U. maritimus* (polar bear) reveals that

Figure 1



Phylogenetic distance tree showing the placement of the cave bear sequences when the 139 bp control region fragment is used. The tree was calculated by the NJ method. Bootstrap values obtained after 1000 replicates are indicated for each branch. The tree is rooted with two *U. americanus* sequences. The various populations of brown bear are designated as in [7], as follows: PYR (Pyrenees, France), CAN (Cantabrian mountains, Spain), NOR (Norway), DAL (Dalarna, Sweden), CRO (Croatia), SLO (Trentino, Italy), ABR (Abruzzo, Italy), BUL (Bulgaria), GRE (Greece), EST (Estonia), RUS (Russia, Slovakia, Lapland, and Finland), RO (Romania).

Figure 2

(a)

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                    71                                                  120
U.arctos PYR        CCTCGAATAC----------TTTTTT---CCCCCCCTATGTATATCG
U.arctos CRO        ..........----------....-----..........................
U.arctos RO         ..........----------C.....TTT..........................
U.maritimus         ..........----------....-----..........................
U.spelaeus TAB2     ...TA.....TTTTTCCTTTATT......T--..........................
U.spelaeus TAB15    ...TA.....TTTTTCCTTTATT......T--..........................
U.spelaeus CLA      ...TA.....TTTTTCCTTTATT.....----..........................
U.spelaeus SC3500   ...TA.....TTTTTCCTTTATT......---..........................
U.spelaeus SC3800   ...TA.....TTTTTCCTTTATT......---..........................
U.americanus        TT.TA.....TTTCCTCTTTTAT.....C---.T..........CG...
U.thibetanus        T..T....T.CCTCTCCTTTATT....-----..........................
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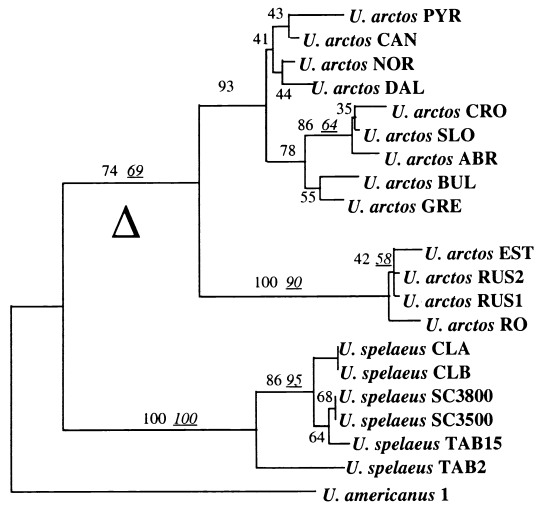
(b)

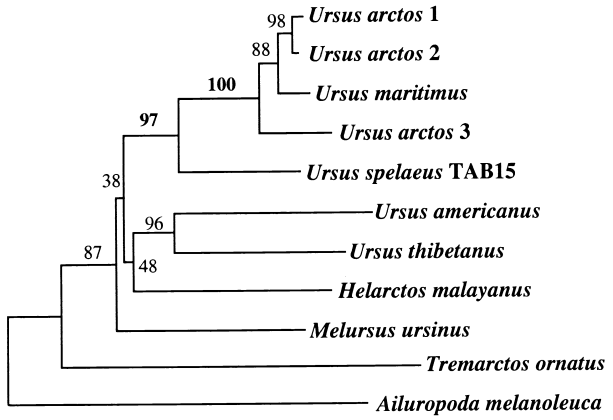


(a) Sequence of the portion of the mt DNA control region containing a specific 13 bp deletion in *U. arctos* and *U. maritimus*. The numbering refers to the first base of the control region. The region located approximately 6 bp downstream of the 13 bp deletion is difficult to align and is variable even between highly related populations. Gaps are shown by hyphens, whereas positions identical to those in the first sequence are replaced by dots. **(b)** Phylogenetic distance tree showing the placement of the cave bear sequences when the 282 bp fragment is used. Bootstrap values obtained with the MUST or PAUP packages [6, 13] after 1000 replicates are indicated for each branch. The bootstrap values above 50 obtained by a parsimony analysis are indicated by italicized and underlined numbers on the relevant branches. The tree is rooted with a *U. americanus* sequence. The various populations of brown bears are as in Figure 1. The 13 bp deletion occurred in the branch leading to *U. arctos* and *U. maritimus* and is indicated by the Greek character delta.

the *U. spelaeus* sequences contain an insertion of 13 bp when they are compared to those of brown and polar bears. The resulting phylogenetic tree has the same topology as that obtained with the 139 bp fragment alone, but it has much higher bootstrap support. The two lineages of brown bears are supported by 93% and 100%, respectively. The cave bears are monophyletic with 100% support. These high values are also found with the parsimony analysis (Figure 2). When the 13 bp insertion is placed in this tree, we notice that it corresponds to a region that was initially present in most bears, including *U. americanus* and *U. thibetanus*. We thus interpret these results as evidence of a specific deletion that occurred only in the brown/polar-bear lineage. Such evidence supports an early split of the cave bear from the brown/polar-bear lineage. This feature will provide a unique molecular marker that will enable researchers to discriminate between brown and cave bear samples.

In order to confirm these results, we analyzed another mt DNA gene, *cyt b*, for which many bear sequences are known. In particular, various lineages of brown bears from North America were recently described by the use of this marker [10–12]. We obtained sequences from the complete *cyt b* gene in one cave bear sample, TAB 15, which always produced very good PCR amplifications. Because it is impossible to obtain a 1140 bp fragment by using ancient DNA templates, we successively amplified seven short, overlapping PCR fragments of the *cyt b* gene. We sequenced all of these fragments and compiled their sequences to generate the full-length sequence. The *cyt b* gene from *U. spelaeus* exhibits 93.5% identity with the *cyt b* gene from *U. arctos*, 90.2% with *U. americanus*, and 85.5%–91.4% with other bears. Interestingly, the cave bear sequence exhibits the same strong bias on nucleotide composition as do the brown bears [11]. This argues in favor of the authenticity and reliability of the ancient cave bear *cyt b* sequence.

Figure 3



Phylogenetic distance tree showing the placement of the cave bear sequence when the complete *cyt b* sequence is used. The three *U. arctos* sequences used were taken from [17]. Bootstrap values obtained after 1000 replicates are indicated for each branch. We obtained similar topologies and bootstrap values by using only the third codon position or by correcting the distance matrix with the Kimura correction.

In a phylogenetic tree, the TAB15 sample clusters with the *U. arctos* and *U. maritimus* sequences, although its relation to these sequences is distant (Figure 3). The cave bear joins brown and polar bears with 97% support on distance analysis and 69% on parsimony analysis (Figure 3 and data not shown). The *cyt b* gene does not resolve the relationships between the other species with high support. Taken together, these results support the notion that the cave bear was an early offshoot of the brown bear lineage and originated long before the split between the two lineages of brown bears.

Authenticity of sequences remains the central issue in ancient DNA studies [3, 4, 15]. Several lines of evidence suggest that the sequences described in this paper are bona fide cave bear sequences. (*i*) Many sequences were independently found by two teams (C. H. and P. T.). Furthermore, modern bear DNA was never introduced in the laboratories used by the C. H. team. This criterion of reproducibility is obviously the most important and is often critical in assessing previous work [16]. (*ii*) During this study, we also extracted ancient bones of brown bears in parallel with our cave bear samples, and we always obtained cave bear sequences for cave bear bones and vice versa. Indeed, all the cave bear sequences obtained in this study cluster together on phylogenetic trees. (*iii*) Each position of the sequences has been verified from at least two amplifications by direct sequencing and cloning. We noticed the presence of a small number of artifactual mutations between the sequences of different clones for the same bone. Given their rarity and their position in conserved sites of the sequence, it was easy to discriminate between artifactual mutations and real sequence differences between samples. These mutations support the ancient origin of the PCR product because it is known that the *Taq* polymerase is particularly error prone in work on ancient samples. (*iv*) In the obtained sequences we found mostly transitions and no transversion, as expected for mt DNA. (*v*) The mutations are clustered in a few highly variable sites corresponding to positions known to vary in other bear species. These last two arguments stress the fact that the mutations were not created at random by PCR but that they indeed represent the results of an evolutionary process. (*vi*) From the same deposit level as CLA and CLB bones, a *Cervus* bone was extracted and analyzed. PCRs with bear-specfic oligonucleotides (H1–H3) gave no positive result, whereas *Cervus*-specific oligonucleotides (C. Donne-Goussé, personal communication) gave a sequence of *Cervus elaphus*; this argues against cross-contamination either in the deposit or during the extraction procedure.

Taken together, these observations strongly support the authenticity of our results, i.e., that the extracted and sequenced DNA comes from extinct specimens rather than from contamination.

Our results clearly indicate that the cave bear emerged much earlier than the split between the eastern and western lineages of European brown bear. This is supported by the mt DNA control region and, even more clearly, by the complete *cyt b* gene. The specific deletion of 13 bp in the 5′ part of the mt DNA control region is a synapomorphy clustering brown bears and polar bears and excluding the cave bears. It fits perfectly with our phylogenetic trees and provides an independent confirmation. This early split of cave bears is in accordance with Kurten's view, which suggests that the cave bear is an early offshoot inside the brown bear lineage [2]. Our findings contradict the views of other authors who suggest that cave bears diverged at about the same time as the two lineages of *U. arctos* [17].

From the distances used to calculate the phylogenetic trees, we can estimate the date of divergence between brown and cave bears (see Supplementary material and Table S2). For the control region, we assume that the two European lineages of brown bears are separated by 7.03% difference at the DNA level and that they diverged 850,000 years ago [7]. We thus calculate a date of divergence of 1.2–1.4 million years for the cave bear by using a genetic distance of 11% between the cave and brown bears. For *cyt b*, we used as a calibration the split between the ABC lineage from southeastern Alaska and the other North American brown bears. Talbot and Shields dated this split at 550,000–750,000 years ago [9, 11]. The genetic distance at the third codon position yields a divergence date of 1.2–1.6 million years ago. The control region might

not be an adequate marker for molecular clock studies [22], but the congruence between divergence times that we obtained by using *cyt b* and the control region is in favor of its validity here. Thus, the split probably occurred in the long, cold period that arose before the major warm climatic period that started approximately 1.2 million years ago [17]. A similar role for climatic fluctuation was proposed to explain the separation of the European brown bear lineages that took place 350,000 years later [7]. Thus, the evolution of bears and their radiation in various independent lineages were shaped by the succession of contrasted climatic periods that took place during the Quaternary Period, as has been observed for other species [19]. Such a scenario may be in accordance with the refuge theory [20, 21], although this remains to be established by the analysis of cave bear samples from a wider geographical distribution.

Bears are now well known in terms of phylogeography of living populations and species, both in Europe and in America [8, 9, 11]. Nevertheless, a number of questions remain open concerning the origin of these populations and their relationships with fossil remains. A recent analysis of ancient brown bear samples from North America suggests that the last glaciation may have resulted in a decrease of the genetic diversity of this species [23]. Since fossils of bears are known from many European deposits and are apparently a good source for ancient DNA, bears appear to be a promising model for the study of species differentiation through time as well as of the establishment of living populations. Even though very ancient DNA sequences are not currently available [15], we believe that the study of ancient remains of the Quaternary Period will be an important source of DNA and will uncover a large amount of relevant information on populations and species of the past.

Supplementary material

Supplementary material including a Materials and methods section as well as 13 supplementary figures is available with the electronic version of this article at http://current-biology.com/supmatin.htm.

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