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Ancient DNA evidence for the loss of a highly divergent brown bear clade during historical times

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The genetic diversity of present-day brown bears (*Ursus arctos*) has been extensively studied over the years and appears to be geographically structured into five main clades. The question of the past diversity of the species has been recently addressed by ancient DNA studies that concluded to a relative genetic stability over the last 35 000 years. However, the post-last glacial maximum genetic diversity of the species still remains poorly documented, notably in the Old World. Here, we analyse Atlas brown bears, which became extinct during the Holocene period. A divergent brown bear mitochondrial DNA lineage not present in any of the previously studied modern or ancient bear samples was uncovered, suggesting that the diversity of *U. arctos* was larger in the past than it is now. Specifically, a significant portion (with respect to sequence divergence) of the intraspecific diversity of the brown bear was lost with the extinction of the Atlas brown bear after the Pleistocene/Holocene transition.

Keywords: ancient DNA, brown bear, Holocene, mtDNA, North Africa, phylogeography

Introduction

Among the large carnivores, the brown bear (*Ursus arctos*) is certainly the one exhibiting the broadest distribution, ranging from North America to Western Europe, comprising populations in Japan, Tibet or even Iran (Waits *et al.* 1999). This holarctic range, associated with a total number of brown bears approaching 250 000 individuals, can be taken as a good proxy for the species survival (Waits *et al.* 1999).

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Nevertheless, brown bears currently live mainly in restricted areas, which is principally due to habitat fragmentation resulting from human activities (Waits *et al.* 1999). Isolation of these small populations is thought to be responsible for large decreases in genetic diversity (Paetkau *et al.* 1998; Miller & Waits 2003), and can result in the extinction of some lineages, as it was recently observed with the death of the last female Pyrenean brown bear in autumn 2004 (Taberlet & Bouvet 1992). As a consequence, numerous studies were performed during the 1990s to assess the brown bear genetic diversity, at a continental (Taberlet & Bouvet 1994; Kohn *et al.* 1995; Talbot & Shields 1996; Waits *et al.* 1998) or even at a more local scale (Taberlet & Bouvet 1992; Randi *et al.* 1994; Masuda *et al.* 1998; Matsushashi *et al.* 1999), notably to improve the understanding of the phylogeography of large mobile species and to guide re-introduction programmes.

From the analysis of the mitochondrial DNA control region (mtCR), it was soon deduced that contrary to other mobile carnivores such as wolves or coyotes (Vilà *et al.* 1999), *U. arctos* populations exhibit a worldwide genetic structure in five mitochondrial lineages (Waits *et al.* 1999). Summarizing and following Waits (Waits *et al.* 1998), clade I comprises populations living exclusively on three coastal Alaskan islands (Archipelago, Banhoff and Chicagoff), which are closely related to the species *Ursus maritimus*; clade II includes those living in Eastern Europe and in the Beringian zone; clade III comprises bears living in eastern Alaska and Northern Canada; clade IV includes bears living in southern Canada and the Lower 48 States and clade V comprises populations settled in Western Europe, which are themselves divided into two geographically distinct subclades, respectively, called the Iberian and the Balkanian subclades (Taberlet & Bouvet 1992; Taberlet & Bouvet 1994; Talbot & Shields 1996; Waits *et al.* 1998; Emerson & Hewitt 2005). So far, all the present-day individuals that have been genetically characterized fall within this scheme (Randi *et al.* 1994; Kohn *et al.* 1995; Masuda *et al.* 1998; Matsuhashi *et al.* 1999).

More recently, genetic studies have been performed on ancient populations of *U. arctos* (Leonard *et al.* 2000; Barnes *et al.* 2002; Hofreiter *et al.* 2004), to check the stability through time of the genetic structure. Although brown bears have been extensively hunted for centuries (Sorensen 1990), the lines of evidence drawn from these analyses suggest that the genetic diversity (understood as the number of haplogroups) was stable over the last 60 000 years. However, admixed populations were recently submitted to extensive lineage sorting that finally led to the present phylogeography (Leonard *et al.* 2000; Barnes *et al.* 2002). In Europe, the four ancient individuals that have been sequenced (and that spanned 40 000 years) are all representatives of clades II or V (Hänni *et al.* 1994; Barnes *et al.* 2002; Hofreiter *et al.* 2004) but some changes in their geographical distribution may have occurred (Barnes *et al.* 2002; Hofreiter *et al.* 2004). In North America, 33 of the 44 Upper Pleistocene and Holocene brown bears that were analysed belong to clade I, II, III or IV (Leonard *et al.* 2000; Barnes *et al.* 2002; Matheus *et al.* 2004). The remaining 11 individuals, that all lived more than 35 000 years ago, can be partitioned into two subclades which are related to clades I and II (Barnes *et al.* 2002). The absence of these two subclades in the latest populations suggests a limited decrease of the genetic diversity of *U. arctos* more than 35 000 years ago, but since then, the genetic diversity of the species appears similar to that observed today. Thus, little changes are observed in North American brown bears since the last glacial maximum (LGM). The species seems to have maintained both genetic diversity (no clade loss) and phylogeographical patterns (Barnes *et al.* 2002).

However, many different subspecies or populations disappeared between the LGM and historic times and have

never been studied (Blyth 1841; Kurten 1965; Hamdine *et al.* 1998). In North America, for example, brown bear remains are known as far south as Mexico but no genetic information about the local subspecies (*Ursus arctos nelsoni* Merriam, 1914) is available (IUCN 1996). In the Mediterranean zone, two extinct subspecies have also been described, one in the Near East (*Ursus arctos syriacus* Hemprich & Ehrenberg, 1828) (Kurten 1965), the other one in the Atlas Mountains of North Africa (Blyth 1841). This last one, often referred to as the Atlas bear (*Ursus arctos crowtheri* Schinz, 1844), is thought to have disappeared during the mid-19th century, although the most recent skeletal remains were recently radiocarbon dated back to 1600 Before Present (BP) (Hamdine *et al.* 1998).

To enhance our knowledge about recent (post-LGM) variations in the genetic diversity of brown bears, we focused on this last population. Mitochondrial DNA sequences obtained from seven North African subfossils from the Holocene period sustain the existence of an extinct, divergent clade of brown bears that was never observed before and hence suggest that the genetic diversity of the brown bear was larger in the past, even in recent periods.

Materials and methods

Samples description

A total of seven *Ursus arctos* bones belonging to seven distinguishable individuals (complete skulls or skeletons) were analysed: (i) four from the Takouatz cave, Algeria: TAK1, right mandible; TAK2, skull fragment [TAK1 and 2 samples were found together with a scapula dated 9620 ± 200 years BP, Ly-1805 (Auboire & Gillon 1995)]; TAK3 (7650 ± 40 years BP, Oxa-11035), parietal bone and TAK4 (7345 ± 40 years BP, Oxa-11006), parietal bone; (ii) two from the Akouker cave, Algeria: AKO1 (1679 ± 35 years BP, Oxa-10750), mandible fragment and AKO2 [1550 ± 40 years BP, Oxa-6805 (Hamdine *et al.* 1998)], mandible fragment; (iii) one from the El-Ksiba cave, Morocco: ELK1 (1285 ± 30 years BP, Oxa 14058), femur fragment.

To complete the cytochrome *b* (cyt *b*) data set of extant bear sequences, two samples from clade V, Iberic subclade bears were also obtained from Pierre Taberlet [Laboratoire d'Écologie alpine (LECA), Grenoble].

DNA extraction, amplification and sequencing

Ancient samples treatment. Extractions and manipulations were performed in specific ancient DNA facilities. Bones were scratched with a sterile scalpel and then reduced to a powder with a sterile hammer. The powder obtained (0.2–1 g) was then digested for 18 h with proteinase K (1 mg/mL) in buffer (0.5 M EDTA, 0.5% N-lauroyl-sarcosine) under constant agitation at 55 °C as in Loreille *et al.* (2001). Following this treatment, samples were centrifuged (1200 g)

and supernatants washed three times with phenol-chloroform-isoamylalcohol (25:24:1) (Orlando *et al.* 2002). Finally, DNA was concentrated on a Centricon 30 column and eluted in an ultra-pure water volume of between 80 μ L and 100 μ L. A further DNA purification was carried out using a QIAquick kit (QIAGEN) when too much inhibition of the polymerase chain reaction (PCR) was detected (Hänni *et al.* 1995). Three overlapping fragments were amplified, resulting in a 269-bp fragment of the 5' end of the mtCR, with the following primer pairs: H3/H4 (206 bp), H5/DR500 (174 bp); H16143/H16299 (179 bp) (Loreille *et al.* 2001; Orlando *et al.* 2002). For TAK3, ELK1 and AKO1, a supplementary 278-bp fragment located at the 5' end of the cyt *b* gene was amplified in two times using the following primer pairs: SCcytb1F (5'-GCTAAAATCATCAACAACCTC-3') and SCcytb1R (5'-GTCTCGGCAAATGTGGGTGAC-3') (185 bp); SCcytb2F (5'-ACACAACCACAGCYTTTTCATC-3') and SCcytb2R (5'-GCCAATGTTTCATGTTTCTG-3') (174 bp). All amplifications were performed from 0.25 to 1 μ L of DNA extract in a total volume of 25 μ L using AmpliTaq Gold (PerkinElmer). Cycling conditions were: 94 °C for 10 min; 50–60 cycles at 94 °C for 30 s, 44–55 °C for 30 s and 72 °C for 45 s; 72 °C for 10 min. PCR products were then cloned using TOPO TA cloning kit for sequencing (Invitrogen). To test insert size, clones were picked and amplified by PCR with Mastermix (Eppendorf) using the following cycling conditions: 40 cycles at 94 °C for 30 s, 44–55 °C for 30 s and 72 °C for 30 s; 72 °C for 5 min. Products of the expected size were sequenced on both strands by Genome Express.

Ancient DNA controls. Contaminations were monitored during the extraction and PCR processes by blank controls (one blank for a maximum of five samples) and cross-contamination tests. Moreover, during each brown bear extraction, additional samples from other species (*Ursus spelaeus* and *Megaloceros giganteus*) were treated and the extracts used in amplification attempts with bear primers to serve as cross-contamination tests. The PCR controls were a classical negative control and an aerosol control which was kept open throughout the manipulation (Loreille *et al.* 2001).

For six out of the seven bear samples, two independent extractions were carried out in the laboratory in Lyon. Two to three independent PCR products/individual were then obtained and subsequently cloned (2–11 clones/PCR product), leading to the analysis of more than 400 clones. Consensus sequences were then determined for each fragment and each individual from the clone alignment.

Finally, a TAK4 bone fragment, which had never been in Lyon, was also independently analysed in Dijon. It yielded sequences identical to that obtained in Lyon.

Modern sample treatment. DNA extraction of hair samples from two extant bears belonging to the clade V was

performed in an independent facility with a QIAGEN DNeasy kit. A 278-bp cyt *b* sequence was amplified using the primer pairs described above. DNA was amplified using Mastermix (Eppendorf), with 5 μ L extract in a total volume of 40 μ L. Conditions were: 40 cycles at 94 °C for 30 s, 44–55 °C for 30 s and 72 °C for 30 s; 72 °C for 5 min. Products were sequenced directly by Genome Express.

Data sets

Modern brown and polar bear sequences were obtained from Taberlet & Bouvet (1994), Waits *et al.* (1998), Delisle & Strobeck (2002), and Marshall & Ritland (2002) in the case of mtCR sequences, and from Loreille *et al.* (2001), Talbot & Shields (1996), and Stone & Cook (2000) in the case of cyt *b* ones. The data set also included *Ursus americanus* (Stone & Cook 2000; Marshall & Ritland 2002) and *U. spelaeus* (Loreille *et al.* 2001) sequences, which served as outgroups for the analyses. Only mtCR sequences covering entirely our fragment of 269 bp were included in this data set. Sequences obtained from both extant and ancient bears were deposited in GenBank under accession nos AM411397–AM411406. All sequences were aligned by eye using SEAVIEW (Galtier *et al.* 1996), each haplotype being only represented once in our final alignments. The mtCR and cyt *b* data sets used in this study are available as Supplementary material (Table S1).

Observed divergence and phylogenetic analyses

Observed divergence (p-distance) was calculated among and within clades using PAUP version 4.0b10 (Swofford 2000). Because of the reduced sampling, only mean values are presented as well as the maximum p-distances for each clade. Phylogenetic analyses were performed to infer relationships of the different brown bear sequences. The appropriate model of evolution was determined for each of the two data sets (mtCR, cyt *b*) using MRMODELTEST 2.0 (Nylander 2004), a MODELTEST (Posada & Crandall 1998) modified version adapted to options available with Bayesian analysis softwares. According to MRMODELTEST, the best-fitting model of DNA substitution was HKY + I + Γ for control region data set and HKY + Γ for cyt *b* data set. Maximum-likelihood (ML) analyses were performed on each data set with PHYML (Guindon & Gascuel 2003) using the online interface <http://atgc.lirmm.fr/phyml/> (Guindon *et al.* 2005). For each analysis, the transition/transversion ratio, the proportion of invariable sites as well as the gamma distribution parameter were estimated and the starting tree was determined by a BioNJ analysis of the data sets (default settings). Using optimization options, 500 bootstrap (Bp) replicates were performed. Bayesian analyses (BA) were performed using MRBAYES version 3.0 (Ronquist & Huelsenbeck 2003). Three independent runs of 1 000 000 generations each were performed under the same models

as above. A burn-in period of 50 000 generations was determined graphically using TRACER 1.2 (Rambaut & Drummond 2003), a software that allows easy plotting of all parameters against the number of generations. For each data set, all three runs gave similar tree topologies and posterior probability (P_p) values. Alternative topologies were finally tested using the Shimodaira–Hasegawa test (SH test (Shimodaira & Hasegawa 1999) implemented in PAUP version 4.0b10 (Swofford 2000), with full optimization option and 1000 bootstrap replicates.

Results

To investigate the genetic history of North African brown bears, seven North African individuals, all dated from the Holocene, were analysed (see Table S2, Supplementary material). Among these samples, which covered most of this period (9620–1280 BP), ELK1 revealed to be the most recent brown bear subfossil ever discovered in North Africa (1280 BP). All the seven bones allowed the amplification of ancient DNA sequences.

Ancient DNA authentication

Authenticating the sequences obtained from subfossils is an important challenge. According to the international standards (Hofreiter *et al.* 2001b), we do believe that the sequences presented here are authentic ones, because (i) each result was confirmed by at least two independent PCR products that were always cloned; (ii) on average, seven clones were sequenced for each PCR product (more than 400 clones for the entire study) allowing us to determine consensus sequences; (iii) degradation was observed for all samples as expected from ancient DNA (see below); (iv) and, for one sample (TAK4), the experiments were independently duplicated in Dijon and identical sequences were found.

Degradation patterns

From a technical point of view, the possibility of a cross-contamination of samples yielding the same sequences (TAK1–4, AKO1 and 2) can be ruled out because (i) none of these samples were extracted together, (ii) all cross-contamination tests were negative, and (iii) specific degradation patterns identified each sample (see Table S2, Fig. S3, S4, Supplementary material). As observed elsewhere (Hansen *et al.* 2001; Hofreiter *et al.* 2001a; Gilbert *et al.* 2003; Smith *et al.* 2003), the most prominent degradations were transitions and the patterns of artefactual mutations observed could not be linked to the sample age. Our findings therefore support once again the key role of taphonomy in allowing DNA preservation (Smith *et al.* 2003).

Phylogenetic analyses

Control region. Using three overlapping short fragments, a 269-bp mtCR was obtained from the seven specimens of ancient North African brown bears that were analysed. Three mtCR haplotypes were determined from this sampling, each cave delivering a unique haplotype, whatever the sample age.

Phylogenetic analyses demonstrated that all ancient bears analysed clustered with brown bears (the monophyly was sustained in all our analyses: bootstrap (Bp) 90, posterior probability (P_p) 97, Fig. 1(a). Furthermore, mtCR sequences of ancient bears all exhibited a typical 15-bp deletion, a synapomorphy of brown and polar bears (Loreille *et al.* 2001).

Sequence divergences between ancient and extant bears were calculated and revealed that: (i) the two Akouker sequences (AKO1 and AKO2 samples) only displayed a weak average divergence from clade V bears (West European ones) and especially from bears belonging to the Iberic subclade as defined in (Taberlet & Bouvet 1994) (< 1.5%, which is the intrapopulation maximum variability, Table 1), even sharing perfect identity with some extant Cantabrian bears (Taberlet & Bouvet 1994); (ii) on the contrary, the five remaining sequences from Takouatz and El-Ksiba samples (TAK1–4 and ELK1), which exhibited limited divergence from each other (1.9%, a value in the range of other intrapopulation maximum variabilities, Table 1), were divergent from all known modern bear sequences and from the Akouker one (> 5.2%, Table 1). Interestingly, numerous unique substitutions were observed in Takouatz and El-Ksiba bear sequences, accounting for a significant part of the divergence (see Fig. S5, Supplementary material).

When performing phylogenetic analyses, ML and Bayesian analysis (BA) methods gave similar results. They all placed the Akouker bears inside the clade V (Bp 67, P_p < 50, Fig. 1a), whereas El-Ksiba and Takouatz bears (forming together a robust group, Bp 98, P_p 100, Fig. 1a) appeared to be related to the clade I (Alaskan island bears), although this grouping receives rather weak support (Bp 55, P_p 75, Fig. 1a). The comparison of alternative topologies with a SH test (Fig. 1a) firmly excluded the North African bears from Takouatz and El-Ksiba from the super-haplogroup comprising clades II, III and IV (Bp 91, P_p 100). However, the test was not able to reject the two other alternative topologies assessed [belonging to clade V (but the P value is near the threshold, $P = 0.08$) or basal branching ($P = 0.86$)].

Cytochrome b. In order to improve the phylogenetic signal and to clarify Takouatz and El-Ksiba bear relations with other bears, a 278-bp cyt b region was also amplified using two overlapping fragments. Since the four samples from

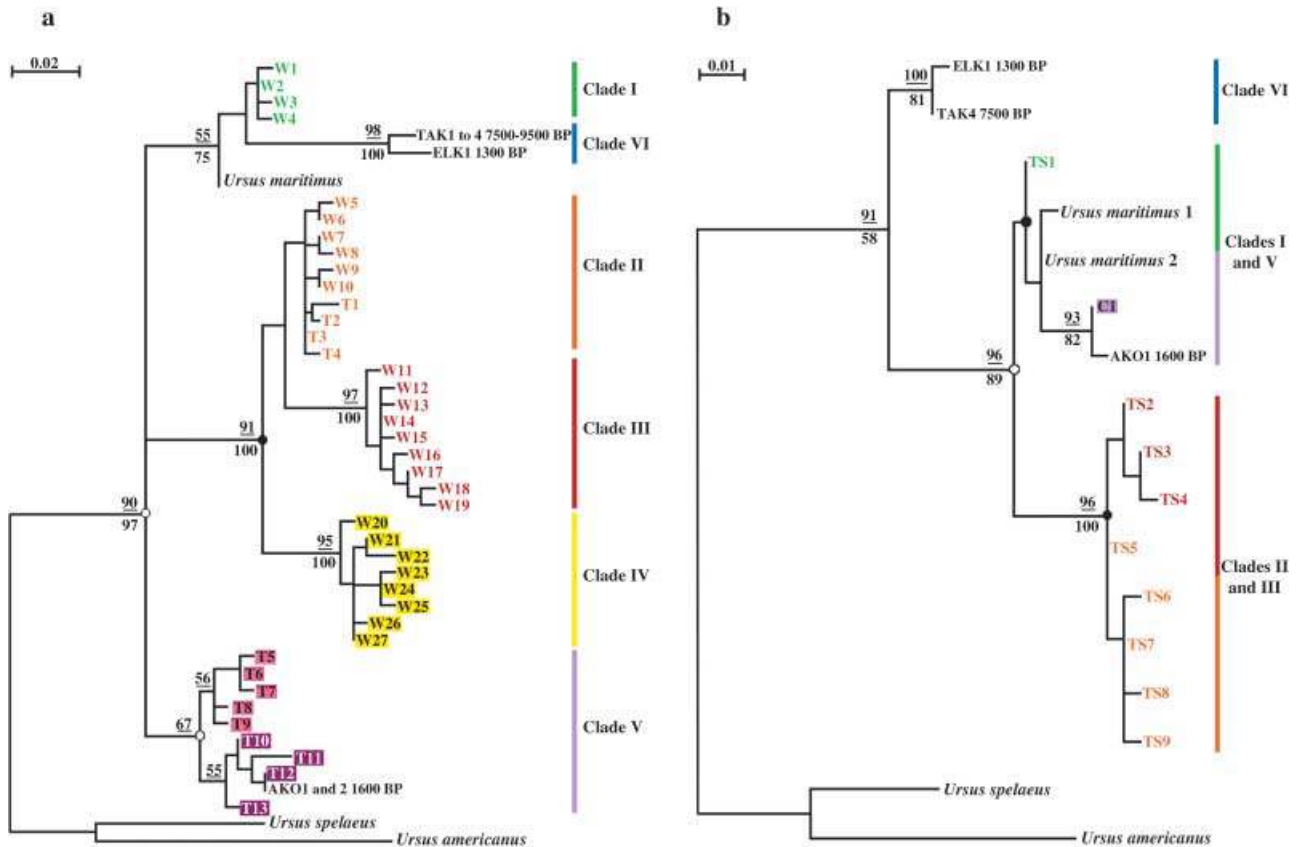


Fig. 1 African brown bear population was heterogeneous and included an unknown sixth clade of brown bear. (a) Phylogenetic tree from the ML analysis of the mtCR data set (269 bp) under the HKY + I + G model. (b) Phylogenetic tree from the ML analysis of cyt *b* data set (278 bp) under the HKY + G model. Underlined numbers above branches show bootstrap values, while numbers below correspond to the mean posterior probabilities obtained from Bayesian analyses. Only values greater than 50 are shown. Open circles indicate alternative branching hypotheses for the clade VI that were not rejected by an SH test ($P > 0.05$); full circles indicate alternative branching hypotheses for the clade VI that were rejected by an SH test ($P < 0.05$). For both panels, haplotype initials are those of authors that first described the haplotype (Taberlet & Bouvet 1994; Talbot & Shields 1996; Waits *et al.* 1998).

Takouatz and the two from Akouker delivered identical control region sequences, the cyt *b* fragment was only amplified from one individual of each cave (namely TAK4 and AKO1). Divergence values followed the same trend as that observed in the mtCR case but were lower, as expected for this coding sequence. As already observed with mtCR sequences, the Akouker bears shared the closest vicinity with the extant bears of clade V. El-Ksiba and Takouatz bears were (i) closely related to each other, only diverging by 0.4% (see Table 1); (ii) divergent from all known lineages with values oscillating between 3.6% and 5.9% according to the compared clades, while intrapopulation divergence observed in clades II and III over this fragment never outscored 0.7% (Table 1). Once again, some specific substitutions were detected but were, as expected, mostly observed in third position of codons, hardly affecting the protein sequence (one valin to methionin replacement detected).

Phylogenetic analyses of cyt *b* sequences placed the Akouker bear sequence inside clade V (Bp 93, pp 82, Fig. 1b). On the other hand, Takouatz and El-Ksiba bear sequences did form a robust monophyletic group placed as a sister group of all other brown bears (Bp 100, P_p 82, Fig. 1b). This time, SH test rejected the belonging of Takouatz and El-Ksiba bears to clade II or III ($P = 0.01$), or to clade I or V ($P = 0.04$), but did not exclude a basal multifurcation ($P = 0.07$).

Discussion

The analyses of cyt *b* and mtCR sequences obtained in this study support the hypothesis that the extinct North African brown bears were not forming a homogeneous population. Our findings show that North Africa had been occupied by at least two different clades that co-existed during at least a part of the first millenary AD (Akouker, 1680 and 1550 BP; El-Ksiba, 1280 BP).

Table 1 Some African brown bears could constitute a divergent lineage. Mean observed divergence (p-distance, in percentage) between ancient and extant brown bears. Mitochondrial DNA CR values are under the diagonal and cyt *b* ones are above the diagonal and are italicized. Comparisons have been made among and within clades. Diagonal cells, corresponding to the maximum intrapopulation variability, are in grey. The number of different mitochondrial haplotypes used in the p-distance calculations is indicated in parentheses for each clade. p-distance values inferior to the maximum intrapopulation variability are marked with a*

mtCR/cyt <i>b</i>	Clade I (1)	Clade II (5)	Clade III (3)	Clade IV	Clade V (1)		Akouker (1)	Takouatz (1)	El-Ksiba (1)
						Iberic (1) Balkanian			
Clade I (4)	0.8/-	2.7	2.6	—	1.4	—	1.8	3.6	4
Clade II (10)	7	1.6/0.7	1.1	—	4.1	—	4.5	5.5	5.9
Clade III (9)	8.3	3.7	2.3/0.7	—	3.4	—	3.7	4.8	5.2
Clade IV (8)	8.5	4.1	6	2.3/-	—	—	—	—	—
Clade V (9)	Iberic (4)	5.5	6.5	7.1	7.5	—	0.4*	3.6	4
	Balkanian (5)				2.4	1.5/-	—	—	—
Akouker (2)	5.8	6.1	7	7.2	0.9*	2.4	0/-	4	4.3
Takouatz (4)	5.2	9.7	10.7	10.3	7.3	7.5	7.5	0/-	0.4
El-Ksiba (1)	5.7	10.6	11.3	10.5	8	8.2	8.2	1.9	-/-

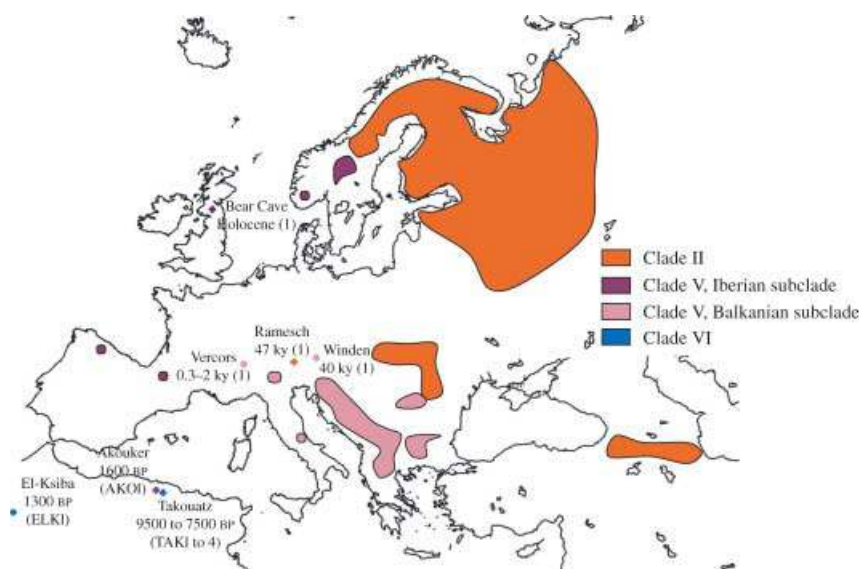


Fig. 2 Distribution of extant and ancient brown bear clades in Europe. Map showing the location of European brown bear clades. Ancient brown bears that had already been genetically characterized are highlighted by crosses of the colour of the clade they belong to (Hänni *et al.* 1994; Barnes *et al.* 2002; Hofreiter *et al.* 2004). The number of bears sequenced for each site is indicated in parentheses. Names and datings of the samples for each site are indicated in parentheses. North African sites are also shown on the map.

Evidence for an extinct brown bear clade

The mtCR and cyt *b* sequences obtained from seven individuals identify two different lineages. The genotyping of two of the most recent brown bear specimens ever found in Africa (AKO1, 1680 BP and AKO2, 1550 BP from Akouker; Hamdine *et al.* 1998) shows the presence of clade V bears on this continent. A divergent lineage describes a second population, which is represented by individuals coming from the Takouatz and El-Ksiba caves. These bears, found several hundreds of kilometres away from each other, were present at least from 9620 BP to 1280 BP (Fig. 2). The mtCR sequences from Takouatz and El-Ksiba cluster together in all trees (Fig. 1a). If this well supported clade of North African bears can be excluded from clades II, III or IV

(Fig. 2), the amplified mtCR fragment is not able to exclude a basal positioning or a clustering with the clade I or V (SH test results). Cyt *b*, with a slower rate of evolution, provides congruent results: the African clade does not belong to clades II or III (Fig. 1b). Furthermore, the cyt *b*, as well as the concatenation of both cyt *b* and mtCR, support a more refined tree (Fig. 1b and Fig. S6, Supplementary material) where all extant clades cluster in a well-supported super-haplogroup (comprising all extant brown bears) and Takouatz and El-Ksiba bears appear as an ancient offshoot. Considering the high bootstrap and posterior probability values as well as the SH test conclusions, we do believe that these North African brown bears should now be considered as belonging to a true, basal clade, defined here as clade VI.

Where did African bears come from?

Akouker specimens exhibit an mtCR sequence that is identical to that of extant Cantabrian bears. This implies (i) either a unique recent colonization event, or (ii) a continuous genetic flow, across the Gibraltar Straits. However, this second hypothesis seems unlikely. Indeed, North Africa has been physically isolated from Europe for more than 5 million years, many examples of vicariance illustrating this fact (Cheylan 1990; Dobson & Wright 2000). Furthermore, water barriers, even if limited in size, are known to efficiently maintain isolation of terrestrial mammals (Dobson & Wright 2000). Importantly, this has also been demonstrated in the case of *Ursus arctos* (Paetkau *et al.* 1998b).

Thus, the introduction of a clade V haplotype in the North African population certainly results from a rare event. This could result from a natural dispersal event, for example crossing the Gibraltar Straits when numerous brown bears were living in the southernmost part of Spain (Sommer & Benecke 2005), or from an event linked to human activities (Michaux *et al.* 2003; Brandli *et al.* 2005), such as an introduction by Romans or Carthaginians who are known to have organized wild beast arena battles in North Africa (Bourguignat 1867). Whatever the cause of their presence, North African bears from the clade V attest that, although exceptional, crossing the Gibraltar Straits was possible (or was made possible) in the recent past.

North African clade VI brown bears are not necessarily native to another continent. It could well stand for an ancient allopatric event, which would have been followed by progressive divergence. If not, the North Africa-restricted presence of clade VI brown bears could be explained by extensive lineage sorting among ancestral European brown bears, that resulted in the loss of this particular lineage everywhere but in North Africa. Although lineage sorting seems to account for a large part of today and past brown bear phylogeography (Taberlet & Bouvet 1994; Leonard *et al.* 2000; Barnes *et al.* 2002; see also Avise 2000 and references therein), in our mind, the total extirpation of clade VI bears from all continental European populations does not retain the highest likelihood. Indeed, European brown bears are thought to have used at least three different refugia in Southern Europe along Great Glaciation events (Sommer & Benecke 2005). Clade VI would so be the only known clade to have been lost in all of these refugia, since no brown bear belonging to this clade has ever been found in Europe, where hundreds of living individuals are yet genetically characterized. Postulating that clade VI bears are not native to Africa, a migration through the North Sahara from the Near or Middle East is certainly more likely. Besides, it would be consistent with numerous studies describing zoogeographical affinities of Northwestern Africa mammals with Near Eastern ones (Cheylan 1990; Dobson & Wright 2000). All the more so that brown bears

were living in the Near East until the beginning of the 20th century, occupying territories corresponding to present Syria, Israel and Lebanon. Naturalists identified these bears as a particular subspecies, named *Ursus arctos syriacus*. Thus, Atlas bears and Syrian bears could well be close relatives. Unfortunately, little is known of the Syrian subspecies up to now.

Clade VI illustrates a reduction of the post-LGM genetic diversity

The clade VI is the first example of a recently extinct brown bear clade (these bears were still living in North Africa just 1280 BP). Its divergence from all living brown bears is particularly striking: at the maximum 11.3% for the mtCR sequences and 5.9% for the cyt *b* ones, values which are to compare to the minimum values of divergence between the brown bear and its sister species (the cave bear), respectively, 9% for the mtCR and 5.7% for the cyt *b* sequences. In fact, to our knowledge, they represent a unique example of post-LGM loss of intraspecific genetic diversity (Barnes *et al.* 2002). Interestingly, this loss of diversity took place after the Pleistocene/Holocene transition (PH transition). Taken together with the results of Barnes *et al.* (whose samples spanned the entire late Pleistocene until the PH transition), it would therefore suggest that intraspecific variability was more affected by the PH transition than by the LGM itself (Barnes *et al.* 2002). This is coherent with the observation that many large mammal species (the so-called megafauna) did survive the LGM but did not survive the PH transition (Stuart *et al.* 2004). This last point will undoubtedly need further investigations.

Conclusion

Finally, the situation in Europe and North Africa contrasts with that observed in the mean time among North American populations. Although experiencing local lineage sorting, the latter indeed succeeded in maintaining the genetic diversity (understood as the number of haplogroups) at the continental scale. The extinction of clade VI bears instead proves a reduction of brown bear intraspecific diversity in Europe after the LGM, and even, after the PH transition. It suggests that stability over the last 35 000 years of the different lineages forming today the *Ursus arctos* species is not an absolute rule, even for periods as recent as the Holocene.

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Sébastien Calvignac PhD thesis has been focused on depicting the recent evolutionary history of the brown bear thanks to ancient DNA analyses. Sandrine Hughes research focuses on goat and sheep domestication as well as on brown bear phylogeography through the use of ancient DNA techniques. Christelle Tougard research is focused on the evolutionary history of mammals, in particular rodents. Jacques Michaux is a palaeontologist interested in rodents, evolution and biogeography. Michel Thevenot is a specialist of the North African bird and mammal faunas. Watik Hamide has been interested in recent mammals of Algeria. Catherine Hänni has coordinated this work and focus on ancient DNA analyses and late Pleistocene and Holocene megafauna.

Supplementary material

The following supplementary material is available for this article:

Table S1 List of sequences used in this study.

Table S2 Description of the samples and degradation patterns in the mtCR.

Fig. S3 Alignment of mtCR clones (attached).

Fig. S4 Alignment of cyt *b* clones (attached).

Fig. S5 Comment: clade VI mtCR sequences are not nuclear mitochondrial insertions (*numt*).

Fig. S6 Phylogenetic analyses of the concatenated sequences.

Supplementary data

S1. List of Sequences Used in this Study.

mtCR dataset

Name in the tree	Name when published	Published in	Accession number
W1	lin.8	Waits et al., 1998	Sequences available from L. Waits
W2	lin.10		
W3	lin.9		
W4	lin.11		
W5	lin.20		
W6	lin.28		
W7	lin.26		
W8	lin.27		
W9	lin.31		
W10	lin.32		
W11	lin.21		
W12	lin.23		
W13	lin.54		
W14	lin.58		
W15	lin.57		
W16	lin.53		
W17	lin.22		
W18	lin.56		
W19	lin.59		
W20	lin.37		
W21	lin.55		
W22	lin.60		
W23	lin.39		
W24	lin.40		
W25	lin.52		
W26	lin.51		
W27	lin.38		
T1	L-Romania2	Taberlet et al., 1994	X75873
T2	L-Romania1		X75872
T3	MNOPQ-Russia		X75875
T4	N-Estonia		X75869
T5	F-Abruzzo		X75862
T6	EGHI-Slovenia		X75877
T7	H-Croatia		X75867
T8	J-Greece		X75870
T9	K-Bulgaria		X75864
T10	C-Norway		X75871
T11	A-Pyrenees		X75878
T12	B-Cantabria		X75865
T13	D-Dalarna		X75868
<i>Ursus maritimus</i>	<i>Ursus maritimus</i>	Delisle et al., 2002	AF303111
<i>Ursus spelaeus</i>	TAB15	Loreille et al., 2001	AY149273
<i>Ursus americanus</i>	Isolate 4971n	Marshall et al., 2002	AF305485

5 *Cytochrome b* dataset

Name in the tree	Name when published	Published in	Accession number
TS1	GB01	Talbot and Shields, 1996	U18870
TS2	GB08		U18877
TS3	GB10		U18879
TS4	GB12		U18881
TS5	GB14		U18883
TS6	GB11		U18880
TS7	GB18		U18887
TS8	GB06		U18875
TS9	GB15		U18884
<i>Ursus maritimus 1</i>	<i>Ursus maritimus</i>	Talbot and Shields, 1996	U18898
<i>Ursus maritimus 2</i>	<i>Ursus maritimus</i>	Delisle et al., 2002	AF303111
<i>Ursus spelaeus</i>	TAB15	Loreille et al., 2001	AF264047
<i>Ursus americanus</i>	AF21119	Stone and Cook, 2000	AF268270

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7 Color codes are the same than table 1.

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9 S2. Description of the samples and degradation patterns in the mtCR.

10 The last column stands for the average number of modified bases/1000 nucleotides
 11 sequenced/PCR product.

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Individual	Location	Sample type	Dating (years BP)	Dating reference	Modified bases per 1000 nucleotides sequenced
TAK1	Takouatz cave, Algeria	right mandible	9620±200	Auboire and Gillon, 1995	1,12
TAK2	Takouatz cave, Algeria	skull fragment	9620±200	Auboire and Gillon, 1995	2,84
TAK3	Takouatz cave, Algeria	temporal bone	7650±40	This study	8,40
TAK4	Takouatz cave, Algeria	temporal bone	7345±40	This study	10,32
AKO1	Akouker cave, Algeria	mandible	1680±35	This study	3,56
AKO2	Akouker cave, Algeria	mandible	1550±40	Hamdine <i>et al.</i> , 1998	3,08
ELK1	El-Ksiba cave, Morocco	humerus or femur	1285±60	This study	4,77

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14 S3. Alignment of mtCR Clones (attached).

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16 S4. Alignment of *cyt b* Clones (attached).

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18 S5. Comment : Clade VI mtCR Sequences Are Not *Nuclear Mitochondrial Insertions (numt)*.

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20 Clade VI mtCR sequences display numerous unique sites among brown bears as
 21 shown in the alignment below:

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Takouatz	ACTACTATTT	TACTCCATGT	CCTATTCATT	TCATATATAC	CATTCTATGT	ACTGTACTAT
A-PyreneesC..GC..T...CT..C..C..		
B-CantabriC..GC..T...CT.....C..		
C-NorwayC..G...T...CT.....C..		
D-Dalarna-C..C..G...T...CT.....C..		
F-Abruzzo-G...T...CT.....G.C..		
EGHI-SloveG...T...CT.....G.C..		
H-CroatiaG...T...CT.....G.C..		
J-GreeceG...T...CT.....G.C..		
K-BulgariaG...T...CT.....G.C..		
MNOPQ-RussC.....	.T.....CT.....C..		
N-EstoniaC.....	.T.....CT.....C..		
L-Romania1C.....	.T.....CT.....C..		
L-Romania2C.....	.T.....CT.....C..		
Lineage.20C.....	C.....C..		
Lineage.26C.T....	.T.....CT.....C..		
Lineage.27C.T....	.T.....TCT.....C..	
Lineage.28C.....	.T.....C.....C..		
Lineage.31C.....	.T.....TCT.....C..	
Lineage.32C.....	.T.....TCT.....C..	
Lineage.21C.....	CT.....	G...G.C..		
Lineage.22C.....	CT.....	G...G.C..		
Lineage.23C.....	CT.....	G...G.C..		
Lineage.53C.....	CT.....	G...G.C..		
Lineage.54C.....	CT.....	G...G.C..		
Lineage.56C.....	CT.....	G...G.C..		
Lineage.57C.....	CT.....	G...G.C..		
Lineage.58C.....	CT.....	G...G.C..		
Lineage.59C.....	CT.....	G...G.C..		
Lineage.37T.....	.T.....CT.....TC..		
Lineage.38T.....	.T.....CT.....TC..		
Lineage.39T.....	.T.....CT.....C..		
Lineage.40T.....	.T.....CT.....TC..		
Lineage.51T.....	.T.....CT.....TC..		
Lineage.52T.....	.T.....CT.....TC..		
Lineage.55T.....	.T.....CT.....TC..		
Lineage.60T.....	.T.....CT.....TC..		
Lineage.8C..	C.....C..		
Lineage.9C..	C.....G.C..		
Lineage.10C..	C.....C..		
Lineage.11C..	C.....C..		
AF303111		C.....C..		
Umar4		C.....C..		
AkoukerC..GC..T...CT.....C..		
El-Ksiba		A.....			
TAB15	--C.T.....T..ACT...CTC....C..	
AF305485	..C.T.....	..C.TG...	C.....C..	

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75      61
76 Takouatz  CACAGTATGT CCTCGAATAC TTT-----C CCCCCCTAT GTATATCGTG
77 A-Pyrenees .....TTT.....- .....
78 B-Cantabri .....TT.....- .....
79 C-Norway .....TTTTT.....- .....
80 D-Dalarna- .....TTT.....- .....
81 F-Abruzzo- .....T.....- .....
82 EGH1-Slove .....T.....- .....
83 H-Croatia .....T.....- .....
84 J-Greece .....TT.....- .....
85 K-Bulgaria .....T.....- .....
86 MNOPQ-Russ .G.....C..T.....- .....
87 N-Estonia .G.....C..T.....- .....
88 L-Romania1 .G.....C..TTTTTT.....- .....
89 L-Romania2 .G.....C..TTT.....- .....
90 Lineage.20 .G.....---.....- .....
91 Lineage.26 .G.....---.....- .....
92 Lineage.27 .G.....---.....- .....
93 Lineage.28 .G.....---.....- .....
94 Lineage.31 .G.....---.....- .....
95 Lineage.32 .G.....---.....- .....
96 Lineage.21 .G.....---.....- .....
97 Lineage.22 .G.....---.....- .....
98 Lineage.23 .G.....---.....- .....
99 Lineage.53 .G.....---.....- .....
100 Lineage.54 .G.....---.....- .....
101 Lineage.56 .G.....---.....- .....
102 Lineage.57 .G.....---.....- .....
103 Lineage.58 .G.....---.....- .....
104 Lineage.59 .G.....---.....- .....
105 Lineage.37 .G.....---.....- ...CG....
106 Lineage.38 .G.....---.....- ...CG....
107 Lineage.39 .G.....---.....- ...CG....
108 Lineage.40 .G.....---.....- ...CG....
109 Lineage.51 .G.....---.....- ...CG....
110 Lineage.52 .G......C.....---.....- ...CG....
111 Lineage.55 .G.....---.....- ...CG....
112 Lineage.60 .G.....---.....- ...CG....
113 Lineage.8 .....---.....- .....
114 Lineage.9 .....---.....- .....
115 Lineage.10 .....---.....- .....
116 Lineage.11 .....---.....- .....
117 AF303111 .....
118 Umar4 .....T.....- .....
119 Akouker .....TT.....- .....
120 El-Ksiba .....T...TTTT.....- .....
121 TAB15 ..T.....TA.....TTCCTTT ATTTTTTTTT .....
122 AF305485 ..T.....T..TA.G...CC.CCTCTTT TATTTTTTTTT .T.....CG....
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Takouatz	CATTAATGGC	GTGCCCCATG	CATATAAGCA	TGTACATACT	GTGCTTGGTC	TTACATGAGG	
A-PyreneesT.	A.....	
B-CantabriT.	
C-NorwayT.	
D-Dalarna-T.	
F-Abruzzo-T	
EGHI-SloveT.T	
H-CroatiaT.T	
J-GreeceT.	
K-BulgariaT.G..	
MNOPQ-RussT.	AC.....A..	
N-EstoniaT.	AC.....A..	
L-Romania1T.	AC.....A..	
L-Romania2T.	AC.....A..	
Lineage.20T.	AC.....A..	
Lineage.26T.	AC.....A..	
Lineage.27T.	AC.....A..	
Lineage.28T.	AC.....A..	
Lineage.31T.	AC.....A..	
Lineage.32T.	AC.....A..	
Lineage.21TT.	AC.....	..G...A..	
Lineage.22G...TT.	AC.....A..	
Lineage.23TT.	AC.....A..	
Lineage.53TT.	AC.....A..	
Lineage.54TGT.	AC.....A..	
Lineage.56G...TT.	.C.....A..	
Lineage.57TT.	AC.....A..	
Lineage.58TT.	AC.....A..	
Lineage.59G...TT.	AC.....	..G...A..	
Lineage.37T.	AC.....A..	
Lineage.38T.	AC.....C.A..	
Lineage.39T.	AC.....C.A..	
Lineage.40T.	AC.....C.A..	
Lineage.51T.	AC.....C.A..	
Lineage.52T.	AC.....C.A..	
Lineage.55T.	AC.....C.A..	
Lineage.60T.	AC.....A..	
Lineage.8	A.....A..	..G.....	
Lineage.9A..	..G.....	
Lineage.10A..	..G.....	
Lineage.11A..	..G.....	
AF303111A..	..G.....	
Umar4A..	..G.....	
AkoukerT.	
El-KsibaC.	..G.....	
TAB15A.....G...T.	A.....A..	
AF305485	

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Takouatz	ACCTGCATTT	TAGAAGTTTA	TCTCAGGTGT	ATAGTCTGCA	AGCATGTATT	TCACTTAGTC
A-PyreneesA.G..C	C.A.....G	.T.....T.
B-Cantabri	..T.A.G..C	C.A...C..G	.T.....T.
C-Norway	..T.A.G..C	C.A.....G	.T.....T.
D-Dalarna-	..T.A.G..C	C.A.....G	.T....C..T.
F-Abruzzo-	..T.A.G..C	CGA.....G	.T.....T.
EGHI-Slove	..T.A.G..C	CGA.....G	.T.....T.
H-Croatia	..T.A.G..C	.GA.....G	.T.....T.
J-Greece	..T.A.G..C	CGA...C..G	.T....C..T.
K-Bulgaria	..T.A.G..C	CGA.....G	.T....C..T.
MNOPQ-Russ	..T.A.G..C	CGA...C...	.T....C..	..G....T.
N-Estonia	..T.A.G..C	CGA...C...	.T....C..	..G.....
L-Romania1	..T.A.G..C	CGA...C...	.T....C..	..GA....T.
L-Romania2	..T.A.G..C	CGA...C...	.T....C..	..GT....T.
Lineage.20	..T.A.G..C	CGA...C...	.T....C..	..G....T.
Lineage.26	..T.A.G..C	CGA...C...	.T....C..	..G....T.
Lineage.27	..T.A.G..C	CGA...C...	.T....C..	..G....T.
Lineage.28	..T.A.G..C	CGA...C...	.T....C..	..G....T.
Lineage.31	..T.A.G..C	CGA...C...	.T....C..	..G....T.
Lineage.32	..T.A.G..C	CGA...C...	.T....C..	..G....T.
Lineage.21	..T.A.G..C	CGA...C...	.T.....	..G....T.
Lineage.22	..T.A.G..C	CGA...C...	.T....C..	..G....T.
Lineage.23	..T.A.G..C	CGA...C...	.T.....	..G....TG
Lineage.53	..T.A.G..C	CG...C...	.T....C..	..G....T.
Lineage.54	..T.A.G..C	CGA...C...	.T.....	..G....T.
Lineage.56	..T.A.G..C	CGA...C...	.T....C..	..G.C...T.
Lineage.57	..T.A.G..C	CGA...C...	.T.....	..G.C...T.
Lineage.58	..T.A.G..C	CGA...C...	.T.....	..G....T.
Lineage.59	..T.A.G..C	CGA...C...	.T....C..	..G.C...T.
Lineage.37	..T.A.G..C	CGA...C...	.T.....	..G....T.
Lineage.38	..T.A.G..C	CGA...C...	.T.....	..G....T.
Lineage.39	..T.A.G..C	CGA...C...	.T...A..A.	..G....T.
Lineage.40	..T.A.G..C	CGA...C...	.T...A..A.	..G....T.
Lineage.51	..T.A.G..C	CGA...C...	.T.....	..G....T.
Lineage.52	..T.A.G..C	CGA...C...	.T...A..A.	..G....T.
Lineage.55	..T.A.G..C	CGA...C...	.T.....	..GA....T.
Lineage.60	..T.A.G..C	CGAG..C...	.T.....	..GA....T.
Lineage.8A.G..C	.GA.....	.T....C..
Lineage.9A.G..C	.GA.....	.T....C..
Lineage.10A.G..C	.GA.....	.T....C..
Lineage.11A.G..C	.GA.....	.T....C..
AF303111	..T.A.G..C	CGA.....	.T....C..
Umar4	..T.A.G..C	CGA.....	.T....C..
Akouker	..T.A.G..C	C.A...C..G	.T.....T.
El-KsibaT.....
TAB15A...C.	C.A.....	.T...A...T.
AF305485A.....	C.A...C..G	.T.T.....T..T.

226
227
228 241
229 Takouatz CGGGAGCTTA ATCACCAGGC CTCGAGAAAC CATCAATCCT TGC
230 A-Pyrenees G..... ..G.....
231 B-Cantabri G..... ..G.....
232 C-Norway G..... ..G.....
233 D-Dalarna- G..... ..G.....
234 F-Abruzzo- G..... ..G.....
235 EGH1-Slove G..... ..G.....
236 H-Croatia G..... ..G.....
237 J-Greece G..... ..G.....
238 K-Bulgaria G..... ..G.....
239 MNO PQ-RussGG...C...
240 N-EstoniaGG...C...
241 L-Romania1GG...C...
242 L-Romania2GG...C...
243 Lineage.20GG...C...
244 Lineage.26GG...C...
245 Lineage.27GG...C...
246 Lineage.28GG...C...
247 Lineage.31G G..... ..G...C...
248 Lineage.32GG...C...
249 Lineage.21 ..A..... ..G...C...
250 Lineage.22 ..A..... ..G...C...
251 Lineage.23 ..A..... ..G...C...
252 Lineage.53 ..A..... ..G...C...
253 Lineage.54 ..A..... ..G...C...
254 Lineage.56 ..A..... ..G...C...
255 Lineage.57 ..A..... ..G...C...
256 Lineage.58 ..A..... ..G...C...
257 Lineage.59 ..A..... ..G...C...
258 Lineage.37 .A..... ..G.....
259 Lineage.38G.....
260 Lineage.39G.....
261 Lineage.40G.....
262 Lineage.51GG.....
263 Lineage.52G.....
264 Lineage.55G.....
265 Lineage.60G.....
266 Lineage.8G.....
267 Lineage.9G.....
268 Lineage.10G.....
269 Lineage.11 G..... ..G.....
270 AF303111G.....
271 Umar4G.....
272 Akouker G..... ..G.....
273 El-Ksiba
274 TAB15G...C...
275 AF305485G...C...
276

277

278 Several lines of evidence permit to state that sequences obtained are authentic mitochondrial
279 sequences and not *numts*: (i) the most variable part of the mtCR (showing four unique
280 substitutions among brown bears concentrated in a 20 bp long zone) have been amplified
281 using two sets of primers (Hofreiter *et al.*, 2001) ; (ii) an extensive number of clones has been
282 sequenced without ever leading to a sequence looking like clade I to V sequences ; (iii) two
283 different haplotypes sharing many unique substitutions among brown bears have been
284 identified in one and four different individuals coming from two different caves; (iv) attempts
285 to amplify nuclear DNA for molecular sexing only succeeded in identifying one of the
286 individuals from Takouatz (TAK4, M. Pagès personal communication) suggesting that
287 extracts only contain low quantities of nuclear DNA ; (v) *cyt b* sequences from clade VI
288 individuals, though being divergent, are encoding a functional protein only differing from
289 other brown bear *cyt b* in 1 out of 92 amino acids (a methionin replaces a valin in the case of
290 Takouatz, these two amino acids being hydrophobic ones).

291 Thus, we are considering clade VI mtCR sequences as being authentic mitochondrial
292 sequences.

293

294 S6. Phylogenetic analyses of the concatenated sequences.

295

296 Sequences constituting the mtCR and *cyt b* datasets were not directly concatenable as
297 they were mostly obtained from different (or unidentifiable) individuals. We hence
298 determined a strict consensus for each marker and each clade and concatenated the resulting
299 sequences. This resulted in an eight taxon dataset (Clade I, *Ursus maritimus*, Clade II, Clade
300 III, Clade V, Clade VI, *Ursus spelaeus*, *Ursus americanus*).

301 Maximum Parsimony (MP), ML and Bayesian analyses were performed on this
302 dataset. The MP analyses were carried out using the software Paup v4.0b10 (Swofford, 2000).
303 MP analysis was performed with equal weighting schemes using the tree bisection-
304 reconnection (TBR) branch swapping method with 100 random sequence additions. Prior to
305 the ML and Bayesian analysis, the best-fitting model of nucleotide substitution for the
306 concatenated sequences (HKY+ Γ) was chosen with MrModeltest 2.0 (Nylander, 2004).
307 Maximum Likelihood (ML) analysis was performed with PHYML (Guindon, Gascuel, 2003)
308 using the online interface <http://atgc.lirmm.fr/phyml/> (Guindon *et al.*, 2005). The
309 transition/transversion ratio, the proportion of invariable sites as well as the gamma
310 distribution parameter were estimated and the starting tree was determined by a BioNJ

analysis of the datasets (default settings). Using optimization options, 500 bootstrap (Bp) replicates were performed. Bayesian analyses (BA) were performed using MrBayes v3.0 (Ronquist, Huelsenbeck, 2003). Three independent runs of 500 000 generations each were performed under the same model as above. However, parameters of the model were allowed to vary according to the gene partition. A burn-in period of 25 000 generations was determined graphically using Tracer1.2 (Rambaut, Drummond, 2003). All three runs gave similar tree topologies and posterior probability (pp) values.

All three methods gave similar topologies that are summarized in the following tree. This tree is a Bayesian phylogenetic tree of the concatenated mtCR and cyt b datasets (561 bp). Underlined numbers above nodes are ML Bp values, italic numbers below nodes are MP Bp values, numbers shown under the nodes are pp values.

