

## FAST-TRACK ARTICLE

### Staying out in the cold: glacial refugia and mitochondrial DNA phylogeography in ancient European brown bears

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#### Abstract

**Models for the development of species distribution in Europe typically invoke restriction in three temperate Mediterranean refugia during glaciations, from where recolonization of central and northern Europe occurred. The brown bear, *Ursus arctos*, is one of the taxa from which this model is derived. Sequence data generated from brown bear fossils show a complex phylogeographical history for western European populations. Long-term isolation in separate refugia is not required to explain our data when considering the palaeontological distribution of brown bears. We propose continuous gene flow across southern Europe, from which brown bear populations expanded after the last glaciation.**

*Keywords:* ancient DNA, expansion contraction model, gene flow, last glacial maximum, phylo-geography, recolonization

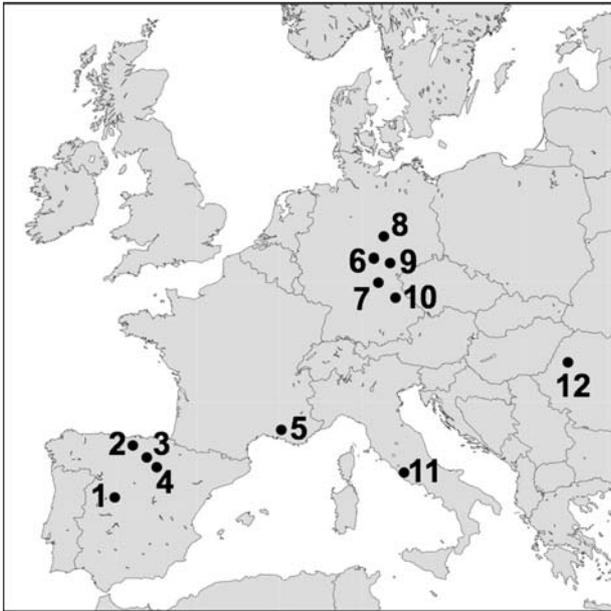
#### Introduction

Extensive climatic fluctuations during the late Quaternary have influenced the demographic history, genetic diversity, and present-day distribution of many species (Webb & Bartlein 1992). Current models propose that the onset of maximal glacial conditions restricted many temperate plant and animal species into unglaciated refugia. Three main European refugia have been proposed for the Pleistocene: the Iberian, Italian and Balkan peninsulas (Bennett *et al.* 1991; Taberlet & Bouvet 1994; Hewitt 1996). According to the expansion/contraction model (E/C), populations survived in these southern refugia during Pleistocene glaciations and expanded into mainland Europe as the glaciers retreated (Hewitt 1996). Both the confinement of populations to

southern refugia and the subsequent range expansion following glacial retreat should result in population bottlenecks that reduce genetic variation in the recolonized areas (Hewitt 1996; Taberlet *et al.* 1998; Hewitt 1999; Randi 2003; Rowe *et al.* 2004). Over multiple glaciations, including the last glacial maximum (LGM) 23 000–18 000 years ago (Kukla *et al.* 2002), refugial populations would have been isolated from one another for extended periods of time and consequently would be expected to exhibit significant genetic divergence from each other (Hewitt 1996; Hewitt 2000). However, the general application of this model to different taxa has been questioned because of palaeontological evidence (Stewart & Lister 2001), DNA sequence data (Rowe *et al.* 2004; Kotlik *et al.* 2006) and pollen records (Willis *et al.* 2000).

As the modern phylogeographical distribution of brown bear haplogroups is consistent with origins in the three major European refugia, the brown bear has served as one of the model species supporting a scenario of glacial refugia

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**Fig. 1** Map of geographical distribution of sample localities. Sites of sampled fossil/subfossil *Ursus arctos*: 1, Navacepeda; 2, Cantabria; 3, Cuevas del Somo; 4, Atapuerca/Cueva Major; 5, Mont Ventoux; 6, Mühlberg; 7, Wysburg near Weisbach; 8, Bad Frankenhausen; 9, Dienstedt; 10, Hohle Fels Cave; 11, Grotta Beatrice; 12, Pestera Baltagul.

and postglacial recolonization of central and northern Europe (Taberlet *et al.* 1994, 1998; Hewitt 1999, 2000, 2001, 2004). There is a clear division into two main mitochondrial lineages in modern European brown bear populations. These populations are divided into those carrying an eastern lineage (clade IIIa, Leonard *et al.* 2000), which is composed of Russian, northern Scandinavian and eastern European populations, and those carrying a western lineage (clade I, Leonard *et al.* 2000), which is composed of two subgroups, one believed to originate from the Iberian Peninsula, including southern Scandinavian bears and the Pyrenean populations; and the other from the Italian–Balkan peninsulas (Taberlet *et al.* 1994; see however Kohn *et al.* 1995). In addition, based on the subfossil record in northwestern Moldova and mitochondrial DNA data from modern populations, a Carpathian refuge has also been proposed (Sommer & Benecke 2005; Saarma *et al.* 2007). Two contact zones have been identified where the two main lineages meet each other, one in Sweden (Taberlet *et al.* 1995) and the other one in Romania (Kohn *et al.* 1995). However, because of historical human activity (Taberlet *et al.* 1994; Waits *et al.* 1999), the current geographical distribution of brown bears in Europe is fragmented and reduced to just a few small populations in the West and some larger ones in the East. Furthermore, brown bears are now extinct in Central Europe, thus interpretation of the present-day phylogeographical

pattern may reveal little about the glacial demographic history. Therefore, analyses of bear fossil remains throughout their historical range can provide direct information of the past population structure.

Previous ancient DNA work on European brown bears (two individuals from Austria) has been used to suggest a different phylogeographical structure in brown bears before the LGM, to that in modern-day populations (Hofreiter *et al.* 2004). In this study, we focus on glacial and postglacial populations, particularly those from Western Europe.

Assuming (i) that the E/C model is a major factor in the formation of the phylogeographical structure seen in modern brown bear populations (Taberlet *et al.* 1994; Hewitt 1996), and (ii) the classical view of Mediterranean refugia for temperate species, from which recolonization of central and northern Europe took place, two predictions can be made for postglacial genetic patterns of brown bears: (i) haplotypes not associated with the traditional refugia should not occur in mainland Europe after the LGM, and (ii) populations in the refugia should be reciprocally mono-phyletic due to long-term isolation. Other temperate species of small mammals, currently inhabiting Central and northern Europe, such as the pygmy (*Sorex minutus*) and common shrews (*Sorex araneus*), and the bank vole (*Clethrionomys glareolus*) have proven not to have a Mediterranean peninsular (i.e. Iberian, Italian or Balkan) origin (Bilton *et al.* 1998), and thus do not fit the traditional E/C model.

Significant differences between major clades, and clear spatial patterning of these clades in modern (Taberlet *et al.* 1994) and past (Barnes *et al.* 2002) populations, attributed to maternal philopatry, make brown bears a tractable and extensively studied taxon for ancient DNA studies (Leonard *et al.* 2000; Barnes *et al.* 2002; Hofreiter *et al.* 2004). In order to investigate the role played by ancient glacial refugia in shaping the current haplotype distributions, we analysed mitochondrial DNA haplotype distribution and nucleotide diversity in ancient brown bear samples from Spain, Southern France, Germany, Italy and Romania, with dates ranging from the Late Pleistocene to Late Holocene (Fig. 1).

## Materials and methods

### DNA extraction

A total of 66 bones and teeth of brown bear specimens from different sites (Fig. 1) were collected from several museum collections (see Supplementary material) and extracted together with 43 water negative controls.

The following procedure was employed to all samples:

Pieces of bone and tooth of about 1 cm<sup>3</sup> were cleaned on the surface with 1 M HCL, and then ground to powder under liquid nitrogen using a Spex 6700 Freezer Mill according to the manufacturer's instructions. Samples were extracted twice, using both solvent and silica-binding approaches

**Table 1** Samples analysed. Ancient brown bear samples that yielded the complete sequence of 193 bp, geographical site, assigned date/cultural context and haplotype. Samples from Romania marked with a \* were not AMS dated directly but could be associated with other bear remains dated from the same site to 3rd century BC

Sample	Site	C14 date BP	Haplotype	Accession no.
Vallecampo	Cuevas del Somo (Burgos, Spain)	7500 ± 55	Ua8	EF488487
C.Motas 33-2	Cuevas del Somo (Burgos, Spain)	—	Ua8	EF488502
C.Motas 33-1	Cuevas del Somo (Burgos, Spain)	4624 ± 45	Ua9	EF488503
Mv4 K3 99	Mont Ventoux (Vaucluse, France)	1570 ± 35	Ua7	EF488495
Atapuerca	Atapuerca-Cueva Mayor (Burgos, Spain)	17 440 ± 425	Ua20	EF488504
Gbcm2	Grotta Beatrice Cenci (Abruzzo, Italy)	16440 ± 65	Ua4	EF488488
Asturias	Cantabria Cave (Cantabria, Spain)	—	Ua10	EF488489
GEE	Cuevas del Somo (Burgos, Spain)	5380 ± 45	Ua8	EF488490
Mv4 L6 714	Mont Ventoux (Vaucluse, France)	4645 ± 40	Ua13	EF488496
Mv4 L5 1184	Mont Ventoux (Vaucluse, France)	3845 ± 40	Ua12	EF488492
Mv4 Mr-204–48	Mont Ventoux (Vaucluse, France)	1750 ± 30	Ua17	EF488493
Mv4 M5 162	Mont Ventoux (Vaucluse, France)	1790 ± 55	Ua7	EF488491
Mv4 L6 851	Mont Ventoux (Vaucluse, France)	6525 ± 50	Ua11	EF488494
Mv4 Remanie	Mont Ventoux (Vaucluse, France)	3445 ± 40	Ua15	EF488505
Hem	Navacerrada (Avila, Spain)	350 ± 40	Ua6	EF488497
A3	Dienstedt (Thuringia, Germany) Mühlberg	1665 ± 35	Ua18	EF488501
A5	(Thuringia, Germany)	1770 ± 35	Ua22	EF488498
A9	Bad Frankenhausen (Thuringia, Germany)	5210 ± 35	Ua15	EF488499
A12	Wysburg (Thuringia, Germany)	XII–XIV century	Ua15	EF488500
Romania1	Pestera Baltagul (Romania)	III century*	Ua23	EF488506
Romania2	Pestera Baltagul (Romania)	III century*	Ua23	EF488507

(Yang *et al.* 1998; Leonard *et al.* 2000). Finally, an independent replication was carried out using the method of Anderung *et al.* (2005) in five samples (see below). In every extraction, 150–250 mg of bone powder was used, except for the Atapuerca sample, where 500 mg were used.

#### DNA amplification

Amplification was carried out using two sets of primers designed to amplify 111-bp and 135-bp fragments (primers not included) from the control region of the mitochondrial genome. Primer sequences are as follows: for the short

fragment (111 bp) designed in this study: URSUSF1\_136–156 CAGCACCCAAAGCTAATGTTTC and URSUSR1\_273–290 GCACGAKMTACATAGGGG; for the long fragment (135 bp), we used primers L16164 and H16299 from (Hänni *et al.* 1994). Polymerase chain reaction (PCR) amplifications were carried out with 1 µL of DNA extract and 1 U of Platinum *Taq* HiFi Polymerase (Invitrogen) when using phenol–chloroform DNA extraction protocol and 5 µL of DNA extract and 2 U of HotStar *Taq* (QIAGEN) when using the silica-based protocol. Reaction conditions were performed as in Leonard *et al.* (2000). PCR products were purified using a MiniElute PCR Purification Kit according to the manufacturer's instructions before cycle sequencing. The sequencing reaction was purified with isopropanol (once at 80% and then 70%) and formamide before PCR products were screened with an ABI PRISM 3100 genetic analyser.

Extractions were carried out in an ancient DNA laboratory at the University College London and repeated in a different ancient DNA laboratory at Centro Mixto UCM-ISCIII de Evolución y Comportamiento Humanos in Madrid. Four out of five samples were successfully replicated in an independent laboratory in Uppsala, Sweden (MV4 L6 714, MV4 L5 1184, MV4 L6 851, Cueva de las motas 33-1). A fifth sample (Vallecampo) replicated in Uppsala University showed sequence variation; in this case, the sample was re-extracted twice. Every sample, from which there was enough material and gave both short and long DNA sequences, was sent for radiocarbon dating except the two bears from Romania, which were indirectly dated, that is associated with multiple other remains from the same site (Table 1); all dates are represented in radiocarbon years. Every sample was extracted and amplified at least twice, in addition to the independent laboratory replication. In four cases, we observed sequence variation in the same sample. In those cases, we applied the majority rule consensus (Krause *et al.* 2006) considering two out of three sequences (from three different amplifications) the correct one for samples MV4 L6714 and MV4162 and three out of four sequences for samples Remanie and Vallecampo.

#### Phylogenetic analyses

Phylogenetic relationships were estimated using maximum likelihood and Bayesian inference. Maximum-likelihood

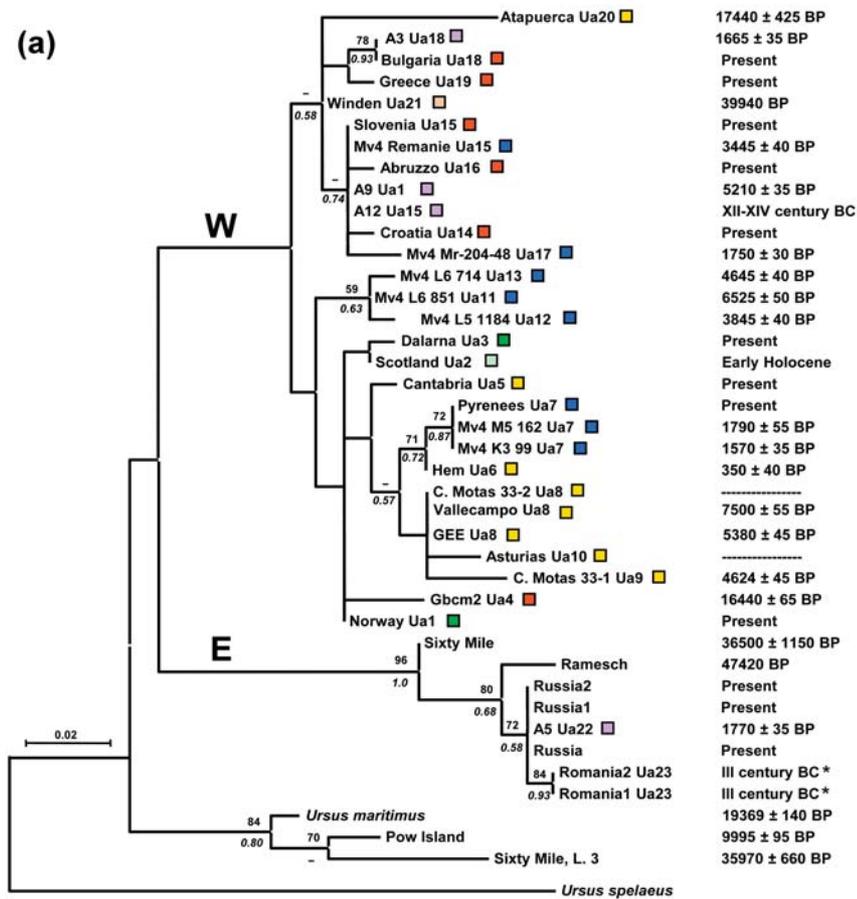
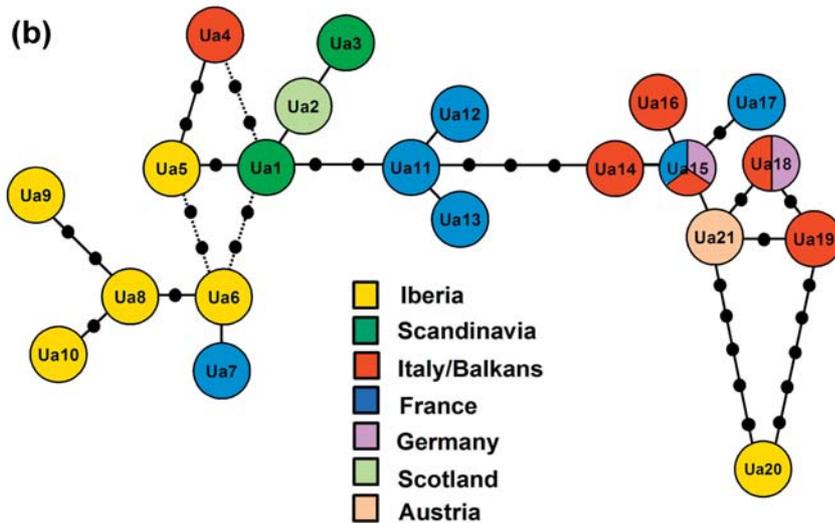


Fig. 2 (a) Maximum-likelihood tree of ancient and modern brown bear sequences. Numbers above nodes represent bootstrap values, below nodes are posterior probabilities. (b) Minimum-spanning network for haplotypes in the western (W) clade. Missing haplotypes are shown with a dot and colours correspond to geographical origin.



trees and all model parameters ( $Ti/Tv = 100.0$ ,  $I = 0.344$ ,  $\alpha = 0.452$ ) were estimated with PHYML (Guindon *et al.* 2005). Substitution models were compared with likelihood-ratio tests when nested, and with the Aikake Information Criterion. The Hasegawa-Kishino-Yano + invariant sites + gamma model of sequence evolution was used to generate Bayesian posterior probabilities. Markov chain Monte Carlo

sampling was performed as implemented in the phylogenetic analysis software MRBAYES (Huelsenbeck *et al.* 2001; Ronquist & Huelsenbeck 2003), using 5 000 000 iterations, sampling every 1000, with the first 1250 samples (25%) discarded as burn-in. Node support was calculated according to 500 bootstrap replicates (maximum likelihood) and posterior probabilities (Bayesian inference) (Fig. 2a).

'Mainland' Europe group	Age	Peninsular group	Age
Dalarna (Sweden)	Present	Abruzzo (Italy) Bulgaria	Present
Norway	Present	Croatia	Present
Pyrenees	Present	Greece	Present
Mv4 M5162 (France)	1790 ± 55 BP	Cantabria (Iberia)	Present
Mv4 L51184 (France)	3845 ± 40 BP	Slovenia	Present
Mv4 MR 204–48 (France)	1750 ± 30 BP	Asturias (Iberia) GEE	Present
Mv4 L6714 (France) Mv4	4645 ± 40 BP	(Iberia)	—
L6851 (France) Mv4	6525 ± 50 BP	HEM (Iberia)	5380 ± 45 BP
Remanie (France) Mv4	3445 ± 40 BP	C.Motas 33–1 (Iberia)	350 ± 40 BP
K3 99 (France)	1570 ± 35 BP	C.Motas 33–2 (Iberia)	4624 ± 45 BP
A5 (Germany)	1770 ± 35 BP	Atapuerca (Iberia)	—
A9 (Germany)	5210 ± 35 BP	Gbcm2 (Italy)	17 440 ± 425 BP
A3 (Germany)	1665 ± 35 BP	Vallecampo (Iberia)	16 440 ± 65 BP
A12 (Germany) Scotland	XII–XIV century	Romania1 Romania2	7500 ± 55 BP
Ramesch (Austria)	Late Holocene	—	III century
Winden (Austria)	47420 BP	—	III century
	39940 BP	—	—

**Table 2** Samples used for  $\pi$  test. Geographical site and age of the samples assigned to 'mainland' Europe and 'Peninsular Groups' used to compare nucleotide diversity, respectively

A minimum-spanning network based on pairwise differences among haplotypes in 30 sequences was constructed using ARLEQUIN (version 2.000) (Fig. 2b) (Schneider *et al.* 2000).

To test for a possible founder effect due to recolonization events (Edmonds *et al.* 2004) in the central European bears, we used estimated pairwise difference nucleotide diversity ( $\pi$ , Nei 1987; Nei & Miller 1990). The sequence data were grouped into two data sets (peninsular  $n = 16$  and main-land  $n = 17$ ) (Table 2). A value for  $\pi$ , with confidence intervals, was estimated through nonparametric bootstrapping with 10 000 bootstrap replicates.

## Results

From 66 samples (see Supplementary material) of bone or tooth, 21 yielded reproducible sequences for both targeted fragments (Accession GenBank nos EF488487–EF488507) (Table 1). Contamination was not detected in any of the 43 water negative controls. We obtained 16 radiocarbon dates for samples from The Ångström Laboratory, Uppsala University, Sweden, using the accelerator mass spectrometry (AMS) method (insufficient sample was available for specimens A12, C. Motas 33-2 and Asturias) (Table 1). One more date (sample gbcm2) was generated at Centro di Datazione e diagnostica at the Università degli Studi di Lecce and provided by the Soprintendenza per i Beni Archeologici dell'Abruzzo, Chieti, Italy. The two Romanian samples were dated through association with other samples from the same site that yielded largely overlapping AMS dates.

### Phylogenetic analysis

The 21 ancient sequences obtained in this study (Fig. 1), together with 19 previously published sequences obtained

from the GenBank database (see Supplementary material) (Taberlet *et al.* 1994; Barnes *et al.* 2002; Hofreiter *et al.* 2004), were used to infer a phylogenetic tree. We used the cave bear (*Ursus spelaeus*), the evolutionary closest distinct species as an outgroup (Loreille *et al.* 2001; Hofreiter *et al.* 2002; Fig. 2a). Using Bayesian, maximum-likelihood and neighbour-joining approaches, we obtained a tree topology similar to that previously inferred (Leonard *et al.* 2000; Barnes *et al.* 2002); eastern and polar bear clades (IIIa and IIb) were well differentiated from the western clade. However, the western European populations showed a complex glacial and post-glacial phylogeographical structure, making it difficult to separate haplogroups according to their geographical origin and showed very low levels of population differentiation as opposed to the strong phylogeographical structure expressed in present-day populations (Taberlet *et al.* 1994). For example, one sample originating in Iberia (dated to 17 440 ± 425 BP) yielded a haplotype (Ua20) that falls into the clade believed to originate in Italy. Similarly, another sample originating in Italy (dated to 16 440 ± 65 BP) yielded a haplotype (Ua4) grouping with the clade hypothesized to originate from Iberia (Taberlet *et al.* 1994). Moreover, western (Ua15, Ua18) and eastern (Ua22) haplotypes dating to 5210 ± 35, 1665 ± 35, and 1770 ± 35 BP, respectively, were identified in Germany (Fig. 2b).

### Minimum-spanning network

Because of the complexity shown during the LGM and post-LGM in the phylogeographical structure of western European populations and the difficulty to associate haplo-groups to a specific peninsular origin, in order to develop a better understanding for the haplotype distribution of this clade, we constructed a minimum-spanning network

of modern ( $n = 10$ ) (Taberlet *et al.* 1994; Kohn *et al.* 1995) and ancient ( $n = 20$ ) sequences originating from different geographical regions across Europe: Scotland (Barnes *et al.* 2002), Austria (Hofreiter *et al.* 2004) Iberia, Italy, France, Germany and Romania (Fig. 2b). A total of 21 haplotypes were obtained, spanning a time range from  $17\,440 \pm 425$  to the present.

In the southern French site of Mont Ventoux 4 (Crégut-Bonnoure *et al.* 2005), there are six haplotypes (Ua7, Ua11–13, Ua15 and Ua17) distributed throughout the network, spanning a time range of  $6525 \pm 50$ – $1570 \pm 35$  BP (Fig. 2a, b). Interestingly, the three oldest samples yielded haplotypes that form a monophyletic group (Ua11–13) positioned between the hypothesized Iberian and Italian/Balkan clades in the minimum-spanning network. These three novel haplotypes dated to  $6525 \pm 50$ ,  $3845 \pm 40$ , and  $4645 \pm 40$  BP, respectively, form a unique and previously undescribed, post-LGM European lineage.

#### Nucleotide diversity

We could not find any significant difference in estimated  $\pi$  ( $P = 0.28$ ; determined through bootstrapping 10 000 replicates) between the three combined peninsulas (Iberia, Italy and Balkans,  $\pi = 0.0388$ ) and the mainland ( $P = 0.0455$ ).

#### Discussion

We did not observe the patterns of phylogeographical structuring that are expected for a peninsular origin for populations inhabiting mainland Europe after the LGM. Instead, we found high diversity in the samples from Mont Ventoux 4 in southern France as well as in four German sites (Bad Frankenhausen, Dienstedt, Wysburg, Mühlberg). We found no evidence for reciprocal monophyly in the peninsulas during or after the LGM, as would be expected following the long-term isolation of these populations. Thus, we fail to provide support for the two predictions made based on an E/C model; our data indicated that the classic glacial refugium model is insufficient to explain the postglacial genetic history of European brown bears. There have been objections to the general application of the refugium model to other taxa. Palaeontological evidence (Stewart & Lister 2001), as well as DNA sequence data (Rowe *et al.* 2004; Kotlik *et al.* 2006) and pollen records (Willis *et al.* 2000) are not always in agreement with an E/C scenario.

In a previous study, two pre-glacial brown bear sequences, among other Late Pleistocene species, were used to argue for a lack of phylogeographical structure shortly before the last glacial maximum (Hofreiter *et al.* 2004). The sequences were retrieved from two cave sites in Austria, Winden (a western haplotype) and Ramesch (an eastern haplotype). Interestingly, the Winden cave is located at the East of the Ramesch cave, this latter one located at the west of the

Romanian contact zone. The lack of phylogeographical structure has been explained as the consequence of extensive mixing between populations following a postglacial expansion, whereby the phylogeographical structure formed during long-term isolation in southern glacial refugia was eroded during postglacial expansion by extensive gene flow. Our data show a similar mixing of mitochondrial types, however, this time during the LGM within glacial refugia populations in the Iberian and Italian peninsulas. These two findings together suggest that any lack of phylogeographical structure before the last glaciation continued during the last glacial maximum. Moreover, during the Holocene, Mont Ventoux 4 (in southern France) presents three different haplogroups at different time periods, each comprising mitochondrial haplotypes of hypothesized Iberian (Ua7 dated to  $1790 \pm 55$  BP and  $1570 \pm 35$  BP) and Italian (Ua15 and Ua17 dated to  $3445 \pm 40$  BP,  $1750 \pm 30$  BP, respectively) populations, and three more haplotypes (Ua11–Ua13 dated to  $6525 \pm 50$ ,  $3845 \pm 40$ , and  $4645 \pm 40$  BP, respectively) that are not associated with any of the three glacial refugia from where the mainland European populations are suggested to have originated. Similarly, high genetic diversity is found in Germany, with haplotypes belonging to the eastern clade and the Italian/Balkan clade present in German populations during the Holocene.

Finding two different lineages (eastern and western) in the ancient German samples could be explained by the existence of a contact zone in this area. This does not, however, apply to the site of Mont Ventoux 4, where not only the two known western (Iberian and Italy/Balkans) lineages are present, but also one that has no counterpart in studied ancestral refugia. As the three lineages do not have a temporal overlap, one can speculate that the presence of this novel lineage could be due to population replacement, with a refugium in southern France and an extinction event during the early Holocene. However, the number of observations is insufficient to test the serial distribution at this site.

An alternative scenario, more compatible with these data and the anomalies associated with each of the predictions under the E/C model, is that brown bears were not restricted to Mediterranean peninsulas during the LGM, but also survived in mainland southern Europe. Although our data do not indicate a clear phylogeographical structure, there is a tendency for haplotypes to group according to a specific geographical area in an east-west cline. Such a pattern would be expected under an isolation by distance model over a continuous range, as has been suggested previously for European brown bears (Randi *et al.* 1994). The lack of a clear phylogeographical structure before (Hofreiter *et al.* 2004), during and after the LGM is compatible with restricted but continuous long-term gene flow among geographical regions within southern mainland Europe and the Mediterranean peninsulas (Avice *et al.* 1987). This scenario would also explain the lack of monophyly in the

proposed refugial populations, the existence of highly divergent haplotypes (Ua4 and Ua20) in the peninsulas and the presence of apparently unique lineages outside the peninsulas. Finally, this is more in concordance with not finding significantly lower diversity in mainland Europe would be expected following a recolonization event, creating a founder effect.

There are other forms of evidence that are also in concordance with this scenario. Floral evidence from France (Renault-Miskovsky & Leroi-Gourhan 1981) as well as macroscopic charcoal fossils from central Europe (Willis *et al.* 2000) have led to the suggestion of pockets of thermophilous trees in Europe during the LGM, an environment that could well sustain bear populations. Although there is little evidence of brown bears in the fossil record during the LGM, some fossil remains have been reported from a number of caves: in the Western Carpathians, Lisková, Važec and Vyvieranie caves, attributed to late glacial period (Sabol 2001) while Moravany Lopata II (Slovakia) (Musil 2003), Cosăuți I (David *et al.* 2003) and Ciuntu (Borziac *et al.* 1997) (Moldova) yielded *Ursus arctos* remains between 24 100 ± 800 BP and 17 030 ± 180 BP. In France, six localities have yielded remains of *U. arctos*: Faurous (Gironde; with Magdalenian industry; Gilbert 1984), Bois Ragot (Goueix, Vienne: level b dated 11 030 ± 140 BP), Duruthy (Sordel'Abbaye, Landes, level 3 with Magdalenian industry; Delpech 1983), Harzabaletako Karbia (Aussurucq, Pyrénées-Atlantiques; 29 200 ± 100 BP; Clot & Duranthon 1990), Oillasco (Saint-Michel, Pyrénées-Atlantiques; 18 720 ± 350 BP; Clot *et al.* 1990), Le Rond du Barry (Solignac, Haute-Loire) and Solutre, an open cave system in southern France (layer 6; 21 600 ± 700, 22 650 ± 500, and 23 200 ± 700 BP; Evin *et al.* 1994). In Austria, stratigraphic layers with brown bear presence have been dated to 22 180 ± 190 BP at Willendorf II (Vogel & Zagwijn 1967; Haesaerts *et al.* 1996) and between 18 890 ± 140 BP and 19 380 ± 90 BP at Grubgraben (Dambon *et al.* 1996; Terberger & Street 2002; Musil 2003). Brown bear remains have also been recovered in Paviland Cave (Goat's Hole), UK, dated to 17 670 ± 140 cal. BP (Aldhouse-Green & Pettitt 1998), these latter suggesting a cold tundra-steppe environment. Thus, the fossil record does indicate a wide geographical range of brown bears across mainland Europe before and during the LGM. Moreover, the geographical range of 47 LGM sites containing temperate mammal elements, such as roe deer, red deer and red fox during 23 000–16 000 BP, clearly shows a distribution which differs from the classical view of populations restricted to glacial Mediterranean refugia (Sommer & Nadachowski 2006).

Brown bears, which have served as a model species for the refugium model, were probably never dependent on a refugium system. During the LGM, permafrost and cold tundra-steppe dominated the region between the ice sheet covering northern Europe and the mountains separating the Mediterranean peninsulas from the rest of the continent

(Hewitt 1999). This environment is similar to the habitats occupied by brown bears today in Alaska, Canada and Siberia (McLoughlin *et al.* 2000). Furthermore, there is evidence in the fossil record of brown bears inhabiting Beringia during the LGM (Leonard *et al.* 2000; Barnes *et al.* 2002). If this was indeed a suitable habitat during the LGM, then the cold tundra-steppe of southern mainland Europe is likely to have been as well. Considering that brown bears represent one of the most adaptable species in Holarctic ecosystems and the flexibility of their diet (Pasitschniak 1993), which can vary from nearly strict vegetarian to full carnivore, it seems probable that bears could have occupied a similar ecological niche in France, northern Italy and Eastern Europe during the LGM.

Theoretical studies have shown that phylogeographical breaks can evolve without barriers to gene flow, especially when dispersal distances are low and population size is decreasing (Irwin 2002). Rather than being the result of repeated isolation in peninsular refugia, the appearance of phylogeographical structure in modern-day brown bear populations may thus be the result of the high degree of female philopatry in brown bears (Randi *et al.* 1994) and the severe reduction in population size during the Late Holocene (Servheen 1990). Following this, the different brown bear lineages in Europe would not represent evolutionary significant units, and would instead be the result of a recent fragmentation caused by human activities.

This study highlights the problem with using mitochondrial DNA data to identify units for conservation, since the current distribution of haplotypes may not accurately relate to species' history. Indeed, there is increasing evidence that phylogeographical inference from modern data conflicts with results obtained from ancient DNA (Leonard *et al.* 2000; Barnes *et al.* 2002; Hofreiter *et al.* 2004; Haak *et al.* 2005; Dalén *et al.* 2007; Leonard *et al.* 2007). Complex demographic histories, including bottlenecks and replacements, are difficult to infer using modern genetic data alone. The pre-glacial (Hofreiter *et al.* 2004) and postglacial history of bear populations in Europe reflects this problem, and is best studied with a temporal data set, including samples dating to periods before major anthropogenic manipulation took place, and also connected to important climatic changes. The extent to which this is applicable to other taxa should be explored.

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