

Post-glacial colonization of Western Europe brown bears from a cryptic Atlantic refugium out of the Iberian Peninsula

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ABSTRACT

The European brown bear (*Ursus arctos*) shows a particular phylogeography that has been used to illustrate the model for contraction-expansion dynamics related to glacial refugia in Southern European peninsulas. Recent studies, however, have nuanced the once generally accepted paradigm, indicating the existence of cryptic refugia for some species further north. In this paper we collected available data on chronology and mitochondrial haplotypes from Western European brown bears, adding new sequences from present day individuals from the Cantabrian (North Iberia) area, in order to reconstruct the dynamics of the species in the region. Both genetics and chronology show that the Iberian Pleistocene lineages were not the direct ancestors of the Holocene ones, the latter entering the Peninsula belatedly (around 10,000 years BP) with respect to other areas such as the British Isles. We therefore propose the existence of a cryptic refugium in continental Atlantic Europe, from where the bears would expand as the ice receded. The delay in the recolonization of the Iberian Peninsula could be due to the orographic characteristics of the Pyrenean-Cantabrian region and to the abundant presence of humans in the natural entrance to the Peninsula.

KEYWORDS

Ursus arctos; brown bear; LG M; postglacial colonization; Iberian Peninsula; cryptic refugium

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Introduction

The paradigm of glacial refugia in southern peninsulas

During Pleistocene glacial phases, southern European peninsulas were the refugia for temperate species that followed a model of contraction-expansion forced by climatic oscillations. This was confirmed through phylogeographic studies of a variety of species, including plants, insects or vertebrates (Hewitt 1996), which showed that many extant populations in northern Europe are derived from southern regions (Hewitt 1996, 1999, 2000; Stewart et al. 2010). Micro or meso mammals, such as the water vole *Arvicola sapidus* (Centeno-Cuadros et al. 2009), the field vole *Microtus agrestis* (Jaarola and Tegelström 1995) or the hedgehog *Erinaceus sp.* (Santucci et al. 1998), and large mammals such as the red deer *Cervus elaphus* (Meiri et al. 2013) or the brown bear *Ursus arctos* (Taberlet and Bouvet 1994), showed this pattern of endurance in the South during glacial pulses, followed by postglacial re-colonization of northern regions (Hewitt 2001). However, recent studies have nuanced the once generally accepted paradigm, indicating the existence of cryptic refugia further north of the peninsulas (Stewart and Lister 2001; Stewart et al. 2010) or even, as in the case of the brown bear, the survival of the species during the cold seasons outside the traditional refugia (Valdiosera et al. 2008).

Phylogeography of modern brown bear in Western Europe

The European brown bear (*Ursus arctos*) shows a particular phylogeography that has been used to illustrate the contraction-expansion model elaborated by Hewitt (1999, 2000). In the past, brown bears occupied a wide geographical range in Europe (Sommer and Benecke 2005; García-Vázquez 2015), but at present some of the most threatened populations are restricted to isolated regions in the South (Cantabrian Mountains and Pyrenees in the Iberian Peninsula, Abruzzi in the Italian Peninsula, Trentino in the Italian Alps; the bears of Trentino and most of the Pyrenean bears were recently reintroduced from Slovenia), while the North eastern Europe populations show a continuous distribution (Taberlet and Bouvet 1994; Korsten et al. 2009; Keis et al. 2012; Salomashkina et al. 2014).

Based on genetic evidences from modern populations of brown bears, Taberlet and Bouvet (1994) proposed the existence of two major clades in Europe: the Western and the Eastern clade. The Western clade, named clade 1 by Leonard et al. (2000), is divided in two sisters subclades: the Iberian clade, or sub-clade 1a (Davison et al. 2011), formed by the bears from the Iberian (Cantabrian Mountains and Pyrenees) and the South of the Scandinavian (Norway and Sweden) Peninsula; and the Italian-Balkan clade, subclade 1b (Davison et al. 2011), that includes the bears from Italy (Abruzzi and Trentino), Slovenia,

Bosnia, Croatia, Greece and Bulgaria. Following the model of contraction-expansion, Taberlet and Bouvet (1994) proposed that this phylogeographic pattern resulted from past episodes of population contraction towards the refugia areas in South European Peninsulas around the last glacial maximum (LGM) and the subsequent recolonization of the North that started from the refugia after the ice melted.

The East clade instead is formed by Russian and North Scandinavian bears and it expands through a wide geographical area from Eurasia to Alaska, showing more continuous and less structured populations than the ones includes in the Western clade (Davison et al. 2011). This clade was described by Leonard et al. (2000). Distribution of clades and subclades in Western Europe, fossil or current, are in Figure 1.

Ancient DNA phylogeographic pattern in fossil brown bears

Ancient DNA (aDNA) is strongly contributing to reconstruct the phylogeography of ancient populations of extinct (Stiller et al. 2014; Enk et al.

2016; Fortes et al. 2016) and extant species (Valdiosera et al. 2008; Rodríguez-Varela et al. 2015; Skoglund et al. 2015). In brown bears, the analysis of mitochondrial DNA (mtDNA) from Pleistocene individuals, revealed the presence of clade 1 in East Europe and suggested a lack of phylogeography in this species before the LGM (Hofreiter et al. 2004). Later on, Valdiosera et al. (2007, 2008) analysed the phylogenetic relationships of 21 ancient remains and 24 modern individuals of brown bears from Spain together with other ancient and modern samples from elsewhere in Europe. They concluded that clade 1 had a complex glacial and postglacial structure without a clear geographic distribution of the subclades 1a and 1b. Furthermore, they found clade 3 in central Europe, corroborating the absence of phylogeographic structure before the LGM. Interestingly, the Iberian Pleistocene bears in their data-set do not cluster directly with Iberian Holocene brown bears. From here on, we are referring to this lineage as Pleistocene clade 1.

Ancient DNA studies have also expanded the geographical range of occupation of clade 1, which has been found in museum samples from the North of Africa and the Lebanon (Calvignac et al. 2008, 2009).

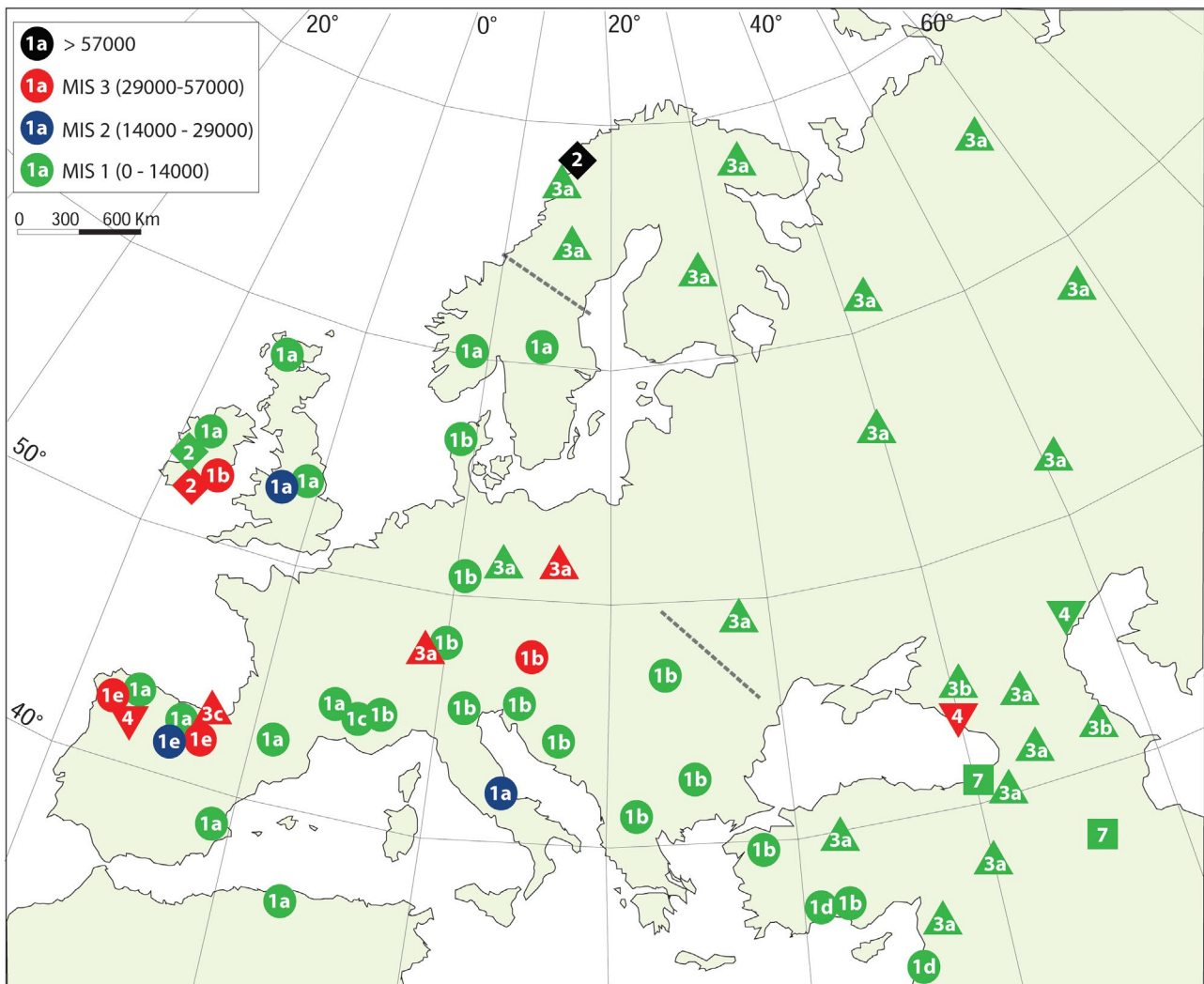


Figure 1. Geographic situation of brown bear clades in western Eurasia since Late Pleistocene to nowadays.

Notes: Circle – clade 1, diamond – clade 2, triangle – clade 3, inverted triangle – clade 4 and square – clade 7. Contact zones are represented by a dashed line. Time periods are from Lisiecki and Raymo (2005). References: Taberlet and Bouvet (1994), Taberlet et al. (1995), Leonard et al. (2000), Hofreiter et al. (2004), Valdiosera et al. (2007), Bon et al. (2008), Calvignac et al. (2008), Valdiosera et al. (2008), Calvignac et al. (2009), Korsten et al. (2009), Bray (2010), Murtskhaladze et al. (2010), Edwards et al. (2011), Münzel et al. (2011), Valdiosera et al. (2011), Bray et al. (2013), Baca et al. (2014), Edwards et al. (2014), García-Vázquez (2015), Xenikoudakis et al. (2015), Ashrafzadeh et al. (2016), Çilingir et al. (2016).

This lineage from the Lebanon is still present in modern populations from Turkey and has been renamed as subclade 1d (Çilingir et al. 2016).

A chronological timeframe for the occurrence of brown bear clades

New studies in recent years have provided a considerable amount of radiocarbon data from fossil brown bears in Western Europe. Some of these studies also include mtDNA analysis from these fossils, bringing together molecular and geographical data that can be clue to reconstruct the past population dynamics of this species.

Edwards et al. (2011, 2014) reported the ^{14}C dates of 27 fossil remains of brown bears from the British Isles together with mtDNA sequences from a total of 32 ancient specimens living before and after the LGM. Xenikoudakis et al. (2015) analysed 60 historical (nineteenth and twentieth centuries) and 47 contemporary remains of Scandinavian brown bears, providing new data on the population dynamics and geographical distribution of the subclade 1a. In the Iberian Peninsula, García-Vázquez et al. (2015) described the locations and ^{14}C dates of 26 Late Pleistocene and Holocene brown bears from Spain. Later on, some of these specimens were also analysed at mtDNA level in Fortes et al. (2016) and González-Fortes et al. (2017), providing 15 complete mitogenomes from middle Holocene samples and 26 short sequences from Pleistocene and Holocene bears respectively.

In the present study, we collected and studied together all the biomolecular data (^{14}C dates and mtDNA sequences) published up to date from fossil and modern brown bears and we generated new mtDNA sequences from modern and historical specimens from the Iberian Peninsula. By integrating the molecular data with the geographical location of paleontological remains of brown bears, we aim to elucidate the pre- and post-LGM phylo-geography of subclade 1a in Western Europe and to review the role of the Iberian Peninsula as a refugium for the brown bear.

Material and methods

New DNA sequences of modern bears from the Iberian Peninsula

In order to study the genetic legacy of the Pleistocene and ancient Holocene bears in the Iberian Peninsula we increased the data-set of genetic information by generating new mtDNA sequences from 11 Cantabrian and 3 Pyrenean samples of Spanish brown bears. Hair samples of 10 modern Cantabrian individuals were collected in the field by the Spanish NGO Fondo para la Protección de los Animales Salvajes (FAPAS) between the years 2007–2012. The other modern sample (individual Ua211) come from hairs from a skin seized from poachers. The museum samples were hair of brown bear from the Pyrenees preceding the introduction of Eastern European bears in the area, donated by the Museu de Ciències Naturals de Barcelona. Our sampling increased the number of individuals sequenced of the modern Cantabrian populations from 24 (Valdiosera et al. 2008) to 35 and the Pyrenees ones from 4 (Taberlet and Bouvet 1994) to 7.

Extraction and sequencing of modern biological samples was carried out by AllGenetics & Biology S.L. (<http://allgenetics.eu>).

All DNA isolations were carried out using the RealPure Microspin kit (Duviz) following the manufacturer's instructions, and resuspended in a final volume of 30 μL . A negative control was included in each round of extraction to check for contamination during the experiments.

Two regions of the mitochondrial DNA were amplified in the 12 samples of modern bears: a fragment of the cytochrome B flanked by the primers CbM1 (5' CTC ACA TGG AAT CTA ACC ATG AC) and CbMR2 (5' AGG GAA TAG TTT AAA TAG AAT TTC AGC) (Matsushashi et al. 1999); and a fragment of the control region covered by the primer pair Ua_CR5_F (5' AGA CCT ACT AAC ACT AAC AT) and Ua_CR5_R (5' TAA AAG ATA CCA AAT GCA TG) (AllGenetics desing). In the museum samples we expect the DNA to be degraded and broken into short fragments due to post mortem DNA damage (Pääbo et al. 2004; Sawyer et al. 2012), thus we use two primer pairs covering short overlapping fragments from which we reconstructed a total sequence of 135 bp. The primers used were URSUSF1 (5' CAG CAC CCA AAG CTA ATG TTC) and URSUSR1 (5' GCA CGA KMT ACA TAG GGG) (Valdiosera et al. 2007), and L16164 (5' GCC CCA TGC ATA TAA GCA TG) and H16299 (5' GGA GCG AGA AGA GGT ACA CGT) (Hänni et al. 1994).

In both ancient and modern samples, the PCRs were carried out in a final volume of 25 μL , containing 6.50 μL of Supreme NZYTaq Green PCR Master Mix (NZYTech), 0.5 μM of each primer, 25 ng of template DNA, and PCR-grade water up to 25 μL . The thermal cycling conditions were as follows: an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s; annealing at 49 °C (Ua CR5 primers); at 48 °C (CbM primers); or at 56 °C (for URSUSF1, URSUSR1, L16164 and L16164); extension at 72 °C for 45 s; and a final extension step at 72 °C for 5 min.

A negative control was included in every PCR round. PCR products were run on 1% agarose gels stained with Real Safe (Duviz), and imaged under UV light. All PCR products were purified using the PCRExtract Mini Kit (5 Prime) following the instructions provided by the manufacturer. All amplifications were replicated once and all PCR products were sequenced using both forward and reverse PCR primers on an ABI 3730XL (Applied Biosystems). Electropherogram analysis and sequence assembly was conducted in Geneious 7.1.5 (<http://www.geneious.com>). During electropherogram analysis, the primer annealing regions and the low quality regions at both ends of each electropherogram were trimmed (error probability limit of 0.03).

Data-set

To evaluate the phylogeography of Western European brown bears, we compiled a database of 132 published mitochondrial DNA sequences of bears belonging to subclade 1a that covered a wide geographic range, including the Italian, Iberian and Scandinavian Peninsulas, southeast France and the British Isles (Supplemental online material, Table S-1). The data-set include 60 modern, 38 historical (the last 2000 years) and 40 ancient brown bears. ^{14}C dates were available for 32 of these individuals and they range from Late Pleistocene to historical times.

We aligned published sequences to the ones generated in this study, compiling a data-set of 137 bears of subclade 1a (Table S-1). Our alignment includes 177 base

pairs (bp) of the mitochondrial genome between positions 16317–16378, 16461–16525, 16533–16582 in the reference sequence EU497665 (Bon et al. 2008). For convenience of the study, 6 sequences from subclade 1b (Taberlet and Bouvet 1994; Hofreiter et al. 2004) and 3 from subclade 1c (Valdiosera et al. 2007) were also included.

The alignment of mtDNA sequences was performed using MUSCLE algorithm in MEGA 6.06 (Tamura et al. 2013). Haplotypes were determined by DnaSP v5 (Librado and Rozas 2009). Aligned sequences were imported in FASTA format to the software PopART (available at <http://popart.otago.ac.nz>) where the genetic relationship was calculated by a median joining analysis (Bandelt et al. 1999) with default parameter (Epsilon = 0). For temporal analysis of subclade 1a, a network has been made using TempNet, an *R*-script (Prost and Anderson 2011).

Table 1. Mitochondrial DNA sequences obtained from hair of modern/historical brown bears from the Cantabrian Mountains and Pyrenees.

Lab number	Collection site	Population	Year	Sex	Bp	ADNmt region	Gen bank
MZB 82-7821	–	Pyrenees	1920	F	–	No DNA	–
MZB 82-7822	Caldes de Boí (Lleida)	Pyrenees	1919	M	–	No DNA	–
MZB 82-7388	Civís (Lleida)	Pyrenees	1922	F	135	CR	MF927781
F-1	Somiedo (Asturias)	Cantabrian	2010	–	381	CR	MF927782
F-2	Teverga (Asturias)	Cantabrian	2011	–	–	No DNA	–
Ua63	Teverga (Asturias)	Cantabrian	2009	M	867	CYB + CR	MF927783
Ua110	Proaza (Asturias)	Cantabrian	2007	–	986	CYB + CR	MF927784
Ua134	Proaza (Asturias)	Cantabrian	2009	F	1604	CYB + CR	MF927785
Ua136	Proaza (Asturias)	Cantabrian	2009	M	455	CR	MF927786
Ua141	Teverga (Asturias)	Cantabrian	2009	M	1636	CYB + CR	MF927787
Ua142	Proaza (Asturias)	Cantabrian	2009	F	–	No DNA	–
Ua151	Proaza (Asturias)	Cantabrian	2009	F	1635	CYB + CR	MF927788
Ua211	Somiedo (Asturias)	Cantabrian	1991	F	–	No DNA	–
Ua230	Piedras Lueñas (Palencia)	Cantabrian	2012	M	–	No DNA	–

Results

Modern samples sequences

Of the 14 modern/historical individuals analysed in this study, 8 produced DNA sequences, 4 sequences between 867 and 1636 bp (cytochrome B and control region) and 4 sequences between 135 and 455 bp (control region) (Table 1). The longest sequences belong to the modern Cantabrian haplotype, being all of them equal in the shared areas. The only sequence obtained from the Pyrenees (MZB 82-7388) is identical to the modern published sequences (X75878 from Taberlet and Bouvet 1994) in the area that both sequences overlap. The sequences reported in this paper have been deposited in the GenBank database (accession Nos. MF927781 – MF927788).

Mitochondrial haplotypes

Of all the samples sequenced in this paper, 5 (Ua63, Ua 134, Ua141, Ua151) shared the 177 bp region of the data-set. Modern Cantabrian samples of this paper were identical to those from Taberlet and Bouvet (1994) and Valdiosera et al. (2008). Valdiosera et al. (2008) included two modern Cantabrian bears (EU400205 and EU400202) that diverged on a single base, but those sequences contained also an unknown nucleotide each. Due to this uncertainty, we have not taken them into consideration in this analysis. Of the 137 samples included in this study, 28 different haplotypes for subclade 1a were obtained, as shown in the network in Figure 2.

Scandinavian and British brown bears form up the central part of the network, with the haplotype 15 being the central one. Haplotypes from bears of other geographical regions are located peripheral to this central Scandinavian-British group.

The Iberian bears fall in two different and distant clusters. The sequences from Iberian Pleistocene bears are separated from the Iberian Holocene haplotypes by the Scandinavian bears. It is worth to notice that the Iberian Pleistocene haplotypes are farther away from subclade 1a and 1b than both of them from each other.

The Iberian Holocene brown bears fell in two related groups. One of them is composed by 18 fossil individuals, all of them from sites in the Iberia North Corridor, the area situated between the Cantabrian mountains and the coast that acted as a faunal corridor during the LGM, when the sea level was lower and the glacial development prevented the fauna crossing the mountains further south, towards the Iberian plateau (Grandal-d'Anglade et al. 1997). All of them are of Holocene age, from 4836 ± 15 to $10,404 \pm 81$ years cal BP, but there are representatives of this group of haplotypes till 1523 ± 61 cal BP (Valdiosera et al. 2008), that were not included in the main data-set, because they are shorter, but can be seen in Online supplemental Table S-2. The haplotypes forming this cluster are not present in any other part of the Iberian Peninsula or outside it. The second set of haplotypes is formed by brown bears from Pyrenees, centre of the Iberian Peninsula and South East France, all of them younger than 2000 years BP (when age is known).

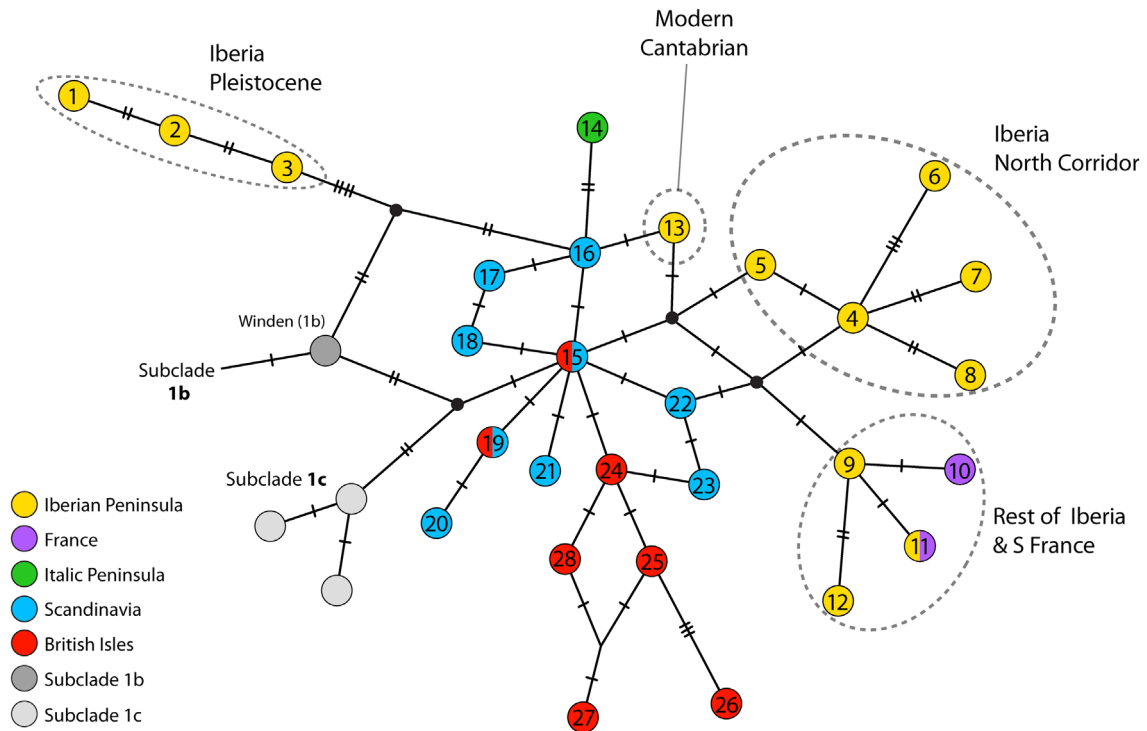


Figure 2. Network of the 177 bp data-set of subclade 1a mtDNA plus subclade 1b and 1c.

Chronology

An exhaustive survey in the literature produced a data-set of 100 direct radiocarbon ages of Western European brown bears and for 78 of these individuals mtDNA sequences were also available (Figure 3 and Table S-2). In Figure 3 we have represented the distribution of these samples over time, their geographic origin and their subclade assignment when genetic information was available.

Based on the ^{14}C dates and the geographic origin of the samples, towards the end of the Pleistocene and before the LGM, we observe the presence of brown bears in the British Isles (mainly in Ireland), Continental Western Europe and the Iberian Peninsula. The mtDNA sequences available from some of the Irish bears were assigned either to Clade 1b or to Clade 2, related to the polar bears (*Ursus maritimus* PHIPPS 1774). In the Iberian Peninsula, most of the Pleistocene bears belong to the Pleistocene clade 1, which is included within clade 1 but considerably distant at mtDNA level from the Holocene haplotypes reported in Iberian bears from more recent periods (as showed in Figure 2 and reported by Valdiosera et al. (2008)). Furthermore, two Pleistocene individuals from the Iberian Peninsula were found to belong to clades 3c (Valdiosera et al. 2008; Bray 2010) and clade 4 (García-Vázquez 2015).

Going forward on time and around the LGM, there are several Pleistocene fossils with radiometric dating from both Ireland and Great Britain (Jacobi 1980; Aldhouse-Green and Pettitt 1998; Jacobi and Higham 2008; Edwards et al. 2011), but there is not genetic information for all of those samples. The oldest radio-carbon age of British brown bears in the MIS 2 (14–29 ky BP) (Lisiecki and Raymo 2005) is from Goat's Hole cave (South Wales), and

dates back to $21,076 \pm 344$ cal BP (Aldhouse-Green and Pettitt 1998), but mitochondrial sequences are lacking. The same occurs with the scarce representation of mainland Pleistocene brown bears.

After the LGM, the oldest brown bear record ($17,381 \pm 402$ cal BP) is in Great Britain, then connected to the continent by substantial areas of the today North Sea and English Channel roughly between 20,000 and 15,000 years ago (Brooks et al. 2011; Clark et al. 2012; Sturt et al. 2013). Based on the genetic information available, clade 1b seems to have disappeared from the British Isles after the LGM, as all fossils analysed so far belongs either to clade 1a or to clade 2. Most of the dated subclade 1a bears are concentrated in the very Late Pleistocene and Early Holocene (between 15,000 and 10,000 BP). The oldest subclade 1a sample in Britain is from Kinsey Cave and dates back to $14,869 \pm 293$ cal BP, already in central England and this subclade persisted in the fossil record until around 2500 yBP (Barnes et al. 2002). Clade 2 instead was present in the British Isles since around 37,000 yBP until 9600 yBP.

In the Iberian Peninsula it seems to be a gap in the fossil record around the LGM, as only one sample has been dated to this period (around 21,000 cal BP). The oldest Iberian record after the LGM is $10,404 \pm 81$ cal BP, four thousand years later than in Great Britain and well after the Younger Dryas. Most of Iberian dates coincide with the Holocene climatic optimum (9000–5000 BP) (Railsback et al. 2011). Interestingly, all Iberian samples from Holocene times belong to clade 1a, which is genetically very distant from Pleistocene Clade 1, as showed in the network in Figure 2. Considering the intensive sampling of the fossil record in Iberia, the absence of the Pleistocene Clade 1 seems to indicate that these old maternal lineages did not persist in the Iberian Peninsula after the LGM.

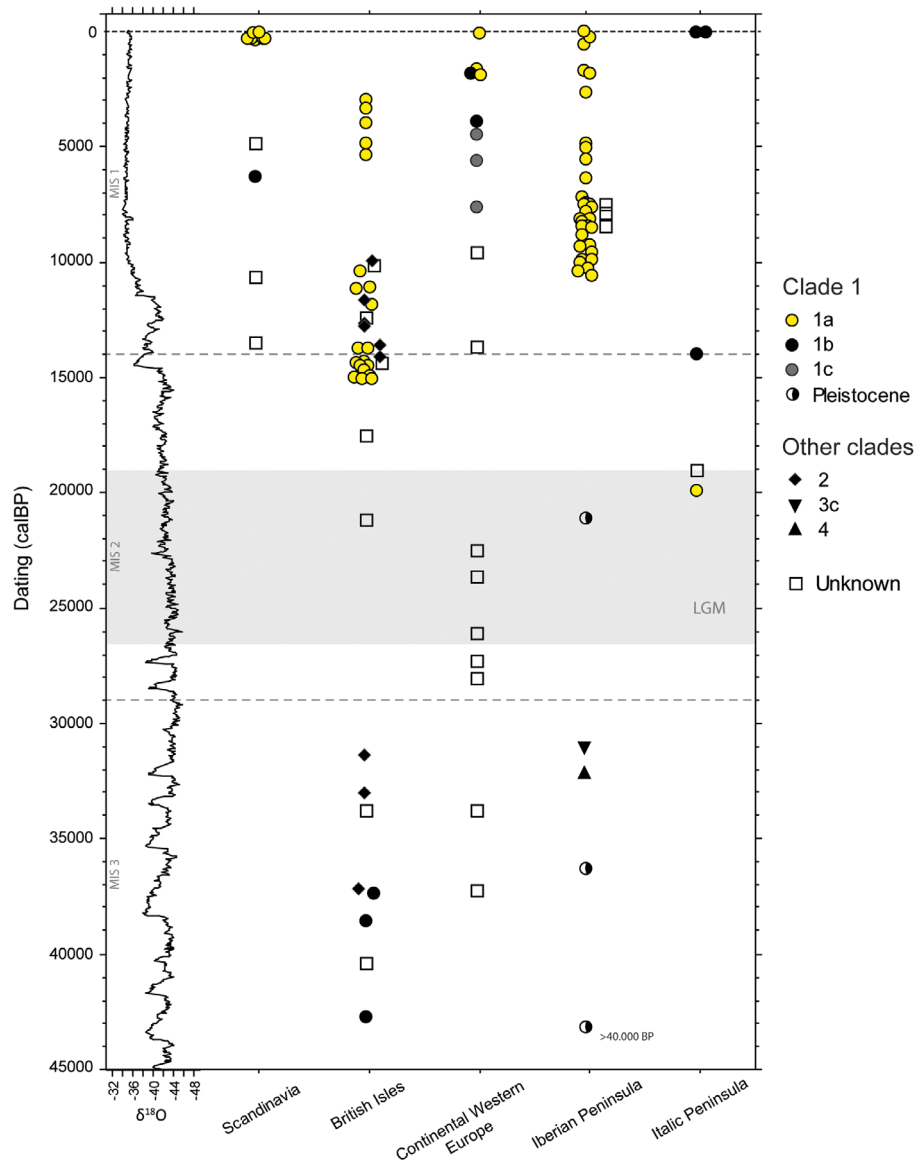


Figure 3. Presence of the different brown bear clades in Western Europe through time. Notes: Clade 1 – circle, clade 2 – diamond, clade 3 – inverted triangle, clade 4 – triangle. For climate and time information, $\delta^{18}\text{O}$ curve is added (Andersen et al. 2004). For more information, see supplementary Table S-2.

About other areas in Western Europe, there is a general lack of fossil information. In south Scandinavia there are some Holocene records, but only modern/historical bears have been analysed at mtDNA level and all of them belong to clade 1a (Xenikoudakis et al. 2015). Something similar happens in France and other areas from Continental Western Europe, where most of the fossil records date to post-LGM times and there is little information at mtDNA level. As reported in Figure 2 and Table S-2, the samples from these areas have been assigned to three different mitochondrial subclades: 1c, 1b, and 1a. Finally, from the Italian Peninsula there is only radiocarbon date information from three fossil samples of brown bears, two of them have been analysed at genetic level (Valdiosera et al. 2007; Bray 2010). The oldest sample from this region (dated around 20,000 y BP) belongs to clade 1a, while all the post-LGM samples belong to clade 1b.

The Clade 1a through space and time

In order to investigate the temporal and geographical distribution of the maternal lineages of brown bear in Western Europe, we have reconstructed several networks, one for each MIS period plus historical times, showing which haplotypes were present in Europe at each time period.

The Figure 4 clearly shows that the Pleistocene haplotypes of the Iberian Peninsula have no continuity in MIS 1. No representative of this clade was found after the LGM either in Northern Iberia or in the rest of Europe.

The first individuals in subclade 1a date from MIS 2 were found in Abruzzi, Italy (Grotta Beatrice Beatrice Cenci, $19,747 \pm 65$ cal BP) and then in North Yorkshire, Great Britain (haplotype 24 in Kinsley cave, $14,869 \pm 293$ cal BP and haplotype 15 in Victoria cave, $14,322 \pm 262$ cal BP, Edwards et al. 2014). From haplotype 24 derived the more recent Holocene bears from Ireland, while haplotype 15 persists in the British Isles during

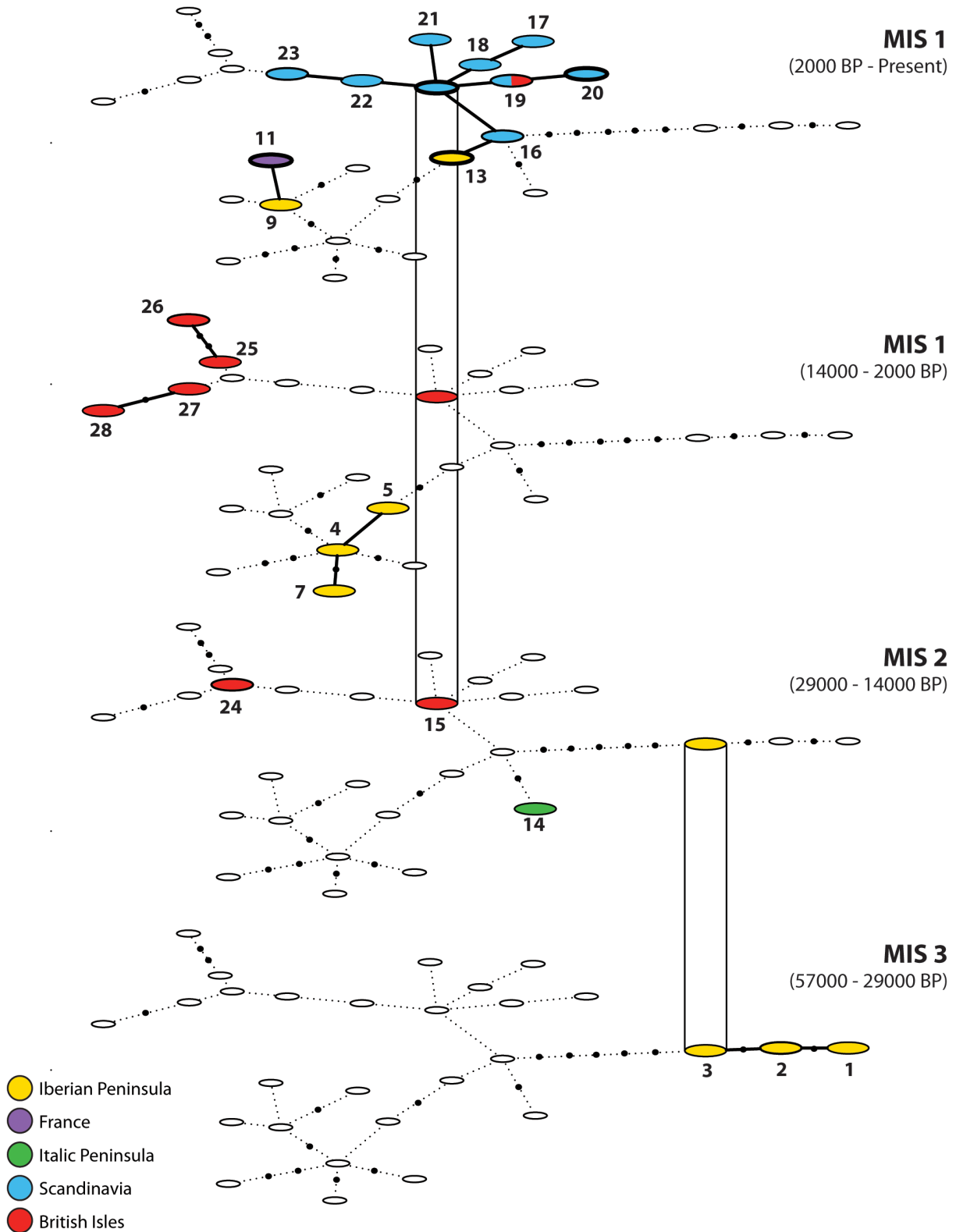


Figure 4. Network of subclade 1a from MIS 3 to nowadays.
Notes: Modern haplotypes are in bold line.

MIS 1 and is found in historical times in Southern Scandinavia (Taberlet and Bouvet 1994; Xenikoudakis et al. 2015), where it is greatly diversified.

The absence of fossil samples from the early Pleistocene or Holocene of Scandinavia precludes the interpretation of the history of brown bear

diversification in this area of northern Europe. However, there is no indication that the Iberian bears, neither the Pleistocene nor the Holocene ones, were directly related to the bears that colonized the British Isles and southern Scandinavia after the LGM. In the North of the Iberian Peninsula,

clade 1a bears appear for the first time during MIS 1 occupying peripheral positions in the network, clearly distant from the Iberian Pleistocene bears and from haplotype 15, the central one in the network and continuous along the post-glacial stage.

Discussion

Western European brown bear dynamics after LGM

British Isles

All pre-LGM brown bear remains from the British Isles that have been analysed at genetic level belong to the subclades 1b and 2. Subclade 1b did not persist during the MIS 2, while clade 2 bears reappeared in the Holocene in Ireland (Edwards et al. 2011). The Irish clade 2 bears probably did not survive in a proposed refugium in southwest Ireland (Leonard et al. 2013), although Montgomery et al. (2014) proposed that they persisted during the LGM in cryptic refugia, probably on exposed areas of the Celtic Sea shelf, from where they re-entered Ireland when suitable areas were available.

Irish clade 2 bears could have hybridized with polar bears and in fact, their isotope signatures show a high input of protein in their diet, although not solely a marine protein input (Edwards et al. 2011). If these bears were hybrids of brown and polar bears, they could have found suitable habitats in these coastal areas. Between approximately 20,000–15,000 years BP, the Celtic Sea region was an archipelago of small islands that were surrounded by very shallow (<20 m) waters. An extensive sea ice cover would have flanked these ice sheets, and many of the small islands present in the Celtic Sea are likely to have become connected (Brooks et al. 2011). In such a scenario it would not be rare that clade 2 brown bears could have reached southern latitudes during the coldest phases. We have not found genetic data of brown bears in the Bay of Biscay area (Aquitaine, Basque Country), but the intriguing cave art in Ekain cave (Guipuzcoa, Basque Country) depicting two bears with a body profile resembling that of polar bear (Altuna and Barandiarán 1969; Figures 51 and 52) could actually represent polar bears or their hybrids. Another possible polar bear representation comes from Isturitz cave in the northern flank of the Pyrenees (Atlantic Pyrenees, Aquitaine). It is a Magdalenian portable art piece carved on a large bovid rib showing two individuals that somehow resemble polar bears (Esparza San Juan and Mujika Alustiza 2003, Figure 1).

Postglacial brown bears inhabiting Britain belong to subclade 1a. In fact, according to radiocarbon dates, Britain was the first area occupied by this subclade after the LGM. The origin of those bears is unknown; if they were traditionally supposed to come from the Iberian refugium (Hewitt 1999), the current data, both chronological and genetic, make this hypothesis unrealistic. Barriers such as the Pyrenees or the Fleuve Manche would have acted as a barrier for the expansion of the Iberian bears, as suggested by Montgomery et al. (2014), while the extensive emerged continental shelf of Doggerland would allow an easier way between Britain and North France, Belgium or the Netherlands.

Scandinavia

Modern populations from southern Scandinavia show only two haplotypes, one in the Norwegian population, and another in the Swedish one (Taberlet and

Bouvet 1994; Kohn et al. 1995) due to a recent bottleneck (Xenikoudakis et al. 2015). Subfossil or historical individuals (nineteenth and twentieth century) show more genetic diversity, sharing two haplotypes with the Britain postglacial bears.

There are only 2 direct dated fossils from south Scandinavia and one indirectly dated from Denmark. The oldest one ($13,330 \pm 190$ cal BP) is from Nørre Lyngby, in Jutland Peninsula (Denmark) (Bondesen and Lykke-Andersen 1978; Aaris-Sørensen and Petersen 1984) and represent and early colonization at the Late Glacial. The oldest date from south Sweden is 10,500 BP (Iregren et al. 1990). These bears record the expansion into ice-free areas as soon as favourable habitats were established (Aaris-Sørensen 1992). However, there is not genetic data from these ancient samples.

The colonization of Scandinavia by Clade 1a brown bears likely happened via Jutland and Southern Scandinavia. The Yoldia Sea, precursor of the current Baltic, separated Southern Scandinavia from North Scandinavia. The lithostatic discharge caused by the gradual melting of the ice cap caused lithosphere uprising, which resulted in a land bridge between today Denmark and today Southern Sweden. This bridge lasted from 9500 to 7500 BP, a time lapse during which brown bears – and other mammals too – colonized Southern Scandinavia (Andersen and Borns 1994; Figure 2–6.7). In the absence of data from bears, human remains support this pathway colonization in southern Norway, dating from between 10,000 and 9000 years (Gjørstad 2014).

However, remnants of ice sheet were still isolating the south and the north of the Scandinavian Peninsula. This barrier must have prevented subclade 1a bears to expand to northern Scandinavia for millennia. Later, when the ice disappeared (Andersen and Borns 1994; Figure 6.2–6.9), the North was already colonized by bears of clade 3 from Eastern Europe. The suture zone between both clades is still present (Taberlet et al. 1995).

Iberian Peninsula

As mentioned previously, among the 8 Iberian Pleistocene bears with genetic data there is no putative ancestor of the bears that populated the region after LGM. The old Pleistocene clade 1 did not persist in the Iberian Peninsula after the LGM. Furthermore, two Pleistocene individuals from the Iberian Peninsula were found to belong to clades 3c (Valdiosera et al. 2008; Bray 2010) and clade 4 (García-Vázquez 2015), respectively. No other representatives of these clades in the Iberian Peninsula or in Western Europe are known, neither fossil nor alive. Clade 3c has only been found in fossil bears from North America (Barnes et al. 2002). Clade 4 is present nowadays in south Canada and contiguous United States in North America, and the nearest members of this clade in Europe are historical individuals from Caucasus (Bray 2010). Valdiosera et al. (2008) suggested that the presence of Clade 3c in Iberia demonstrated a lack of phylogeographic structure in preglacial brown bears. But according to their chronology, the individuals of foreign clades (3c and 4) reached the Peninsula during a cold pulse, towards the end of MIS 3. Based on the genetic information available from fossil and modern individuals, it seems that these two individuals represent a minor population of vagrant bears that expanded westwards, instead eastwards, with the expansion of the ice sheets towards the end of MIS 3.

The effective population size of both lineages would have been small, since finally they faded out without contributing to the gene pool of the Iberian bears, neither in the Holocene populations nor in the present day Iberian bears.

First postglacial records in North Iberia are dated around 5000 years later than those from the British Isles (Jacobi 1980; Barton 1999; Edwards et al. 2011, 2014). These radiocarbon data and also genetic evidence suggest a late entry in the Peninsula respecting the early recolonization of the British Isles. After an extensive survey in the literature, and sampling effort for finding samples for radiocarbon dating (García-Vázquez 2015), none of the 29 brown bear dated samples from North Iberia can be assigned to the early postglacial epoch, with a long gap from 20,900 BP until ca. 10,000 BP, well after the Younger Dryas. We could argue that this hiatus is an artefact caused by the almost total absence of direct dated samples in the many sites of Cantabria and the Basque Country, that is the natural entrance of the North Corridor. In fact, the intense archaeological studies in the East Cantabrian area yielded up to 6 sites with brown bear remains (with a scarce minimum number of individuals, if more than one) stratigraphically related to Solutrean and Magdalenian contexts, 4 of them in the Basque Country and 2 in Cantabria (for a review, see García-Vázquez et al. 2015). However, up to 40 sites in the same area did not yield any Solutrean or Magdalenian bear remain, but related to older or younger archaeological contexts. While it would be desirable to have more direct dated samples from this region, it does not seem possible that hypothetic early incoming bears had concentrated exclusively in that area, since no geographical or climatic barrier would prevent them to extend westwards along the Northern Corridor.

This late entry has several possible explanations. On one hand, the continental shelf of the margin of Iberian Bay of Biscay is narrow, constrained by the Capbreton canyon. In addition, the proximity of the Atlantic Pyrenees to the coast means a barrier for the bears to entry in the peninsula. Studies on glacial forms and deposits, and marine and continental pollen records (as reviewed in Jalut et al. 2010) points to an early local glacial maximum in the Iberian Peninsula, preceding the global LGM. In the most important glacial basins of the Pyrenean–Cantabrian range, glaciers were still present at middle elevation ca. 18–20 kiloyears (ky). The final deglaciation did not start until around 16 ky, and at that time there was still ice around 1300 m a.s.l. (Jalut et al. 2010). Several phases of advance and retreat of glaciers occurred, leading to a rapid early Holocene deglaciation between 11.7 and 10.1 cal ky BP (Jambrina-Enríquez et al. 2014).

During this time, the marine transgression would be dismantling the continental shelf exposed during the maximum glacial advance, while the recovery of vegetation in the mountain area after the melting of glaciers must have been delayed due to the step topography of the area, with an extended post-deglacial evolution of mountain slopes and recurrent climatic fluctuations. In addition to this, the most favourable areas of the Cantabrian coast, at low altitude or on valley bottoms, were already occupied by the human groups that inhabited the region densely and continuously (Straus et al. 2000), this being perhaps more decisive cause to prevent the expansion of the bears across the region. Despite their great ecological plasticity, which is an advantage over the obligate herbivores, bears need to hibernate for several months, so they are more prone to disturbance by human

groups (Swenson et al. 1997; Linnell et al. 2000; Petram et al. 2004; Elfström et al. 2008). According to this hypothesis, only when the mountain areas were habitable again, with a suitable and well-developed vegetation cover, bears entered the Peninsula, preferably occupying the highest areas. In fact, the oldest Cantabrian records (around 10 ky cal BP) are already in caves at high altitude above sea level. More to the northwest, in Galicia, where the Holocene brown bear remains are numerous and human settlements are scarce, we observed a rapid colonization from the lowest to the highest caves between 9000 and 8000 years Cal BP (García-Vázquez et al. 2011; García-Vázquez 2015).

Throughout the Holocene, the record of brown bears in the N of the Iberian Peninsula become scarcer, probably in response to the anthropic pressure culminating in the almost total extinction of the species. Brown bears began to reduce their population drastically since the appearance of firearms in sixteenth century, from south to north. Between 0.4 and 0.3 ky ago, Cantabrian and Pyrenean populations split up (Nores and Naves 1993). Cantabrian population continued to decline till the decade of 1950, when two separated subpopulations were established (Nores and Naves 1993) with a total of 40 individuals (Couturier 1954). In that decade, Naves and Palomero (1993) describe a re-colonization in some councils of Asturias (NW of Spain).

All Cantabrian modern bears (the oldest living in the 1960s, Valdiosera et al. 2008) share the same haplotype, both the ones sequenced in this work and those previously published (Valdiosera et al. 2008). This haplotype is missing in the Holocene samples. This is the result of a strong bottleneck caused by the anthropic pressure, like in Scandinavia in the last 200 years (Xenikoudakis et al. 2015), but for the moment it is not possible to know if the modern haplotype was just not found in the Holocene samples, or if it arrived more recently from the central or south Iberian Peninsula. For instance, a small population of brown bears was recorded in León and Zamora (Spain), near Portugal boundary, at least until the 80s (Grande del Brío et al. 2002). Such small populations could had reoccupied the Cantabrian Mountains in recent times and contribute genetically to modern Cantabrian population. This case of sub-refugia into the refugia was also proposed for the water vole *Arvicola sapidus* (Centeno-Cuadros et al. 2009) or for the roe deer *Capreolus capreolus* (Royo et al. 2007) in the Iberian Peninsula.

In the mid-nineteenth century the brown bear was distributed throughout the Pyrenees, but in the early twentieth century had three nuclei with sporadic appearances including: Western, Central and Eastern (Alonso and Toldra 1993; Caussimont et al. 1993). In the 50's of the twentieth century, the population consisted of 70 animals (Couturier 1954), but in 1991 and there were only 11 bears in the western nucleus and 1 or 2 in the central (Caussimont et al. 1993). The Pyrenean haplotype sequenced by Taberlet and Bouvet (1994), has been identified in this paper in another individual of nearly 100 years old. This haplotype belongs to a group formed by individuals more recent than 2000 years old, however, not all individuals in the group are dated, so, coupled with the lack of data from other parts of the Peninsula southwards from the North Corridor, it hinders the phylogeographic interpretation of this haplotypes.

France

Most of the brown bear remains around the LGM period (Figure 3) come from South and South East of France, but unfortunately there is not genetic data from the French brown bears until mid-dle Holocene. It is remarkable the presence of brown bear remains in Oilascoa cave, in the Atlantic Pyrenees (France), whose date ($22,400 \pm 545$ cal BP) places it in the LGM (Clot and Duranthon 1990). This occurrence gives evidence that brown bears were present in SW France, at the gates of the Iberian Peninsula, during the cold pulse. Also in the western part of the continent, but further north, a direct dated brown bear ($37,125 \pm 710$ cal BP) is recorded in Goyet cave (Belgium) (Naito et al. 2016). None of these bears have been sequenced, thus precluding their interpretation, but their presence suggest that these areas could have been a central point for the expansion towards the Iberian Peninsula, the British Isles and Scandinavia when the climatic conditions became favourable.

At genetic level, the best known site in France is Mont Ventoux (South West France), but the brown bear remains from this site are quite recent, dating from the mid Holocene to historical periods (Valdiosera et al. 2007). There is a succession of different haplotypes/subclades in Mont Ventoux over time, which points to this area as a confluence zone of different migrations. The oldest remains from this site (from 7434 ± 50 to 4276 ± 85) are the only representatives of subclade 1c until now, even if subsequent analysis have related these 1c haplotypes with post-glacial individuals from subclade 1b (Bray 2010). Individuals assigned to subclade 1b have been identified in this site and dated around 3730 ± 72 y Cal BP (Valdiosera et al. 2007). Finally, also subclade 1a appears in this area around 1700 years ago.

Italian Peninsula

Genetic data from Italian bears are scarce. Italian subclade 1a bear is from about $19,747 \pm 293$ cal BP, and is the only Pleistocene bear from Italy sequenced. The next one is postglacial ($13,784 \pm 161$ cal BP) and is from subclade 1b, like nowadays Italian bears (Taberlet and Bouvet 1994; Bray 2010). Preglacial 1a bears could be replaced by subclade 1b bears after the LGM, or could have been lost more recently, during the population decline. However, only two cases are not enough for a reliable reconstruction of the brown bear dynamics in this peninsula.

So where was the refugium, if any?

After the LGM the first brown bears remains belonging to sub-clade 1a are from the British Isles (Figure 3). Their haplotypes are situated in the central part of the network, as well as the modern Scandinavian ones (Figure 2). However, it is difficult to assume that Britain or Scandinavia could act as a refugium area during the LGM, since they were practically covered by the ice sheet, and this assumption would violate directly the theory of the southern glacial refugia.

Sommer and Nadachowski (2006) analysed 47 LGM archaeological sites from Europe with several temperate mammal species, concluding that their range showed a distribution that differs from the hypothesized traditional refugia areas of the temperate fauna during the LGM. The authors propose Dordogne in SW France as glacial refugia besides the Iberian Peninsula (North and Portugal) for Western Europe.

Despite the Iberian peninsula is traditionally considered a glacial refugium, and that the model works for some large mammals such as red deer (Meiri et al. 2013) and even some human mitochondrial haplogroups (Soares et al. 2010), neither the genetic data nor the chronology support the model, at least for the northern Iberian Peninsula, for the brown bears.

All these data allow us to hypothesize that the glacial refugia would not be located in the Iberian Peninsula, but somewhere of the Atlantic continental Europe (probably present-day France), where populations would expand as climate warmed, reaching first the British Isles, and eventually, through the emerged North Sea continental shelf (Doggerland), Jutland and South Scandinavia. The entrance in North Iberia would be retarded by the causes already described: a too narrow corridor between France and the Cantabrian mountains, besides the high density of humans that occupied the most favourable areas. However, with the current data we cannot propose a specific area as the main refugium. Mont Ventoux bears in the SE of France show high genetic variability, but are too recent, and the large number of haplotypes recorded seems to respond better to a secondary contact following postglacial recolonization.

Another possibility is that the western brown bears were not restricted to a particular refugium during the LGM. In fact, the maximum advance of glaciers was not contemporary in all regions (Jalut et al. 2010; Cossart et al. 2012; Darnault et al. 2012; Jambrina-Enríquez et al. 2014), nor was the post-deglacial development of mountain slopes and vegetal recolonization. If local LGM in Britain and the Cantabrian-Pyrenees system preceded the global LGM, as was postulated (Jalut et al. 2010; Clark et al. 2012), the brown bear populations could have been moving from one region (mainland Western Europe) to another (Britain first, North Iberia later) following the different deglaciation timing and onset of available ecospace.

Conclusions

After an exhaustive compilation of available genetic and radiometric data from Western European brown bears, and adding new sequences of current bears from the Iberian Peninsula, we try to reconstruct the postglacial dynamics of this species in West Europe, mainly in the Iberian Peninsula.

In the north of the Iberian Peninsula a variety of haplotypes is observed during the Pleistocene period. This could be the natural situation, as it is nowadays in Russia. However, two of these haplotypes are represented by only one individual each one and both of them are unknown in Europe, both in present-day bears and fossils. Their chronology coincides with the cooling towards the end of MIS 3, suggesting in our opinion the occasional presence of small vagrant populations, rather than the existence of a continuous population from which no descendants remain. It would be necessary to obtain more mitochondrial sequences of bears from other regions in Western Europe to clarify if the presence of these haplotypes is casual or, on the contrary, other representatives were not found due to the scarcity of data.

However, there is no continuity of any of the Pleistocene lineages in the Holocene of the Iberian Peninsula. All defined haplotypes were extirpated during the LGM at least in the North. After a gap in the record that coincides with the LGM, radiometric dating shows a sudden abundance of Cantabrian bears that we interpret as a late recolonization of the peninsula

with respect to Britain. In Britain the same pattern of sudden abundance is observed coinciding with the melting of the glacier caps, about 15,000 BP. In Iberia, this expansion occurred after the Younger Dryas.

The Iberian Peninsula was considered traditionally as a glacial refugium for large mammals and humans, but with the available data, neither the ¹⁴C ages nor the mtDNA data support an Iberian refugium for the brown bears during the LGM, at least for those that recolonized the British Isles and south Scandinavia. We therefore propose the existence of a cryptic refuge in continental Atlantic Europe, from where the bears would expand as the ice receded. The delay in the recolonization of the Iberian Peninsula could be due to the orographic characteristics of the Pyrenean-Cantabrian region and to the abundant presence of humans in the natural entrance to the Peninsula.

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