MITOCHONDRIAL DNA ANALYSIS OF THE EVOLUTION AND GENETIC DIVERSITY OF ANCIENT AND EXTINCT BEARS

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THESIS DECLARATION

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THESIS ABSTRACT

Mitochondrial phylogeographic analyses of modern populations can be used to make inferences about the impacts of the last glacial maximum (LGM) and anthropogenic disruption on late Pleistocene and Holocene ancestral populations. However, it is becoming more and more evident that ancient DNA studies greatly augment traditional mtDNA studies based only on extant lineages, and can reveal more complex scenarios than those hypothesised from modern data alone.

Ancient DNA studies allow us to trace historic and ancient gene flow through time, giving a dynamic temporal and geographic understanding of genetic diversity. This is particularly informative when molecular data can be coupled with environmental or chronological information (such as radiocarbon dated specimens) allowing links to be made between climatic or anthropogenic disruptions and the genetic response of populations or species.

This PhD research used ancient DNA techniques to investigate a number of biogeographic scenarios in relation to the phylogeography of brown bears (*Ursus arctos*) in Europe (Chapter 2- 4) and across the Eurasian continent (Chapter 5) throughout the Late Pleistocene and Holocene periods. Similarly, a study of the genetic diversity and phylogeography of the extinct giant short-faced bear (*Arctodus simus*) in North America was undertaken (Chapter 6) to provide a comparison with the dynamic phylogeographic history of contemporaneous Beringian brown bears (Barnes *et al.*, 2002). Additionally, the deeper evolutionary history of the extinct Tremarctine bears was investigated using a molecular approach (Chapter 7) in an attempt to clarify the phylogenetic relationships of this lineage which have remained unresolved by morphological analyses.

The research presented in this PhD thesis reinforces the important role that ancient DNA can play in understanding the mtDNA population dynamics and movements of taxa in response to environmental or anthropogenic changes through time. It stresses once again that the use of modern data alone is likely to lead to over-simplified or inaccurate views of past evolutionary history. Ancient DNA studies such as those presented here allow us to develop a more complex understanding of Quaternary phylogeographic patterns in a small number of taxa for which a sufficient number of samples can be obtained, and may guide future research to determine if similar patterns exist for other less-well studied species.

Barnes I, Matheus P, Shapiro B, Jensen D, Cooper A (2002) Dynamics of Pleistocene Population Extinctions in Beringian Brown Bears. *Science* **295**, 2267-2270.

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CHAPTER 1
INTRODUCTION

1.1 THE ROLE OF QUATERNARY CLIMATE FLUCTUATIONS & HUMANS ON GLOBAL BIODIVERSITY

It is now increasingly evident from fossil, palaeoecological and genetic data that Quaternary climate fluctuations played an important role in shaping the evolution and distribution of a diverse range of animal and plant taxa around the globe (Hewitt, 2000; Jansson, Dynesius, 2002). More recently, global biodiversity has additionally been influenced by a variety of anthropogenic factors such as habitat fragmentation, resource competition and direct hunting (Chapin *et al.*, 2000). However, the effects of climate change and humans on individual species can be difficult to disentangle, with changes to population size, distribution, and genetic diversity occurring through time.

Quaternary climate change impacted biodiversity by altering the global temperature and sea levels which subsequently caused shifts in habitat and environmental niches. For example, during the Pleistocene fluctuations of up to 15 ºC could occur in as little as a few decades (Rahmstorf, 2002), resulting in temperatures during glacial maxima as much as 21 ºC cooler than the current climate (Cuffey *et al.*, 1995). These extreme changes in temperature were also associated with fluctuations in sea levels due to the large volume of water involved in the formation of glaciers and polar ice caps. The formation of vast ice sheets such as the Laurentide and Cordilleran in North America and the Fenno-Scandian and Alpine in Europe during periods of glacial maxima resulted in sea level drops of as great as 120 m below current levels (Arnold *et al.*, 2002; Cuffey *et al.*, 1995; Rahmstorf, 2002). These lower sea levels allowed land-bridges to form, connecting regions that would be separated by water at warmer stages of the glacial cycle for example the Bering land bridge (Beringia) linking Eurasia with North America, or those joining the British Isles and the southern tip of Scandinavia with the rest of the European continent.

In response to the changing climate and environmental conditions, it is believed that many species attempted to track their suitable habitat (Eldredge, 1989), resulting in dynamic range contractions and expansions through time and across the landscape during the Quaternary glacial cycles. Species or populations unable to track suitable habitat needed to be able to adapt to their new environment or faced extinction, as has recently been demonstrated for the arctic fox (Dalen *et al.*, 2007) in northern Europe.

Changes in sea levels and the formation and subsidence of land-bridges have been recognised to have had important effects on species distributions around the globe not only throughout the Pleistocene but over millions of years. A key example is the formation of the Panamanian Isthmus which occurred approximately three million years ago, linking the North and South American continents for the first time and resulting in the exchange of a large number of previously isolated taxa in what is described as the Great American Biotic Interchange – GABI (Webb, 1976).

In Europe, a glacial refugial model has been developed based on the modern distribution and genetic diversity of a number of key taxa (Hewitt, 1996; Hewitt, 1999). This model suggests that during periods of glacial maximum the range of temperate European species contracted

southwards leading to restriction in one or more of the Mediterranean peninsula refugia (Iberia, and Italy/Balkans). In inter-glacial (warmer) periods the model proposes these refugial species expanded northwards, resulting in a genetic diversity gradient where populations from refugial areas are more diverse than those at the leading edge of the expansion. This increased genetic diversity in the southern peninsulas is presumed to result from the persistence of particular haplogroups in refugial areas during multiple glacial maxima, allowing time for accumulation of genetic variation (Hewitt, 2001). The northern regions of Europe are believed to display lower genetic diversity due to being a subset of the refugial genepool, the rapid nature of the subsequent recolonisation and sequential founder effects from the southern refugia during the interglacial periods (Hewitt, 1996).

While there is no doubt that climatic and environmental change had a huge impact on species distribution and genetic diversity throughout the Quaternary, an unprecedented mass megafaunal extinction (Alroy, 1999) occurred during the last glacial cycle. This resulted in more than 60 % of vertebrate land animals greater than 44 kg becoming extinct by the end of the Pleistocene (Barnosky *et al.*, 2004). A key question therefore remains unresolved - what was different about the conditions in the last glacial cycle as opposed to those preceding it that resulted in the mass megafaunal extinctions? The causes of the megafaunal extinctions are still hotly debated, although most researchers either believe that they were caused by climate change or anthropogenic factors or a combined effect of both climate change and human hunting (Alroy, 2001; Barnosky *et al.*, 2004; Brook, Bowman, 2002; Koch, Barnosky, 2006). Others have suggested that catastrophic meteorite impacts (Firestone *et al.*, 2007; Haynes, 2008) played a crucial role.

Was the arrival and expansion of human populations the critical factor in the equation, tipping the balance for a number of taxa already hit by the climatic and environmental fluctuations? In many island environments, humans undoubtedly mediated the extinction of a whole suite of animals and birds, for example the giant ratite Moa in New Zealand (Holdaway, Jacomb, 2000). In Australia evidence is also mounting to suggest human involvement in the extinction of the megafauna (Brook *et al.*, 2007; Gillespie, 2008). However, in North America and Europe it remains much harder to disentangle the timing and effects of human expansion and climate change (Hofreiter, Stewart, 2009). Evidence is emerging that rather than being a single mass extinction ‘event’, different populations and species were affected at different times, leading to a staggered range of extinctions, difficult to correlate with any single climatic or anthropogenic event. For example American hemionid horses are believed to have gone extinct as early as >30,000 years ago (Guthrie, 2003) before the LGM, similarly European cave bears died out following a protracted genetic decline approximately 25,000 years ago (Pacher, Stuart, 2009; Stiller *et al.*, 2010), while other megafaunal species such as mammoths survived up until 4,000-6,000 years ago in some island refuges (Enk *et al.*, 2009; Guthrie, 2004; Vartanyan *et al.*, 1993). Periods of population turnover and demographic declines pre-dating the LGM and the arrival and expansion of humans have now been identified for a number of North American taxa, for example late Pleistocene horses, bison and mammoth from Beringia (Debruyne *et al.*, 2008; Guthrie, 2003; Shapiro *et al.*, 2004). This may suggest that factors pre-dating both the LGM and the arrival and expansion of humans contributed to the genetic decline and subsequent extinction of a number of the megafauna in Eurasia and North America.

In more recent times (from the start of the Holocene period 10,000 ybp to the present), there are clear examples where humans have been instrumental in altering the distribution and genetic diversity of particular species thus determining their ultimate survival and expansion or decline and extinction. A recent genetic study of New Zealand penguin species provides a good example of human-mediated extinction of one species which appears to have allowed

the subsequent range expansion and survival of a sister-species, the endangered yellow-eyed penguin (Boessenkool *et al.*, 2009).

1.2 USING GENETICS TO STUDY THE EFFECTS OF CLIMATE CHANGE & HUMAN IMPACTS IN THE QUATERNARY

The term 'phylogeography' was coined in the late 1980s (Avise *et al.*, 1987), and involves studying the geographical distribution of genealogical lineages, usually at the intra-specific level, and frequently utilising mitochondrial DNA sequences (mtDNA). The relatively rapid rate of mitochondrial evolution (compared to the nuclear genome) combined with its (essentially) non-recombining maternal mode of inheritance often makes it ideally suited to intra-specific phylogeographic studies spanning the Quaternary period (Avise *et al.*, 1998; Hewitt, 2001), allowing mtDNA studies to dominate the field.

Mitochondrial phylogeographic analyses of modern populations can be used to make inferences about and test the impacts of the last glacial maximum (LGM) and anthropogenic disruption on late Pleistocene and Holocene ancestral populations. For example, a recent mtDNA study investigated the effects of the last glacial cycle and human colonisation of Mediterranean islands during the Holocene on the phylogeographic distribution of modern European weasels (Lebarbenchon *et al.*, 2010). This study found evidence of a phylogeographic structure consistent with Pleistocene glacial refugia, but also found support for the suggestion that weasels colonised at least four Mediterranean islands through Holocene human intervention rather than in response to Pleistocene glacial cycles.

Mitochondrial DNA phylogeographic studies of a number of extant taxa have been used to suggest the location of the previously mentioned southern European glacial refugia in the Iberian (Spanish), Italian and Balkan peninsulas (Hewitt, 2001; Hewitt, 1996; Hewitt, 1999) and to infer subsequent post-glacial colonisation routes. Many species have current distributions and phylogeographic structuring consistent with those of the three paradigm species (the grasshopper, hedgehog and brown bear) used by Hewitt to develop the expansion/contraction (E/C) model of European interglacial-glacial phylogeographic patterns (Figure 1.1). In general, the range of many temperate species appear to have been restricted to the southern peninsulas during one or more glacial periods, allowing genetic diversity to be preserved and over time, accumulate, in refuge areas. In central and northern regions of Europe temperate species frequently possess lower levels of mtDNA diversity than found in the southern refugia. This pattern of decreased genetic diversity in the north is thought to be a result of rapid post-glacial recolonisation from one or more refugia and repeated founder events during interglacial periods (Hewitt, 2004). Hybrid zones or contact zones between different mtDNA lineages can result when haplotypes originating from more than one refugial location are united during the recolonisation process (Hewitt, 2004). For example Scandinavia appears to have been recolonised from the south by brown bears from the Iberian peninsula (Clade 1) and from Russia in the north (Clade 3a) with the two lineages forming a contact zone where they meet in central Scandinavia (Taberlet *et al.*, 1995). Studying mtDNA haplotype diversity can thus be used to deduct plausible hypotheses for the presence of glacial refugia and post-glacial recolonisation routes.

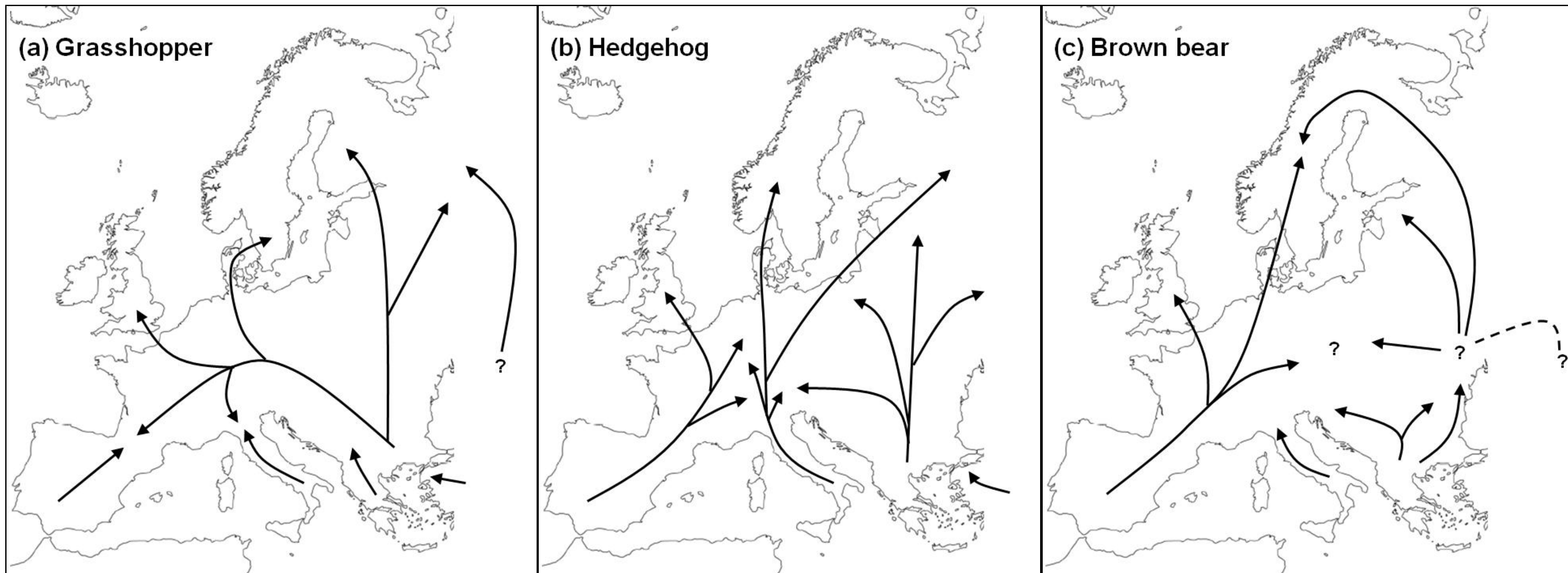

Figure 1.1 Post-glacial recolonisation routes postulated for three paradigm taxa based on modern mtDNA phylogeographic structure. The grasshopper *Chorthippus parallelus* (a), the hedgehog *Erinaceus europaeus/concolor* (b), and the brown bear *Ursus arctos* (c). Figure modified from Hewitt 2001

1.3 OVERVIEW OF ANCIENT DNA

The study of ancient DNA (aDNA) or ancient genetics emerged almost three decades ago, made possible through the advent of cloning techniques and PCR. The very first studies were published during the 1980s (Higuchi *et al.*, 1984; Paabo, 1985), and although many of the more spectacular claims are now considered as unreliable or contaminated (Austin *et al.*, 1997; Binladen *et al.*, 2007; Willerslev, Cooper, 2005), ancient DNA techniques and applications have continued to be developed and improved upon at an amazing rate. New methodological advances such as the application of multiplex PCR to ancient DNA templates (Krause *et al.*, 2006; Krause *et al.*, 2008; Rompler *et al.*, 2006; Sanchez, Endicott, 2006), the use of next generation sequencing techniques such as the 454 and Solexa platforms (Ermini *et al.*, 2008; Gilbert *et al.*, 2007b; Green *et al.*, 2006; Noonan *et al.*, 2006; Poinar *et al.*, 2006), and improved measures to detect, quantify or avoid contamination (Cooper, Poinar, 2000; Leonard *et al.*, 2007a; Linderholm *et al.*, 2008; Pruvost *et al.*, 2005; Sampietro *et al.*, 2006), have contributed greater power, sensitivity and credibility to the field of aDNA.

Ancient DNA studies allow us to trace historic and ancient gene flow through time, giving a dynamic temporal and geographic understanding of genetic diversity. This can be particularly informative when coupled with environmental data or chronological information (such as radiocarbon dated specimens) allowing links to be made between climatic or anthropogenic disruptions and the genetic response of populations or species. Ancient DNA analyses have been performed on a wide range of taxa including Beringian brown bears (Barnes *et al.*, 2002; Leonard *et al.*, 2000), Steppe bison (Shapiro *et al.*, 2004), woolly mammoth (Barnes *et al.*, 2007), and moa (Baker *et al.*, 2005), and from a range of sample types such as bones and tooth roots (Rohland, Hofreiter, 2007), hair (Gilbert *et al.*, 2004), feathers (Rawlence *et al.*, 2009), coprolites (Wood *et al.*, 2008) and sediments (Haile *et al.*, 2007; Willerslev *et al.*, 2003). Through the diverse range of these studies it has been demonstrated that ancient DNA analyses possess the power and sensitivity to detect dynamic population movements, bottlenecks, extinctions and repopulations from as far back as the late Pleistocene. Ancient DNA is most commonly retrieved from samples dating from approximately the last 50,000 years, although in rare cases DNA sequences have been obtained from samples >100,000 years (See Willerslev *et al.*, 2007; Willerslev *et al.*, 2004).

Another advantage of an ancient DNA dataset over the traditional modern dataset is the ability to identify extinct lineages and evolutionary relationships that would be impossible to detect with samples from a single point in time (See Figure 1.2) as in the case of a purely modern dataset. This is particularly important when the population of interest has passed through an historical or ancient genetic bottleneck prior to the time of sampling. For example, in some cases modern datasets reveal a low level of genetic diversity, yet it is not possible to determine conclusively whether this is due to an ancient or historical bottleneck or due to a low effective population size or some other factor, as seen in a mtDNA study of killer whales (Hoelzel *et al.*, 2002). As killer whales display a pattern of strict maternal philopatry with almost no dispersal, some populations were found to have zero mtDNA diversity, making it

impossible to determine if this was merely due to the social behaviour of the modern populations or if an historical bottleneck was the cause of the low genetic diversity observed.

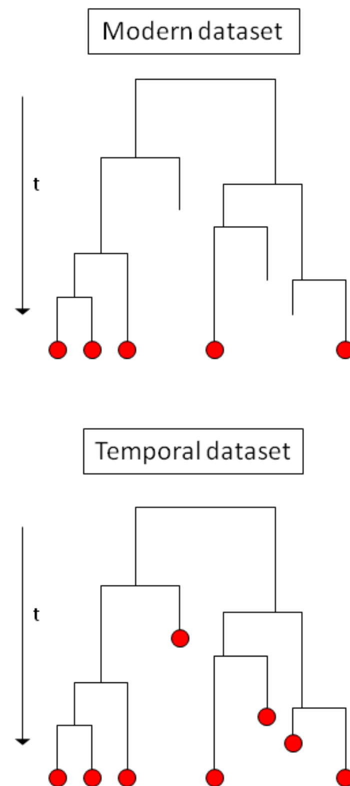

Figure 1.2 Schematic comparison of a modern and a temporal (e.g. ancient DNA) dataset. The arrow marked with 't' indicates time, while the red circles represent individual genetic haplogroups/lineages. Whereas the modern dataset only allows those lineages that survive to the present day to be detected, the temporal dataset can detect both extant and extinct lineages.

1.3.1 Problems and challenges with working on ancient DNA

Ancient DNA techniques provide us with a very powerful toolkit, with the ability to track genetic changes through time rather than relying on inferences from a more limited time-slice such as a purely modern dataset. However, working with ancient DNA is very challenging, and requires the adherence to rigorous standards in order to obtain authentic and reliable sequence results. Many of the challenges with working on ancient DNA templates and the precautions required to mitigate them have been reviewed in detail (Willerslev, Cooper, 2005) and a list of authenticity guidelines followed while undertaking this PhD research are summarised in Table 1.1. Generally, when ancient DNA molecules are present they are usually limited to mtDNA rather than nuclear, and are found in very low copy numbers (frequently <100 molecules), are highly fragmented (typically <100bp – 500bp mtDNA), they can exhibit *post-mortem* modifications or damage (Brotherton *et al.*, 2007; Gilbert *et al.*, 2007a), and can be cross-linked (Sutlovic *et al.*, 2008).

Many of the difficulties of working with ancient DNA (such as degradation and low template number) arise through the particular preservation environment the sample was subjected to - either from the natural environment or handling and storage after collection for analysis (Hofreiter *et al.*, 2001; Wayne *et al.*, 1999; Willerslev, Cooper, 2005). In brief, temperature and moisture levels are thought to play an important role in determining the relative preservation of DNA in many ancient samples. For instance samples from hot, humid or wet sites are less likely to yield well-preserved endogenous aDNA compared to samples from cold dry places. Similarly, humic acids (Sutlovic *et al.*, 2008) and other minerals and compounds can be present in some samples at a level that causes inhibition of DNA extraction or subsequent PCR amplification.

Compared to the vast quantities and pervasive nature of modern DNA ubiquitous in the environment (particularly from bacteria, humans and domestic animals) the limited survival and degraded nature of ancient DNA molecules make research anything but routine. All work on ancient samples (pre-PCR amplification) must be performed in a dedicated ancient DNA laboratory, physically separated from any PCR facility, and with personnel movement restricted to ensure unidirectional travel from pre- to post- PCR areas to eliminate contamination from previously amplified PCR products. Within the ancient DNA facility, all personnel should wear full-body suits, face shields, masks and gloves in order to minimise contamination from modern environmental sources and from the lab personnel themselves. The laboratory should be routinely irradiated with UV lights, and equipment, work surfaces and consumables treated with bleach and/or UV irradiation to destroy unwanted DNA molecules.

Even if suitable precautions are followed in handling samples in the ancient DNA lab, contamination of PCR reagents with modern human and animal DNA during their manufacturing processes can be an issue, as highlighted recently by Leonard *et al.*, 2007. The scale of the potential for contamination can be hard to appreciate. For example, one aerosol droplet from a single PCR reaction can contain more than one thousand times the amount of amplifiable mtDNA found in an entire gram of ancient starting material (Cooper *et al.*, 2001; Handt *et al.*, 1996), thereby constituting a very real contamination threat to an ancient DNA facility. New methods and guidelines for detecting and/or preventing contamination continue to be suggested to combat the problems associated with contamination, for example the use of bleach to remove contaminating DNA from the exterior surface of ancient samples (Kemp, Smith, 2005).

Table 1.1 Summary list of authenticity criteria & guidelines applied to the ancient DNA studies presented in this thesis. Modified from (Cooper, Poinar, 2000; Rompler *et al.*, 2006; Willerslev, Cooper, 2005)

- ✓ Physically isolated pre-PCR laboratory dedicated to ancient DNA research with strict adherence to ancient DNA protocols to prevent contamination.
- ✓ Full body suit, gloves, face mask, face shield and other protective gear to be worn in the pre-PCR ancient DNA facility to minimise contamination.
- ✓ Multiple negative (blank) extraction and PCR controls included per experiment.
- ✓ Only process a small number of samples per experiment (e.g. two extraction blanks plus eight samples per extraction).
- ✓ Independent extraction replications of a subset of samples.
- ✓ Cloning or direct sequencing of multiple independent PCR amplifications of a small subset of samples if independent replication of extraction is not possible due to limited quantity of original sample.
- ✓ Decontamination of reagents and specimens (by exposure to bleach or UV light as appropriate).
- ✓ Appropriate molecular behaviour is observed (e.g. DNA sequence length obtained reflects expected preservation conditions and age of sample).

1.3.2 Ancient phylogeographic studies

Despite the wealth of knowledge obtained through modern phylogeographic studies, it is becoming increasingly apparent that relying on inferences based on modern data alone can lead to misleading reconstructions of historic or ancient phylogenies and phylogeographic patterns (see Hofreiter & Stewart 2009 for a recent review). This is due to purely modern datasets being generally biased to reflect very recent or extreme genetic changes while ancient or temporal datasets have more power to detect complex and dynamic changes through time (Ramakrishnan, Hadly, 2009). For example, a study comparing mtDNA diversity of Pleistocene and modern European salmon (*Salmo salar*) revealed that the most common haplogroups of the Iberian refugia during the LGM were very rare or absent from modern populations (Consuegra *et al.*, 2002). Another recent study combining morphometric and isotopic analyses with ancient DNA data found evidence of an extinct ecomorph of wolf in Beringia which was both genetically and ecologically different from the modern Alaskan gray wolf (Leonard *et al.*, 2007b). This study not only showed that modern genetic diversity of North American wolves is much lower than was seen in the

Pleistocene, but also detected the presence of a unique ecomorph, which would not have been possible through analysis of modern genetic material alone.

While many ancient DNA studies continue to suffer from low numbers of suitably well-preserved samples, there are examples where even a single specimen has lead to a new understanding of the phylogeographic range or presence of a species or population. For instance, until recently extinct cave bears (*Ursus spelaeus*) were believed to have been restricted to Europe and could be divided into two main genetic lineages (Hofreiter *et al.*, 2004a). In 2009, a new study of Asian bear samples revealed not only the presence of a third previously unidentified lineage in the Caucasus Mountains, but also identified a single related specimen approximately 6000 km further east in Siberia – suggesting a much more widespread and complex phylogenetic history and distribution than previously imagined for cave bears (Knapp *et al.*, 2009).

Extraction of ancient DNA from late Pleistocene and Holocene material is emerging as a valuable source of information not only for evolutionary and phylogenetic analysis but also for fine scale demographic analysis (for example Beringian bison, Shapiro *et al.* 2004) – allowing detection of population bottlenecks, crashes or expansions which can then be temporally linked with potential causative events to tease apart the relative roles of climatic and human impacts. A recent study of Pleistocene musk oxen (Campos *et al.*, 2010) provides a good example of an ancient DNA analysis being able to exclude anthropogenic influences as a cause for dynamic population fluctuations in this species.

While it is not always possible to determine whether climatic or anthropogenic changes are to blame, many ancient DNA studies reveal more complex or genetically diverse population histories compared with modern populations, such as for three kiwi bird species in New Zealand (Shepherd, Lambert, 2008), brown bears in North America (Barnes *et al.*, 2002) and Holarctic musk oxen (MacPhee *et al.*, 2005). In contrast, a few ancient DNA studies have proposed stable genetic diversity through time. For example, a study of Holocene bowhead whales surprisingly failed to detect any significant loss of diversity through time in spite of the historical population bottleneck believed to have occurred due to hunting by humans (Borge *et al.*, 2007).

It is important to remember that ancient DNA studies are not limited to extinct taxa (for example the woolly mammoth; Barnes *et al.* 2007), but can also reveal important information from historic or ancient populations of extant taxa (for example arctic foxes; Dalen *et al*. 2007), allowing inferences previously based on modern samples alone to be tested. Some results from ancient DNA studies of past populations of extant taxa can even be applied to the conservation of modern populations, as reviewed recently by Leonard *et al*. (2008). For example gene-flow management of Yellowstone brown bears (Miller, Waits, 2003) and the endangered Florida panther population (Culver *et al.*, 2000).

1.4 BEARS

1.4.1 Genetic diversity and phylogeography of brown bears

Modern brown bears (*Ursus arctos*) have a widespread (although not continuous) Holarctic distribution, and are currently found from western Europe, across the Eurasian continent to North America. Based on mtDNA control region sequences, brown bear populations have traditionally been divided into four main distinct extant lineages or clades (1, 2, 3 and 4) displaying strong phylogeographic structuring (Figure 1.3). The clade nomenclature used in this thesis is consistent with that used by Leonard *et al.* (2000) and built on by subsequent studies including those by Barnes *et al.* (2002) and Miller *et al*. (2006). Within the major clades several subclades have also been identified. For instance Clade 2 consists of an isolated group of bears from the Alaskan ABC Islands (designated Clade 2a) and polar bears (Clade 2b), while Clade 3 includes a common and geographically widespread group (Clade 3a) found in Europe, Eurasia, Japan and North America, and a less well studied group (Clade 3b) found in Japan and parts of North America and Canada. Similarly, a number of apparently extinct subclades have been revealed through ancient DNA studies such as clade 2c and 3c from the late Pleistocene in North America (Barnes *et al.*, 2002).

Modern European brown bear populations can be split into two distinct phylogenetic lineages – the 'western' clade 1 and the 'eastern' clade 3a (Taberlet, Bouvet, 1994). The western clade 1 is currently found in Spain, France, Italy and southern Scandinavia, while bears of the eastern clade 3a are found in eastern Europe, northern Scandinavia and Russia. These distinct geographically structured mtDNA clades observed in modern European brown bears are believed to be a result of their response to the last glacial cycle combined with the effects of recent anthropogenic hunting and habitat fragmentation (Swenson *et al.*, 1995; Taberlet, Bouvet, 1994; Taberlet *et al.*, 1998). Interestingly, contact zones between the two lineages have only been identified in Scandinavia (Taberlet *et al.*, 1995) and in Romania (Kohn *et al.*, 1995). The strong mtDNA phylogeographic pattern observed in brown bears is believed to be further enhanced by female philopatry (Randi *et al.*, 1994).

The brown bear was one of the paradigm species used by Hewitt to develop the expansion/contraction (E/C) model of European interglacial-glacial phylogeographic patterns (Hewitt, 1996; Hewitt, 1999). While the modern brown bear mtDNA phylogeographic structure is generally considered to be consistent with the E/C theory (Hewitt, 2001; Taberlet *et al.*, 1998), recent evidence from the field of ancient DNA is beginning to raise doubts about the existence and/or genetic isolation of the southern glacial refugia, with suggestions of continuous gene-flow between brown bear populations across southern Europe before (Hofreiter *et al.*, 2004b), during and after the last glacial maximum (Valdiosera *et al.*, 2008; Valdiosera *et al.*, 2007). Evidence from other sources (reviewed by Stewart, Lister, 2001) are also beginning to question the restriction of temperate species to southern refugia, with several plant studies suggesting the existence of cryptic central or northern European refugia during the LGM (Magri *et al.*, 2006; Naydenov *et al.*, 2007; Willis *et al.*, 2000).

Similarly, an ancient DNA study of Pleistocene Beringian brown bears (Barnes *et al.*, 2002) revealed a complex background to the modern phylogeographic structuring of North American brown bears. Rather than North America being colonised by a small founding population from Eurasia, ancient DNA data revealed a dynamic Pleistocene history of colonisation followed by local extinctions and repopulation by a series of distinct mtDNA clades through time. Each reciprocally monophyletic colonising clade already encompassed considerable mtDNA diversity, presumably subsets of established Eurasian clades (Barnes *et al.*, 2002). Additionally, this study suggested that the current phylogeographic patterns had developed over thousands of years, possibly due to repeated population isolations and local extinctions in response to climatic shifts, rather than having evolved on the American continent as the result of recent population barriers and low dispersal rates.

The widespread distribution of brown bears combined with their strong mtDNA phylogeographic structuring makes them an ideal candidate for a range of studies addressing the effects of Quaternary climatic fluctuations on large terrestrial mammals. Additionally, the species is of concern in terms of wildlife conservation management, due to hunting and habitat destruction by humans (Kohn *et al.*, 1995; Miller, Waits, 2003; Swenson *et al.*, 1995; Waits *et al.*, 1998). As a result, both modern and ancient brown bear populations continue to be a subject of interest, with numerous new genetic datasets and phylogeographic interpretations being presented in recent years. Some key examples include the identification of additional mtDNA clade diversity such as the extinct North African Atlas bear lineage (Calvignac *et al.*, 2008; Clade 7, Figure 1.3b), and two less well characterised clades from the middle east (Clade 5 and 6) described in Miller *et al.*, (2006).

Studies of the genetic diversity of endangered North American brown bear populations (Jackson *et al.*, 2008; Miller *et al.*, 2006) have the potential to impact conservation management of this species in the future, while the publication of the first mtDNA analyses of modern Eurasian (Korsten *et al.*, 2009) and extinct North African (Calvignac *et al.*, 2008) brown bear populations have begun to extend our focus to these continents, paving the way for a more global understanding of brown bear phylogeography.

The last three years has also seen the emergence of a more complex phylogeographic picture of late Pleistocene and Holocene European brown bears, with two ancient DNA studies reporting continuous geneflow across southern Europe during and after the last glacial maximum (Valdiosera *et al.*, 2008; Valdiosera *et al.*, 2007). These studies are in direct contrast to Hewitt's traditional glacial refugial model developed based solely on modern phylogeographic patterns (Hewitt, 2001; Taberlet *et al.*, 1998).

Figure 1.3 Distribution of major brown bear mtDNA clades and subclades. (a) shows the approximate distribution of extant clades and subclades only, including the recently described middle eastern clades 5 and 6 (Miller *et al.*, 2006). (b) shows the approximate distribution of both extinct and extant clades and subclades. The clades considered to be extinct (2c, 3c and 7) have been circled in black on the map.

1.4.2 Unresolved issues within the Tremarctine bear lineage

While brown bears - and even their extinct relative, the cave bear (*U. spelaeus*) -are relatively well studied, another lineage of the bear family, the Tremarctine bears are much less well understood. The Tremarctine bears are a subfamily endemic to the New World, with several extinct genera from North and South America and only a single extant species – the Andean spectacled bear (*Tremarctos ornatus*) surviving in South America. Resolving the evolutionary relationships of the Tremarctine bears has been problematic in the past partly due to the fragmentary nature of the South American fossil record (Soibelzon *et al.*, 2005) and the wide range of individual variation seen within bear species (Kurten, 1966; Kurten, 1967; Kurten, Anderson, 1980; Trajano, Ferrarezzi, 1994). Genetic analyses using whole mtDNA genomes has recently revealed that *T. ornatus* is a basal sister taxon to the Ursine bears, with only the giant panda (*Ailuropoda melanoleuca*) falling more basal in the phylogenetic tree (Krause *et al.*, 2008; Yu *et al.*, 2007). One of these recent studies (Appendix 7) also

included the sequence of the mtDNA genome of an extinct Tremarctine bear (Krause *et al.*, 2008), the North American giant short-faced bear (*Arctodus simus*) and confirmed it as a sister taxa to the extant spectacled bear (Figure 1.4). The evolutionary relationships of the other extinct Tremarctine bears, including the extinct South American short-faced bear (*Arctotherium* sp.), remains unresolved.

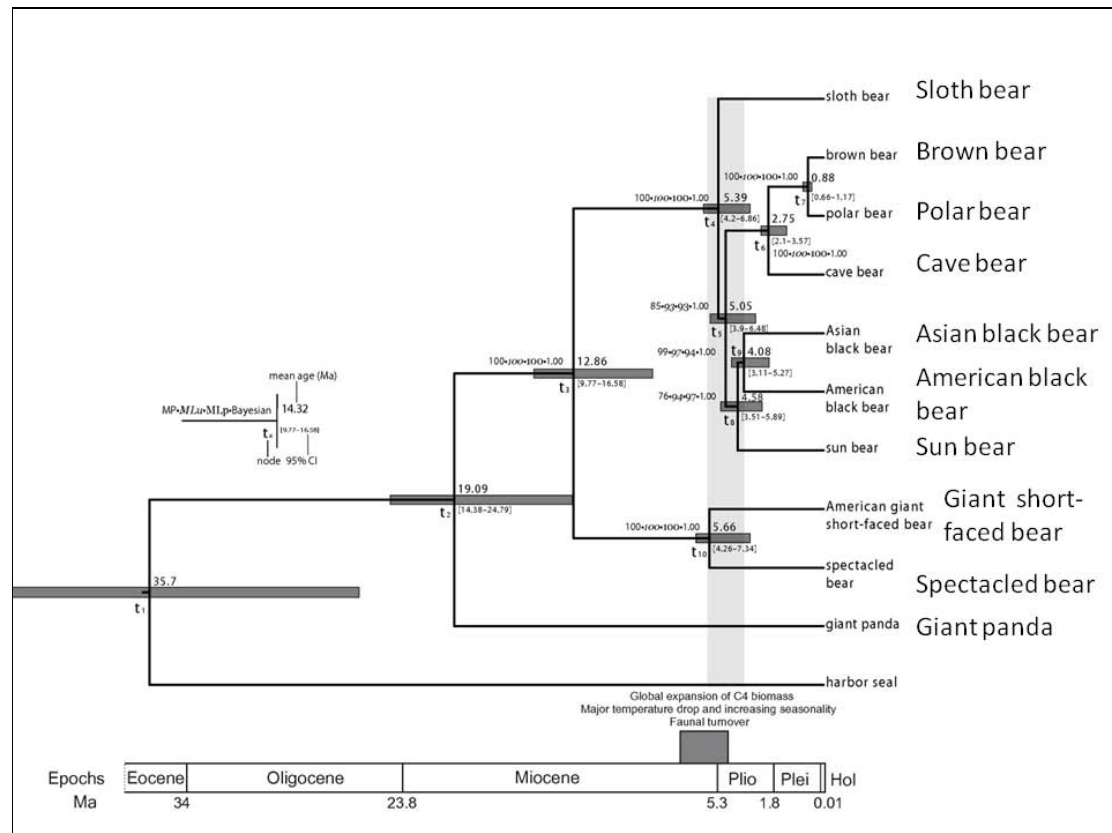

Figure 1.4 Phylogeny of bears based on Bayesian analysis of whole mitochondrial genomes. Figure adapted from Krause *et al.*, 2008 (Appendix 7).

Interestingly, the New World endemic, *A. simus,* became extinct in Beringia during the LGM (approximately 21,000 years ago) and from the ice-free lower states of North America approximately 10,000 years ago, while in contrast the Old World coloniser, the brown bear, survives to the present day. Ancient DNA analyses of Beringian brown bears from the Pleistocene period revealed it to be a period of dynamic population extinctions and repopulations (Barnes *et al.*, 2002). To date no genetic studies have been published on the mtDNA diversity or phylogeographic structure of *A. simus,* preventing a comparison with patterns observed in contemporaneous brown bear populations. Such a study would increase understanding of the diversity within the Tremarctine bear populations, and may shed some light into why *A. simus* became extinct while *U. arctos* survived.

1.5 SCOPE OF THIS THESIS

Despite the rapid increase in the publication of bear mtDNA studies in recent years (Table 1.2), many interesting questions remain unanswered. This PhD research aims

to help fill some of these gaps and to answer specific phylogeographic and evolutionary questions which currently remain unresolved for brown bears and Tremarctine bears. Two chapters in this thesis are in manuscript format prepared for submission to Molecular Ecology (Chapter 2) and Current Biology (Chapter 3). The remaining chapters are conventional thesis chapters, to be converted to manuscripts for submission to journals at a later stage. Below is an overview of the scope of this thesis.

Table 1.2 Summary of published brown bear publications (1994 – 2009) highlighting the number of different studies and variable size and position of mtDNA sequences analysed in each publication. Table does not include recent publications using whole mtDNA genomes. Table compiled based on Davison *et al.* (2010).

Reference	mtDNA nucleotides	Approximate alignment in relation to Cytochrome b and Control region
Calvignac *et al.* 2009	549 bp	
Korsten *et al.* 2009	1953 bp	
Jackson *et al.* 2008	589 bp	
Valdiosera *et al.* 2008	177 bp	
Calvignac *et al.* 2008	547 bp	
Valdiosera *et al.* 2007	193 bp	
Saarma *et al.* 2007	388 bp	
Saarma & Kojola 2007	388 bp	
Miller *et al.* 2006	229 bp	
Matheus *et al.* 2004	195 bp	
Hofreiter *et al.* 2004	270 bp	
Barnes *et al.* 2002	195 bp	
Matsuhashi *et al.* 2001	966 bp	
Leonard *et al.* 2000	675 bp	
Shields *et al.* 2000	286 bp	
Matsuhashi *et al.* 1999	1815 bp	
Masuda *et al.* 1998	270 bp	
Waits *et al.* 1998	294 bp	
Talbot & Shields 1996	1275 bp	
Kohn *et al.* 1995	213 bp	
Taberlet & Bouvet 1994	269 bp	
		Cytochrome b Control region

1.5.1 Chapter 2: Ancient DNA analysis of post-glacial Scandinavian brown bears

As previously described, modern European brown bear populations exhibit a strong mtDNA phylogeographic structure generally assumed to be related to their post-glacial recolonisation from southern refugia in the Iberian, Italian and Balkan peninsulas, and from eastern refugia in the Carpathian mountains. Currently in Scandinavia members of the two divergent mtDNA lineages form a contact zone, with bears from northern Scandinavia belonging exclusively to the eastern European clade 3a, while those in southern Scandinavia belong to the clade associated with the Iberian (Spanish) glacial refugium. The timing and formation of this contact zone and

the low levels of genetic diversity currently observed within the modern populations has major implications for brown bear conservation in Scandinavia.

Chapter 2 of this thesis uses ancient DNA to investigate the genetic diversity and phylogeographic structure of Holocene Scandinavian brown bears spanning a period of approximately the last 6,000 years, and attempts to determine whether the phylogeographic patterns and low levels of diversity seen in modern populations are a result of post-glacial recolonisation or alternatively due to more recent anthropogenic effects.

1.5.2 Chapter 3: From Iberia to Alaska: ancient DNA links late Pleistocene brown bears across the Holarctic

A recent ancient DNA study of European brown bears reported the presence of a pre-glacial bear from the Iberian peninsula (Asieko, 40-80,000 ybp, GENBANK# EU400180) belonging to the eastern European clade 3a (Valdiosera *et al.*, 2008). This finding was seen to strengthen the argument for a continuity of putative refugial regions across southern Europe (Valdiosera *et al.*, 2007) and also represents the most geographically distant group that may have influenced the Iberian brown bear population (Valdiosera *et al.*, 2008). Chapter 3 reports a reanalysis of this study, and reveals that rather than a common clade 3a haplotype, the Aseiko specimen belonged to the extinct clade 3c, previously only known from Beringia prior to 35,000 ybp. The implications of this finding are discussed in the light of global brown bear phylogeography.

1.5.3 Chapter 4: Post-glacial phylogeography of European brown bears

As demonstrated in Chapter 3, while the combination of ancient DNA and radiocarbon dating provides a powerful tool for testing phylogenetic and temporal aspects of Quaternary phylogeography, care is needed. Chapter 4 combines previously published modern and ancient brown bear mtDNA sequence data with newly generated sequences from 28 ancient European bears to comprehensively evaluate the post-glacial phylogeography of the western clade 1 lineage, and to test some of the recent hypotheses (Valdiosera *et al.*, 2007) that question the expansion/contraction model developed by Hewitt.

1.5.4 Chapter 5: Ancient Eurasian brown bear phylogeography

Fossil evidence suggests that brown bears evolved in Eurasia (Kurten, 1968), yet until the recent study by Korsten *et al.*, (2009), almost nothing was known about the genetic diversity or phylogeographic structure of brown bears across the Eurasian continent. Korsten *et al.*, (2009) surveyed the mtDNA diversity of 205 modern brown bears from across northern continental Eurasia and the Kamchatka peninsula. Remarkably, they only identified a single lineage (Clade 3a) and over 50 % of the samples belonged to a single haplotype (designated EA1) distributed across the entire width of the continent from northern Scandinavia to the Russian Far East and Kamchatka. Korsten *et al.,* (2009) suggest a sudden expansion of 3a bears across Eurasia after the LGM, and infer a general post-glacial expansion model for mammals on this continent.

Chapter 5 of this thesis is the first study of the genetic diversity and phylogeography of ancient and historic Eurasian brown bears. The research presented in this chapter increases the distribution of mtDNA brown bear sequences from Eurasia – including samples from the Caucasus, Urals and Altai mountain ranges among other regions, with radiocarbon dated specimens ranging from >46,000 ybp to historic specimens (collected during the 19th and 20th centuries). The data allows several of the conclusions presented by Korsten *et al.,* (2009) to be tested.

1.5.5 Chapter 6: Ancient DNA reveals different phylogeographic histories for the extinct New World giant short-faced bear (Arctodus simus) and the brown bear (U. arctos)

While brown bears have been the focus of a great deal of study, nothing is known of the genetic diversity or phylogeographic structure of the extinct giant short-faced bear, *A. simus*, a member of the Tremarctine bear lineage and a Pleistocene contemporary of the brown bear in North America. Chapter 6 is the first study to analyse mtDNA control region sequences from *A. simus*, and 23 specimens spanning a period of 33,000 years from 44,240 ± 930 – 11,619 ± 40 ybp were examined. The results of this study are then discussed in comparison to the Beringian brown bears (Barnes *et al.*, 2002) in an attempt to identify possible reasons for the differential survival of the Old World bear species (*U. arctos*) compared to the New World endemic (*A. simus*).

1.5.6 Chapter 7: Evolutionary relationships of the Tremarctine bears

Following the study of *A. simus* phylogeography and genetic diversity, Chapter 7 investigates the deeper evolutionary relationships of two extinct Tremarctine bears – the South American short-faced bear (*Arctotherium* sp.) and the North American giant short-faced bear (*A. simus*).

1.5.7 Chapter 8: Conclusions

Chapter 8 discusses the importance of the results presented as part of this PhD research, and suggests directions for future research, particularly in light of recent advances in technology that are transforming ancient DNA studies to the genomic level.

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CHAPTER 2

ANCIENT DNA ANALYSIS OF POST-GLACIAL SCANDINAVIAN BROWN BEARS

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CHAPTER 2

ANCIENT DNA ANALYSIS OF POST-GLACIAL SCANDINAVIAN BROWN BEARS

2.1 ABSTRACT

Brown bear populations in modern Europe display a strong mtDNA phylogeographic structure, assumed to be a result of post-Last Glacial Maximum (LGM) recolonisation from southern European refugia in the Iberian, Italian and Balkans peninsulas, and from eastern European refugia in the Carpathian mountains. Currently in Scandinavia members of two divergent mtDNA lineages form a contact zone, with bears from northern Scandinavia belonging exclusively to the eastern European clade, while those in the south belong to the clade associated with the Iberian glacial refugium. The timing and formation of this contact zone and the low levels of genetic diversity found within the modern populations has major implications for brown bear conservation in Scandinavia.

We used ancient DNA techniques to analyse mtDNA control region sequence from 20 post-glacial Norwegian and Danish brown bears to investigate the phylogeographic structure and genetic diversity of Scandinavian bears over the last 6000 years. We found that the lack of mtDNA diversity in northern Scandinavian populations is most likely due to an ancient founder effect during post-glacial recolonisation rather than the result of a recent anthropogenic bottleneck. Additionally, in southern Scandinavia we identified the most northerly record of an Italian/Balkans mtDNA haplotype in Europe. This demonstrates that, in contrast to previous interpretations, the recolonisation of southern Scandinavia was not limited to bears from the Iberian refugium. Our results suggest either genetic turnover with complete replacement through time in post-glacial southern Scandinavia, or alternatively raises the possibility that the Italian/Balkans haplogroup has been overlooked in modern Scandinavian populations.

2.2 INTRODUCTION

The brown bear (*Ursus arctos*) exhibits strong mitochondrial DNA (mtDNA) phylogeographic structure across its Northern hemisphere range. In modern European populations brown bears can be differentiated into two divergent mtDNA lineages – western (clade 1) and eastern (clade 3a) (Taberlet & Bouvet, 1994). Bears of the western clade can be further split into two subclades (Iberian and Italian/Balkans) which are believed to originate from separate glacial refugia during the Last Glacial Maximum (LGM) (Taberlet *et al.*, 1998; Hewitt, 1999; Hewitt, 2000), whereas the eastern clade is believed to be Eurasian in origin, possibly expanding into Europe from a refugium in the Carpathian mountains (Sommer & Benecke, 2005; Saarma *et al.*, 2007).

Directly after the LGM, bears from the Iberian peninsula are thought to have expanded northwards into central and northern Europe, while the glaciated Alps continued to constitute a barrier for bears of the Italian/Balkans refugia, delaying their northward expansion until after much of Europe had been recolonised (Taberlet & Bouvet, 1994; Hewitt, 1999; Sommer & Benecke, 2005). While post-glacial and modern Iberian haplotypes have been identified across Western Europe from Spain, France, Germany and as far afield as Scotland and southern Scandinavia (Taberlet & Bouvet, 1994; Taberlet *et al.*, 1995; Barnes *et al.*, 2002; Valdiosera *et al.*, 2007; Valdiosera *et al.*, 2008), bears of the Italian/Balkans group are thought to have experienced a more restricted expansion, to Mont Ventoux, in the south-east

of France 3445 ± 40 C^{14} ybp and central Germany 5210 ± 35 C^{14} ybp (Valdiosera *et al.*, 2007).

Currently in Scandinavia members of the western and eastern lineages form a contact zone, with bears from northern Scandinavia belonging exclusively to the eastern clade, while those in the south belong to the western subclade associated with the Iberian refugium (Taberlet *et al.*, 1995). The mtDNA diversity in modern Scandinavian bears is very low, with only two haplotypes from the western (Iberian) clade (Genbank Accession: X75871 and X75868) and a single haplotype (Genbank Accession: X75874) from the eastern clade being reported from southern and northern populations, respectively (Taberlet & Bouvet, 1994; Taberlet *et al.*, 1995; Waits *et al.*, 2000).

The low mtDNA diversity seen in Scandinavian brown bears (along with wolverines, wolves and lynx) (Walker *et al.*, 2001; Flagstad *et al.*, 2003; Rueness *et al.*, 2003) may have been caused by a major population bottleneck during predator removal programmes and hunting in the mid-1800s (Swenson *et al.*, 1995). Recent management efforts by Swedish and Norwegian governments (since 1927 in Sweden and 1972 in Norway) have allowed brown bear populations to increase dramatically (from as few as an estimated 130 individuals in the 1930s to approximately 1000 individuals at the start of the 21st century)(Swenson *et al.*, 1995; Waits *et al.*, 2000). The low levels of genetic diversity within modern populations has major implications for brown bear conservation in Scandinavia (Tallmon *et al.*, 2004). This has led to questions about whether the present lack of mtDNA diversity and contact zone is the result of historical anthropogenic effects or alternatively reflects the post-glacial recolonisation of brown bears approximately 5,000-9,000 ybp into Scandinavia (Siivonen, 1982; Taberlet *et al.*, 1995; Waits *et al.*, 2000). We employed ancient DNA techniques to investigate the timing and potential causes of these patterns.

2.3 MATERIALS & METHODS

2.3.1 Samples

Intact bone, pre-powdered bone or tooth root material was obtained from 46 Holocene Scandinavian brown bears (*Ursus arctos*) (see Appendix Table 1.1 for sample details). Norwegian material was collected as previously described (Østbye *et al.*, 2006) while Danish samples were obtained from the Natural History Museum of Denmark, University of Copenhagen, and a Swedish sample from the Geological Survey of Sweden in Lund. Thirty-nine specimens were from limestone caves in northern Norway, six specimens originated from Denmark and the remaining specimen from Ugglarp, in southern Sweden. Radiocarbon dated ancient bear specimens ranged in age from 420 ± 90 to 6210 ± 100 C^{14} ybp (see Appendix Table 1.1 for details).

2.3.2 Ancient DNA Extractions

All pre-PCR analyses were performed in the dedicated Australian Centre for Ancient DNA (ACAD), under positive HEPA-filtered air-pressure, with nightly UV decontamination. This facility is geographically isolated from any modern molecular biology laboratories, and follows strict ancient DNA protocols to prevent contamination (Willerslev & Cooper, 2005). No work on modern bears has been conducted previously in this facility.

The exterior surface of intact bone samples was removed using a Dremel tool to eliminate surface contamination. 0.18-1.13 g of the cleaned bone or tooth root was powdered using an 8 mm tungsten ball bearing in a Mikro-Dismembrator (Sartaurius) at 3000 rpm in sterilised stainless steel canisters. Bone/tooth root powder was then decalcified overnight in 10-20 ml of

0.5M EDTA (pH 8) on a rotary mixer at room temperature. The decalcified material was collected by centrifugation and digested in 3 ml of 100 mM Tris-HCl (pH 8), 100 mM NaCl, 0.5 mg/ml proteinase K, 10 mg/ml dithiothreitol (DTT), and 1% sodium dodecyl sulphate (SDS), overnight at 55 °C on a rotary mixer. Following digestion, an equal volume (3 ml) of Tris-saturated phenol was added and mixed on a rotary mixer for 10 minutes at room temperature, followed by centrifugation at 1500 g for 5 minutes. The aqueous phase was then transferred to a new tube. This process was repeated twice, once with an equal volume of Tris-saturated phenol, and once with an equal volume of chloroform. The final aqueous phase was de-salted with sequential additions of DNA-free water to an Amicon Ultra-4 Centrifugal Filter Unit (Millipore), and concentrated to a final volume of 100-200 µl. DNA extractions were performed in batches of eight samples, with two negative extraction controls (containing no bone powder).

2.3.3 PCR Amplifications

A carnivore-specific primer pair L16164 and H16299 (Hänni *et al.*, 1994), designed to amplify a hypervariable 135-bp fragment of the mitochondrial control region (CR), was used in order to facilitate comparison with previous studies of both modern and ancient European brown bears and to differentiate all major brown bear mtDNA lineages (Taberlet & Bouvet, 1994; Valdiosera *et al.*, 2007). A second primer pair, URSUSF1_136-156 and URSUSR1_273-290 (Valdiosera *et al.*, 2007) was used to amplify an additional 111-bp CR fragment from a Danish individual (Virksund ZMK9/1861), which produced an unexpected result, to allow more accurate phylogenetic placement of the specimen and to identify any connection to glacial refugial populations. (Taberlet & Bouvet, 1994; Valdiosera *et al.*, 2007).

PCR amplifications were performed in 25 µl reactions with 1-2 µl of DNA extract, 1-1.25 U Platinum *Taq* DNA Polymerase High Fidelity and 1X buffer (Invitrogen), 2 mg/ml rabbit serum albumin (RSA; Sigma), 2 mM $MgSO_4$, 250 µM of each dNTP, and 1 µM of each primer. PCR thermal cycling reactions consisted of 94 °C 1 min, followed by 50-55 cycles of 94 °C denaturation for 15 sec, annealing for 15-20 sec at 55 °C (52 °C for the URSUSF1_136-156 and URSUSR1_273-290 primers), and extension at 68 °C for 30 sec, followed by a final extension at 68 °C for 10 min. PCR products that yielded bands of the expected size were purified using ExoSAP-IT or Agencourt AMPure PCR purification kit according to manufacturers instructions. PCR products were sequenced in both directions using Big Dye chemistry and an ABI 3130XL Genetic Analyzer. Sequences were edited and contigs formed using Sequencher 4.7, and then aligned in MEGA 3.1 (Kumar *et al.*, 2004).

2.3.4 Verification/Validation of aDNA results

Samples were extracted in small batches (eight or less) bracketed with at least two accompanying extraction blanks. In addition, bear samples from other geographic regions (expected to belong to divergent mtDNA clades than those found in modern European populations) were included in each extraction set to monitor for contamination between samples during the extraction process. No cross-contamination was identified and all extraction blanks were negative. All PCR amplifications included PCR blanks, which were also negative.

Sequence from the Danish sample (Virksund ZMK9/1861) was amplified and sequenced independently three times in both directions for the 111bp fragment to confirm the initial result (Hofreiter *et al.*, 2001). Duplicate samples from five Norwegian specimens were independently replicated at Centro Mixto UCM-ISCIII de Evolución y Comportamiento Humanos, Madrid, Spain (See Appendix 1 for details).

2.3.5 Phylogenetic Analysis

To investigate the phylogenetic relationship of Scandinavian sequences obtained in this study to representative sequences from Holocene and modern European *Ursus arctos* eastern and western haplogroups (Appendix Table 1.2) we used 131 bp of homologous CR sequence to generate a distance-based tree with 1000 bootstrap replicates in PAUP* (Swofford, 2000) using the K80+G model chosen according to the Aikake Information Criterion in Modeltest (Posada & Crandall, 1998). The same dataset was also used to construct a maximum parsimony tree using CNI searches with 100 random sequence addition replicates in MEGA 3.1 (Kumar *et al.*, 2004). An extinct cave bear (*Ursus spelaeus* AJ300176) (Hofreiter *et al.*, 2002) sequence was used as an outgroup.

To further assess the phylogeographic identity of the ancient Danish sample we generated a haplotype network in TCS (Clement *et al.*, 2000) using a dataset of longer sequences (193 bp of CR) for bears belonging only to the western lineage (Clade 1).

The genetic diversity of the Holocene Norwegian sequences was calculated using the program Arlequin 3.1.1 (Schneider *et al.*, 2000) (See Appendix Table 1.3).

2.4 RESULTS

We obtained 135 bp of mtDNA control region sequence from 21 out of the 46 ancient *Ursus* specimens from Scandinavia. 20 out of 21 were genetically identified as brown bears (Table 2.1) with the one exception being a polar bear (Appendix Table 1.1). The majority of the samples from which DNA was successfully amplified originated from northern Norway, with only one of six ancient Danish bears yielding DNA (See Figure 2.1 for map showing origin of these samples). We obtained an additional 111bp mtDNA from the control region of the Danish individual (Virksund ZMK9/1861) giving a total of 246 bp. We were unable to obtain any result from the Swedish sample.

Five samples were extracted, amplified and sequenced independently to give a total of three independent sequences for each of these samples, and a sixth sample was internally replicated by sequencing the same fragment from three individual amplification products. Two of these six samples showed C-T substitutions in one of the three sequence replicates (See Appendix 1). This was interpreted as typical aDNA damage (Brotherton *et al.*, 2007), and the best of three sequences rule (Hofreiter *et al.*, 2001) was applied to obtain a consensus sequence.

Table 2.1 Samples analysed. Ancient brown bear samples that yielded a minimum of 135bp of sequence with radiocarbon dates, location and mtDNA clade information.

Sample	Location	Uncalibrated C^{14} date (ybp)	Clade	Accession No.
RM-893	Northern Norway	3530 ± 110	3a	
RM-418B	Northern Norway	undated Holocene	3a	
B-77-N-9-4	Northern Norway	1310 ± 30	3a	
RS-030986	Northern Norway	6210 ± 100	3a	
B-78-B-1	Northern Norway	2870 ± 80	3a	
B-77-G-5-7	Northern Norway	4420 ± 70	3a	
B-81-S-1A	Northern Norway	undated Holocene	3a	
RM-3188A	Northern Norway	3980 ± 180	3a	
ROG-01A	Northern Norway	undated Holocene	3a	
FAU-01A	Northern Norway	undated Holocene	3a	
TROLL-4A	Northern Norway	undated Holocene	3a	
TROLL-3A	Northern Norway	undated Holocene	3a	
B-81-S-1B	Northern Norway	3970 ± 60	3a	
B-1979-N-320	Northern Norway	2800 ± 160	3a	
82-9-11-5a	Northern Norway	3150 ± 120	3a	
HA-88-2	Northern Norway	undated Holocene	3a	
RM-894Virvel	Northern Norway	undated Holocene	3a	
RM3188	Northern Norway	undated Holocene	3a	
BU-1982-2	Northern Norway	undated Holocene	3a	
ZMK 9/1861	Virksund Denmark	5310 ± 20* (CURL-10287)	1	

*See Appendix 1 for details of this radiocarbon date.

All ancient brown bear samples from northern Norway (uncalibrated C^{14} ages: 1310-6210 ybp) belonged to the eastern clade 3a (See Figure 2.2 and Appendix Figure 1.1) with 17 of 19 sequences identical to the previously published modern northern Scandinavian haplotype (GenBank accession: X75874) (Taberlet & Bouvet, 1994). The remaining two ancient Norwegian samples (RS-030986 and B-77-N-9-4) differed by one C-T substitution each, and this was confirmed through independent replication and application of the best of three rule (Hofreiter *et al.*, 2001). The overall nucleotide diversity (π) was 0.001607 ± 0.002277 for the northern Norwegian Holocene brown bears (see Appendix Table 1.3). Although this is higher than the modern northern population in which all known mtDNA sequences are identical (π = 0), it is a result of only two individuals differing by 1 nucleotide each over a period of 6000 years, and so is not considered to be substantially different to the current lack of genetic diversity.

Figure 2.1 Map of Europe showing the location of samples that yielded genetic data. The location of the Danish (Virksund) specimen is indicated by an arrow, while the box depicts the region where the northern Norwegian specimens were collected. The exact sites of the Norwegian samples are not given, however all were collected from limestone caves situated inland (Østbye *et al.*, 2006). The star indicates the most northerly location that a bear possessing an Italian/Balkans haplotype had previously been reported (Germany, 5210 ± 35 C^{14} ybp, EF488499). The approximate locations of the putative glacial refugia in Iberia, Italy and the Balkans are also shown.

The Danish sample (Virksund ZMK9/1861, uncalibrated C^{14} age: 5310+/-20 ybp) belongs to the Italian/Balkans subclade of the western lineage (Clade1) (Figure 2.2 and Appendix Figure 1.1). 193 bp of the 246 bp CR obtained from the Virksund specimen could be compared with homologous sequence from representatives of both Iberian and Italian/Balkans western haplotypes (Holocene and modern) due to relative alignments of the fragments. The Virksund sequence is identical to three Italian/Balkans sequences (modern Slovenia X75877, 3445±40 C^{14} ybp France EF488505, and 5210±35 C^{14} ybp Germany EF488499) (Taberlet & Bouvet, 1994; Valdiosera *et al.*, 2007). The TCS haplotype network (Figure 2.3) confirmed the placement of the Virksund sequence within the Italian/Balkans subclade, with four key single nucleotide polymorphisms (SNPs) at positions 16,620, 16,662, 16,740, and 16,762 (numbers refer to position in the *Ursus arctos* mt genome sequence AF303110) separating all Italian/Balkans bears from Iberian bears included in this analysis. The sample from Virksund, Denmark, represents the most northerly known record for the Italian/Balkans subclade.

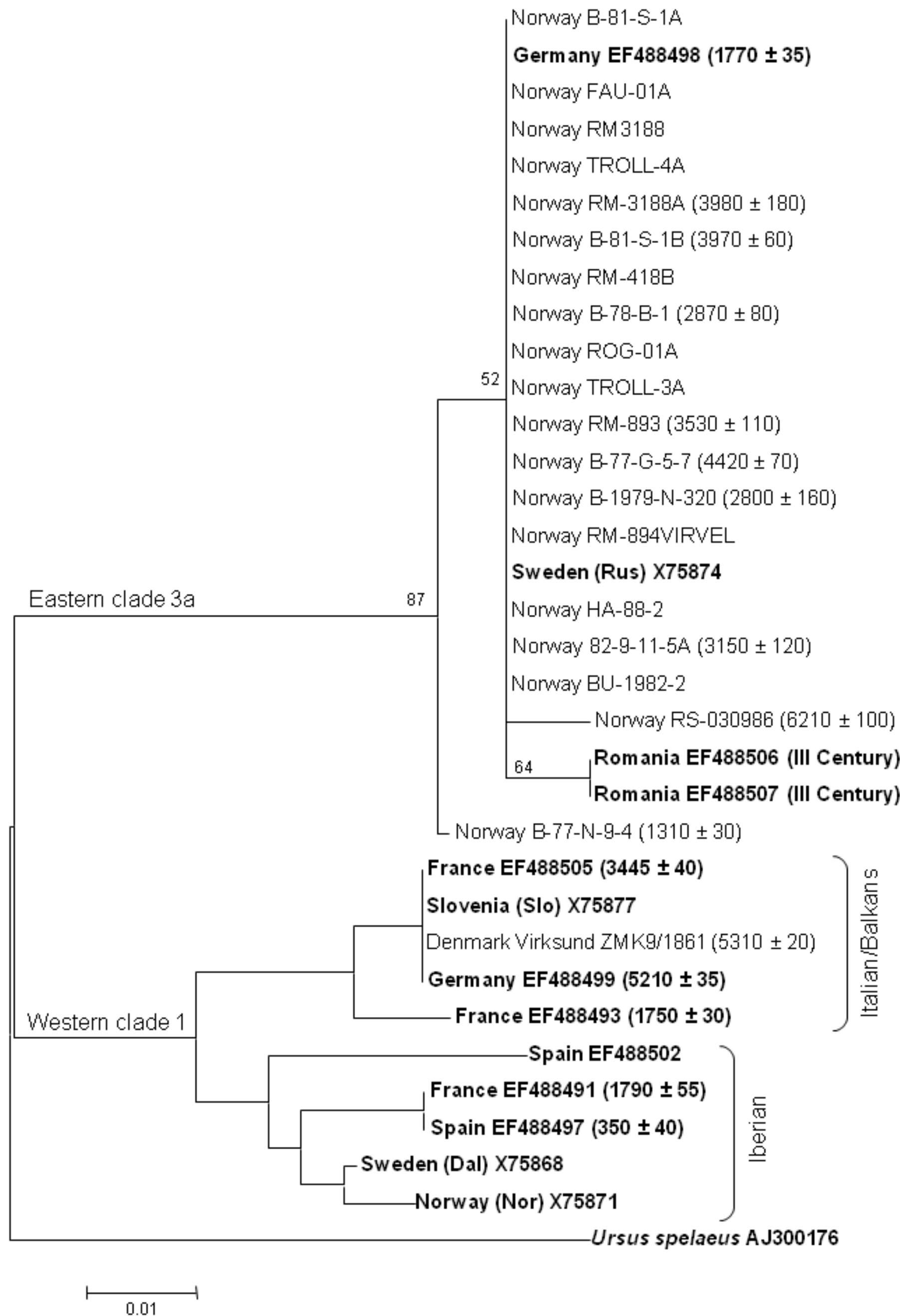

Figure 2.2 Neighbor-joining phylogeny showing the placement of Holocene Scandinavian sequences in relation to key European brown bear haplogroups. All northern Norwegian sequences fall within the Eastern clade 3a. The Virksund specimen from Denmark falls within the Italian/Balkans group of the Western clade 1. Numbers above nodes represent bootstrap values ≥ 50 % using the K80+G model. Labels indicate the Location / Sample name / (Age) for sequences generated in this study, and Location / GenBank Accession No. / (Age) in bold for previously published sequences included in this analysis. All ages are uncalibrated C^{14} ybp. Analysis is based on 131-bp control region mtDNA with a cave bear (*Ursus spelaeus*) sequence used as an outgroup.

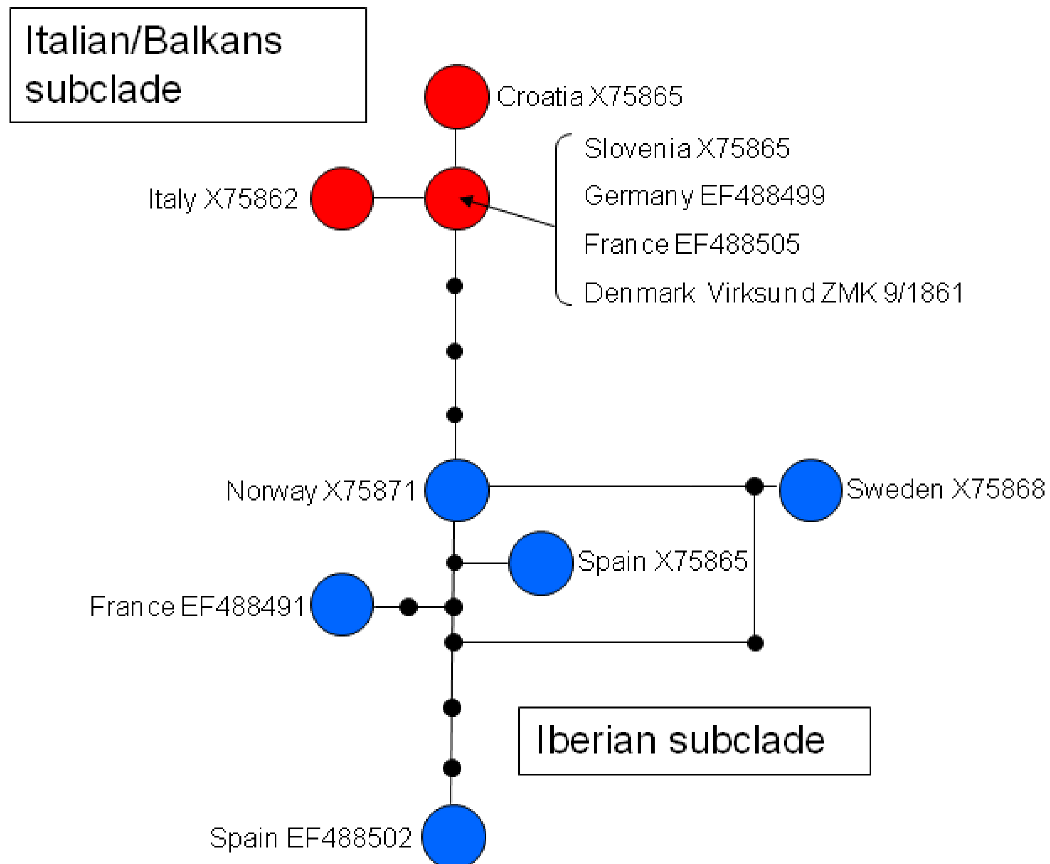

Figure 2.3 Haplotype network of western European bears (clade 1) based on 193-bp control region mtDNA. Sequences from modern and Holocene western European bears (clade 1) have been included to demonstrate that the ancient Virksund specimen from Denmark falls within the Italian/Balkans group in contrast to the modern Norwegian and Swedish samples which belong to the Iberian group. The Virksund sequence is identical to three previously published Italian/Balkans sequences: Slovenia X75865, Germany EF488499 and France EF488505. Haplotypes have been coloured according to their putative refugial origin – Italian/Balkans (blue) and Iberian (red). Previously published sequences are named according to country of origin and GenBank Accession number.

2.5 DISCUSSION

The 19 Holocene brown bear samples from limestone cave deposits in northern Norway all belong to the eastern clade 3a. The extraordinarily low level of genetic diversity found in these Holocene specimens (89% of sequences were identical, with only two individuals showing variation in 1-2 bp of the 135bp hypervariable CR fragment) is comparable with modern populations (all modern northern Norwegian sequences are identical) and suggests that the eastern clade 3a has been present in northern Scandinavia for at least the last 6210 ± 100 C^{14} ybp. These results imply that the current distribution and lack of genetic diversity of clade 3a in northern Scandinavia are the result of an ancient founder event during the post-glacial colonisation of Scandinavia from Eurasia, rather than an artefact of the recent anthropogenic bottleneck.

It was not possible (due to a lack of well preserved cave sites containing Holocene bear remains in southern Norway) to date the arrival of the western clade via the Danish-Swedish

land-bridge route (Björck, 1995; Björck, 1996), nor to determine whether the eastern 3a clade previously extended further south than the current contact zone. However, the western lineage was present in southern Scandinavia (Virksund, Denmark) at least as early as 5310 ± 20 C^{14} ybp. This finding supports the hypothesis that bears of the western lineage colonised southern Scandinavia during the Late Glacial-Boreal timeframe via the southern land-bridge following the retreat of the ice caps (Björck, 1996; Hewitt, 1999; Sommer & Benecke, 2005).

It is also worth noting that the recolonisation of Scandinavia via the southern route was unlikely to be a single event, so the presence of more than one haplogroup should not necessarily be surprising. While southern Sweden was connected to the Danish peninsula via a land-bridge for approximately 400 years from 11,200 ybp and again for approximately 1,100 years from 10,300 ybp (Björck & Møller, 1987; Björck, 1995; Jaarola *et al.*, 1999), island-hopping or ice-crossings at other times may have been plausible for mobile large mammals such as brown bears (Heaton *et al.*, 1996). An additional colonisation route between the North Sea Continent (Doggerland) and ice-free areas on the west and north Norwegian coasts may have been possible as early as 13,000 ybp (Houmark-Nielsen, 1989). The first appearance of post-LGM bears in Scandinavia has been documented from Allerød deposits (dated to c. 13.4 cal. kyr BP) in Denmark (Aaris-Sørensen; unpublished data). Staggered local extinctions have been seen on the Island of Sjælland (Zealand) and Fyn (Funen) approximately 8-7 cal. kyr BP, and on the Jylland (Jutland) Peninsula in northern continental Europe c. 4.8-4.4 cal. kyr BP (Aaris-Sørensen; unpublished data), potentially providing an opportunity for genetic turnover or increased diversity in subsequent recolonising populations.

To date, all mtDNA sequences reported from southern Scandinavian bears are from the Iberian subclade of the western lineage. It has been postulated that bears from the Iberian refuge recolonised central Europe and southern Scandinavia directly following the end of the LGM (Taberlet & Bouvet, 1994; Björck, 1996; Hewitt, 1999; Sommer & Benecke, 2005), while the bears of the Italian/Balkans refugia were unable to spread northwards during this same period, trapped behind the ice caps of the Alps (Taberlet & Bouvet, 1994; Hewitt, 1999). Here we present the first known record of a bear of the Italian/Balkans subclade in Scandinavia, (Virksund, ZMK9/1861) dated to 5310 ± 20 C^{14} ybp. This finding not only represents the most northerly record of an Italian/Balkans haplotype in Europe, but also suggests that the recolonisation of southern Scandinavia by the western lineage was not limited to bears of the Iberian refugium.

Bears of the Italian/Balkans subclade have previously been described from Germany 5210 ± 35 C^{14} ybp (Figure 1) and southern France 3445 ± 40 C^{14} ybp (Valdiosera *et al.*, 2007), however the specimen from Denmark predates these. These suggest that the Italian/Balkans lineage recolonised large parts of Europe prior to or coincident with the Iberian lineage. Support for the existence of cryptic refugia in central and northern Europe is also increasing (Stewart & Lister, 2001), and while most evidence is based on plant studies (Willis *et al.*, 2000; Willis & van Andel, 2004; Magri *et al.*, 2006; Naydenov *et al.*, 2007), it might be worth considering whether the presence of temperate refugia in central or northern Europe could help explain the ability of some vertebrate taxa such as brown bears to recolonise Scandinavia.

The presence of an Italian/Balkans haplotype in Holocene southern Scandinavia, where only the Iberian haplogroup is found in modern populations, does suggest dynamic mtDNA turnover in bear populations similar to those reported for ancient Beringian brown bears (Barnes *et al.*, 2002), and in contrast with the current stasis of mtDNA populations in northern Norway. However it is not yet possible to differentiate between several alternative scenarios. It is not clear whether members of both the Iberian and Italian/Balkans lineages co-existed in

southern Scandinavia during the Holocene, with the Italian/Balkans haplogroup extirpated during the predator removal programs of the mid-1800s, or whether the Italian/Balkans haplogroup in southern Scandinavia was locally replaced by members of the Iberian haplogroup sometime earlier during the Holocene post-glacial recolonisation. Further analysis of historic material from bears collected during the last 200 years will be required to address this issue.

Although it is widely accepted that all modern southern Scandinavian bears belong to one of two Iberian haplotypes (X75868 and X75871) (Taberlet & Bouvet, 1994), it is possible that bears possessing the Italian/Balkans haplogroup do in fact survive in southern Scandinavia today. Recent studies (Taberlet *et al.*, 1995; Waits *et al.*, 2000) have used methods capable only of differentiating between the eastern (clade 3a) and western (clade 1) lineages (Taberlet *et al.*, 1995; Waits *et al.*, 2000), and in most cases cannot identify subclades within the western lineage. Reanalysis of the mtDNA control region of modern Scandinavian clade 1 bears using direct sequencing methods may reveal the persistence of the Italian/Balkans haplogroup in modern populations.

The data presented here supports the general hypothesis that Scandinavia was recolonised from the north by bears of the eastern clade 3a and from the south by bears of the western lineage following the end of the LGM (Taberlet & Bouvet, 1994; Taberlet *et al.*, 1995; Hewitt, 1999; Sommer & Benecke, 2005), and suggests that the contact zone and low mtDNA diversity is a signature of post-glacial population processes rather than an artefact of recent human activity. Similar post-glacial recolonisation routes resulting in contact zones between northern and southern populations have been described for other Scandinavian taxa, for example common shrews (Narain & Fredga, 1996), bank voles (Tegelström, 1987) and European field voles (Jaarola & Tegelström, 1995). The lack of diversity in northern Scandinavian populations appears to be a feature not only of modern populations but throughout at least the last 6000 years.

The situation in southern Scandinavia appears to be more complex than previously realised, and this study reinforces the important role that ancient DNA can play in understanding the population dynamics and post-glacial movements of taxa through time. It stresses once again that the use of modern data alone is likely to lead to over-simplified or inaccurate views of past evolutionary history. Ancient DNA studies continue to help produce a clearer picture of the post-glacial recolonisation of European biota, and may also have impacts on future conservation efforts (Leonard, 2008) by highlighting diversity that may have remained hitherto undetected in modern populations.

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CHAPTER 3

FROM IBERIA TO ALASKA: ANCIENT DNA LINKS LATE PLEISTOCENE BROWN BEARS ACROSS THE HOLARCTIC

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Performed initial phylogenetic analysis to identify the phylogenetic position of the Aseiko sample within global brown bear haplogroups. Co-wrote the manuscript, edited figures and acted as corresponding author.

I hereby certify that the statement of contribution is accurate.

Signed……………………………………………………………………………Date…………………

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Performed all the subsequent analyses. Co-wrote the manuscript, created figures and acted as corresponding author.

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Supervised coalescent simulations and dating analyses.

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Signed…………………… …………………Date…08/01/2009……

CATHERINE HANNI

I shared some results of our lab with the other authors, and I gave some comments on the manuscript.

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CHAPTER 3

FROM IBERIA TO ALASKA: ANCIENT DNA LINKS LATE PLEISTOCENE BROWN BEARS ACROSS THE HOLARCTIC

3.1 SUMMARY

Studies of European taxa have linked modern phylogeographic patterns with late Pleistocene glacial cycles suggesting restriction to, and subsequent expansion from, a small number of southern refugia (Hewitt, 1996; Hewitt, 1999). The striking mitochondrial (mtDNA) phylogeographic structure observed in European brown bears (*Ursus arctos*) has been used to support this contraction/expansion model (Hewitt, 1996; Hewitt, 1999). Modern brown bear mtDNA distributions are consistent with post-glacial recolonisation from the Iberian and Italian/Balkan peninsulas (western clade 1) and western Russia (eastern clade 3a) (Taberlet & Bouvet, 1994; Sommer & Benecke, 2005; Saarma *et al.*, 2007). However, ancient DNA studies have revealed a more complex and dynamic picture, with evidence of population connectedness between separate European refugia (Valdiosera *et al.*, 2007; Valdiosera *et al.*, 2008) at odds with the strict contraction/expansion model (Hewitt, 1996; Hewitt, 1999). The report of an apparently eastern European clade 3a bear in Iberia (Asieko, 40-80 kya, EU400180) strengthens the argument for a continuity of putative refugia across southern Europe prior to the last glacial maximum (LGM) and represents the most geographically distant group to have genetically influenced the Iberian population (Valdiosera *et al.*, 2008). We have reanalysed 601 mtDNA sequences from modern and ancient brown bears across their historical and current range to reveal that the sequence obtained from a pre-glacial Iberian bear (Asieko, 40-80 kya, EU400180) is of far greater significance than realised – falling within an apparently extinct clade, 3c, previously known only from Beringia prior to 35 kya.

3.2 RESULTS AND DISCUSSION

Median-joining network (Figure 3.1), maximum likelihood and Bayesian analyses (Appendix Figure 2.1) of the 601 global brown bear mtDNA sequences available for the 177bp mtDNA control region sequence clearly place the pre-glacial Iberian bear (Asieko, 40-80 kya, EU400180) within clade 3c, formerly only known from Alaska prior to 35 kya. This clade is characterised by a unique T-C SNP at position 16,882 of the brown bear mt genome (AF303110). The position of the Asieko sequence suggests that rather than being a localised Beringian clade, the 3c haplogroup was found more widely (but not necessarily continuously) across the northern hemisphere during the Pleistocene. These analyses provide the first genetic evidence to link Pleistocene brown bears of North America with lineages found in Western Europe, and exhibit a similar pattern to the current distribution of clade 3a bears from eastern Europe to North America (Figure 3.2). Our results suggest a pre-glacial turnover of brown bear mtDNA lineages across their Holarctic range further highlighting the complexities of inferring past population events from modern mtDNA phylogeographic patterns (Barnes *et al.*, 2002). This echoes the recent finding of a completely new haplogroup in ancient North African brown bears with no extant equivalent (clade 7 in Figure 3.1 and 3.2; Calvignac *et al.*, 2008).

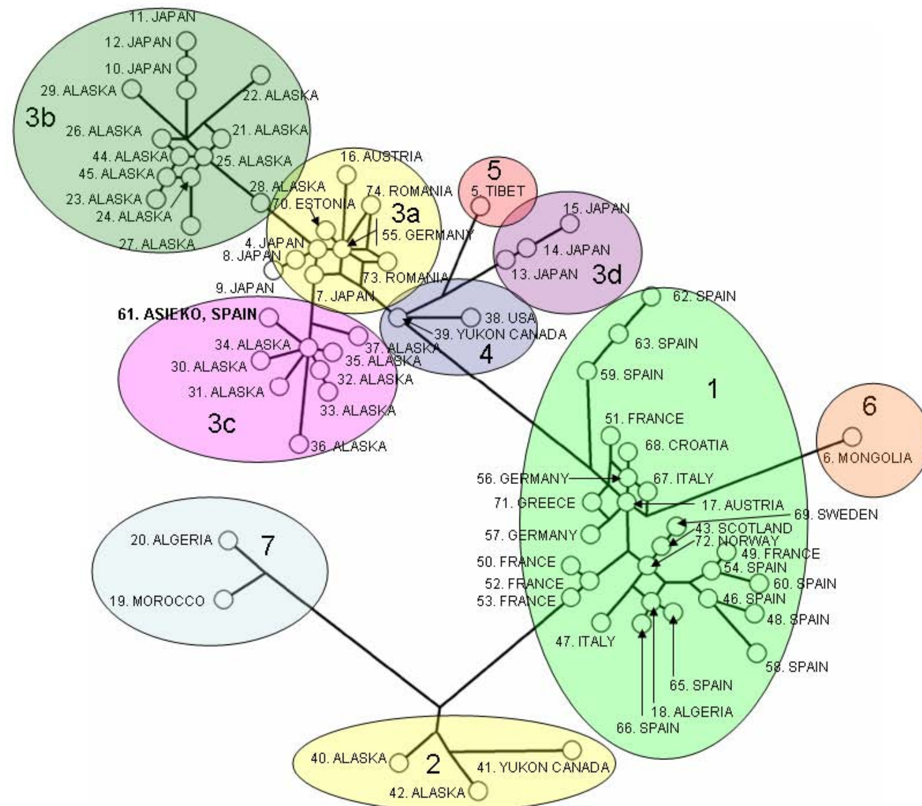

Figure 3.1 Median-joining network of global brown bear mtDNA control region haplotypes. The network was produced from the dataset of haplotypes containing the 177-bp control region sequence. Haplotype and sample details are provided in Appendix Table 2.1. Clade numbering system is as per (Barnes *et al.*, 2002; Miller *et al.*, 2006), The Asieko bear (EU400180, haplotype 61) falls clearly within the clade 3c.

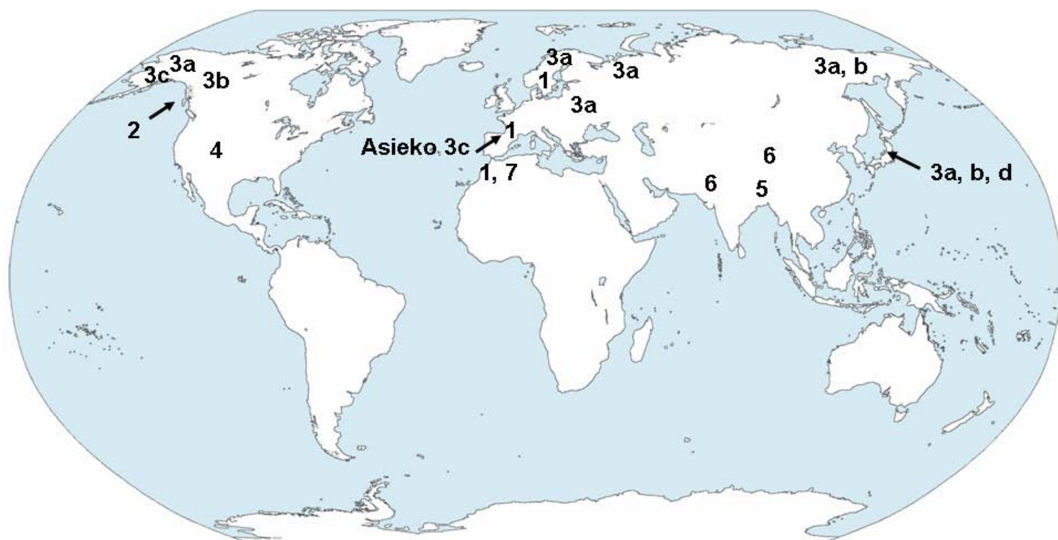

Figure 3.2 Map showing the location of the Asieko 3c individual. The approximate geographical distribution of other brown bear mtDNA clades have been included to highlight the potentially similar distribution of Pleistocene 3c to modern 3a. Figure adapted from (Miller *et al.*, 2006).

The Bayesian analysis of the global brown bear dataset (including 54 dated bones) generated a new mutation rate estimate (64.68% substitutions/site/million years), which is considerably faster than those used in (Valdiosera *et al.*, 2008) (i.e. 10% (Saarma *et al.*, 2007) and 29.8% (Taberlet & Bouvet, 1994) substitution per site per million years). Divergence dates for each clade of the brown bear phylogeny were calculated using this new rate (Table 3.1), accepting that it is likely to represent an average due to the effect of rate curves (Ho *et al.*, 2008). Interestingly, this analysis suggests that the Iberian subgroup of the western European lineage (clade 1) originated around 35 kya, at around the same time as the most recent 3c bear specimens in both Beringia and Iberia. Additional bear remains >35 kya can be tested to confirm that this was a period of turnover, with 3c being replaced by the 'western' clade 1. Further sampling of modern and ancient populations across Eurasia is required to determine whether clade 3c had a broader distribution and to confirm it as an extinct late Pleistocene lineage.

Table 3.1 Divergence dates of brown bear clades. BEAST analysis of the estimated divergence times for each clade. Samples were drawn every 10,000 MCMC steps from a total of 100 million steps, after a burn-in of 25 million steps was discarded. Divergence dates reported are an average with a [95% lower – 95% upper] confidence interval. The nodes A-F reported here correspond to Appendix Figure 2.1.

Divergence Date	Confidence interval	Nodes	Clades
88, 510	[69, 680 – 109, 100]	F	3a 3b 3c
74, 880	[63, 390 – 87, 460]	E	3c
101, 700	[75, 080 – 131, 300]	D	3a 3b 3c 3d 4 5
35, 590	[23, 120 – 49, 680]	C	1 (Iberian clade)
116, 000	[87, 910 – 154, 400]	B	1 (Iberian, Italian/Balkan clade)
206, 900	[144, 100 – 278, 900]	A	Origin of brown bear diversity

The presence of the single highly divergent clade 3c sequence in Iberia does not affect the previously reported demographic reconstructions of Valdiosera *et al.* (2008). Our reanalysis of serial coalescent simulations of the brown bear mtDNA dataset either including or excluding the Asieko individual supports the original conclusion of a population bottleneck at the Pleistocene-Holocene transition (as long as the effective population size was >1,000-2,000 individuals; Appendix Table 2.2).

Combined with the previously published evidence of geneflow between the Iberian and Italian/Balkan peninsulas (Valdiosera *et al.*, 2007; Valdiosera *et al.*, 2008), this new result indicates that the dynamic nature of Pleistocene brown bear population genetics was not a phenomenon limited to Beringia (Barnes *et al.*, 2002) but was also occurring simultaneously in southern Europe, and may even have been taking place on a global scale. Importantly, our results are further evidence that it is only possible to extrapolate the structure of past populations from modern genetic data to a very limited extent. Ancient DNA analyses are

clearly indispensable to fully understand animal population dynamics over time, and studies of additional taxa are urgently needed to determine whether such dynamic evolutionary histories are common.

3.3 EXPERIMENTAL PROCEDURES

All brown bear sequences available in Genbank for the mtDNA control region (CR) were aligned manually, and those exhibiting less than 177-bp of sequence information were removed. The sequence dataset was reduced to the 71 unique haplotypes using Fabox 1.32 (Villesen, 2007) to avoid redundancy, and each haplotype was annotated with the Genbank accession number, geographic origin and the age of each specimen. Aligned sequences were used to construct a median-joining network (Network; Bandelt *et al*., 1999), maximum-likelihood (PHYML online; Guindon & Gascuel, 2005) and Bayesian trees (MrBayes 3.1.2; Huelsenbeck *et al.*, 2001) with the GTR+G4 model of molecular evolution identified as the best fit by the AIC criterion of Modeltest (Posada & Crandall, 1998). Three cave bear sequences were used as outgroups (Genbank accession numbers: AY149268, AY149271, AY149273). The strength of the phylogenetic signal was assessed via nonparametric bootstrapping (1000 pseudo-replicates for ML analyses) and posterior probabilities (25 million generations, sampled every 5,000 generations, with a burn-in value of 1250). The known ages of the different brown bear sequences present in the dataset were used to re-estimate the substitution rate of the 177-bp section of the control region using BEAST 1.4.6 (http://beast.bio.ed.ac.uk/). When sequences of the same haplotype were obtained from specimens with different ages, the haplotype was duplicated in the dataset and minimal and maximal ages were given to the two duplicates, respectively. A global constant demographic model was selected for the worldwide brown bear population. A strict clock model was rejected by Likelihood Ratio Test using PAML4 (LRT = 192.37, df = 72, p-value $< 10^{-3}$; (Yang, 2007), http://abacus.gene.ucl.ac.uk/software/paml.html). Bayesian analyses were performed using BEAST with a relaxed-clock framework (Drummond *et al.*, 2006; Drummond & Rambaut, 2007). Mutation rates were assumed *a priori* to be uncorrelated between neighbouring branches and to follow a lognormal distribution (Drummond *et al.*, 2006). Analyses were also performed assuming an exponential distribution and led to highly similar results (data not shown). The posterior distribution of the substitution rate (64.7% substitutions/site/MY, with a 95% Posterior Distribution of 36.8% - 96.5% substitutions/site/MY) was estimated by Markov chain Monte Carlo (MCMC) sampling in BEAST. Samples were drawn every 10,000 MCMC steps from a total of 100 million steps, after a burn-in of 25 million steps was discarded. Tracer (http://beast.bio.ed.ac.uk/Tracer) was used to inspect the posterior samples and confirm convergence to a stationary distribution and sufficient sampling. The new substitution rate estimate is significantly higher than that reported in (Saarma *et al.*, 2007), but in agreement with more recent analyses (39% in Ho *et al.*, 2008, or 69% in Ho *et al.*, 2007). The divergence times of the different nodes were estimated using BEAST with the new rate (using a normal distribution) as above. Finally, the serial coalescent analyses of (Valdiosera *et al.*, 2008) were re-evaluated using the new rate estimate assuming either a possible age of 40 kya or 80 kya for the Asieko specimen, or removing this specimen from the analysis entirely. The gene diversity of the Spanish brown bear population for the Pleistocene period was estimated with Arlequin 3.1 (Schneider *et al.*, 2000), following the methods described in (Valdiosera *et al.*, 2008).

3.4 ACKNOWLEDGEMENTS

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CHAPTER 4

POST-GLACIAL PHYLOGEOGRAPHY OF EUROPEAN BROWN BEARS

4.1 INTRODUCTION

Late Quaternary fluctuations in climate are believed to have played an important role in shaping the genetic diversity, distribution and phylogeographic structure of many plant and animal species. The most prevalent model used to explain the current phylogeographic patterns in Europe is the Expansion/Contraction (E/C) model, championed by Godfrey Hewitt since the 1990‟s (Hewitt, 2000; Hewitt, 1996; Hewitt, 1999; Hewitt, 2004). The E/C model proposes repeated cycles of range contraction into temperate southern refugia (in the Iberian, Italian and Balkans peninsulas) during periods of glacial maxima, followed by a northward range expansion during the warmer interglacials (Hewitt, 1996; Hewitt, 1999; Taberlet *et al.*, 1998) resulting in the formation of genetically distinct refugial populations through allopatric genetic drift. Subsequent recolonisation of central and northern Europe during the interglacials allowed expansion of southern refugial haplotypes, with the populations in recently recolonised regions displaying lower levels of genetic diversity than those in the refugial regions due to founder effects (Hewitt, 1996).

The mtDNA phylogeographic structure of modern European brown bears (*Ursus arctos*) has generally been considered to be consistent with Hewitt‟s E/C model, and in fact was one of the key taxa used to develop the model (Hewitt, 2000; Hewitt, 2001; Hewitt, 1999) based largely on the earlier mtDNA work of Pierre Taberlet and colleagues (1994, 1995). In addition, brown bears are female philopatric and are known to exhibit strong mtDNA phylogeographic structure (Randi *et al.*, 1994; Waits *et al.*, 2000) making them ideal for tracing post-glacial recolonisation histories following the Last Glacial Maximum (LGM; 23,000-18,000 ybp; Hewitt, 1996 and Kukla *et al.*, 2002).

In Europe, modern brown bear populations are split into two divergent mtDNA groups referred to as the „western‟ (Clade 1) and „eastern‟ (Clade 3a) lineages reflecting their approximate distribution on the continent (Kohn *et al.*, 1995; Taberlet, Bouvet, 1994). The „western‟ (Clade 1) bears can be further subdivided into two groups, the „Iberian‟ haplogroup found in Spain, France and southern Scandinavia, and the „Italian/Balkan‟ haplogroup found in Italy and the Balkans (Taberlet, Bouvet, 1994).

The phylogeographic subdivision of bears of the „western‟ (Clade 1) lineage was hypothesised to reflect their glacial isolation in southern refugia in either the Iberian, or the Italian/Balkans peninsulas during the LGM, followed by northerly post-glacial expansion. It has been proposed that bears originating from refugia in the Iberian peninsula would have been able to recolonise central and northern Europe relatively early following the glacial retreat at the end of the LGM, along with bears of the „eastern‟ lineage (Clade 3a) expanding from putative „eastern‟ refugia (Hewitt, 1999; Kohn *et al.*, 1995; Taberlet, Bouvet, 1994), possibly located in the Carpathian Mountains (Saarma *et al.*, 2007; Sommer, Benecke, 2005). Expansion from the Italian and Balkans refugia was assumed to have been impeded by the geographic barrier of the glaciated Alps until after much of Europe was already recolonised by bears of Iberian or eastern (Clade 3) haplogroups (Hewitt, 1999; Kohn *et al.*, 1995; Taberlet, Bouvet, 1994). Contact (or hybrid) zones between the eastern and western lineages have only been detected in Scandinavia (Taberlet *et al.*, 1995) and Romania (Kohn *et al.*, 1995) possibly resulting from the meeting of separate post-glacial recolonisation routes

(Taberlet, Bouvet, 1994; Taberlet *et al.*, 1995). Figure 4.1 gives an overview of the E/C model and post-glacial recolonisation routes proposed by Hewitt.

NOTE:
This figure is included on page 51 of the print copy of the thesis held in the University of Adelaide Library.

Figure 4.1 Hewitt's proposed post-glacial recolonisation routes for European brown bears based on genetic and fossil evidence. Image adapted from Hewitt (1999) with the identity of the mtDNA clades added.

While the E/C model offers a plausible explanation for the current phylogeographic structure observed in brown bears, ancient DNA evidence has recently been put forward to question both the existence of glacial refugia and their genetic isolation, with claims of continuous gene flow between brown bear populations across southern Europe before (Hofreiter *et al.*, 2004), during and after the last glacial maximum (Valdiosera *et al.*, 2008; Valdiosera *et al.*, 2007).

Specifically, Hofreiter *et al.*, 2004 presented two divergent pre-glacial brown bear sequences from the Austrian cave sites of Winden (39,940 ybp) and Ramesch (47,420 ybp). These aDNA sequences revealed that the bear from Winden belonged to the „western" lineage while the bear from Ramesch (approximately 200 km to the west of Winden) belonged to the „eastern" lineage. Although only based on two specimens, they interpreted these results as evidence for a lack of phylogeographic structure prior to the LGM, or at least as representing a different phylogeographic structure than is observed in modern populations.

Valdiosera et al., 2007 presented 21 ancient (post-glacial) European brown bear sequences, 16 of which were dated between 17,440 ± 425 and 350 ± 40 ybp. After combining these ancient sequences with modern sequences from GenBank they reported the presence of an Iberian haplotype in Italy at 16,440 ± 65 ybp and an Italian/Balkan haplotype in Iberia (Spain) at 17,440 ± 425 ybp, as well as identifying previously unrecognized „western" haplogroups in France during the Holocene that did not appear to belong to either Iberian or Italian/Balkans refugial groups. Contrary to the predictions of the E/C model, this was interpreted as being consistent with gene flow between the peninsulas and suggestive that bears were not restricted to southern refugia during the LGM. In addition to this, Valdiosera et al. 2008 presented pre-glacial ancient bear sequences from the Iberian peninsula and serial simulations of population sizes through time from the Pleistocene to the present. From these analyses they concluded

that bears from Iberia were exposed to exotic genetic influences and population size fluctuations throughout the past and only became isolated very recently in the Holocene.

An ancient DNA study of extinct African bears recently revealed not only the presence of a previously undescribed mtDNA clade (Atlas bears), but also detected the first evidence of a western European (Clade 1 – Iberian haplogroup) outside of Europe in Algeria, northern Africa (Calvignac *et al.*, 2008). As discussed by Calvignac and colleagues, it is unclear whether these late Holocene specimens (dated to 1,679 ± 35 and 1,550 ± 40 ybp) arrived in northern Africa as a result of human activities or due to a rare crossing of the Gibraltar Straits. However, the recent discovery of a new clade and expansion of the range of another does highlight the speed at which our understanding of the phylogeographic history of brown bears is changing, and that new discoveries and advances are still being made.

While ancient DNA sequence data combined with radiocarbon dating has the potential to provide a powerful tool for testing the phylogenetic and temporal aspects of post-glacial recolonisation hypotheses, care is needed. For example, a close inspection of the results and subsequent interpretations of Valdiosera *et al*. 2007 and 2008 used to refute the E/C model reveals a number of flaws, indicating that a more thorough re-evaluation of post-glacial European brown bears is critical before dismissing the traditional glacial refugial model. In this chapter I combine previously published modern (Bon *et al.*, 2008; Taberlet, Bouvet, 1994) and ancient (Calvignac *et al.*, 2008; Valdiosera *et al.*, 2007) brown bear sequence data with newly generated sequences from ancient European bears in order to more comprehensively evaluate the post-glacial phylogeography of the „western" lineage (Clade 1).

4.2 MATERIALS & METHODS

4.2.1 Samples

Sub-fossil bone and tooth root samples were collected from 38 post-glacial brown bear specimens (late Pleistocene – Holocene) originating from Germany, Switzerland, Austria, Scotland and Italy. Twenty-one of the samples were also radiocarbon dated to provide temporal data in key locations and time periods. For details of the samples and radiocarbon data please refer to Appendix Table 3.1.

4.2.2 Ancient DNA extractions

Ancient samples were extracted in the dedicated ancient DNA facility at the Australian Centre for Ancient DNA according to the ancient DNA extraction technique described previously (Chapter 2.3.2) and following protocols to minimise contamination by modern and PCR amplified DNA (see Chapter 1, section 1.3.1).

4.2.3 PCR Amplifications

Polymerase chain reaction (PCR) amplifications targeted a hypervariable 135-bp fragment of the mitochondrial control region (CR) using carnivore-specific primers (L16164 and H16299; Hänni *et al.*, 1994) which allow differentiation between the major brown bear lineages (see Barnes *et al.*, 2002). These primers were also chosen to facilitate comparison with previous studies of both modern and ancient Clade 1 (western European) brown bears (Bon *et al.*, 2008; Calvignac *et al.*, 2008; Taberlet, Bouvet, 1994; Valdiosera *et al.*, 2007).

PCR amplifications were performed in 25 µl reactions with 1-2 µl of DNA extract, 1-1.25 U Platinum *Taq* DNA Polymerase High Fidelity and 1X buffer (Invitrogen), 2 mg/ml rabbit

serum albumin (RSA; Sigma), 2 mM $MgSO_4$, 250 µM of each dNTP, and 1 µM of each primer. PCR thermal cycling reactions consisted of 94 °C 1 min, followed by 50-55 cycles of 94 °C denaturation for 15 sec, annealing for 15-20 sec at 55 °C and extension at 68 °C for 30 sec, followed by a final extension at 68 °C for 10 min.

PCR amplification success was assessed following electrophoresis of a 1/10 volume of PCR product on 2 % agarose TBE gels, post stained with ethidium bromide and visualised with UV light in a Dolphin-Doc gel documentation system. Gels included a Hyperladder IV or V DNA ladder to provide size and qualitative concentration estimates. PCR products that yielded bands of the expected size were purified using ExoSAP-IT (Fermentas) or AMPure (Agencourt) according to manufacturer"s instructions. PCR products were sequenced in both directions using Big Dye 3.1 chemistry using the same primers as the PCR and an ABI 3130XL Genetic Analyzer. Sequences were edited and contigs formed using Sequencher 4.7 (Genecodes corporation).

4.2.4 Validation of aDNA results

Two samples (ACAD3647, Switzerland, 9,700 ± 90 ybp and ACAD3651, Austria, 2,935 ± 35 ybp) were independently replicated in a second ancient DNA facility (Ludovic Orlando, unpublished data). The replication included independent ancient DNA extractions using a silica-based method (essentially as described in Rohland and Hofreiter, 2007), multiple independent PCR amplifications and cloning. Consensus sequences were created from the clones and compared to the original sequences obtained via direct sequencing in Adelaide.

4.2.5 Phylogeographic analyses

All Clade 1 (western European lineage) brown bear sequences available in GenBank encompassing at least 131-bp homologous mtDNA control region fragment were aligned manually in MEGA 4 (Tamura *et al.*, 2007) with the Clade 1 sequences generated in this study and in Chapter 2 of this thesis. The sequences generated in this study are 135-bp in length, however a number of relevant DNA sequences from ancient European bears deposited in GenBank (for example Valdiosera *et al.*, 2007) only incorporate 131 out of 135-bp of homologous sequence. The additional four bases in my sequences occur directly after the end of the primer sequence and reflect the high quality of the direct sequencing method employed. These first four base pairs in my dataset are invariable between individuals, therefore it was decided to limit the phylogeographic analyses to the shorter homologous 131-bp. Some Western European brown bear sequences available on GenBank could not be included as they were either from a different mitochondrial region or did not incorporate the full 131-bp fragment and so would reduce the strength of the analyses (for example Hoss & Pääbo, 1993, Kohn et al., 1995 and Valdiosera *et al.*, 2008). Sequences known to originate prior to the last glacial maximum (e.g. AJ809334 Winden, Austria 39,940 ybp) (Hofreiter *et al.*, 2004) were removed from the analysis to avoid artificially increasing genetic diversity in the dataset or interfering with reconstructions of post-LGM phylogeographic relationships. One previously published DNA sequence (Barnes et al 2002, RSM1962/63, GenBank accession: AY082845, late Holocene, Bear Cave, Scotland), is thought to originate from one of the specimens extracted in this study (ACAD338 or 339), and was therefore also excluded to avoid duplication.

The remaining 108 aligned sequences of 131-bp (including sequences generated in this study and those from GenBank) were used to construct a statistical parsimony haplotype network using the program TCS (Clement *et al.*, 2000) with a 95% connection limit. As the aligned sequences did not contain any insertions or deletions, the decision to treat gaps as missing

data in TCS would have no impact on the analysis. The 108 sequences used in the network analysis were collapsed into unique haplotypes using Fabox 1.35 (http://www.birc.au.dk/fabox/).

4.2.6 Statistical genetic measures

Standard measures of genetic diversity (haplotype diversity, nucleotide diversity, and mean pairwise differences) were calculated in DnaSP (Rozas *et al.*, 2003) and Arlequin (Schneider *et al.*, 2000) separately for western (Clade 1) Iberian haplogroup and western (Clade 1) Italian/Balkans haplogroup individuals. Tajima‟s D, Fu and Li‟s F* and D* and Fu‟s FS test statistics were also calculated for both groups in DnaSP (Rozas *et al.*, 2003) to test for signs of neutrality and demographic expansion.

Table 4.1 List of new and previously published post-glacial (<18,000 ybp) Clade 1 (Western European lineage) bear sequences included in the analysis. Sequences listed by haplotype number based on alignment of the 135-bp control region mtDNA fragment. Only those previously published sequences which included the corresponding 131-bp fragment were included. Genbank accession numbers are listed for previously published sequences. Sequences with ACAD numbers were generated in this study. Ages given as „Modern", „Holocene" (undated) or in uncalibrated radiocarbon years before present (ybp). Haplotype numbers also correspond to those used in the TCS haplotype network (Figure 4.2).

Haplotype	Sample name	Geographic location	Age	Haplogroup	GenBank Accession	Number of individuals†
1	Guzet	Guzet shelter, Ariege, France	Modern	Iberian	EU497665	1
2	AK02	Akouker cave, Algeria	1,550 ± 40	Iberian	AM411398	1
	AK01	Akouker cave, Algeria	1,679 ± 35	Iberian	AM411397	1
	Can1	Cantabria, Spain	Modern	Iberian	X75866	1
	Can	Cantabria, Spain	Modern	Iberian	X75865	1
3	Mv4 Remanie	Mont Ventoux, France	3,445 ± 40	Italian/Balkans	EF488505	1
	A12	Wysburg, Germany	XII-XIV century	Italian/Balkans	EF488500	1
	A9	Bad Frankenhausen, Germany	5,210 ± 35	Italian/Balkans	EF488499	1
	Slo	Slovenia	Modern	Italian/Balkans	X75877	34
	ACAD103	Brunnenschacht, Austria	Holocene	Italian/Balkans		1
	ACAD107	Seekarlhohle, Austria	Holocene	Italian/Balkans		1
	ACAD109	Notentalhohle, Austria	5,325 ± 40	Italian/Balkans		1
	ACAD112	Burianhohle, Austria	Holocene	Italian/Balkans		1
	ACAD113	Barenkammer, Austria	Holocene	Italian/Balkans		1
	ACAD114	Grubenlocher, Austria	1,805 ± 35	Italian/Balkans		1
	ACAD116	Rabenmaurhohle, Austria	3,643 ± 41	Italian/Balkans		1
	ACAD118	Gemsenhohle, Austria	Holocene	Italian/Balkans		1
	ACAD120	Windorgel, Austria	3,321 ± 40	Italian/Balkans		1
	ACAD123	Barenloch, Austria	2,485 ± 20	Italian/Balkans		1
	ACAD127	Gemsenhohle, Austria	3,130 ± 35	Italian/Balkans		1
	ACAD3643	Allander Tropfsteinhohle, Austria	10,870 ± 80	Italian/Balkans		1
	ACAD3645	Neue Laubenstein-Barenhohle, Germany	11,872 ± 92	Italian/Balkans		1
	ACAD3649	Barengraben hintersilberen, Switzerland	3,275 ± 50	Italian/Balkans		1

Haplotype	Sample name	Geographic location	Age	Haplogroup	GenBank Accession	Number of individuals†
3	ACAD3651	Feistringhohle, Austria	2,935 ± 25	Italian/Balkans		1
	ACAD3931	Virksund, Denmark	5,310 ± 20	Italian/Balkans	(Chapter 2)	1
4	Atapuerca	Atapuerca, Spain	17,440 ± 425	Iberian	EF488504	1
5	C.Motas 33-1	Cuevas del Somo, Spain	4,624 ± 45	Iberian	EF488503	1
	GEE	Cuevas del Somo, Spain	5,380 ± 45	Iberian	EF488490	1
	Vallecampo	Cuevas del Somo, Spain	7,500 ± 55	Iberian	EF488487	1
6	C.Motas 33-2	Cuevas del Somo, Spain	-----------	Iberian	EF488502	1
7	A3	Dienstedt, Germany	1,665 ± 35	Italian/Balkans	EF488501	1
	Bul	Bulgaria	Modern	Italian/Balkans	X75864	8
8	Mv4 L6 714	Mont Ventoux, France	4,645 ± 40	Italian/Balkans	EF488496	1
9	HEM	Navacepeda, Spain	350 ± 40	Iberian	EF488497	1
	Mv4 K3 99	Mont Ventoux, France	1,570 ± 35	Iberian	EF488495	1
	Mv4 M5 162	Mont Ventoux, France	1,790 ± 55	Iberian	EF488491	1
	Pyr	Pyrenees, France	Modern	Iberian	X75878	4
10	Mv4 L6 851	Mont Ventoux, France	6,525 ± 50	Italian/Balkans	EF488494	1
	ACAD117	Napflucke, Austria	9,985 ± 57	Italian/Balkans		1
	ACAD130	Laufenberg, Austria	9,810 ± 70	Italian/Balkans		1
	ACAD3646	Grotta d'Ernesto, Italy	11,900 ± 33	Italian/Balkans		1
	ACAD3647	Hohle 92/2 Barenloch, Switzerland	9,700 ± 80	Italian/Balkans		1
11	Mv4 Mr-204-48	Mont Ventoux, France	1,750 ± 30	Italian/Balkans	EF488493	1
12	Mv4 L5 1184	Mont Ventoux, France	3,845 ± 40	Italian/Balkans	EF488492	1
13	Asturias	Cantabria Cave, Spain	-----------	Iberian	EF488489	1
14	Gbcm2	Grotta Beatrice Cenci, Italy	16,440 ± 65	Iberian	EF488488	1
15	Nor	Norway	Modern	Iberian	X75871	1
16	Dal	Dalarna, Sweden	Modern	Iberian	X75868	7
	ACAD388	Bear Cave, Scotland	2,673 ± 54	Iberian		1
	ACAD389	Bear Cave, Scotland	2,673 ± 54	Iberian		1
17	Cro	Croatia	Modern	Italian/Balkans	X75867	1
18	Abr	Abruzzo, Italy	Modern	Italian/Balkans	X75862	1

Haplotype	Sample name	Geographic location	Age	Haplogroup	GenBank Accession	Number of individuals†
19	ACAD115	Barenhohle, Austria	2,264 ± 40	Italian/Balkans		1
20	ACAD121	Schoberbergschacht, Austria	Holocene	Italian/Balkans		1
	ACAD126	Knochenrohre, Austria	Holocene	Italian/Balkans		1
	ACAD128	Turkenloch, Austria	Holocene	Italian/Balkans		1
21	ACAD3644	Wolfhohle, Austria	6,615 ± 45	Italian/Balkans		1
22	ACAD3650	Barengraben Hintersilberen, Switzerland	4,135 ± 50	Italian/Balkans		1
23	Gre	Greece	Modern	Italian/Balkans	X75870	1

† Some GenBank Accession numbers represent multiple individuals possessing an identical haplotype. Where more than one individual is known to be accessioned under the one GenBank number the frequency is indicated and taken into account for genetic diversity estimates.

4.3 RESULTS

I successfully obtained 135-bp control region mtDNA sequence from 28 of 38 post-glacial European brown bears (Appendix Table 3.1). Twenty-seven of these bears belong to the western European lineage (Clade 1), while a single sample (ACAD119, Barenloch, Austria, 1,985 ± 35 ybp) belongs to the eastern European lineage (Clade 3a). The sequence obtained for this sample is identical to a common eastern haplotype („Rus" Sweden, GenBank X75874) that was also detected in ancient northern Norwegian bears (Chapter 2; Figure 2.2).

Radiocarbon dates were available or were generated for 21 of the 28 samples from which DNA was successfully extracted to provide important temporal data. Of these 21 samples, four were dated at the Institut für Radiumforschung und Kernphysik (VERA) in Austria, four others were dated at the Erlangen AMS Facility (ERL) in Germany and another sample was dated at two independent laboratories - once at the University of California Irvine (UCI) facility and again at the University of Colorado INSTARR Laboratory for AMS Radiocarbon Preparation and Research (CURL). The remaining twelve samples were dated previously by collaborators or as part of other studies. Radiocarbon dates and references for all twenty-one samples are provided in Appendix Table 3.1. All radiocarbon dates discussed in this study are presented in uncalibrated radiocarbon years before present (ybp), where „present" refers to the calendar year 1950 (van der Plicht, Hogg, 2006), unless otherwise stated. Appendix Table 3.2 provides calibrated dates converted from the uncalibrated radiocarbon years before present using the CalPal_2007_HULU calibration curve implemented through CalPal online (www.calpal-online.de/).

Two samples were independently replicated in a second ancient DNA facility (ACAD3647, Switzerland and ACAD3651, Austria). Both samples were independently extracted, amplified in three separate PCR reactions, then cloned. A total of 22 clones were sequenced from ACAD3647, and 21 from ACAD3651 (Ludovic Orlando, unpublished data). The consensus sequences of the clones were identical to the sequences obtained in Adelaide through direct sequencing (see Appendix 3 for details of the independent replication).

From the 108 post-glacial Western European (Clade 1) bear sequences (27 generated in this study, 81 obtained from GenBank) 23 unique haplotypes were identified. The statistical parsimony network (Figure 4.2) revealed that these 23 haplotypes can be divided into two groups separated by one fixed substitution (A/G) at position 16,762 (in relation to *U. arctos* mt genome AF303110). This substitution differentiates between bears of two previously identified haplogroups corresponding to putative glacial refugia in the Iberian and Italian/Balkans peninsulas (Hewitt, 1996; Hewitt, 1999; Taberlet, Bouvet, 1994). Bears from the Iberian haplogroup possess an "A" at this site, while those with Italian/Balkans haplogroup possess a "G".

Based on this diagnostic single nucleotide polymorphism (SNP), 29 samples belong to the western (Clade 1) Iberian haplogroup and 79 bears belong to the western (Clade 1) Italian/Balkans haplogroup. Of the 27 new sequences generated in this study belonging to the Western (Clade 1) lineage, 25 fall within the Italian/Balkans haplogroup while two samples (ACAD338 and 339) were placed within the Iberian haplogroup.

Only one sample (Abruzzo Gbcm2 Italy, EF488488, 16,440 ± 65) out of 108 analysed was found to possess a SNP from a refugial population different to that expected based on geographical location. This sample is reported to originate from Italy (Valdiosera *et al.*, 2007), yet it possesses the Iberian SNP. Another sample (Atapuerca, Spain, EF488504,

17,440 ± 425) previously reported to be evidence of an Italian/Balkans haplotype in Spain (Valdiosera *et al.*, 2007) was found to possess the Iberian SNP.

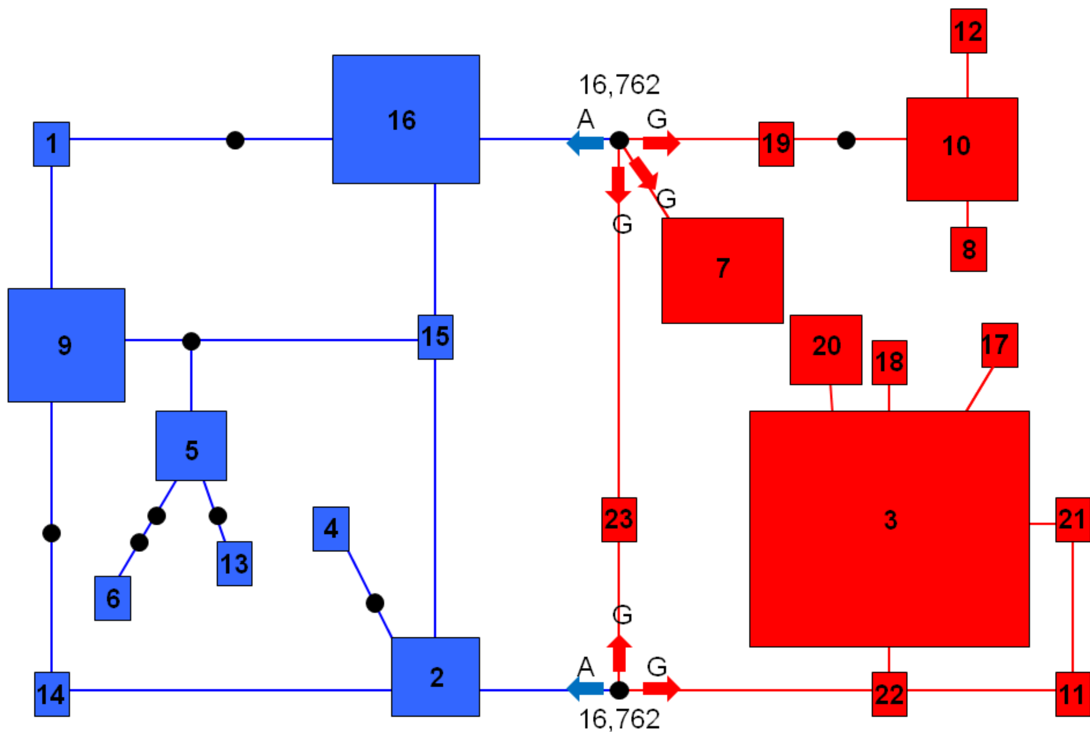

Figure 4.2 Haplotype network of Western European (Clade 1) brown bears. Statistical parsimony network created using TCS, based on 131-bp CR mtDNA sequence from 108 sequences, including 27 new sequences (this study) and 81 obtained from GenBank. Numbers indicate haplotype number (see Table 4.1 for haplotype list). The sizes of the boxes are proportional to the number of individuals sharing that haplotype. All sequences of Iberian haplotypes (blue boxes) possess an "A" at position 16,762 (in relation to *U. arctos* mtDNA genome AF303110), while sequences of Italian/Balkans haplotypes (red boxes) possess a "G" at this site. Unsampled/missing haplotypes are indicated by small black circles.

Table 4.2 Genetic diversity measures for 108 post-glacial western European (Clade 1) brown bears based on 131-bp control region mtDNA sequences. Calculations performed in DnaSP (Rozas *et al.*, 2003) and Arlequin (Schneider *et al.*, 2000).

Group	**No. of samples***	**No. of haplotypes**	**Mean pairwise differences**	**Haplotype diversity (h) ± SD**	**Nucleotide diversity (π) ± SD**
Iberian	29	10	2.487685± 1.382226	0.837 ± 0.043	0.01899 ± 0.00200
Italian/Balkans	79	13	1.446284 ± 0.889410	0.537 ± 0.065	0.01071 ± 0.00730

* Sample size is inclusive of the multiple individuals accessioned under some GenBank numbers as indicated in Table 4.1

The statistical parsimony analysis of post-glacial European brown bears also revealed that the three „unique" haplotypes (EF488492, EF488494 and EF488496; Valdiosera et al 2007) of Mont Ventoux in southern France reported to fall between the Iberian and Italian/Balkans groups, all possess a "G" at the SNP position 16,762, placing them within the Italian/Balkans haplogroup (Figure 4.2 and Table 4.1).

Bears of the Clade 1 Italian/Balkans haplogroup were present in central/northern Europe at least as early as 11,872 ± 92 in Germany (ACAD3645), 10,870 ± 80 (ACAD3643) and 9,985 ± 57 (ACAD117) in Austria, 9,700 ± 80 (ACAD3647) in Switzerland and 11,900 ± 33 (ACAD3646) in northern Italy. With the exception of the single sample discussed previously (Abruzzo Gbcm2 Italy, EF488488, 16,440 ± 65), the earliest record of bears of the Iberian haplogroup outside of the Iberian peninsula occur much more recently, at 2,673 ± 54 ybp in Scotland (ACAD338 and 339) and at 1,790 ± 55 and 1,570 ± 35 in southern France (EF488491 and EF488495; Valdiosera *et al.*, 2007).

Two specimens from a single cave site in Austria (Barenloch) were found to belong to divergent mtDNA lineages: ACAD119 dated to 1,985 ± 35 ybp belongs to the eastern lineage (Clade 3a), and ACAD123 dated to 2,530 ± 20 ybp belongs to western (Clade 1) Italian/Balkans haplogroup.

Nucleotide diversity calculations for post-glacial western European (Clade 1) bears are reported in Table 4.2. The calculations were based on 29 post-glacial bears of the Iberian haplogroup and 79 bears of the Italian/Balkans haplogroup (Refer to Table 4.1). Tajima"s D values and Fu and Li"s F* and D* statistics were not significantly different to zero for either Iberian ($D = -0.07354$ $P > 0.10$, $F^* = -0.61575$ $P > 0.10$, $D^* = -0.72648$ $P > 0.10$) or Italian/Balkans ($D = -0.94653$ $P > 0.10$, $F^* = -0.75484$ $P > 0.10$, $D^* = -0.47251$ $P > 0.10$) haplogroups. Fu"s Fs statistic was also not significant for Iberian ($Fs = -2.299$, $P > 0.05$) or Italian/Balkans ($Fs = -5.545$, $P > 0.05$) haplogroups.

4.4 DISCUSSION

In contrast to the recent hypothesis of continuous gene-flow throughout the last glacial cycle (Valdiosera *et al.*, 2007), analysis of 27 new and 81 existing post-glacial western European (Clade 1) bears is consistent with expansion and recolonisation from at least two distinct southern glacial refugia following the end of the LGM. All 108 post-glacial (ancient and modern) Clade 1 brown bears analysed can be subdivided into two haplogroups based on a single nucleotide polymorphism (SNP) at position 16,762 (in relation to the whole mtDNA genome of *U. arctos* AF303110) corresponding to the putative refugial populations previously identified (Hewitt, 1996; Hewitt, 1999; Taberlet, Bouvet, 1994) in the Iberian and Italian/Balkans peninsulas (Figure 4.2 and Table 4.1).

Two DNA sequences (Atapeurca, Spain, EF488504, 17,440 ± 425 and Abruzzo Gbcm2, Italy, 16,440 ± 65) have previously been used as evidence supporting glacial gene-flow between the southern peninsulas based on the report that the Spanish sample belonged to an Italian/Balkan haplogroup while the Italian sample belonged to an Iberian haplogroup (Valdiosera *et al.*, 2007). However, my reanalysis of these sequences shows that the Spanish sample possesses an „A" at the SNP position 16,762, placing it within the Iberian haplogroup rather than the Italian/Balkan group as previously reported. The hypothesis for continuous gene-flow between the peninsulas during the LGM now relies on a single post-glacial specimen (Abruzzo Gbcm2, Italy, 16,440 ± 65) and becomes less tenable. I propose a number of

alternative explanations that do not contradict the E/C model to explain the presence of an Iberian haplogroup bear in Italy, including limited pre-glacial dispersal of Iberian haplogroup bears into Italy with subsequent lineage extinction during or immediately after the LGM (as predicted by the E/C model), sample misidentification and post-mortem DNA damage. This sample, critical to Valdiosera *et al.*'s argument, was not independently replicated in the original study, and so sample misidentification and post-mortem DNA damage cannot be ruled out. DNA damage is possible because the „A‟ at the SNP position for the Abruzzo Gbcm2 specimen reflects the most prevalent form of ancient DNA damage (G>A and C>T, Brotherton *et al.*, 2007) and unrecognised DNA damage has been shown to negatively impact demographic and phylogenetic analyses (Axelsson *et al.*, 2008).

The three DNA sequences (EF488492, EF488494 and EF488496) from Mont Ventoux, in southern France previously reported to belong to a „unique‟ lineage positioned between the Iberian and Italian/Balkan groups (Valdiosera *et al.*, 2007) were found to possess a „G‟ at the SNP position 16,762, placing them within the Italian/Balkans group. When my new sequences were included in the alignment it also became apparent that the oldest of these three samples (Mv4 L6 851, EF488494, 6525 ± 50) belongs to a haplotype which includes four other ancient Italian/Balkans bears including ACAD3646 Grotta d‟Ernesto, Italy 11,900 ± 33 (See haplotype 10, Table 4.1). Regardless, the presence of Iberian and Italian/Balkans haplogroup bears in southern France during the mid-late-Holocene is predicted under the E/C model, as Mont Ventoux is geographically intermediate between the two peninsula refuges.

Currently no DNA sequences are available from brown bear samples originating from glacial refugial locations during the LGM (approximately 23,000-18,000 ybp, Hewitt, 1996 and Kukla *et al.*, 2002). This makes it impossible to test one of the key assumptions of the Expansion/Contraction (E/C) model that post-glacial recolonising populations should be a genetically less diverse subset of their original monophyletic refugial populations. Despite the absence of DNA sequences from the LGM period (See Figure 4.3), Valdiosera *et al.* (2007) attempted to test this by dividing sequences into „peninsula‟ and „mainland‟ groups and comparing the nucleotide diversity for each. They reported no significant difference in nucleotide diversity between the three combined peninsulas (Iberia, Italy and the Balkans) and the mainland and interpreted this as evidence to refute the glacial refugia model since both groups were similarly diverse and the mainland diversity was not a smaller subset of the peninsula diversity. However, I believe there are a number of flaws in this interpretation, as not only are there no sequences from refugial populations dating to the LGM period with which to compare, two pre-glacial Pleistocene sequences (Ramesch, Austria, AJ809333, 47,420 ybp and Winden, Austria, AJ809334, 39,940 ybp) were included in the „mainland‟ group. Additionally, although ostensibly investigating the western European lineage (associated with the putative peninsula refugia), three DNA sequences included in the analysis (Ramesch, Austria, AJ809333; Romania1, EF488506; Romania2, EF488507) belong to the divergent eastern Clade 3a (associated with putative refugia in the Carpathian mountains; Sommer & Benecke 2005, Saarma *et al.*, 2007). By including these sequences it artificially increases the nucleotide diversity, and this particularly affects the interpretation that mainland Europe is no less diverse than the putative refugial populations when extra diversity has been added to the mainland group.

While there is still insufficient sequence data from LGM bears to perform this analysis, it is possible to calculate nucleotide diversity values for post-glacial brown bears of the Iberian and Italian/Balkans groups (Table 4.2). The values I obtained are lower than either the combined peninsula or mainland nucleotide diversities calculated by Valdiosera *et al.* (2007), partly due to exclusion of the inappropriate sequences discussed above and partly because I chose to divide the dataset by refugial haplogroup rather than combining all the separate

peninsulas together. The lack of significance associated with the neutrality and expansion statistics (Section 4.4) is presumably due to the lack of sequences available to test from LGM refugial bears rather than suggesting a real lack of expansion.

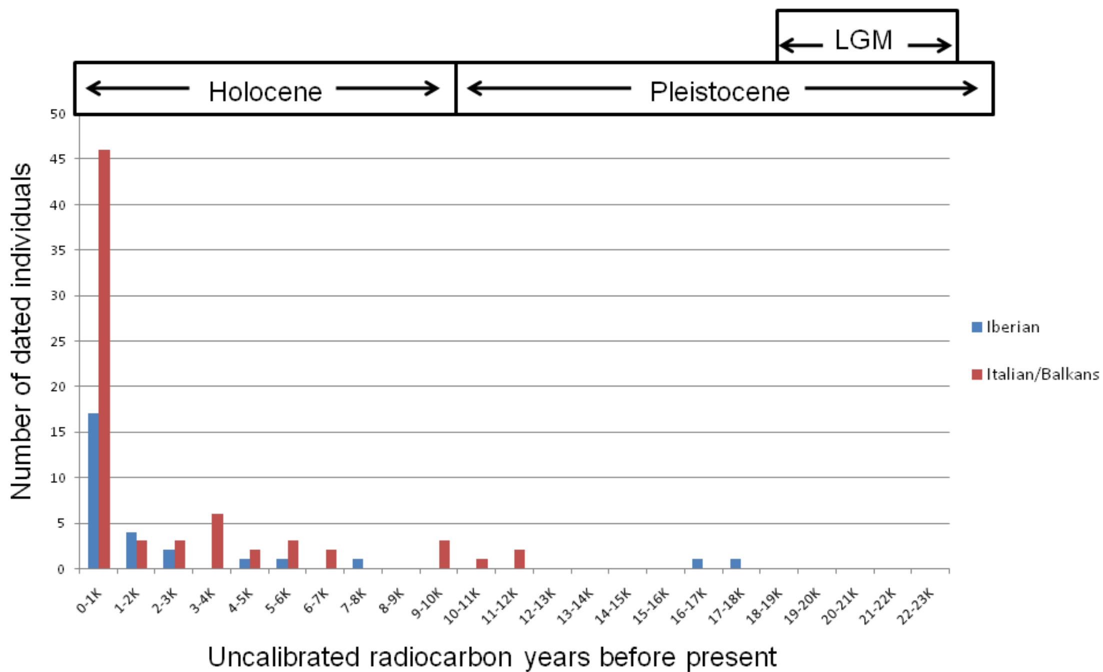

Figure 4.3 Frequency distribution of dated Clade 1 brown bear mtDNA sequences. Sequences with Iberian haplotypes are shown in blue, while sequences with Italian/Balkans haplotypes are shown in red. The LGM period (18,000-23,000 ybp) is shown to highlight the lack of sequences from glacial bear samples. The majority of mtDNA sequences are dated to the Holocene (0-10,000 ybp) period.

Although the results of my analysis are consistent with at least two previously identified southern European glacial refugia - the Iberian peninsula and the Italian/Balkans peninsulas - it is interesting to note the differential temporal phylogeographic patterns of these two haplogroups (Figure 4.4). According to the E/C model, the bears of the Iberian peninsula were the first to expand from their refugium and recolonise central and northern Europe (See Figure 4.5A; Hewitt, 1999). It is suggested that the glaciated Alps presented a barrier to the bears of the Italian/Balkans refugia, delaying their expansion into central Europe, so that once the geographical obstacle was overcome most of Europe was already occupied by bears of the Iberian and Eastern (Clade 3a) lineages (Hewitt, 1999; Taberlet, Bouvet, 1994).

By combining radiocarbon dates with the ancient mtDNA sequences it is possible to test both the genetic identity and the timing of the expansions. In contrast to the predictions of Hewitt"s model (Figure 4.5A), the ancient data suggests that bears of the Italian/Balkans haplogroup expanded into central and northern Europe as early as 12,000 ybp, with bears of the Italian/Balkans group appearing in Germany at least as early as 11,872 ± 92 ybp. With the exception of the one previously published sample from Abruzzo bearing the Iberian haplogroup SNP, I did not observe any bears of the Iberian group outside of Iberia prior to 2,673 ± 54 ybp (Scotland) and 1,790 ± 55 (France). The earliest post-glacial observations of bears of the eastern lineage (Clade 3a) are in Northern Scandinavia (~6,000 ybp; Chapter 2), Austria (1,985 ± 35 ybp) and Germany (1,770 ± 35 ybp). These data strongly suggest that it

was bears of the Italian/Balkan haplogroup that recolonised central Europe first, with a delayed expansion by bears of the Iberian and Eastern groups at a much later date, perhaps as recently as the last 2,000 years. Other unpublished data suggesting bears of the Iberian group arrived in Ireland approximately 4,000-5,000 ybp (Edwards *et al.*, 2008) corroborates my new post-glacial recolonisation model, although possibly extending the timing of the Iberian expansion back to approximately 5,000 years ago (Figure 4.4B). Future work may shed light on both the causes of the delayed expansion of the Iberian (and Eastern) bears, the trigger that allowed the change in phylogeography seen at 2,000-5,000 years ago, and whether this can be correlated with human activities.

It remains unclear from the available data whether the early expansion by the Italian/Balkans bears is indicative that the Alps did not pose such an insurmountable barrier to their dispersal as previously expected, or whether there may have been cryptic refugia for bears of the Italian/Balkan haplogroup in a more central location (for example Austria). Evidence is mounting, particularly from plant studies (Magri *et al.*, 2006; Naydenov *et al.*, 2007; Willis *et al.*, 2000; Willis, van Andel, 2004), to suggest the existence of cryptic refugia in central and northern Europe (Stewart, Lister, 2001), and my data is not inconsistent with this hypothesis. Although there does not appear to be any discussion about this in the literature, another alternative might be that bears from the Balkans were able to recolonise central Europe by taking a route between the Alps and Carpathian Mountains following a similar post-glacial pathway to that described for the meadow grasshopper, *Chorthippus parallelus* (Hewitt, 1999).

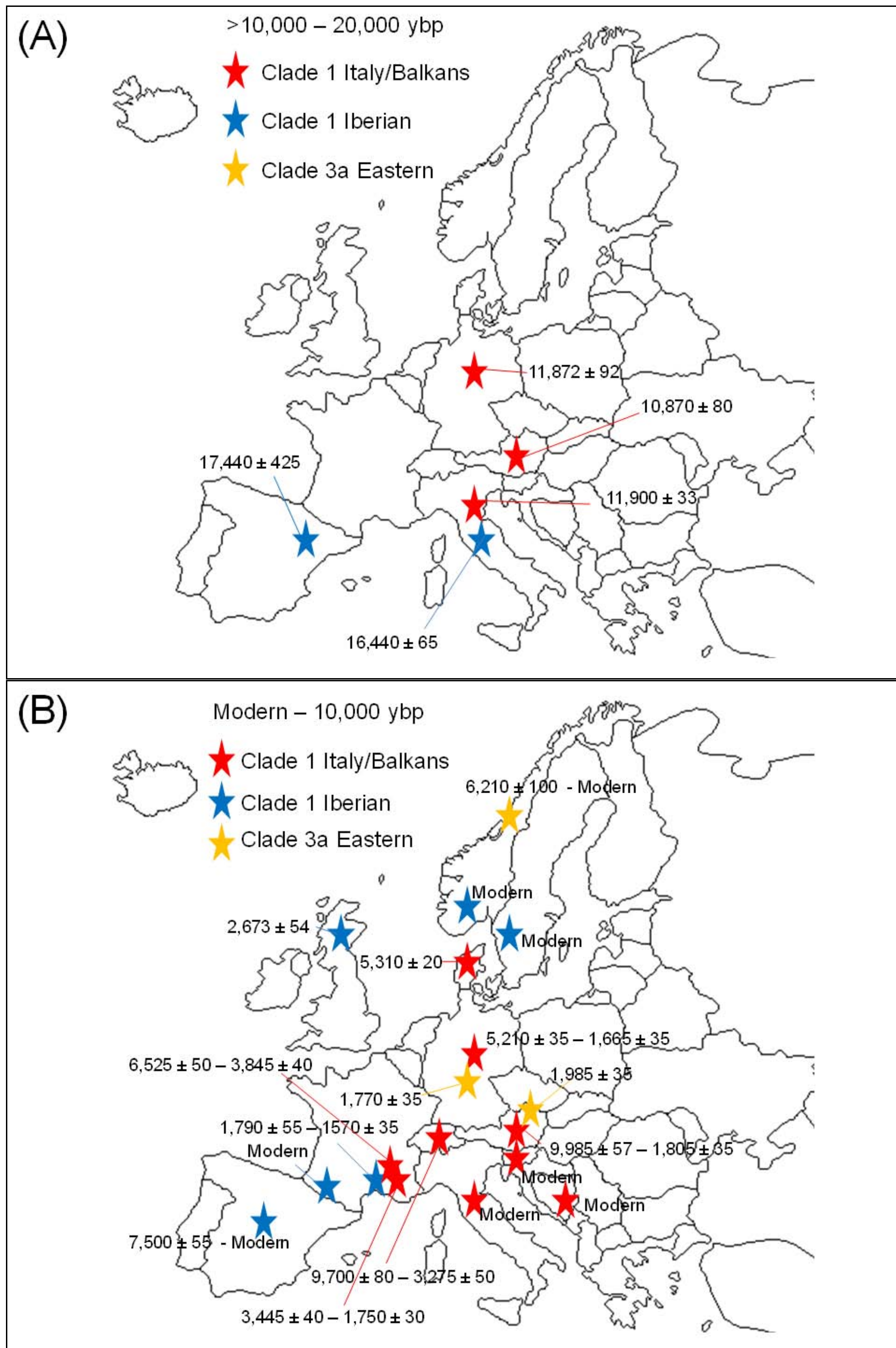

Figure 4.4 Map of Europe showing the approximate distribution of post-glacial brown bear mtDNA clades. Panel (A) shows brown bear specimens dated from >10,000 ybp – 20,000 ybp. Panel (B) shows brown bear specimens from 10,000 ybp – modern day. Dates indicated refer to uncalibrated radiocarbon years before present (ybp). Where more than one specimen from the same region and same mtDNA clade was identified the range of radiocarbon dates are indicated from the oldest and youngest specimen. Undated specimens were excluded from the distribution map. Coloured stars indicate mtDNA clade identity: Clade 1: Italian-Balkans (red), Clade 1: Iberian (blue) and Clade 3a: eastern European lineage (yellow).

From my new dataset I have identified two samples from one site in Austria (Barenloch) which belong to divergent mtDNA lineages. One sample, ACAD123, radiocarbon dated to 2,530 +/-20 ybp, belongs to the Italian/Balkans haplogroup. The other sample, ACAD119 dated to 1,985 +/- 35 ybp, belongs to the eastern (Clade 3a) lineage. This could be further evidence of dynamic local extinctions and replacements of mtDNA haplotypes observed previously in bears from Beringia (Barnes *et al.*, 2002) and Mont Ventoux, France (Valdiosera *et al.*, 2007) where both Iberian and Italian/Balkans haplotypes have been found at different (non-overlapping) times during the Holocene. Alternatively, these results could be due to sampling from a mixed population, suggestive of contact zones between different mtDNA haplogroups and lineages, as seen in modern bear populations in Scandinavia (Taberlet *et al.*, 1995) and Romania (Kohn *et al.*, 1995).

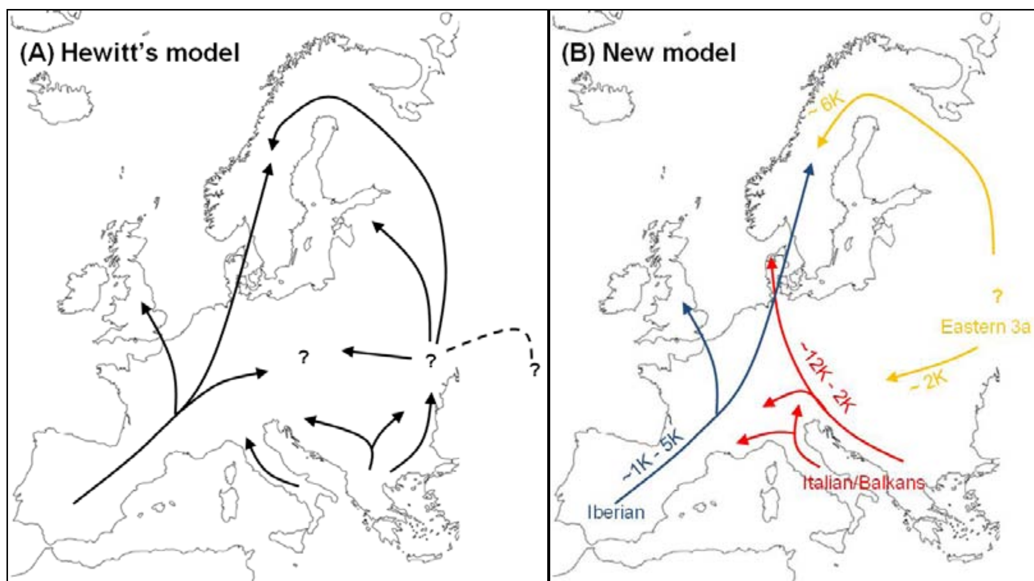

Figure 4.5 Models for post-glacial brown bear recolonisation of Europe.
Panel (A) is the traditional Hewitt „E/C" model of glacial expansion/contraction in which the Clade 1 Iberian bears expand first, and the Italian/Balkans bears expansion is delayed by the glaciated Alps and earlier expansion by the Iberian bears. Panel (A) is adapted from (Hewitt, 1999). Panel (B) is the new model that I propose based on my data and previously published post-glacial bear mtDNA sequences. In this model Clade 1 Italian/Balkans bears expand first at least 12,000 ybp, and it is not until the very latest Holocene that Clade 1 Iberian and Clade 3a are able to expand into central and northern Europe. In panel (B) I have colour-coded the mtDNA clades: Clade 1 Italian/Balkans (red), Clade 1 Iberian (blue) and Clade 3a (yellow). The approximate uncalibrated radiocarbon years indicate estimated timing of expansions by the different lineages based on the available data. The "?" indicates the uncertainty associated with the putative refugial location of the eastern European Clade 3a.

4.5 CONCLUSIONS

By combining my additional 28 ancient European brown bear sequences with modern and ancient sequences obtained from GenBank it was possible to identify post-glacial phylogeographic patterns of Clade 1 bears consistent with expansion from at least two southern European refugia (Iberia and Italian/Balkans) following the LGM. I found no evidence to support the claims of Valdiosera *et al.* (2007) of continuous gene-flow between the southern peninsula refugia during the LGM, and instead identified several flaws overlooked by that study.

This investigation is also the first to reveal that contrary to Hewitt‟s E/C model, the Italian/Balkans haplogroup recolonised central and northern Europe at least as early as 12,000 ybp, and that the expansion of the Iberian and Eastern lineages did not occur until much more recently (possibly in the last 2,000-5,000 years).

In terms of conservation it suggests that in contrast to claims that Iberian bears have only become isolated very recently (Valdiosera *et al.*, 2008), they may in fact have been a lot more geographically restricted than the Italian/Balkans bears since the LGM. The finding of a bear of the Italian/Balkans haplogroup in Denmark approximately 5,000 years ago (Chapter 2) combined with the data presented in this study showing the timing and extent of the Italian/Balkans post-glacial recolonisation of Europe suggests that Italian/Balkans bears should be considered a valid option for restocking other central and northern European populations rather than considering bears only from Iberia (Randi *et al.*, 1994; Taberlet *et al.*, 1995).

This study highlights that while ancient DNA can be a powerful tool in clarifying post-glacial phylogeography and recolonisation patterns not possible from modern data alone, it shows that caution is also required when interpreting aDNA data, especially when working with very small sample sizes.

4.6 ACKNOWLEDGEMENTS

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CHAPTER 5

EURASIAN BROWN BEAR PHYLOGEOGRAPHY

5.1 INTRODUCTION

While the phylogeography of brown bears has been relatively well studied at the extremes of their Holarctic range in Europe and North America/Beringia (Taberlet & Bouvet, 1994; Taberlet *et al.*, 1995; Waits *et al.*, 1998; Leonard *et al.*, 2000; Barnes *et al.*, 2002; Valdiosera *et al.*, 2007), relatively little is known about the mtDNA diversity or phylogeographic structure of brown bears from central, northern and eastern Eurasia. Korsten *et al.* (2009) recently attempted to fill this gap by surveying mtDNA diversity in 205 modern Eurasian brown bears. However, sampling across Eurasia was uneven, with 93% of samples sourced from west of the Urals Mountains (western Russia, Estonia and Finland) and the Kamchatka Peninsula (in the east). Only eighteen individuals have been sampled in the approximately 6000 km intervening gap; four from Siberia and fourteen from the Russian Far East (Korsten *et al.* 2009, Waits unpublished described in Miller *et al.* 2006). Despite the lack of samples across central Eurasia, Korsten *et al.* summarised the distribution of major mtDNA clades across the Holarctic. Their map (Figure 5.1) suggests a continuous distribution of clade 3a from western Europe to Alaska.

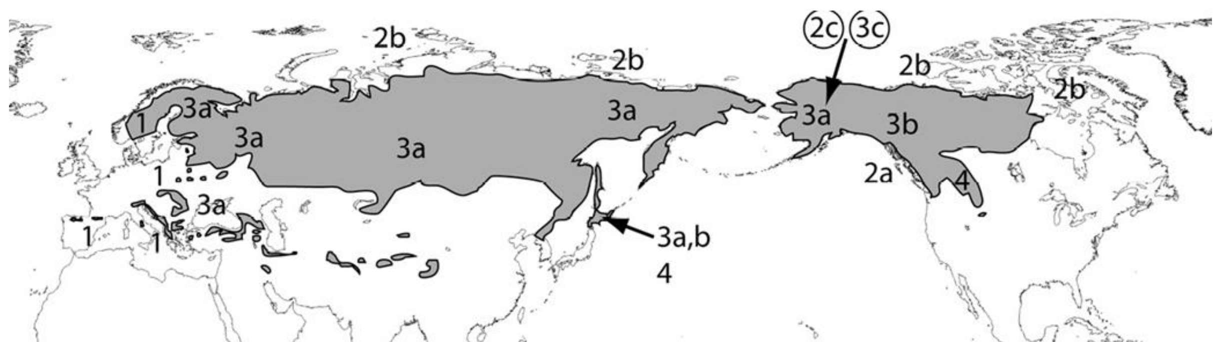

Figure 5.1 Approximate current distribution of brown bear haplogroups (extant without circles, extinct with circles) as presented in Korsten *et al.*, (2009).

Remarkably, Korsten *et al.*"s 2009 survey combined with an expanded dataset including bears from Eurasia, Alaska and Japan identified only a single haplogroup (Clade 3a) on continental Eurasia from Estonia and Finland in the west to Beringia in the east (Korsten *et al.*, 2009). Of 50 Eurasian haplotypes detected within Clade 3a, two account for almost 50% of the individuals sampled. One of these, (designated haplotype EA1 in Korsten *et al.* 2009) represented by 66/205 individuals and a second related haplotype (EB1) were found across the entire width of Eurasia from Scandinavia to the Russian Far East and the Kamchatka peninsula.

Combined with coalescent estimates of divergence times, Korsten *et al.* (2009) interpreted these findings as evidence for a sudden demographic expansion of Clade 3a brown bears across northern continental Eurasia following the last glacial maximum (LGM) and suggested that this may represent a general model for the phylogeography of Eurasian mammals. Specifically they concluded that:

1) Brown bears underwent a sudden and quite recent expansion across Eurasia, most likely occurring immediately after the LGM.

2) The founder population underwent a severe bottleneck prior to the expansion.

3) The founder population was most likely carrying haplotype EA1 and EB1 (the two most common haplotypes identified in their dataset).

4) Coalescent analyses revealed the timing of the most recent common ancestor (MRCA) of the extant Clade 3a bears to be approximately 26,000 ybp (95 % highest posterior density interval of 13-46 ybp).

5) Northern continental Eurasian populations expanded from a single glacial refuge (of unknown location).

6) There were no significant barriers to this post-LGM expansion.

Based on Korsten *et al.*'s conclusions, a number of predictions can be made about past and present mtDNA diversity of Eurasian brown bears:

1. mtDNA diversity prior to the LGM was higher than the present.

2. Immediately after the LGM bottleneck all individuals should belong to the hypothesised founder haplotypes (EA1 and EB1).

3. Any non-EA1 or EB1 haplotypes that were present before the LGM bottleneck should not be found after the LGM.

4. The pre-LGM distribution of samples belonging to EA1 and EB1 should indicate the location of the glacial refuge.

The fossil record suggests that brown bears evolved on the Eurasian continent (Kurten, 1968), and only reached North America via the Beringian land-bridge during the late Pleistocene approximately 100,000 ybp (Kurten & Anderson, 1980). However, in contrast to the apparently continuous distribution of Clade 3a bears from Europe to North America, bears of Clades 3b and 4 have only been found in North America and Japan where they appear to have arrived relatively recently (Matsuhashi *et al.*, 1999; Barnes *et al.*, 2002; Miller *et al.*, 2006) and it is unlikely that they have evolved there *in situ*. To date, the only example of a bear of either of these haplogroups being found outside North America and Japan is a single continental Eurasian Clade 3b sample reported by Miller *et al*. (2006) from the Russian Far East (sequence not available on Genbank). It is likely that the bears from North America and Japan (Clades 3b and 4) derived from common ancestral Eurasian populations, however as neither Clade 3b or 4 were detected in the recent analysis of modern Eurasian bears it has

been postulated that these groups are no longer present in continental Eurasia (Korsten *et al.*, 2009).

Given the current picture of brown bear mtDNA haplogroup distribution, the Eurasian continent is likely to hold the key to understanding the origin, evolution and expansion of the „eastern" brown bear mtDNA clades (Clades 3 and 4). The limited number of samples analysed to date from central, northern and eastern Eurasia and the lack of any ancient Eurasian brown bear mtDNA sequences creates an important gap in our current knowledge of bear mtDNA evolution and phylogeography.

In this study, I aimed to analyse a large collection (approximately 100 samples) of ancient (Pleistocene) and historic (<200 years) brown bear specimens obtained from across the Eurasian continent to gain an insight into the genetic diversity of Eurasian bears through time, allowing some of the hypotheses and conclusions presented by Korsten *et al.* (2009) to be tested. In addition, the new sequences generated from ancient specimens from across Eurasia would be able to help fill in a major temporal and geographical gap in brown bear biogeographic research and subsequently pave the way for the first comprehensive global analysis of brown bear phylogeography from the Pleistocene to modern times.

5.2 MATERIALS & METHODS

5.2.1 Samples

I obtained 49 ancient and 47 historic brown bear (*Ursus arctos*) bone and tooth root samples from sites across Eurasia. The historic samples were from museum specimens, collected during 1878-1883 (provided by VNHM, sampled by Gregor Larson) and 1903-1989 (provided by the Zoological Museum of Moscow University, sampled by Ian Barnes, 2001). Two ancient specimens from North America were also included in this investigation, as well as two modern samples. I obtained the modern tissue samples from a Syrian brown bear (*Ursus arctos syriacus*) individual from the Adelaide Zoo, and a brown bear from the Yukon, Canada (See Appendix Table 4.1 for sample and extraction details). The Syrian brown bear was included as a modern representative of a Eurasian brown bear. Additionally, at the time of this investigation, no mtDNA sequences from *U. arctos syriacus* had been reported in the literature or deposited on GenBank. The brown bear from the Yukon was also included as a modern representative, predicted to belong to the 3b haplogroup based on geographic location (see Barnes *et al*. 2002 and Miller *et al*. 2006), providing a modern 3b sequence to compare to any 3b bears that might be detected in the ancient or historic Eurasian samples.

5.2.2 Ancient DNA extractions

Ancient and historic samples were extracted in the ancient DNA facility at the Australian Centre for Ancient DNA (ACAD) either as previously described (Chapter 2.3.2) or using a Qiagen DNeasy Blood and Tissue kit according to manufacturers instructions with modifications described by Boessenkool *et al.* (2009).

Appendix Table 4.1 indicates which method was used for each extraction.

5.2.3 Modern DNA extractions

The modern tissue samples were extracted using a Qiagen DNeasy Blood and Tissue kit according to manufacturers instructions at the South Australian Museum EBU lab, geographically isolated from the ancient DNA facility at ACAD.

5.2.4 PCR amplifications

A carnivore-specific primer pair L16164 and H16299 (Hänni *et al.*, 1994), designed to amplify a hypervariable 135-bp fragment of the mitochondrial control region (CR), was used in order to facilitate comparison with previous studies of both modern and ancient brown bears and to differentiate all major brown bear mtDNA lineages (Taberlet & Bouvet, 1994; Barnes *et al.*, 2002; Valdiosera *et al.*, 2007; Korsten *et al.*, 2009).

PCR amplifications were performed in 25 µl reactions with 1-2 µl of DNA extract, 1-1.25 U Platinum *Taq* DNA Polymerase High Fidelity and 1X buffer (Invitrogen), 2 mg/ml rabbit serum albumin (RSA; Sigma), 2 mM $MgSO_4$, 250 µM of each dNTP, and 1 µM of each primer. PCR thermal cycling reactions consisted of 94 °C 1 min, followed by 50-55 cycles of 94 °C denaturation for 15 sec, annealing for 15-20 sec at 55 °C, and extension at 68 °C for 30 sec, followed by a final extension at 68 °C for 10 min.

PCR products that yielded bands of the expected size were purified using ExoSAP-IT or Agencourt AMPure PCR purification kit according to manufacturers instructions. PCR products were sequenced in both directions using Big Dye chemistry and an ABI 3130XL Genetic Analyzer. Sequences were edited and contigs formed using Sequencher 4.7, and then aligned in MEGA 3.1 (Kumar *et al.*, 2004).

5.2.5 Independent replications

Nine ancient samples were independently replicated in a second ancient DNA facility (Ludovic Orlando, unpublished data). The replication included independent ancient DNA extractions using a silica-based method (essentially as described in Rohland and Hofreiter, 2007), multiple independent PCR amplifications and cloning. Consensus sequences were created from the clones and compared to the original sequences obtained through direct sequencing at ACAD (See Appendix 4 for more details of the independent replications).

5.2.6 Radiocarbon dating

Ten specimens (including the nine that underwent independent genetic replication) were sent for radiocarbon dating at the University of California Irvine (UCI) and the University of Colorado INSTARR Laboratory for AMS Radiocarbon Preparation and Research as part of this study (See Table 5.1 for details). Other samples already had dates associated with them, or dating information was obtained from sampling notes and databases (Alan Cooper, pers. comms. 2006-2009). All available date information is provided in Table 5.1 and Appendix Table 4.1.

5.2.7 Phylogenetic analysis

The sequences generated in this study were aligned with the 1943-bp modern Eurasian brown bear sequences (205 individual sequences represented by GenBank Accession Numbers EU526765-EU526814) generated in the recent study by Korsten *et al*. (2009).

The aligned sequences were cropped down to the homologous 135-bp CR fragment used in this study, and collapsed into unique haplotypes using Fabox 1.35. The aligned 135-bp sequences were also used to construct two separate minimum spanning mtDNA haplotype networks using TCS (Clement *et al.*, 2000) in order to investigate phylogeographic origin and relationships of sequences generated in this study only, and sequences from this study combined with the modern sequences of Korsten *et al.* (2009).

Table 5. 1 Radiocarbon dates and associated isotope data for the bear samples dated as part of this study. Dates are reported as uncalibrated radiocarbon years before present. UCIAMS numbers refer to the University of California Irvine, CURL numbers refer to the University of Colorado Radiocarbon Lab.

Sample	Origin	Delta C^{13}	Delta N^{15}	Carbon date (ybp)	Reference number
ACAD 1927	Irtysh River, Urals, Russia	-19.5	10.3	43,600 ± 1500	UCIAMS-56989
		-19.5	N/A	42,400 ± 1500	CURL-10290
ACAD 1933	Tain Cave, Urals, Russia	-19.2	6.2	>46,700	UCIAMS-56990
		-16.5	N/A	>46,700	CURL-10278
ACAD 1939	Denisova Cave, Altai Mountains, Russia	-20.3	5.4	>46,000	UCIAMS-56997
		-18.7	N/A	50,100 ± 3900	CURL-10280
ACAD 1941	Smuggler"s Cave, Altai Mountains, Russia	-19.1	4.3	16,450 ± 60	UCIAMS-56991
		-13.7	N/A	16,410 ± 60	CURL-10267
ACAD 1942	Smuggler"s Cave, Altai Mountains, Russia	-19.3	2.8	16,440 ± 60	UCIAMS-56992
		-19.9	N/A	16,470 ± 70	CURL-10265
ACAD 1943	Smuggler"s Cave, Altai Mountains, Russia	-19.0	2.2	16,380 ± 60	UCIAMS-56993
		-18.5	N/A	16,420 ± 70	CURL-10288
ACAD 1944	Smuggler"s Cave, Altai Mountains, Russia	-18.9	3.2	13,925 ± 40	UCIAMS-56994
		-19.9	N/A	13,915 ± 50	CURL-10285
ACAD 1945	Smuggler"s Cave, Altai Mountains, Russia	-19.2	2.5	15,370 ± 100	UCIAMS-56995
		-18.0	N/A	15,370 ± 60	CURL-10289
ACAD 1946	Smuggler"s Cave, Altai Mountains, Russia	-18.8	3.1	13,830 ± 40	UCIAMS-56996
		-12.9	N/A	13,995 ± 45	CURL-10277
ACAD 4098	Mezmaiskaya Cave, Caucasus Mountains, Russia	-18.5	N/A	42,900 ± 1600	CURL-10273 *

* See Appendix 4 for more details about this date.

5.3 RESULTS

5.3.1 Mitochondrial DNA diversity in Eurasia

I successfully extracted and sequenced 135-bp control region mtDNA from 24 out of 51 ancient, 35 out of 47 historic and both modern brown bear specimens from across the Eurasian and North American continents (61/100 total samples; See Appendix Table 4.1).

These 61 new sequences represent 19 haplotypes of which three are identical to previously published haplotypes from Eurasia and North America (haplotypes 1, 5 and 12 in Table 5.2). Sixteen haplotypes are newly identified in Eurasian brown bears (Table 5.2, haplotypes 2-4, 6-11, 13-19). Forty-seven samples (including modern, historic and ancient specimens from across the entire Eurasian sample range) belong to Clade 3a. Of these, 32 belong to a single haplotype - identical to a modern Swedish brown bear sequence X75874 „Rus" (Taberlet & Bouvet, 1994), and to the EA1 common clade 3a haplotype of Korsten *et al*. (2009) and haplotype 29 of Miller *et al*. (2006). Eight samples (including the modern Yukon specimen and three historic and four ancient specimens from the Kuril Isles, Altai Mountains, and the Ussury Region) belong to Clade 3b. The two Holocene specimens from central North America (ACAD 195 and 1731) belong to Clade 4. The remaining four ancient and historic samples (ACAD 137, 154, 1927 and 4098) are related to Clade 4 and represent new mtDNA diversity.

5.3.2 Temporal and geographic distribution of mtDNA diversity

Of the 61 new sequences presented here, three originate from the North American continent and one from a captive Syrian brown bear. The 57 remaining sequences originate from continental Eurasia and islands off the east coast of Russia (Figure 5.2) and their distribution, age and genetic diversity will be described below briefly and then in more detail, based on geographic origin of the specimens.

Briefly, the new sequences presented in this study suggest that Clade 3a has been present in western, central and eastern Eurasia since at least 46,000 ybp, Clade 3b has been present in southern Eurasia from at least 46,000 ybp until historic times, and Clade 4 (or a close relative) has been present in western Eurasia (in the Urals and Caucasus Mountain ranges) from at least 43,000 ybp until historic times.

Seven sequences were obtained from the **Caucasus Mountains**, ranging in age from historic to approximately 43,000 ybp. Of these seven sequences, four belong to Clade 3a (but not to the most common 3a haplotype 1), and three belong to Clade 4 or a previously undetected close relative of the Clade 4 individuals from North America.

Ten sequences were obtained from the **Ural Mountains** (or in the surrounding region), ranging in age from historic to >46,000 ybp. These mostly belong to the common 3a haplotype 1, with two main exceptions. One Clade 3a sequence dated to >46,000 ybp belongs to haplotype 12 and is identical to the extant EA28 haplotype described by Korsten *et al*. (2009) from Finland. The other sequence dated to >46,000 ybp belongs to Clade 4 (or a previously undetected close relative).

Three historic sequences were obtained from **far western Russia** (in an area heavily sampled by Korsten *et al*. 2009) and all belong to the common 3a haplotype 1.

Thirteen sequences were obtained from **central and southern Russia** including eight from the **Altai Mountains** ranging in age from historic to >46,000 ybp. Eight belong to the common 3a haplotype 1 (five historic and three immediately following the LGM). Five belong to Clade 3b (consisting of four different haplotypes), one of which is dated to >46,000 ybp, three from the period immediately after the LGM, and one historic individual. To the north of the Altai Mountains five historic individuals (all belonging to the common 3a haplotype) were detected.

From **eastern Russia** five sequences were obtained ranging in age from historic to >30,000 ybp. Four of these belong to the common 3a haplotype 1 (three historic specimens and one individual dated to 30,660 ± 180 ybp). A single 3b sequence was obtained from an historic individual originating from the **far south-east of Russia** on the border with China.

Nine historic sequences were obtained from the **Kamchatka peninsula**. One of these individuals belongs to the common 3a haplotype 1, while the remaining eight belong to Kamchatka-specific haplotypes.

The remaining six sequences (all belonging to the common 3a haplotype 1) lacked sufficient location details to pinpoint their origin within the Eurasian continent.

Table 5.2 Haplotype list of Eurasian brown bear sequences generated in this study (haplotypes 1-19) with equivalent haplotypes from Korsten *et al*. (2009) indicated. Haplotypes identified in the modern Korsten *et al*. (2009) dataset but not in my historic and ancient dataset are also included in this table (haplotypes 20-30) to allow correlation with Figure 5.3 panel B.

Haplotype	Frequency in this study	Sample name	Geographic location	Age *	Equivalent haplotypes from Korsten *et al.*	Frequency of equivalent haplotypes from Korsten *et al.*
1	32	ACAD 95	Rodnichnaya, Russia	P/H	EA1-4, EA6-13, EA15-17, EA19-25, EA27, EB1, EB3-5, EB8, KA1 and KA2	167
		ACAD 99	Shaitansky, Russia	P/H		
		ACAD 100	Ignodiveskaya, Russia	P/H		
		ACAD 101	Lakeiskaya, Russia	P/H		
		ACAD 102	Shaitansky, Russia	P/H		
		ACAD 131	Pechoro-Illych Nature Reserve, Russia	Historic		
		ACAD 132	Evenkia, Russia	Historic		
		ACAD 133	Pechoro-Illych Nature Reserve, Russia	Historic		

Haplotype	**Frequency in this study**	**Sample name**	**Geographic location**	**Age ***	**Equivalent haplotypes from Korsten *et al.***	**Frequency of equivalent haplotypes from Korsten *et al.***
1		ACAD 134	Belomor-Baltic canal, Russia	Historic		
		ACAD 139	Kuril Isles, Russia	Historic		
		ACAD 140	Vologda province, Russia	Historic		
		ACAD 141	Kamchatka, Russia	Historic		
		ACAD 142	Murmansk, Russia	Historic		
		ACAD 144	Upper Kosogol lake, NW Mongolia	Historic		
		ACAD 149	Enisei River, Russia	Historic		
		ACAD 150	Pechoro-Illych Nature Reserve, Russia	Historic		
		ACAD 151	Yakutia, Russia	Historic		
		ACAD 153	Yakutia, Russia	Historic		
		ACAD 159	Sakhalin Island, Russia	Historic		

Haplotype	Frequency in this study	Sample name	Geographic location	Age *	Equivalent haplotypes from Korsten *et al.*	Frequency of equivalent haplotypes from Korsten *et al.*
1		ACAD 162	Baikal lake, Russia	Historic		
		ACAD 164	Krasnoyarsk region Aginsky district, Russia	Historic		
		ACAD 165	Magadan province, Russia	Historic		
		ACAD 347	Russia	P/H		
		ACAD 360	Russia	P/H		
		ACAD 407	Indigirka area, Russia	30,660 ± 180 ybp (Oxford-14944)		
		ACAD 410	Russia	P/H		
		ACAD 1921	Russia	P/H		
		ACAD 1924	Urals, Russia	Pleistocene		
		ACAD 1928	Irtysh River, Urals, Russia	P/H		

Haplotype	**Frequency in this study**	**Sample name**	**Geographic location**	**Age ***	**Equivalent haplotypes from Korsten *et al.***	**Frequency of equivalent haplotypes from Korsten *et al.***
1		ACAD 1941	Smuggler‟s cave, Altai Mountains, Russia	16,450 ± 60 ybp (UCIAMS-56991)		
		ACAD 1942	Smuggler‟s cave, Altai Mountains, Russia	16,440 ± 60 ybp (UCIAMS-56992)		
		ACAD 1943	Smuggler‟s cave, Altai Mountains, Russia	16,380 ± 60 ybp (UCIAMS-56993)		
2	3	ACAD 136	Northern Caucasus, Russia	Historic		
		ACAD 161	Northern Caucasus, Russia	Historic		
		ACAD 163	Northern Caucasus, Russia	Historic		
3	1	ACAD 137	Caucasian Biosphere Nature Reserve, Russia	Historic		

Haplotype	**Frequency in this study**	**Sample name**	**Geographic location**	**Age ***	**Equivalent haplotypes from Korsten *et al.***	**Frequency of equivalent haplotypes from Korsten *et al.***
4	1	ACAD 143	Kuril Isles, Russia	Historic		
5	8	ACAD 146	Kamchatka, Russia	Historic	KA5-8, KA11 and KA12	21
		ACAD 167	Kamchatka, Russia	Historic		
		ACAD 5390	Kamchatka, Russia	Historic		
		ACAD 5391	Kamchatka, Russia	Historic		
		ACAD 5392	Kamchatka, Russia	Historic		
		ACAD 5394	Kamchatka, Russia	Historic		
		ACAD 5396	Kamchatka, Russia	Historic		
		ACAD 5397	Kamchatka, Russia	Historic		
6	2	ACAD 152	Bolshoy Shantar Island, Russia	Historic		
		ACAD 166	Nth Caucasus, Chechnya, Russia	Historic		

Haplotype	**Frequency in this study**	**Sample name**	**Geographic location**	**Age ***	**Equivalent haplotypes from Korsten *et al.***	**Frequency of equivalent haplotypes from Korsten *et al.***
7	1	ACAD 154	Caucasian Biosphere Nature Reserve, Russia	Historic		
8	1	ACAD 155	Ussury Region, Russia	Historic		
9	1	ACAD 156	Altai Mountains, Russia	Historic		
10	1	ACAD 1731	Mineral Hill, Nevada, USA	9,960 ± 50 ybp (SR-5293)		
11	1	ACAD 1927	Irtysh River, Russia	43,600 ± 1500 ybp (UCIAMS-56989)		
12	1	ACAD 1933	Tain Cave, Urals	>46,700 ybp (UCIAMS-56990)	EA28	1
13	2	ACAD 1939	Denisova Cave, Altai Mountains	>46,000 ybp (UCIAMS-56997)		
		ACAD 1944	Smuggler‟s cave, Altai Mountains, Russia	13,925 ± 40 ybp (UCIAMS-56994)		

Haplotype	Frequency in this study	Sample name	Geographic location	Age *	Equivalent haplotypes from Korsten *et al.*	Frequency of equivalent haplotypes from Korsten *et al.*
14	1	ACAD 1945	Smuggler"s cave, Altai Mountains, Russia	15,370 ± 100 ybp (UCIAMS-56995)		
15	1	ACAD 1946	Smuggler"s cave, Altai Mountains, Russia	13,830 ± 40 ybp (UCIAMS-56996)		
16	1	ACAD 195	Natural Trap Cave, Kansas, USA			
17	1	ACAD 4098	Mezmaiskaya Cave, Caucasus Mountains	42,900 ± 1600 ybp (CURL-10273)		
18	1	ACAD 5634	Yukon, Canada	Modern		
19	1	ACAD 6728	Adelaide Zoo (Syrian brown bear)	Modern		
20					KA10	2
21					KA9	1
22					KA4	2
23					KA3	1

Haplotype	Frequency in this study	Sample name	Geographic location	Age *	Equivalent haplotypes from Korsten *et al.*	Frequency of equivalent haplotypes from Korsten *et al.*
24					EC1 and EC2	3
25					EB7	1
26					EA14	1
27					EB2	1
28					EA26	1
29					EA18	1
30					EA5	1

* undated ancient samples believed to be from the Pleistocene-Holocene are indicated as „P/H", radiocarbon dated samples are indicated in uncalibrated radiocarbon years before present (ybp) followed by radiocarbon lab reference number, historic samples (from the 1800s and 1900s) are reported as „Historic" and modern tissue samples are indicated as „Modern". Where a sample was radiocarbon dated more than once, only the UCIAMS date is presented.

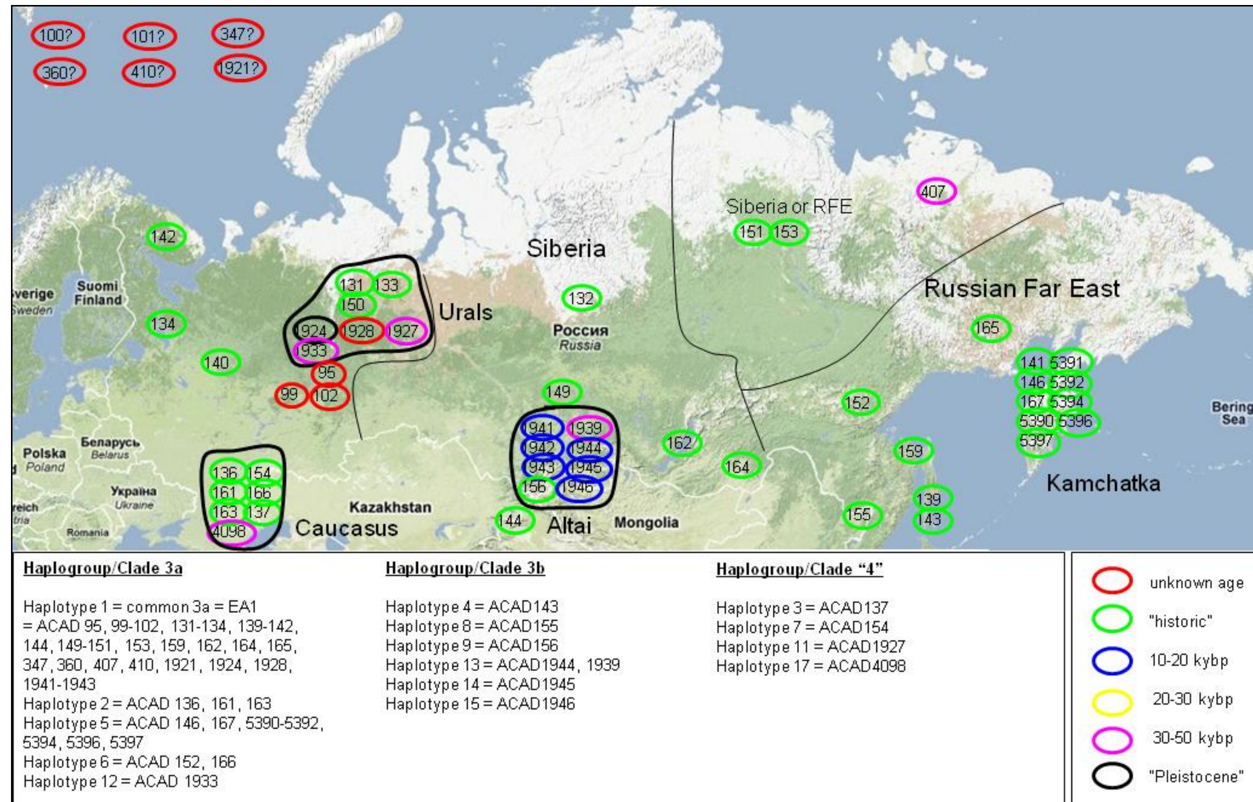

Figure 5.2 Approximate distribution of ancient and historic Eurasian brown bear samples from which DNA was obtained in this study. The numbers refer to the ACAD number of the sample and can be cross-referenced with the appropriate Haplogroup and Haplotype below the map. The coloured circles around the sample numbers indicate the age of the specimen. Samples without detailed location information e.g. sample ACAD410 "Russia" have been included in the top left-hand corner of the figure. The basic map was downloaded from Google Maps (http://maps.google.com) with key Eurasian mountain ranges and regions indicated by the addition of black lines and labels e.g Urals, Caucasus and Altai Mountains

5.3.3 Independent DNA replications

Nine ancient specimens were independently extracted, amplified, cloned and sequenced (Ludovic Orlando, unpublished data), and the consensus sequences of these clones were compared to the 135-bp sequences obtained by direct sequencing (Appendix 4). Seven out of nine consensus clone sequences were identical to direct sequencing result. One of the nine consensus clone sequences had a single base pair difference when compared to the direct sequencing result (ACAD#1939, G-A base change at a single site), while one other consensus sequence differed from the direct sequencing result by two base pairs (ACAD#1946, C-T base changes at two sites). In both cases the „best of three" rule (Hofreiter *et al.*, 2001) was applied and the consensus clone sequence (from a minimum of two independent PCR amplifications) was used in subsequent analyses (Appendix 4).

5.3.4 Radiocarbon dating results

Carbon dating of ten new ancient samples from the Urals, Altai and Caucasus mountains returned ages between 13,000 and >46,000 C^{14} ybp, although dates are clustered into two groups >42,000 C^{14} ybp and 13,000-16,000 C^{14} ybp. Nine out of ten of these specimens were sent for radiocarbon dating at both the University of Colorado and the University of California Irvine as part of this study. The remaining sample (ACAD#4098) was radiocarbon dated at only one laboratory (the University of Colorado INSTARR Laboratory for AMS Radiocarbon Preparation and Research) due to the small amount of bone material available for dating. See Table 5.1 for details of samples dated as part of this study, and Appendix Table 4.1 for dates recorded during sampling trips. Samples dated at both laboratories returned consistent dates, so for clarity I will use the UCIAMS dates (where available) while discussing the results in this chapter.

5.3.5 Sequence alignment & phylogenetic analysis

Alignment of new sequences generated in this study, with 205 existing brown bear control region sequences from modern Eurasian bears recently produced by Korsten *et al.* (2009) identified a single-base anomaly in the Korsten *et al.* (2009) sequences (an insertion between positions 16,789 and 16,790 in the brown bear complete mtDNA genome). This insertion is not present in any sequences previously produced by five different research groups (Taberlet & Bouvet, 1994; Barnes *et al.*, 2002; Hofreiter *et al.*, 2004; Miller *et al.*, 2006; Valdiosera *et al.*, 2007). To allow an unbiased comparsion between datasets, this insertion was removed from all of the Korsten *et al.* sequences. When the sequences generated in this study were aligned with the cropped Korsten *et al.*(2009) sequences and analysed in TCS (Clement *et al.*, 2000), the combined dataset consisted of 30 haplotypes; 16 unique to my dataset (haplotypes 2-4, 6-11, 13-19), 10 unique to Korsten *et al.*"s modern Eurasian dataset (haplotypes 20-30), and three haplotypes (haplotypes 1, 5 and 12) were shared by both datasets (See Table 5.2 for a haplotype list and Figure 5.3 panel A and B for the results of the minimum spanning mtDNA haplotype network).

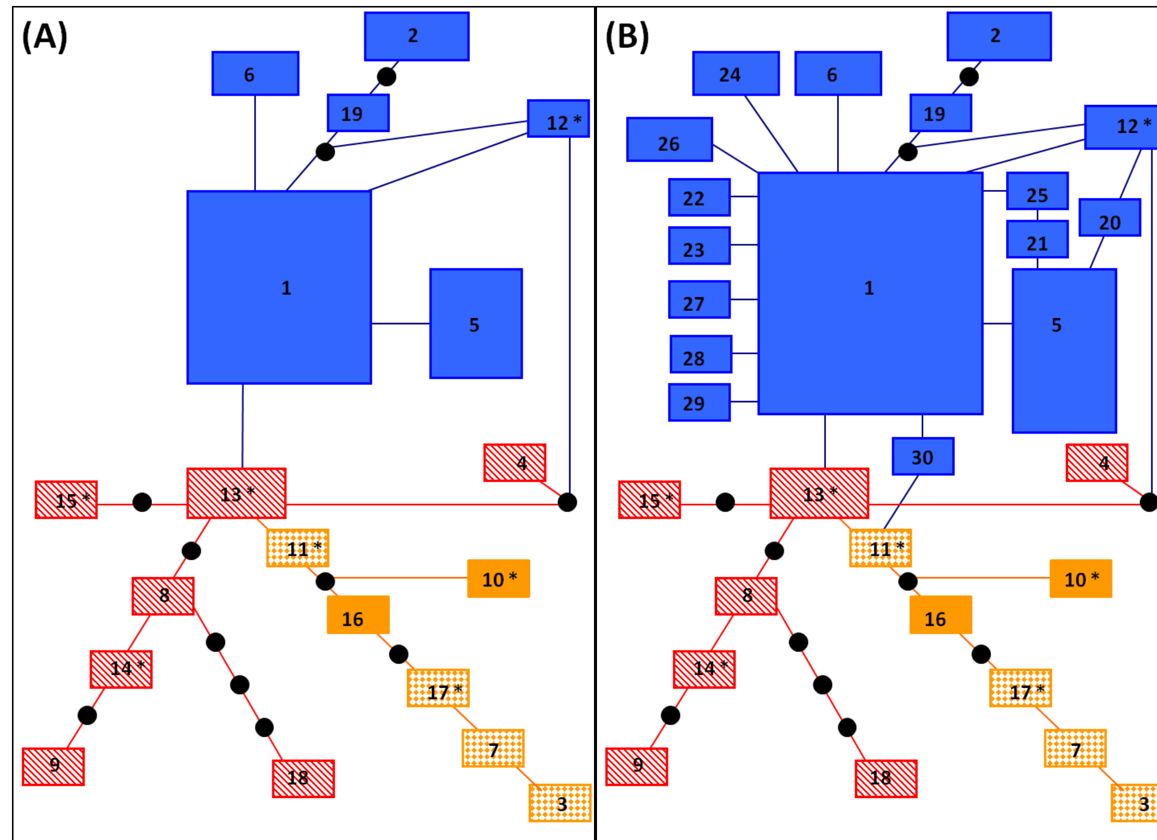

Figure 5.3 Minimum spanning mtDNA Haplotype network for n=61 Eurasian brown bear sequences generated in this study (Panel A) and this study combined with the homologous 135-bp sequences from n=205 additional modern Eurasian sequences reported by Korsten *et al*. (Panel B). Numbers within the squares indicate the haplotype number (refer to Table 5.2 for haplotype list) while the size of the squares are approximately proportional to the relative frequency of the haplotype. Haplogroups are colour-coded as follows: Clade 3a (solid blue), Clade 3b (stripy red), Clade 4 (solid orange), unusual sequences potentially related to Clade 4 (orange checkers). Missing or unsampled haplotypes indicated by small black circles. Haplotypes only detected in ‚ancient' samples are indicated by an asterix (*).

5.4 DISCUSSION

The 61 new sequences obtained in this study provide valuable new information about the phylogeography of Eurasian brown bears from the Pleistocene until historic times, and give insight into the temporal and geographic distribution of brown bear mtDNA lineages across the continent.

5.4.1 Spatial and temporal distribution of Clade 3a

Similar to Korsten *et al.* (2009), a single Clade 3a haplotype (haplotype 1; Table 5.2, Figure 5.2) was found to be the most common and widespread geographically across the Eurasian continent, being found in the far west, far east, northern, central and southern regions sampled as part of this study (Figure 5.4). By increasing the brown bear sample size in the ~6000 km gap between the Urals and the Kamchatka Peninsula, support is increased for the continuous modern distribution of a single (or small number) of Clade 3a haplotypes from Scandinavia to Alaska.

This new dataset provides evidence for two extant clade 3a haplotypes (ACAD407, haplotype 1, Indigirka region, and ACAD1933 haplotype 12, Urals) at both the eastern and western edges of the continent at least 46,700-30,000 ybp, suggesting that Clade 3a was already widespread before the last glacial maximum. In addition, three specimens from the Altai mountains (dated to approx. 16,000 ybp) and a single „Pleistocene" specimen from the Urals all belong to Clade 3a adding weight to the idea that this haplogroup was already well established and prevalent during the Pleistocene both before and after the LGM.

Korsten *et al*. (2009) reported that modern Kamchatka populations appear to have remained somewhat separate from those found in the rest of continental Eurasia. Only a single haplotype (the common widespread Clade 3a haplotype 1) from continental Eurasia is detected in Kamchatka, with all other sequences from modern Kamchatka bears being found uniquely on the Kamchatka peninsula. This new study included nine historic bears from the Kamchatka peninsula. Consistent with the modern data, these historic bears belonged to either the common Clade 3a haplotype 1 (a single individual) or to haplotype 5 (eight individuals), a Kamchatka-specific haplotype previously detected in the modern populations (Table 5.2).

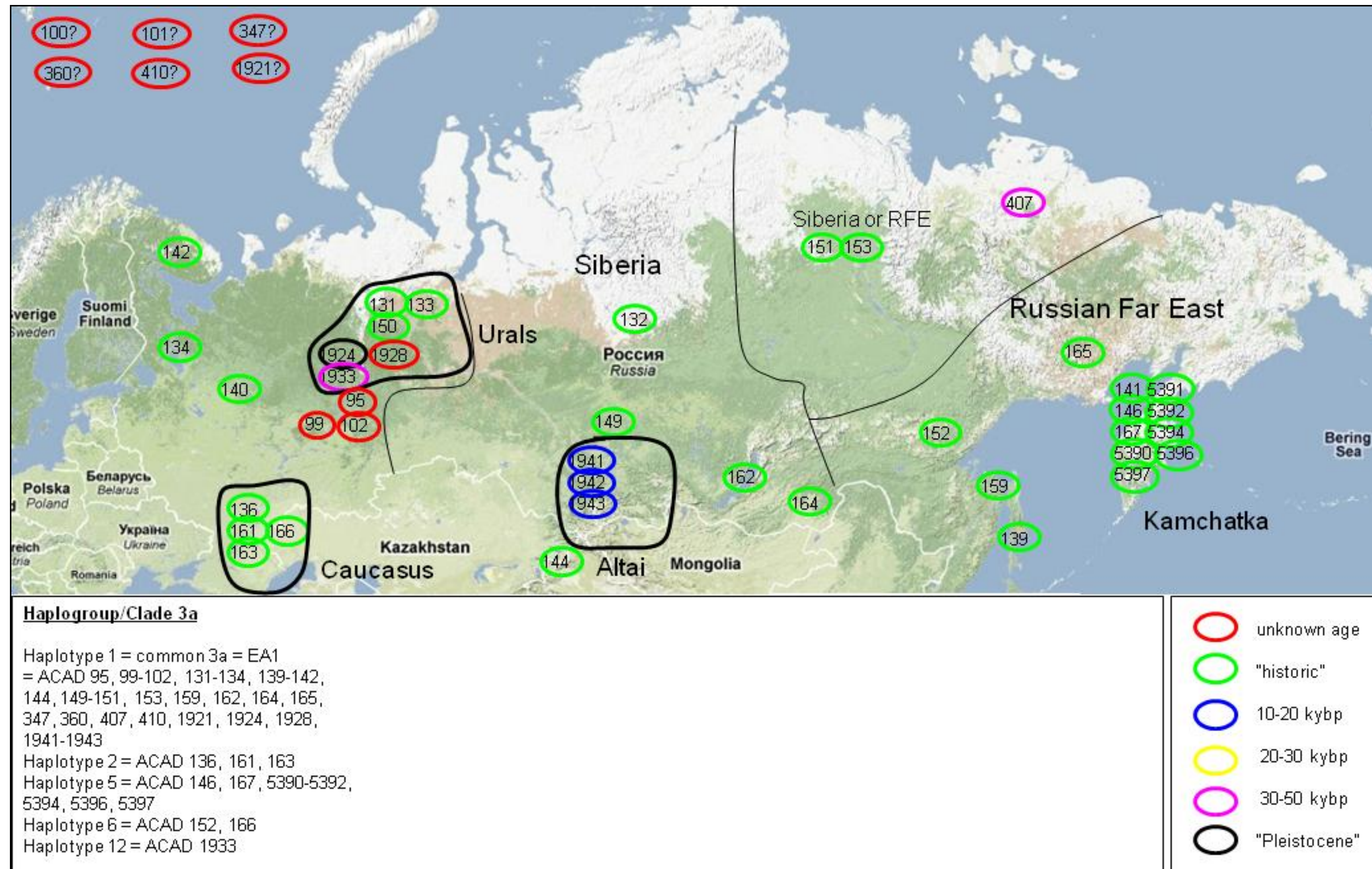

Figure 5.4 Approximate distribution of Haplogroup/Clade 3a samples in this study. The numbers refer to the ACAD number of the sample and can be cross-referenced with the appropriate Haplotype below the map. The coloured circles around the sample numbers indicate the age of the specimen. Samples without detailed location information e.g. sample ACAD410 “Russia” have been included in the top left-hand corner of the figure. The basic map was downloaded from Google Maps (http://maps.google.com) with key Eurasian mountain ranges and regions indicated by the addition of black lines and labels e.g Urals, Caucasus and Altai Mountains.

5.4.2 Spatial and temporal distribution of Clade 3b

Contrary to Korsten *et al*. (2009), this dataset also reveals a southern Eurasian presence for Clade 3b (Figure 5.5) with eight individuals from the Altai mountains to the Kuril Isles (near Japan) ranging from approximately 46,000 ybp to historic times. The existence of Clade 3b on continental Eurasia was indicated by Miller *et al.* (2006) but was only based on a single sample (not submitted to GenBank) and was subsequently questioned by Korsten *et al.* (2009). The new data presented here indicates that although Clade 3a has dominated northern Eurasia, Clade 3b has survived at the southern margins of the Eurasian continent, at least until historic times. Whether this is a continuous distribution from the Altai Mountains to Japan, or whether the distribution of Clade 3b ever extended further north than the Altai Mountains remains to be tested. The route that 3b bears took to migrate to north America across the Bering land bridge also remains to be investigated, to determine whether the 3b bears distribution extended northward along the eastern coast of the Eurasian continent or further inland through central Eurasia and Siberia. As the recent study of modern Eurasian bears failed to detect a single individual belonging to Clade 3b (Korsten *et al.*, 2009), the question of whether any 3b bears survive in modern Eurasian populations remains, however my study confirms that they were present in the Altai mountains from >46,000 ybp until at least the 1950s (although not necessarily continuously present during this period). Another point to note with respect to the 3b bears is that from eight individuals I obtained six haplotypes. From the Altai Mountains five 3b individuals were observed representing four different haplotypes. This is in contrast to the picture seen with 3a bears, where a single haplotype is found across the entire Eurasian continent, and suggests a different phylogeographic and demographic history for the two subclades.

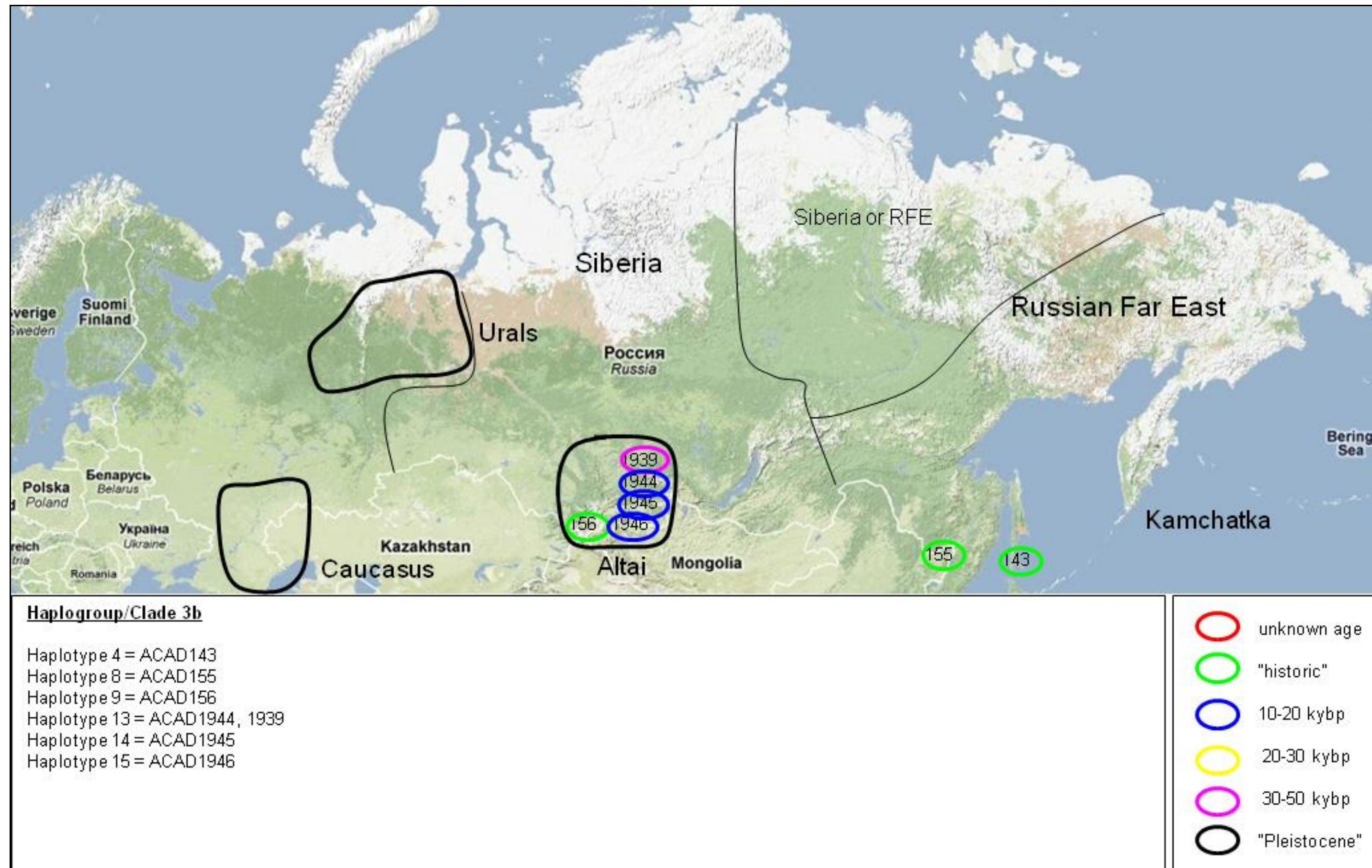

Figure 5.5 Approximate distribution of Haplogroup/Clade 3b samples from this study. The numbers refer to the ACAD number of the sample and can be cross-referenced with the appropriate Haplotype below the map. The coloured circles around the sample numbers indicate the age of the specimen. The basic map was downloaded from Google Maps (http://maps.google.com) with key Eurasian mountain ranges and regions indicated by the addition of black lines and labels e.g Urals, Caucasus and Altai Mountains.

5.4.3 Detection of Clade 4 in Continental Eurasia

In addition to the widespread distribution of Clade 3a and the southern fringe distribution of 3b specimens described above, the new dataset reveals the presence of four Eurasian bear specimens with unique mtDNA haplotypes which appear to represent previously undetected diversity within or closely related to the Clade 4 bears of modern North American populations (Figure 5.6).

Almost all North American Clade 4 bears are reported from modern populations (Waits *et al.*, 1998; Miller *et al.*, 2006), with three exceptions – a Pleistocene individual from Sixtymile in the Yukon radiocarbon dated to 35,970 ± 660 ybp (Barnes *et al.*, 2002), another Pleistocene specimen from Edmonton, Canada (approx. 26,000 ybp; Matheus *et al.*, 2004), and one from Ice Cave in Wyoming from the early-Holocene (Barnes *et al.*, 2002). It is assumed that these are all decendents of an unsampled Eurasian clade that crossed the Bering land bridge during the Pleistocene (Barnes *et al.*, 2002). Sequences from Japan (Masuda *et al.*, 2001) dated between 1,000-1,400 ybp have variously been clustered with Clade 3 bears (designated 3d in Miller *et al*. 2006) and with the North American Clade 4 bears in a recent Bayesian analysis (Korsten *et al.*, 2009). My study provides the first evidence of Pleistocene relatives of Clade 4 in Eurasia, and also extends the genetic diversity and temporal distribution of this group. The MRCA of Clade 4 was recently calculated to be 49,000 BP (Korsten *et al.*, 2009), and the evidence of two different Clade 4 haplotypes present at two disparate Eurasian locations (Caucasus Mountains and Urals Mountains) at 42,900 ± 1600 ybp and 43,600 ± 1500 ybp is consistent with this timeframe.

Two ancient specimens (ACAD1927, 43,600 ± 1500 ybp, Urals) and (ACAD4098, 42,900 ± 1600 ybp, Caucasus) with unique sequences suggest that Clade 4 (or related) bears harboured some level of diversity in Eurasia during the Pleistocene (before the LGM) and were present in more than one region during that time. The two historic specimens (ACAD137 and 154) also belong to different haplotypes, suggesting that similar to Clade 3b, this group may not have been affected by a past population bottleneck and subsequent expansion, with genetic diversity present before and after the LGM. Further sampling concentrating on the Caucasus and Urals mountains may reveal whether Clade 4 bears continue to survive in modern Eurasian populations, or whether they are limited to the North American continent and Japan in modern times.

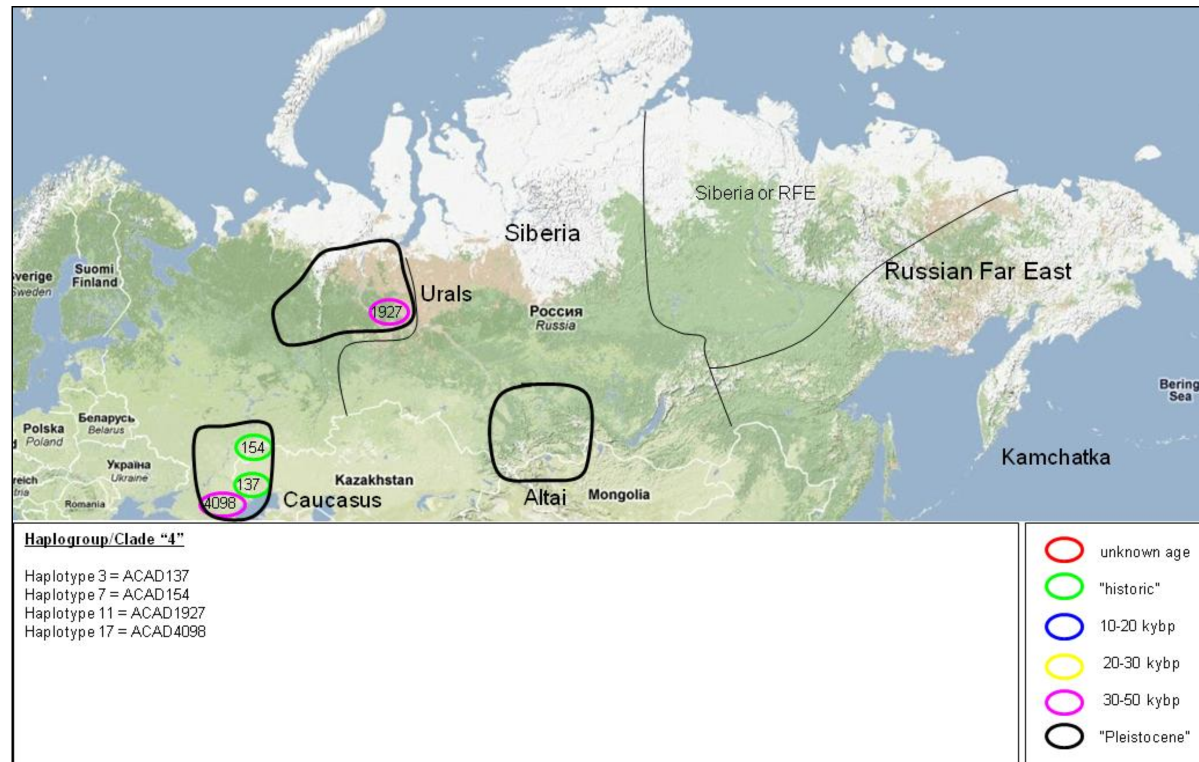

Figure 5.6 Approximate distribution of Haplogroup/Clade 4 samples in this study. The numbers refer to the ACAD number of the sample and can be cross-referenced with the appropriate Haplotype below the map. The coloured circles around the sample numbers indicate the age of the specimen. The basic map was downloaded from Google Maps (http://maps.google.com) with key Eurasian mountain ranges and regions indicated by the addition of black lines and labels e.g Urals, Caucasus and Altai Mountains.

5.4.5 Addressing the predictions based on the conclusions of Korsten et al. (2009)

The ancient and historic mtDNA sequences presented in my study provide new information for interpreting the hypotheses and conclusions of the recent study of modern Eurasian brown bear mtDNA phylogeography (Korsten *et al.*, 2009), outlined in section 5.1 of this chapter.

1). Consistent with the bottleneck hypothesis of Korsten *et al.* (2009), mtDNA diversity prior to the LGM was found to be higher than the present. While 50% of all modern individuals sampled by Korsten *et al*. (2009) belonged to only one of two Clade 3a haplotypes, I detected pre-LGM specimens belonging to Clades 3a, 3b and 4. However, while all specimens sampled in the modern study were found to belong to Clade 3a, my study detected the presence of Clade 3a, 3b and 4 both prior to, and following the LGM, and the proposed bottleneck only appears to dramatically reduce the post-LGM diversity of Clade 3a.

2). Following the predictions based on the modern data alone, immediately after the LGM bottleneck all samples should belong to the hypothesised founder haplotypes (Clade 3a haplotypes EA1 and EB1). All the Clade 3a sequences obtained from ancient bears dated from 10,000-20,000 ybp did indeed belong to the hypothesised founder haplotype EA1, however I also detected Clade 3b haplogroups immediately after the LGM (10,000-20,000 ybp), suggesting the bottleneck was not as severe as predicted from the modern data alone, or perhaps only involved bears of Clade 3a.

3). Consistent with the hypotheses of Korsten et al. (2009), all Clade 3a non-EA1 or non-EB1 haplotypes that were present before the LGM bottleneck were not detected after the LGM.

4). The pre-LGM distribution of samples belonging to EA1 and EB1 should indicate the location of the glacial refuge, however my study only revealed the presence of a single pre-LGM specimen (ACAD407, Indigirka area, 30,660 ± 180 ybp) belonging to the Clade 3a haplotype 1 (EA1), and it is not possible to make any conclusions about location of potential glacial refugia from a single specimen. While it is not possible to confirm the glacial refugial location for Clade 3a haplotype 1 (EA1) from this specimen, ACAD407 (Indigirka area, 30,660 ± 180 ybp) is important as it implies that the most recent common ancestor (MRCA) of this group is at least as early as 30,000 ybp, prior to the LGM and pre-dating the Bayesian estimation of 26,000 ybp calculated by Korsten *et al*. (2009). Additionally, while the oldest sequence of Clade 3a haplotype 1 (EA1) is dated to 30,660 ± 180 ybp, another Clade 3a sample (haplotype 12, non-EA1) was found to be even older, dated to >46,000 ybp (ACAD1933, Urals). Similarly, the oldest Clade 3b sample in my study was dated to >46,000 ybp (ACAD1939, Altai) which suggests that the MRCA for the split between Clade 3a and 3b is at least >46,000 ybp.

5.5 CONCLUSIONS

Based on modern Eurasian brown bear mtDNA sequences Korsten *et al* (2009) hypothesised the occurrence of a sudden demographic post-LGM expansion of Eurasian brown bears preceeded by a severe genetic bottleneck during or just prior to the last ice age. My new dataset supports some of the hypotheses of Korsten *et al* (2009), however it also brings into question other aspects of their conclusions. For instance, in contrast to the widespread

domination by Clade 3a haplotype 1 (EA1) observed in the modern dataset, I found evidence of extinct and extant haplotypes from Clades 3a, 3b and 4 pre-LGM, immediately post-LGM (3a and 3b) and in historic populations (3a, 3b and 4).

The data presented here suggests that either the pre-expansion bottleneck was much less severe than predicted, or that it did not affect all Eurasian regions or brown bear clades equally, with genetically diverse individuals from Clade 3b and 4 existing from >46,000 ybp until historic times in multiple regions across the Eurasian Continent (Urals, Caucasus, Altai Mountains). It could be speculated that Clade 3b inhabited the southern regions (e.g. Altai Mountains - Japan), Clade 4 inhabited western regions (e.g. Caucasus and Urals) and that bears of these clades maintained their diversity within mountainous refuge locations. Perhaps in contrast to this scenario, Clade 3a bears may have had a more widespread pre-LGM distribution without a single discrete refugium and yet may have been the only haplogroup to undergo a population bottleneck and subsequent expansion. Various putative glacial refugial locations have been suggested as the source of the Clade 3a expansion into Europe following the end of the LGM, for example from the Carpathian Mountains or Caucasus Mountains (Taberlet *et al.*, 1998; Hewitt, 2000; Hofreiter *et al.*, 2004; Sommer & Benecke, 2005; Saarma *et al.*, 2007) but it is still far from clear whether there was a single refugium or multiple refugia for Clade 3a to expand into Europe and other parts of Eurasia. The most ancient representative of Clade 3a haplotype 1 (EA1) originates from the Indigirka region (far north east of the continent) dated to approximately 30,000 ybp, however it is not possible to confirm a refugial location with a single individual specimen that predates that LGM period. Perhaps as Clade 3a was already widespread across Eurasia 46,700-30,000 ybp, individuals from a number of distant regions/refuges could have harboured the common Clade 3a haplotype 1 and thus expanded from multiple locations following the LGM. This could explain the apparent rapidity of recolonisation and lack of effective barriers to the expansion of 3a following the last glacial maximum (Korsten *et al.*, 2009).

A similar scenario has also been suggested for Eurasian badgers (*Meles meles*): A recent study of modern Eurasian populations found that this species can be divided into four phylogeographic groups based on mtDNA diversity: Europe, North & East Asia, Southwest Asia and Japan, thought to have originated due to the combined effects of Pleistocene glacial stages and geographic barriers such as mountain ranges and deserts (Marmi *et al.*, 2006). This study determined that the European, North & East Asian, and Southwest Asian badger groups evolved separately since the end of the Pliocene, with the Japanese group splitting off from continental Eurasian populations during the middle Pleistocene. A sudden demographic growth during the Middle Pleistocene was detected for European and Southwest Asian badgers but not for badgers of the other phylogeographic groups (Marmi *et al.*, 2006). This sudden expansion of particular badger haplogroups in Eurasia could be driven by similar factors as those affecting the expansion of Clade 3a brown bears. Similarly, as observed for Eurasian brown bear Clade 3b and 4, not all Eurasian badger haplogroups underwent the same sudden demographic expansion as Clade 3a bears or European and Southwest Asian badgers, suggesting that a single factor or event did not affect all Eurasian populations equally.

5.5.1 Future directions

The sequences generated as part of this study should be incorporated in more detailed Bayesian analyses in the future to re-calculate the MRCAs presented in Korsten *et al.* (2009). The addition of radiocarbon dated specimens from continental Eurasia including sequences from Clade 4 may greatly improve the estimations. These sequences should also be included in future analyses to develop a more comprehensive global understanding of brown bear phylogeography.

This study provided the first strong evidence of the presence of both Clade 3b and Clade 4 in continental Eurasia in ancient and historic times. Future studies may extend this study by sampling further to:

1) determine the relationships between the ancient continental Clade 3b and 4 bears and their modern counterparts in North America and Japan and gain insight into the timing and colonisation routes into these regions.

2) determine whether Clade 3b and 4 exist in modern Eurasian populations. Clade 3b and 4 were not detected in the recent study of 205 modern Eurasian bears (Korsten *et al.*, 2009), however this needs to be confirmed through further sampling as it could either reflect sampling bias in that study or alternatively suggest that Clade 3b and 4 are no longer present in continental Eurasia.

It would also greatly benefit this study to increase the length of sequences analysed from the small 135 bp CR fragment possibly up to the level of whole mtDNA genomes, particularly on a few of the key specimens, for example the four Eurasian samples that appear to belong to Clade 4. As more mtDNA genome sequences from modern brown bears continue to become available on GenBank, it would be of interest to compare an mtDNA genome from a modern North American Clade 4 bear with mtDNA genomes of ancient Eurasian Clade 4 bears. However, at this time, the 135 bp sequences are still informative as this is the most commonly sequenced fragment across numerous brown bear phylogeographic studies (as summarised in Table 1.2 of this thesis Introduction chapter 1) allowing comparative analysis with a greater number of modern and ancient sequences.

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CHAPTER 6

ANCIENT DNA REVEALS DIFFERENT PHYLOGEOGRAPHIC HISTORIES FOR THE EXTINCT NEW WORLD GIANT SHORT-FACED BEAR (*Arctodus simus*) AND THE BROWN BEAR (*Ursus arctos*)

6.1 INTRODUCTION

The late Pleistocene (approx. 50-10 kya) was a period of major global climatic change which culminated in mass megafaunal extinctions (Alroy, 1999). While more than 60 % of known animal genera >44 kg became extinct by the Holocene (approx. 10 kya) (Barnosky *et al.*, 2004), either as a result of climate change or human hunting (Alroy, 2001; Brook, Bowman, 2002; Koch, Barnosky, 2006), many others experienced profound changes to their distribution, population connectedness and demography (Hewitt, 2004). Evidence is increasing from ancient DNA (aDNA) studies to suggest that events during the Marine Isotopic Stage (MIS) 3 (65,000-25,000 ybp) prior to the start of last glacial maximum (LGM; approx. 21,000-18,000 ybp) played a defining role in reducing the genetic diversity of many species (for example brown bears, horses, bison and woolly mammoths; Barnes *et al.* 2002, Guthrie *et al*. 2003, Shapiro *et al.*, 2004, Barnes *et al.*, 2007), which in turn may have lead to an increased susceptibility to the effects of the LGM or human population expansion (Spielman *et al.*, 2004).

Brown bears colonised North America from the Old World across the Bering land bridge during the Wisconsinan stage (approx.100 kya) (Kurten, Anderson, 1980), and populations still persist in modern times. Ancient DNA studies of Beringian (Siberian, Alaskan and western Canadian) brown bears have already demonstrated a high level of genetic diversity and a dynamic phylogeographic turnover in this species throughout the late Pleistocene (Barnes *et al.*, 2002), making them a good example of a megafaunal species that experienced genetic turnover yet still survived the period of mass extinctions.

In contrast, nothing is known of the genetic structure of the contemporaneous giant short-faced bear (*Arctodus simus*), a member of the endemic New World Tremarctine bear subfamily, which became extinct in Beringia during the LGM (21,000 ybp) and from the ice-free lower states of North America approximately 10,000 ybp (Kurten, Anderson, 1980; Harington, 2003). Why an endemic bear species would become extinct during this period while a relatively recent colonist from the Old World would survive is an interesting question which remains to be resolved.

A variety of factors such as competition (Kurten, Anderson, 1980; Barnes *et al.*, 2002), diet and (putative) inability to hibernate (Matheus, 1995) may have played a role in the demise of *A. simus* although no consensus has been reached. Radiometric data suggests that *A. simus* became extinct in Beringia approximately 21,000 ybp, coincident with the recolonisation of the region by brown bears, which might be suggestive of competition. The giant short-faced bear has been previously described as a hyper-carnivore in comparison with the more omnivorous brown bear (Kurten, 1967; Matheus, 1995), however isotopic evidence that Beringian brown bears shifted to a more carnivorous diet following the extinction of the giant short-faced bears (Barnes *et al.*, 2002) lends some support to the idea that competition may have been a factor.

Similarly, it has been argued that the giant short-faced bear was constrained within a much narrower trophic niche than the brown bear, being restricted to terrestrial carnivory, while brown bears are more omnivorous, capable of surviving on herbivorous diets supplemented with meat or salmon whenever available (Matheus, 1995). Thus the more plastic ecology of the brown bear may have been a factor in their survival over the more specialised hyper-carnivorous giant short-faced bear, particularly if abundance of available terrestrial prey species was limited. However, this restriction of *A. simus* to hyper-carnivory has been questioned (Emslie, Czaplewski, 1985; Sorkin, 2006; Figueirido *et al.*, 2009) and the most recent evidence from cranio-dental morphology strongly suggests that *A. simus* was omnivorous (Figueirido *et al.*, 2009; Figueirido *et al.*, 2010) rather than a specialised scavenger or hyper-carnivore as previously depicted.

It has also previously been speculated that the ability to hibernate evolved only within the *Ursus* genus lineage (Matheus, 1995; Pages *et al.*, 2008) (i.e. *U. arctos*, *U. spelaeus*, *U. maritimus* and *U. americanus*), and that this may have given brown bears an advantage over giant short-faced bears in times of dietary shortage (Matheus, 1995).

In this study, the first to investigate the intra-specific mitochondrial genetic structure of a Tremarctine bear species (extinct or extant), I will analyse the genetic diversity and phylogeographic structure of the giant short-faced bear (*A. simus*) and compare these findings with those of the Pleistocene Beringian brown bears (Barnes *et al.*, 2002) to determine if genetic differences could shed any light on the differential survival of the two bears through the climatic fluctuations of the MIS3.

6.2 MATERIALS & METHODS

6.2.1 Samples

Forty-two bone or tooth root samples were obtained from *Arctodus*, Ursid and carnivore specimens from a variety of museum and field collections (See Appendix Table 5.1). The samples are from specimens distributed across the range of *A. simus*, from Alaska and Canada in the north west, to Ohio in the east of the lower 48 states of America (south of the maximum extent of the LGM ice sheets).

6.2.2 Ancient DNA extractions

The exterior surface of each sample was removed using a Dremel tool to eliminate surface contamination. Up to 0.70 g of the cleaned bone or tooth root was powdered using an 8 mm tungsten ball bearing in a Mikro-Dismembrator (Sartaurius) at 3000 rpm in sterilised stainless steel canisters. Bone/tooth root powder was then decalcified overnight in 10-20 ml of 0.5M EDTA (pH 8) on a rotary mixer at room temperature. The decalcified material was collected by centrifugation and digested in 3 ml of 100 mM Tris-HCl (pH 8), 100 mM NaCl, 0.5 mg/ml proteinase K, 10 mg/ml dithiothreitol (DTT), and 1% sodium dodecyl sulphate (SDS), overnight at 55 °C on a rotary mixer. Following digestion, an equal volume (3 ml) of Tris-saturated phenol was added and mixed on a rotary mixer for 10 minutes at room temperature, followed by centrifugation at 1500 g for 5 minutes. The aqueous phase was then transferred to a new tube. This process was repeated twice, once with an equal volume of Tris-saturated phenol, and once with an equal volume of chloroform. The final aqueous phase was de-salted with sequential additions of DNA-free water to an Amicon Ultra-4 Centrifugal Filter Unit (Millipore), and concentrated to a final volume of 100-200 µl. DNA extractions were

performed in batches of up to eight samples, with two negative extraction controls (containing no bone powder).

Figure 6.1 Location of *Arctodus simus* samples used in this study. Geographical location and number of samples from which DNA was successfully obtained are marked in black (Alaska and Ohio from USA, Yukon and Alberta from CANADA). Grey shading indicates approximate extent of glaciation at 21,000-18,000 ybp (Dyke *et al.*, 2002). Blue shading indicates approximate distribution of *A. simus* based on fossil sites (Kurten, Anderson, 1980).

6.2.3 PCR amplifications

All aDNA extracts were initially tested (as described in Chapter 2) using carnivore-specific primers designed to amplify a 135 base pair (bp) fragment of the hypervariable region of the mitochondrial (mtDNA) control region (CR) (Hänni *et al.*, 1994). Additionally, for the aDNA extracts performed or stored at the Australian Centre for Ancient DNA, new primers were designed and a multiplex PCR protocol (Rompler *et al.*, 2006) was applied yielding a total of 135-364 bp of CR sequence and up to 137 bp protein-coding ATP8 sequence (See Appendix Table 5.2 for amplification and sequence details and Appendix Table 5.3 for primer details). Amplification products of the expected size were cleaned and sequenced as described previously (Chapter 2, section 2.3.3).

6.2.4 Validation of aDNA results

All ancient DNA extractions and pre-PCR steps were performed in one of the following geographically isolated, dedicated ancient DNA facilities: the Australian Centre for Ancient DNA, Adelaide; the Oxford University Museum of Natural History, Oxford; and The Henry Wellcome Ancient Biomolecules Centre, Oxford.

Guidelines for the authentication of aDNA results were followed (Cooper, Poinar, 2000; Willerslev, Cooper, 2005; Rompler *et al.*, 2006), including multiple extraction blanks, negative PCR controls, independent repeat extractions, repeat amplifications and cloning. Four samples (sample numbers 3, 7, 9 and 12; Table1) were independently extracted and amplified in separate ancient DNA facilities, and one sample (sample number 15) was cloned and a consensus sequence from 14 clones (Jacobo Weinstock, unpublished data, see Appendix 5) was used for subsequent analyses. Seven samples (sample numbers 2, 3, 4, 5, 9, 11, and 12) were each sequenced from more than one independent PCR amplifications.

Additionally, an aliquot of the DNA extract from sample 7 was sent to the ancient DNA facility at the Max Planck Institute for Evolutionary Anthropology in Leipzig and underwent a series of independent amplification tests before being used in a whole mtDNA genome study (Krause *et al.*, 2008).

6.2.5 Phylogenetic analyses

Sequences were visually inspected using Sequencher (Genecodes corporation), and aligned using Clustal within the MEGA 4.1 program (Tamura *et al.*, 2007). Three datasets were generated: 1) Short dataset – 135 bp hypervariable Control Region fragment from 23 individuals 2) Concatenated dataset – using all available non protein-coding data for each individual (135-364 bp) and incorporating gaps as missing data 3) ATP8 dataset containing 137 bp of ATP8 for six individuals from across the geographic range of *A. simus* samples.

The low information content of the *A. simus* sequences (due to the small number of variable sites even within the hypervariable control region), precludes analysis by parameter-rich methods such as Bayesian phylogenetic inference. Neighbor-joining (NJ) and maximum parsimony (MP) analyses were therefore selected as more suitable methods for analysis of these datasets. MP analyses with 1000 bootstrap replicates were performed on all 3 datasets separately using MEGA 4.1 (Tamura *et al.*, 2007). The MP trees were obtained using the Close-Neighbor-Interchange (CNI) algorithm in which the initial trees were obtained with the random addition of sequences (100 replicates). The NJ analyses with 1000 bootstrap replicates were performed on the 135 bp control region dataset and the 137 bp ATP8 dataset, but not the concatenated dataset as NJ analyses do not account for sequences of varying length or missing data.

To further investigate the genetic diversity within the *A. simus* sequences and to attempt to identify any patterns of phylogeographic structure, a minimum-spanning haplotype network was constructed in TCS based on the 135 bp hypervariable control region fragment.

An haplotype network using the short 135 bp dataset was constructed in TCS (Clement *et al.*, 2000) using an input file generated by FaBox (Villesen, 2007). A connection limit of 95% was used and as there are no indels in this dataset, and missing data is only the result of a very small number of ambiguous bases or missing bases at the start of the sequence at invariable sites, therefore any gaps were treated as missing data.

6.2.6 Statistical genetic measures

Standard measures of genetic diversity (haplotype diversity, nucleotide diversity, and mean pairwise differences) were calculated in DnaSP (Rozas *et al.*, 2003) and Arlequin (Schneider *et al.*, 2000) using 135 bp Control Region sequence from 23 *A. simus* individuals and also for 27 contemporaneous Pleistocene Beringian brown bears (See Appendix Table 5.4 and (Barnes *et al.*, 2002)) as a comparison. Tajima's D, Fu and Li's F* and D* and Fu's FS test statistics

were also calculated for both *A. simus* and *U. arctos* datasets in DnaSP (Rozas *et al.*, 2003) to test for signs of neutrality and demographic expansion.

6.3 RESULTS

135 – 364 bp Control Region mtDNA was successfully extracted, amplified and sequenced from 23 extinct *A. simus* specimens (Table 6.1) from Alaska, Canada and central North America, including samples from areas north and south of the maximum extent of LGM ice cover (Figure 6.1). The samples from which DNA was successfully sequenced span a period of approximately 33,000 years (44,240 ± 930 -11,619 ± 40 ybp), from pre-LGM Alaska and Canada to post-LGM from south of the ice.

Table 6.1 List of ancient bone samples used in this study from which a minimum of 135-bp Control Region sequence was obtained.

Sample no.	Geographic origin of Specimen	Museum/ Collection ID †	C^{14} date (ybp) ‡	DNA extraction reference no. *
1	CANADA – Edmonton, Alberta, consolidated pit #48	P96.2.38	N/A	ACAD346A
2	CANADA – Yukon, Ophis Creek	YG-24.1	20,210 ± 110 (Harington, 2001)	ACAD434A
3	CANADA – Sixty Mile	CMN 42388	44,240 ± 930 (Harington, 2001)	ACAD438A + IB 187
4	OHIO, USA – Sheriden Pit	CMNHS VP8289	11,619 ± 40 [#]	ACAD1734A
5	ALASKA – Goldstream	#850 575 UCLA	N/A	ACAD437A
6	CANADA – Quartz Creek	YT03/134	26,940 ± 570 (ANUA-38615)	ACAD1954A
7	CANADA – Dawson area, Eldorado Creek Loc.45	CMN 37957	22,417 ± 452 (Wk20235)	ACAD424A + IB 253
8	ALASKA	AMNH ‘ALASKA Bx35’	25,264 ± 650 (Wk20236)	ACAD450A
9	CANADA – Dawson area, Hester Creek Loc.57	CMN 49874	26,720 ± 270 (OxA-9259)	ACAD330A + AC 688
10	CANADA – Hester Creek	76.4 (JS)	N/A	ACAD344A
11	CANADA – Gold Run	CMN 34556	N/A	ACAD428A
12	ALASKA – Ester Creek	FAM 95656	N/A	ACAD441A + IB 202
13	ALASKA – No.2 G-strip area	AMNH A-82-1039	N/A	ACAD443A
14	ALASKA – Goldstream	AMNH A-1828	N/A	ACAD436A
15	CANADA – Hester Creek	YT03/288 Cat. No. 129.1 (JS)	N/A	JW 131
16	ALASKA – Dawson Cut	AMNH A-676-5625	N/A	IB 191
17	CANADA – Cripple Creek	AMNH A-217-	N/A	IB 195

Sample no.	Geographic origin of Specimen	Museum/ Collection ID †	C^{14} date (ybp) ‡	DNA extraction reference no. *
		2297		
18	ALASKA – Eva Creek Mine	PM-97-001-100	N/A	BS 3
19	CANADA – Dawson area	CMN 36236	N/A	BS 72
20	CANADA – Dawson, Hunker Creek, 80-Pup	CMN 44566	N/A	BS 71
21	CANADA – Dawson, Hunker Creek	CMN 42335	N/A	BS 73
22	ALASKA – Lillian Creek	UAF/Paleo V-55-524	N/A	BS 74
23	CANADA – Dawson	CMN 37577	N/A	IB 255

† Museum/Collection abbreviations as follows: Canadian Museum of Nature (CMN), American Museum of Natural History (AMNH), Frick Collection, American Museum (FAM), University of Alaska, Fairbanks (UAF/Paleo), Yukon Territories (YT) field collection, Yukon Government collections at Whitehorse (YG), University of California, Los Angeles (UCLA) and Cincinnati Museum of Natural History & Science (CMNHS). Other samples were personally collected by Paul E. Matheus (PM) or John Storer (JS).
‡ Radiocarbon date followed in parentheses by laboratory reference number (if dated in this study) or source from which the date was obtained.
# Personal communication: Greg McDonald 2007. Weighted average of CAMS-12837, 12839 and 12845.
* Extraction performed by Sarah Bray (ACAD), Ian Barnes (IB), Beth Shapiro (BS), Alan Cooper (AC) or Jacobo Weinstock (JW). For samples that were independently extracted in separate laboratories, both extraction references are given.

Maximum parsimony and neighbor-joining phylogenetic analyses of the control region datasets placed all 23 individuals in a strongly supported monophyletic clade together with the whole mtDNA genome sequence of *A. simus* (GenBank accession NC011116), the extant Andean Spectacled bear (*Tremarctos ornatus*) falling out as the sister taxon. As both MP and NJ analyses produced largely concordant results only the maximum parsimony phylogeny is presented here (Figure 6.2) although nodal bootstrap support values from both MP and NJ analyses are indicated.

The control region sequences fall into two moderately-well supported groups/clades however there is no discernable geographic or temporal partitioning and all internal branches are very short. An additional 137 bp of the mtDNA protein-coding ATP8 gene was amplified and sequenced from six individuals (including representative samples from Alaska, Canada and Ohio). Although this fragment was found to possess only a single parsimony informative site, it differentiated the samples into the same two groups/clades revealed by the more variable control region dataset (See Appendix Figure 5.1).

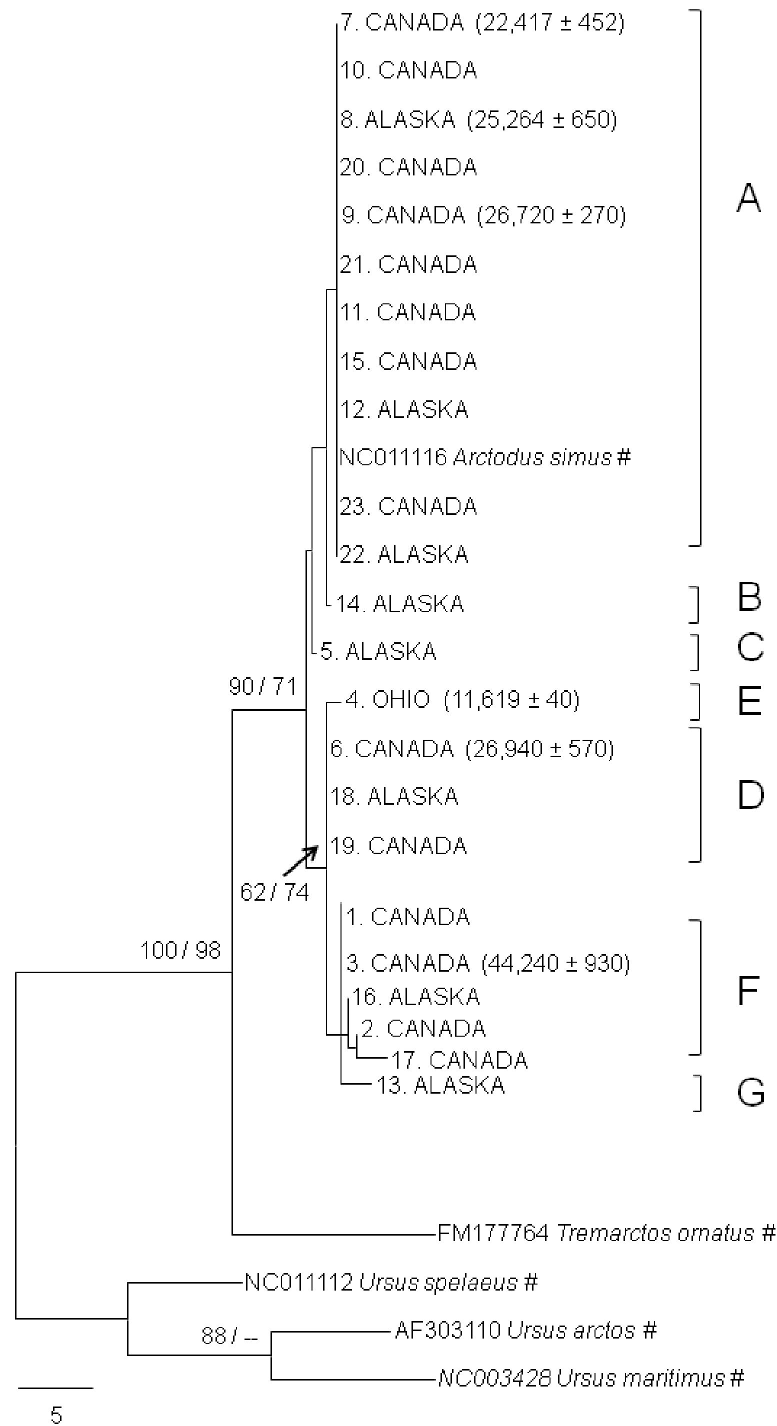

Figure 6.2 Maximum parsimony analysis of *Arctodus simus*. Analysis performed using all available non-protein-coding sequence data for each specimen (135-364 bp incorporating tRNA Pro/Thr – hypervariable control region). See Appendix Table 5.2 for specific fragments and sequence lengths used for each individual specimen. Numbers above branches are Maximum parsimony bootstrap values (>60%) based on 1000 replicates, followed by bootstrap values (>60%) obtained in a Neighbour-Joining analysis based on 1000 replicates using the 135 bp dataset. Taxa are labelled with sample number (refer to Table 6.1 for details), geographic origin and radiocarbon date (if available). Letters A – G correspond to the seven haplotypes identified from the 135 bp CR dataset. Homologous 364 bp sequence from a cave bear (*U. spelaeus*), brown bear (*U. arctos*), polar bear (*U. maritimus*), and a spectacled

bear (*T. ornatus*) obtained from GenBank (indicated on the figure by # and Genbank accession number) were used as outgroups.

The haplotype network (Figure 6.3) confirmed the extremely low level of genetic diversity within *A. simus* observed in the MP and NJ analyses, with only 7 haplotypes identified from the 23 individuals across their geographic range. There are no obviously divergent clades or haplogroups apparent, with each haplotype only differentiated from the next by 1-2 single nucleotide substitutions/polymorphisms. The network does not reveal any notable phylogeographic patterns with all haplotypes represented by more than a single individual being found in both Canada and Alaska.

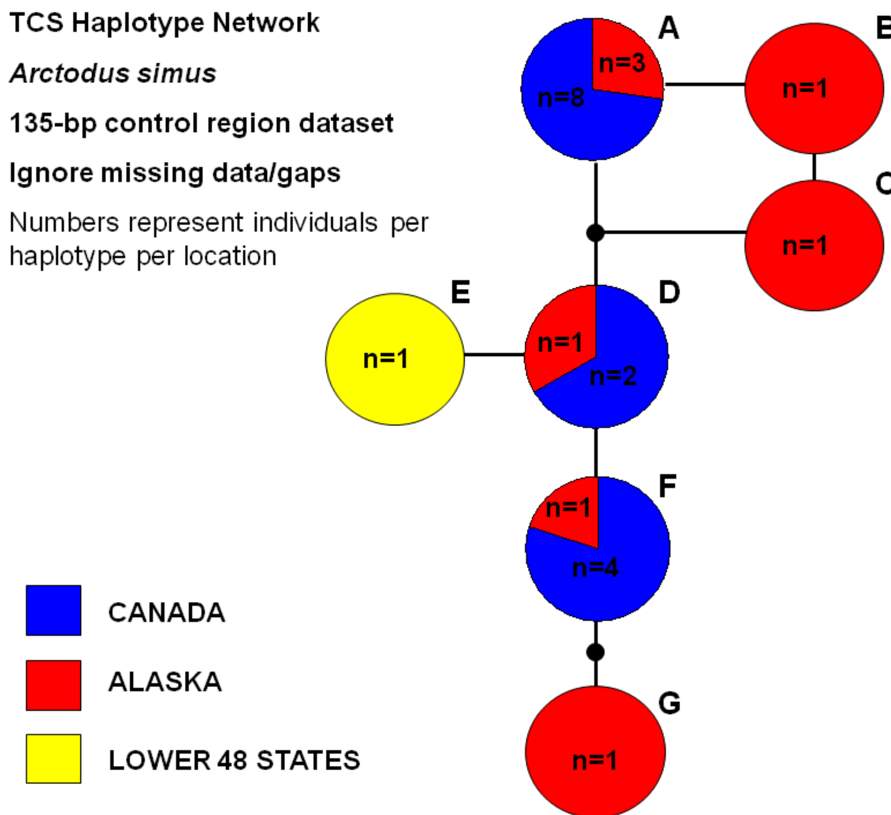

Figure 6.3 Minimum spanning mtDNA haplotype network of *Arctodus simus*.
The haplotype network was constructed using the statistical parsimony method of TCS based on the 135-bp control region dataset. Gaps were treated as missing data, and a 95% confidence level was applied. The large circles represent the different haplotypes, while the small black circles represent undetected intermediate haplotype states or mutational steps. The geographical origin of samples used in the haplotype network is indicated by different colours (blue - Canada, red - Alaska, yellow – Ohio, lower 48 states of USA). Numbers within the circles indicate the haplotype frequencies per geographic location. Letters A – G designate the seven haplotypes identified from the 135 bp CR dataset.

To allow some level of quantification to be made between the amount of control region variation in *A. simus* and their contemporaneous Beringian brown bears (*U. arctos*), standard measures of genetic diversity were calculated using the 135 bp CR sequences generated in this study and the homologous 135 bp CR sequence from 27 Pleistocene brown bears (See Appendix Table 5.4 and (Barnes *et al.*, 2002)). In concordance with the phylogenetic analyses, the results of the population genetic analyses (Table 6.2) highlight the extreme lack of variation within the *A. simus* sequences, particularly in comparison to those of Pleistocene Beringian brown bears.

Tajima's D values and Fu and Li's F* and D* statistics were found to be not significantly different to zero for either *A. simus* (D = -0.05165 P > 0.10, F* = -0.56262 P > 0.10, D* = -0.66182 P > 0.10) or *U. arctos* (D = -1.24412 P > 0.10, F* = -1.23036 P > 0.10, D* = -0.95528 P > 0.10). Fu's Fs statistic was significant for *U. arctos* (Fs = -4.692, P < 0.05) but not for *A. simus* (Fs = -1.138, P > 0.05). The significant Fu's Fs value for brown bears but lack of significance for the other test statistics suggests range expansion (rather than selection) (Fu, 1997) for Beringian brown bears but not for giant short-faced bears.

6.4 DISCUSSION

Loss and turnover of mtDNA diversity in Eurasian and New World megafauna predating the LGM has been identified in bison (Shapiro *et al.*, 2004), mammoth (Barnes *et al.*, 2007), and lions (Barnett *et al.*, 2009). A common theme among many of these species seems to be a late Pleistocene decrease in population size from a previously genetically diverse population, followed either by some level of recovery through repopulation events from source populations in the Old World (or vice versa in the case of Mammoths; Barnes *et al*. 2007), or extinction by the start of the Holocene period.

Consistent with this pattern, brown bears underwent a period of dynamic population changes, with local extinctions of some mtDNA clades followed by repopulation by bears of other mtDNA clades from the Eurasian continent across the Bering land bridge (Barnes *et al.*, 2002). In direct contrast to the relatively high levels of genetic diversity and dynamic phylogeographic patterns of extinction and repopulation observed in Beringian brown bears (*U. arctos*) during the late Pleistocene (Leonard *et al.*, 2000; Barnes *et al.*, 2002), the giant short-faced bear (*A. simus*) appears to have been genetically impoverished throughout this time with a lack of discernable phylogeographic structure. This could be explained if *A. simus* underwent a genetic bottleneck prior to 44,000 years ago, or alternatively if a low level of genetic diversity was a feature of this New World bear.

The low levels of genetic diversity observed in the mtDNA control region dataset for *A. simus* (Table 6.2) is of a comparable level with a number of endangered and threatened modern taxa for example the brown kiwi *Apteryx australis* (Shepherd, Lambert, 2008) and including the infamously genetically depauperate African cheetah (no. of samples = 20, no. of haplotypes = 8, nucleotide diversity = 0.0131) (Freeman *et al.*, 2001). Possessing such a reduced level of genetic diversity is thought to influence long-term survival and population fitness by limiting a species ability to respond to changing environmental conditions (O'Brien, Evermann, 1988; Hedrick, Kalinowski, 2000; McCallum, 2008). As a lack of genetic diversity and associated inability to adapt to disruptions of their ecological niche is believed to have contributed to the endangerment of many other extant highly specialised carnivorous species including lions (Burger *et al.*, 2004; Barnett *et al.*, 2006) and the marsupial Tasmanian devil (Jones *et al.*, 2004), similarly low levels of mtDNA variation in *A. simus* might indicate that the New World bears were more vulnerable to the changing conditions in the lead up to the Pleistocene/Holocene boundary than the more genetically diverse Old World bears (*U.arctos*).

Table 6.2 Genetic diversity measures of late Pleistocene giant short-faced bears and contemporaneous Beringian brown bears. Calculations performed in DnaSP (Rozas *et al.*, 2003) and Arlequin (Schneider *et al.*, 2000) using the homologous 135-bp control region sequence from 23 Pleistocene *A. simus* specimens (this study) and 27 Pleistocene *U. arctos* specimens (Barnes *et al.*, 2002) ranging in age from10,015 ± 62 to >59,000 (refer to Appendix Table 5.4 for detailed list of sequences used).

Species	**No. of samples**	**No. of haplotypes**	**Mean pairwise differences**	**Haplotype diversity (h) ± SD**	**Nucleotide diversity (π) ± SD**
Arctodus simus	23	7	1.865613 ± 1.107947	0.731 ± 0.078	0.01492 ± 0.00204
Ursus arctos	27	16	4.678063 ± 2.365045	0.840 ± 0.071	0.03742 ± 0.00922

However, there is also some evidence to suggest that brown bears became locally extinct in North America 35,000-25,000 ybp (Barnes *et al.*, 2002), indicating that they might have been equally effected as *A. simus* by climatic or other events occurring at that time, with the only major difference between the bears being the presence of genetically diverse source populations of *U. arctos* in Eurasia which could recolonise North America following local population bottlenecks, while the New World *A. simus* did not.

The 135 bp sequence fragment used in this study falls within the hypervariable control region of mtDNA, and has been found to be informative in intra-specific phylogenetic and phylogeographic studies in both modern and ancient Ursine bears (Hänni *et al.*, 1994; Wooding, Ward, 1997; Waits *et al.*, 1998; Leonard *et al.*, 2000; Barnes *et al.*, 2002). However, as mtDNA diversity studies are lacking for the only extant member of the Tremarctine lineage, the Andean Spectacled bear (*T. ornatus*), it is unknown whether the lack of diversity observed in *A. simus* is a result of a reduction in genetic diversity of this species due to a bottleneck predating the LGM, (as seen in Beringian bison, mammoths and cave lions (Shapiro *et al.*, 2004; Barnes *et al.*, 2007; Barnett *et al.*, 2009)), or instead is a feature of the New World bears in general.

Although low levels of genetic diversity combined with an absence of phylogeographic structure are often associated with population bottlenecks, there are several other possible scenarios to consider in explaining the differential variation of the control region observed in *A. simus* and *U. arctos*.

The strong mtDNA phylogeographic signal observed in brown bears is believed to be strengthened and maintained as a result of female philopatry exhibited in this species (Randi *et al.*, 1994; Taberlet, Bouvet, 1994; Talbot, Shields, 1996). Perhaps the comparative lack of phylogeographic structuring in *A. simus* suggests that *A. simus* was not female philopatric, at least not to the same extent as seen in brown bears, although this doesn't necessarily lead to a lower mtDNA diversity. A comparatively lower genetic diversity in *A. simus* than *U. arctos* might be expected if the species was primarily a solitary animal with a large dispersal range and relatively small effective population size. Morphologically there is some evidence which would be supportive of this scenario. *A. simus* fossils possess characters suggestive of a highly efficient gait well suited to wide range dispersal (Matheus, 1995) with some resemblance to felids; for instance the fore- and hind feet are turned forwards in contrast to

the toe-in position seen in most bears (Kurten, Anderson, 1980). A similar lack of diversity of mtDNA haplotypes across large geographic areas can also be seen in other large scavenger species such as the striped hyena (*Hyaena hyaena*) and brown hyena (*Parahyaena brunnea*) in Africa (Rohland *et al.*, 2005). It is also interesting to note that even the *A. simus* individual from Ohio (sample 4) only differed by one nucleotide substitution from Alaskan and Canadian sequences, despite possibly being separated from the northern bear populations by the Laurentide and Cordilleran ice sheets during the LGM. This is in contrast to the lack of gene flow observed between Pleistocene American lion populations from north and south of the ice sheets (Barnett *et al.*, 2009), and suggests that the ecological or competitive barriers operating for lions did not restrict *A. simus* in the same way, perhaps due to greater dispersal range.

There are also disparities evident in the evolutionary histories of the two bears which could contribute to the difference observed in their genetic diversity and phylogeographic structure. As a member of the Tremarctine bear lineage, *A. simus* was a New World endemic, and may have always had a long-term low effective population size. Nuclear microsatellite studies of the extant spectacled bear suggest that although being genetically impoverished, no signal of a recent population bottleneck could be detected (Ruiz-Garcia, 2003; Ruiz-Garcia *et al.*, 2005) – perhaps indicating that a lack of diversity is a feature of Tremarctine bears possibly through small effective population sizes. On the other hand, brown bears are Old World in origin, and only entered North America relatively recently via the Bering land bridge. The existence of a diverse sympatric source population in Eurasia has been demonstrated to be important for a number of species including brown bears (Barnes *et al.*, 2002), allowing reinvasions/repopulations following local extinctions in the New World. If *A. simus* had similarly passed through a population bottleneck or experienced local extinctions prior to the LGM, they would be unable to supplement their reduced genetic diversity with new colonists in the way that *U. arctos* could.

6.5 CONCLUSION

This study identifies differences in genetic diversity and phylogeographic structuring between two late Pleistocene bears in North America (the Old World colonist *U. arctos* and the New World endemic *A. simus*), and provides insight into the factors involved in the differential genetic response of these two species in the lead up to the mass megafaunal extinctions of the Pleistocene/Holocene boundary. It appears that in addition to being (possibly) less ecologically plastic than brown bears, the giant short-faced bear was at a disadvantage genetically with very low levels of genetic diversity and a lack of a source population to replenish/supplement from following a population bottleneck.

The low levels of genetic diversity and the lack of phylogeographic structure revealed in late Pleistocene giant short-faced bear populations adds to the growing body of evidence that reductions in genetic diversity predated both the LGM and arrival of humans for a number of megafaunal taxa in North America. As more population-level studies of both Old and New World Pleistocene species are completed, it should become possible to identify patterns in their genetic responses to climate change and to differentiate between factors that contributed to the extinction of some, but not all megafaunal species.

Additionally, this study represents the only known investigation of mtDNA diversity within any member of the New World Tremarctine bears, allowing population level comparisons with the Ursine bears for the first time, and raising questions about the differences in evolutionary responses of these two bear subfamilies to changing climatic conditions of the

late Pleistocene. It is apparent that a comprehensive study of the mtDNA diversity of the extant spectacled bear is now well overdue, and would provide valuable information not only in light of conservation management but also for understanding the evolutionary history of the New World Tremarctine bears.

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CHAPTER 7

EVOLUTIONARY RELATIONSHIPS OF THE TREMARCTINE BEARS

7.1 INTRODUCTION

The Tremarctinae are an endemic New World subfamily of bears which have been divided into two subgroups based on morphology: the basal spectacled bears and the more derived short-faced bears (Kurten, 1967; Kurten, Anderson, 1980; Tedford, Martin, 2001; Soibelzon, 2002; Soibelzon *et al.*, 2005). The spectacled bears include the earliest known Tremarctine bear, *Plionarctos* (originating in North America during the late Miocene-early Pliocene, Tedford, Martin, 2001) and *Tremarctos* (*T. floridanus* described in North America from the Pliocene-Pleistocene and *T. ornatus* in South America during Recent times; Tedford, Martin, 2001; Kurten, Anderson, 1980). The short-faced bears include two North American species from the Pliocene-Pleistocene –*Arctodus simus* (giant short-faced bear) and *Arctodus pristinus* (lesser short-faced bear) (Kurten, Anderson, 1980) and up to five species of *Arctotherium* from South America's Pleistocene (Soibelzon, 2002). The only surviving member of the Tremarctine lineage is the endangered South American spectacled bear (*T. ornatus*), a mainly herbivorous inhabitant of the Andean cloud forests. Mitochondrial (mtDNA) (Yu *et al.*, 2007; Krause *et al.*, 2008) and nuclear (Pages *et al.*, 2008) genetic studies have recently confirmed the Tremarctinae as the basal sister group to the rest of the Ursine bears, yet the systematics within the subfamily remains unclear.

According to the current morphologically-based phylogeny (Figure 7.1), the two short-faced bear genera form a monophyletic group consisting of the North American *Arctodus* and the South American *Arctotherium* species. Similarly, within the spectacled bear group, the genus *Tremarctos* forms a monophyletic group consisting of the North American *T. floridanus* and South American *T. ornatus*. However, the evidence supporting these groupings is far from conclusive – with one cladistic analysis (Trajano, Ferrarezzi, 1994) unable to identify a single unequivocal synapomorphy to link the two *Tremarctos* species, and only one character (molar width) and a general similarity in body morphology uniting the two short-faced bear genera.

Palaeontological evidence suggests that Tremarctine bears dispersed into South America at least twice (Prevosti *et al.*, 2003). The first dispersal event is thought to have occurred during the early Pleistocene as part of the Great American Biotic Interchange (GABI) following the formation of the Panamanian Isthmus approximately 2.5-3.0 million years ago (mya) (Webb, 1976; Cione, Tonni, 1995). Fossil records of *Arctotherium angustidens* from the Pampean region of Argentina and Bolivia dated to the Ensenadan Age (Pampean geological epoch approximately equivalent to the early-mid Pleistocene) (Soibelzon *et al.*, 2005) are believed to provide some of the earliest evidence of bears on the South American continent, supporting the first early dispersal event.

The second dispersal event is believed to have occurred much more recently - during the late Pleistocene/Holocene - by *Tremarctos*. While the extant spectacled bear *T. ornatus* has been present in the Andes from Recent times, the complete absence of this genus from the fossil record of South America does suggest a very recent arrival from North America (Trajano, Ferrarezzi, 1994; Soibelzon *et al.*, 2005) presumably as a descendent of the Pleistocene

Florida bear *T. floridanus*. Alternatively this raises the possibility that *T. ornatus* evolved from the earlier South American short-faced bears.

However, the relatively poor record of stratigraphy and fragmentary nature of many South American Tremarctine fossils (Soibelzon *et al.*, 2005) combined with a confusing taxonomic history and the great range of individual variation observed within bear species (Kurten, 1966; Kurten, 1967; Trajano, Ferrarezzi, 1994) has lead others to question whether there is sufficient evidence to differentiate between the roles of dispersal and *in situ* speciation in the evolutionary history of the Tremarctine bears (Trajano, Ferrarezzi, 1994).

NOTE:
This figure is included on page 118 of the print copy of the thesis held in the University of Adelaide Library.

Figure 7.1 Hypothesised phylogeny of the Tremarctine bears based on morphological characteristics. Figure adapted from (Trajano, Ferrarezzi, 1994), with key inferred synapomorphies highlighted: (a) relatively wider molars (b) P4 molar even wider than in *Arctodus* (c) upper pre-molars compressed. It is worth noting that in this figure the *Tremarctos* branch is differentiated from the others only by overall smaller size rather than any unequivocal synapomorphy, while the more derived *Arctodus* and *Arctotherium* branches are grouped monophyletically based primarily on the relatively larger size of their molar teeth.

Accurate dating of the divergence events within the Tremarctinae might provide insight into whether the South American bears evolved in North America and subsequently dispersed into South America, or whether speciation may have occurred *in situ* on the South American continent. A mitogenomics study (see Appendix 7) recently estimated the divergence between the extinct North American giant short-faced bear (*A. simus*) and the extant South American spectacled bear (*T. ornatus*) at 5.33-6.98 million years ago (mya) (Krause *et al.*, 2008). This would be consistent with a split occurring between these genera in North America, before the formation of the Panamanian Isthmus approximately 2.5-3.0 mya (Webb, 1976), but the timing of the split between the two short-faced bear genera *Arctodus* and *Arctotherium* remains unknown, as does the divergence date between the South American short-faced bears and the extant Andean spectacled bear *T. ornatus*.

To date no genetic data has been presented from the extinct South American short-faced bears, *Arctotherium sp.* which might help to clarify the evolutionary relationships between North and South American short-faced bears and the extant South American spectacled bear. This study aims to examine both the evolutionary relationships and timing of divergence

between two of the late Pleistocene short-faced bears (*A. simus* and *Arctotherium tarijense*) and the endangered spectacled bear (*Tremartos ornatus*) using a molecular approach.

7.2 MATERIALS & METHODS

7.2.1 Samples

Bone or tooth root samples (0.04 – 0.55 g) were obtained from four specimens of the extinct South American short-faced bear genus (*Arctotherium*) of which three were identified tentatively to the species level as *Arctotherium tarijense*, based on morphological characteristics (Martin *et al.*, 2004) and/or geographic origin and age (Leopoldo Soibelzon, personal communication; 2007). Ancient samples from which DNA could successfully be retrieved were radiocarbon dated - either at Waikato Radiocarbon lab (this study) or as part of a previous study (Martin *et al.*, 2004).

Additionally I obtained one historic bone sample and one modern tissue sample from specimens of the Andean spectacled bear, *T. ornatus*, the only extant member of the Tremarctinae. See Table 7.1 for details of the samples extracted in this study.

7.2.2 Ancient DNA extractions

All ancient DNA extractions were performed in the dedicated ancient DNA facility at the Australian Centre for Ancient DNA (ACAD) as described below:

For intact bone or tooth root samples the exterior surface was removed using a Dremel tool to eliminate surface contamination. The cleaned bone or tooth root was then powdered using an 8 mm tungsten ball bearing in a Mikro-Dismembrator (Sartaurius) at 3000 rpm in sterilised stainless steel canisters. Bone/tooth root powder was then decalcified overnight in 10-20 ml of 0.5M EDTA (pH 8) on a rotary mixer at room temperature. The decalcified material was collected by centrifugation and digested in 3 ml of 100 mM Tris-HCl (pH 8), 100 mM NaCl, 0.5 mg/ml proteinase K, 10 mg/ml dithiothreitol (DTT), and 1% sodium dodecyl sulphate (SDS), overnight at 55 °C on a rotary mixer. Following digestion, an equal volume (3 ml) of Tris-saturated phenol was added and mixed on a rotary mixer for 10 minutes at room temperature, followed by centrifugation at 1500 g for 5 minutes. The aqueous phase was then transferred to a new tube. This process was repeated twice, once with an equal volume of Tris-saturated phenol, and once with an equal volume of chloroform. The final aqueous phase was de-salted with sequential additions of DNA-free water to an Amicon Ultra-4 Centrifugal Filter Unit (Millipore), and concentrated to a final volume of 100-200 µl. DNA extractions were performed in batches of eight samples, with two negative extraction controls (containing no bone powder).

7.2.3 Modern DNA extractions

The modern tissue from the spectacled bear specimen (ACAD#3597, La Plata Zoo) was extracted with a Qiagen DNeasy Blood and Tissue kit according to manufacturer's instructions in a modern DNA extraction laboratory at the South Australian Museum EBU geographically isolated from the ancient DNA facility.

Table 7.1 South American bear samples extracted for this study.

Extract no.	Bear identity	Location	Museum Accession no.	Sample details	mtDNA sequenced (bp)	Age
ACAD 340	*Arctotherium tarijense*	Cueva del Milodon, Chile ¥	Malmo Katal 216, Sweden	0.44 g bone (femur)	365	13,257 ± 147 ybp *
ACAD 3599	*Arctotherium tarijense*	Cueva del Puma, Chile	Punta Arenas CEHA	0.55 g bone (femur)	694	10,345 ± 75 ybp †
ACAD 3577	*Arctotherium tarijense*	Unknown	La Plata 9645	0.11 g bone (tibia)	Fail	Unknown
ACAD 3578	*Arctotherium sp.*	Lujanese, Camet Norte, Argentina	La Plata	0.04 g tooth root (molar)	Fail	Unknown
ACAD 157	*Tremarctos ornatus*	unknown	MMZ S-35539 Moscow	0.28 g	135	Historic
ACAD 3597	*Tremarctos ornatus*	La Plata Zoo, Argentina	La Plata Zoo, Argentina	Toe pad tissue	135	Modern

¥Also known as Mylodon Cave or Ultima Esperanza, Chile; (52º 05' 37" S; 69º 44' 31" W)
*Uncalibrated radiocarbon date: 13,257 ± 147 ybp, dN15: 6.92, dC13: -19.12 Waikato Ref # WK20234.
†Uncalibrated radiocarbon date: 10,345 ± 75 ybp, dC13: -19.5 (Ua21033) (Martin *et al.*, 2004).

7.2.4 PCR amplifications

All aDNA extracts were initially tested using carnivore-specific primers designed to amplify a 135 base pair (bp) fragment of the hypervariable region of the mitochondrial (mtDNA) control region (CR) (Hänni *et al.*, 1994) as described previously (Chapter 2.3.3).

Additionally, for the aDNA extracts from which the 135 bp CR fragment was successfully amplified and sequenced, new primers were designed (either manually or using the PrimerBLAST function on GenBank) and a multiplex PCR protocol (Rompler *et al.*, 2006) was applied yielding a total of 135-364 bp of CR sequence and up to 137 bp protein-coding ATP8 sequence and 192 bp Cyt*b* sequence.

Many of the primers were designed manually before the *A. simus* mtDNA genome was available for use as a template, and the multiplex system and the newly designed primers required extensive troubleshooting. Through the troubleshooting process some primer pairs were found to be more suitable than others at amplifying DNA from *Arctotherium* sp, and some worked under normal PCR conditions but not in the multiplex setup. Details of the primers that I found to be most effective and which I used to amplify the sequences in this chapter can be found in Table 7.2.

For this study, multiplex reactions were set up in the ancient DNA facility at the Australian Centre for Ancient DNA. Primer mix A consisted of the following primer pairs: L16164/H16299, L16455/H16574, L8951/H9089. Primer mix B consisted of

L16852/H16968, L15994/H16105 and L15338/H15460. Each 20 µl multiplex reaction contained the following: 1 µl DNA extract, 2 U Platinum *Taq* DNA Polymerase High Fidelity and 1X buffer (Invitrogen), 1 mg/mL rabbit serum albumin (RSA; Sigma), 8 mM $MgSO_4$, 250 µM of each dNTP, 3 µl of primer mix A or B (containing the three primer pairs at concentrations of 1 µM each). Multiplex PCR thermal cycling reactions consisted of 94 ºC for 1 min, followed by 30 cycles of 94 ºC denaturation for 15 sec, annealing for 20 sec at 55 ºC, and extension at 68 ºC for 30 sec, followed by a final extension at 68 ºC for 10 min.

The PCR mastermix for the singleplex reactions was set up and aliquoted into PCR tubes in the ancient DNA facility at the Australian Centre for Ancient DNA, then transferred to the geographically separate South Australian Museum EBU lab where the diluted multiplex PCR product (containing amplified PCR product) was added to each tube. Each 20 µl singleplex reaction contained the following: 5 µl 1/20 dilution of multiplex PCR product, ¼ U Platinum *Taq* DNA Polymerase High Fidelity and 1X buffer (Invitrogen), 1 mg/mL rabbit serum albumin (RSA; Sigma), $MgSO_4$, 250 µM of each dNTP, 0.75 µM forward primer, 0.75 µM reverse primer. Singleplex PCR thermal cycling reactions consisted of 94 ºC for 1 min, followed by 30 cycles of 94 ºC denaturation for 15 sec, annealing for 20 sec at 55 ºC, and extension at 68 ºC for 30 sec, followed by a final extension at 68 ºC for 10 min.

As the whole mtDNA genome is available on GenBank for *T. ornatus*, yet nothing is known of the genetic diversity of mtDNA Control Region of this bear, I chose to target only the short hypervariable CR fragment (135 bp)(Hänni *et al.*, 1994) from one historic and one modern *T. ornatus* specimen to give a preliminary gauge of control region diversity in this species. The PCR amplifications on the historic specimen were performed in the Ancient DNA facility at the Australian Centre for Ancient DNA, whereas DNA from the modern specimen was amplified in the South Australian Museum EBU lab, physically isolated from the ancient DNA facility.

Amplification products (from normal PCRs or singlexplex PCRs described above) of the expected size were purified using ExoSAP-IT or Agencourt AMPure PCR purification kit according to the manufacturer's instructions. Purified PCR products were sequenced in both directions using Big Dye chemistry and an ABI 3130XL Genetic Analyzer.

Table 7.2 Primer sequences, primer combinations, annealing temperatures (Ta) and approximate size of product.

Primer pair	Target†	Forward primer (5' – 3')	Reverse primer (5' – 3')	Ta (ºC)	Product length (bp)¥
L16164 H16299*	CR	GCCCCATGCATATAAGC ATG	GGAGCGAGAAGAGGTA CACGT	55	~135
L16455 H16574	CR + tRNA Pro Thr	GTGAAGAGTCTTTGTAG	GGAATAATGGTATCAGG GAA	55	~119
L16852 H16968	CR	CCTCGAGAAACCAGCAA CCCTTG	CCCATTTGAAGGGTTAG TAG	55	~115
L8951 H9089	ATP8	AACTAGACACATCAACA TGG	GAGGCAAATAAATTTTC GTT	55	~137
L15994 H16105	Cyt*b*	CCGACCTCTAAGCCAAT GC	TGTGAAGTAGAGGATGG AGGCT	58	~89
L15338 H15460	Cyt*b*	GACGGGGCCTGTATTAC G	GACGGTTGCTCCTCAAA AG	58	~103

* (Hänni *et al.*, 1994)
† Mitochondrial Control Region (CR), ATP synthase subunit 8 (ATP8), Cytochrome *b* (Cyt*b*), tRNA proline (Pro) and Threonine (Thr).
¥ Fragment length does not include primer sequence.

7.2.5 Validation of ancient DNA results

Work on the ancient specimens was performed in the dedicated ancient DNA facility at the Australian Centre for Ancient DNA (ACAD) under positive HEPA-filtered air-pressure, with nightly UV decontamination. This facility is geographically isolated from any modern molecular biology laboratories, and follows strict ancient DNA protocols to prevent contamination (Gilbert *et al.*, 2005; Willerslev, Cooper, 2005). No work on any modern tissue samples including those from bears has been conducted previously in this facility.

One specimen (ACAD#3599; *Arctotherium tarijense*; Cueva del Puma) was sampled on two separate occasions, and one of these two samples was extracted, amplified, cloned and sequenced in a second independent ancient DNA laboratory at the Henry Wellcome Ancient Biomolecules Centre, Oxford (Jacobo Weinstock; unpublished data).

7.2.6 Phylogenetic analysis of the evolutionary relationships of the Tremarctine bears

To determine the evolutionary relationships of the Tremarctine bears, the six mtDNA sequence fragments (totalling 694 bp) of the extinct South American short-faced bear (ACAD#3599; *Arctotherium tarijense*; Cueva del Puma, Chile) generated in this study were aligned with the whole mitochondrial genomes of the extinct giant short-faced bear (*A. simus*) and the extant spectacled bear (*T. ornatus*) in MEGA 4 (Tamura *et al.*, 2007). The analysis was restricted to one individual per genus in order to maximise sequence length. The whole mtDNA genomes of six Ursine bears and the giant panda were also included in the alignment to provide a phylogenetic context/framework for the Tremarctinae analysis (See Table 7.3 for a list of the sequences obtained from Genbank which were included in my analysis).

Once aligned, the six sequence fragments from each individual were concatenated (with each fragment separated from the next by 'NNN') and the remaining parts of the mtDNA genome sequences removed, leaving homologous sequences of equal length for analysis. The concatenated mtDNA sequence fragments were analysed without partitioning the data. It was decided to leave the data as unpartitioned mainly due to the short sequence length and also due to reports of phylogenetic conflict between different mtDNA genes in previous mitogenomic studies in which partitioning was employed (Rohland *et al.*, 2007; Krause *et al.*, 2008).

A maximum parsimony (MP) analysis was performed using CNI searches with 100 random sequence addition replicates and 1000 bootstrap replicates in MEGA 4 (Tamura *et al.*, 2007).

A TrN+G substitution model was selected according to the Aikake Information Criterion (AIC) in Modeltest (Posada, Crandall, 1998), and implemented as the HKY + gamma model in BEAST 1.4.8 (Drummond, Rambaut, 2007) to generate a Bayesian inference (BI) phylogeny. Three independent runs of ten million Markov chain Monte Carlo (MCMC) generations sampled every 1000 steps were performed in BEAST 1.4.8 (Drummond, Rambaut, 2007) using a relaxed molecular clock (uncorrelated lognormal)(Drummond *et al.*, 2006), the HKY + gamma substitution model, and a Yule speciation prior on the tree. The results of the three independent runs were combined. Results of the individual and combined runs were visualised in Tracer 1.4 (http://tree.bio.ed.ac.uk/software/tracer) to check for convergence and to ensure all ESS scores were >200 (suggesting sufficient sampling and run length). The first 1 million steps (10%) of each run were discarded as burnin. Trees were annotated and were visualised in Figtree 1.1.2 (http://tree.bio.ed.ac.uk/software/figtree).

Table 7.3 List of previously published bear mtDNA genome sequences used in this study.

Common name	Current taxonomic name	GenBank accession no.	Reference
Polar bear	*Ursus maritimus*	NC_003428	(Delisle, Strobeck, 2002)
Brown bear	*Ursus arctos*	AF303110	(Delisle, Strobeck, 2002)
Cave bear †	*Ursus spelaeus*	NC_011112	(Krause *et al.*, 2008)
American black bear	*Ursus americanus*	NC_003426	(Delisle, Strobeck, 2002)
Sun bear	*Ursus malayanus*	FM177765	(Krause *et al.*, 2008)
Sloth bear	*Ursus ursinus*	FM177763	(Krause *et al.*, 2008)
Spectacled bear	*Tremarctos ornatus*	FM177764	(Krause *et al.*, 2008)
Giant short-faced bear †	*Arctodus simus*	NC_011116	(Krause *et al.*, 2008)
Giant panda	*Ailuropoda melanoleuca*	NC_009492	(Peng *et al.*, 2007)

† extinct

7.2.7 Estimation of divergence dates of the Tremarctine bears

Dating of the divergence events of the Tremarctine bear lineage was done in BEAST 1.4.8 (Drummond, Rambaut, 2007) using a relaxed molecular clock approach (Drummond *et al.*, 2006) combined with fossil calibration points similar to those described previously (Krause *et al.*, 2008). To summarise, a normal prior (mean=5.65 and st. dev=0.9) was placed on the root.height of the Ursine bear clade to represent the range between the minimum age for the oldest described *Ursus* fossils (*U. minimus* and *U. ruscinensis*) at 4.2 MYA and the maximum age for the youngest fossils from the genus *Ursavus* (*U. depereti* and *U. ehrenbergi*) which is believed to have given rise to the *Ursus* lineage at 7.1 MYA (Krause *et al.*, 2008). The prior based on these calibration points was used to obtain the posterior distribution of the estimated divergence times for the Tremarctine lineage and also for the divergence events within the Ursine bears to verify if the shorter 694 bp sequences used in this study were sufficient to obtain divergence estimates close to or within the error range of those calculated using whole mt genomes (Krause *et al.*, 2008).

7.2.8 Variation within South American bear species

The control region sequences of the modern and historic *T. ornatus* specimens were aligned in MEGA 4 (Tamura *et al.*, 2007) and compared to the homologous region of the *T. ornatus* mtDNA genome on Genbank. Nucleotide diversity (π) was calculated for the three individual sequences using DnaSP (Rozas *et al.*, 2003).

The control region sequences obtained from the ancient *A. tarijense* specimens were aligned in MEGA 4 (Tamura *et al.*, 2007) and compared to each other for nucleotide variation between individuals. Nucleotide diversity (π) of homologous sequences was calculated using DnaSP (Rozas *et al.*, 2003).

7.3 RESULTS

I successfully extracted and amplified mtDNA from two out of four ancient specimens of the extinct South American short-faced bear, *Arctotherium tarijense*, and from both the historic

and the modern specimens of the extant spectacled bear, *T. ornatus* (See Table 7.1). The two *Arctotherium* specimens that I was unable to obtain DNA from may have failed due to poor DNA preservation although the small amount of bone material available for these extractions (0.04-0.11 g) may have also had an impact on success.

For the extinct *A. tarijense* specimens I obtained a total of 694 bp of mtDNA (365 bp CR, 137 bp ATP8 and 192 bp Cyt*b*) from one specimen (ACAD#3599, Cueva del Puma), and 365 bp CR from the other specimen (ACAD#340, Cueva del Milodon). The Cueva del Puma specimen was independently extracted, amplified, cloned and sequenced for a 135 bp CR fragment (Jacobo Weinstock, unpublished data) and the consensus of these clones was found to be identical to the same fragment amplified and directly sequenced at ACAD (See Appendix 6 for replication details). In order to maximise sequence length in the phylogenetic analyses and divergence dating estimates only sequences obtained from the Cueva del Puma (ACAD#3599) specimen were used.

The radiocarbon date for the *A. tarijense* specimen (ACAD#3599) from Cueva del Puma has previously been reported as 10,345 ± 75 ybp (Martin *et al.*, 2004).

The *A. tarijense* specimen (ACAD#340) from Cueva del Milodon was radiocarbon dated to 13,257 ± 147 ybp (uncalibrated) as part of this study with the following results for stable nitrogen (dN^{15}: 6.92) and carbon (dC^{13}: -19.12) dietary isotopes (Waikato radiocarbon lab reference WK20234).

The nucleotide diversity (π) for the two *A. tarijense* specimens for the 135 bp CR fragment was found to be 0.00741 with a standard deviation of 0.00370, and for the larger 365 bp CR fragment 0.00542 with standard deviation of 0.00271. This corresponds to two variable sites in the 365 bp.

The nucleotide diversity (π) for the three *T. ornatus* specimens for the homologous 135 bp CR fragment was found to be 0.01975 with a standard deviation of 0.00931. This corresponds to four variable sites in the 135 bp CR fragment. All four of these variable sites occurred in the sequence obtained from Genbank. The two sequences obtained in this study were identical, despite one being a modern specimen and the other being historic.

7.3.1 Evolutionary relationships of the Tremarctine bears

The overall topology of the phylogenetic tree (Figure 7.2 and Appendix Figure 6.1) constructed using the 694 bp dataset is very similar to that obtained in previous studies using nuclear genes or whole mitochondrial genomes. The only incongruence is the position of the sloth bear (*U. ursinus*), which is the most basal of the Ursine bears in the mtDNA genome analysis but is sister to the American black bear (*U. americanus*) in the smaller 694 bp phylogeny.

The three New World bears form a monophyletic Tremarctine clade, forming a sister group basal to all the Ursine bears. This is consistent with previous analyses based on both nuclear genes (Pages *et al.*, 2008) and whole mtDNA genomes of *T. ornatus* (Peng *et al.*, 2007; Yu *et al.*, 2007; Krause *et al.*, 2008) and *A. simus* (Krause et al., 2008).

Within the Tremarctine clade, my analysis places the giant short-faced bear (*A. simus*) in the most basal position, falling as the sister taxon to the two South American genera, the extinct South American short-faced bear (*Arctotherium tarijense*) and the extant spectacled bear (*T. ornatus*).

7.3.2 Divergence estimates

I calculated the divergence date between Tremarctine (New World) and Ursine (Old World) bears to be 8.81 MYA (4.71-13.44 95% HPD confidence interval). The posterior mean divergence of the three New World bears was estimated to be 4.92 MYA (1.98-7.82 95% HPD confidence interval), with the basal giant short-faced bear (*A. simus*) diverging first followed by the most recent common ancestor (MRCA) of the two South American genera (extinct *Arctotherium* and extant *Tremarctos*) at 4.09 MYA (1.48-6.79 95% HPD confidence interval). Divergence dates and confidence intervals are reported in Figure 7.2

7.4 DISCUSSION

The overall topology of my MP (Appendix Figure 6.1) and BI trees (Figure 7.2) is in agreement with previous mitogenomic bear phylogenies (Yu *et al.*, 2007; Krause *et al.*, 2008) with the exception of the placement of the sloth bear in the BI analysis (See Figure 7.3). The position of the sloth bear has been problematic in many previous molecular phylogenetic analyses of bears (Zhang, Ryder, 1993; Zhang, Ryder, 1994; Talbot, Shields, 1996; Waits *et al.*, 1999; Pages *et al.*, 2008) and has even been omitted entirely in one recent mitogenomic analysis (Peng *et al.*, 2007) - it is therefore unsurprising that it is also unresolved in my study. In my analysis this is probably partially due to the short sequence length used (approximately 700 bp), however it is important to note that many of the internodes in the analyses incorporating the whole mtDNA genome data are also extremely shallow (See Figure 7.3).

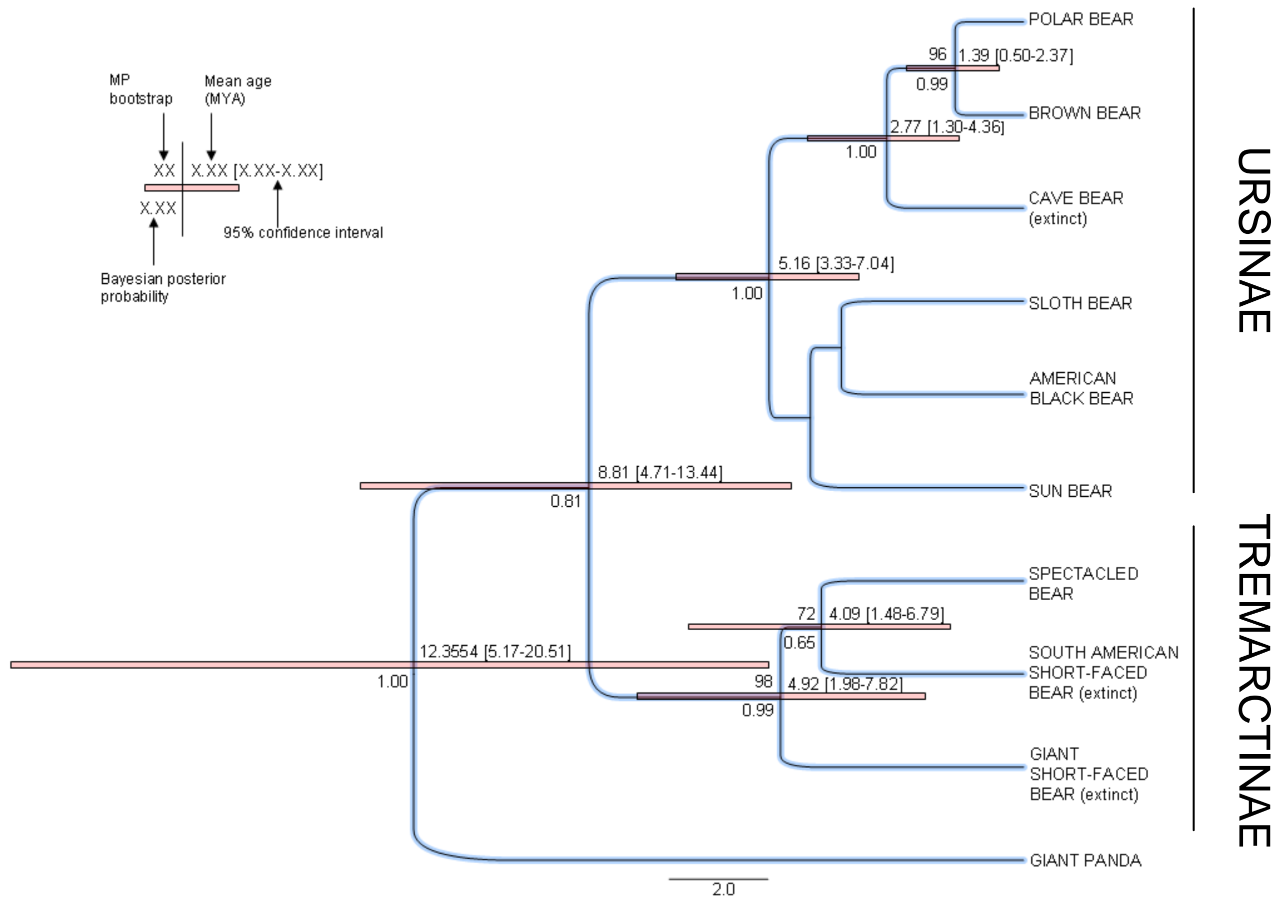
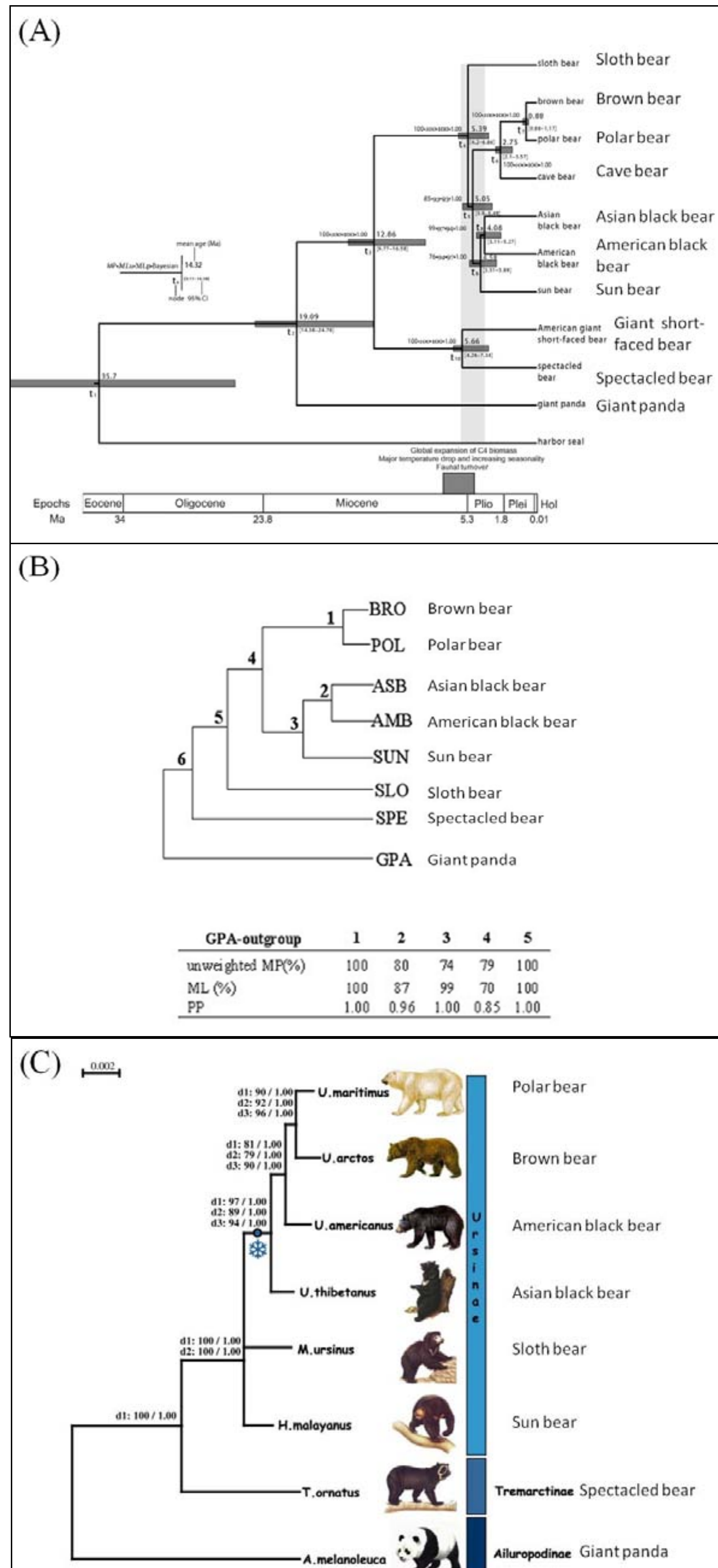


Figure 7.2 Maximum clade probability tree displayed as a chronogram from the BEAST analysis of the unpartitioned 694 bp mtDNA alignment. All lineages evolved according to a relaxed clock and the HKY+Gamma substitution model. Numbers above the node on the right show the posterior mean estimate of the divergence time followed by the 95% confidence interval in square brackets. Node bars (red) illustrate the width of the 95% highest posterior density (95% HPD). Numbers above the node on the left indicate maximum parsimony bootstraps ≥65%, while numbers below the nodes indicate the Bayesian posterior probability ≥0.65.



GPA-outgroup	1	2	3	4	5
unweighted MP(%)	100	80	74	79	100
ML (%)	100	87	99	70	100
PP	1.00	0.96	1.00	0.85	1.00

Figure 7.3 Topology comparison of the bear phylogeny based on whole mitochondrial genome studies (A) Krause *et al.*, 2008 and (B) Yu *et al.*, 2007 and fourteen nuclear genes (C) Pages *et al.*, 2008. Figures adapted from those sources. The sloth bear is most basal of the Ursine bears in the mtDNA genome analyses (A and B) but is unresolved by the nuclear analysis (C). All analyses place the Tremarctine lineage outside the Ursine bears. Note the very shallow branching of the internodes in the panel (A) despite being based on whole mtDNA genome sequences.

7.4.1 Evolutionary relationships within the Tremarctine bears

My molecular analysis suggests that the two South American genera (represented here by *A. tarijense* and *T. ornatus*) are each other's closest relatives, forming a monophyletic South American clade (Figure 7.2). While this is in contrast to the current palaeontological view (Figure 7.1) that places the two short-faced bear genera (*Arctodus* and *Arctotherium*) in a monophyletic clade sister to the extinct (*Plionarctos* and *T. floridanus*) and extant (*T. ornatus*) spectacled bears (Soibelzon, 2002; Soibelzon, 2004; Soibelzon *et al.*, 2005), the monophyly of the short-faced bears has been questioned previously (Trajano, Ferrarezzi, 1994) and my molecular data provides some evidence to substantiate/validate this earlier line of questioning.

As with most ursid species, Tremarctine bears exhibit a wide range of individual variation and notable sexual dimorphism in size and shape of skulls (males up to two times larger than females) (Kurten, 1966; Kurten, 1967; Trajano, Ferrarezzi, 1994). When you consider that most of the Tremarctine bear genera are differentiated on size characters (either overall size or molar teeth) and that the fossil record of the South American bears is so fragmentary it is not surprising that the systematics of the subfamily based on morphological characteristics are problematic.

While my molecular data casts doubt on the current evolutionary relationships constructed according to morphological/palaeontological characters, it is still not possible to differentiate between a number of alternate evolutionary scenarios (See Figure 7.4) as I was unable to include *T. floridanus* in my analyses. It is therefore impossible to determine whether the closest sister taxa to the South American bears is *T. floridanus* (Figure 7.4 a or b) or *Arctodus* (Figure 7.4 c and d). It also remains unclear whether *Arctotherium* and *Tremarctos* arose in North America followed by two independent dispersals into South America (*Arctotherium sp.* in the early-mid Pleistocene followed by *T. ornatus* in the late Pleistocene/Holocene; Figure 7.4 a or c) or whether there was a single dispersal event by the ancestor of *Arctotherium sp.* and *T. ornatus* followed by speciation in South America (Figure 7.4 b or d). My data is not consistent with the current palaeontological model (Figure 7.4 e).

A scenario consistent with my data is that *T. ornatus* evolved *in situ* in South America either from *Arctotherium* sp. or their MRCA. If this is the case then it might prompt a revision of the taxonomic designations of the South American Tremarctine bears, with *Arctotherium* falling within the same lineage as *T. floridanus* and *T. ornatus*, or alternatively *T. ornatus* falling within the *Arctotherium* genus rather than being directly descendent from *T. floridanus*. Unfortunately *T. floridanus* specimens were not available for inclusion in this study, although ancient DNA analysis of suitably preserved *T. floridanus* specimens in the future might help to resolve this issue and allow further differentiation between the competing hypotheses shown in Figure 7.4.

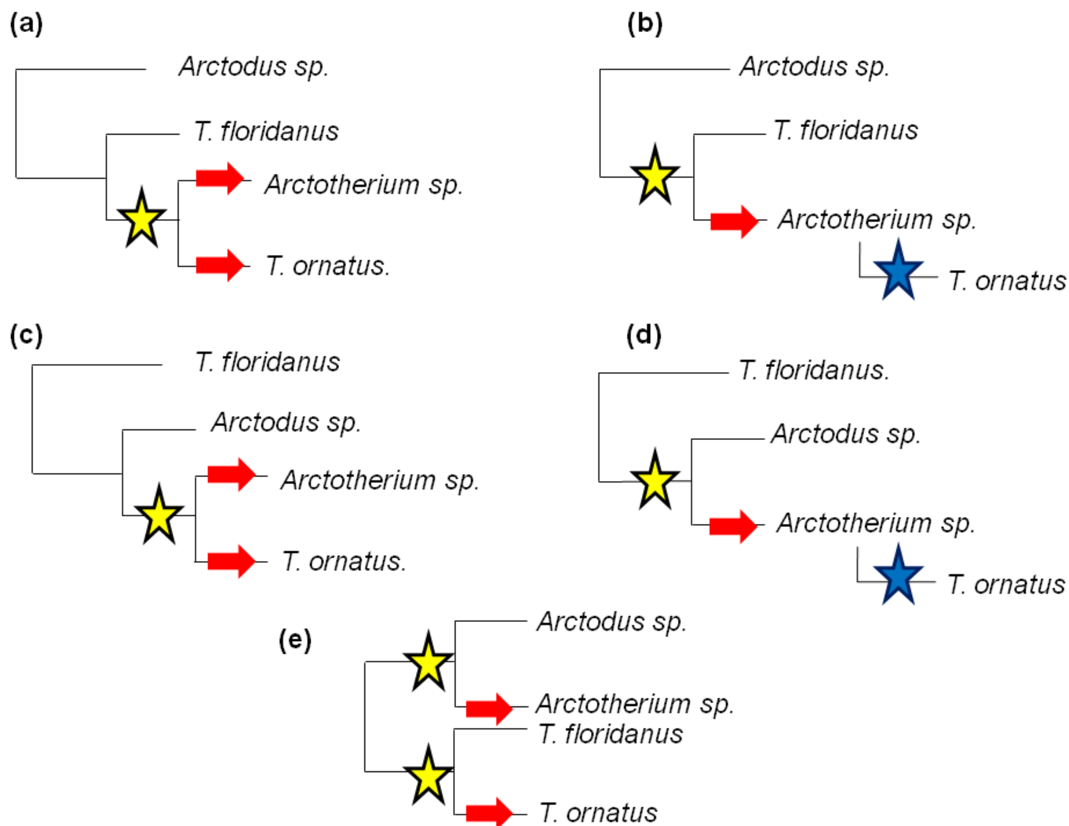


Figure 7.4 Five alternative hypotheses for the evolutionary relationships of the Tremarctine bears. Hypotheses (a-d) are all consistent with my aDNA analysis, in which the two South American genera are most closely related to each other and the North American giant short-faced bear is a sister taxa to the South American bears. From my data it is not possible to differentiate between the first four hypotheses due to the absence of aDNA material from the extinct North American Florida bear *T. floridanus*. It is not possible to differentiate between *Arctodus* sp or *T. floridanus* being the MRCA of the South American bears, and it is also not possible to determine whether speciation occurred in North America followed by two dispersal events into South America (hypothesis a and c), or whether there was a single speciation event followed by *in situ* speciation of *T. ornatus* in South America (hypothesis b and d). Hypothesis (e) is not consistent with the genetic data, but it represents the current model favoured by palaeontological/morphological data, with the short-faced bears forming one monophyletic clade, and the spectacled bears forming a sister monophyletic clade. Red arrows indicate dispersal events from North America to South America. Yellow stars indicate speciation events occurring in North America, while blue stars indicate *in situ* speciation in South America.

7.4.2 Tremarctine bear divergence date estimates

The mean divergence estimates for the three Tremarctine bears included in my analysis fall within the period of rapid speciation observed for the Old World bears at the Miocene-Pliocene boundary (Yu *et al.*, 2007; Krause *et al.*, 2008) which has also previously been identified as a period of rapid speciation in other mammalian groups such as in African primates (including chimpanzees and human lineages) (Kumar *et al.*, 2005) and felids (Johnson *et al.*, 2006).

I found that within the Tremarctinae, the North American giant short-faced bear *A. simus* diverged first at 4.92 mya (1.98-7.82 95% HPD confidence interval), followed by the

divergence of the two South American genera (*Arctotherium sp.* and *T. ornatus*) at 4.09 MYA (1.48-6.79 95% HPD confidence interval). Due to the short length of mtDNA sequence (~700 bp) used in these analyses, the confidence intervals on the divergence dates are large, although it is worth noting that in most cases the mean divergence dates estimated in this study fall close to or within the confidence intervals estimated previously based on whole mtDNA genomes (Krause *et al.*, 2008).

It is not possible from the available data to determine whether the divergence of *Arctotherium* and/or *Tremarctos* pre- or post-dated the formation of the Panamanian land-bridge (2.5-3.0 MYA) as the 95% confidence intervals on the divergence dates estimated here overlap this period however it is possible/likely that one or both of these genera arrived in South America as part of the Great American Interchange (GABI) across the Isthmus of Panama.

7.4.3 Palaeobiology of Arctotherium tarijense

Interestingly, fossil records of *Arctotherium sp.* are mainly found on the east side of the South American continent (Venezuela, Brazil, Uruguay, Argentina, Bolivia and Chile) whereas *T. ornatus* is distributed on the west (Venezuela, Colombia, Ecuador, Peru and Bolivia) (Ruiz-Garcia *et al.*, 2005; Soibelzon *et al.*, 2005). These different patterns of distribution (See Figure 7.5) may be evidence of alternate colonisation/migration routes into South America by *Arctotherium sp.* and *T. ornatus* or their most recent common ancestors, or could reflect different adaptations to environmental niches in South America – *Arctotherium* more adapted to the open grasslands of the east, – with *T. ornatus* better adapted to the denser forests of the west.

Radiocarbon dating of the Cueva del Mylodon specimen returned a date of 13,257 ± 147 ybp. During the LGM (20,000-18,000 ybp) glacial ice covered south-western Patagonia (Clapperton *et al.*, 1995). Deglaciation of this area began between 16,000-14,000 ybp (Clapperton *et al.*, 1995; Heusser, 1995; Rabassa *et al.*, 2000), therefore the radiocarbon date of 13,257 ± 147 for the *A. tarijense* specimen from Mylodon cave suggests that this species arrived relatively soon after the retreat of the ice, and was possibly the first carnivore species to occupy this cave post-LGM, as radiocarbon dates for large felid species from this site occur more recently (11-12,000 ybp; Ross Barnett, unpublished data).

The stable Nitrogen isotope results obtained for the Cueva del Mylodon specimen were 6.92. This is at the lower end of the scale when compared to the typical nitrogen isotope scores reported for the giant short-faced bear *A. simus* which had an average stable nitrogen isotope value of 8.5 (reported values ranged from 6.60 – 10.31)(Matheus, 1995), and suggest a similarly omnivorous-carnivorous diet for *Arctotherium* as for its North American counterpart (Matheus, 1995; Sorkin, 2006; Figueirido *et al.*, 2009). When the isotope data for this *A. tarijense* specimen was compared to isotope data obtained from known carnivorous felids such as *Puma concolor*, *Smilodon*, and *Panthera onca mesembryna* from the same site, they were found to range from 6.7-11.1 (Ross Barnett, unpublished data), confirming this specimen of *A. trajense* was consuming meat on levels comparable with felids. Morphological analysis of a skull and mandible from another member of the South American short-faced bear genus, *Arctotherium brasiliense*, revealed a comparatively shallower premasseteric fossa than *T. ornatus*, which was interpreted to either suggest a diet consisting of softer non-fibrous plant material or alternatively a less herbivorous diet (i.e more carnivorous) than the spectacled bear (Trajano, Ferrarezzi, 1994). This morphological interpretation combined with the isotopic data suggest the diet of *Arctotherium* was at the more carnivorous end of the omnivore range similar to *A. simus* (Matheus, 1995; Sorkin, 2006; Figueirido *et al.*, 2009) but in contrast to the primarily herbivorous diet of *Tremarctos* (Peyton, 1980).

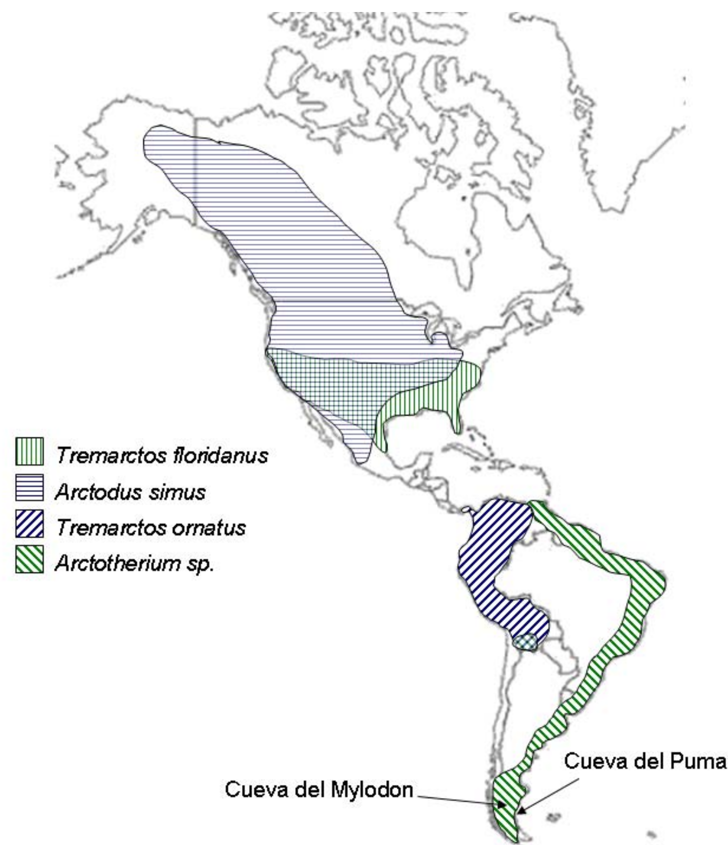


Figure 7.5 Map showing approximate distributions of *T. floridanus*, *A. simus*, *Arctotherium sp*. and *T. ornatus*. Distributions of fossil sites for *T. floridanus* and *A. simus* were based on those described in (Kurten, Anderson, 1980). Fossil distribution of *Arctotherium sp*. was based on (Soibelzon *et al.*, 2005). Current distribution of *T. ornatus* is based on (Ruiz-Garcia *et al.*, 2005). The location of Cueva del Puma and Cueva del Milodon are indicated.

7.4.4 Genetic variation among A. tarijense individuals

The sequences obtained from the two *A. tarijense* specimens as part of this study are the first DNA sequences from the South American short-faced bear genus *Arctotherium*. From the 365 bp of homologous control region mtDNA obtained from these specimens there are only two variable sites. While a sample size of two is too small to make any informative claims about the genetic diversity of this species, it does provide another measure of confidence in the validity of the Cueva del Puma ancient DNA sequence used in the divergence dating estimates and Tremarctine bear phylogeny, since it would be highly unlikely to obtain two such similar sequences by chance.

Fossil sites suggest that *Arctotherium* sp. were distributed over the entire eastern part of the South American continent (Figure 7.5) since the early-mid Pleistocene. Both specimens of *A. tarijense* sequenced in this study originate from the very southern tip of their hypothesised range, and are among the most recent fossils of this genus separated by less than 3,000 years. Therefore it is perhaps not surprising that their mtDNA control region sequences show such little variation. As many large terrestrial mammals (for example brown bears; Taberlet, Bouvet, 1994, and spotted hyenas; Rohland *et al*., 2005) typically exhibit strong phylogeographic structuring over continental ranges it would be interesting to assess both the temporal and range-wide mtDNA diversity of *Arctotherium* sp. in the future, if suitably preserved fossil material can be located. This would also allow a valuable comparison with the lack of mtDNA phylogeographic structuring observed in the North American giant short-

faced bear (See Chapter 6), and might provide insight into whether this is a feature of the New World bears in general, or a trait specific to *A. simus*.

7.4.5 Genetic variation among T. ornatus individuals

Although only based on three individuals, this analysis of CR variation is still of interest, as there is a complete lack of knowledge of the genetic diversity of the mtDNA of *T. ornatus*, despite its endangered status. Two recent studies utilising microsatellite markers found that the spectacled bear is genetically depauperate (Ruiz-Garcia, 2003; Ruiz-Garcia *et al.*, 2005), with very low levels of microsatellite heterozygosity compared to Ursine bears. Despite the low genetic diversity, neither study managed to detect any signal of a population bottleneck, interpreted to reflect the bears colonisation history into South America and suggesting they always had a very low effective population size. Although a sample size of three individuals is not significant statistically, the lack of CR variation in these two modern and one historic specimens is in agreement with the low microsatellite diversity. It is also interesting to note that all four variable sites detected in the 135 bp CR fragments analysed here occurred within the *T. ornatus* sequence obtained from the whole mtDNA genome sequence on Genbank (FM177764). The historic and modern sequences obtained in this study were found to be identical for all 135 bp.

7.5 CONCLUSIONS

The first DNA sequences from the extinct South American short-faced bear questions the current palaeontological models of the evolutionary relationships of the Tremarctine bears. The genetic data implies a common ancestor for the two South American genera (*Arctotherium sp*. and *T. ornatus*) - possibly the extinct Florida bear *T. floridanus* - rather than finding support for a monophyletic short-faced bear clade uniting the North American *A. simus* and South American *Arctotherium* groups.

Despite the very small sample sizes for the South American bears – two individuals of *Arctotherium tarijense*, and three of *T. ornatus* - our preliminary analysis provides the first evidence to suggest that very low levels of genetic diversity might not just be a trait of the North American giant short-faced bears and instead may be a general feature of the New World bears.

Future ancient DNA work building on this study should attempt to include genetic material from the extinct Florida bear (*T. floridanus*) to further investigate the evolutionary relationships within the Tremarctinae. It would also be valuable to explore the genetic diversity and phylogeographic structure of *Arctotherium sp*. across the South American continent, providing suitably preserved fossil material can be obtained.

In addition to this, our preliminary examination of the mtDNA diversity of the endangered spectacled bear should be expanded to include a much larger sample size from across its current distribution to complement the existing microsatellite datasets (Ruiz-Garcia, 2003; Ruiz-Garcia *et al.*, 2005) to guide future conservation management of the only surviving member of the Tremarctine bears.

7.6 ACKNOWLEDGEMENTS

I would like to thank Leopoldo Soibelzon for providing samples of *Arctotherium* sp. for ancient DNA analysis, as well as for advice on identifying the Cueva del Milodon specimen to species level based on geographic location/provenance and age. I would also like to thank Fabiana Martin, Flavia Morello and Alfredo Prieto for providing the samples from the Cueva

del Puma specimen. Thanks to Alan Cooper for taking samples from the South American bears and for bringing back the modern tissue from the *T. ornatus* specimen from La Plata Zoo, and for allowing me access to his sample collection and the ACAD facilities. Thanks to Jaco Weinstock for performing the independent replication of the aDNA work on the Cueva del Puma specimen. I would like to thank Ross Barnett for sharing some of his unpublished isotope data from the Mylodon cave felid specimens. I would like to thank the Waikato Radiocarbon lab for the dating of the Milodon cave specimen. I would like to thank Jeremy and Alan for helpful discussions and feedback. I would like to thank Trevor Worthy and Aaron Camens for lots of helpful paleo/bear/isotope discussions.

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CHAPTER 8

GENERAL DISCUSSION

8.1 THESIS SUMMARY

Ancient DNA analyses have contributed greatly to our understanding of Quaternary phylogeography, revealing evolutionary lineages and processes which would remain hidden from genetic analyses of extant populations alone. The research presented in this thesis used ancient DNA techniques to investigate a number of questions relating to the phylogeography and genetic diversity of large mammal populations - specifically brown bears (*Ursus arctos*; Chapters 2-5) and giant short-faced bears (*Arctodus simus*; Chapter 6) - during the Quaternary period. Additionally, ancient DNA was extracted from two extinct genera of the New World Tremarctine bears (*Arctodus* and *Arctotherium*; Chapter 7) in an attempt to clarify their deeper evolutionary relationships which have eluded conclusive resolution by traditional morphological methods.

8.1.1 Post-glacial phylogeography of Scandinavian brown bears

Modern brown bear populations in Scandinavia are phylogeographically divided into two divergent mtDNA clades. Brown bears in the north belong to Clade 3 while those in the south belong to Clade 1. Within each of the two divergent groups the levels of mtDNA diversity are very low in both extant populations (Taberlet, Bouvet, 1994; Taberlet *et al.*, 1995; Waits *et al.*, 2000), and the Holocene specimens analysed in Chapter 2. It is unknown whether the current pattern observed is due to recent anthropogenic effects (such as hunting and habitat destruction in the last two centuries) or whether it can be traced back to the post-glacial recolonisation of Scandinavia. The timing and formation of the contact zone between the two clades combined with the low levels of genetic diversity seen in the modern Scandinavian populations are therefore seen to be important in terms of guiding conservation efforts (Manel *et al.*, 2004; Manel *et al.*, 2007; Swenson *et al.*, 1995; Taberlet *et al.*, 1995; Tallmon *et al.*, 2004; Waits *et al.*, 2000).

In Chapter 2 of this thesis, ancient DNA techniques were employed to obtain mtDNA control region sequences from 20 post-glacial Norwegian and Danish brown bear specimens to investigate the genetic diversity and phylogeographic patterns of Scandinavian brown bears over the last 6,000 years. The ancient DNA analysis of these sequences determined that the lack of mtDNA diversity in northern Scandinavian populations is most likely due to an ancient founder effect during post-glacial recolonisation rather than simply the result of a recent anthropogenic bottleneck. Additionally, this study identified the most northerly record of a Clade 1 Italian/Balkans mtDNA haplotype in Europe. This important finding demonstrates that, in contrast to previous interpretations, the recolonisation of southern Scandinavia was not limited to Clade 1 bears from the Iberian refugium. The results presented in Chapter 2 suggest either genetic turnover with complete replacement through time in post-glacial southern Scandinavia, or alternatively raises the possibility that the presence of the Italian/Balkans haplogroup has been overlooked in modern Scandinavian populations.
This study revealed a more complex post-glacial population history than previously imagined for brown bears, however it may also suggest that southern Scandinavian populations of other large vertebrates have a similarly complex past that could be revealed through ancient DNA studies, rather than relying on analysis of purely modern or historic specimens. Other candidate Scandinavian populations that might benefit from extension of their modern and

historic datasets with the addition of some ancient specimens include the wolverine (Walker *et al.*, 2001) and the grey wolf (Flagstad *et al.*, 2003).

8.1.2 From Iberia to Alaska: ancient DNA links late Pleistocene brown bears across the Holarctic

In Chapter 3, a single nucleotide polymorphism (SNP) was identified at position 16,882 of the brown bear mt genome (GenBank accession number AF303110) which differentiates between bears belonging to the extinct Clade 3c and the more widespread extant Clade 3a. A reanalysis of 601 brown bear mtDNA sequences subsequently revealed that a previously published Pleistocene brown bear sequence from Iberia initially reported to belong to the 'eastern' Clade 3 actually possesses this SNP placing it within the extinct Clade 3c. This was a significant finding as it suggested that the extinct Clade 3c was not limited to Pleistocene Beringia as previously believed, but was present in both the far west and far east of the Eurasian continent at that time, possibly with a phylogeographic range similar to that of modern Clade 3a. This chapter also provides another example of ancient DNA revealing an unexpected phylogeographic structure and extended range for large mammals in the Pleistocene, as was shown recently with Eurasian cave bears (Knapp *et al.*, 2009) and suggested for European brown bears (Hofreiter *et al.*, 2004).

8.1.3 Post-glacial phylogeography of European brown bears

Hewitt's glacial Expansion/Contraction (E/C) model offers a plausible explanation for the current phylogeographic structure observed in modern European brown bear populations. However, ancient DNA evidence has recently been put forward to question both the existence of southern glacial refugia and their genetic isolation, with claims of continuous gene flow between brown bear populations across southern Europe before (Hofreiter *et al.*, 2004) during and after (Valdiosera *et al.*, 2008; Valdiosera *et al.*, 2007) the last glacial maximum.

To further investigate the post-glacial phylogeography in Europe, mtDNA sequences were successfully retrieved from 28 ancient brown bear specimens (ranging in age from late Pleistocene to Holocene). By combining these additional ancient European brown bear sequences with modern and ancient sequences obtained from GenBank it was possible to identify post-glacial phylogeographic patterns of Clade 1 bears consistent with expansion from at least two southern European refugia (Iberia and Italian/Balkans) following the LGM (Chapter 4). However, contrary to Hewitt's E/C model, this investigation is the first to reveal that the Italian/Balkans haplogroup recolonised central and northern Europe at least as early as 12,000 ybp, and that the expansion of the Iberian and Eastern lineages did not occur until much more recently (possibly in the last 2,000-5,000 years).

This analysis found no evidence to support the recent claims of Valdiosera *et al.* (2007) of continuous gene-flow between the southern peninsula refugia during the LGM, and instead identified several flaws overlooked by that study. While it was beyond the scope of our study to investigate the possible existence and location of cryptic refugia in central and northern Europe, evidence from a number of plant-based studies is increasing (Magri *et al.*, 2006; Naydenov *et al.*, 2007; Willis *et al.*, 2000; Willis, van Andel, 2004), and future studies combining the phylogeographic data presented in Chapter 4 and palaeo-climate niche modelling (as recently employed for mammoths; Nogues-Bravo *et al.*, 2008) may shed light on potential cryptic refugia suitable for bears in central and northern Europe.

As part of the research presented in Chapter 4, a single nucleotide polymorphism (SNP) was also identified at position at position 16,762 (in relation to *U. arctos* mt genome AF303110)

which differentiates between Clade 1 bears of the Iberian and the Italian/Balkans refugia. The identification of such a SNP may be valuable in the future for determining the refugial origin of Clade 1 bear samples particularly when working with very degraded ancient samples. A similar SNP-based approach has been used previously in a study of Pleistocene cave bear specimens (Valdiosera *et al.*, 2006), in which the targeting of small SNP fragments allowed the researchers to extend the temporal limits of DNA recovery in these degraded specimens. It could also be of use in studies of modern populations as in the case of Scandinavia, where the presence of bears of the Italian/Balkans group may have been overlooked. SNP testing may represent a cheaper or more efficient way to screen a large group of specimens already known to belong to Clade 1.

8.1.4 Genetic diversity of ancient Eurasian brown bears

Until very recently, almost nothing was known about the genetic diversity or phylogeography of Eurasian brown bears. In 2009, Korsten *et al.* published a survey of 205 modern Eurasian brown bears. Amazingly, this study identified only a single haplogroup (Clade 3a) on continental Eurasia from Estonia and Finland in the west to Beringia in the east. In contrast, my analysis of approximately 100 ancient and historic Eurasian brown bears (Chapter 5) identified individuals belonging to both Clade 3a and 3b, including previously unpublished haplotypes within these groups. Additionally my study identified two ancient and two historic individuals (from the Caucasus and Ural Mountains) with unique mtDNA haplotypes which appear to represent previously undetected diversity within or closely related to the Clade 4 bears of modern North American populations.

This study (Chapter 5) increases our understanding of Eurasian brown bear phylogeography in a number of ways. Firstly, it greatly increases the number of ancient Eurasian brown bear mtDNA sequences, providing 58 new control region sequences. Secondly, it extends the coverage of samples across Eurasia both geographically and temporally, helping to fill in some of the regions that have previously not been sampled in the study by Korsten *et al.* (2009). Thirdly, this study reveals the presence of a much greater genetic diversity in the ancient and historic samples than was detected in modern populations, with the presence of individuals from Clades 3a, 3b and 4. The new sequences presented in this study combined with the radiocarbon results suggest that Clade 3a has been present in western, central and eastern Eurasia since at least 46,000 ybp which pre-dates the MRCA of Clade 3a calculated by Korsten *et al.* 2009 to be 26,000 ybp). The new data presented in Chapter 5 also suggests that Clade 3b has been present in southern Eurasia from at least 46,000 ybp until historic times, and Clade 4 (or a close relative) has been present in western Eurasia (in the Urals and Caucasus Mountain ranges) from at least 43,000 ybp until historic times.

8.1.5 Genetic diversity and phylogeography of the extinct giant short-faced bears

While brown bears have been the focus of a great deal of study, nothing is known of the genetic diversity or phylogeographic structure of the extinct giant short-faced bear, *A. simus*, a member of the Tremarctine bear lineage and a Pleistocene contemporary of the brown bear in North America. Chapter 6 is the first study to address this knowledge gap, and attempts to do this by analysing mtDNA control region sequences from 23 extinct *A. simus* specimens spanning a period of 33,000 years from 44,240 ± 930 – 11,619 ± 40 ybp. This investigation revealed that in contrast to Pleistocene brown bears from Beringia, *A. simus* possessed very low levels of genetic diversity and lacked the strong phylogeographic structure seen in their contemporaneous brown bears. It is plausible that low levels of genetic diversity and absence of a source population from which to replenish stocks following population bottlenecks may have put the giant short-faced bear at a disadvantage. In comparison, the brown bear was able

to recolonise North America from a genetically diverse Eurasian source population. The low levels of genetic diversity and the lack of phylogeographic structure revealed in late Pleistocene giant short-faced bear populations also adds to the growing body of evidence that reductions in genetic diversity predated both the LGM and arrival of humans for a number of megafaunal taxa in North America, for example as shown for hemionid horses, bison and mammoths (Debruyne *et al.*, 2008; Guthrie, 2003; Shapiro *et al.*, 2004).

8.1.6 Evolutionary relationships of the extinct Tremartcine bears

Due to a combination of factors including the relatively poor record of stratigraphy and fragmentary nature of many South American Tremarctine fossils, a confusing taxonomic history, and the great range of individual variation observed within bear species, the evolutionary relationships and times of divergence between the members of the extinct Tremarctine bears remain unresolved by morphological/palaeontological methods. Chapter 7 represents the first ancient DNA study of an extinct South American short-faced bear, *Arctotherium tarijense*. This study attempts to clarify the evolutionary relationships and timing of divergence of the extinct Tremarctine bears *A. simus* and *Arctotherium tarijense* with respect to the extant spectacled bear *T. ornatus,* using molecular methods.

These first DNA sequences from the extinct South American short-faced bear question the current palaeontological models of the evolutionary relationships of the Tremarctine bears. The genetic data implies a common ancestor for the two South American genera *Tremartos ornatus* and *Arctotherium* sp. rather than finding support for a monophyletic short-faced bear clade uniting the North American *Arctodus* and South American *Arctotherium* groups. It is still not possible from the available data to differentiate between *Arctodus* sp or *T. floridanus* being the MRCA of the South American bears, nor is it possible to determine whether speciation occurred in North America followed by two dispersal events into South America or whether there was a single speciation event followed by in-situ speciation of *T. ornatus* in South America. However our genetic data is not consistent with the current model favoured by palaeontological/morphological data, with the short-faced bears forming one monophyletic clade, and the spectacled bears forming a sister monophyletic clade.

The questions surrounding the origin and diversification of the Tremarctine bears are shared by other South American vertebrates and therefore the clarification of the timing of divergence events within the Tremarctinae may be more broadly relevant. For example, the timing of the arrival of the first Sigmodontine rodents in South America has also been the subject of debate since the middle of last century (Engel *et al.*, 1998), with two main models proposed. The first suggests an ‘early arrival’ approximately 20 MYA by waif dispersal over water followed by extensive diversification in South America and subsequent migrations back to North America during the formation of the Panamanian Isthmus in the Pliocene (Hershkovitz, 1966; Hershkovitz, 1972). The second model suggests a ‘late arrival’ in which a diverse range of Sigmodontines evolved in North America and then a large number of these genera invaded South America relatively recently during the Great American Biotic Interchange approximately 3.5 MYA, and further diversification then occurred in South America (Patterson, Pascual, 1968; Simpson, 1950; Simpson, 1969). A genetic study of extant Sigmodontine rodents attempted to clarify this but did not find support for either of these two models, instead estimating the arrival of Sigmodontines in South America at approximately 5-9 MYA (Engel *et al.*, 1998). Clearly the timing and origin of divergence events of South American vertebrates is a complex area that requires further work, and molecular approaches are likely to prove critical in resolving a number of the outstanding questions.

8.2 FUTURE DIRECTIONS

This research has increased our knowledge about the genetic diversity of ancient brown bears and short-faced bears and addressed a number of interesting questions to do with the evolution and phylogeographic histories of these taxa. However, during the course of this research and with consideration for the findings presented here, still more questions have arisen. In this final section of my thesis I would like to suggest several directions for future research that could be taken to extend the findings presented in this thesis, and to highlight some specific questions that could be addressed with further time and funding.

8.2.1 Brown bear mitogenomics

New technological advances such as the emergence of next generation sequencing are now seen as a realistic tool to be applied to aDNA studies (see Ho & Gilbert 2010 for a summary of the emergence of ancient mitogenomics). When the majority of the laboratory component of this PhD research was being conducted (2006-2008), the first aDNA papers using next generation or high throughput sequencing were only starting to be published and the technology was beyond the scope of this project. Some of the early reservations in relation to next generation sequencing of aDNA samples were due partially to cost and ability to process the huge amount of data generated by the next generation sequencing process, and partially due to the high proportion of contaminating sequences generated compared to the ancient endogenous sequences. For example, only approximately 45 % of the DNA extracted from a well-preserved deep-frozen mammoth bone could be mapped to the draft Elephant genome, and many specimens are much less well preserved than this, with the endogenous DNA making up less than 10 % of the total extract (Ho, Gilbert, 2010; Poinar *et al.*, 2006).

While entire mtDNA genomes/mitogenomes of extinct mammals are beginning to appear on GenBank there are still relatively few species represented, often with more than one research group targeting the same few taxa almost simultaneously. The extinct mammalian (excluding hominids) mitogenomic list currently consists of the woolly mammoth (Gilbert *et al.*, 2007a; Gilbert *et al.*, 2008; Gilbert *et al.*, 2007b; Krause *et al.*, 2006; Poinar *et al.*, 2006; Rogaev *et al.*, 2006), mastodon (Rohland *et al.*, 2007), woolly rhinoceros (Willerslev *et al.*, 2009), cave bear (Bon *et al.*, 2008; Krause *et al.*, 2008), giant short-faced bear (Krause *et al.*, 2008), Tasmanian tiger (Miller *et al.*, 2009) and the aurochs *Bos primigenius* (Edwards *et al.*, 2010). Despite this relatively short list, we are beginning to see the first intraspecific phylogenetic and phylogeographic studies of extinct mammals based on multiple complete ancient mtDNA genomes (for example woolly mammoths; Gilbert *et al*. 2008 and cave bears; Stiller *et al*. *In Press* described in Ho & Gilbert 2010).

Brown bears would be an ideal candidate for such studies, due to the relative abundance/availability of specimens and their widespread distribution across the northern hemisphere throughout the Pleistocene and Holocene periods. Key questions on the phylogenetic relationships of some of the less well understood brown bear clades and subclades could no doubt be further clarified with analysis of whole mt genomes. Initially mtDNA genomes should be analysed from modern individuals from each major group for example Clade 1a, 1b, 2a, 2b, 3a, 3b, 4, and some middle eastern specimens, in order to generate a scaffold phylogeny. Later this could be augmented with key ancient/extinct mtDNA genomes if preservation of the samples allow, for example individuals belonging to Clade 3c from Beringia and Iberia, 2c from the ABC Islands, extinct north African bears and the the bears identified in Eurasia (Chapter 5) that appear to be related to Clade 4.

Another related grey area that could potentially be resolved with analysis of whole mtDNA genomes would be the phylogenetic placement of the polar bear within the brown bear Clade

2. A complete mtDNA genome has recently been published from an ancient polar bear specimen from Svalbard and from modern Clade 2a brown bears from the ABC Islands (Lindqvist *et al.*, 2010) but a whole mtDNA genome from the extinct brown bear Clade 2c is still required to clarify this phylogeny.

Advances in molecular dating are also allowing theoretically more reliable dating estimates (Ho *et al.*, 2008), contributing to an improved understanding of the timing of divergence of the major brown bear clades, which in turn will lead to a more comprehensive global understanding of brown bear evolution and phylogeography (Davison *et al.*, 2010). Obtaining complete mtDNA genomes of many of the clades and subclades of brown bears will not only improve our knowledge of the phylogenetic relationships but will also be important for calculating more reliable molecular clocks and estimating divergence dates, which subsequently may allow identification and correlation with climatic events which may have played a role in the dynamics of Pleistocene brown bear phylogeography.

However, ancient mitogenomics may not always be the only solution or the best way to answer the outstanding questions of bear phylogeographic history. There are instances when analyses of even short mtDNA sequence fragments manage to detect the same evolutionary patterns as studies employing whole mtDNA genomes, for example the position of the extinct Tremarctine bear *A. simus* in the bear family tree (Chapter 7). In some cases the identification of important SNPs can impact on our understanding of phylogeographic scenarios (e.g. Chapter 3 and 4). Multiplexed mitochondrial SNP analysis is another powerful alternative to whole mitochondrial genome sequencing (Endicott *et al.*, 2006). There are also situations where even obtaining complete mtDNA genomes fails to clarify evolutionary relationships, as recently discovered in an attempt to elucidate the phylogeny of the extinct and extant rhinoceros species using mtDNA genomes (Willerslev *et al.*, 2009).

Another important area for future research would be to analyse nuclear DNA markers or SNPs and compare the results of these to the mitochondrial DNA studies which currently dominate bear phylogeographic studies. As the strong mtDNA phylogeographic structuring observed in modern (and ancient) brown bears is believed to be enhanced by female philopatry (Randi *et al.*, 1994), it would be particularly informative to compare phylogeographic signals from both male and female lineages in this species. There are few published studies of brown bears in which nuclear DNA has been analysed, and most of these use the nuclear data to investigate deeper evolutionary questions - for example Ursine evolutionary relationships (Nakagome *et al.*, 2008; Pages *et al.*, 2008) - rather than looking at the population or phylogeographic level. Brown bear nuclear DNA microsatellites have been analysed at a population level from the Yellowstone National Park in North America (Miller, Waits, 2003) and from Scandinavia (Waits *et al.*, 2000), and both these studies found greater than expected nuclear diversity compared to mtDNA diversity. Further nuclear DNA studies of modern brown bears from other regions would be desirable, and as aDNA techniques improve it may also be possible to retrieve nuclear SNPs from key well-preserved ancient specimens, as recently demonstrated for ancient cattle (Svensson *et al.*, 2007).

8.2.2 Genetic diversity of modern Clade 1 brown bears in Scandinavia

The results from Chapter 2 revealed for the first time that Clade 1 bears of the Italians/Balkans refugia reached southern Scandinavia (at least as far north as Denmark) during their post-glacial expansion. It is currently believed that in southern Scandinavia all modern Clade 1 bears belong to the haplogroup associated with the Iberian glacial refugia. However, of the 49 modern Clade 1 bears previously analysed from southern Scandinavian populations (Taberlet *et al.*, 1995), the mtDNA control region was sequenced from just eight,

with only two haplotypes identified (GenBank Accession X75868 and X75871). The rest of the bears were analysed using a restriction enzyme technique which only allowed the differentiation between bears of Clade 1 and Clade 3, without the power to determine the subclade or putative refugial origin.

It would therefore be interesting to re-analyse the samples from the original restriction enzyme study by PCR amplification and direct sequencing of the control region and look for the SNP at position 16,762 (described in Chapter 4) to confirm whether they do all belong to the Iberian haplogroup as previously assumed, or alternatively reveal the presence of bears of the Italian/Balkans haplogroup which has previously not been detected in modern Scandinavian populations. If the samples from the original study are no longer available for re-analysis, perhaps collection of new samples from southern Scandinavian populations is warranted, as the results of such a study may influence future conservation management.

8.2.3 Extending the Eurasian brown bear study

My study of ancient Eurasian brown bears (Chapter 5) revealed the presence of Clades and haplogroups previously not detected in modern Eurasian populations, for example Clade 3b. This may suggest further sampling is required across Eurasia to determine if these haplogroups have been lost from modern populations altogether or whether sampling bias is an issue (Korsten *et al.* (2009) only detected Clade 3a across all of continental Eurasia). My study of ancient Eurasian brown bears also detected four unique previously undescribed haplotypes in the Caucasus and Ural mountain ranges which appear to be related to modern Clade 4 bears of North America. Future research should include increasing the sequence length from these specimens (ideally obtaining complete mtDNA genomes if the preservation status allows) to more accurately determine the phylogenetic placement of these bears. It is possible that these individuals represent the ancestors of the Clade 4 bears which colonised North America and so obtaining more information on this previously undetected Eurasian group may be important for clarifying routes and timing for the colonisation of these regions. The levels of diversity seen in the ancient specimens from Eurasia (Chapter 5) was particularly high in the mountainous regions that were sampled, such as the Altai Mountains, the Urals and the Caucasus. This may suggest that they acted as refugia, and would be key areas for future studies (of both modern and ancient samples) to target for collecting additional specimens.

8.2.4 Future directions for the Tremarctine bears

Chapter 6 was the first investigation into the mtDNA genetic diversity of any member of the Tremarctine lineage. The lack of mtDNA data from the only extant member of this lineage, *T. ornatus*, is conspicuously lacking. *T. ornatus* is listed by CITES as endangered, currently threatened by habitat destruction, hunting, illegal trade and conflict with cattle ranchers (Mondolfi, 1989; Peyton, 1980; Rodriguez-Clark, Sanchez-Mercado, 2006). Microsatellite studies have found a low level of diversity but failed to detect any evidence of a population bottleneck in this species and have concluded that *T. ornatus* populations therefore maintained a small effective population size since colonisation (Ruiz-Garcia, 2003; Ruiz-Garcia *et al.*, 2005). A mitochondrial analysis would complement the earlier microsatellite studies, and could shed light on the maternal lineages and their diversity as well as suggest colonisation routes if mtDNA phylogeographic patterns are revealed. It would also be of interest to compare the mtDNA genetic diversity of *T. ornatus* with that of the extinct North American Tremarctine bear, *A. simus* (Chapter 6), to see if the lack of mtDNA diversity and phylogeographic structure observed in *A. simus* is a feature of the Tremarctine bears. Considering its conservation status and the relatively easy extraction and amplification of

modern mtDNA this should be undertaken in the near future. DNA samples from 155 *T. ornatus* have previously been collected for the microsatellite analyses (Ruiz-Garcia *et al.*, 2005), so it may even be possible to utilise these samples for an mtDNA study, without the difficulty of obtaining new samples.

The evolutionary relationships of the South American Tremarctine bears are still incompletely understood, as is the case for many other South American taxa for which the timing and origin of diversification is unclear (for example the Sigmodontine rodents; Engel *et al*., 1998 and the South American camelid species such as vicuna and guanaco; Marin *et al*., 2007). Future work to extend the findings presented in Chapter 7 should firstly concentrate on determining the MRCA of the South American taxa *Arctotherium sp.* and *T. ornatus*. If a suitably well-preserved bone sample of the extinct Florida bear (*T. floridanus*) could be made available for ancient DNA analysis it may be possible to resolve this issue. Although preservation of Pleistocene samples from South America is likely to be problematic, it would also be of interest to obtain whole mtDNA genomes of *Arctotherium* (and possibly *T. floridanus*) to compare to those already obtained for *A. simus* and *T. ornatus*, allowing a better resolution of the timing of divergence of the Tremarctine bears.

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CHAPTER 9

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APPENDIX 1

Supporting information for Chapter 2

“Ancient DNA analysis of post-glacial Scandinavian brown bears”

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Appendix Table 1.1 Comprehensive list of all samples analysed in Chapter 2. Showing extraction details, bone types, museum/collection details and radiocarbon dates. Green indicates successful extraction, amplification and sequencing of *Ursus arctos* mtDNA. Red indicates that no endogenous DNA could be extracted. Blue indicates samples from which DNA was successfully extracted, amplified and sequenced but DNA result indicated the sample was not from an *Ursus arctos* specimen and so were not included in the analyses in this chapter.

ACAD Extraction Number	Sample Accession Number	Specimen	Sample details	Mass	Radiometric age	Radiocarbon reference number	Sample provided by	Extraction details	DNA result
1744A	BU-1982-2	*U. arctos*	mandibula	0.19g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 26/10/2006 Sarah Bray	No DNA
1745A	Bu-1982-1	*U. arctos*	femur	0.28g	3600 +/- 80	T-6264	Lauritzen & Østbye	Phenol/Chloroform 26/10/2006 Sarah Bray	No DNA
1746A	4045	*U. arctos*	femur	0.38g	420 +/- 90	T-8088	Lauritzen & Østbye	Phenol/Chloroform 26/10/2006 Sarah Bray	No DNA
1747A	JS-455	*U. arctos*	calvarium	0.57g	2550 +/- 100	T-8087	Lauritzen & Østbye	Phenol/Chloroform 26/10/2006 Sarah Bray	No DNA
1748A	HM 50	*U. arctos*	calvarium	0.56g	1839 +/- 90	Ua-1925	Lauritzen & Østbye	Phenol/Chloroform 26/10/2006 Sarah Bray	No DNA
1749A	RM-893	*U. arctos*	mandibula	0.43g	3530 +/- 110	Ua-1921	Lauritzen & Østbye	Phenol/Chloroform 26/10/2006 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1750A	RM-2392	*U .arctos*	mandibula	0.44g	2440 +/- 20	Ua-1920	Lauritzen & Østbye	Phenol/Chloroform 26/10/2006 Sarah Bray	No DNA
1751A	RM-418 B	*U. arctos*	mandibula	0.56g			Lauritzen & Østbye	Phenol/Chloroform 21/03/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1752A	B-77-N-9-4	*U. arctos*	atlas	0.43g	1310 +/- 30		Lauritzen & Østbye	Phenol/Chloroform 21/03/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a

ACAD Extraction Number	Sample Accession Number	Specimen	Sample details	Mass	Radiometric age	Radiocarbon reference number	Sample provided by	Extraction details	DNA result
1753A	B-77-N-8-1	*U. arctos*	ulna	0.30g	1660 +/- 100	Ua-1926	Lauritzen & Østbye	Phenol/Chloroform 26/10/2006 Sarah Bray	No DNA
1754A	RS-030986	*U. arctos*	humerus	0.30g	6210 +/- 100	T-7024	Lauritzen & Østbye	Phenol/Chloroform 26/02/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1755A	B-78-B-1	*U. arctos*	scapula	0.37g	2870 +/- 80	T-4155	Lauritzen & Østbye	Phenol/Chloroform 26/02/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1756A	B-77-G-5-7	*U. arctos*	femur	0.24g	4420 +/- 70	T-4157	Lauritzen & Østbye	Phenol/Chloroform 07/05/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1757A	RM-1858	*U. arctos*	calvarium	0.46g	4370 +/- 40	T-4654	Lauritzen & Østbye	Phenol/Chloroform 26/02/2007 Sarah Bray	No DNA
1758A	RM-903	*U. arctos*	calvarium	0.45g	2240 +/- 190	Ua-1924	Lauritzen & Østbye	Phenol/Chloroform 26/02/2007 Sarah Bray	No DNA
1759A	B-81-S-1 A	*U. arctos*	scapula	0.18g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 26/02/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1760A	RM-418A	*U. arctos*	mandibula	0.20g	2340 +/- 200	Ua-1918	Lauritzen & Østbye	Phenol/Chloroform 22/11/2006 Sarah Bray	No DNA
1761A	RM-3188 A	*U. arctos*	radius	0.21g	3980 +/- 180	Ua-1923	Lauritzen & Østbye	Phenol/Chloroform 22/11/2006 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1762A	KJ-01A	*U. arctos*		0.64g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 24/04/2007 Sarah Bray	No DNA
1763A	ROG-01A	*U. arctos*		0.41g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 22/11/2006 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a

ACAD Extraction Number	Sample Accession Number	Specimen	Sample details	Mass	Radiometric age	Radiocarbon reference number	Sample provided by	Extraction details	DNA result
1764A	FAU-01A	*U. arctos*		0.51g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 22/11/2006 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1765A	TROLL-4A	*U. arctos*		0.78g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 10/08/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1766A	TROLL-2A (290795-4)	*U. arctos*		0.55g			Lauritzen & Østbye	Phenol/Chloroform 26/02/2007 Sarah Bray	No DNA
1767A	TROLL-3A	*U. arctos*		0.36g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 21/03/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1768A	TROLL-1A	*U. arctos*		0.61g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 10/08/2007 Sarah Bray	No DNA
1769A	B-80-G-1-A	*U. arctos*	scapula	0.66g	820 +/- 80	T-4156	Lauritzen & Østbye	Phenol/Chloroform 26/02/2007 Sarah Bray	No DNA
1770A	B-81-S-1 B	*U. arctos*	ulna	0.61g	3970 +/- 60	T-4655	Lauritzen & Østbye	Phenol/Chloroform 22/11/2006 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1771A	BU-1982-3	*U. arctos*	calvarium	0.70g	4340 +/- 200	Ua-1919	Lauritzen & Østbye	Phenol/Chloroform 10/08/2007 Sarah Bray	No DNA
1772A	B-1979-N-320	*U. arctos*	tibia	0.59g	2800 +/- 160	Ua-1927	Lauritzen & Østbye	Phenol/Chloroform 22/11/2006 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1773A	82-9-11-5a	*U. arctos*	fragments	0.44g	3150 +/- 120	T-4901	Lauritzen & Østbye	Phenol/Chloroform 21/03/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
1774A	HA-88-2	*U. arctos*	tarsal bone	0.66g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 22/11/2006 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a

ACAD Extraction Number	Sample Accession Number	Specimen	Sample details	Mass	Radiometric age	Radiocarbon reference number	Sample provided by	Extraction details	DNA result
1775A	?	*U. spelaeus*	mandibula	0.73g			Lauritzen & Østbye	Phenol/Chloroform 22/11/2006 Sarah Bray	No DNA
2309A	KJ91-2a	*U. maritimus*		0.56g	Pleistocene		Lauritzen & Østbye	Phenol/Chloroform 24/04/2007 Sarah Bray	Success *Ursus maritimus* Clade 2b*
2310A	B-19??-N-1a	*U. arctos*		1.13g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 07/05/2007 Sarah Bray	No DNA
2311A	FAU-05	*U. arctos*		0.49g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 12/07/2007 Sarah Bray	No DNA
2312A	RM-894 Virvel	*U. arctos*		0.93g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 12/07/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
2313A	PU96-6 Hulebjorn	*U. arctos*		0.80g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 12/07/2007 Sarah Bray	No DNA
2314A	RM3188	*U. arctos*		0.86g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 12/07/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
2315A	BU-1982-2	*U. arctos*		0.70g	undated Holocene		Lauritzen & Østbye	Phenol/Chloroform 07/05/2007 Sarah Bray	Success *Ursus arctos* Eastern Clade 3a
3927A	ZMK 52/1942 Dyrhojgards Mose Denmark	*U. arctos*		0.59g			Kim Aaris-Sørensen	Phenol/Chloroform 22/01/2008 Sarah Bray	Success *Phoca vitulina*
3928A	Denmark Faurbo Knold ZMK 170/1980	*U. arctos*		0.502g			Kim Aaris-Sørensen	Phenol/Chloroform 11/02/2008 Sarah Bray	No DNA

ACAD Extraction Number	Sample Accession Number	Specimen	Sample details	Mass	Radiometric age	Radiocarbon reference number	Sample provided by	Extraction details	DNA result
3929A	ZMK 1/1846 Hormested Sogn Denmark	*U. arctos*		0.48g			Kim Aaris-Sørensen	Phenol/Chloroform 22/01/2008 Sarah Bray	No DNA
3930A	ZMK 2/1918 Svaerdborg I. Denmark	*U. arctos*		0.52g			Kim Aaris-Sørensen	Phenol/Chloroform 22/01/2008 Sarah Bray	No DNA
3931A	Denmark Virksund ZMK 9/1861	*U. arctos*		0.548g	5310 +/- 20	CURL-10287**	Kim Aaris-Sørensen	Phenol/Chloroform 11/02/2008 Sarah Bray	Success *Ursus arctos* Western Clade1 Italy/Balkans subclade
3932A	Denmark Oreso Molle ZMK 17/1980	*U. arctos*		0.784g			Kim Aaris-Sørensen	Phenol/Chloroform 11/02/2008 Sarah Bray	No DNA

DNA sequences obtained from ACAD 2309A are included in Davison et al. (2010, manuscript submitted to QSR).

*****Radiocarbon dating of Virksund, Denmark (ZMK 9/1861) – CURL-10287***

Powdered bone from the Danish Virksund (ZMK 9/1861) specimen was prepared for Accelerator Mass Spectrometry by amino acid purification using ion exchange chromatography (Stafford Jr *et al.*, 1988) at the University of Colorado INSTARR Laboratory for AMS Radiocarbon Preparation and Research. This sample was included in a batch of 26 samples prepped in July of 2008. Of the four bone standards included with these samples, one bone standard was ~650 radiocarbon years too old (INSTARR date 10,200 vs. expected date 9,500 radiocarbon years before present), which was outside of statistical bounds. Due to a suspected dead carbon contaminant in the INSTARR procedure, twenty-two of the twenty six samples had enough material remaining to repeat, and were subsequently prepared from the collagen stage using the ultrafiltration technique (Brown *et al.*, 1988) at the University of California Irvine (UCI) Keck CCAMS facility and re-dated. One of the twenty-two samples was different by ~625 years (INSTARR date 10255 ± 30 vs. UCI 9630 ± 30), which confirmed a potential contaminant in the INSTARR procedure, which may result in miss-dating bone samples <15,000 radiocarbon years old on the scale of hundreds of years. The Virksund (ZMK 9/1861) specimen could not be re-dated due to a lack of remaining bone material. Therefore, the

date of 5310 ± 20 radiocarbon years for Virksund (ZMK 9/1861) should be interpreted with caution as being potentially as young as 4600 radiocarbon years. However, we can still be confident that the sample is not modern or historic.

Appendix Table 1.2 List of previously published samples obtained from GenBank and included in these analyses.

Accession No.	Country	Sample ID	Age	Clade	Reference
X75874	Sweden	Rus	modern	3a	(Taberlet & Bouvet, 1994)
X75877	Slovenia	Slo	modern	1	(Taberlet & Bouvet, 1994)
X75868	Sweden	Dal	modern	1	(Taberlet & Bouvet, 1994)
X75871	Norway	Nor	modern	1	(Taberlet & Bouvet, 1994)
EF488498	Germany	A5	1770 ± 35	1	(Valdiosera *et al.*, 2007)
EF488506	Romania	Romania1	III Century	3a	(Valdiosera *et al.*, 2007)
EF488507	Romania	Romania2	III Century	3a	(Valdiosera *et al.*, 2007)
EF488505	France	Mv4 Remanie	3445 ± 40	1	(Valdiosera *et al.*, 2007)
EF488491	France	Mv4_162	1790 ± 55	1	(Valdiosera *et al.*, 2007)
EF488493	France	Mv4Mr204-48	1750 ± 30	1	(Valdiosera *et al.*, 2007)
EF488499	Germany	A9	5210 ± 35	1	(Valdiosera *et al.*, 2007)
EF488502	Spain	C.Motas 33-2	undated	1	(Valdiosera *et al.*, 2007)
EF488497	Spain	Hem	350 ± 40	1	(Valdiosera *et al.*, 2007)
AJ300176	Germany	*U.spelaeus*	Pleistocene	-	(Hofreiter *et al.*, 2002)

Verification/Validation of aDNA results

1) Duplicate samples from five Norwegian specimens were independently replicated at Centro Mixto UCM-ISCIII de Evolución y Comportamiento Humanos, Madrid, Spain. The independent extractions were performed using QIAquick Spin columns (Qiagen). Each extract was PCR amplified twice using the L16164/H16299 135bp CR primers and sequenced. The PCR conditions used are described below:

PCR reaction mix

PCR buf	2.5 µl
MgCl	1.0 µl
dNTPs	0.5 µl
P1	2.5 µl
P2	2.5 µl
BSA	2.5 µl
Taq	0.4 µl
H_20	8.1 µl
DNA	5.0 µl

<u>PCR conditions :</u>
95ºC for 11min,
(94ºC for 30sec, 52ºC for 30sec, 72ºC for 30sec) x 54 cycles
72ºC for 10min,
10ºC Hold.

The sequences obtained from the two independent PCR amplifications performed in Madrid (Sequences 2 and 3 below) were then compared to the sequences generated in Adelaide (Sequence 1 below) from the original PCR amplifications. The best of three rule was used to determine a consensus sequence for use in further analyses (Hofreiter *et al.*, 2001).

Norway B-77-G-5-7 (Sequence1)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway B-77-G-5-7 (Sequence2)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway B-77-G-5-7 (Sequence3)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway B-77-N-9-4 (Sequence1)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGTGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway B-77-N-9-4 (Sequence2)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGTGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway B-77-N-9-4 (Sequence3)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGTGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway B-81-5-11-BJ (Sequence1)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway B-81-5-11-BJ (Sequence2)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway B-81-5-11-BJ (Sequence3)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway RSO30986 (Sequence1)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway RSO30986 (Sequence2)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTTCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway RSO30986 (Sequence3)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTTATTTAGTTCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway RM-3188-A (Sequence2)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway RM-3188-A (Sequence2)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

Norway RM-3188-A (Sequence3)
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCT
CGAGAAACCAGCAACCCTTGCGAGT

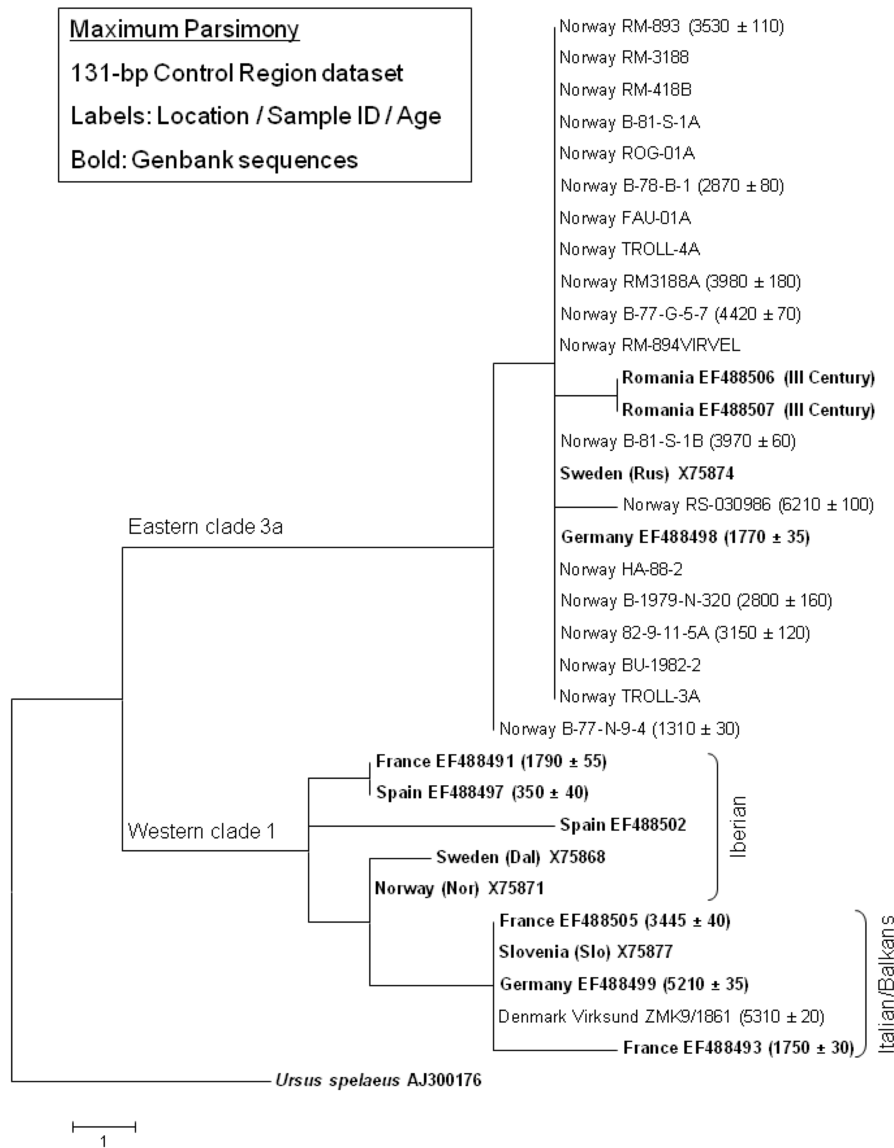
2) Three independent PCR amplifications were performed in Adelaide on the Danish sample (Virksund ZMK9/1861) using the 111bp primers. All three PCR products were directly sequenced in both directions. The best of three rule was used to determine a consensus sequence for use in further analyses (Hofreiter *et al.*, 2001).

>Virksund_Rep1
GGGAAAAGTATTCGAGGACATACTGTGATGGCACAGTACATAAGATGGTATATATGAAATAAATAGGACACGGAGTAAAATAGTAGTATGTACCAGGGAATAGTTTA
AATA

>Virksund_Rep2
GGGAAAAGTATTCGAGGACATACTGTGATGGCACAGTACATAAGATGGTATATATGAAATAAATAGGACACGGAGTAAAATAGTAAATATGTACCAGGGAATAGTTTA
AATA

>Virksund_Rep3
GGGAAAAGTATTCGAGGACATACTGTGATGGCACAGTACATAAGATGGTATATATGAAATAAATAGGACACGGAGTAAAATAGTAGTATGTACCAGGGAATAGTTTA
AATA

Appendix Figure 1.1 Maximum Parsimony analysis. Showing that all the ancient northern Norwegian samples fall within the Eastern clade 3a, while the Danish sample (Virksund ZMK9/1861) falls within the Italian/Balkans group of the Western clade 1.



Appendix Table 1.3 Genetic diversity measures for the 135bp control region sequence of the Holocene northern Norwegian brown bear population. Values calculated using Arlequin 3.1.1 (Schneider *et al.*, 2000).

No. of samples	No. of haplotypes	Mean pairwise differences	Haplotype diversity (h) ± SD	Nucleotide diversity (π) ± SD
19	3	0.210526 ± 0.266959	0.2047 ± 0.1191	0.001607 ± 0.002277

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APPENDIX 2

Supporting information for Chapter 3

"From Iberia to Alaska: ancient DNA links late Pleistocene brown bears across the Holarctic"

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Text in manuscript 2009.

Appendix Figure 2.1 Phylogenetic tree of global brown bear Control Region haplotypes. Above nodes: posterior probabilities for rooted / unrooted phylogenetic analyses. Below nodes: boostrap supports for rooted / unrooted phylogenetic analyses. Divergence dates for nodes A-F are reported in Chapter 3, Table 3.1.

Appendix Table 2.1 Haplotype list and sample details.

Haplotype number	Label on network or tree	Sample name	GenBank No.	Age of sample	No. of identical sequences	Length of haplotype (bp)	Sample name	GenBank No.	Age of sample
1	*Ursus spelaeus*	SC3800	AY149268	Pleistocene	1	177			
2	*Ursus spelaeus*	CLA	AY149271	Pleistocene	1	177			
3	*Ursus spelaeus*	TAB15	AY149273	Pleistocene	1	177			
4	JAPAN	JAPAN-1	AB010725		13	177	JAPAN-2	AB010726	
							HB-01-834 Hokkaido	AB013040	
							HB-02-823 Hokkaido	AB013041	
							HB-02-2078 Hokkaido	AB013042	
							HB-02-1137 Hokkaido	AB013043	
							HB-02-3092 Hokkaido	AB013044	
							HB-02-3091 Hokkaido	AB013045	
							HB-03-1171 Hokkaido	AB013046	
							HB-04-2225 Hokkaido	AB013047	
							HB-04-3076 Hokkaido	AB013048	
							HB-04-826 Hokkaido	AB013049	
							HB-07-427 Hokkaido	AB013053	
5	TIBET	TIBET-1	AB010727		1	177			
6	MONGOLIA	GOBI-1	AB010728		1	177			
7	JAPAN	HB-05-910	AB013050		3	177	HB-05-835 Hokkaido	AB013051	
							HB-06-4293 Hokkaido	AB013052	

Haplotype number	Label on network or tree	Sample name	GenBank No.	Age of sample	No. of identical sequences	Length of haplotype (bp)	Sample name	GenBank No.	Age of sample
8	JAPAN	HB-08-520	AB013054		1	177			
9	JAPAN	HB-09-421	AB013055		1	177			
10	JAPAN	HB-10-820	AB013056		2	177	HB-11-617 Hokkaido	AB013057	
11	JAPAN	HB-12-822	AB013058		1	177			
12	JAPAN	HB-13-4162	AB013059		2	177	HB-13-811 Hokkaido	AB013060	
13	JAPAN	HB-14-2098	AB013061		1	177			
14	JAPAN	HB-15-1119	AB013062		1	177			
15	JAPAN	HB-16-4008	AB013063		8	177	HB-16-4028 Hokkaido	AB013064	
							HB-17-5051 Hokkaido	AB013065	
							HB-17-602 Hokkaido	AB013066	
							HB-17-518 Hokkaido	AB013067	
							HB-17-2026 Hokkaido	AB013068	
							HB-17-515 Hokkaido	AB013069	
							HB-17-4025 Hokkaido	AB013070	
16	AUSTRIA	Ramesch-1	AJ809333	47420 ybp	1	177			
17	AUSTRIA	Winden-1	AJ809334	39940 ybp	1	177			
18	ALGERIA	AKO1	AM411397	1679 ± 35 ybp	25	177	AKO2 Algeria	AM411398	1550 ± 40 ybp
							Oso1 Spain	EU400184	
							Oso2 Spain	EU400185	
							Oso3 Spain	EU400186	
							Oso4 Spain	EU400187	
							Oso5 Spain	EU400188	
							Oso6 Spain	EU400189	

Haplotype number	Label on network or tree	Sample name	GenBank No.	Age of sample	No. of identical sequences	Length of haplotype (bp)	Sample name	GenBank No.	Age of sample
							Oso7 Spain	EU400190	
							Oso8 Spain	EU400191	
							Oso9 Spain	EU400192	
							Oso10 Spain	EU400193	
							Oso11 Spain	EU400194	
							Oso12 Spain	EU400195	
							Oso13 Spain	EU400196	
							Oso15 Spain	EU400198	
							Oso17 Spain	EU400199	
							Oso18 Spain	EU400200	
							Oso20 Spain	EU400201	
							Oso22 Spain	EU400203	
							Oso23 Spain	EU400204	
							Oso25 Spain	EU400206	
							PEU Spain	EU400207	~ 40 ya
							Cantabria	X75865	
							Cantabria1	X75866	
19	MOROCCO	ELK1	AM411399	1285 ± 30 ybp	1	177			
20	ALGERIA	TAK1	AM411400	9620 ± 200 ybp	4	177	TAK2 Algeria	AM411401	9620 ± 200 ybp
							TAK3 Algeria	AM411402	7650 ± 40 ybp
							TAK4 Algeria	AM411403	7345 ± 40 ybp
21	ALASKA	FAM30771 Fairbanks Alaska	AY082810 + AY082846	20080 ± 160 ybp	1	177			

Haplotype number	Label on network or tree	Sample name	GenBank No.	Age of sample	No. of identical sequences	Length of haplotype (bp)	Sample name	GenBank No.	Age of sample
22	ALASKA	AMNH30421 Fairbanks Alaska	AY082811 + AY082847	undated	1	177			
23	ALASKA	FAM95612 Fairbanks Alaska	AY082816 + AY082852	10015 ± 62 ybp	1	177			
24	ALASKA	AMNH95665 Fairbanks Alaska	AY082819 + AY082855	13760 ± 50 ybp	7	177	FAM95632 Fairbanks Alaska	AY082820 + AY082856	14810 ± 80 ybp
							FAM95628 Fairbanks Alaska	AY082822 + AY082858	12310 ± 65 ybp
							FAM95670 Fairbanks Alaska	AY082823 + AY082859	15830 ± 100 ybp
							FAM95653 Fairbanks Alaska	AY082853 + AY082817	9535 ± 75 ybp
							FAM95597 Fairbanks Alaska	AY082854 + AY082818	undated
							AMNH95630 Fairbanks Alaska	AY082857 + AY082821	14980 ± 60 ybp
25	ALASKA	FAM95598 Fairbanks Alaska	AY082824 + AY082860	12320 ± 90 ybp	2	177	AMNH30422 Fairbanks Alaska	AY082825 + AY082861	19027 ± 132 ybp
26	ALASKA	FAM95596 Fairbanks Alaska	AY082826 + AY082862	undated	1	177			
27	ALASKA	FAM95595 Fairbanks Alaska	AY082827 + AY082863	12441 ± 75 ybp	1	177			

Haplotype number	Label on network or tree	Sample name	GenBank No.	Age of sample	No. of identical sequences	Length of haplotype (bp)	Sample name	GenBank No.	Age of sample
28	ALASKA	AMNH95679 Fairbanks Alaska	AY082828 + AY082864	15370 ± 60 ybp	1	177			
29	ALASKA	FAM95671 Fairbanks Alaska	AY082829 + AY082865	20820 ± 120 ybp	1	177			
30	ALASKA	FAM95609 Fairbanks Alaska	AY082830 + AY082866	50800 ± 1900 ybp	1	177			
31	ALASKA	FAM95640 Fairbanks Alaska	AY082831 + AY082867	>53900 ybp	1	177			
32	ALASKA	FAM95601 Fairbanks Alaska	AY082832 + AY082868	36137 ± 783 ybp	1	177			
33	ALASKA	FAM95666 Fairbanks Alaska	AY082833 + AY082869	47100 ± 3100 ybp	1	177			
34	ALASKA	FAM95639 Fairbanks Alaska	AY082870 + AY082870	>56900 ybp	1	177			
35	ALASKA	FAM95681 Fairbanks Alaska	AY082835 + AY082871	>59000 ybp	1	177			
36	ALASKA	FAM95634 Fairbanks Alaska	AY082836 + AY082872	41787 ± 212 ybp	1	177			
37	ALASKA	FAM95664 Fairbanks Alaska	AY082837 + AY082873	42600 ± 850 ybp	1	177			
38	USA	KU23034 Wyoming USA	AY082838 + AY082874	Holocene	1	177			
39	YUKON	CMN42381 Sixty Mile, Yukon, Canada	AY082839 + AY082875	35970 ± 660 ybp	1	177			
40	ALASKA	FAM95657 Fairbanks Alaska	AY082840 + AY082876	19360 ± 140 ybp	1	177			
41	YUKON	CMN38279 Sixty Mile, Yukon, Canada	AY082842 + AY082878	36500 ± 1150 ybp	1	177			

Haplotype number	Label on network or tree	Sample name	GenBank No.	Age of sample	No. of identical sequences	Length of haplotype (bp)	Sample name	GenBank No.	Age of sample
42	ALASKA	AK-374-V-8 PoW Island, Alaska	AY082844 + AY082880	9995 ± 95 ybp	1	177			
43	SCOTLAND	RSM1962/63 Scotland	AY082845 + AY082881	Holocene	1	177			
44	ALASKA	FAM95641 Fairbanks Alaska	AY082848 + AY082812	11940 ± 100 ybp	1	177			
45	ALASKA	FAM95599 Fairbanks Alaska	AY082849 + AY082813	14310 ± 100 ybp	3	177	FAM95659 Fairbanks Alaska	AY082850 + AY082814	13415 ± 70 ybp
							FAM95642 Fairbanks Alaska	AY082851 + AY082815	14150 ± 90 ybp
46	SPAIN	Vallecampo Spain	EF488487	7500 ± 55 ybp	6	177	GEE Spain	EF488490	5380 ± 45 ybp
							C.Motas 33.2 Spain	EF488503	
							Machorras Spain	EU400176	Holocene
							Gal Spain	EU400177	Holocene
							Hortiguela Spain	EU400179	6325 ± 50 ybp
47	ITALY	Gbcm2 Italy	EF488488	16440 ± 65 ybp	1	177			
48	SPAIN	Asturias Spain	EF488489		1	177			
49	FRANCE	MV4-162 France	EF488491	17909 ± 55 ybp	3	177	MV4-K3-99 France	EF488495	1570 ±35 ybp
							Pyrenees France	X75878	
50	FRANCE	MV4-1184 France	EF488492	3845 ± 40 ybp	1	177			
51	FRANCE	MV4-204-48 France	EF488493	1750 ± 30 ybp	1	177			

Haplotype number	Label on network or tree	Sample name	GenBank No.	Age of sample	No. of identical sequences	Length of haplotype (bp)	Sample name	GenBank No.	Age of sample
52	FRANCE	MV4-851 France	EF488494	6525 ± 50 ybp	1	177			
53	FRANCE	MV4-714 France	EF488496	4645 ± 40 ybp	1	177			
54	SPAIN	HEM Spain	EF488497	350 ± 40 ybp	1	177			
55	GERMANY	A5 Germany	EF488498	1770 ± 35 ybp	4	177	Sweden (Rus)	X75874	
							Rus' Slovakia Russia	X75875	
							Rus" Slovakia Russia	X75876	
56	GERMANY	A9 Germany	EF488499	5210 ± 35 ybp	4	177	A12 Germany	EF488500	XII-XIV Century
							REMANIE France	EF488505	3445 ± 40 ybp
							Slovenia (Slo)	X75877	
57	GERMANY	A3 Germany	EF488501	1665 ± 35 ybp	2		Bulgaria (Bul)	X75864	
58	SPAIN	C.Motas 33-1 Spain	EF488502	4624 ± 45 ybp	1	177			
59	SPAIN	Atapuerca Spain	EF488504	17440 ± 425 ybp	2	177	Vdgba Spain	EU400181	80,000 ybp
60	SPAIN	O.Pal Spain	EU400178	Holocene	1	177			
61	ASIEKO SPAIN	Asieko Spain	EU400180	Pleistocene 40000-80000 ybp	1	177			
62	SPAIN	Vb88T6 Spain	EU400182	80,000 ybp	1	177			
63	SPAIN	VbL13 Spain	EU400183	80,000 ybp	1	177			
64	SPAIN	Oso14 Spain	EU400197		1	177			
65	SPAIN	Oso21 Spain	EU400202		1	177			

Haplotype number	Label on network or tree	Sample name	GenBank No.	Age of sample	No. of identical sequences	Length of haplotype (bp)	Sample name	GenBank No.	Age of sample
66	SPAIN	Oso24 Spain	EU400205		1	177			
67	ITALY	Abruzzo (Abr) Italy	X75862		1	177			
68	CROATIA	Croatia (Cro)	X75867		1	177			
69	SWEDEN	Dalarna (Dal) Sweden	X75868		1	177			
70	ESTONIA	Estonia (Est)	X75869		1	177			
71	GREECE	Greece (Gre)	X75870		1	177			
72	NORWAY	Norway (Nor)	X75871		1	177			
73	ROMANIA	Romania1 (Ro1)	X75872		1	177			
74	ROMANIA	Romania2 (Ro2)	X75873		1	177			

Appendix Table 2.2 Serial coalescent simulations of the brown bear dataset. Either including or excluding the 3c individual (40-80 kya, Asieko, EU488488), the data supports the conclusion of (Valdiosera *et al.* 2008) that a population bottleneck occurred at the Pleistocene-Holocene transition, as long as the effective population was >1000-2000 individuals.

	Without Asieko	**Asieko at 40 Kya**	**Asieko at 80 KYA**
Ne	**H/M – P/H – P/M**	**H/M – P/H – P/M**	**H/M – P/H – P/M**
Observed shift	0.387 - 0.166 – 0.554	0.387- 0.233 - 0.621	0.887- 0.233 - 0.621
Probabilities:			
100	0.234 - 0. 493 – 0.155	0.247- 0.532 - 0.195	nd - nd - nd
500	0.090 - 0. 206 – 0.021	0.092- 0.245 - 0.032	0.100- 0.159 - 0.019
1000	0.021 - 0. 082 – 0.001	0.028- 0.102 - 0.005	0.029- 0.070 - 0.002
2000	0.003 - 0. 020 – 0.000	0.003- 0.013 - 0.000	0.000- 0.000 - 0.000
5000 - 45000	0.000 - 0. 000 – 0.000	0.000- 0.000 - 0.000	0.000- 0.000 - 0.000

APPENDIX 3

Supporting information for Chapter 4

"Post-glacial phylogeography of European brown bears"

Appendix Table 3.1 List of European brown bear samples extracted for analysis in Chapter 4. Samples from which 135bp mtDNA was successfully amplified and sequenced are highlighted in green. Samples from which DNA could not be successfully amplified are highlighted in red.

Sample name	Geographic location	Museum Accession number	Sample details	Sample provided by	Age *	Radiocarbon date reference	Amount extracted	mtDNA result
ACAD103	Brunnenschacht, Austria	VNMH H1977-18-2	Canine root	Doris Nagel	Holocene	N/A	0.37 g	Clade1
ACAD104	Flunkerhohle. Austria	VNMH H90-64	Tibia	Doris Nagel	Holocene	N/A	0.47 g	No DNA
ACAD105	Barenhohle, Austria	VNMH H1988-62b	Ulna	Doris Nagel	Holocene	N/A	0.44 g	No DNA
ACAD106	Schwabenreithhohle, Austria	VNMH H1975-43-1	Radius	Doris Nagel	Holocene	N/A	0.57 g	No DNA
ACAD107	Seekarlhohle, Austria	VNMH H1990-62-1	Canine root	Doris Nagel	Holocene	N/A	0.67 g	Clade1
ACAD108	Felis-Schacht, Austria	VNMH H1978-15-1	Molar root	Doris Nagel	Holocene	N/A	0.60 g	No DNA
ACAD109	Notentalhohle, Austria	VNMH H1997-21-2	Tibia	Doris Nagel	5,325 ± 40	VERA-4523	0.84 g	Clade1
ACAD110	Dixlucke, Austria	VNMH H1976-7	Humerus	Doris Nagel	Holocene	N/A	0.48 g	No DNA
ACAD111	Turkenloch, Austria	VNMH H83-1-4	Scapula	Doris Nagel	Holocene	N/A	1.0 g	No DNA
ACAD112	Burianhohle, Austria	VNMH (no number)	Ulna	Doris Nagel	Holocene	N/A	0.32 g	Clade1
ACAD113	Barenkammer, Austria	VNMH H1983-47-1	Canine root	Doris Nagel	Holocene	N/A	0.21 g	Clade1
ACAD114	Grubenlocher, Austria	VNMH 3724	Radius	Doris Nagel	1,805 ± 35	VERA-4524	0.46 g	Clade1
ACAD115	Barenhohle, Austria	VNMH H-74-13-1	Humerus	Doris Nagel	2,264 ± 40	Erl-13095	0.41 g	Clade1
ACAD116	Rabenmaurhohle,	VNMH	Humerus	Doris Nagel	3,643 ± 41	Erl-13092	0.46 g	Clade1

	Austria	H1974-10-3						
ACAD117	Napflucke, Austria	VNMH H1964-9	Canine root	Doris Nagel	9985 ± 57	Erl-13094	0.31 g	Clade1
ACAD118	Gemsenhohle, Austria	VNMH H93-12	Canine root	Doris Nagel	Holocene	N/A	0.81 g	Clade1
ACAD119	Barenloch, Austria	VNMH H86-178-1	Canine root	Doris Nagel	1,985 ± 35	VERA-4522	0.40 g	Clade 3a
ACAD120	Windorgel, Austria	VNMH H90-7-3	Canine root	Doris Nagel	3,321 ± 40	Erl-13093	0.81 g	Clade1
ACAD121	Schoberbergschacht, Austria	VNMH H89-27-1	Molar root	Doris Nagel	Holocene	N/A	0.60 g	Clade1
ACAD122	Kohlerwandhohle, Austria	VNMH H1964-29-5	Phalange	Doris Nagel	Holocene	N/A	0.50 g	No DNA
ACAD123	Barenloch, Austria	VNMH H1985-98-1	Tibia	Doris Nagel	2,530 ± 20 2,485 ± 20	CURL-10276 UCIAMS - 56988	0.19 g	Clade1
ACAD124	Sinterkamin, Austria	VNMH H1973-19-3	Humerus	Doris Nagel	Holocene	N/A	0.37 g	No DNA
ACAD125	Turkenloch, Austria	VNMH H1983-6-3	Tibia	Doris Nagel	Holocene	N/A	0.34 g	No DNA
ACAD126	Knochenrohre, Austria	VNMH H90-103-11	Radius	Doris Nagel	Holocene	N/A	0.45 g	Clade1
ACAD127	Gemsenhohle, Austria	VNMH H93-12	Tibia/ Humerus	Doris Nagel	3,130 ± 35	VERA-4525	0.70 g	Clade1
ACAD128	Turkenloch, Austria	VNMH H1985-19-6	Phalange	Doris Nagel	Holocene	N/A	0.25 g	Clade1
ACAD130	Laufenberg, Austria	VNMH		Doris Nagel	9,810 ± 70	GrN-22339	0.68 g	Clade1
ACAD388	Bear Cave, Scotland	Royal Scot. Museum NMS.Z.1962 .63 (juvenile)	-		2,673 ± 54	BM-724	0.37 g	Clade1

ACAD389	Bear Cave, Scotland	Royal Scot. Museum NMS.Z.1962 .63 (adult)	-		2,673 ± 54	BM-724	0.22 g	Clade1
ACAD3643	Allander Tropfsteinhohle, Austria	-	PH II	Doris Döppes	10,870 ± 80	(Döppes & Pacher, 2005)	0.68 g	Clade1
ACAD3644	Wolfhohle, Austria	-	Mc II d	Doris Döppes	6,615 ± 45	VERA-0836	0.62 g	Clade1
ACAD3645	Neue Laubenstein-Barenhohle, Germany	-	-	Doris Döppes	11,872 ± 92	Erl-7851	0.53 g	Clade1
ACAD3646	Grotta d‘Ernesto, Italy	-	Fibula	Doris Döppes	11,900 ± 33	Gd-6182	0.40 g	Clade1
ACAD3647	Hohle 92/2 Barenloch, Switzerland	-	Femur dex	Doris Döppes	9,700 ± 80	ETH-12785	1.15 g	Clade1
ACAD3648	Hohle 92/2, Switzerland	-	-	Doris Döppes	9,700 ± 80	ETH-12785	0.68 g	No DNA
ACAD3649	Barengraben hintersilberen, Switzerland	-	-	Doris Döppes	3,275 ± 50	ETH-31380	0.40 g	Clade1
ACAD3650	Barengraben Hintersilberen, Switzerland	-	-	Doris Döppes	4,135 ± 50	ETH-31320	0.64 g	Clade1
ACAD3651	Feistringhohle, Austria	-	-	Doris Döppes	2,935 ± 25	VERA-2193	0.42 g	Clade1

* Age is given in uncalibrated radiocarbon years before present (if known).
† Sample dated as part of this study.

Appendix Table 3.2 Radiocarbon calibration comparison.
All radiocarbon dates in this chapter are presented and discussed as uncalibrated radiocarbon years before present (ybp), however for those who prefer calibrated dates, this table provides a comparison between the uncalibrated dates used in this chapter to those calibrated using the CalPal_2007_HULU calibration curve implemented through CalPal online (www.calpal-online.de/). The calibrated dates are shown as Calendric Age cal BP, while the uncalibrated dates are shown as uncalibrated radiocarbon years before present (ybp).

Sample name	Museum Accession number	Uncalibrated C^{14} ybp	Radiocarbon date reference	Calibrated Date (Calendric Age cal BP)
ACAD109	VNMH H1997-21-2	5,325 ± 40	VERA-4523	6,107 ± 71
ACAD114	VNMH 3724	1,805 ± 35	VERA-4524	1,752 ± 47
ACAD115	VNMH H-74-13-1	2,264 ± 40	Erl-13095	2,262 ± 68
ACAD116	VNMH H1974-10-3	3,643 ± 41	Erl-13092	3,983 ± 70
ACAD117	VNMH H1964-9	9,985 ± 57	Erl-13094	11,468 ± 130
ACAD119	VNMH H86-178-1	1,985 ± 35	VERA-4522	1,942 ± 38
ACAD120	VNMH H90-7-3	3,321 ± 40	Erl-13093	3,554 ± 56
ACAD123	VNMH H1985-98-1	2,530 ± 20	CURL-10276	2,641 ± 82
ACAD123	VNMH H1985-98-1	2,485 ± 20	UCIAMS-56988	2,599 ± 81
ACAD127	VNMH H93-12	3,130 ± 35	VERA-4525	3,354 ± 38
ACAD130	VNMH	9,810 ± 70	GrN-22339	11,241 ± 51
ACAD338	NMS.Z.1962.63 (juvenile)	2,673 ± 54	BM-724	2,803 ± 40
ACAD339	NMS.Z.1962.63 (adult)	2,673 ± 54	BM-724	2,803 ± 40
ACAD3643	-	10,870 ± 80	(Döppes & Pacher, 2005)	12,841 ± 90
ACAD3644	-	6,615 ± 45	VERA-0836	7,512 ± 43
ACAD3645	-	11,872 ± 92	Erl-7851	3,784 ± 161
ACAD3646	-	11,900 ± 33	Gd-6182	13,794 ± 140
ACAD3647	-	9,700 ± 80	ETH-12785	11,036 ± 152
ACAD3648	-	9,700 ± 80	ETH-12785	11,036 ± 152
ACAD3649	-	3,275 ± 50	ETH-31380	3,510 ± 56
ACAD3650	-	4,135 ± 50	ETH-31320	4,686 ± 99
ACAD3651	-	2,935 ± 25	VERA-2193	3,099 ± 50

Independent replication results.

Independent ancient DNA extractions were performed by Ludovic Orlando at the following ancient DNA facility:
Paléogénétique et Evolution moléculaire
UMR 5242 du CNRS, INRA, UCB Lyon1
Institut de Génomique Fonctionnelle de Lyon
IFR128 BioSciences Lyon-Gerland
Ecole Normale Supérieure de Lyon
France.

The extraction method used was silica-based, as described (Orlando *et al.*, 2009) – essentially a modified version of previously described methods (Rohland & Hofreiter, 2007). Following extraction by this method, each ancient DNA extract was PCR amplified in three independent reactions, then cloned. A consensus of the clone sequences was created for each sample and compared to the original sequences obtained at ACAD through direct sequencing. In both cases the clone consensus sequence was identical to the original sequence obtained by direct sequencing.

The original sequence from direct sequencing of 1 PCR product (ACAD#3647):

```
TACATATTGTGCTTGGTCTTACATGAGGACTTACGTTCTGAAAGTTTGTTTCAGGTGTATAG
TCTGCAAGCATGTATTTCACTTAGTCCGGGAGCTTAGTCACCAGGCCTCGAGAAACCAGCAA
TCCTTGCGAGT
```

Consensus sequence of 22 clones from 3 independent PCR products of ACAD#3647 (Ludovic Orlando):

```
TACATATTGTGCTTGGTCTTACATGAGGACTTACGTTCTGAAAGTTTGTTTCAGGTGTATAG
TCTGCAAGCATGTATTTCACTTAGTCCGGGAGCTTAGTCACCAGGCCTCGAGAAACCAGCAA
TCCTTGCGAGT
```

The original sequence from direct sequencing of 1 PCR product (ACAD#3651):

```
TACATATTGTGCTTGGTTTTACATGAGGACTTACGTTCCGAAAGTTTGTTTCAGGTGTATAG
TCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTAGTCACCAGGCCTCGAGAAACCAGCAA
TCCTTGCGAGT
```

Consensus sequence of 21 clones from 3 independent PCR products of ACAD#3651 (Ludovic Orlando):

```
TACATATTGTGCTTGGTTTTACATGAGGACTTACGTTCCGAAAGTTTGTTTCAGGTGTATAG
TCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTAGTCACCAGGCCTCGAGAAACCAGCAA
TCCTTGCGAGT
```

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APPENDIX 4

Supporting information for Chapter 5

“Eurasian brown bear phylogeography”

Appendix Table 4.1 List of Eurasian brown bear samples extracted for analysis in Chapter 5. Successful DNA extractions from which at least 135-bp control region mtDNA could be amplified and sequenced are highlighted in green, while extractions from which it was not possible to amplify this fragment are highlighted in red.

Sample extraction number (ACAD#)	Sample details	Extraction method*	Amount extracted	Age (if known)	Age reference (if available)
95A	Bear Ekat 719/449 Rodnichnaya Phal 1.	Phen/Chlor 07/03/2008	0.23g	Pleistocene / Holocene	
96A	Bear humerus Irtysh River EKAT 915/869	Phen/Chlor 23/04/2008	0.98g	Pleistocene / Holocene	
97A	Bear Ekat 871/1 Ukku-Tash humerus.	Phen/Chlor 07/03/2008	0.68g	Pleistocene / Holocene	
98A	Bear Ekat 178/440 Ulna Nizhnaya Taida	Phen/Chlor 08/04/2008	0.94g	Pleistocene / Holocene	
99A	Bear Dentes. Russia, Shaitansky. 107/06.	Phen/Chlor 11/02/2008	0.67g	Pleistocene / Holocene	
100A	Bear Ekat 253/843. mp. Ignodiveskaya	Phen/Chlor 07/03/2008	0.69g	Pleistocene / Holocene	
101A	Bear Ekat 86/297 dentes. Lakseiskaya,	Phen/Chlor 07/03/2008	0.83g	Pleistocene / Holocene	
102A	Bear Dentes. Russia, Shaitansky. 621/9	Phen/Chlor 08/04/2008	0.26g	Pleistocene / Holocene	
131A	*Ursus arctos* MMZ S-66359 Pechoro-Ilych Nature Reserve. Female	Qiagen DNeasy 23/11/2007	0.2g	Historic 1953	
132A	*Ursus arctos* MMZ S113733 Evenkia Vanavara	Qiagen DNeasy 23/11/2007	0.2g	Historic 1974	

Sample extraction number (ACAD#)	Sample details	Extraction method*	Amount extracted	Age (if known)	Age reference (if available)
133A	*U. arctos* MMZ S66362 Pechoro-Ilych Nature Reserve	Qiagen DNeasy 28/01/2008	0.17g	Historic 1947	
134A	*U. arctos* MMZ S34946 Environs Poventsa, Belomor-Baltic canal. Male.	Qiagen DNeasy 10/03/2008	0.12g	Historic 1934	
135A	*U. arctos* MMZ S51937 Turkmenia, Ashkhabad - Zoo	Qiagen DNeasy 28/01/2008	0.11g	Historic 1935	
136A	*U. arctos* MMZ S14882 Northern Caucasus , Terskaya province (Daghestan, Stavropol, Chechnya). Male.	Qiagen DNeasy 10/03/2008	0.10g	Historic 1911	
137A	*U. arctos* MMZ S22359 Caucasian Biosphere Nature Reserve, Pshekshi Mt., Kisha river. Male.	Qiagen DNeasy 10/03/2008	0.11g	Historic 1933	
138A	*U. arctos* MMZ S34969 Krasnoyarsk region, Mana river, Taiga forests	Qiagen DNeasy 20/03/2008	0.11g	Historic 1911	
139A	*Ursus arctos* MMZ S60149 Kuril Isles	Qiagen DNeasy 05/12/2007	0.10g	Historic 1957	
140A	*Ursus arctos* MMZ S2073 Vologda province, Velsk district	Qiagen DNeasy 05/12/2007	0.13g	Historic 1910	

Sample extraction number (ACAD#)	Sample details	Extraction method*	Amount extracted	Age (if known)	Age reference (if available)
141A	*U. arctos* MMZ S1395 Kamchatka	Qiagen DNeasy 08/02/2008	0.19g	Historic 1946	
142A	*U. arctos* MMZ S34945 St. Selemgo, Kirovsk (Murmansk). Male.	Qiagen DNeasy 10/03/2008	0.16g	Historic 1935	
143A	*U. arctos* MMZ S60146 Kuril isles, Iturup island.	Qiagen DNeasy 10/03/2008	0.18g	Historic 1955	
144B	*Ursus arctos* MMZ S3007 Russian-Chinese Border, upper Kosogol lake (Khubsugol) NW Mongolia	Qiagen DNeasy 23/11/2007	0.16g	Historic 1903	
145A	*U. arctos* MMZ S6039 Okhotsk region, Burgalgana	Qiagen DNeasy 20/03/2008	0.13g	Historic 1929	
146A	*U. arctos* MMZ S1373 Kamchatka	Qiagen DNeasy 08/02/2008	0.13g	Historic 1946	
147A	*U. arctos* MMZ S3077 Vologda province	Qiagen DNeasy 20/03/2008	0.11g	Historic 1913	
148A	*U. arctos* MMZ S14933 Baikal lake, Barguzin Nature Reserve, Sosnovka. Male.	Qiagen DNeasy 10/03/2008	0.14g	Historic 1925	
149A	*Ursus arctos* MMZ S59248 Enisei River, Verkhniy Imbak River 16.VI.Male	Qiagen DNeasy 23/11/2007	0.15g	Historic 1956	

Sample extraction number (ACAD#)	Sample details	Extraction method*	Amount extracted	Age (if known)	Age reference (if available)
150A	*Ursus arctos* MMZ S66341 Pechoro-Ilych Naure Reserve .Female juv.	Qiagen DNeasy 05/12/2007	0.16g	Historic 1947	
151A	*U. arctos* MMZ S84887 Yakutia, Elgiyay settlement.	Qiagen DNeasy 10/03/2008	0.14g	Historic 1969	
152A	*U. arctos* MMZ S34934 Bolshoy Shantar island, Raduzhnyi cape	Qiagen DNeasy 08/02/2008	0.11g	Historic 1926	
153A	*U. arctos* MMZ S84888 Yakutia, Elgiyay settlement	Qiagen DNeasy 08/02/2008	0.17g	Historic 1969	
154A	*U. arctos* MMZ S 22367 Caucasian Biosphere Nature Reserve, Dudugule Mt., Kosaya meadow	Qiagen DNeasy 08/02/2008	0.2g	Historic 1936	
155A	*U. arctos* MMZ S34958 Ussury Region	Qiagen DNeasy 08/02/2008	0.29g	Historic 1932	
156A	*Ursus arctos* MMG S34972 Altai Mts	Qiagen DNeasy 23/11/2007	0.19g	Historic 1934	
158A	*U. arctos* MMZ S66352 Pechoro-Ilych Nature Reserve	Qiagen DNeasy 08/02/2008	0.35g	Historic 1954	
159A	*U. arctos* MMZ S580867 Sakhalin island	Qiagen DNeasy 28/01/2008	0.12g	Historic 1967	

Sample extraction number (ACAD#)	**Sample details**	**Extraction method***	**Amount extracted**	**Age (if known)**	**Age reference (if available)**
160A	*U. arctos* MMZ S34951 Krasnoyarsk region, Mana river, Taiga forests	Qiagen DNeasy 20/03/2008	0.11g	Historic 1911	
161A	*U. arctos* MMZ S14938 Northern Caucasus , Terskaya province (Daghestan, Stavropol, Chechnya). Male	Qiagen DNeasy 10/03/2008	0.22g	Historic 1912	
162A	*U. arctos* MMZ S20651 Baikal lake, Barguzin Nature Reserve, Sosnovka	Qiagen DNeasy 28/01/2008	0.20g	Historic 1934	
163A	*U. arctos* MMZ S14896 Northern Caucasus , Terskaya province (Daghestan, Stavropol, Chechnya).	Qiagen DNeasy 28/01/2008	0.07g	Historic 1913	
164A	*U. arctos* MMZ S34955 Krasnoyarsk region, Aginsk district	Qiagen DNeasy 28/01/2008	0.10g	Historic 1911	
165A	*U. arctos* MMZ S159009 Magadan province, upper stream of Anadyr river, Balaganchik river	Qiagen DNeasy 08/02/2008	0.34g	Historic 1989	
166A	*Ursus arctos* MMZ S14939 Nth Caucasus Chechnya.Female	Qiagen DNeasy 05/12/2007	0.12g	Historic 1913	
167A	*U. arctos* MMZ S1396 Kamchatka	Qiagen DNeasy 28/01/2008	0.03g	Historic 1946	

Sample extraction number (ACAD#)	**Sample details**	**Extraction method***	**Amount extracted**	**Age (if known)**	**Age reference (if available)**
168A	*Ursus arctos* MMZ S34928 Shantarsiy isles.female	Qiagen DNeasy 05/12/2007	0.13g	Historic 1925	
191A	*Ursus arctos* Cave "Pobeda" near UFA Region Urals, Institute of Zoology Almaty	Phen/Chlor 24/04/2007	0.30g	Upper Pleistocene	
195A	*Ursus arctos*? Natural Trap Cave (NTC) Kansas USA KU-42725	Phen/Chlor 24/04/2007	0.6g		
347A	*U. arctos* EKAT 250/13 Russia	Qiagen DNeasy 19/05/2008	0.12g	Pleistocene / Holocene	
350A	*U. arctos* EKAT 86/297 incisor Russia	Qiagen DNeasy 19/05/2008	0.03g	Pleistocene / Holocene	
360A	*U. arctos* EKAT 88/1419 molar Pl/Hol ? Russia	Qiagen DNeasy 19/05/2008	0.09g	Pleistocene / Holocene	
407A	*U. arctos* PIN Moscow 2002, 3657-153 permafrost. Indigirka area 4/4	Qiagen DNeasy 28/01/2008	N/A	30,660 +/-180 ybp	Oxford-14944
409A	*U. arctos* EKAT Phalange	Qiagen DNeasy 19/05/2008	0.20g	Early Holocene	
410A	*U. arctos* EKAT 543/63 Phalange	Qiagen DNeasy 19/05/2008	0.02g	Pleistocene / Holocene	

Sample extraction number (ACAD#)	Sample details	Extraction method*	Amount extracted	Age (if known)	Age reference (if available)
411A	*U. arctos* EKAT 821/9	Qiagen DNeasy 19/05/2008	0.04g	Pleistocene?	
1731A	*Ursus arctos*/Arctodus? Mineral Hill, Nevada, USA (B.Hockett, BLM)	Phen/Chlor 07/09/2006	0.05g	9960+/-50 ybp	SR-5293
1916A	Ursus Ekat IPAE 802/699, ACS 197, Zylische Sokola, Urals, phalange	Phen/Chlor 07/03/2008	0.5g	>50K?	
1917A	*Ursus arctos* EKAT 915/393 cranium Irtysh River kholotry ACS115	Phen/Chlor 23/04/2008	0.62g	Pleistocene / Holocene	
1918A	Ursus Ekat 705/514 tibia ACS185	Phen/Chlor 08/04/2008	0.6g	Pleistocene / Holocene	
1919A	*Ursus arctos* Ekat IPAE No #. SW Siberia, Jalutazousk.Urals ACS 114. femur	Phen/Chlor 07/03/2008	0.89g	Pleistocene / Holocene	
1920A	*U. arctos* EKAT 915/870 tibia Irtysh R Kholotry ACS116	Phen/Chlor 23/04/2008	0.35g	Pleistocene / Holocene	
1921A	Ursus Ekat 915/1015 ACS202	Phen/Chlor 08/04/2008	0.64g	Pleistocene / Holocene	
1922A	Ursus bone frag Zylische sokola ACS198	Phen/Chlor 23/04/2008	0.68g	>50K?	
1923A	*Ursus arctos* EKAT 915/1471 humerus Irtysh R Kholotry ACS118 1-2/4	Phen/Chlor 23/04/2008	0.81g	Pleistocene / Holocene	

Sample extraction number (ACAD#)	Sample details	Extraction method*	Amount extracted	Age (if known)	Age reference (if available)
1924A	*U. arctos* Urals, Russia Ekaterinberg IPAE 705/517	Phen/Chlor 06/12/2006	0.42g	Pleistocene	
1925A	Ursus. Usolcevskaya Cave. Limb frag ACS196. >50K Urals Russia. IPAE	Phen/Chlor 11/02/2008	0.39g	>50K?	
1927A	*Ursus arctos* Ekat 915/869 ACS117, humerus Irtysh R. Urals.	Phen/Chlor 08/04/2008	N/A	43,600+/-1500	UCIAMS 56989
1928A	*Ursus arctos* Ekat IPAE 915/1185 Irtysh River, Kholotny, Urals, ACS 119, Ulna	Phen/Chlor 07/03/2008	0.66g	Pleistocene / Holocene	
1929A	Ursus EKAT 915/1014 humerus ACS200	Phen/Chlor 23/04/2008	0.53g	Pleistocene / Holocene	
1930A	Ursus Usokeushaya cave ACS 195	Phen/Chlor 08/04/2008	0.57g	Pleistocene / Holocene	
1931A	Ursus EKAT ACS193 Usokevskriya cave phalange(small)	Phen/Chlor 23/04/2008	0.42g	Pleistocene / Holocene	
1932A	Ursus ACS 191, 178/316, ulna	Phen/Chlor 08/04/2008	0.42g	Pleistocene / Holocene	
1933A	*Ursus arctos* Tain Cave Ekat 07-10-04 ACS 135 3/4. Urals Russia IPAE 705/517	Phen/Chlor 11/02/2008	0.56g	>46,700	UCIAMS 56990
1934A	Ursus EKAT ACS194 Usokevskriya cave phalange	Phen/Chlor 23/04/2008	0.32g	Pleistocene / Holocene	
1935A	Ursus bone frag Zylische sokola >50K	Phen/Chlor 08/04/2008	0.33g	>50K	Alan Cooper's notes

Sample extraction number (ACAD#)	**Sample details**	**Extraction method***	**Amount extracted**	**Age (if known)**	**Age reference (if available)**
1936A	Ursus sp? Ekat IPAE 178/604 Nizhnyaya Tavda, Urals	Phen/Chlor 07/03/2008	1.15g	Pleistocene / Holocene	
1937A	*U. arctos* Denisova Cave, Altai Mts	Phen/Chlor 15/02/2007	0.28g	Pleistocene / Holocene	
1938A	*U. arctos* Denisova Cave, Altai Mts	Phen/Chlor 15/02/2007	0.45g	Pleistocene / Holocene	
1939A	*U. arctos* Denisova Cave, Altai Mts	Phen/Chlor 15/02/2007	0.71g	>46,000	UCIAMS 56997
1940A	*U. arctos* Denisova Cave, Altai Mts	Phen/Chlor 15/02/2007	0.21g	Pleistocene	
1941A	*U. arctos* Robbers Cave/Smuggler's Cave, Altai Mts	Phen/Chlor 15/02/2007	0.71g	16,450+/-60	UCIAMS 56991
1942A	*U. arctos* Robbers Cave/Smuggler's Cave, Altai Mts	Phen/Chlor 15/02/2007	0.30g	16,440+/-60	UCIAMS 56992
1943A	*U. arctos* Robbers Cave/Smuggler's Cave, Altai Mts	Phen/Chlor 06/12/2006	0.40g	16,380+/-60	UCIAMS 56993
1944A	*U. arctos* Robbers Cave/Smuggler's Cave, Altai Mts	Phen/Chlor 06/12/2006	0.35g	13,925+/-40	UCIAMS 56994
1945A	*U. arctos* Robbers Cave/Smugglers Cave, Altai Mts 1977 a 15-60cm	Phen/Chlor 06/12/2006	0.31g	15,370+/-100	UCIAMS 56995
1946A	*U. arctos* Robbers Cave/Smuggler's Cave, Altai Mts	Phen/Chlor 06/12/2006	0.66g	13,830+/-40	UCIAMS 56996

Sample extraction number (ACAD#)	Sample details	Extraction method*	Amount extracted	Age (if known)	Age reference (if available)
1948A	*U. arctos* Denisova Cave, Altai Mts	Phen/Chlor 15/02/2007, Silica 19/02/07	0.25g	Pleistocene	
1949A	*U. arctos* Denisova Cave, Altai Mts	Phen/Chlor 15/02/2007, Silica 19/02/07	0.46g	Pleistocene	
4098A	Mezmaiskaya Cave, Layer 2A, Caucasus Mountains, Russia	Kefei Chen extract 2008	N/A	42,900 +/- 1600	CURL-10273**
5390A	*Ursus arctos* Kamchatka VNMH40604	Qiagen DNeasy 19/06/2008	0.15g	Historic 1898-1883	
5391A	*Ursus arctos* Kamchatka VNMH40648	Qiagen DNeasy 19/06/2008	0.25g	Historic 1898-1883	
5392A	*Ursus arctos* Kamchatka VNMH40627	Qiagen DNeasy 19/06/2008	0.13g	Historic 1898-1883	
5393A	*Ursus arctos* Kamchatka VNMH40601	Qiagen DNeasy 19/06/2008	0.27g	Historic 1898-1883	
5394A	*Ursus arctos* Kamchatka VNMH40626	Qiagen DNeasy 19/06/2008	0.19g	Historic 1898-1883	

Sample extraction number (ACAD#)	Sample details	Extraction method*	Amount extracted	Age (if known)	Age reference (if available)
5395A	*Ursus arctos* Kamchatka VNMH40610	Qiagen DNeasy 19/06/2008	0.15g	Historic 1898-1883	
5396A	*Ursus arctos* Kamchatka VNMH40667	Qiagen DNeasy 19/06/2008	0.13g	Historic 1898-1883	
5397A	*Ursus arctos* Kamchatka VNMH40642	Qiagen DNeasy 19/06/2008	0.16g	Historic 1898-1883	
5398A	*Ursus arctos* Kamchatka VNMH40650	Qiagen DNeasy 19/06/2008	0.19g	Historic 1898-1883	
5399A	*Ursus arctos* Kamchatka VNMH40628	Qiagen DNeasy 19/06/2008	0.25g	Historic 1898-1883	
5634A	*Ursus arctos*. Yukon. Canada. Uni of Alaska Museum. UAM70670, ABTC 103077	Qiagen DNeasy 2009	N/A	Modern	
6728A	*Ursus arctos* (Syrian brown bear - Eli - Adelaide Zoo) Frozen muscle tissue	Qiagen DNeasy (Modern tissue) 10/02/2009	N/A	Modern	

* DNA Extraction method: All samples were extracted by either a phenol/chloroform-based method (Phen/Chlor) as described in Chapter 2.3.2 or by a modified Qiagen DNeasy silica extraction kit as described in Chapter 5.2.2. The date indicates when the sample was extracted. All extractions were performed by S.Bray with the exception of one sample: ACAD#4098 which was extracted by Dr. Kefei Chen (ACAD postdoc) but all subsequent work on this extract including PCR amplifications was performed by S.Bray.

*****Radiocarbon dating of ACAD#4098 (Mezmaiskaya Cave) – CURL-10273***

Powdered bone from the Mezmaiskaya Cave (ACAD#4098) specimen was prepared for Accelerator Mass Spectrometry by amino acid purification using ion exchange chromatography (Stafford Jr *et al.*, 1988) at the University of Colorado INSTARR Laboratory for AMS Radiocarbon Preparation and Research. This sample was included in a batch of 26 samples prepped in July of 2008. Of the four bone standards included with these samples, one bone standard was ~650 radiocarbon years too old (INSTARR date 10,200 vs. expected date 9,500 radiocarbon years before present), which was outside of statistical bounds. Due to a suspected dead carbon contaminant in the INSTARR procedure, twenty-two of the twenty six samples had enough material remaining to repeat, and were subsequently prepared from the collagen stage using the ultrafiltration technique (Brown *et al.*, 1988) at the University of California Irvine (UCI) Keck CCAMS facility and re-dated. One of the twenty-two samples was different by ~625 years (INSTARR date 10255 ± 30 vs. UCI 9630 ± 30), which confirmed a potential contaminant in the INSTARR procedure, which may result in miss-dating bone samples <15,000 radiocarbon years old on the scale of hundreds of years. The Mezmaiskaya Cave (ACAD#4098) specimen could not be re-dated due to a lack of remaining bone material. Therefore, we were advised to treat the date of 42,900 ± 1600 radiocarbon years for Mezmaiskaya Cave (ACAD#4098) with caution, however the date is much older than 15,000 radiocarbon years, and the sample originates from a stratigraphic layer (2A) from which other bones have been dated previously with a mean age of 40,800 ka (Skinner *et al.*, 2005), which is consistent with the radiocarbon date we obtained.

Independent replication results.

Independent ancient DNA extractions were performed by Ludovic Orlando at the following ancient DNA facility:
Paléogénétique et Evolution moléculaire
UMR 5242 du CNRS, INRA, UCB Lyon1
Institut de Génomique Fonctionnelle de Lyon
IFR128 BioSciences Lyon-Gerland
Ecole Normale Supérieure de Lyon
France.

The extraction method used was silica-based, as described (Orlando *et al.*, 2009) – essentially a modified version of previously described methods (Rohland & Hofreiter, 2007). Following extraction by this method, each ancient DNA extract was PCR amplified in two-three independent reactions, then cloned. A consensus of the clone sequences was created for each sample and compared to the original sequences obtained through direct sequencing at ACAD. In both cases the clone consensus sequence was identical to the original sequence. Only two samples (ACAD# 1939 and ACAD#1946) showed any variation between the original sequence and the consensus sequence, and these discrepancies are highlighted below in red. As the clones were from a

minimum of 2 independent PCR amplifications, in the case of discrepancies the „best of three" rule (Hofreiter *et al.*, 2001) was applied and the clone consensus sequence was used for the analyses.

The original sequence from direct sequencing of 1 PCR product (ACAD#1927):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGTGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTAATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

Consensus sequence of 15 clones from 2 independent PCR products of ACAD#1927 (Ludovic Orlando):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGTGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTAATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

The original sequence from direct sequencing of 1 PCR product (ACAD#1933):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGATCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

Consensus sequence of 22 clones from 3 independent PCR products of ACAD#1933 (Ludovic Orlando):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGATCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

The original sequence from direct sequencing of 1 PCR product (ACAD#1939):
TACATATTACGCTTGGTCTTACATAAGGACTTACATTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTAATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

Consensus sequence of 20 clones from 2 independent PCR products of ACAD#1939 (Ludovic Orlando):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTAATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

The original sequence from direct sequencing of 1 PCR product (ACAD#1941):

TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

Consensus sequence of 21 clones from 3 independent PCR products of ACAD#1941 (Ludovic Orlando):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

The original sequence from direct sequencing of 1 PCR product (ACAD#1942):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

Consensus sequence of 14 clones from 2 independent PCR products of ACAD#1942 (Ludovic Orlando):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

The original sequence from direct sequencing of 1 PCR product (ACAD#1943):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

Consensus sequence of 26 clones from 3 independent PCR products of ACAD#1943 (Ludovic Orlando):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

The original sequence from direct sequencing of 1 PCR product (ACAD#1944):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTAATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

Consensus sequence of 20 clones from 3 independent PCR products of ACAD#1944 (Ludovic Orlando):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTAATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

The original sequence from direct sequencing of 1 PCR product (ACAD#1945):
TACATATTACGCTTGGTCTTACATAAGGACCTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGAGAGCTTAATCAC
CGGGCCTCGAGAAACCAGCAACCCTTGCGAGT

Consensus sequence of 22 clones from 3 independent PCR products of ACAD#1945 (Ludovic Orlando):
TACATATTACGCTTGGTCTTACATAAGGACCTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGAGAGCTTAATCAC
CGGGCCTCGAGAAACCAGCAACCCTTGCGAGT

The original sequence from direct sequencing of 1 PCR product (ACAD#1946):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTAATCAC
CAGGCCTCGAGAAACCAGCAACCCTTGCGAGT

Consensus sequence of 17 clones from 2 independent PCR products of ACAD#1946 (Ludovic Orlando):
TACATATTACGCTTGGTCTTACATAAGGACTTACGTTCCGAAAGCTTATTTCAGGCGTATGGTCTGTAAGCATGTATTTCACTTAGTCCGGGAGCTTAATCAC
CAGGCCTCGAGAAACCAGCAACTTTTGCGAGT

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APPENDIX 5

Supporting information for Chapter 6

"Ancient DNA reveals different phylogeographic histories for the extinct New World Giant Short-faced bear (*Arctodus simus*) and the brown bear (*Ursus arctos*)."

Appendix Table 5.1 List of samples extracted for analysis in Chapter 6. Successful DNA extractions from which at least 135-bp control region mtDNA could be amplified and sequenced are highlighted in green, while extractions from which it was not possible to amplify this fragment are highlighted in red.

DNA extract no.*	Geographic origin of specimen	Museum/ Collection ID †	Morphology ID	Sample type	C^{14} date (ybp) ‡	Genetic ID	ACAD DNA extraction date
ACAD 183A	CANADA – Sixty Mile	Unknown	*Ursus arctos*? *Arctodus*?	r. ulna	N/A	N/A	07/09/2006 SB
ACAD 330A & AC688	CANADA – Dawson area, Hester Creek Loc.57	CMN 49874	*Arctodus*	ulna	26,720 ± 270 (OxA-9259)	*A. simus*	02/02/2007 SB
ACAD 341A	CANADA? Sheriden Cave	CMN VP3866	*Ursus* sp.	l. tibia juv	N/A	N/A	02/02/2007 SB
ACAD 344A	CANADA – Hester Creek	76.4 (JS)	*Arctodus simus*	r. radius	N/A	*A. simus*	02/02/2007 SB
ACAD 346A	CANADA – Edmonton, Alberta, consolidated pit #48	P96.2.38	*Ursus*/Ursidae sp.	r. radius	N/A	*A. simus*	02/02/2007 SB
ACAD 420A	KANSAS, USA – Natural Trap Cave	KS30	*Arctodus*	femur	N/A	N/A	13/10/2006 SB
ACAD 421A	ALASKA - Goldstream	Unknown	*Arctodus*	metatarsal	N/A	N/A	10/08/2007 SB
ACAD 422A	CANADA - Old Crow loc.67	CMN 27831	*Arctodus*	r. humerus	N/A	N/A	13/10/2006 SB
ACAD 424A & IB253	CANADA – Dawson area, Eldorado Creek Loc.45	CMN 37957	*Arctodus simus*	r. calcaneum	22,417 ± 452 (Wk20235)	*A. simus*	26/07/2006 SB
ACAD 425A	ALASKA – Cleary Creek	A147-30493	*Arctodus*	ramus	N/A	N/A	16/08/2007 SB
ACAD 428A	CANADA – Gold Run	CMN 34556	*Arctodus*	l. femur	N/A	*A. simus*	16/08/2007 SB
ACAD 429A	ALASKA – Cripple Creek	A225-2625	*Arctodus*	ulna	N/A	N/A	16/08/2007 SB

DNA extract no.*	Geographic origin of specimen	Museum/ Collection ID †	Morphology ID	Sample type	C^{14} date (ybp) ‡	Genetic ID	ACAD DNA extraction date
ACAD 430A	ALASKA - North Slope	Paul Matheus T99-016	*Arctodus*	phalange	N/A	N/A	26/07/2006 SB
ACAD 434A	CANADA – Yukon, Ophis Creek	YG-24.1	*Arctodus simus*	skull	20,210 ± 110 (Harington, 2001)	*A. simus*	26/07/2006 SB
ACAD 435A	ALASKA – Ester Creek	FAM 127693	*Arctodus*	ulna (pathology)	N/A	N/A	13/10/2006 SB
ACAD 436A	ALASKA – Goldstream	AMNH A-1828	*Arctodus*	ulna (limited pathology)	N/A	*A. simus*	10/08/2007 SB
ACAD 437A	ALASKA – Goldstream	#850 575 UCLA	*Arctodus simus*	l. radius	N/A	*A. simus*	26/07/2006 SB
ACAD 438A & IB187	CANADA – Sixty Mile	CMN 42388	*Arctodus*	l. metacarpal	44,240 ± 930 (Harington, 2001)	*A. simus*	07/09/2006 SB
ACAD 439A	CANADA – Old Crow loc.22	CMN 26864	*Arctodus*	l. radius	N/A	N/A	13/10/2006 SB
ACAD 440A	CANADA - Cripple Creek	Unknown	*Arctodus*	radius	N/A	N/A	07/09/2006 SB
ACAD 441A & IB202	ALASKA – Ester Creek	FAM 95656	*Arctodus*	humerus	N/A	*A. simus*	10/08/2007 SB
ACAD 442A	CANADA – Cripple Creek	A203-2808	*Arctodus*	humerus	N/A	N/A	10/08/2007 SB
ACAD 443A	ALASKA – No.2 G-strip area	AMNH A-82-1039	*Arctodus*	ramus	N/A	*A. simus*	16/08/2007 SB
ACAD 444A	ALASKA – Cripple Creek	A340-2950	*Arctodus*	ulna	N/A	N/A	16/08/2007 SB
ACAD 445A	KANSAS, USA – Kaw River bank	KU81230	*Arctodus*	vertebrae	N/A	N/A	10/08/2007 SB
ACAD 447A	CANADA – Old Crow loc.23	CMN 43461	*Arctodus*	l. humerus	N/A	N/A	13/10/2006 SB
ACAD	CANADA - Hunker	CMN 37577	*Arctodus*	r. humerus	N/A	N/A	26/07/2006 SB

DNA extract no.*	Geographic origin of specimen	Museum/ Collection ID †	Morphology ID	Sample type	C^{14} date (ybp) ‡	Genetic ID	ACAD DNA extraction date
448A	Creek, Dawson area		*simus*				
ACAD 450A	ALASKA	AMNH „ALASKA Bx35"	*Arctodus*	humerus	25,264 ± 650 (Wk20236)	*A. simus*	26/07/2006 SB
ACAD 1732A	OHIO, USA – Sheriden Pit	Unknown	*Arctodus simus*	fibula fragment	N/A	N/A	07/09/2006 SB
ACAD 1733A	OHIO, USA – Sheriden Pit	Unknown	Carnivora	incisor	N/A	N/A	07/09/2006 SB
ACAD 1734A	OHIO, USA – Sheriden Pit	CMNHS VP8289	Ursid?Black bear?*Arctodus* ?	phalange	11,619 ± 40 #	*A. simus*	07/09/2006 SB
ACAD 1735A	OHIO, USA – Sheriden Pit	Unknown	Ursidae?	incisor	N/A	N/A	07/09/2006 SB
ACAD 1953A	CANADA? Irish Gulch	YT03/48	Carnivore	tibia	N/A	N/A	02/02/2007 SB
BS3	ALASKA – Eva Creek Mine	PM-97-001-100	*Arctodus*	femur	N/A	*A. simus*	N/A
BS71	CANADA – Dawson, Hunker Creek, 80-Pup	CMN 44566	*Arctodus*	unknown	N/A	*A. simus*	N/A
BS72	CANADA – Dawson area	CMN 36236	*Arctodus*	r. tibia	N/A	*A. simus*	N/A
BS73	CANADA – Dawson, Hunker Creek	CMN 42335	*Arctodus*	unknown	N/A	*A. simus*	N/A
BS74	ALASKA – Lillian Creek	UAF/Paleo V-55-524	*Arctodus*	humerus	N/A	*A. simus*	N/A
IB191	ALASKA – Dawson Cut	AMNH A-676-5625	*Arctodus*	fibula	N/A	*A. simus*	N/A
IB195	CANADA – Cripple Creek	AMNH A-217-2297	*Arctodus*	tibia	N/A	*A. simus*	N/A
IB255	CANADA – Dawson	CMN 37577	unknown	unknown	N/A	*A. simus*	N/A
JW131	CANADA – Hester Creek	YT03/288 Cat. No. 129.1 (JS)	*Arctodus*	r. ulna	N/A	*A. simus*	N/A

† Museum/Collection abbreviations as follows: Canadian Museum of Nature (CMN), American Museum of Natural History (AMNH), Frick Collection, American Museum (FAM), University of Alaska, Fairbanks (UAF/Paleo), Yukon Territories (YT) field collection, Yukon Government collections at Whitehorse (YG), University of California, Los Angeles (UCLA) and Cincinnati Museum of Natural History & Science (CMNHS). Other samples were personally collected by Paul E. Matheus (PM) or John Storer (JS).
‡ Radiocarbon date followed in parentheses by laboratory reference number (if dated in this study) or source from which the date was obtained.
# Personal communication: Greg McDonald 2007. Weighted average of CAMS-12837, 12839 and 12845.
* Extractions performed by Sarah Bray (ACAD), Ian Barnes (IB), Beth Shapiro (BS), Alan Cooper (AC) or Jacobo Weinstock (JW). For samples that were independently extracted in separate laboratories, both extraction references are given.

Appendix Table 5.2 List of fragments amplified from each specimen. Success=amplification attempted and sequenced successfully, Failure=amplification attempted but no authentic DNA sequence was obtained, N/A=amplification not performed. Initials indicate who performed the amplification: Sarah Bray (SB), Ian Barnes (IB), Beth Shapiro, Jacobo Weinstock (JW) or Alan Cooper (AC).

Sample number	**135-bp CR L16164/H16299**	**115-bp CR L16455/H16574**	**119-bp CR L16852/H16968**	**137-bp ATP8 L8951/H9089**
1	Success (SB)	Failure (SB)	Success (SB)	Success (SB)
2	Success (SB)	Success (SB)	Success (SB)	N/A
3	Success (SB+IB)	Success (SB)	Success (SB)	Success (SB)
4	Success (SB)	Failure (SB)	Success (SB)	Success (SB)
5	Success (SB)	Success (SB)	Success (SB)	Success (SB)
6	Success (SB)	Success (SB)	Success (SB)	Success (SB)
7	Success (SB)	Success (SB)	Failure (SB)	Success (SB)
8	Success (SB)	Success (SB)	Success (SB)	N/A
9	Success (SB+AC)	Success (SB)	Success (SB)	N/A
10	Success (SB)	Success (SB)	Success (SB)	N/A
11	Success (SB)	Success (SB)	Success (SB)	N/A
12	Success (SB)	Success (SB)	Success (SB)	N/A
13	Success (SB)	Success (SB)	Failure (SB)	N/A
14	Success (SB)	Failure (SB)	Failure (SB)	N/A
15	Success (JW)	N/A	N/A	N/A
16	Success (IB)	N/A	N/A	N/A
17	Success (IB)	N/A	N/A	N/A
18	Success (BS)	N/A	N/A	N/A
19	Success (BS)	N/A	N/A	N/A
20	Success (BS)	N/A	N/A	N/A
21	Success (BS)	N/A	N/A	N/A
22	Success (BS)	N/A	N/A	N/A
23	Success (SB)	N/A	N/A	N/A

NB. Fragment length does not include primers.

Appendix Table 5.3 List of primers used in Chapter 6.

Primer combination	**Target**	**Forward primer (5' – 3')**	**Reverse primer (5' – 3')**	**Ta (ºC)**	**Product length (bp) †**
L16164/H16299* (A128/A129)	Control Region	GCCCCATGCATATAAGCATG	GGAGCGAGAAGAGGTACACG T	55	135
L16455/H16574# (A373/A372)	Control Region + tRNA Pro/Thr	GTGAAGAGTCTTTGTAG	GGAATAATGGTATCAGGGAA	55	119
L16852/H16968# (A198/A200)	Control Region	CCTCGAGAAACCAGCAACCC TTG	CCCATTTGAAGGGTTAGTAG	55	115
L8951/H9089# (A367/A368)	ATP8	AACTAGACACATCAACATGG	GAGGCAAATAAATTTTCGTT	55	137

* Previously published in Hanni *et al.* 1994. PNAS vol. 91 p.12336-12340.
# Primers designed by Sarah Bray as part of this study.
† Fragment length does not include primer sequence.

Appendix Table 5.4 List of 27 previously published homologous Pleistocene Beringian brown bear control region sequences (135bp) used for genetic diversity calculations displayed in Chapter 6 Table 6.2. Only *Ursus arctos* samples with Pleistocene radiocarbon dates were used in these analyses to ensure they were contemporaneous to the Pleistocene *Arctodus simus* samples in this study.

GenBank Accession	Museum/Collection ID	C^{14} date (ybp)	mtDNA Clade
AY082810	FAM 30771	20,080 ± 160	3b
AY082812	FAM 95641	11,940 ± 100	3b
AY082813	FAM 95599	14,310 ± 100	3b
AY082814	FAM 95659	13,415 ± 70	3b
AY082815	FAM 95642	14,150 ± 90	3b
AY082816	FAM 95612	10,015 ± 62	3b
AY082819	AMNH 95665	13,760 ± 50	3b
AY082820	FAM 95632	14,810 ± 80	3b
AY082821	AMNH 95630	14,980 ± 60	3b
AY082822	FAM 95628	12,310 ± 65	3b
AY082823	FAM 95670	15,830 ± 100	3b
AY082824	FAM 95598	12,320 ± 90	3b
AY082825	AMNH 30422	19,027 ± 132	3b
AY082827	FAM 95595	12,441 ± 75	3b
AY082828	AMNH 95679	15,370 ± 60	3b
AY082829	FAM 95671	20,820 ± 120	3b
AY082830	FAM 95609	50,800 ± 1900	3c
AY082831	FAM 95640	>53,900	3c
AY082832	FAM 95601	36,137 ± 783	3c
AY082833	FAM 95666	47,100 ± 3100	3c
AY082834	FAM 95639	>56,900	3c
AY082835	FAM 95681	>59,000	3c
AY082836	FAM 95634	41,787 ± 212	3c
AY082837	FAM 95664	42,600 ± 850	3c
AY082839	CMN 42381	35,970 ± 660	4
AY082841	PM Collected	48,164 ± 3224	2c
AY082842	CMN 38279	36,500 ± 1150	2c

Cloning results.

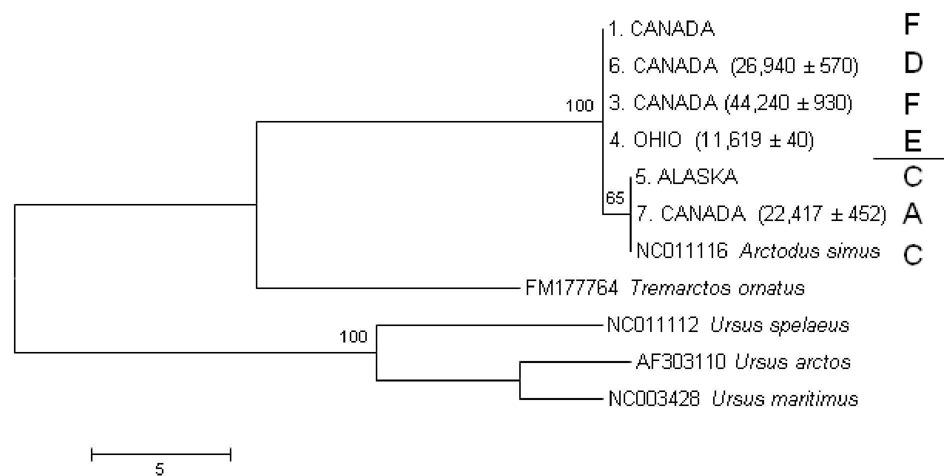
Sample 15: JW 131 Hester Creek was extracted, 135-bp CR fragment PCR amplified, cloned and sequenced by Jacobo Weinstock (unpublished data; see below). From fourteen clones, five are identical and were taken as the consensus sequence. One clone had a missing base, other deviations from the identical clones were A-G, T-C or ambiguous bases and were interpreted as DNA damage and therefore excluded from analyses.

1aJW_131_H TACATGTTGT GCTTGGTTTT ACATGAGGAC ANATTCTTCA AAAGCTTGTT
1bJW_131_H TACATATTGT GCTTAATTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
1cJW_131_H TATATGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
1dJW_131_H TACATGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
1fJW_131_H TACATGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
1hJW_131_H TACATGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
2bJW_131_H TACATGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
2cJW_131_H TAYATGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
2dJW_131_H TACATGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
2fJW_131_H TACATGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
2gJW_131_H TACATGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
2aJW_131_H TACATGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
1eJW_131_H TACAGGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTATT
1gJW_131_H TACATGTTGT GCTTAGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTATT

1aJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT
1bJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT
1cJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT
1dJW_131_H TTGAAGGTAT GGTCTGTA-G CATGTATTTC ACTTAGTCCGGGAGCTTAGT
1fJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT
1hJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT
2bJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT
2cJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT
2dJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT
2fJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT
2gJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT

2aJW_131_H TTGAAGGTAT GGTCTGTGAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT
1eJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAGT
1gJW_131_H TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAATCCG GGAGCTTAGT

1aJW_131_H CACCAGGCCT CGAGAAACCA GCAACCCTTG CGAGT
1bJW_131_H CACCAGGCCT CGAGAAACCA GCAGCCCTTG CAAGT
1cJW_131_H CACCAGGCCT CGAGAAACCA GCAACCCTTG CAAGT
1dJW_131_H CACCAGGCCT TGAGAAACCA GCAACCCTTG CGAGT
1fJW_131_H CACCAGGCCT CGAGAAACCA GCAACCCTTG CGAGT
1hJW_131_H CACCAGGCCT CGAGAAACCA GCAACCCTTG CGAGT
2bJW_131_H CACCAGGCCT CGAGAAACCA GCAACCCTTG CGAGT
2cJW_131_H CACCAGGCCT CGAGAAACCA GCAACCCTTG CGAGT
2dJW_131_H CACCAGGCCT CGAGAAACCA GCAACCCTTG CGAGT
2fJW_131_H CACCAGGCCT CGAGAAACCA GCAACCCTCG CGAGT
2gJW_131_H CACCAGGCCT CGAGAAACCA GCAACCCTTG CGAGT
2aJW_131_H CACCAGGCCT CAAGAAACCA GCAACCCTTG CGAGT
1eJW_131_H CACCAGGCCT CGAGAAACCA GCAACCCTTG CGAGT
1gJW_131_H CACCAGGCCT CGAGAAACCA GCAACCCTTG CGGGT



Appendix Figure 5.1 Maximum parsimony analysis of 137 bp ATP8 sequences from *Arctodus simus*. Sequences from this study (see Table 6.1 for details) are labelled with sample number, geographic origin and radiocarbon date (if available). Sequences obtained from GenBank are labelled by GenBank accession number and species name. A single base substitution (C-T at position 107/137 bp sequence) separates *Arctodus* samples 1,3,4,6, from the other *Arctodus* samples. Numbers above branches are bootstrap support values (>60%) based on 1000 replicates. Letters A, C, D, E and F correspond to the control region haplotypes for each sample.

APPENDIX 6

Supporting information for Chapter 7

“Evolutionary relationships of the Tremarctine bears”

Independent replication results.

Independent replication results of the Cueva del Puma *Arctotherium tarijense* specimen (ACAD#3599):

The directly sequenced 135 bp CR product (ACAD3599) is identical to 12/16 clones from the independent extraction, amplification, cloning and sequencing performed by Jaco Weinstock in Oxford (as described in Chapter 7.2.5 and 7.3).

```
               ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50

ACAD3599       TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
3aJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
3bJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
3cJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
3eJW_257_C     TACATACTAT GCTTGGTTTT ACATAAAGAC ATATAATCCA AAAGTTCGTC
3gJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
3hJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
4aJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
4bJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
4cJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
4dJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
4eJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
4fJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
4gJW_257_C     TACATACTAT GCTTGGTTTT ACATAAGGAC ATATAATCCA AAAGTTCGTC
3dJW_257_C     TACATGTTGT GCTTGGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTGTT
3fJW_257_C     TACATGTTGT GCTTAGTTTT ACATGAGGAC ATACTCTTCA AAAGCTTATT

               ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
ACAD3599       TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
3aJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
3bJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
3cJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
3eJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
3gJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
3hJW_257_C     TTGAAGACAT ATTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
4aJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
4bJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
4cJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
4dJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
4eJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
4fJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
4gJW_257_C     TTGAAGGCAT AGTCTGTAAG CATGCACTTC ACTTAGACCG AGAGCTTAAT
3dJW_257_C     TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTTAGTCCG GGAGCTTAAT
3fJW_257_C     TTGAAGGTAT GGTCTGTAAG CATGTATTTC ACTCAGTCCG GGAGCTTAGT
```

```
               ....|....| ....|....| ....|....| ....|
                       110        120        130
ACAD3599       CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
3aJW_257_C     CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
3bJW_257_C     CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
3cJW_257_C     CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
3eJW_257_C     CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
3gJW_257_C     CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
3hJW_257_C     CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
4aJW_257_C     CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
4bJW_257_C     CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
4cJW_257_C     CACCAAGCCT CGAGAAGCCA GCAATCCTTG CGAGT
4dJW_257_C     CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
4eJW_257_C     CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
4fJW_257_C     CACCAAGCCT CGAGAAACCA GCAACCCTTG CGAGT
4gJW_257_C     CATTAAGCCC CGAGAAACCA GCAACCCTTG CGAGT
3dJW_257_C     CACCAGGCCT CGAGAAACTA GCAACCCTTG CGAGT
3fJW_257_C     CACCAGGCCT CGAGAAACCA GCAACCCTTG CGAGT
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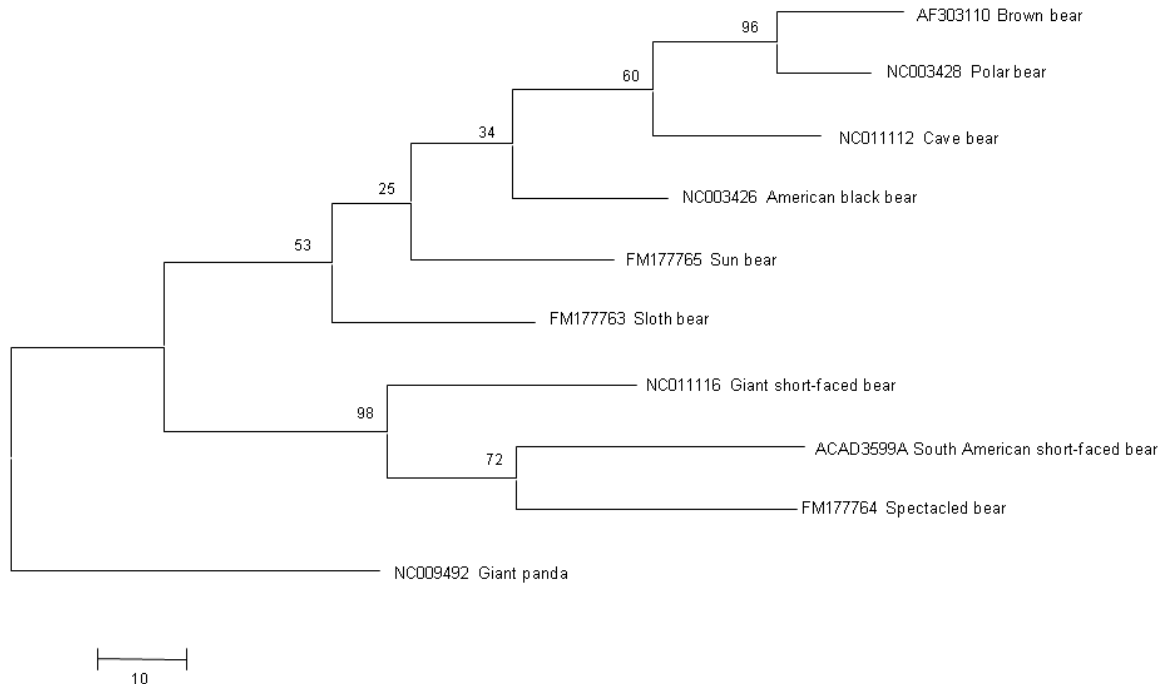

Appendix Figure 6.1 Maximum Parsimony analysis. Results of the maximum parsimony analysis based on 694 bp mtDNA performed as described in Chapter 7.2.6. Numbers above nodes are bootstrap values. The overall topology is the same as that obtained using whole mtDNA genomes (Yu *et al.*, 2007; Krause *et al.*, 2008), although many of the bootstrap values are extremely low.

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APPENDIX 7

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Mitochondrial genomes reveal an explosive radiation of extinct and extant bears near the Miocene-Pliocene boundary

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Abstract

Background: Despite being one of the most studied families within the Carnivora, the phylogenetic relationships among the members of the bear family (Ursidae) have long remained unclear. Widely divergent topologies have been suggested based on various data sets and methods.

Results: We present a fully resolved phylogeny for ursids based on ten complete mitochondrial genome sequences from all eight living and two recently extinct bear species, the European cave bear (*Ursus spelaeus*) and the American giant short-faced bear (*Arctodus simus*). The mitogenomic data yield a well-resolved topology for ursids, with the sloth bear at the basal position within the genus *Ursus*. The sun bear is the sister taxon to both the American and Asian black bears, and this clade is the sister clade of cave bear, brown bear and polar bear confirming a recent study on bear mitochondrial genomes.

Conclusion: Sequences from extinct bears represent the third and fourth Pleistocene species for which complete mitochondrial genomes have been sequenced. Moreover, the cave bear specimen demonstrates that mitogenomic studies can be applied to Pleistocene fossils that have not been preserved in permafrost, and therefore have a broad application within ancient DNA research. Molecular dating of the mtDNA divergence times suggests a rapid radiation of bears in both the Old and New Worlds around 5 million years ago, at the Miocene-Pliocene boundary. This coincides with major global changes, such as the Messinian crisis and the first opening of the Bering Strait, and suggests a global influence of such events on species radiations.

Background

The bear family (Ursidae) is one of the most studied families within the order Carnivora. Members of this family are present on most continents and occupy a wide range of ecological niches from the arctic ice shelves to tropical rainforests (see Additional File 1, Figure S1a). Despite numerous morphological and molecular studies on the phylogenetic relationship among Ursidae members, no consensus exists with regard to either their phylogeny or their taxonomic nomenclature (Table 1). Most analyses have concentrated on the eight extant bear species: brown bear, American black bear, Asian black bear, polar bear, sun bear, sloth bear, spectacled bear and giant panda (for species names see Table 1). Molecular studies based on mitochondrial and nuclear DNA from modern bears have recently provided convincing evidence about several of the controversial relationships among the bears, such as the basal positions of the giant panda and the spectacled bear in the bear tree [1-4] and the position of the polar bear within the brown bear tree making the later paraphyletic [5,6]. However, molecular studies for a long time failed to conclusively resolve the phylogenetic relationships among the members of the bear subfamily *Ursinae* [5], which includes all living bear species except the giant panda and the spectacled bear, from here on referred to as ursine bears. The phylogenetic uncertainty has resulted in major taxonomic confusion. Based on both morphological and molecular data up to six different genera (*Ursus*, *Helarctos*, *Euarctos*, *Selenartos*, *Thalarctos* and *Melursus*; i.e. one for each species) have been suggested for the extant ursine bears (Table 1).

Recently, a study on mitochondrial genome sequences (mtDNAs) of all extant bears presented for the first time an almost completely resolved bear phylogeny with support for most of the problematic nodes in the bear family tree, except for the position of the sloth bear [4]. This shows that longer sequences are necessary for reconstructing a robust phylogeny [4,6-9]. Such large data sets also facilitate the molecular dating of divergence events within a phylogeny [12-14]. To resolve the relationships between the extant and extinct members of the bear family and to date the various divergence events among them, we used the complete mtDNA (consisting of ~17 kb) from ten different bear species. In addition to three published modern mtDNAs [10], we amplified and sequenced five modern bear mtDNAs using a 2-step multiplex PCR approach [9,16]. We also amplified and sequenced entire mtDNAs from the extinct European cave bear (*Ursus spelaeus*), believed to belong to the ursine bears [11], and the extinct North American giant short-faced bear (*Arctodus simus*) (see Additional File 1, Figure S1b), thought to be related to the spectacled bear [12].

Results

Sequence retrieval

We retrieved complete mtDNAs from GenBank for three extant bear species: brown bear, American black bear and polar bear (GenBank: NC003427, GenBank: NC003428, GenBank: NC003426). For the remaining five living bear species, we sequenced the entire mtDNA in overlapping fragments using a 2-step multiplex PCR approach [7] and a mixture of direct sequencing and sequencing multiple clones (EMBL:FM177759, EMBL:FM177761, EMBL:FM177763, EMBL:FM177764, EMBL:FM177765). We also obtained the complete mtDNA from the extinct European cave bear using a 44,000 year old bone found in Gamssulzen Cave, Austria. Again, we used a 2-step multiplex approach, but in this case, all PCR products were cloned and multiple clones were sequenced (EMBL:FM177760). Moreover, to ensure sequence accuracy, we determined each sequence position from at least two independent primary PCRs [13]. When we observed a discrepancy between the consensus sequences from each of the two amplifications we performed a third amplification and used the consensus sequence from all three amplifications (see Additional File 1). We used the same approach to sequence the extinct American giant short-faced bear mtDNA, using a 22,000 year-old calcaneum bone from Eldorado Creek, Canada (EMBL:FM177762). In order to further ascertain that the results obtained are reproducible, samples of both extinct bears were extracted, amplified and sequenced each in an additional laboratory that did not have access to the results obtained

Table 1: Taxonomic designations for the bears.

Common name	Eisenberg [72]	Ewer [73]; Corbet & Hill [74]	Zhang & Ryder [75]	Thenius [76]; Wozencraft [77]	Hall [28]; Nowak [29]; Yu [4], this study
giant panda	*Ailuropoda melanoleuca*	*A. melanoleuca*	*A. melanoleuca*	*A. melanoleuca*	*A. melanoleuca*
spectacled bear	*Tremarctos ornatus*	*T. ornatus*	*T. ornatus*	*T. ornatus*	*T. ornatus*
Asian black bear	*Selenarctos thibethanus*	*S. thibethanus*	*S. thibethanus*	*Ursus thibethanus*	*U. thibethanus*
sloth bear	*Melursus ursinus*	*M. ursinus*	*M. ursinus*	*M. ursinus*	*Ursus ursinus*
sun bear	*Helarctos malayanus*	*H. malayanus*	*H. malayanus*	*H. malayanus*	*Ursus malayanus*
polar bear	*Thalarctos maritimus*	*T. maritimus*	*Ursus maritimus*	*U. maritimus*	*U. maritimus*
American black bear	*Ursus americanus*	*Euarctos americanus*	*E. americanus*	*U. americanus*	*U.americanus*
brown bear	*Ursus arctos*	*U. arctos*	*U. arctos*	*U. arctos*	*U. arctos*

in Leipzig. For the cave bear a total of 3,520 bp were independently reproduced in Cambridge and for the American giant short-faced bear a total of 395 bp was replicated in the Australian Centre for Ancient DNA in Adelaide. The consensus sequences for all fragments determined in Cambridge were identical to those determined in Leipzig. The replicated fragments at the Australian Centre for Ancient DNA were identical to the sequence obtained in Leipzig except for a single deletion close to the 5'-end of the light strand in the first fragment. The sequence for this fragment was obtained by direct sequencing in just one 5' to 3' direction on the light strand. Given that sequence accuracy immediately downstream the sequencing primer is low, it is likely that this deletion represents a sequencing artifact.

Phylogenetic analyses

All ten bear mtDNAs were aligned using the harbor seal (*Phoca vitulina*) mtDNA as outgroup. Phylogenetic trees were reconstructed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference. We recovered the same topology using all above-mentioned optimality criteria (Figure 1 and Table 2). Our results confirm the giant panda's basal position in the bear phylogeny [1-4,14-17] and also place the spectacled bear outside ursine bears. In contrast to previous studies our data was sufficient to resolve the phylogenetic relationships among ursine bears with statistical support for all nodes. The sloth bear falls basal to all other ursine bears, which form a monophyletic group with 85%, 93%, and 100% support (MP symmetric resampling, ML bootstrap, and 1.00 Bayesian posterior probability (PP), respectively). The hypothesis suggesting that the sloth bear is basal to the sun bear and black bear clade (hypothesis 6, see Additional File 1, Figure S2) did not have a significantly worse likelihood (AU test, $p = 0.147$) than the topology favored by our data (sloth bear as the most basal ursine bear; hypothesis 9, see Additional File 1, Figure S2) although it had a slightly higher homoplasy index and a less parsimonious tree ($HI_{best} = 0.389$, $HI_{competing} = 0.393$; $TL_{best} = 10092$, $TL_{competing} = 10157$). In a MP analysis, however, this hypothesis (hypothesis 6, see Additional File 1, Figure S2) received significantly less support (Wilcoxon signed-ranks and sign tests, $p < 10^{-4}$). Thus, we suggest that ursine bears are separated into two sister clades, comprised of three species each with the sloth bear forming the basal branch. The first clade contains cave, brown, and polar bears and is supported by all methods. The second clade is composed of the sun and black bears (American and Asian) and receives varying support values, depending on the tree reconstruction method (76% MP symmetric resampling, 97% ML bootstrap, and 1.00 Bayesian PP). Finally, the placement of the extinct American giant short-faced bear as a sister taxon to the spectacled bear is supported in all analyses (100% bootstrap/symmetric resampling and 1.00 PP in all analyses).

Table 2: Node support values for the mitogenomic phylogeny of the bears.

Node	Unpartitioned			Partitioned	
	MP	ML	Bayesian	ML	Bayesian
t_3	100	100	1.00	100	1.00
t_4	100	100	1.00	100	1.00
t_5	85	93	1.00	93	1.00
t_6	100	100	1.00	100	1.00
t_7	100	100	1.00	100	1.00
t_8	76	94	1.00	97	1.00
t_9	99	97	1.00	94	1.00
t_{10}	100	100	1.00	100	1.00

Unpartitioned and partitioned phylogenetic analyses in maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference of phylogeny. In partitioned analyses every partition was allowed to evolve under a separate unlinked GTR+Γ substitution model and distinct base frequencies in ML and Bayesian inference of phylogeny.

The phylogenetic reconstruction makes evident a difficulty in resolving the relationships within ursine bears. As a matter of fact, most of the internal branches are very short. This observation (Fig. 2) makes it likely that individual genes (or short sequences) may exhibit different tree topologies, as shown for humans, chimpanzees and gorillas [18]. In our case, we notice that individual loci of the mtDNA support different topologies. Genes such as 12S rRNA, ND4L, ND5, and ND3 exhibited phylogenetic incongruence with two to four other mitochondrial genes (ILD test, $p \leq 0.05$). The highest amount of phylogenetic conflict emerged from partitioning the mtDNA to individual genes and the tRNAs of the two strands ($tRNA^-$ and $tRNA^+$), but only two nodes showed evidence of hidden conflict in a MP setting (node t_3 HBS = -10, node t_5 HBS = -5). Emerging support was evident for all terminal nodes including the ursine branching point (HBS range: 7–21). Intra-ursine nodes t_5, t_8, and t_9 were the only nodes to show <100% replicate-based support values, a fact that may be owed to the lack of consistent support provided by those genes (PHBS range: -1 to -7).

Estimation of divergence times

We used several fossil calibration points to estimate the mtDNA divergence dates within the bears (see Materials and Methods). The posterior mean of the divergence time between bears and the harbor seal was estimated at 36 million years ago (Ma), which agrees with previous estimates based on both molecular and paleontological data [12,19]. However, due to the wide uniform priors we used for the calibration points, the confidence interval (26.5–47.4 Ma, Table 3) remains large, depending on the type of analysis. The initial divergence within bears occurred between the giant panda and all remaining bears, esti-

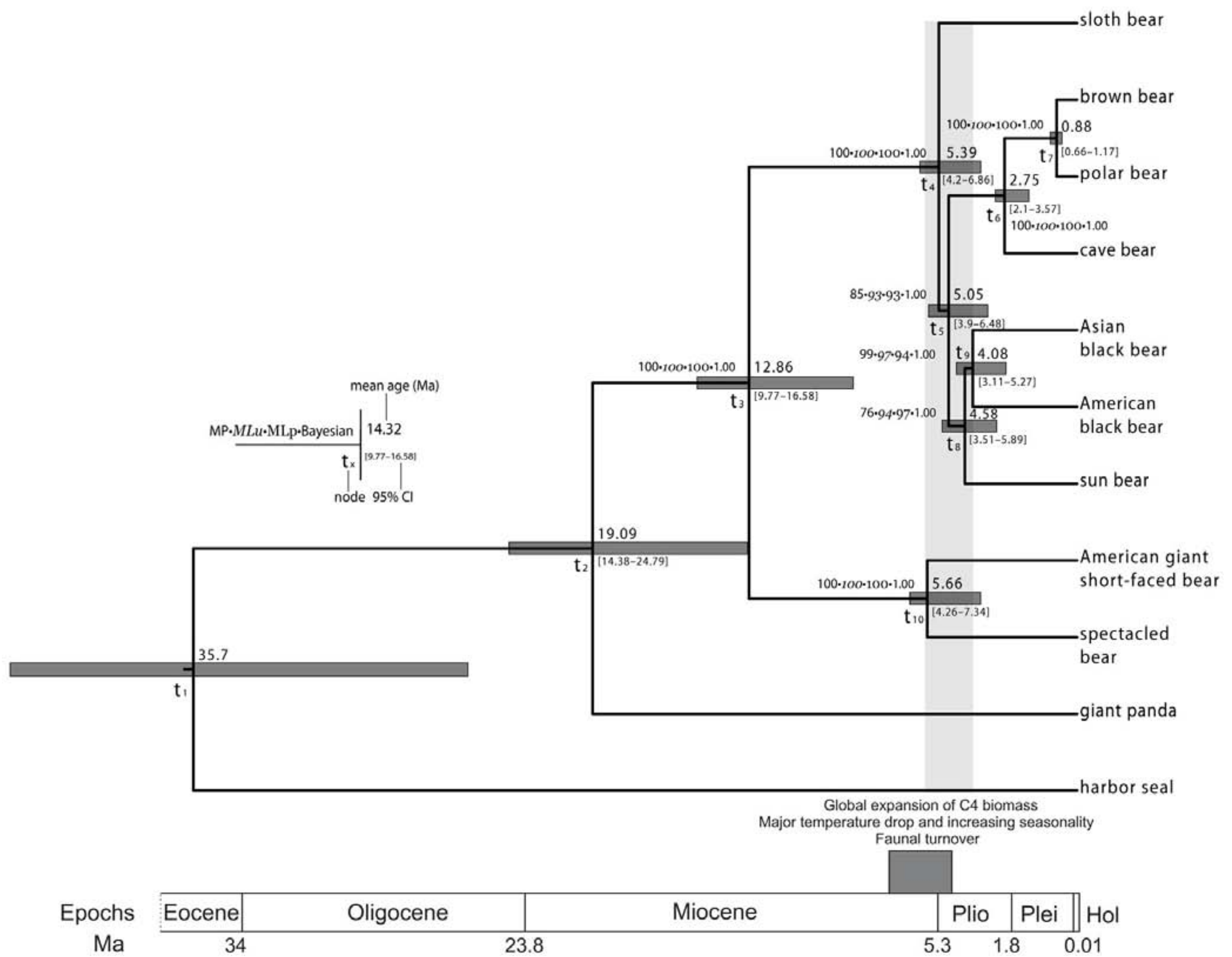

Figure 1

Maximum clade probability tree displayed as a chronogram from the BEAST analysis of the unpartitioned mitochondrial genome alignment. All lineages evolved according to a strict clock and the GTR+Γ_4 substitution model. Numbers above the nodes indicate phylogenetic support measures. Node bars illustrate the width of the 95% highest posterior density. Numbers in bold indicate the posterior mean estimates of divergence times.

mated to have happened between 17.9 and 22.1 Ma (range of posterior means across analyses). The next divergence was that of the New World spectacled bear group, which separated from the main ursine bear lineage about 12.4 to 15.6 Ma. The posterior mean divergence of the two New World species, the extant spectacled and the extinct American giant short-faced bear, was estimated to have happened between 5.3 and 7 Ma. Within the remaining ursine bears, the estimated divergence times all show overlapping confidence intervals, except for those within the brown bear clade.

Within the brown bear clade, we dated the divergence event between the cave and brown bear mtDNA to 2.4–3.1 Ma. The origin of the polar bear is more difficult to determine, as partial mtDNA sequences suggest that polar bears actually fall within the genetic diversity spectrum of brown bears [20,21], where they constitute a monophyletic clade closely related to a clade of brown bears from the ABC Islands in Alaska. Unfortunately, the published brown bear mtDNA [10] does not originate from this ABC Island clade, and therefore our estimated divergence for polar bears and brown bears is not a minimum date for this event, but rather the divergence date for different brown bear clades. As a consequence, the estimated divergence date of 2.7 and 1.3 Ma for brown and polar bears obtained in two recent studies [4,19] using the same complete mtDNAs should be interpreted with care. A

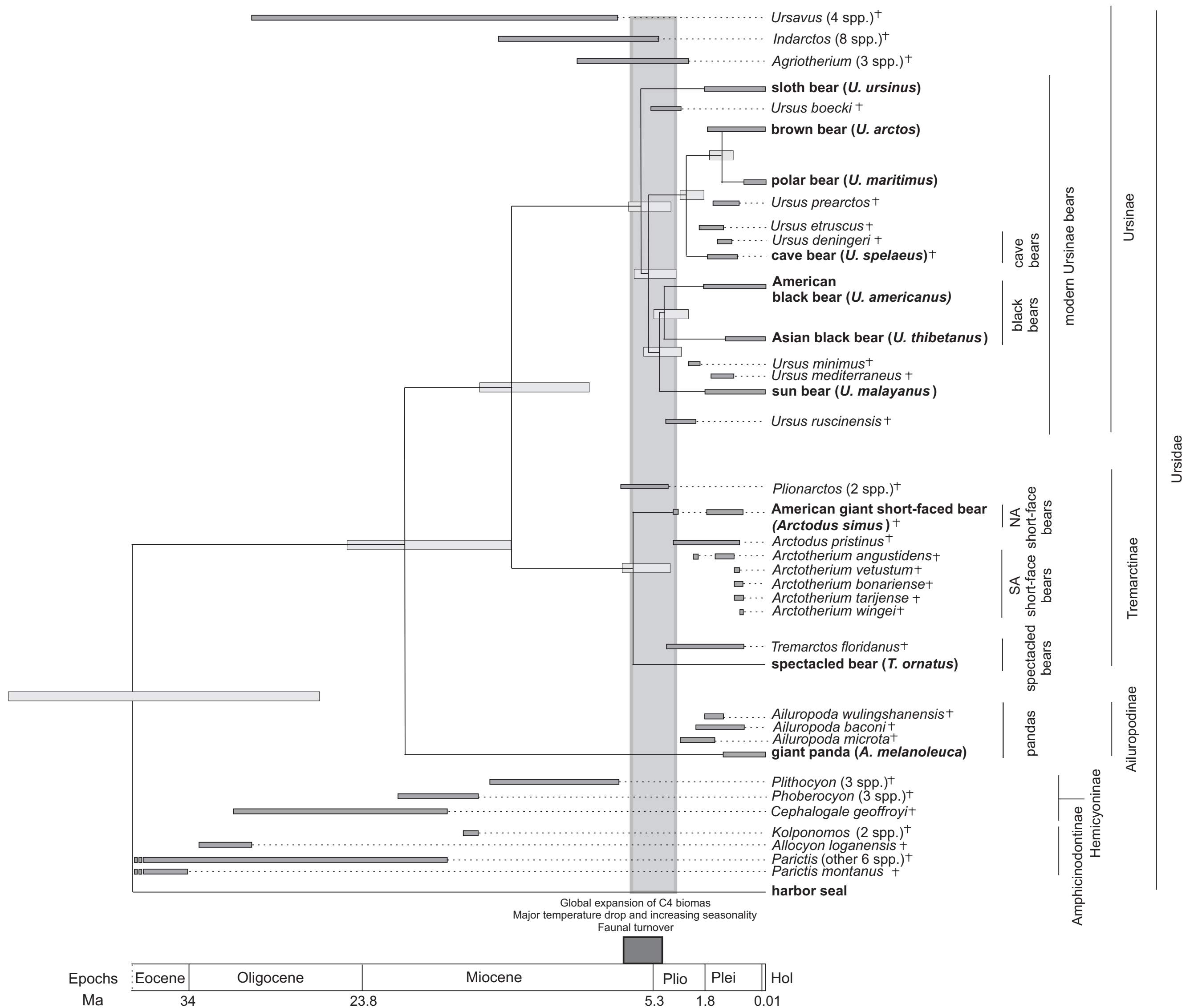

Figure 2

Temporal ranges of extant and fossil bears. Extinct genera and species are denoted with a cross (†). Species used in this study are written in bold. Horizontal dark grey bars indicate temporal range based on fossil evidence. Horizontal light grey bars show width of the 95% credibility interval for the molecular divergence time (see also Figure 1). The vertical grey bar illustrates the range of the posterior mean estimate of divergence times for all extant ursine bears (expect polar and brown bear) as well as American giant short-faced bear and spectacled bear. The dark grey box illustrates a time interval of massive global changes around the Miocene- Pliocene boundary.

mtDNA from an ABC island brown bear will be required to date the actual speciation event of polar bears more accurately.

We also examined whether more sequence data would improve our estimates on divergence times by plotting the posterior means of divergence times against the width of their corresponding 95% credibility interval (see Additional File 1, Figure S3), following Yang and Rannala [22]. We found significant correlations for both the unpartitioned and partitioned datasets ($p < 2.2 \times 10^{-16}$). This linear relationship strongly suggests that longer sequences or more taxa than those examined here are unlikely to increase the precision of the divergence time estimates. Therefore, in order to narrow the confidence intervals for the divergence date estimates within the bear phylogeny, more precisely dated fossil calibration points would be required.

Table 3: Posterior estimates of divergence times.

Node	Unpartitioned				Partitioned	
	BEAST		mcmctree		mcmctree	
	Mean	95% HPD	Mean	95% HPD	Mean	95% HPD
t_1	35.69	26.55–46.51	36.59	30.71–42.63	36.49	31.20–47.40
t_2	19.09	14.38–24.79	22.05	18.37–25.57	17.88	15.13–23.23
t_3	12.86	9.77–16.58	15.57	12.93–17.99	12.36	10.44–15.97
t_4	5.39	4.20–6.86	6.31	5.23–7.14	4.88	4.17–6.37
t_5	5.05	3.90–6.48	5.80	4.81–6.64	4.55	3.85–5.99
t_6	2.75	2.10–3.57	3.11	2.55–3.63	2.41	2.01–3.23
t_7	0.88	0.66–1.17	0.97	0.78–1.16	0.75	0.61–1.00
t_8	4.58	3.51–5.89	5.31	4.39–6.11	4.11	3.44–5.38
t_9	4.08	3.11–5.27	4.69	3.86–5.43	3.66	3.07–4.84
t_{10}	5.66	4.26–7.34	6.98	5.73–8.18	5.33	4.43–7.00
rate	1.199	0.896–1.50	1.07	0.92–1.28	AF	AF
κ	-	-	35.72	33.01–38.63	AF	AF
α	0.155	0.146–0.163	0.20	0.19–0.21	AF	AF

The two mcmctree columns correspond to the unpartitioned and partitioned analyses. The divergence time estimates are in millions of years before present. HPD, highest posterior density; rate, evolutionary rate ($\times 10^{-8}$ substitutions/site/year); κ, transition-transversion parameter of the HKY model; α, shape parameter of the rate heterogeneity Γ-distribution. AF, see Additional File 1.

The estimated substitution rate of approximately 10^{-8} substitutions/site/year was more similar to a mitogenomic dataset from primates than to the rate from extant and extinct proboscideans [9]. This evolutionary rate was also higher than that for parts of the nuclear IRBP gene in bears (0.139×10^{-8} substitutions/site/year; [17]).

Discussion

Our study represents the first comprehensive sampling of mtDNAs for recent bears, including all living and two recently extinct bear species. The cave bear and the American giant short-faced bear are the third and fourth Pleistocene species for which mtDNAs have been determined. Moreover, the cave bear genome is the first determined from a Pleistocene sample obtained from a non-permafrost environment. Compared to the extinct moas from which complete mtDNAs have previously been determined from non-permafrost specimens [23], the cave bear genome extends the time frame by an order of magnitude, showing that complete mtDNA analysis can be performed using a wide range of samples. As is common in large scale ancient DNA analyses [7,9,24], we found a number of consistent differences between independent primary PCRs, all of which were either C to T or G to A substitutions (see Additional File 1). This confirms previous reports that deamination of cytosine is one of the most common, and probably the only type of miscoding lesion in ancient DNA [13,24-26]. Moreover, the high number of consistent substitutions (81) observed in the cave bear genome sequences shows that each sequence position needs to be replicated when performing such large scale analyses.

This analysis has allowed the phylogenetic topology of the bear family to be resolved with high support values. Interestingly, it places the sloth bear basal to all other ursine bear species and the sun bear in a sister group related to the two black bear species. The latter observation coincides with paleontological information [27] and previous mtDNA studies [4,17,21]. An earlier study analysing six mtDNA fragments, also placed the sloth bear basal to all other members of the ursine bears [3]. However, this study found weak support for the sun bear as being basal to the brown bear – polar bear clade rather than to the two black bear species.

The phylogenetic reconstruction also reveals the reasons for previous problems in resolving the relationships among ursine bears, as most of the internal branches for their phylogenetic tree are very short. Such a short internal branch structure (Figure 1) makes it likely that individual nuclear genes (or short sequences) may exhibit different tree topologies, as shown for nuclear loci from humans, chimpanzees and gorillas [18]. Furthermore it was previously shown that despite being a non-recombining single genetic locus, individual genes on the mtDNA might produce different tree topologies [6,8,9,18].

The mitogenomic data also has implications for bear taxonomy. Six ursine bears and the sloth bear are monophyletic with absolute support, which agrees with Hall and Nowak's inclusion (Table 1) of the Asian black bear, American black bear, sun bear, polar bear and brown bear within the genus *Ursus* [28,29] and confirms the mitogenomic study by Yu et al [4]. Given the short divergence

time of the six ursine bears and the sloth bear we suggest, following Hall 1981, Nowak 1991 and Yu et al 2007 [4,28,29], that the sloth bear is grouped together with the other ursine bears in the genus *Ursus* and that the other genus names previously suggested for members of this radiation are discarded (Table 1).

Using this data set and multiple fossil calibration points, we have dated the various mtDNA divergence events during bear evolution with reasonable confidence. Strikingly, the divergence of the giant panda is estimated at about 19 Ma (95% HPD: 14.4–24.8 Ma, HPD: highest posterior density). This estimate is much earlier than previously reported for the divergence of the panda lineage from the *Ursavus* lineage based on teeth morphology of *Agriarctos* fossils (12–15 Ma) [30]. The latter divergence date has been used in several studies as a calibration point for dating bear radiations [2,4,35]. We decided not to use this date as a calibration point, since the oldest known panda fossil, *Ailuropoda microta*, is less than 2.4 million years old [31], and therefore allows no inference about the date of divergence of this lineage. Moreover, the fossil record for both *Ailuropoda* and its potential ancestral species from the genus *Agriarctos* is sparse, making an early Miocene divergence date for the giant panda's lineage plausible. Interestingly, the next divergence event is not until 13 Ma (spectacled and American giant short-faced bear) followed by a gap until 6 Ma when a rapid radiation occurs. The American giant short-faced and spectacled bears diverged around 5.7 Ma, and the five ursine lineages diverged between 5.4 and 4.1 Ma (posterior mean age estimates) (Figures 1 and 2).

Thus, taking the confidence intervals for the molecular dating into account, seven lineages radiated between 3.7 and 7 Ma. Such rapid radiations are also observed in other mammals, such as the cats [32] and procyonids [33], as well as in bird families like the woodpeckers [34]. Strikingly, the major radiation wave for these families also occurred at the end of the Miocene. In combination with the fossil record, the mtDNA divergence estimates suggest that the rapid radiation of the bear family around the Miocene-Pliocene boundary followed a major extinction of some of the main bear genera such as *Ursavus*, *Indarctos*, *Agriotherium*, and the *Hemicyoninae* (Figure 2). Similar species turnover events were also observed for other mammals over a limited time span near the Miocene-Pliocene boundary resulting in a massive extinction of more than 60–70% of all Eurasian genera and 70–80% of North American genera [35]. The cause of this widespread species turnover during this time period remains unclear. Some studies suggest that the initial opening of the Bering Strait at the beginning of the Pliocene around 5.3 Ma caused a major separation of northern hemisphere habitats [36]. Major climatic changes occurred during that time, such as the Messinian crisis during which the Mediterranean Sea lost its connection to the world ocean system and became desiccated [37]. These changes resulted in forest cover decline and the spread of arid habitats in Northern America and Eurasia [38,39] as well as a global increase in C4 biomass [40]. During that time, open grassland habitats, which were exploited by an entirely new suite of mammals [40], replaced the earlier less seasonal woodland forest habitats. Thus, it is possible that the environmental changes associated with the Miocene-Pliocene boundary and the following emergence of new ecological niches such as open grasslands caused an adaptive radiation in Old and New World bears similar to a number of other species groups [34]. This could explain the divergence of the *Tremarctinae* with the spectacled bear adapted to closed habitats and the American giant short-faced bears being predators dwelling in open habitats [12,27]. The latter adaptation was also described in other predator species that evolved around the Miocene-Pliocene boundary and were built for hunting in open habitats such as the cats [32,35]. Other events such as the opening of the Bering Strait could have additionally promoted allopatric speciation in black bears. Our divergence time estimates suggest that the American black bear could have spread to America before the Bering Strait opened around 5.3 Ma [36]. An early migration of ursine bears into the Americas is also supported by the oldest known *Ursus* fossil in North America, *Ursus abstrusus* [41], which was dated at 4.3 Ma, suggesting that *U. abstrusus* may be ancestral to the American black bear lineage.

Obviously, the Miocene-Pliocene global changes had a major impact on the radiation of bears and other species, both between and within the Old and New Worlds. It is interesting to note that African apes experienced a similar species turnover at the end of the Miocene, including the divergence of the chimpanzee and human lineages [42]. This latter event has been attributed to a magnified climatic variability starting at the end of the Miocene [43]. More studies are necessary to address the relationships between global changes and species radiations at the beginning of the Pliocene. Our results strongly support the idea of a major wave of bear radiations during that time.

Our data also indicate a much earlier divergence for the cave bear and brown bear lineages than those previously assumed, with a mean estimate at 2.8 Ma. This date agrees with recent results suggesting the existence of representatives of the brown bear lineage in Europe as early as 1.5 Ma (G. Rabeder, personal observation). Nevertheless, it questions other studies suggesting a later divergence time for this species pair at around 1.2–1.4 Ma based on the fossil record [27] and molecular data [44]. Loreille et al. [44], however, used Taberlet & Bouvet's estimated diver-

gence date for the two European brown bear lineages (Western and Eastern) of 850 ka [45], which in turn was based on an application of Vigilant et al.'s [46] intraspecific human rate of 8.4×10^{-8} substitutions/site/year – Taberlet & Bouvet cautioned that their estimates could be prone to uncertainty as they imported a human evolutionary rate. Given recent reports of problems in estimated intraspecific divergence times based on interspecific calibrations and *vice versa*, the implicit use of indirectly extrapolated evolutionary rates is not recommended [51,52].

Most of the youngest fossils for *Ursus etruscus*, the assumed ancestor of the cave and brown bear, have been dated to 2–2.7 Ma [47], suggesting that a late divergence for the two lineages around 1.2 Ma is rather unlikely. These dates also partially overlap with the divergence date we obtained (range of posterior means across methods: 2.4–3.1 Ma). A greater number of reliably dated fossils from early members of both the cave bear and brown bear lineages are necessary to date the divergence of *U. spelaeus*. However, around 2.8 Ma, the climate again changed dramatically with the onset of the first major cooling events and climatic oscillations at the end of the Pliocene that eventually led to the Pleistocene glaciations [48]. Thus, if bear speciation events were influenced by climate change, cave bears and brown bears may indeed have separated as early as 2.8 Ma.

Conclusion

Using complete mitochondrial genome sequences from both extinct and extant bears, we found evidence for a rapid radiation of bears at the Miocene – Pliocene Boundary 5–6 million years ago within the Old and New worlds. As rapid radiations were also observed in other species groups around this time [37-39], we suggest that climate change played an important role during bear evolution and animal speciation in general.

Our results clearly demonstrate the power of mitogenomic analyses for resolving complicated phylogenetic relationships among both extant and extinct species, using samples obtained not only from permafrost, but also from non-permafrost environments.

Methods

Ancient and modern DNA samples

The modern DNA samples of the Asian black bear, sloth bear and sun bear were obtained from DNA stocks held at the National Cancer Institute, Laboratory of Genomic Diversity in Frederick, Maryland (USA). The DNA samples of the giant panda and spectacled bear were obtained from the National Fish & Wildlife Forensic Lab in Ashland, Oregon (USA).

In Leipzig, cave bear DNA was extracted from 640 mg of bone powder taken from a femur found in Gamssulzen cave (Austria) that was dated to 44.160 +1.400/-1.190 BP (KIA 25287). The extraction was performed as described previously [49], yielding 70 μl of DNA extract. In Cambridge, 500 mg of cave bear bone was extracted using the same protocol as in Leipzig. Details for the American giant short-faced bear DNA extraction performed in the Australian Centre for Ancient DNA can be found in Additional File 1.

Multiplex amplification and sequencing

Primer pairs were designed by aligning the three published mtDNAs of brown bear, polar bear and American black bear [10], and partial mtDNAs from various bear species retrieved from GenBank. The revised Cambridge reference sequence for the human mtDNA [50] was also included in the alignment. For the primers, regions were chosen that are highly conserved among bears, but carry substitutions compared to the modern human sequence, to minimize the risk of human contamination. As previously described for the 2-step multiplex protocol [7,51], the primer pairs for the first and second step were divided into two sets, ODD and EVEN, to avoid amplifying the overlapping fragments between adjacent products. The two primer sets were used in separate 2-step multiplex PCRs, as previously described [7]. The first amplification step was performed in a total volume of 20 μl. Each reaction contained a final concentration of 1x PCR-buffer, 4 mM $MgCl_2$, 250 μM of each dNTP, 150 nM of each primer from one set and 2 U *AmpliTaq*® Gold DNA polymerase plus 5 μl of the DNA extract. PCRs were initiated by exposure to 94°C for 9 min, followed by 25 cycles of 20 s at 94°C, 30 s at 52°C and 1 min at 72°C. At the end, a final 4-min extension at 72°C was performed. This amplification was then diluted 40 fold and 5 μl of the dilution were used as a template in each of the single amplification reactions. Reagent concentrations were as described above, except that a single primer pair was used at a concentration of 1.5 μM for each primer, and only 0.5 U of DNA polymerase were used in each reaction. The PCR temperature profile was the same as in the first amplification step. Amplification products of the correct size for the two extinct bears were cloned using the TOPO TA cloning kit (Invitrogen), and a minimum of three clones were sequenced on an ABI3730 capillary sequencer (Applied Biosystems). For the modern samples, PCR products were either sequenced from both directions, or multiple clones were sequenced to ensure sequence accuracy. Primers for fragments that gave no product in the first amplification attempts were redesigned if the adjacent fragments showed substitutions in the primer site. The resulting primers were then used to amplify the remaining segments of the bear genomes. For the two extinct bear species, each position of the mtDNA was amplified at least

twice from independent primary amplifications to ensure the authenticity of the sequence [13]. For the cave bear a nested primer design was chosen where the primers in the singleplex amplification are shifted inwards compared to the primers used in the multiplex step. This design ensures specificity of the singleplex PCR and reduces the risk of contamination of the multiplex PCR since only products from the singleplex reaction are amplified to high copy numbers [51]. All primer sequences used can be found in Additional File 1. For the sequenced modern bears and the cave bear primer sets EVEN and ODD are comprised of 20 primer pairs each. A single primer pair, EVEN21, spanning a repeat region within the D-loop, was excluded from both sets and only used in singleplex PCRs. For the Giant short faced-bear 81 primer pairs were designed in total and split into two sets; all amplification attempts spanning the repeat region within the D-loop for the American giant short-faced bear failed.

In Cambridge, amplifications were completed using the same PCR conditions as in Leipzig, but with a reduced number of primer pairs. Both water controls and an extraction control consisting of a mammoth DNA extract were negative for cave bear-specific products. Eighteen amplification products, originating from independent primary PCRs, were sequenced in both directions for 9 different fragments distributed throughout the whole mtDNA. A total of 3,520 bp were amplified and sequenced. The consensus sequences for all fragments were identical to the corresponding sequences produced in Leipzig.

For the American giant short-faced bear in total 395 bp of the mtDNA were replicated in two fragments at the Australian Centre for Ancient DNA. Details can be found in Additional File 1.

Mitochondrial genome sequence alignment and annotation

The newly sequenced mtDNAs for the two extinct and five extant bear species, as well as the four publicly available genomes (three bears and a harbor seal) were aligned in MUSCLE 3.6 using the default parameters [52]. The D-loop was removed for all analyses, as it is too variable for interspecific comparisons and could partially not be determined from the American short-faced bear. We employed nine sequence data partitioning schemes in the following order: the transcription process; the three codon positions on each strand, the tRNAs on each strand and the rRNA genes. A few nucleotides were duplicated in the partitioned dataset because of the overlap of some loci, and a small number of non coding nucleotides were excluded. The annotation was completed using the program DOGMA [53] and modified manually to avoid overlap of tRNA and protein-coding genes.

Phylogenetic analyses

The substitution model was selected using Akaike's Information Criterion on all models available in the baseml program of PAML 3.15 [54]. For both the partitioned and the unpartitioned datasets, the GTR+Γ [55-57] was found to be the best-fit model. This model was used in all subsequent analyses unless specified otherwise.

The phylogeny of the mtDNAs was reconstructed using a thorough maximum parsimony (MP) search that is implemented in TNT [58], with 500 random-addition sequences and a variety of tree space exploration techniques. We also employed maximum likelihood (ML) in RAxML 2.2.3 [59], as well as a Bayesian inference (BI) of phylogeny in MrBayes 3.1.2 [60]. The GTR+Γ_4 substitution model was used for both ML and BI analyses. Phylogenetic support was provided with 1000 bootstrap pseudoreplicates in ML and 5,000 replicates of symmetric resampling in MP. MrBayes was run twice for 3 million generations with a burn-in of 2,500 steps. For details, see Additional File 1.

Incongruence between individual partitions was evaluated in an MP framework employing variations of Bremer support measures, as implemented in Automated Simultaneous Analysis of Phylogenies [61], as well as with the ILD test [62]. Agreement or disagreement between individual partitions at each node in the mtDNA tree was expressed through positive and negative hidden branch support (HBS) values, respectively [63]. See Additional File 1 for further details.

Contrasting alternative phylogenetic hypotheses

We collected 10 alternative topologies on the phylogenetic relationships of bears from the available literature (see Additional File 1, Figure S2) and compared them in an ML framework using the approximately unbiased test (AU) [64] in CONSEL [65], along with a comparison of homoplasy indices and tree lengths.

Estimation of divergence times

Dating of the divergence events within bears was done using a molecular clock approach and several fossil calibration points. The minimum for the divergence of bears and seals was set to 33.9 Ma, based on the fossil species *Parictis montanus* [66] and *Parictis parvus* [67] both dated to 38–33.9 Ma, and the first well-described members on the bear lineage. As a second calibration point, the minimum age for the oldest described *Ursus* fossils, *U. minimus* and *U. ruscinensis*, at 4.2 Ma [47] was used, and the maximum for the youngest fossils from the genus *Ursavus*, *U. depereti* and *U. ehrenbergi*, which gave rise to the *Ursus* lineage [11], at 7.1 Ma [47].

The above mentioned calibration points were used as priors to obtain the posterior distribution of the estimated divergence times. Evolutionary rate constancy according to a molecular clock for all bear mtDNAs, including the harbor seal outgroup, was tested using a likelihood ratio test (LRT) in baseml [68]. The assumption of a molecular clock at the 1% level under a GTR+Γ model for the whole mtDNA alignment excluding the D-loop for the partitioned ($-2\delta L = 14.3$, $p = 0.112$) and unpartitioned ($-2\delta L = 18.0$, $p = 0.036$) dataset could not be rejected.

We estimated divergence times using two Bayesian approaches implemented in the programs mcmctree [54] and BEAST 1.4.4 [69]. Mcmctree was run using the HKY85+Γ_8 substitution model [57,70], the most parameter-rich model available in this program. A total of 10^5 generations were sampled every 5 steps after discarding 10^4 initial steps as burn-in. The more parameter-rich model GTR+Γ_4 was used in BEAST with the following priors: Yule speciation prior on the tree, siteModel.alpha (initial = 0.2, exponential prior with mean 1.0 and 95% CI of 0.05129–2.996), clock.rate (initial = 0.015, uniform prior of 0–10), root.height of ursine bear clade (uniform prior of 7.1–4.2 Ma based on the basal ursine bear radiation fossil data). Thirty million Markov chain Monte Carlo (MCMC) steps were sampled every 1,000 generations. Convergence was assessed in Tracer v1.3 [71] after excluding the first 5 million samples as burn-in. All effective sample size values exceeded 20,000, suggesting a sufficient run length. The strict clock was implemented in all divergence time estimations, as suggested by the LRT.

Authors' contributions

JK, TU, AN, AWB, SJB, PHD, HS and MS were responsible for the experimental work. JK, ASM and SOK performed the sequence and evolutionary analyses. GR and PM obtained the ancient bear specimens and arranged the dating. LS contributed paleontological information on the bear fossil record. SJO provided extant bear DNA. AC, SP, JK, MSlatkin and MH conceived ideas for this project. JK, ASM, SOK and MH wrote the manuscript. All authors read and approved the final draft.

Additional material

Additional File 1
Supplementary materials.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2148-8-220-S1.doc]

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