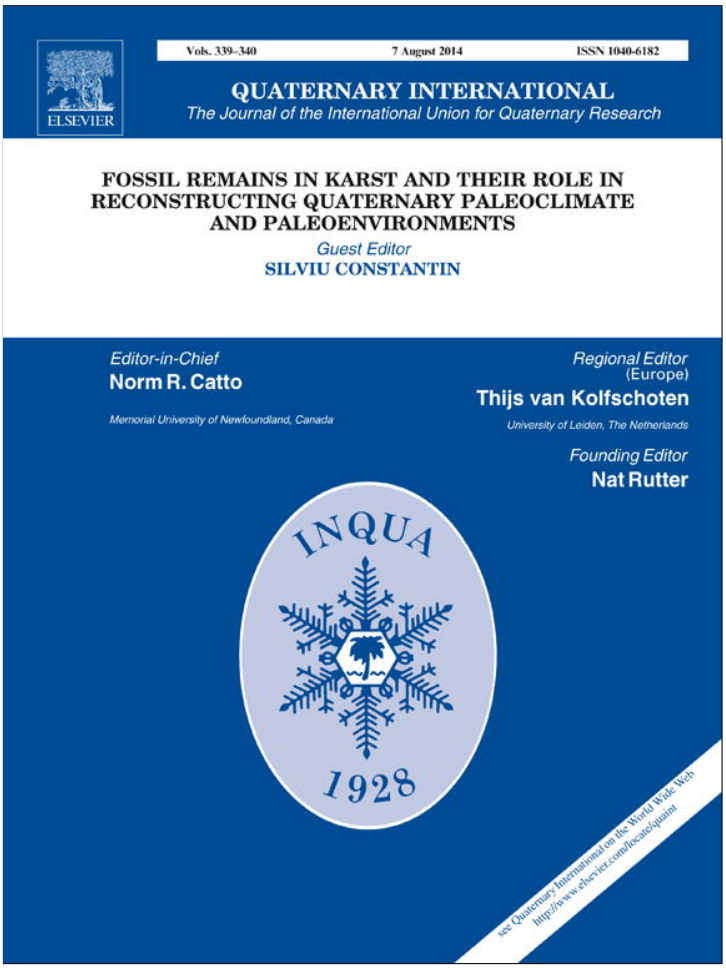


Quaternary International 339-340 (2014) 224–231

Contents lists available at ScienceDirect

Quaternary International

journal homepage: www.elsevier.com/locate/quaint

Mitochondrial DNA diversity and evolution of the Pleistocene cave bear complex

Mathias Stiller [a,b,1], Martyna Molak [c,1], Stefan Prost [b,d,e], Gernot Rabeder [f], Gennady Baryshnikov [g], Wilfried Rosendahl [h], Susanne Münzel [i], Hervé Bocherens [j], Aurora Grandal-d'Anglade [k], Brigitte Hilpert [l], Mietje Germonpré [m], Oleh Stasyk [n], Ron Pinhasi [o,p], Andrea Tintori [q], Nadin Rohland [r], Elmira Mohandesan [s], Simon Y.W. Ho [c], Michael Hofreiter [b,t,*,1], Michael Knapp [b,d,u,**,1]

[a] Department of Ecology and Evolutionary Biology, University of California Santa Cruz, Santa Cruz, CA 95064, USA
[b] Max Planck Institute for Evolutionary Anthropology, Deutscher Platz 6, D-04103 Leipzig, Germany
[c] School of Biological Sciences, University of Sydney, New South Wales 2006, Australia
[d] Allan Wilson Centre for Molecular Ecology and Evolution, Department of Anatomy, University of Otago, Dunedin, New Zealand
[e] Department of Integrative Biology, University of California, Berkeley, CA, USA
[f] Department of Palaeontology, University of Vienna, 1090 Vienna, Austria
[g] Zoological Institute, Russian Academy of Sciences, 199034 St. Petersburg, Russia
[h] Reiss-Engelhorn-Museen, Department for World Cultures and Environment, 68159 Mannheim, Germany
[i] Institute for Archaeological Sciences, Archaeozoology, University of Tübingen, 72070 Tübingen, Germany
[j] Department of Geosciences, Biogeology, University of Tübingen, 72074 Tübingen, Germany
[k] Instituto Universitario de Xeoloxía, Universidade da Coruña, 15081 A Coruña, Spain
[l] Geo-Center of Northern Bavaria, Research Group Palaeontology, Friedrich-Alexander University Erlangen-Nürnberg, 91054 Erlangen, Germany
[m] Operational Direction 'Earth and History of Life', Royal Belgian Institute of Natural Sciences, 1000 Brussels, Belgium
[n] Institute of Cell Biology, National Academy of Sciences of Ukraine, Drahomanov Str. 14/16, Lviv 79005, Ukraine
[o] School of Archaeology, University College Dublin, Belfield, Dublin 4, Dublin, Ireland
[p] Conway Institute of Biomolecular & Biomedical Research, University College Dublin, Belfield, Dublin 4, Dublin, Ireland
[q] Dipartimento di Scienze della Terra A. Desio, Università degli Studi di Milano, Italy
[r] Department of Genetics, Harvard Medical School, Boston, MA, USA
[s] Institute of Population Genetics, University of Veterinary Medicine of Vienna, Veterinärplatz 1, A-1210 Vienna, Austria
[t] Department of Biology, University of York, Wentworth Way, Heslington, York YO10 5DD, UK
[u] Molecular Ecology and Fisheries Genetics Laboratory, School of Biological Sciences, Bangor University, Deiniol Road, Bangor, UK

ARTICLE INFO

Article history:
Available online 5 November 2013

ABSTRACT

Cave bears are among the most well known extinct Pleistocene mammals. Their biogeography and taxonomy, along with the factors that led to their extinction, have been subject to long-standing controversy. Here, we reconstruct the phylogeography as well as the temporal and spatial population dynamics of cave bears across their range using mitochondrial DNA control region sequences from 77 published as well as 65 new cave bear samples, Our analyses reveal a dramatic loss of genetic diversity in cave bear populations after 30,000 years before present and provide evidence for a range decline from east to west towards the onset of the last glacial maximum. Our results also suggest that the three major haplogroups within cave bears, which may correspond to distinct species, were previously more widespread, with relict populations in remote and alpine areas still harbouring haplotypes that have disappeared from most of their previous range. Applying a phylogenetic dating approach, we estimated the age of the oldest of our samples, originating from the Yana River region in north-eastern Siberia, to be around 178,000 years, which confirms a previous estimate of a Middle Pleistocene age based on its stratigraphic position. Our results extend our knowledge about the evolutionary history of cave bears,

* Corresponding author. Department of Biology, University of York, Wentworth Way, Heslington, York YO10 5DD, UK.
** Corresponding author. Molecular Ecology and Fisheries Genetics Laboratory, School of Biological Sciences, Bangor University, Bangor, UK
E-mail addresses: michi@palaeo.eu (M. Hofreiter), m.knapp@bangor.ac.uk (M. Knapp).
[1] These authors contributed equally to this work.

1040-6182/$ – see front matter
http://dx.doi.org/10.1016/j.quaint.2013.09.023

but they also show that to unravel the complexities of cave bear evolution future ancient DNA studies on this Pleistocene species will need to go beyond short mitochondrial DNA fragments, including full mitochondrial genomes as well as nuclear DNA sequences.

1. Introduction

Among the large mammal species that went extinct at the end of the Pleistocene, cave bears (*Ursus spealeus* sensu lato) have one of the most extensive fossil records (Kurtén, 1976; Musil, 1980; Baryshnikov, 1998; Rabeder, 1999; Rabeder et al., 2000; Baryshnikov, 2007). There have been numerous studies on both the morphology and genetics of cave bears. Cave bears displayed extensive morphological diversity both within and among cave sites (Rabeder, 1995; Rabeder and Hofreiter, 2004; Rabeder et al., 2008). At least six different Late Pleistocene morphotypes have been identified, albeit with controversial taxonomic status (Baryshnikov and Puzachenko, 2011 and references therein; Hofreiter et al., 2004; Knapp et al., 2009; Rabeder et al., 2004). The cave bear was also one of the first species from which Pleistocene DNA sequences were obtained (Hänni et al., 1994).

Recent studies have provided significant new insights into cave bear phylogeography, evolution and extinction. Cave bears have long been regarded as an exclusively European species (Kurtén, 1968, 1976; Musil, 1980; Kahlke, 1994), with their easternmost distributions in the Ural and Caucasus Mountains. Recent studies, however, have shown that they occurred as far east as the Altai Mountains (Knapp et al., 2009) and North-Eastern Siberia (Sher et al., 2011). Moreover, genetic analyses of mitochondrial (mt) DNA have shown that cave bears from the Caucasus comprise a group genetically distinct from those found in Europe, and, more generally, that cave bears displayed substantial genetic diversity across their range (Knapp et al., 2009; Stiller et al., 2009).

Other studies have shed new light on the extinction of the cave bear, which was previously believed to coincide with the end of the Pleistocene (Martin and Steadman, 1999; Barnosky et al., 2004). Extensive radiocarbon dating now suggests that the extinction happened much earlier, around 24,000 ^{14}C years before present (BP; 27,800 calibrated (cal.) BP), at the onset of the last glacial maximum rather than at its end (Pacher and Stuart, 2009; Münzel et al., 2011; Bocherens et al., 2014). Furthermore, a demographic analysis of mtDNA sequences suggested that cave bear population size declined over an extended period of about 25,000 years before their eventual extinction (Stiller et al., 2010). However, all of these genetic studies were based on relatively small datasets. In fact, despite a large body of research on various aspects of cave bear biology, the amount of DNA sequence data available for them is still smaller than for many large herbivores such as mammoth, bison, musk ox, horse and reindeer (see Lorenzen et al., 2011 for an overview). For these other species, numerous, well-preserved specimens have been recovered from permafrost environments. In contrast, nearly all of the specimens of cave bears have been recovered from non-permafrost environments, resulting in comparatively low DNA quality and quantity in the specimens. Thus, our knowledge of cave bear evolution, distribution and specifically genetics is surprisingly incomplete.

Here we analyse 77 published and 65 new cave bear mitochondrial DNA sequences, representing six of the species/subspecies from the Late Pleistocene cave bear complex. Using recently developed techniques, including a phylogenetic method for estimating the ages of ancient DNA sequences in the absence of radiocarbon dates (Shapiro et al., 2011), we investigate the evolution, extinction, and geographical range of different cave bear taxa and mitochondrial haplogroups during the last few hundred thousand years. We discuss the implications of our findings for future research on cave bear ecology and extinction.

2. Materials and methods

2.1. Samples and DNA extraction

We obtained 65 cave bear samples from across the currently recognized range of the cave bear (Fig. 1). DNA was extracted from bone or tooth sample material (100–500 mg) following the protocols described by Hofreiter et al. (2004) and Rohland and Hofreiter (2007a, 2007b). A further 77 control region sequences of 251 bp in length were obtained from GenBank (accession numbers in Table S1). In total, our data set consisted of 142 cave bear samples (Table S1).

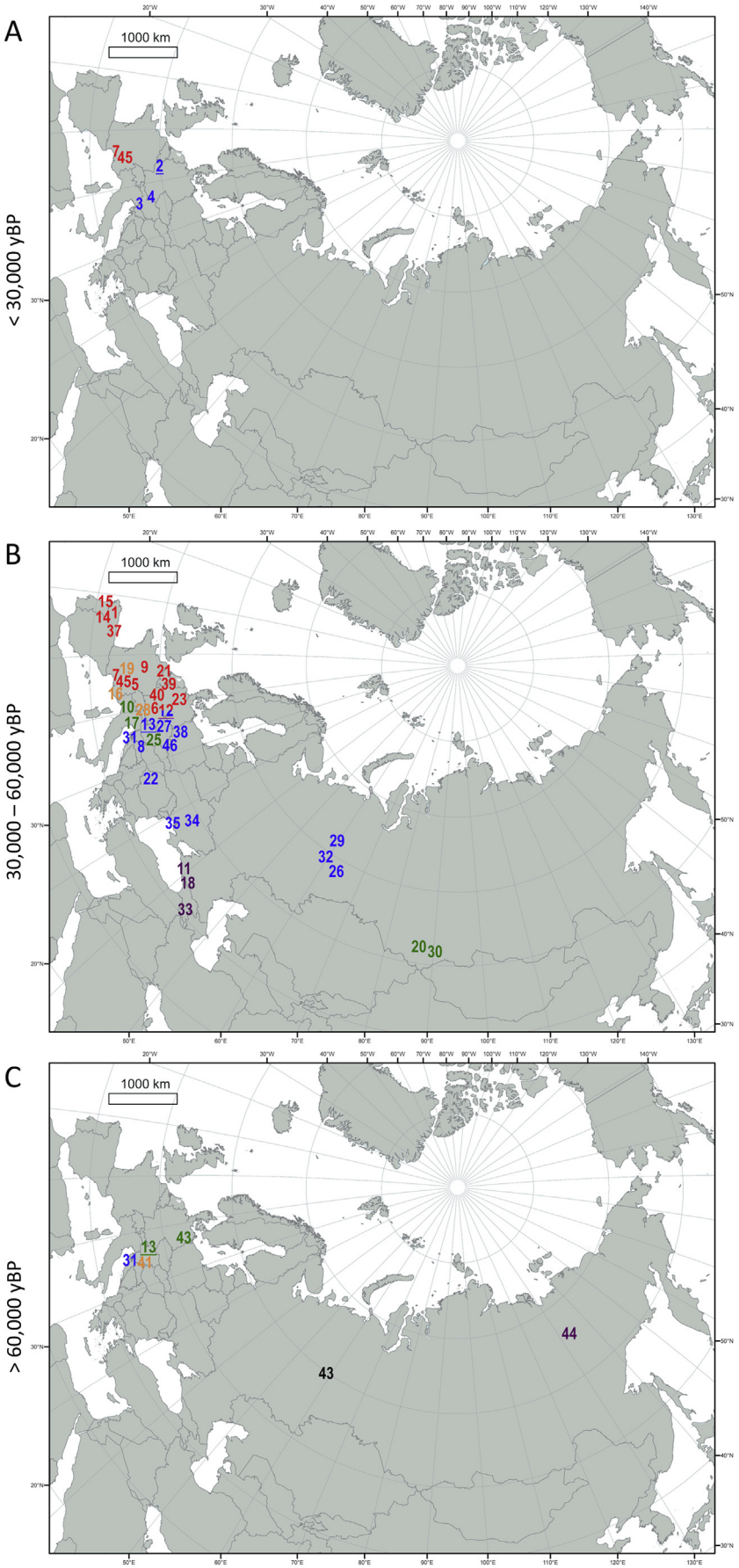
2.2. DNA amplification and sequencing

For all samples as well as all extraction no-template controls and PCR no-template controls we attempted amplification of an approximately 285-bp fragment of the mitochondrial D-loop (Hofreiter et al., 2002). We used primers from Hofreiter et al. (2002) as well as primers that amplify shorter fragments (Hofreiter et al., 2004; Knapp et al., 2009). Amplifications were performed using either standard simplex PCR or multiplex PCR (Römpler et al., 2006). Amplification conditions and annealing temperatures were adopted from Hofreiter et al. (2002). All no-template controls were clean. Amplification products were cloned into the pCR2.1-TOPO vector (Life Technologies) following the supplier's instructions. A minimum of three clones per PCR product was sequenced on an ABI 3730 sequencer using the BigDye Terminator v1.1 Cycle Sequencing Kit and M13 universal primers. Clone sequences were visually aligned using the program package BioEdit (Hall, 1999) and consensus sequences were called for all individuals. A long pyrimidine stretch was removed from all sequences, since this region could not be aligned unambiguously. This resulted in an alignment of 251 bp in length.

Each sequence position was determined from two independent PCR amplifications to avoid sequence errors caused by template damage (Hofreiter et al., 2001). When we found consistent nucleotide differences between two independent amplifications we performed a third amplification and called a consensus. We also observed characteristic C-to-T and G-to-A changes resulting from cytosine deamination, typical for ancient DNA.

2.3. Network and neighbor-joining analyses

To investigate the relationships among different cave bear haplotypes through time we constructed a temporal statistical parsimony network using the R script TempNet v1.4. (Prost and Anderson, 2011; http://www.stanford.edu/group/hadlylab/tempnet/; Fig. 2). We also reconstructed a Neighbor-Joining tree in MEGA4 (Tamura et al., 2007) based on corrected (Tamura and Nei, 1993) nucleotide distances from all sequences available, including those for which we could not obtain reliable phylogenetic estimates of dates. Individual node support was estimated via 1000 bootstrap replicates.



2.4. Bayesian phylogenetic analyses

The data set included 142 sequences, of which 90 had ages estimated by stratigraphic or radiocarbon dating (if not stated otherwise, we use radiocarbon, rather than calibrated ages). To estimate the ages of the remaining 52 sequences, we used a Bayesian phylogenetic approach based on a molecular clock (Shapiro et al., 2011). We began by testing this approach using leave-one-out cross-validation based only on the dated sequences. In this test, the age of each sequence was individually estimated in turn, with the ages of the remaining 89 dated sequences used as calibrations. The best-fitting model of nucleotide substitution was chosen using the Bayesian Information Criterion. Analyses were performed using BEAST v1.7.5 (Drummond and Rambaut, 2007), with a uniform prior on evolutionary rate ($0–10^{-4}$ mutations/site/year) and a $1/x$ prior on the constant population size. A uniform prior ($0–10^{6}$ years) was used for the age of the sequence being estimated. Posterior distributions of parameters were estimated using Markov chain Monte Carlo (MCMC) sampling, with samples drawn every 3000 steps over a total of at least 30 million steps. Some chains were extended to ensure sufficient sampling and effective sample sizes above 100 for all parameters.

For all but 6 of the ages estimated using this technique, the radiocarbon age was within the 95% credibility interval of the estimate (Table S2). We then used this phylogenetic approach to estimate the ages of the 52 undated sequences. Our analyses yielded unimodal, non-zero age estimates for 45 of these sequences. We used an empirical Bayesian approach in which these estimates were used to specify prior distributions of the sequence ages in subsequent phylogenetic analyses.

To test whether the data set contained sufficient temporal information for calibrating estimates of the rate and timescale, we conducted a date-randomization test (Ramsden et al., 2009; Ho et al., 2011). In this test, phylogenetic analyses are performed using 10 replicates of the data set in which the ages of sequences are randomly reassigned. If the mean rate estimate for the original data set is not included in the 95% credibility intervals of the estimates from the date-randomized replicates, the sequence ages are considered to provide sufficient calibrating information. This was the case for the data set comprising only samples with stratigraphic and radiocarbon age estimates, as well as by the full data set comprising 135 sequences (including the 45 sequences for which ages were successfully estimated phylogenetically).

Bayesian phylogenetic inference was performed with BEAST, using all of the sequences that could be dated reliably. Owing to the intraspecific nature of the data set, we used a strict-clock model. Estimates of posterior distributions of parameters were obtained using MCMC sampling, with samples drawn every 2×10^{4} steps over 2×10^{8} steps. We checked for convergence and sufficient sampling using Tracer v1.5 (Drummond and Rambaut, 2007). Comparison of coalescent models using Bayes factors revealed support for an extended Bayesian skyline plot over a constant-size model. The Bayesian Skyline Plot shows a population decline from about 50,000 years ago, consistent with Stiller et al. (2010) (Fig. S2).

3. Results

3.1. Cave bear phylogeography and systematics

The cave bear specimens in this study were morphometrically assigned to six different species or subspecies including *Ursus spelaeus spelaeus*, *Ursus spelaeus eremus*, *Ursus spelaeus ladinicus*, *Ursus ingressus*, *Ursus rossicus* and *Ursus kudarensis* (Table S1; Baryshnikov, 1998; Rabeder and Hofreiter, 2004; Baryshnikov and Puzachenko, 2011). Our phylogenetic analyses of the samples confirmed genetic distinctiveness consistent with a taxonomic designation for *U. spelaeus*, *U. ingressus*, *U. rossicus* and *U. kudarensis* and the differentiation of these four groups was well supported (Fig. 3). *U. s. eremus* and *U. s. ladinicus* were also found to be genetically distinct from their closest relative *U. s. spelaeus*. However, clades within the *ladinicus/eremus* complex were very poorly resolved by Bayesian and Neighbor-Joining approaches and topologies differ significantly depending on the tree building approach (Fig. 3; Fig. S1). The cave bears from the Altai region, which had genetically been assigned to *U. s. eremus* (Knapp et al., 2009) and the specimen from Baumann's cave in the Harz Mountains (Germany) formed a separate clade but the systematic assignment of the clade differs between both tree building approaches and is not well supported by either approach (Fig. 3; Fig. S1). Both Bayesian and Neighbor-Joining approaches confirmed the deep divergence of the Caucasus cave bears (*U. kudarensis*) from all other cave bears, as in Stiller et al. (2009), as well as their sister-group relationship to the single individual from the Siberian Yana River region as described by Knapp et al. (2009).

For the purpose of describing the origin of the samples, we split Europe into Eastern Europe and Western Europe, with the border running from the Adriatic Sea along the eastern borders of Italy, Austria and Germany to the Baltic Sea. All *U. s. spelaeus* samples came from Western Europe while *U. ingressus* had a more Eastern European distribution, including the Balkans and the Ural mountains. *U. ingressus* overlapped with *U. s. spelaeus* on the western margin of its distribution (Fig. 1). The *U. s. ladinicus/eremus* complex was found only in the Alps (including northern Italy with the samples from Monte Generoso and Grotta Rota Imagna, Tables S1 and S2), while the Altai samples previously assigned to *U. s. eremus* formed a sister relationship with the specimen from Baumann's cave in the German Harz mountains. *U. kudarensis* sequences were only found in the Caucasus and in one genetically very distinct individual from the Yana River region in Eastern Siberia.

Small cave bears morphologically comparable to *U. rossicus* have been found in the southern parts of Western Siberia, dating to the Middle Pleistocene (Baryshnikov and Foronova, 2001). Their genetic affinity is unknown but morphological similarity of these small bears to *U. rossicus* from the Late Pleistocene suggests that they belonged to a single phylogenetic lineage. Our data set contains two *U. rossicus* individuals, both coming from Kizel cave in the Ural Mountains. Our phylogenetic analyses suggest that they form a sister group to *U. ingressus* (Fig. 3).

3.2. Phylogenetic dating of samples

To estimate the ages of our 52 undated samples, we analysed their DNA sequences using a Bayesian phylogenetic approach based on a molecular clock. 45 of 52 samples yielded unimodal, non-zero age estimates, with median age estimates ranging from 30,075 years for a sample from Hohle Fels in Germany to 178,300 years for the sample from the Yana River region in Siberia (Table S1). It is noteworthy that all samples younger than 34,550 years were from Western Europe or the Balkans, with the youngest reliably dated sample being from Geissenklösterle (Germany, 26,530 years). All samples from east of the Balkans and Poland, with the exception of the Caucasus samples, are older than 40,000 years. Western European samples ranged in age from 26,530 (Geissenklösterle) to

Fig. 1. Phylogeography of cave bears through time. The numbers correspond to haplotype numbers in Table S1. Colours correspond to different species/subspecies: red: *U.s. spelaeus*; orange: *U.s. ladinicus*; green: *U.s. eremus*; blue: *U. ingressus*; purple: *U. kudarensis*; black: *U. rossicus*.

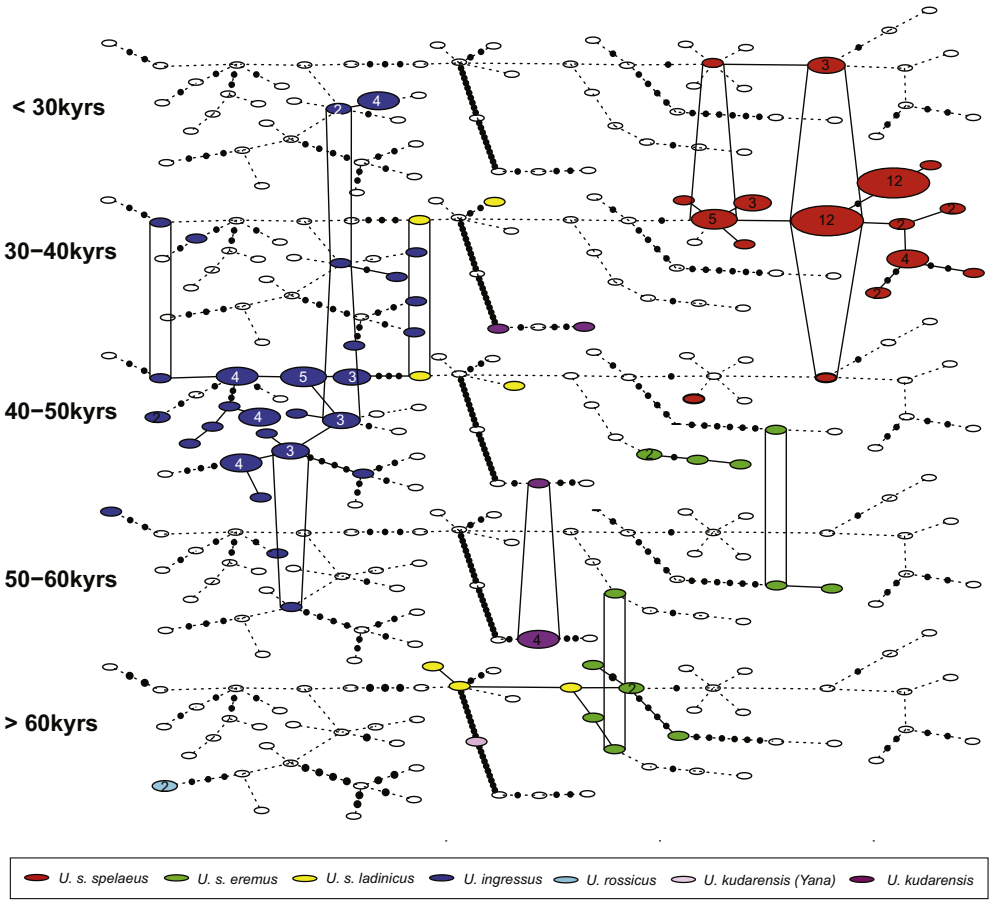


Fig. 2. Temporal haplotype network displaying the relationships of cave bear haplotypes through time. Haplotypes are represented by ellipses. The number of sequences sharing the same haplotype is indicated by the numbers in the ellipses (only numbers greater than 1 are shown). Small white ellipses indicate the absence of a haplotype that is otherwise found in a different time period. Shared haplotypes between time-points are connected by two vertical lines. Within each time period, haplotypes are connected by a line if they are separated by one mutation; each additional mutation is indicated by a small black dot. Red: *U. s. spelaeus*; green: *U. s. eremus*; yellow: *U. s. ladinicus*; blue: *U. ingressus*; cyan: *U. rossicus*; rose: *U. kudarensis (Yana)*; purple: *U. kudarensis*.

96,392 years (Baumann's cave, Germany), while Eastern European samples (including Balkan samples) range from 26,900 (Potocka Zijalka, Slovenia) to 100,648 years (Kizel Cave, Ural Mountains).

Some of the cave bear species/subspecies appear only within limited time frames. The oldest accession of *U. s. spelaeus* was only 40,000–45,000 years old (from Scladina, Belgium). None of the 14 samples assigned to *U. s. eremus* and only 1 of 7 *U. s. ladinicus* samples (Grotte Merveilleuse, 35,610 years) are younger than 40,000 years. Similarly, both *U. rossicus* accessions were estimated to be around 100,000 years old.

3.3. Population dynamics and extinction

The temporal network was constructed with time slices of 10,000 years. It shows temporal continuity for a few major haplotypes belonging to *U. s. spelaeus*, and *U. ingressus* (Fig. 2). Haplotypes of *U. s. eremus*, *U. s ladinicus*, *U. rossicus* and *U. kudarensis* are more short-lived; however relatively few samples have been identified with these haplotypes. Haplotype diversity drops significantly about 30,000 years ago, with only four different haplotypes being younger than 30,000 years. The highest diversity can be observed between 30,000 and 50,000 years ago (25 different haplotypes from 30,000 to 40,000 years ago; 24 different haplotypes from 40,000 to 50,000 years ago, Fig. 2). For the eastern *U. ingressus*, the highest diversity was identified from 40,000 to 50,000 years ago with a sharp drop in more recent times. The greatest haplotype diversity of *U. s. spelaeus* in our study was found from 30,000 to 40,000 years ago.

4. Discussion

The sequence data we have obtained for this study cover only a short fragment of the mitochondrial control region and our results are inevitably associated with relatively large error margins. Nevertheless, our results provide new insights and testable hypotheses regarding cave bear phylogeography and extinction. Our study aims at encouraging discussion and further, more detailed research into cave bears based on the new results presented here.

4.1. Cave bear phylogeography and evolution

The phylogenetic relationships of cave bears across their range have been discussed in numerous publications (Rabeder et al., 2004; Knapp et al., 2009; Stiller et al., 2009; Baryshnikov and Puzachenko, 2011; Sher et al., 2011). Based on morphological features, cave bears were subdivided into at least 6 different groups, including *U. s. spelaeus*, *U. s. eremus*, *U. s. ladinicus*, *U. ingressus*, *U. rossicus* and *U. kudarensis* (Hofreiter et al., 2004; Rabeder and Hofreiter, 2004; Baryshnikov and Puzachenko, 2011), all of which are represented by samples in this study. The taxonomic status of

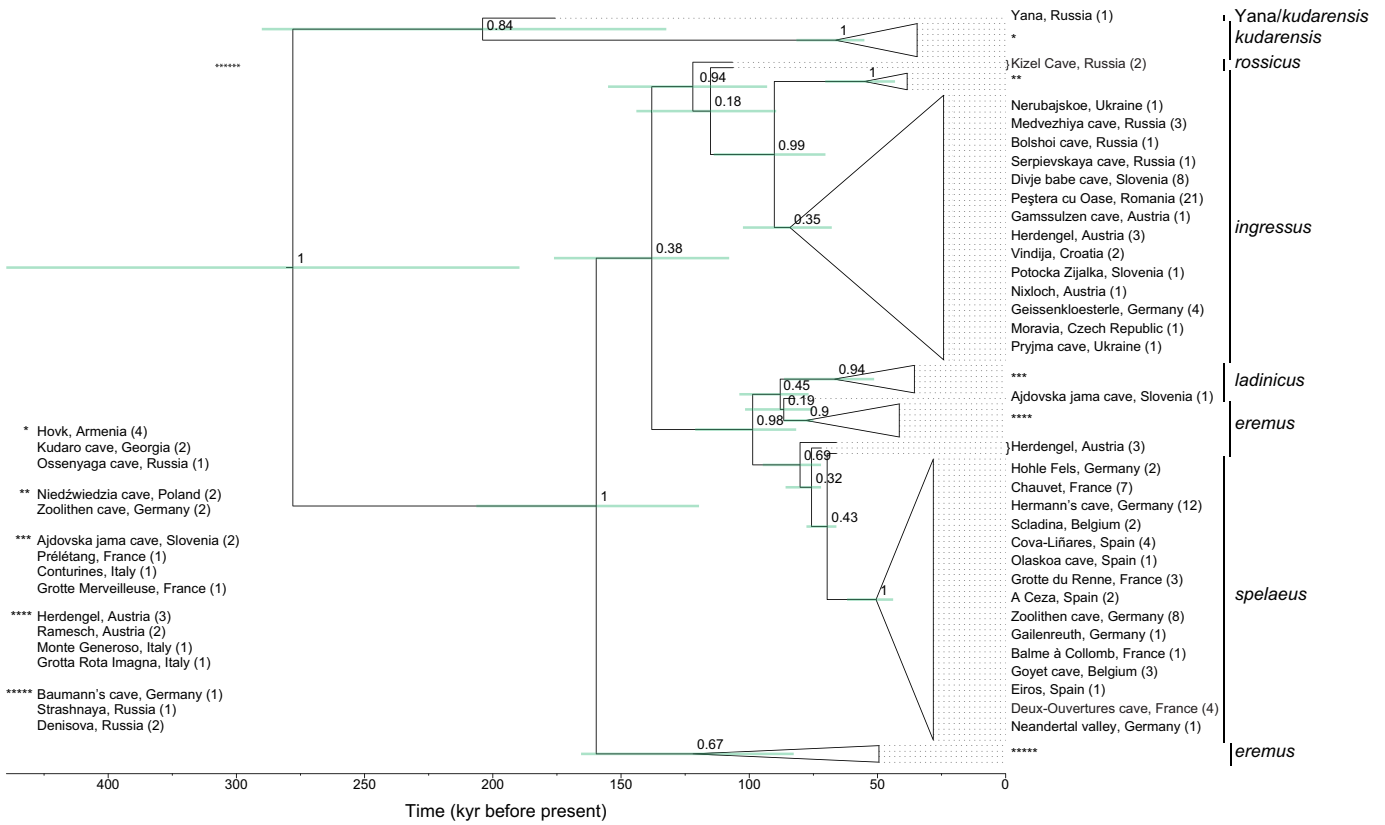


Fig. 3. Bayesian estimate of the cave bear phylogeny, drawn to a timescale. The horizontal time axis is in kiloyears before present. Numbers of samples are given in parentheses. The position of the root was estimated by molecular clock rooting.

the morphologically distinct groups is controversial and it has been argued that they may represent different species (Hofreiter et al., 2004; Rabeder and Hofreiter, 2004), but also that they are not even distinct enough to warrant subspecies status (Baryshnikov and Puzachenko, 2011). The genetic data are consistent with the principal classification above, albeit with variable support. *U. spelaeus* (including *spelaeus*, *ladinicus* and *eremus*), *U. ingressus* (together with *U. rossicus*), and *U. kudarensis* form clades that are consistent with reproductive isolation among the haplogroups. However similar mitochondrial population structure in the brown bear (for example Hirata et al., 2013) warrants caution when identifying putative species status for distinct cave bear haplogroups. As mitochondrial data alone is insufficient to resolve this long-standing question, we will not speculate on the species status of these haplogroups.

Consistent with recent findings of Dabney et al. (2013), [our data suggest that the three main haplogroups found in Late Pleistocene cave bears (*spelaeus*, *ingressus*, *kudarensis*) were already present as early as the Middle Pleistocene (Fig. 3) and that initially their representatives were most likely widely distributed. Interestingly, we found *ladinicus*, *eremus* and *rossicus* haplotypes only in a few samples that were either quite old (*eremus* from Baumann's cave) or from mountain regions. In fact, in our data, there is very little temporal overlap between *U. s. eremus* and *U. s. ladinicus* (mostly older than 40,000 years) and *U. s. spelaeus* (mostly younger than 40,000 years) (Fig. 2; Table S1). Similarly, our two *U. rossicus* samples are substantially older than all our *U. ingressus* samples. Moreover, the specimens from which the older haplotypes were obtained tend to be smaller (*rossicus*) or morphologically more archaic (*ladinicus*) than those from which *ingressus* or *spelaeus* (sensu stricto) sequences were obtained. Although currently speculative, this pattern may indicate that the classical Late Pleistocene cave bear morphology developed locally, possibly two times independently, and that these populations replaced more archaic cave bear populations, including the associated haplotypes, except in remote and/or high altitude areas. Further studies, combining carbon dating, morphological analyses and DNA sequencing of both mitochondrial and nuclear DNA are needed to reveal the details of cave bear evolution.

U. kudarensis sequences were only found in the Caucasus and the Yana River region in Eastern Siberia. In the Caucasus, the earliest findings of cave bears similar in the tooth morphology to *U. kudarensis* are from the Kudaro caves, dating to nearly 400 ky BP (Baryshnikov, 1998, 2006). An earlier subspecies, *U. k. praekudarensis* from the Middle Pleistocene reveals a successive morphological transition to *U. k. kudarensis* from the Late Pleistocene, suggesting a direct relationship as ancestor and descendant. The presence of cave bears genetically resembling *U. kudarensis* in the northern parts of Siberia may be explained by a wide distribution of representatives of this haplogroup in Northern Asia during the Middle Pleistocene and a successive reduction in distribution range during the Late Pleistocene, eventually becoming restricted to the Southern Caucasus and adjacent territories.

4.2. Cave bear population dynamics

Recent studies have provided new insights into the population and extinction dynamics of cave bears. By radiocarbon dating a large number of cave bears from Europe and the Ural mountains, Pacher and Stuart (2009) and Münzel et al. (2011) were able to show that, in contrast to previous beliefs (Kurtén, 1976; Musil, 1980), cave bears went extinct already around 24,000

radiocarbon years ago (about 27,800 cal. BP) rather than towards the end of the last glacial period about 13,000 years ago. We recently used mtDNA sequences to reconstruct the population dynamics of cave bears through time and found evidence for a population decline that started more than 25,000 years prior to their extinction (Stiller et al., 2010). Our new data confirm these results (Fig. S2) and shed further light on the last 30,000 years before cave bear extinction. Most notably, they indicate that cave bear populations may have declined from east to west. As in a previous study by Bon et al. (2011), all samples less than 30,000 years old were found in Western Europe or the Balkans. It should be noted that Pacher et al. (2009) reported several samples from the Ural Mountains that were between 30,000 and 37,000 years old. We estimated one of the Polish samples by Baca et al. (2012) to be 37,590 years old and the youngest dated sample from the Caucasus dates to 34,550 ^{14}C years (Table S1), but as in previous studies (e.g. Pacher and Stuart, 2009), the vast majority of our Eastern European samples are older than 40,000 years.

Figs. 1 and 2 illustrate the significant loss of genetic diversity and range in samples less than 30,000 year old, a pattern observed previously (Bon et al., 2011). A retraction from eastern habitats towards the onset of the last glacial maximum, resulting in a range shift to the west, would certainly be consistent with observations that eastern *U. ingressus* haplotypes replaced the western *U. spelaeus* haplotypes in the Ach Valley at the eastern margin of the *U. s. spelaeus* range about 28,000 ^{14}C years ago (ca. 32,500 cal. BP; Hofreiter et al., 2007; Münzel et al., 2011), but the lineage of *U. s. spelaeus* persisted at least 3000 years longer in Eastern France (Bocherens et al., 2014). Our data show a similar replacement, albeit with less temporal resolution, for cave bears from Herdengel (Austria). All samples from this cave were dated by stratigraphic context and assigned to either *U. s. eremus* or *U. ingressus*. According to their stratigraphic position, all *eremus* samples included in our data set are older than 60,000 years while all included *ingressus* samples from this site are younger than 37,000 years. However, it should be noted that, when we include samples for which only part of the mtDNA fragment analysed here could be amplified, the two groups overlap to some extent in the stratigraphy. The Zoolithen cave represents a third cave in which both *U. ingressus* and *U. s. spelaeus* mtDNA sequences were observed (nine *U. s. spelaeus* samples and two *U. ingressus* samples), but here the *U. s. spelaeus* samples are consistently younger than the *U. ingressus* samples (Tables S1 and S2). Table S1 also shows the absence of *U. ingressus* samples greater than 50,000 year old from the Ural Mountains. This could be a result of limited sample numbers from Eastern Europe, but it could also suggest that the range of *U. ingressus* extended east only temporarily at the time of highest diversity between 40,000 and 50,000 years ago (Baca et al., 2012).

Our analyses revealed two particularly interesting relationships, connecting samples that are both geographically and temporally very distant from each other. The sample from the Yana River region in Siberia was found to be most closely related to the *kudarensis* cave bears from the Caucasus region many thousands of kilometres to the west. The individual from the Yana River was assigned a mid-Pleistocene age from its stratigraphic context by Sher et al. (2011). This is consistent with our molecular estimates (mean: 178,300 years), making this sample much older than the Late Pleistocene samples from the Caucasus to which it is related. Similarly, the oldest Western European sample from Baumann's cave (Germany; mean: 96,392 years) was most closely related to three individuals from the Altai region. Although, as discussed above, these results are suggestive of a formerly wider distribution of the different haplogroups, given the small number of samples from both taxonomic groups, it is impossible to say whether this was indeed the case or whether the observed patterns are instead the result of long-distance migrations and/or range shifts.

Given the wide error margins of our molecular age estimates, additional radiocarbon dating will be required to test the hypotheses proposed above. If shown to be correct, a habitat retraction from east to west would have implications for our understanding of cave bear extinction. It would be highlighting the increasingly continental climate in Europe towards the last glacial maximum as a potential stressor, as has already been suggested by Pacher and Stuart (2009). We have previously suggested that climate alone was not responsible for the extinction of the cave bear and that competition for cave sites between cave bears and an increasing human population was critical factor (Stiller et al., 2010), but our hypothesis also allows for an influence of climate change. Bocherens et al. (2014) suggested a fragmentation of cave bear populations in the northwestern Alpine foreland before the final extirpation of the species in this region, while stable isotope data showed no evidence of ecological change prior to extinction. Furthermore, there is evidence of human hunting of cave bears, which could also have had a significant negative influence on the dwindling populations (Münzel and Conard, 2004; Germonpré and Hämäläinen, 2007; Münzel et al., 2011). A shrinking of the habitat in the east would suggest that cave bears did follow the more marine climate to the west and avoided the increasingly continental climates in the east. It would also mirror the movement of other large mammals adapted to more temperate climates, such as horses (Lorenzen et al., 2011), but more data are clearly required to test the hypothesis of an east-to-west extinction of cave bears. In addition, ecological tracking through stable isotope analyses will provide further possibilities to test the impact of environmental changes on the evolution of cave bear populations in different parts of their distribution (e.g. Bocherens et al., 1994; Bocherens et al., 2011).

Our study suggests that there is still a lot to learn about cave bear biology and extinction. Using molecular tools to estimate the age of previously undated samples, including samples that are beyond the reach of radiocarbon dating, we were able to get a first glimpse at how the range of cave bears changed through time. While these new insights are only tentative because of the small amount of DNA sequence data available for our analyses, they do affect our understanding of the effects of climate change on cave bear populations. Importantly, our study provides new hypotheses, which can hopefully be tested in the near future. Additional (nuclear) DNA and radiocarbon data, in combination with new statistical tools, may finally allow us to reconstruct the evolution as well as the complex of factors that led to the extinction of the cave bear.

Acknowledgements

The study was funded by the Max Planck Society. MS is supported by the National Science Foundation Award #0909456. MM is supported by the University of Sydney International Scholarship. MK was and SP is supported by the Allan Wilson Centre for Molecular Ecology and Evolution and the University of Otago. AGD was funded by the Spanish Ministry of Science and Innovation CICYT – CGL2011-30141. SYWH is supported by the Australian Research Council. We thank Grit Schubert for assistance in the lab.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.quaint.2013.09.023.

References

Baca, M., Stankovic, A., Stefaniak, K., Marciszak, A., Hofreiter, M., Nadachowski, A., Weglenski, P., Mackiewicz, P., 2012. Genetic analysis of cave bear specimens from Niedzwiedzia Cave, Sudetes, Poland. Palaeontological Electron 15.

Barnosky, A.D., Koch, P.L., Feranec, R.S., Wing, S.L., Shabel, A.B., 2004. Assessing the causes of Late Pleistocene extinctions on the continents. Science 306, 70–75.

Baryshnikov, G., 1998. Cave bears from the Paleolithic of the Greater Caucasus. In: Saunders, J.J., Styles, B.W., Baryshnikov, G.F. (Eds.), Quaternary Paleozoology in the Northern Hemisphere, Illinois State Museum Scientific Papers, vol. XXVII. Springfield.

Baryshnikov, G., 2006. Morphometrical Variability of Cheek Teeth of Cave Bears. In: Sci. Annals, School Geol. Aristotle Univ. Thessaloniki.

Baryshnikov, G., 2007. Bears Family (Carnivora, Ursidae), Fauna of Russia and Neighbouring Countries. Nauka Press, St Petersburg, Russia (in Russian).

Baryshnikov, G., Foronova, I., 2001. Pleistocene small cave bear (*Ursus rossicus*) from the South Siberia, Russia Cadernos Lab. Xeolóxico Laxe Coruña 26, 373–398.

Baryshnikov, G.F., Puzachenko, A.Y., 2011. Craniometrical variability in the cave bears (Carnivora, Ursidae): multivariate comparative analysis. Quaternary International 245, 350–368.

Bocherens, H., Bridault, A., Drucker, D.G., Hofreiter, M., Münzel, S.C., Stiller, M., van der Plicht, J., 2014. The last of its kind? Radiocarbon, ancient DNA and stable isotope evidence from a late cave bear from Rochedane (France). Quaternary International 339-340, 179–188.

Bocherens, H., Fizet, M., Mariotti, A., 1994. Diet, physiology and ecology of fossil mammals as inferred from stable carbon and nitrogen isotope biogeochemistry – implications for pleistocene bears. Palaeogeography Palaeoclimatology Palaeoecology 107, 213–225.

Bocherens, H., Stiller, M., Hobson, K.A., Pacher, M., Rabeder, G., Burns, J.A., Tütken, T., Hofreiter, M., 2011. Niche partitioning between two sympatric genetically distinct cave bears (*Ursus spelaeus* and *Ursus ingressus*) and brown bear (*Ursus arctos*) from Austria: isotopic evidence from fossil bones. Quaternary International 245, 238–248.

Bon, C., Berthonaud, V., Fosse, P., Gely, B., Maksud, F., Vitalis, R., Philippe, M., van der Plicht, J., Elalouf, J.M., 2011. Low regional diversity of late cave bears mitochondrial DNA at the time of Chauvet Aurignacian paintings. Journal of Archaeological Science 38, 1886–1895.

Dabney, J., Knapp, M., Glocke, I., Gansauge, M.-T., Weihmann, A., Nickel, B., Valdiosera, C., Garcia, N., Pääbo, S., Arsuaga, J.-L., Meyer, M., 2013. A complete mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultra-short DNA fragments. Proceedings of the National Academy of Sciences of the United States of America. Early online: www.pnas.org/cgi/doi/10.1073/pnas.1314445110.

Drummond, A., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7, 214.

Germonpré, M., Hämäläinen, R., 2007. Fossil bear bones in the Belgian Upper Palaeolithic: the possibility of a proto-bear ceremonialism. Arctic Anthropology 44, 1–30.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 44, 211–232.

Hänni, C., Laudet, V., Stehelin, D., Taberlet, P., 1994. Tracking the origins of the cave bear (*Ursus spelaeus*) by mitochondrial DNA sequencing. Proceedings of the National Academy of Sciences of the USA 91, 12336–12340.

Hirata, D., Mano, T., Abramov, A.V., Baryshnikov, G.F., Kosintsev, P.A., Vorobiev, A.A., Raichev, E.G., Tsunoda, H., Kaneko, Y., Murata, K., Fukui, D., Masuda, R., 2013. Molecular phylogeography of the brown bear (*Ursus arctos*) in Northeastern Asia based on analyses of complete mitochondrial DNA sequences. Molecular Biology and Evolution 30, 1644–1652.

Ho, S.Y.W., Lanfear, R., Phillips, M.J., Barnes, I., Thomas, J.A., Kolokotronis, S.O., Shapiro, B., 2011. Bayesian estimation of substitution rates from ancient DNA sequences with low information content. Systematic Biology 60, 366–374.

Hofreiter, M., Capelli, C., Krings, M., Waits, L., Conard, N., Münzel, S., Rabeder, G., Nagel, D., Paunovic, M., Jambresic, G., Meyer, S., Weiss, G., Pääbo, S., 2002. Ancient DNA analyses reveal high mitochondrial DNA sequence diversity and parallel morphological evolution of late pleistocene cave bears. Molecular Biology and Evolution 19, 1244–1250.

Hofreiter, M., Jaenicke, V., Serre, D., Haeseler Av, A., Pääbo, S., 2001. DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. Nucleic Acids Research 29, 4793–4799.

Hofreiter, M., Münzel, S., Conard, N.J., Pollack, J., Slatkin, M., Weiss, G., Pääbo, S., 2007. Sudden replacement of cave bear mitochondrial DNA in the late Pleistocene. Current Biology 17, R122–R123.

Hofreiter, M., Rabeder, G., Jaenicke-Despres, V., Withalm, G., Nagel, D., Paunovic, M., Jambresic, G., Pääbo, S., 2004. Evidence for reproductive isolation between cave bear populations. Current Biology 14, 40–43.

Kahlke, R.-D., 1994. Die Entstehungs-, Entwicklungs- und Verbreitungsgeschichte des oberpleistozänen Mammuthus-Coelodonta-Faunenkomplexes in Eurasien (Großsäuger). Abhandlungen der Senckenbergischen Naturforschenden Gesellschaft 546, 1–164.

Knapp, M., Rohland, N., Weinstock, J., Baryshnikov, G., Sher, A., Nagel, D., Rabeder, G., Pinhasi, R., Schmidt, H.A., Hofreiter, M., 2009. First DNA sequences from Asian cave bear fossils reveal deep divergences and complex phylogeographic patterns. Molecular Ecology 18, 1225–1238.

Kurtén, B., 1968. Pleistocene Mammals of Europe. Weidenfeld and Nicolson, London.

Kurtén, B., 1976. The Cave Bear Story. Life and Death of a Vanished Animal. Columbia University Press, New York.

Lorenzen, E.D., Nogues-Bravo, D., Orlando, L., Weinstock, J., Binladen, J., Marske, K.A., Ugan, A., Borregaard, M.K., Gilbert, M.T.P., Nielsen, R., Ho, S.Y.W., Goebel, T., Graf, K.E., Byers, D., Stenderup, J.T., Rasmussen, M., Campos, P.F., Leonard, J.A., Koepfli, K.P., Froese, D., Zazula, G., Stafford, T.W., Aaris-Sorensen, K., Batra, P., Haywood, A.M., Singarayer, J.S., Valdes, P.J., Boeskorov, G., Burns, J.A., Davydov, S.P., Haile, J., Jenkins, D.L., Kosintsev, P., Kuznetsova, T., Lai, X.L., Martin, L.D., McDonald, H.G., Mol, D., Meldgaard, M., Munch, K., Stephan, E., Sablin, M., Sommer, R.S., Sipko, T., Scott, E., Suchard, M.A., Tikhonov, A., Willerslev, R., Wayne, R.K., Cooper, A., Hofreiter, M., Sher, A., Shapiro, B., Rahbek, C., Willerslev, E., 2011. Species-specific responses of Late Quaternary megafauna to climate and humans. Nature 479, 359–364.

Martin, P.S., Steadman, D.W., 1999. Prehistoric extinctions on islands and continents. In: MacPhee, R.D.E. (Ed.), Extinctions in Near Time: Causes, Contexts and Consequences. Kluwer Academic/Plenum, New York, pp. 17–55.

Münzel, S.C., Conard, N.J., 2004. Cave bear hunting in the Hohle Fels, a cave site in the Ach Valley, Swabian Jura. Revue de Paleobiologie 23, 877–885.

Münzel, S.C., Stiller, M., Hofreiter, M., Mittnik, A., Conard, N.J., Bocherens, H., 2011. Pleistocene bears in the Swabian Jura (Germany): Genetic replacement, ecological displacement, extinctions and survival. Quaternary International 245, 225–237.

Musil, R., 1980. Ursus spelaeus - Der Höhlenbär. Museum für Ur- und Frühgeschichte Thüringens, Weimar.

Pacher, M., Stuart, A.J., 2009. Extinction chronology and palaeobiology of the cave bear (Ursus spelaeus). Boreas 38, 189–206.

Pacher, M., Stuart, A.J., Baryshnikov, G., Stiller, M., Kosintsev, P., Vorobiev, A., 2009. Cave bears of the Ural Mountains – a survey based on direct radiocarbon dates, aDNA and morphometrical analysis. In: Sabol, M. (Ed.), 15th International Cave Bear Symposium. Department of Geology & Palaeontology. Faculty of Natural Sciences, Comenius University in Bratislava, Slovak Republic, Spišská Nová Ves, Slovakia, pp. 14–17.

Prost, S., Anderson, C.N.K., 2011. TempNet: a method to display statistical parsimony networks for heterochronous DNA sequence data. Methods in Ecology and Evolution 2, 663–667.

Rabeder, G., 1995. Evolutionsniveau und Chronologie der Höhlenbären aus der Gamssulzen-Höhle im Toten Gebirge (Oberösterreich). Mitteilungen der Kommission für Quartärforschung 9, 69–81.

Rabeder, G., 1999. Die Evolution des Höhlenbärgebisses. Verlag der Österreichischen Akademie der Wissenschaften, Wien.

Rabeder, G., Debeljak, I., Hofreiter, M., Withalm, G., 2008. Morphological responses of cave bears to high-alpine habitats. Die Höhle 59, 59–72.

Rabeder, G., Hofreiter, M., 2004. Der neue Stammbaum der alpinen Höhlenbären. Die Höhle 55, 58–77.

Rabeder, G., Hofreiter, M., Withalm, G., 2004. The systematic position of the Cave Bear from Potočka zijalka (Slovenia). Mitteilungen der Kommission für Quartärforschung der Österreichischen Akademie der Wissenschaften 13, 197–200.

Rabeder, G., Nagel, D., Pacher, M., 2000. Der Höhlenbär. Jan Thorbecke Verlag, Stuttgart.

Ramsden, C., Holmes, E.C., Charleston, M.A., 2009. Hantavirus evolution in relation to its rodent and insectivore hosts: no evidence for codivergence. Molecular Biology and Evolution 26, 143–153.

Rohland, N., Hofreiter, M., 2007a. Ancient DNA extraction from bones and teeth. Nature Protocols 2, 1756–1762.

Rohland, N., Hofreiter, M., 2007b. Comparison and optimization of ancient DNA extraction. Biotechniques 42, 343–352.

Römpler, H., Dear, P.H., Krause, J., Meyer, M., Rohland, N., Schöneberg, T., Spriggs, H., Stiller, M., Hofreiter, M., 2006. Multiplex amplification of ancient DNA. Nature Protocols 1, 720–728.

Shapiro, B., Ho, S.Y.W., Drummond, A.J., Suchard, M.A., Pybus, O.G., Rambaut, A., 2011. A Bayesian phylogenetic method to estimate unknown sequence ages. Molecular Biology and Evolution 28, 879–887.

Sher, A.V., Weinstock, J., Baryshnikov, G.E., Davydov, S.P., Boeskorov, G.G., Zazhigin, V.S., Nikolskiy, P.A., 2011. The first record of "spelaeoid" bears in Arctic Siberia. Quaternary Science Reviews 30, 2238–2249.

Stiller, M., Baryshnikov, G., Bocherens, H., d'Anglade, A.G., Hilpert, B., Münzel, S.C., Pinhasi, R., Rabeder, G., Rosendahl, W., Trinkaus, E., Hofreiter, M., Knapp, M., 2010. Withering away-25,000 years of genetic decline preceded cave bear extinction. Molecular Biology and Evolution 27, 975–978.

Stiller, M., Knapp, M., Stenzel, U., Hofreiter, M., Meyer, M., 2009. Direct multiplex sequencing (DMPS)–a novel method for targeted high-throughput sequencing of ancient and highly degraded DNA. Genome Research 19, 1843–1848.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596–1599.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10, 512–526.