

## Cave bears and ancient DNA: a mutually beneficial relationship

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### Abstract

For almost 30 years, cave bears and paleogenetic research have shared a mutually beneficial relationship. Due to the abundance and frequently good preservation of cave bear bones, they have often been the tissue of choice to develop and test molecular approaches aimed at recovering and sequencing DNA from ancient remains. Our understanding of cave bear biology has similarly profited from the molecular data produced through paleogenetic studies. DNA data has complemented morphological data to provide insights into the evolution and phylogeny of cave bears. Molecular population dynamic studies have helped develop hypotheses explaining the extinction of cave bears, and new genomic data is now promising to shed light on evolutionary and population genetic processes that could previously only be obtained from living species. Here we evaluate and review the role cave bears have played in the development of paleogenetic research as well as the role that paleogenetic research has had in understanding cave bear biology. We provide a perspective on where this mutually beneficial relationship is likely to take us in the near future.

### Zusammenfassung

Seit fast 30 Jahren verbindet die Höhlenbären- und paläogenetische Forschung eine, für beide Seiten vorteilhafte, Beziehung. Aufgrund der Fülle und häufig guten Erhaltung von Höhlenbär-Knochen waren sie häufig das Material der Wahl, um molekulare Ansätze zur Extraktion und Sequenzierung von DNA aus Fossilien zu entwickeln und zu testen. Unser Verständnis der Biologie des Höhlenbären hat in ähnlicher Weise von den molekularen Daten aus paläogenetischen Studien profitiert. DNA-Daten haben morphologische Daten ergänzt, um Einblicke in die Evolution und Phylogenie des Höhlenbären zu erhalten. Molekulare Studien zur Populationsdynamik haben dazu beigetragen, Hypothesen zum Aussterben der Höhlenbären zu entwickeln und neue Genomdaten versprechen nun Aufschluss über evolutionäre und populationsgenetische Prozesse, die bisher nur an noch lebenden Arten untersucht werden konnten. In diesem Artikel untersuchen und diskutieren wir die Rolle, die Höhlenbären bei der Entwicklung der paläogenetischen Forschung gespielt haben, wie auch die Rolle, die die paläogenetische Forschung beim Verständnis der Biologie der Höhlenbären gespielt hat. Es wird ein Überblick darüber gegeben, wohin uns diese, für beide Seiten vorteilhafte, Beziehung in naher Zukunft führen wird.

**Key words:** *Ursus spelaeus*, megafaunal extinction, molecular palaeontology, cave bear taxonomy, paleogenomics, paleogenetics

## Introduction

The study of ancient DNA, i.e. DNA mostly derived from subfossil bones less than 1 million years old, has fascinated scientists and the public alike ever since the first reports on its feasibility were published more than 30 years ago (HIGUCHI, BOWMAN, FREIBERGER, RYDER & WILSON, 1984). By now, DNA data from thousands of specimens have been published, ranging from short snippets of mitochondrial DNA to multifold coverage of the nuclear genome. These data have been used to address a variety of questions, including identification of samples to species (DALÉN et al., 2017), phylogenetic relationships of extinct species to their extant relatives (DABNEY et al., 2013, KRAUSE et al., 2006, MEYER et al., 2012), identification of morphological traits and their genetic basis, especially traits that do not fossilize (BURGER, KIRCHNER, BRAMANTI, HAAK & THOMAS, 2007, FORTES, SPELLER, HOFREITER & KING, 2013, OLALDE et al., 2014, RÖMPLER et al., 2006), and various population genetic analyses (SHAPIRO et al., 2004), such as changes in genetic diversity over time (STILLER et al., 2013), gene flow (BARLOW, CAHILL et al., 2018) and population replacements (COLLINS et al., 2014, HOFREITER et al., 2007; RAWLENCE et al., 2017).

However, despite the wide range of research that can be conducted using ancient DNA and the spectacular successes obtained through it, retrieving DNA sequences from fossils is not trivial. DNA degrades rapidly in the fossil record, resulting in three main obstacles for analysis. First, many bones simply contain no DNA of interest (i.e. that from the individual a bone originates from, also called “endogenous DNA”). The older a bone and the warmer the climate in the place it originates from, the less likely it is that a sample still contains DNA (PÄÄBO et al., 2004). The age limit for aDNA retrieval at the moments lies around 400,000 years (DABNEY et al., 2013) outside the permafrost and around 700,000 years for permafrost samples (ORLANDO et al., 2013). Second, even if DNA is preserved, often the endogenous DNA is far rarer than DNA originating from microorganisms that have lived on the bone any time between the death of an animal and the analysis of the bone. And third, ancient DNA molecules are short, often in the range of 30-50 basepairs (KISTLER WARE, SMITH, COLLINS & ALLABY, 2017, ROHLAND, GLOCKE, AXIMU-PETRI & MEYER, 2018) meaning puzzling together useful data sets is a major task even though this is now done by computer programs. In fact, modern high-throughput sequencing datasets contain so many short DNA sequencing reads that assembling them into their respective genome regions can often not be achieved without very powerful “super” computers.

So clearly some issues that need paying attention to if ancient DNA is analysed. Providing this is done, successful ancient DNA analysis are, as in any scientific endeavour, then simply a combination of asking the right questions, obtaining the right samples (as much as this is possible to know in

advance), applying the right methods for analysis (both in the laboratory and at the computer) and having the required amount of luck. Cave bears are a particularly suitable species for ancient DNA analyses, which has to do mainly with where and how they lived. Cave bears probably represent the Pleistocene species with the richest fossil record of all. This is mainly the case since they used karstic caves for hibernation, which means their bones can be found in large numbers and are usually well preserved. They also went extinct only about 27,000 years ago (PACHER & STUART, 2009) and diverged from their sister species, the common ancestor of brown bears and polar bears between 1.2 and 1.6 million years ago (KNAPP et al., 2009), meaning that a substantial part of their evolutionary history falls in the time range for which ancient DNA analyses are possible. Furthermore, within the family Ursidae, there are several quite closely related species, most of which are more or less well studied genetically and genomically, so that comparative genetic data are available (BARLOW, CAHILL et al., 2018, CAHILL et al., 2013). And finally, the cave bear is a well-known and charismatic species, and its evolution as well as the causes that led to its demise are of interest not only to scientists but also to the broader public.

### **Part 1: Contributions of cave bears to ancient DNA**

Our understanding of cave bear evolutionary history has benefitted tremendously from ancient DNA analyses, but at the same time, ancient DNA as a research field has also benefitted tremendously from analyses of cave bear samples. Thus, the first short DNA segments analysed from cave bears and published in 1994 (HÄNNI, LAUDET, STEHELIN, TABERLET, 1994) also represented the first reproducible Pleistocene DNA sequences obtained for any species from outside the permafrost. Although alleged sabretooth cat sequences had been published in 1992 (JANCZEWSKI, YUHKI, GILBERT, JEFFERSON & O'BRIEN, 1992), these have been shown to be incorrect (BARNETT et al., 2005) and are now widely accepted to represent contamination with modern DNA from the lab, in which the analyses were performed.

Cave bears also hold the records for the oldest sample outside of permafrost from which DNA has been firstly recovered, and subsequently sequenced. Both records go back to the same bone, a 400,000 year old cave bear bone from the Spanish site Sima de los Huesos (DABNEY et al., 2013). A 2006 study had already managed to type single nucleotide polymorphisms of the mitochondrial genome from this sample, an achievement clearly ahead of its time (VALDIOSERA et al., 2006). The data was later confirmed by sequencing of the entire mitochondrial genome from this specimen using next generation sequencing in combination with DNA hybridization capture in 2013 (DABNEY et al., 2013).

Moreover, the first Pleistocene mitochondrial genome from samples outside the permafrost was also obtained from a cave bear (KRAUSE et al., 2008). In fact, since one of the current authors was principal

investigator on this study, we are able to point out that the cave bear mitochondrial genome was in fact the first Pleistocene mitochondrial genome obtained ever, even predating the mammoth (KRAUSE et al., 2006) that was published earlier. Publication of the cave bear mitochondrial genome was simply delayed by a lack of comparative data from the related species at the time, and obtaining these required substantial time. Even earlier, studies on larger sets of cave bear bones demonstrated both the feasibility and richness of insights that can be achieved using Pleistocene population genetics based on mitochondrial DNA (HOFREITER et al., 2002, 2007, 2004, LOREILLE et al., 2001, ORLANDO et al., 2002).

Cave bear fossils were not only instrumental in development of mitochondrial DNA studies, the first Pleistocene nuclear DNA sequences were also obtained from a cave bear bone (GREENWOOD, CAPELLI, POSSNERT & PÄÄBO, 1999) as was the first genomic study from ancient DNA (NOONAN et al., 2005). Thus, it was cave bears that started the field of paleogenomics. Within this field of research, thanks to the above mentioned advantages, cave bears continue to play an important role, for example as the first undisputed species for which partial genomic survival in an extant relative, the brown bear, has been demonstrated (BARLOW, CAHILL et al., 2018).

Finally, thanks to their abundance, cave bear fossils have played a pivotal role in the development of molecular techniques for ancient DNA, especially in improving DNA extraction techniques for ancient DNA (DABNEY et al., 2013; ROHLAND & HOFREITER, 2007, ROHLAND, SIEDEL & HOFREITER, 2009). More recently, cave bear bones have been used to develop CT-scanning as a technology for choosing the best region of a bone for ancient DNA sampling (ALBERTI et al., 2018).

Thus, it is no exaggeration to state that cave bear fossils have been absolutely instrumental for progress and numerous – both technical and conceptual – developments in ancient DNA research.

## **Part 2: Contributions of ancient DNA to cave bear biology and evolution**

Just like cave bears have contributed greatly to progress in ancient DNA research, paleogenetic data has had an impact on our understanding of cave bear ecology, biogeography and evolution.

While early genetic analyses of cave bear remains were limited by the amount of DNA sequence data that could be obtained using traditional Sanger sequencing technology, they have been useful in complementing and evaluating information retrieved from morphological analyses. For example, cave bears across Late Pleistocene Europe were phenotypically diverse. Based on morphological data, they were subdivided into at least six different groups, *Ursus spelaeus spelaeus*, *U. s. eremus*, *U. s. ladinicus*, *U. ingressus*, *U. rossicus* and *U. kudarensis* (BARYSHNIKOV & PUZACHENKO, 2011, HOFREI-

TER et al., 2004, RABEDER & HOFREITER 2004). Interpretations of this diversity reached from all types belonging to a single, phenotypically diverse species to cave bears representing multiple different species.

A recent publication (STILLER et al., 2013) summarized previously published and new mitochondrial control region data from 142 cave bear samples representing all of the six different groups listed above. Their phylogenetic reconstruction of relationships between the different groups of cave bears showed that samples identified as *Ursus s. spelaeus*, *U. s. ladinicus*, *U. ingressus*, *U. rossicus* and *U. kudarensis*, respectively, belonged to distinct, reciprocally monophyletic lineages. *Ursus s. eremus*, on the other hand, could not be confirmed as single lineage. Different samples were found to either form a sister clade to just the *U. s. spelaeus* and *U. s. ladinicus* clades or a much earlier diverging sister clade to all European cave bear lineages. The latter placement was confirmed by more recent nuclear genome studies, which also recovered a sister group relationship of *spelaeus* and *ingressus* (BARLOW, CAHILL et al., 2018), in accordance with morphological data (BARYSHNIKO & PUZACHENKO, 2011), suggesting that conflicting mitochondrial data may be a result of incomplete lineage sorting and / or introgression, a phenomenon also observed in polar bears and brown bears (CAHILL et al., 2015).

While these results are consistent with the different cave bear groups forming different subspecies or even species, the conflicting data highlights that studies of mitochondrial lineages alone are not suitable to unequivocally confirm reproductive isolation and therefore species status of the different groups. Mitochondrial DNA is maternally inherited only, precluding any formal evaluation of gene flow or admixture between groups or individuals.

Nevertheless, taken together with morphological data, mitochondrial phylogenies can yield information which, if not unequivocal, is at least strong evidence for reproductive isolation between groups. For example, morphological analyses of phenotypically distinct populations of cave bears in two Austrian caves that were only 10 km apart showed that phenotypic differences between bears in these two caves appeared to be consistent for 15,000 years (HOFREITER et al., 2004). Genetic analyses revealed that the bears represented distinct mitochondrial lineages, *U. s. eremus* and *U. ingressus*, respectively, with no evidence of genetic exchange between the caves for more than 15,000 years. In other words, the mitochondrial lineage characteristic for one cave was never found in the respective other cave. Morphological data did not provide any evidence that phenotypic differences between the two caves diminished through time, as would be expected if gene flow occurred between caves. As the caves were certainly close enough for the cave bears to encounter each other, the observed pattern was most likely explained by reproductive isolation between the two populations, an indication that *U. s. eremus* and *U. ingressus* were at least different subspecies if not species. A speculative,

but not unjustified extension of this conclusion would be to hypothesise that if *U. s. eremus* and *U. ingressus* were reproductively isolated subspecies or species, then *U. kudarensis*, the Caucasus cave bear, which was a distinct sister taxon to the *spelaeus/ingressus* clade, must have also been a separate subspecies or species. This hypothesis is at least consistent with recent genome wide data (BARLOW, CAHILL et al., 2018). However, the authors were cautious about making firm taxonomic conclusions based on their data. An informative amount of nuclear genome data from a broad taxonomic range of samples will be required to shed more light on the relationships within the Pleistocene cave bear complex. Nevertheless, the study raises expectations that state-of-the-art sequencing technologies have brought such studies within reach of cave bear researchers.

Beyond addressing taxonomic questions, molecular data has made significant contributions to reconstructing cave bear population dynamics and potential causes of extinction. For example, STILLER et al. (2010) reconstructed Late Pleistocene population dynamics of brown bears and cave bears and found that only cave bears seem to have suffered a population decline, starting from approximately 50,000 years before present. The study concluded that ecological differences between brown bears and cave bears, and in particular different hibernation strategies, might have contributed to the different fates of the two species. If cave bears were more dependent on caves for hibernation and birth, they would have been more affected by the increased human activity in Eurasian caves starting from about 50,000 years before present. While these conclusions were rather speculative, a recent study found further evidence to support the hypothesis (FORTES et al., 2016). Analysing mitochondrial data, the study found that cave bears, very unlike brown bears, displayed “homing” behavior, returning to the cave of their birth for hibernation year after year. Such behavior would increase the detrimental effects of competition for cave sites with the growing human population.

Taken together with sample age information from radiocarbon dating, ancient DNA has also contributed to reconstructing population movements through time. STILLER et al. (2013) showed that the cave bear range appeared to become increasingly restricted in Asia and Eastern Europe as the last glaciation proceeded. Consistent with these findings, a replacement of the western *U. s. spelaeus* mitochondrial lineage by the eastern *U. ingressus* lineage in several German caves in the Ach valley at about 28,000 years bp had previously been shown (HOFREITER et al., 2007). These results suggest that the loss of range in the east was not only a result of eastern populations dying out, but also of active westwards movements of eastern populations.

Technical improvements now allow for analyses of unprecedented amounts of nuclear genome data from extinct species. While most paleogenomic studies have focused on reconstructing our own history and evolution, first paleogenomic studies into cave bears are now providing insights into cave bear biology that had previously remained hidden. BARLOW, CAHILL et al. (2018) investigated poten-

tial admixture of brown bears and cave bears and found evidence that parts of the cave bear genome have survived to the present day in modern brown bears. The findings mirror those from Neanderthals and modern humans and paint a more complex picture of relationships between the Pleistocene cave bear complex and brown bears as well as within the cave bear complex itself.

### **Part 3: The future of cave bear paleogenomics**

Given the considerable advances in our knowledge of cave bear biology facilitated by ancient DNA thus far, one could imagine that the rate of new discoveries will start to slow in the coming years and decades. However, we argue the opposite: that we are in fact on the brink of a new era of cave bear research facilitated by the emerging field of paleogenomics. Currently, analysis of the petrous bone appears to be the most efficient approach for the recovery of genome-level data from Pleistocene mammals outside of permafrost regions. This requirement for a specific, small skeletal element will be a limiting factor for taxa which are poorly represented in the fossil record; however, the sheer volume of cave bear bones deposited in their hibernation caves means this is unlikely to apply to cave bears. Thus, at least in principal, the potential for paleogenomic research on cave bears is huge.

One area where genome-level data can clearly contribute is our basic understanding of the relationships among cave bear taxa. Although relationships at the mitochondrial level are well established (STILLER et al., 2013), conflicting relationships recently inferred from paleogenomes (BARLOW, CAHILL et al., 2018) call into question the accepted view of cave bear evolution and taxonomy. Priorities for future studies include comprehensive geographic sampling of cave bear morphotypes to allow tests of monophyly as well as phylogeny. The challenge for these studies will be to extract phylogenetic information from typically low coverage and error-rich paleogenomic datasets (BARLOW, HARTMANN, GONZALEZ, HOFREITER & PAIJMANS, 2018), as well as separating population histories from more recent admixture and ancestral genetic polymorphisms (WEÇEK et al., 2017). Nonetheless, such knowledge is essential to identify macroevolutionary processes such as convergent or parallel evolution, as well as allowing the formulation of basic hypotheses on other aspects of cave bear evolution.

In addition to phylogeny, gene flow or admixture between populations provides an additional factor shaping their genetic variation. For cave bears, mitochondrial DNA has been used as evidence for a lack of gene flow (HOFREITER et al., 2004), but this evidence is indirect and cannot unequivocally exclude, for example, very low level or male-biased gene flow. Genome-level data, in contrast, can provide direct evidence of gene flow even involving ancestors occurring hundreds of generations in the past (FU et al., 2015, PRÜFER et al., 2014). Careful analysis can further elucidate the direction of gene flow as well as differences in the frequency that males and females admixed. Given the well-established evidence of admixture occurring within the genus *Ursus* (CAHILL et al., 2015, KUMAR et

al., 2017), including between cave bears and brown bears (BARLOW, CAHIL et al., 2018), it may be tentatively hypothesized that different cave bear taxa also admixed over the course of their evolution. These questions represent priorities for future paleogenomics studies of cave bears, with populations occurring at contact zones between taxa (HOFREITER et al., 2007, MÜNDEL et al., 2011) representing a logical starting point.

As well as improving geographic sampling of cave bear paleogenomes, another goal of future studies should be to extend their temporal sampling. Successful sequencing of Middle Pleistocene cave bear mitochondrial DNA (DABNEY et al., 2013, STILLER et al., 2013) suggests that retrieving paleogenomic data for Middle Pleistocene cave bears may be possible. A fascinating aspect of cave bear evolution is their morphological change through time, for example the small bodied Middle Pleistocene *U. deningeri*, which is thought to have given rise to several Late Pleistocene forms including the very large bodied *U. ingressus* and *U. s. spelaeus* (GARCÍA, ARSUAGA & TORRES, 1997). Sequencing the genomes of ancestor and descendent may thus allow the genomic changes underpinning this morphological evolution to be uncovered. This idea leads to a completely new avenue for cave bear genetic research, which has been largely unexplored, namely identifying evidence of selection on specific genes or genome regions which have facilitated adaptive evolution. In addition to body size, adaptations evolving in response to the cave bear's herbivorous diet, in contrast to their close relatives the carnivorous polar bears and omnivorous brown bears, would be of key evolutionary interest.

A final noteworthy area for future paleogenomics research on cave bears are their population dynamics, encompassing the occurrence of their populations through space and time, the size and composition of these populations, and the factors that shaped them. At a microgeographic scale, a recent study based on mitochondrial DNA found evidence that behavioural processes – specifically homing behaviour – shaped patterns of genetic variation within a single cave bear population in Late Pleistocene Northern Spain (FORTES et al., 2016). In this population, the timing of establishment of the hibernation cave groups, group size, and level (of lack) of migration between cave groups appears to have existed simultaneously at the perfect level to result in the clear segregation of mitochondrial haplotypes among hibernation groups. Critically, any major deviation in any of these population parameters could render this behaviour undetectable using mitochondrial DNA. Paleogenomes, in contrast, provide much higher sensitivity and would allow generally applicable tests for homing behaviour in other cave bear populations.

In terms of global population dynamics, mitochondrial DNA has similarly provided key initial insights, specifically on the timing of the cave bear population decline. With paleogenomes, similar inferences are possible from a high coverage genome of a single individual (LI & DURBIN, 2011). This offers several advantages over demographic inference based on mitochondrial DNA. Paleogenomic



data has the potential to recover older events and, since inferences are based on a single individual, the potential confounding factor of population structure among the sampled individuals is avoided. Moreover, sampling of multiple paleogenomes would allow the demography of different cave bear taxa to be investigated, providing a more complete and complex view of the processes leading to their extinction.

## Conclusions

Cave bear research and ancient DNA research have enjoyed a mutually beneficial relationship for more than two decades. The most significant results with implications for our understanding of evolutionary processes in general and for the evolution of cave bears in particular are likely still to come. In fact, some of the new ancient DNA sequencing technologies that are now promising unprecedented insights into cave bear biology have been developed using cave bear samples. The cave bear might be extinct in nature, but as an essential model species in evolutionary biology, it remains very much alive.

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