

# Evolutionary History of Saber-Toothed Cats Based on Ancient Mitogenomics

## Highlights

- We present the first near-complete mitochondrial genomes from saber-toothed cats
- *Smilodon* and *Homotherium* are estimated to have diverged ca. 18 million years ago
- We find limited genetic divergence between American and European *Homotherium*
- Late Pleistocene *Homotherium* should be considered a single species: *H. latidens*

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## In Brief

Pajmans et al. present ancient DNA from some of the most recognized extinct Pleistocene megafauna: the saber-toothed cats. The results elucidate the evolutionary history of these iconic carnivores and provide genetic evidence that saber-toothed cats existed in Europe over 200,000 years later than previously believed.

# Evolutionary History of Saber-Toothed Cats Based on Ancient Mitogenomics

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## SUMMARY

Saber-toothed cats (*Machairodontinae*) are among the most widely recognized representatives of the now largely extinct Pleistocene megafauna. However, many aspects of their ecology, evolution, and extinction remain uncertain. Although ancient-DNA studies have led to huge advances in our knowledge of these aspects of many other megafauna species (e.g., mammoths and cave bears), relatively few ancient-DNA studies have focused on saber-toothed cats [1–3], and they have been restricted to short fragments of mitochondrial DNA. Here we investigate the evolutionary history of two lineages of saber-toothed cats (*Smilodon* and *Homotherium*) in relation to living carnivores and find that the *Machairodontinae* form a well-supported clade that is distinct from all living felids. We present partial mitochondrial genomes from one *S. populator* sample and three *Homotherium* sp. samples, including the only Late Pleistocene *Homotherium* sample from Eurasia [4]. We confirm the identification of the unique Late Pleistocene European fossil through ancient-DNA analyses, thus strengthening the evidence that *Homotherium* occurred in Europe over 200,000 years later than previously believed. This in turn forces a re-evaluation of its demography and extinction dynamics. Within the *Machairodontinae*, we find a deep divergence between *Smilodon* and *Homotherium* (~18 million years) but limited diversity between the American and European *Homotherium* speci-

mens. The genetic data support the hypothesis that all Late Pleistocene (or post-Villafrancian) *Homotherium* should be considered a single species, *H. latidens*, which was previously proposed based on morphological data [5, 6].

## RESULTS AND DISCUSSION

*Homotherium* and *Smilodon* were large-bodied predators with widespread distributions. The Holarctic genus *Homotherium* has Old World origins, with Pleistocene forms in Eurasia generally being assigned to *H. latidens* and those in North America being assigned to *H. serum* [7, 8]. The New World genus *Smilodon* is thought to have evolved from Old World dirk-toothed cats of the genus *Megantereon*, which may have dispersed into the Americas during the Pliocene (Blancan). Two Late Pleistocene (Rancholabrean) *Smilodon* species are recognized, with *S. fatalis* being confined to areas south of the continental ice sheets in North America, whereas the contemporary, larger, and more robust *S. populator* was restricted to South America. Despite their widespread occurrence, *Homotherium* and *Smilodon* remains are uncommon and generally fragmentary in the fossil record, except in rare cases (e.g., [9, 10]). *Homotherium* in particular is generally only represented by isolated cranial or dental elements, leading to many uncertainties about their taxonomy, demography, and extinction dynamics. Both *Homotherium* and *Smilodon* survived in North America until the Late Pleistocene and went extinct alongside many other megafauna species on the continent (e.g., mammoths and giant sloths [11]). In Eurasia, however, *Homotherium* is generally thought to have gone extinct much earlier, during the Middle Pleistocene around 300,000 years ago [12–15]. To date, there is only a single dated Late Pleistocene *Homotherium* fossil recovered in

**Table 1. Sample Details of *Smilodon* and *Homotherium***

Sample Code	Species	Location	Age	Dating Facility and Number	Skeletal Element	Collection	Reference
SP1007	<i>Homotherium latidens</i>	North Sea, the Netherlands	31,300 ± 400	Utrecht University AMS facility: 10456	mandible	Rotterdam	[4]
SP1007	<i>Homotherium latidens</i>	North Sea, the Netherlands	31,300 ± 400	Utrecht University AMS facility: 10999	mandible	Rotterdam	[4]
SP1007	<i>Homotherium latidens</i>	North Sea, the Netherlands	26,900 ± 400	Utrecht University AMS facility: 10908	mandible	Rotterdam	[4]
SP1007	<i>Homotherium latidens</i>	North Sea, the Netherlands	26,700 ± 240	Utrecht University AMS facility: 11064	mandible	Rotterdam	[4]
SP1007	<i>Homotherium latidens</i>	North Sea, the Netherlands	28,100 ± 220	Utrecht University AMS facility: 11000	mandible	Rotterdam	[4]
SP1007	<i>Homotherium latidens</i>	North Sea, the Netherlands	27,650 ± 280	Utrecht University AMS facility: 11065	mandible	Rotterdam	[4]
SP1714	<i>Homotherium latidens</i>	60-mile, Yukon Territory, Canada	>56,500	Oxford Radiocarbon Accelerator: 10082	left humerus	Canadian Museum of Nature, Ottawa, CMN46442	[3]
YG 439.38	<i>Homotherium latidens</i>	Dominion Creek, Dawson City, Yukon Territory	>47,500	Stafford, UCIAMS: 142835	left humerus	Yukon government collection, Whitehorse	Figure S1; Table S2
ZMA20.042	<i>Smilodon populator</i>	Ultima Esperanza, Chile	11,335 ± 30	Stafford, UCIAMS: 142836	left tibia	Kruimel collection, Naturalis, Leiden	—

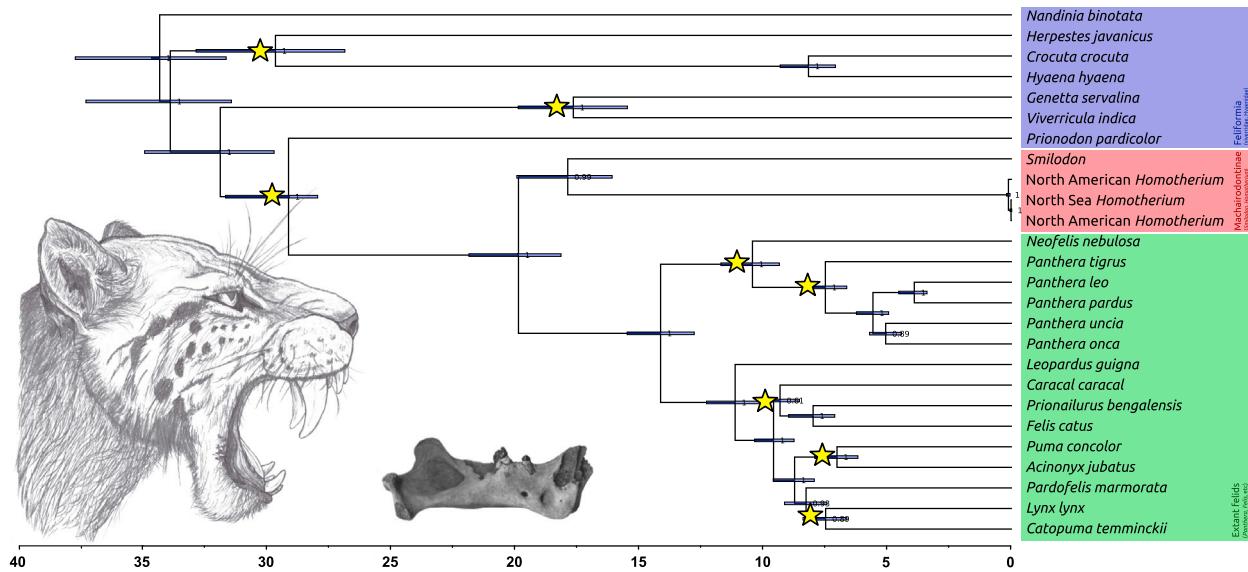
Sample details for the *Smilodon* and *Homotherium* samples included in this study. In this table, only samples for which a (partial) mitogenome could be reconstructed are listed. All radiocarbon ages are given in uncalibrated years before present. See also Figure S1 and Tables S1–S4.

Europe [4]. We used ancient-DNA techniques to retrieve and analyze genetic data from this individual and compared the specimen to two North American *Homotherium* specimens and one South American *Smilodon* specimen, in order to investigate the evolutionary history of the Machairodontinae and the taxonomy, demography, and phylogeography of *Homotherium*.

### Evolutionary History of Machairodontinae

Mitochondrial genome data (mitogenomes) were generated for one *Smilodon* and three *Homotherium* specimens using hybridization capture and were assembled via both an iterative mapping approach using three different mitochondrial sequences as initial reference seeds and a regular read alignment approach (for more details, see STAR Methods; Figure S1; Table S2). The *Smilodon* sample was collected in Chile and is dated to 11,335 years (carbon dates are given as uncalibrated  $^{14}\text{C}$  years; Table 1). Two *Homotherium* fossils were collected in the Yukon Territory (northwest Canada), and both proved to be beyond the limits of radiocarbon dating (>50,000 years; Table 1) [3]. The European *Homotherium* was recovered from the North Sea and is dated to ~28,000 years (Table 1) [4]. The recovered mitogenomes had an average depth of 19× (7–35×), resulting in partial mitogenome sequences spanning 44.5%–92.4% of the expected sequence length (Table S1). The recovered Machairodontinae mitogenomes were aligned with 22 additional carnivoran mitogenomes retrieved from GenBank and subjected to maximum-likelihood (ML) and Bayesian phylogenetic analyses (Table S3). These analyses confirm the placement of *Smilodon* and *Homotherium* as sister lineages in the subfamily Machairodontinae with 94% bootstrap support (BS) and a Bayesian posterior probability (BPP) of 0.99, basal to all extant Felidae species (100% BS, 1.0 BPP; Figure 1; Figure S2). The mitogenome-based

phylogenetic relationship between the *Homotherium* and *Smilodon* data is in agreement with analyses based on morphological evidence [8] and shorter mitochondrial sequences [1, 3]. We then used a time-calibrated Bayesian analysis to estimate divergence times on the ML topology with multiple fossil calibration points (Table 2). The estimated median time to the most recent common ancestor (tMRCA) for all Felidae was 20 million years ago (95% credibility interval: 18.2–22.0 millions years [Ma]). This is in line with earlier estimates of 14.5–21.5 Ma [3]. The tMRCA for extant Felids was found to be 14.2 Ma, also similar to other estimates (e.g., 15.3–17.4 Ma [20]). The calibrated phylogeny indicates a deep divergence between *Smilodon* and *Homotherium* (18.0 Ma; 95% credibility interval: 16.0–20.0 Ma; estimated sequence divergence ~11%), supporting an Early Miocene separation into the tribes Smilodontini and Homotherini, respectively (the latter is sometimes referred to as Machairodontini [21]). The oldest undisputed *Homotherium* fossils from Early Pliocene assemblages in Ukraine and Kenya suggest either a Eurasian or African origin of the genus and a subsequent dispersal into America during the Late Pliocene (Blancan; [22, 23]). *Smilodon* remains have only been recovered on the American continents, from the Miocene-Pliocene boundary to the Late Pleistocene, and have never been found in Eurasia [24]. The deep divergence inferred from our mitogenome data between *Homotherium* and *Smilodon* is congruent with the proposed evolution of these genera around the Miocene-Pliocene transition on separate continents. Within *Smilodon*, there are currently two recognized Late Pleistocene species: *S. populator*, which has so far only been found in South America, and *S. fatalis*, the last surviving *Smilodon* species of North America [8]. The *Smilodon* specimen investigated in this study was recovered in Chile and is dated to 11,335 years before present (Table 1); it thus can be assigned



**Figure 1. Calibrated Phylogeny for *Smilodon* and *Homotherium***

Time-calibrated mitochondrial phylogeny of the Felidae, including the saber-toothed cat *Smilodon* and scimitar-toothed *Homotherium*. Node support is indicated by Bayesian posterior probabilities (see Figure S2 for RAxML phylogeny and bootstrap values). Calibrated nodes are indicated with a star (see also Table 2). Blue node bars indicate the 95% credibility interval of divergence times. The lower axis shows millions of years. *Homotherium* artwork was provided by Binia De Cahsan. The image of the mandible is adapted from [4]. See also Figures S1 and S2.

to the South American Late Pleistocene species *S. populator*. The tMRCA of the three *Homotherium* individuals as inferred from the Bayesian analyses is 144,800 years (95% credibility interval: 77,076–215,970 years; estimated sequence divergence 0.2%–0.04%). This divergence date is relatively recent and is similar to that reported for other felid species (e.g., leopards from Asia [25]).

#### Late Pleistocene Occurrence of *Homotherium* in Eurasia

Our genetic analyses corroborate published radiocarbon dates and morphological descriptions, which together provide conclusive evidence that the specimen recovered from the North Sea represents the first confirmed Late Pleistocene *Homotherium* from Eurasia, forcing a re-evaluation of the traditional view of the demographic processes that preceded extinction of this iconic megafaunal species. Very few other Late Pleistocene *Homotherium* fossils have been recovered in Europe [26, 27], and their age, origin, and species identification are subject to much discussion [28–31]. The *Homotherium* specimen investigated here was found on the Brown Bank region in the North Sea (~80 km off the Dutch Coast), an area where Late Pleistocene and Early Holocene fossils are commonly found from species that existed in Western Eurasia [32]. Furthermore, the fragile state of the North Sea mandible makes it unlikely to have been transported from remote regions, for example through taphonomic processes. Based on morphological characteristics, the specimen was identified as *Homotherium* rather than any other Late Pleistocene felid genus [4]. The Late Pleistocene age of this fossil has been confirmed through six independent radiocarbon dates (~28,000 years old [4]), which makes it the only firmly dated Late Pleistocene fossil in Europe assigned to the

genus *Homotherium*. The occurrence of *Homotherium* in Europe during the Late Pleistocene could be the result of several different demographic scenarios. The Late Pleistocene *Homotherium* population in Eurasia may have existed at low population densities, effectively dropping under the “fossil detection threshold,” with very few remains surviving in the fossil record, which has also been previously proposed as an explanation for the low abundance of *Homotherium* fossils in America [33, 34]. This scenario would not be unique to *Homotherium*; for example, although there are currently only four fossils recovered from the Denisovan hominins from a single cave, genetic data indicate that they occupied large parts of Eurasia during the Late Pleistocene [35–38]. Despite its widespread Holarctic distribution during the Late Pleistocene, *Homotherium*, like other megafaunal species, proved vulnerable to environmental and/or ecological changes, which led to its eventual extinction. Alternatively, it is conceivable that the *Homotherium* found in the North Sea descends from a Late Pleistocene dispersal from a core population in Central Eurasia or Beringia, as has been suggested for other Pleistocene megafauna (e.g., mammoths [39] and wolves [40]). Similar to extant large felids (e.g., [41]), *Homotherium* is likely to have been a highly mobile taxon, and it may have re-colonized Europe during the Late Pleistocene after the resident population went extinct in the Middle Pleistocene. This scenario is consistent with the estimated coalescence timing of the European and American *Homotherium* mitochondrial lineages (95% credibility interval: 77–216 thousand years [Ka]).

In order to identify which of the possible demographic scenarios is applicable to Late Pleistocene *Homotherium*, additional samples have to be recovered and analyzed. However, all of these scenarios point to a situation in which *Homotherium* roamed at least part of the Eurasian continent for hundreds of

**Table 2. Fossil Constraint Used for Calibrated Phylogeny**

Fossil	Fossil Constraint	Calibration Prior	Reference
<i>Genetta</i> fossil: 11.2 Ma	minimum 11.2 Ma	uniform: 50–11.2 Ma	[16, 17]
Hyaenid fossil: 16.4 Ma	minimum 16.4 Ma	uniform: 50–16.4 Ma	[16, 17]
Herpestid fossil: 16.4 Ma	minimum 16.4 Ma	uniform: 50–16.4 Ma	[16, 17]
Felidae stem fossils, <i>Prionodon</i> fossils	minimum 28 Ma	uniform: 50–28 Ma	[16, 17]
<i>Lynx</i> fossil: 5.3 Ma	minimum 5.3 Ma	uniform: 10–5.3 Ma	[16, 17]
<i>Acinonyx</i> fossils: 3.8 Ma	minimum 3.8 Ma	uniform: 10–3.8 Ma	[18, 19]
Caracal and serval fossils: 3.8 Ma	minimum 3.8 Ma	uniform: 16–3.8 Ma	[19]
Oldest <i>Panthera</i> fossil: 3.8 Ma	minimum 3.8 Ma	uniform: 16–3.8 Ma	[8, 19]
Oldest <i>Panthera tigris</i> fossil: 1.5 Ma	minimum 1.5 Ma	uniform: 10–1.5 Ma	[8]

Fossil constraints and calibration priors used in the time-calibrated BEAST analysis [8, 16–19] are shown. Ma, million years.

millennia later than was previously believed. This situation forces a re-assessment of the Late Pleistocene population dynamics and timing of extinction of this large felid species. Some of the general attributes that threaten extant large-bodied felids [42, 43], such as large body size, high trophic level (i.e., hypercarnivory [44–46]), and low population densities and/or fragmented populations may also have placed *Homotherium* at risk. However, our evidence of Late Pleistocene survival of *Homotherium* in Europe suggests that these factors may not have been the sole driving force behind its extinction, since it survived for over 200,000 years at low or fragmented population densities, as suggested by the scarcity of fossils. Thus, gathering additional insights into the population structure and extinction dynamics of *Homotherium* may also help explain why the extinction risks of extant felids are sometimes overestimated [47]. Ultimately, *Homotherium* was unable to survive the climatic and ecological changes that occurred at the end of the Pleistocene, a time during which many other large-bodied mammals, such as mammoths [39] and cave lions [48], also experienced severe population fluctuations and extinction. In order to gain a better understanding of the population dynamics of *Homotherium* during the Late Pleistocene and why it eventually went extinct, more samples will have to be recovered and analyzed from Europe as well as Asia. In light of the morphological and genetic evidence for the Late Pleistocene occurrence of *Homotherium* in Europe, it is conceivable that some Late Pleistocene remains that are currently assigned to one of the more common large cat species (e.g., cave lion) could be re-identified as *Homotherium*.

#### Taxonomic Revision of Holarctic *Homotherium*

Species-level identification of saber-toothed cats has been based on geographical and/or morphological data, which hold a number of inherent limitations [49]. The data presented here allow for a direct comparison at the mitochondrial DNA level between the commonly recognized *Homotherium* species that inhabited the North American and Eurasian continents: *H. serum* and *H. latidens*, respectively [8]. We found low mitogenome diversity among Late Pleistocene representatives of the genus and a tMRCA of ~145,000 years. Previous studies based on short mitochondrial sequences from North American *Homotherium* have also found low levels of genetic diversity, despite considerable geographical (>2,000 km) and temporal (>25,000

years) separation of the fossils [1]. We were unable to compare our North Sea and North American mitogenomes with previously published short mitochondrial sequences from other individuals [1, 3], as we did not have complete sequence coverage for the relevant mitochondrial regions (e.g., 16S, cytB, and ATP8). However, the very recent tMRCA (~145,000 years) for the three *Homotherium* mitogenomic sequences is also indicative of low diversity between the *Homotherium* sequences, particularly considering their geographical distance. Low intraspecific diversity in such a widespread species has been previously reported for other carnivores (e.g., ancient lion sequences [48] and modern wolf sequences [40]). We therefore compared the intraspecific diversity of the three *Homotherium* mitogenomes to the diversity between subspecies of other big cats (tigers, lions, and leopards) and found the *Homotherium* sequence diversity to be lower than those for any extant felid species (STAR Methods). The low mitogenomic genetic diversity is further supported by the low genetic diversity measured between short mitochondrial DNA fragments from two North American *Homotherium* [1]. This degree of genetic similarity suggests that all three *Homotherium* individuals were representatives of a single Late Pleistocene species, thus casting doubt on the validity of the distinct American and Eurasian *Homotherium* species currently recognized (*H. serum* and *H. latidens*, respectively). Furthermore, the European *Homotherium* mitochondrial sequence is nested within the diversity of the two American *Homotherium* sequences in the phylogeny (Figure 1), further supporting the monospecificity of all Late Pleistocene Holarctic *Homotherium* populations.

Since the first *Homotherium* fossil discovery in 1824 [50], multiple *Homotherium* species have been proposed. However, these have typically been based on geographical or temporal separation of fossils, rather than distinguishable morphological characteristics [8]. In North America, older (Pliocene) fossils are considered morphologically distinct from younger Pleistocene finds and are thus generally separated into two species: *H. ischyrus* and *H. serum*, respectively [7, 33, 51]. In the Eurasian fossil record, such distinction between older and younger forms is controversial: although earlier studies recognized two [52] or even three distinct Eurasian species [53], recent finds from Spain suggest that all Pleistocene Eurasian *Homotherium* fossils are more accurately grouped into a single, morphologically variable species, *H. latidens* [7]. These authors

also note that the variation within *H. latidens* is extensive enough to assign North American *H. serum* fossils—if they were found in Europe—to *H. latidens* [7].

The morphological overlap between North American and Eurasian *Homotherium* fossils has been regarded as evidence that all Pleistocene *Homotherium* can be assigned to a single, morphologically variable species [5, 6]. It has also previously been suggested, based on morphological similarities between two Early Pleistocene individuals from France [54] and Oregon, that these individuals should belong to the same species [34]. The high similarity found between mitochondrial DNA fragments recovered from two North American (Yukon and Great Lakes region) *Homotherium* fossils also indicates a very close relationship between the individuals, despite their considerable geographical and temporal distance [1]. Although clearly limited due to small sample size, the mitochondrial DNA evidence that we present here further supports the hypothesis, suggested previously based on morphological data, that at least Late Pleistocene North American and Eurasian *Homotherium* are monospecific, rather than two separate species. For reasons of priority, this taxon should be called *H. latidens* [55]; consequently, *H. serum* [56] is a junior synonym.

## Conclusions

In this study, we present partial mitogenome sequences from two lineages of Machairodontinae, *Smilodon* and *Homotherium*, and confirm the phylogenetic relationships and evolutionary history of these iconic felids. Furthermore, the mitochondrial DNA that we recovered from the North Sea *Homotherium* specimen confirms the Late Pleistocene survival of this enigmatic saber-toothed cat in Eurasia. Much like the Denisovan hominins, the North Sea *Homotherium* represents another striking example of the major gaps in our knowledge of Pleistocene fauna composition on the Eurasian continent and holds important clues about population and extinction dynamics of Pleistocene species. By applying DNA analysis on ancient samples, even a controversial find such as the North Sea *Homotherium* can be firmly identified. The *Homotherium* mitogenome sequences revealed low genetic diversity, which strongly supports the hypothesis based on morphology of a single, widespread Holarctic *Homotherium* species during the Late Pleistocene (*H. latidens*). This study highlights the importance of combining morphological and genetic information for species identification.

## STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- CONTACT FOR REAGENT AND RESOURCE SHARING
- METHOD DETAILS
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- Bioinformatic procedures
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## ● DATA AND SOFTWARE AVAILABILITY

## SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and four tables and can be found with this article online at <https://doi.org/10.1016/j.cub.2017.09.033>.

## AUTHOR CONTRIBUTIONS

Conceptualization, M.H., J.W.F.R., M.T.P.G., R.B., and J.L.A.P.; Methodology, J.L.A.P., M.H., A.B., M.T.P.G., and R.B.; Investigation, J.L.A.P. and R.B.; Formal Analysis, J.L.A.P., R.B., A.B., M.L.Z.-M., and M.V.W.; Resources, J.W.F.R., A.L., N.R., J.A.L., G.B., J.d.V., M.K., G.Z., and D.N.; Writing, J.L.A.P., M.H., A.B., and J.W.F.R.; Discussion, J.L.A.P., M.H., R.B., M.T.P.G., G.B., G.Z., A.B., J.W.F.R., and D.N.; Supervision, M.H. and M.T.P.G.

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Guanidinium hydrochloride 99%, M 95,53 g/mo	Roth	0037.1
Sodium acetate	Sigma Aldrich	S7899-100ml
Isopropanol	A. Hartenstein	CP50
Tween-20	A. Hartenstein	CT20
EDTA	VWR	E177-500MLDB
QIAGEN MinElute kit	QIAGEN	28006
NaCl solution, 5 M	Life Technologies	AM9760G
SDS solution, 20%	Life Technologies	AM9820
1M TrisHCl	Life Technologies	15568-025
20X SSC	Roth	1054.1
HPLC grade water	A. Hartenstein	CW20
QIAGEN QiaQuick kit	QIAGEN	28104
Critical Commercial Assays		
D1000 ScreenTape (Tapestation 2200)	Agilent	5067-5582
dsDNA HS Assay Kit (Qubit 2.0)	Thermofisher	Q32851
Agilent DNA 1000 kit (BioAnalyzer)	Agilent	5067-1504
Deposited Data		
Mitogenome consensus for SP1007 (North Sea <i>Homotherium</i> )	This paper	GenBank: MF871701
Mitogenome consensus for SP1714 (North American <i>Homotherium</i> )	This paper	GenBank: MF871703
Mitogenome consensus for YG 439.38 (North American <i>Homotherium</i> )	This paper	GenBank: MF871702
Mitogenome consensus for ZMA20.042 (South American <i>Smilodon</i> )	This paper	GenBank: MF871700
Oligonucleotides		
Primer inPE1.0	Illumina	
Software and Algorithms		
Illumina bcl2fastq 2.15	Illumina	<a href="https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html">https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html</a>
SeqPrep	John St. John	<a href="https://github.com/jstjohn/SeqPrep">https://github.com/jstjohn/SeqPrep</a>
Cutadapt v1.10	[57]	<a href="http://cutadapt.readthedocs.io/en/stable/index.html">http://cutadapt.readthedocs.io/en/stable/index.html</a>
PINSEQ-lite v0.20.4	[58]	<a href="http://prinseq.sourceforge.net/">http://prinseq.sourceforge.net/</a>
Burrows-Wheeler Aligner (BWA) v0.7.8	[59]	<a href="http://bio-bwa.sourceforge.net/">http://bio-bwa.sourceforge.net/</a>
Samtools v0.1.19	[60]	<a href="https://sourceforge.net/projects/samtools/files/samtools/0.1.19/">https://sourceforge.net/projects/samtools/files/samtools/0.1.19/</a>
mitoBim v1.8	[61]	<a href="https://github.com/chrishah/MITOBIM">https://github.com/chrishah/MITOBIM</a>
Geneious v7.0	[62]	<a href="https://www.geneious.com/">https://www.geneious.com/</a>
MEGA v5.2	[63]	<a href="http://www.megasoftware.net/">http://www.megasoftware.net/</a>
PartitionFinder v1.1.1	[64]	<a href="http://www.robertlanfear.com/partitionfinder/">http://www.robertlanfear.com/partitionfinder/</a>
RaxML-HPC v8.2.4	[65]	<a href="https://sco.h-its.org/exelixis/web/software/raxml/index.html">https://sco.h-its.org/exelixis/web/software/raxml/index.html</a>
CIPRES Science Gateway	[66]	<a href="http://www.phylo.org/">http://www.phylo.org/</a>
BEAUTi v1.8.2	[67]	<a href="http://beast.community/index.html">http://beast.community/index.html</a>
BEAST v1.8.2	[67]	<a href="http://beast.community/index.html">http://beast.community/index.html</a>

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
TreeAnnotator v1.8.2	[67]	<a href="http://beast.community/index.html">http://beast.community/index.html</a>
FigTree v1.4	Andrew Rambaut	<a href="http://tree.bio.ed.ac.uk/software/figtree/">http://tree.bio.ed.ac.uk/software/figtree/</a>
Other		
Proteinase K	Promega	V3021
Zymo-spin V column extension reservoir	Zymo	C1016-50
CircLigase II	Biozym	131406
Endonuclease VIII	NEB	M0299
Afu UDG	NEB	M0279
FastAP	Thermo Scientific	EF0651
PEG-4000	Life Technologies	C1602
MyOne C1	Life Technologies	65001
Buffer Tango	Fermentas	BY5
dNTP	Fisher Scientific	10520651
T4 DNA polymerase	Fermentas/Thermo	EP0062/EP0061
T4 DNA ligase	Fermentas/Thermo	EL0011
Accuprime Pfx	Thermo Fisher	12344024
ATP	Thermo Scientific	R0441
SYBR green PCR MasterMix	Life Technologies	4309155
Buffer Tango	Fermentas	BY5
Bst 2.0	NEB	M0537 S
NEBNext DNA Library Prep Master Mix Set for 454	NEB	E6070S

## CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to, and will be fulfilled by the Lead Contact, Johanna L.A. Paijmans ([paijmans.jla@gmail.com](mailto:paijmans.jla@gmail.com)).

## METHOD DETAILS

### Morphological description for sample YG 439.38 (North American *Homotherium*)

Specimen YG 439.38 from Dominion Creek, Yukon, consists of the distal three-quarters of a left humerus (Figure S1). The specimen is generally well preserved, except for some erosion on the posterior parts of both epicondyles. *Homotherium* specimens are very rarely recovered in eastern Beringia (unglaciated parts of Alaska and Yukon), but their humeri can be readily distinguished from much larger, and more robust *Panthera leo spelaea*, the only other large Pleistocene felid that is also known from the region (Table S2). Some of the key distinguishing characteristics are (1) general slenderness of the humerus shaft; (2) the angle of intersection of the deltoid and medial ridges is relatively more acute; (3) the lateral supracondylar ridge is relatively straight and sharp, while in *Panthera* it is slightly convex and more obtuse crested; (4) the relatively small entepicondylar foramen; (5) the entepicondylar bar is in a more anterior position; and (6) the relative prominence of the lateral epicondyle and weaker development of muscle scar above. The specimen compares well with descriptions and mensurational data from other *Homotherium* material from the Pleistocene of Yukon [68] and areas in midcontinental North America [1, 9]. Morphological characteristics for remaining *Homotherium* specimens have been described elsewhere [3, 4].

### Laboratory procedures

All pre-PCR procedures were performed in dedicated ancient DNA facilities with appropriate contamination precautions in place (e.g., [69]). Experiments for samples YG 439.38 and ZMA20.042 were performed at the Centre for GeoGenetics, University of Copenhagen. Samples SP1714 and SP1007 (Table 1) were processed in ancient DNA facilities of the Evolutionary Adaptive Genomics group at Potsdam University. Preliminary PCR data generation was performed in 2008 at the Max Plank Institute for Evolutionary Anthropology, Leipzig (MPI EVA).

### Preliminary PCR data

DNA was extracted from sample SP1714 in dedicated cleanlab facilities at the MPI EVA using a silica spin column protocol and a vacuum manifold [70]. Primer pairs (Table S4) were split into two pools of non-overlapping fragments. A total of 4 multiplex PCRs

were set up in 25  $\mu\text{L}$  reaction volumes using 5  $\mu\text{L}$  template, containing: 1x AmpliTaq Gold buffer, 4 mM MgCl<sup>2</sup>, 1 mg/mL BSA, 0.2 mM each dNTP, 2U AmpliTaq Gold, and 1  $\mu\text{M}$  of each primer in a pool of non-overlapping primer pairs (even versus odd numbered primer pairs [71]). PCR cycling conditions were as follows: initial denaturation at 94°C for 10 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 15 s, ending with a final extension for 5 min at 72°C. After multiplex PCR, a simplex PCR was carried out for each individual primer pair using the same conditions as described above and 5  $\mu\text{L}$  of a 40-fold dilution of the respective multiplex PCR. PCR products were tagged and built into NGS libraries [72], and sequenced on a 454-GS20. Raw data were demultiplexed using a custom script and aligned to make a final consensus sequence per PCR product. The resulting data were used for validating and extending the captured mitogenome sequences for sample SP1714 generated at the University of Potsdam (described below; Table S4).

### Extractions

All pre-PCR procedures (extraction, library building) were performed in dedicated ancient DNA facilities at the University of Potsdam and the Centre for GeoGenetics, University of Copenhagen, with contamination precautions in place. For specimens YG439.38 and ZMA20.042, samples of cortical bone were taken from long bone element (approx. 1 cm<sup>3</sup>) using a Dremel powertool, reduced to powder in a Mikrodismembrator, and extracted according to the protocol described in Orlando et al. [73]. For the remaining *Homotherium* samples, DNA was extracted according to the protocol by Dabney et al. [74]. All procedures included negative controls that were processed in parallel with the samples.

### Library preparation

For specimens YG 439.38 and ZMA20.042, DNA extract and negative controls were built into genomic libraries using the NEB E6070 kit and a slightly modified version of the protocol as used by Vilstrup et al. [75]. Briefly, extract (30  $\mu\text{L}$ ) was end-repaired and then passed through a MinElute column. The collected flow-through was then adaptor-ligated and passed through a QiaQuick column. Adaptor fill-in reaction was then performed on the flow-through, before final incubation at 37°C (30 min) followed by inactivation overnight at -20°C. For libraries of specimens YG 439.38 and ZMA20.042, we amplified in a 50  $\mu\text{L}$  reaction volume, using 25  $\mu\text{L}$  of library for 12 cycles under the following reaction conditions. Final concentrations were 1.25 U AccuPrime Pfx DNA Polymerase (Invitrogen), 1x AccuPrime Pfx reaction mix (Invitrogen), 0.4mg/mL BSA, 120nM primer in TE, and 120nM of a multiplexing indexing primer containing a unique 6 nucleotide index code (Illumina). PCR cycling conditions consisted of an initial denaturation step at 95°C for 2 min, followed by 12 cycles of 95°C denaturation for 15 s, 60°C annealing for 30 s, and 68°C extension for 30 s. A final extension step at 68°C for 7 min was also included. Library preparation success was checked on a 2% Agarose gel before purification using the QIAquick column system (QIAGEN) and quantification was performed on an Agilent 2100 BioAnalyzer.

For remaining *Homotherium* specimens, libraries were prepared according to the single-stranded library protocol as set out in Gansauge & Meyer [76]. The optimal cycle number for every library was estimated using qPCR [76]. Amplification was performed in 4 parallel reactions of 20  $\mu\text{L}$  each. Final concentrations in the indexing PCR reaction: 0.5 U AccuPrime Pfx DNA Polymerase (Invitrogen), and 1x AccuPrime Pfx reaction mix (Invitrogen), 0.75  $\mu\text{M}$  each of the Illumina indexing primers, with a unique 8 nucleotide index incorporated in the P7 primer. PCR cycling conditions were as follows: initial denaturation step at 95°C for 2 min, followed by the qPCR-estimated number of cycles of 95°C denaturation for 15 s, 60°C annealing for 30 s, and 68°C extension for 60 s, followed by a 3 min final extension at 68°C. Libraries were visualized on the Agilent Tapestation 2200 and measured using Qubit 2.0 Fluorometric quantification.

### Capture

For specimens YG 439.38 and ZMA20.042, two sets of capture experiments were performed. The first method used biotinylated RNA probes transcribed from fresh DNA extract derived from modern lion tissue by MYcroarray (Ann Arbor, MI, USA). The second method used previously published lion genome data [77] to identify exon coding regions and create biotinylated RNA baits that covered these regions. Both sets of baits were used in conjunction with MYbaits genome capture kit to enrich the ancient extracts for endogenous felid DNA. After capture and cleanup, enriched libraries were re-amplified for further sequencing using Phusion polymerase with primers IS5\_reamp.P5 and IS6\_reamp.P7 over 14 cycles [78]. The sequencing data resulted from a pooled product of both the whole-genome enrichment and exon capture. Thus, although the mitochondrial data is likely to have come from the whole-genome enrichment experiment as the exon capture bait set did not contain mitochondrial DNA baits, we could not distinguish between the two in the resulting data.

For *Homotherium* samples SP1714 and SP1007, mitogenome MYbait capture baits were designed from preliminary mitogenome data from sample YG 439.38, using only regions with  $\geq 5$ x coverage. Missing or ambiguous regions were replaced by a reconstructed ancestral felid mitogenome [79]. Capture was performed according to the protocol described by Li et al. [80], at a hybridization temperature of 65°C. Additional European *Homotherium* samples were screened for endogenous content using low-level shotgun sequencing, but due to the low estimated endogenous content, these samples were not used for sequence capture (Table S1).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Bioinformatic procedures

#### Mitogenome assembly

For samples YG 439.38 (*Homotherium*) and ZMA20.042 (*Smilodon*), an iterative mitogenome assembly method was used to reconstruct the mitogenome in the absence of a close reference. Raw sequences were trimmed using cutadapt v1.10 for single-end data (Martin, 2011), using a length cut-off of 25bp. Before mitogenome assembly, duplicate reads were removed from the fastq data using PRINSEQ-lite v0.20.4 [58]. For both YG 439.38 (*Homotherium*) and ZMA20.042 (*Smilodon*), MitoBIM v1.8 [61] was used to reconstruct the partial mitogenomes. MITObim was implemented using three different references as starting bait sequences (*Felis catus* (GenBank: FCU20753), *Crocuta crocuta* (GenBank: JF894377.1) and *Prionodon pardicolor* (GenBank: NC\_024569.1)) with default parameters apart from adjustments to the kmer value (kvalue = 25) and mismatch values (following [81]). We tried different mismatch values, ranging from 0%–8%. For both the *Homotherium* and *Smilodon*, no additional mitogenomic information was recovered using a mismatch value of above 3%. We therefore decided upon 3% as our mismatch value. MITObim output mira files were converted to sam files and then visualized using Geneious. For each starting bait sequence, a reference consensus sequence was constructed using a minimum coverage value of 10x and a base call threshold of 75%. These three sequences were then aligned using Mafftv7.271 and a majority rule consensus base calling was implemented to generate the final *Homotherium* and *Smilodon* mitochondrial sequences.

#### Mitogenome mapping

Remaining *Homotherium* samples were aligned to the mitogenome assembly for YG439.38 (Table S1). Raw sequences were trimmed using SeqPrep (available from <https://github.com/jstjohn/SeqPrep>) for paired-end data, and cutadapt v1.10 for single-end data [57]. All reads shorter than 30 bp were discarded: a more stringent length cut-off than for samples YG 439.38 (*Homotherium*) and ZMA20.042 (*Smilodon*) to ensure reliable read alignment. The Burrows-Wheeler Aligner (BWA) v0.7.8 [59] was used for read mapping, with default values for seed length (32 bp) and mismatch values (0.04). Samtools v1.19 [60] was used to remove reads with a mapping quality < Q30. Duplicates were identified according to both the 5' and 3' end mapping coordinates using MarkDuplicatesByStartEnd.jar (<https://github.com/dariober/Java-cafe/tree/master/MarkDupsByStartEnd>). The consensus sequence was generated using Geneious v7.0 [62], using a minimum sequence depth of 4x and a 75% majority rule for base calling. For sample SP1714, short mitochondrial DNA sequences from earlier published work [3] and preliminary generated PCR data (Table S4) were compared to the mitogenome retrieved using capture, for an independent validation of parts of the mitogenome sequence (over 1,200 bp of the capture consensus sequence). Furthermore, regions where there was no coverage using the capture data could be supplemented using the PCR data (about 600 bp).

### Phylogenetic analysis

#### Alignment

Mitogenome sequences were aligned using ClustalW v2 [82] as implemented in Geneious v7.0. The control region, as well as any positions in the alignment that contained missing data, were removed. The resulting alignment (6,649 bp in length) was manually annotated in Geneious using the domestic cat (GenBank: FCU20753) as reference. All mitochondrial regions except for the control region were present in the alignment, although these were highly fragmented and partially incomplete due to the removal of missing data. For intraspecies comparison between *Homotherium* and other large-bodied felids, mitogenomes for tiger, lion and leopard subspecies were downloaded and aligned with the three *Homotherium* specimens using ClustalW v2. Alignment columns containing missing data were not considered to enable direct comparison of genetic distances within extant species with those estimated from partial *Homotherium* and *Smilodon* assemblies. The alignment contained four tiger subspecies (*Panthera tigris altaica* [GenBank: JF357973], *P. t. amoyensis* [GenBank: HM589215], *P. t. tigris* [GenBank: JF357968], and *P. t. sumatrae* [GenBank: JF357969]), two leopard subspecies (*Panthera pardus orientalis* [GenBank: KX655614], and *P. p. japonensis* [GenBank: KJ866876]) and two lion subspecies (*Panthera leo leo* [GenBank: KP001502] and *P. l. persica* [GenBank: KP001501]). The observed genetic distances (p-distance) were measured in MEGA v5.2 [63] to be 0.006, 0.007, 0.003 and 0.001 for tiger, leopard, lion and *Homotherium*, respectively.

#### Partitionfinder

An optimal set of partitions and substitution models was selected from all possible combinations of genes and tRNAs, considering all substitution models available in BEAST, under the Bayesian Information Criterion (BIC) in PartitionFinder v1.1.1 [64]. The partition-finder analysis used the greedy search algorithm and linked branch lengths. PartitionFinder found best support for a five-partition scheme (BEAST xml input file available upon request).

#### RaxML

The maximum likelihood tree was calculated using RaxML-HPC v8.2.4 [65] CIPRES black box version on the CIPRES Science Gateway [66], with default GTR+CAT substitution models for each partition. RAxML rapid bootstrapping was used with 1000 replicates. The African palm civet (*Nandinia binotata*, belonging to the monotypic family Nandiniidae) was used as outgroup.

#### BEAST

Bayesian analyses were performed in BEAST v. 1.8.2 [67], with the 5 partitions selected by PartitionFinder. First, we tested for rate variation among lineages using a lognormal clock model on each partition (mean 0.05, standard deviation 0.05), with a uniform prior on the mean per-lineage substitution rate of 0 to 20% per million years, under a Birth-Death speciation tree prior. The MCMC chain

was run for a sufficient number of generations to achieve convergence and adequate posterior sampling of all parameters (ESS > 200), checked using Tracer v1.5 (available from <http://beast.community/tracer>). For some partitions, individual parameters of the GTR substitution model selected by PartitionFinder failed to converge, and so the simpler HKY model was used for these partitions in order to achieve convergence. The posterior sample of the ucld.stdev parameter, which describes substitution rate variation among lineages, was found to abut zero, thus not rejecting an absence of rate variation and justifying the use of a strict clock model. The analysis was rerun using a strict clock model with an uninformative uniform prior on the mean per-lineage substitution rate of 0 to 20% per million years, for molecular dating analyses with fossil calibration. The fossil calibrations that were used are listed in [Table 2](#). The BEAUTI-generated XML input file is available upon request. TreeAnnotator v1.8.2 was then used to remove the first 25% of trees as burnin and extract the Maximum Clade Credibility (MCC) tree with nodes scaled to the median heights recovered by the posterior sample.

#### DATA AND SOFTWARE AVAILABILITY

*Homotherium* and *Smilodon* consensus sequences are available on GenBank: MF871700–MF871703.

**Supplemental Information**

**Evolutionary History of Saber-Toothed Cats**

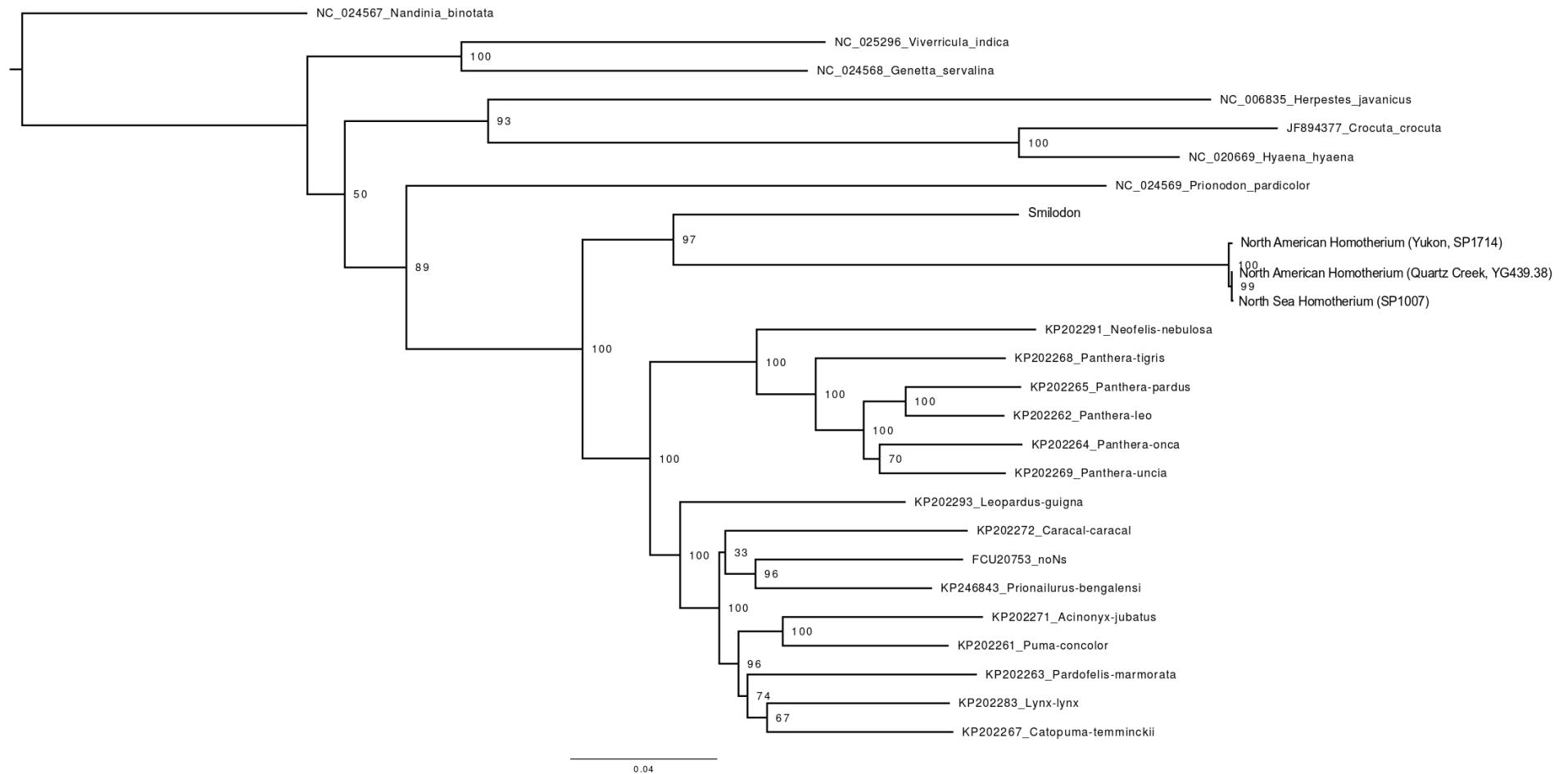
**Based on Ancient Mitogenomics**

**Johanna L.A. Paijmans, Ross Barnett, M. Thomas P. Gilbert, M. Lisandra Zepeda-Mendoza, Jelle W.F. Reumer, John de Vos, Grant Zazula, Doris Nagel, Gennady F. Baryshnikov, Jennifer A. Leonard, Nadin Rohland, Michael V. Westbury, Axel Barlow, and Michael Hofreiter**



**Figure S1: *Homotherium* YG 439.38 from Dominion Creek**

Left humerus of *Homotherium* YG 439.38 from Dominion Creek, Yukon: A) anterior view, B) posterior view, C) medial view, D) lateral view, E) distal view. Related to the morphological description in the STAR methods.



**Figure S2: Maximum likelihood phylogeny**

Majority Rule consensus tree generated using RaxML v8.2.4 [S1]. Node support is indicated as bootstrap percentages. Related to the maximum likelihood analyses described in the STAR methods.

Sample	Code	Total reads	Collapsed reads (used for assembly)	Reference	Assembled reads	mapped BP	read depth samtools	mitogenome
North American <i>Homotherium</i>	YG439.3	18,559,186	16,838,552	<i>Felis catus</i>	4,329	409,623	23.9	
				<i>Crocuta crocuta</i>	3,826	364,645	21.1	
				<i>Prionodon pardicolor</i>	4,061	385,561	22.4	92.4%
				<i>Felis catus</i>	6,981	601,976	35.0	
<i>Smilodon</i>	ZMA20.042	15,556,579	8,922,504	<i>Crocuta crocuta</i>	6,810	587,837	34.0	
				<i>Prionodon pardicolor</i>	6,515	563,288	32.8	76.6%

Sample	Code	Total reads	Trimmed/merged reads (used for mapping)	Reference	Aligned reads	Unique reads	mapped BP	read depth samtools	mitogenome
North Sea <i>Homotherium</i> (captured)	SP1007	72,759,982	47,585,057	YG439.3_consensus	8,881,785	3,160	144762	12.0	58.5%
North American <i>Homotherium</i> (captured)	SP1714	65,926,908	39,029,827	YG439.3_consensus	12,050,089	1,425	74693	6.5	44.5%

#### Unsuccessful samples & negative controls:

Possible European <i>Homotherium</i> (captured)	BAR005	63,503,213	41,914,703	YG439.3_consensus	272,328	104	4701	n/a	n/a
Extraction blank (captured)	eBLK2510	5,425,320	951,394	YG439.3_consensus	0	0	0	n/a	n/a
Library blank (captured)	liBLK2810	291	79	YG439.3_consensus	0	0	0	n/a	n/a
Extraction blank	eBLK-5-10-15	1,489,533	342,595	YG439.3_consensus	0	0	0	n/a	n/a
Library blank	liBLK-8-10-15	1,562,571	298,968	YG439.3_consensus	0	0	0	n/a	n/a
Possible European <i>Homotherium</i> (Hungary)	Pot-Homotherium-1	1,323,175	774,756	YG439.3_consensus	0	0	0	n/a	n/a
Possible European <i>Homotherium</i> (Hungary)	Pot-Homotherium-2	1,252,703	640,629	YG439.3_consensus	0	0	0	n/a	n/a
Possible European <i>Homotherium</i> (Hungary)	Pot-Homotherium-3	1,454,572	895,960	YG439.3_consensus	0	0	0	n/a	n/a
Extraction blank	EB	8,161,815	1,302,642	YG439.3_consensus	0	0	0	n/a	n/a
Library blank	LB	4,777,605	1,439,416	YG439.3_consensus	0	0	0	n/a	n/a
Possible European <i>Homotherium</i> (North Sea)	Y1394	10,662,459	6,806,396	YG439.3_consensus	1	1	42	n/a	n/a
Possible European <i>Homotherium</i> (North Sea)	Y1706	6,602,537	3,981,944	YG439.3_consensus	3	3	114	n/a	n/a
Possible European <i>Homotherium</i> (North Sea)	Y2972	12,305,888	7,470,173	YG439.3_consensus	0	0	0	n/a	n/a

**Table S1: Summarised sequence statistics for all samples included in our study.** Related to the bioinformatic procedures described in the STAR methods.

Collection	Specimen	Side	Locality	Taxon	Greatest length from caput	Minimum breadth of diaphysis	Greatest depth of diaphysis	Greatest breadth of trochlea	Greatest distal breadth	Minimum anteroposterior diameter of articulating surface for ulna	Maximum width of entepicondylar foramen	Maximum length of entepicondylar foramen	Width of entepicondylar bar	Reference	
YG	439.38	L	Dominion Creek, Sailer's	<i>Homotherium serum</i>	26.4	60.0	78.3	24.9	6.7	10.9	10.7	This study			
SMM	P2012:2:3	R	Tyson Spring Cave, Minnesota	<i>Homotherium serum</i>	383.0	30.2	39.7	53.1	72.0	29.7	7.8	12.8	11.5	[S2]	
TMM			Friesenhahn Cave, Texas	<i>Homotherium serum</i>	M	334.1	30.5	44.3	78.8	28.4	7.4	15.4	8.6	[S3]	
					OR	287.4 - 358	27.5 - 33	40.3 - 49	74.3 - 84.4	25.3 - 30.7	6.3 - 8.3	13.2 - 20.9			
					N	3	5	5	9	11	10	10			
LACM			Rancho La Brea, California	<i>Panthera leo atrox</i>	M	36.8	60.3	100.1	32.8					[S4]	
					OR	33.0 - 41.4	53.4 - 70.0	85.7 - 111.3	29.5 - 36.0						
					N	9	9	9	9						
YG	401.410	L	Lower Quartz Creek, YG, CAN	<i>Panthera leo spelaea</i>	28.6	52.1	70.0	86.1	29.1	8.9	15.6	11.5	This study		
YG	545.99	R	Upper Quartz Creek, YT, CAN	<i>Panthera leo spelaea</i>			64.8	74.1 (min)	24.5	6.7	13.7	11.4	This study		
YG	544.250	L	Lower Quartz Creek, YG, CAN	<i>Panthera leo spelaea</i>	351.1	31.4	56.1	92.5	30.9	10.4	20.8	12.5	This study		
YG	489.430	R	Upper Quartz Creek, YT, CAN	<i>Panthera leo spelaea</i>	25.8	61.7	76.9	23.6	7.8	13.8	12.6	This study			
YG	406.472	L	Lower Quartz Creek, YG, CAN	<i>Panthera leo spelaea</i>	31.6	58.6	77.7	94.7	32.1	9.5	17.9	11.5	This study		
YG	205.1	R	Last Chance Creek, YT, CAN	<i>Panthera leo spelaea</i>	27.1	46.9	64.9	77.5	26.3	7.3	12.9	11.9	This study		
YG	130.1	R	Lower Quartz Creek, YG, CAN	<i>Panthera leo spelaea</i>	37.8					6.1	15.8	12.8	This study		
NMC	11706		Dawson Loc. 28, YT, CAN	<i>Panthera leo spelaea</i>	32.5	54.6	90.6	29.1						This study	
NMC	27278		Old Crow Loc. 62, YT, CAN	<i>Panthera leo spelaea</i>	34.8	41.8	88.8	29.2						This study	

**Table S2: Morphometrics for *Homotherium* sample YG 439.38**

Measurements of *Homotherium* sample YG 439.38, in relation to cave lion remains recovered from the same or nearby locations. Abbreviations: YG = Yukon Government, SMM = Science Museum of Minnesota, LACM = Los Angeles County Museum, NMC = Canadian Museum of Nature. Related to the morphological description in the STAR methods.

<b>Species</b>	<b>Common name</b>	<b>Genbank Acc. Number</b>
<i>Acinonyx jubatus</i>	Cheetah	KP202271
<i>Caracal caracal</i>	Caracal	KP202272
<i>Catopuma temminckii</i>	Asian golden cat	KP202267
<i>Crocuta crocuta</i>	Spotted hyena	JF894377
<i>Felis catus</i>	Domestic cat	FCU20753
<i>Genetta servalina</i>	Servaline genet	NC_24568
<i>Herpestes javanicus</i>	Asian mongoose	NC_006835
<i>Hyaena hyaena</i>	Striped hyena	NC_020669
<i>Leopardus guigna</i>	Kodkod	KP202293
<i>Lynx lynx</i>	Eurasian lynx	KP202283
<i>Nandinia binotata</i>	African palm civet	NC_24567
<i>Neofelis nebulosa</i>	Clouded leopard	KP202291
North American <i>Homotherium</i> (SP1714)	Scimitar toothed cat	MF871702 (this study)
North American <i>Homotherium</i> (YG 439.38)	Scimitar toothed cat	MF871703 (this study)
North Sea <i>Homotherium</i>	Scimitar toothed cat	MF871701 (this study)
<i>Panthera leo</i>	Lion	KP202262
<i>Panthera onca</i>	Jaguar	KP202264
<i>Panthera pardus</i>	Leopard	KP202265
<i>Panthera tigris</i>	Tiger	KP202268
<i>Panthera uncia</i>	Snow leopard	KP202269
<i>Pardofelis marmorata</i>	Marbled cat	KP202263
<i>Prionailurus bengalensis</i>	Leopard cat	KP246843
<i>Prionodon pardicolor</i>	Spotted linsang	NC_24569
<i>Puma concolor</i>	Puma	KP202261
<i>Smilodon populator</i>	Dirk toothed cat	MF871700 (this study)
<i>Viverricula indica</i>	Indian civet	NC_025296

**Table S3: Mitogenome sequences used for sequence alignment and phylogenetic inference.**

Related to the phylogenetic analyses described in the STAR methods.

Product name	Sequence	# confirmed positions	# supplemented positions	Forward primer (5' → 3')	Reverse primer (5' → 3')	Notes
NA_Hom2_12S1	TTAGGCCCTAACCCAGACAGTTACCTAAACAAAATGTCGGCCAGAGAACACTAGGAACA	16	46	CTGGGATTAGATACCCCACTATGY	CCAAGTCTTGTAGTTTAAGC	
NA_Hom1_12S1_Sanger	TTAGGCCCTAACCCAGACAGTTACCTAAACAAAATGTCGGCCAGAGAACACTAGGAACA					
NA_Hom1_12S3	ATAATCCCGATACACCTCACCATCCCTTGTAAATCCAGCTTATACCGCCATCTTCAGCAAACCCCTAAAGGAGGAAAGTAAGCACAAGTATCTAACACA/	96	10	GAGGAGCCTGTTCYATAATCR	GCTACACCTTGACCTAACCTTT	
NA_Hom2_12S3	ATAATCCCGATACACCTCACCATCCCTTGTAAATCCAGCTTATACCGCCATCTTCAGCAAACCCCTAAAGGAGGAAAGTAAGCACAAGTATCTAACACA/					
NA_Hom2_12S4	ATCTTAAACAAAAAAGTTAGGTCAAGGTGTAGGCCAATGAGATGGAAGAACATGGCTACATTTCTACAGCT	73	0	AAGGAAGAAAAGTAAGCAMAAGT	GGATCTTCGTRRTTCT	
NA_Hom1_12S4	ATCTTAAACAAAAAAGTTAGGTCAAGGTGTAGGCCAATGAGATGGAAGAACATGGCTACATTTCTACAGCT					
NA_Hom2_12S5	ACAGCTAGAACCTACGAAACATCTTGTAAACTAAGGGTCAAGGGAGATTAGTAGAAATCTGAGAATAGAGAGCTAATTGAATCGGGCA	9	87	AAGCAATGGCTACATTTCT	GGTGTGCGTGCTTC	
NA_Hom1_12S5_Sanger	ACAGCTAGAACCTACGAAACATCTTGTAAACTAAGGGTCAAGGGAGATTAGTAGAAATCTGAGAATAGAGAGCTAATTGAATCGGGCA					
NA_Hom1_12S5	ACAGCTAGAACCTACGAAACATCTTGTAAACTAAGGGTCAAGGGAGATTAGTAGAAATCTGAGAATAGAGAGCTAATTGAATCGGGCA					
NA_Hom2_16S_6	CTTGTCCCTAAATGGGACTCGTATGAACTGGCCACAGGGCTTACTGTCTTACTCC	62	1	CGTGCAAAGGTAGCATAATCA	GAAGGTCATTACGGGATY	
NA_Hom1_16S_6	CTTGTCCCTAAATGGGACTCGTATGAACTGGCCACAGGGCTTACTGTCTTACTCC					
NA_Hom1_16S_7	TCCAATCAGTAAATTGCTTCCCAGAAGGGCGGAAATTAAATAAGCAGAGAACCTATG	46	22	CGAGGGCTTWCAGTCTCTTACT	TTGGGTCGTTAATRAAGCTC	
NA_Hom2_16S_7	TCCAATCAGTAAATTGCTTCCCAGAAGGGCGGAAATTAAATAAGCAGAGAACCTATG					
NA_Hom1_16S_8	TGGAGCTTAAATCAACTAACAGCTTACATGGACATCTACCTTATAGGTAGCA	78	0	ATAATAAAGACGAGAACYCTA	GAGGTACCCCAACCYAAAT	
NA_Hom2_16S_8	TGGAGCTTAAATCAACTAACAGCTTACATGGACATCTACCTTATAGGTAGCA					
NA_Hom1_16S_10	CRAGTGTAAATCTAGACTAACAGTCAAAGCATCATCACTATTGTACAAAAGCTGA	61	4	CTCGGAGAATARAYAACCTC	AGGGTAACTTGTTCCGTTGA	
NA_Hom2_16S_10	CGAGTGTAAATCTAGACTAACAGTCAAAGCATCATCACTATTGTACAAAAGCTGA					
NA_Hom2_16S_11	AAGCTGTACACGGAAACAGTTACCTTGGATAACCGGAATCTTACAGGTTCATATGACAATAGGGTTACGAC	83	0	GTAATACATCACTWATTGATCCAA	GTCTGATCCAACATGAG	
NA_Hom1_ND1_3	GATATGGTTTATCTAACATTAGCAGAAAACCCAGGCTTACATCGACCTAACAGAAGGAGAACACTGCTCCGGATTCAAT	87	0	CCTGCATGACCVYTRGCVAT	GGGCCTGCTGCRVAYTCTAC	G → A mismatch, but only 1 PCR sequence
NA_Hom2_ND1_4	CTCGGATTCAATGTAGAATCGCAGCAGGCCCTTGCCTCTAGCAGAAATGTACATCA	57	0	CTGACAGAAGGAGAACATCAGARYTNGT	GAATAGGATTGTTGAGGMTRTTTATA	N's not supplemented, only 1 PCR sequence
NA_Hom1_ND1_6	ACTATATACCATCACTCTAGTAAACACTTACCTACTTCTGAGATCCACCA	79	0	CCACAGCCCTTAYTDCCVGA	GTGTTAGTGTGTCRATYGRRA	
NA_Hom2_ND1_6	ACTATATACCATCACTCTAGTAAACACTTACCTACTTCTGAGATCCACCA					
NA_Hom1_ND1_9	TCTGATTTAAAGAGTCTTGTAGAGGAAATAATAGGGTTAACCCCTTATTCTAGAATTA	66	0	CCACCTCAAACATAAGAAATATG	AGGATTAGGTTCRANYYCTTA	
NA_Hom1_ND1_10	TTATAGGAATCGAACCTAACTCTAAGAATCCTTCTGCTTACACTTACACCATATTCTACAGTAA	56	18	GGTTAAACCTCTTATTCTAGAA	CGATAGCTTATTGCTGAC	
NA_Hom2_ND1_10	TTATAGGAATCGAACCTAACTCTAAGAATCCTTCTGCTTACACTTACACCATATTCTACAGTAA					
NA_Hom1_ND1_11	ACTATTACACCATATTCTACAGTAAGGTAATAAGCTATGGGCCCATACCCGAAATGTTGGTTATAT	54	0	CCAAAATCTCGTGTCTACC	GGTTGATTAGTAYGGAAAGG	
NA_Hom1_ND1_13	ATAATATCTCCATTGACTTTGTGAAATTGGCTTGAATAATCTAGCCATAATTCCATCTCATAAAAAAATAT	84	0	CCGTTATCTAGGAACATRATYGT	GCTTCTATGGCYCGTGGRTT	
NA_Hom1_ND1_15	TCAAGAACCGCTTCAATCTAACTAACTGAAACCTTACACTCTGACAACTGAGTACAAAGA	48	0	CAGCCACAAATTTYYTRAC	TTGATGCTATGGRTTRAGR	N's not supplemented, only 1 PCR sequence
NA_Hom1_ND1_20	ATATTCCCAATCTCCATTAGATGAACTACAGCTTACACTTACACCCATTAACCTAAACCTCATTTATTATC	0	0	CACAACCTACGAAAATCMTRGC	AATAGTATGAAGGTTGTYTTAT	No coverage for any of Homotherium specimens, sequence not supplemented
NA_Hom2_ND1_21	CACATTAATAATCTAAACCTCATTTATTATCATAACCATACCCATTACATTACATACCTCCGCCATACAACCTATCCCTGTACAAACAA	0	0	CAGCTATCATAAATRATYRYCC	TGATTAGGNGTTTTRTTCA	No coverage for any of Homotherium specimens, sequence not supplemented
NA_Hom1_ND1_21	CACATTAATAATCTAAACCTCATTTATTATCATAACCATACCCATTACATTACATACCTCCGCCACCAACACTATCCCTGTACAAACAA					No coverage for any of Homotherium specimens, sequence not supplemented
NA_Hom1_ATB8_1_Sanger	GGAGTTAAACCTCCCTTAATGATCACAATTAA	0	0	GCATTAACCTTTAAGTTAARAYTG	GATGGATCAGGKTAGYTRTC	No coverage for any of Homotherium specimens, sequence not supplemented
NA_Hom1_ATP8_2	ATTAGACACATCTACCTGATTCACTACTTATCATGATCATAACACTTATTGTATTCTAACCTTAA	0	0	CCCTTAATGRYATGCCACA	GGTTGATGGATATARTRYTTGA	No coverage for any of Homotherium specimens, sequence not supplemented
NA_Hom2_ATP8_2	ATTAGACACATCTACCTGATTCACTACTTATCATGATCATAACACTTATTGTATTCTAACCTTAA					No coverage for any of Homotherium specimens, sequence not supplemented
NA_Hom1_ND4_7	TAATTCTGCTCCACGTAAAAACGTGCTTTCACTTTTATAGGATAGAAGTTACCTCTGGTCTAGGAACAAA	62	0	CGAAAAAGTATGCAAGAACACTGC	ATTTGGAGTGCACCAATT	N's not supplemented, only 1 PCR sequence
NA_Hom1_ND5_4	AATCTCATCTAACTACATTAAGCTTAAACTGTTACTCTTATTCTGGTACCAAGTACAGCT	0	0	ATTATCTAAACACTGACACTGAYTRDC	CTATGATGGYCATGTRACRAA	No coverage for any of Homotherium specimens, sequence not supplemented
NA_Hom2_ND5_7	TATTGGCTGAGGGAGTAGGAGTATATCTCTCTCATCGATGATGACACGGCGAGCAGATGCTAACACTGCC	20	62	CCGCTAACATCTTCTC	CGGGTTGAGGATTGCTGYAR	
NA_Hom1_ND5_7	TATTGGCTGAGGGAGTAGGAGTATATCTCTCTCATCGATGATGACACGGCGAGCAGATGCTAACACTGCC					
NA_Hom2_cytB_1	AACTACAAGAACCTAATGACCAACATTGAAAATCACCCCCCTAATTAAACATCATCAACCAATCATTCTGACCTACCTACCCCTCAACATCTCAGCA	51	0	ATGAAAACCATCGTGTTRYTC	GGAGCCGAARTTYCAYCA	
NA_Hom1_cytB_1	AACTACAAGAACCTAATGACCAACATTGAAAATCACCCCCCTAATTAAACATCATCAACCAATCATTCTGACCTACCTACCCCTCAACATCTCAGCA					
NA_Hom2_cytB_2	AGCATGATGAAACTCGGATCCCTACTAGGGATTCTCTTAACTCTCAACCTCACAGGCTTATTCTAG	0	0	CGCCCCATCYAAYATYTC	GTGTCATGATGTTGTRYATRG	
NA_Hom1_cytB_2	AGCATGATGAAACTCGGATCCCTACTAGGGATTCTCTTAACTCTCAACCTCACAGGCTTATTCTAG					
NA_Hom1_cytB_3	AGGCTTATTCTCTAGCCATACACTACACATCACAGACACAACTGTTCTCATCAATTGCCATTATTGCCGT	52	0	GCCTAACCTYACARATYCTNAC	ATGATTCAACCTARTTDACRTC	C → T mismatch, but only 1 PCR sequence
DQ097176 [S5]	CTCTAAATCATGAAACCTAATCTGACTTACCTACCCCTTCAACATCTGAGCATGAAACACTGGATCCCTACTAGGCATTGGCTAACTTCTCAAATCTCTGATCTCACCATACATCAGACACAACTGCTTCTCATCAATTGCCATTATTGCCGTAGCTGAGGAAATTATGGTGAATTATCCGATATGACAGGCAATGGAGCTCTATATTCTCATCTGATACCTGAGCTGAGGAATTATTAGGGTTCTACACCTTCACTAGAAACATGAAATATGGAAATCTCTAGCATGAGGCTTATTCTCTGAGGGACTACCTTCACTGGGAGCTTATTACCAACCTCT	86	361	See [S5]	See [S5]	

**Table S4: Preliminary and published PCR data for sample SP1714.** Related to the preliminary PCR data and data validation described in the STAR methods.

## **Supplemental References**

- S1. Stamatakis, A. (2014). RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics*, btu033.
- S2. Widga, C., Fulton, T.L., Martin, L.D., and Shapiro, B. (2012). Homotherium serum and Cervalces from the Great Lakes Region, USA: geochronology, morphology and ancient DNA. *Boreas* 41, 546–556.
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- S4. Merriam, J.C., and Stock, C. (1932). The Felidae of Rancho LaBrea (Carnegie Institution of Washington).
- S5. Barnett, R., Barnes, I., Phillips, M.J., Martin, L.D., Harington, C.R., Leonard, J.A., and Cooper, A. (2005). Evolution of the extinct Sabretooths and the American cheetah-like cat. *Curr. Biol.* CB 15, R589-590.