Evolutionary history of sabre-toothed cats based on ancient mitogenomics

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Summary

Sabre-toothed cats (Machairodontinae) are among the most widely recognised 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 sabre-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 sabre-toothed cats (*Smilodon* and *Homotherium*) in relation to living carnivores, and find 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.

41samples, including the only Late Pleistocene *Homotherium* sample from Eurasia [4]. We confirm
42the identification of the unique Late Pleistocene European fossil through ancient DNA analyses,
43thus strengthening the evidence that *Homotherium* occurred in Europe over 200,000 years later than
44previously believed. This in turn forces a re-evaluation of its demography and extinction dynamics.
45Within the Machairodontinae, we find a deep divergence between *Smilodon* and *Homotherium* (~18
46million years), but limited diversity between the American and European *Homotherium* specimens.
47The genetic data support the hypothesis that all Late Pleistocene (or post-Villafrancian)
48*Homotherium* should be considered a single species, *H. latidens,* which was previously proposed
49based on morphological data [5,6].
50

51**RESULTS AND DISCUSSION**

52*Homotherium* and *Smilodon* were large-bodied predators with widespread distributions. The
53Holarctic genus *Homotherium* has Old World origins, with Late Pleistocene forms in Eurasia
54generally assigned to *H. latidens* while those in North America to *H. serum* [7,8]. The New World
55genus *Smilodon* is thought to have evolved from Old World dirk-toothed cats of the genus
56*Megantereon* which may have dispersed into the Americas during the Pliocene (Blancan). Two Late
57Pleistocene (Rancholabrean) *Smilodon* species are recognised, with *S. fatalis* confined to areas
58south of the continental ice sheets in North America, while the contemporary, larger, and more
59robust *S. populator* was restricted to South America. Despite their widespread occurrence,
60*Homotherium* and *Smilodon* remains are uncommon and generally fragmentary in the fossil record,
61except in rare cases, e.g. [9,10]. *Homotherium* in particular is generally only represented by isolated
62cranial or dental elements, leading to many uncertainties about their taxonomy, demography and
63extinction dynamics. Both *Homotherium* and *Smilodon* survived in North America until the Late
64Pleistocene, and went extinct alongside many other megafauna species on the continent (e.g.
65mammoth and giant sloth [11]). In Eurasia, however, *Homotherium* is generally thought to have
66gone extinct much earlier, during the Middle Pleistocene around 300,000 years ago [12–15]. To
67date, there is only a single dated Late Pleistocene *Homotherium* fossil recovered in Europe [4]. We
68used ancient DNA techniques to retrieve and analyse genetic data from this individual, and
69compared the specimen to two North American *Homotherium* and one South American *Smilodon*
70specimen, in order to investigate the evolutionary history of the Machairodontinae, and the
71taxonomy, demography and phylogeography of *Homotherium*.
72

73**Evolutionary history of Machairodontinae**

74 Mitochondrial genome data (mitogenomes) were generated for one *Smilodon* and three
75 *Homotherium* specimens using hybridization capture, and assembled using both an iterative
76 mapping approach using three different mitochondrial sequences as initial reference seeds, as well
77 as regular read alignment approach (for more details see STAR Methods; Figure S1; Table S2). The
78 *Smilodon* sample was collected in Chile, and is dated to 11,335 years (carbon dates are given as
79 uncalibrated ^{14}C years; Table 1). Two *Homotherium* fossils were collected in the Yukon Territory
80 (northwest Canada), and both proved to be beyond the limits of radiocarbon dating (>50,000 years;
81 Table 1; [3]). The European *Homotherium* was recovered from the North Sea, and is dated to
82 ~28,000 years old (Table 1; [4]). The recovered mitogenomes had an average depth of 19x (7 -
83 35x), resulting in partial mitogenome sequences spanning 44.5 - 92.4% of the expected sequence
84 length (Table S1). The recovered Machairodontinae mitogenomes were aligned with 22 additional
85 carnivoran mitogenomes retrieved from GenBank and subjected to Maximum-Likelihood (ML) and
86 Bayesian phylogenetic analyses (Table S3). The resulting ML phylogeny confirms the placement of
87 *Smilodon* and *Homotherium* as sister lineages in the subfamily Machairodontinae with 94%
88 bootstrap support (BS) and a Bayesian Posterior Probability (BPP) of 0.99, basal to all extant
89 Felidae species (100% BS, 1.0 BPP; Figure 1; Figure S2). The mitogenome-based phylogenetic
90 relationship between *Homotherium* and *Smilodon* data is in agreement with analyses based on
91 morphological evidence [8], and shorter mitochondrial sequences [1,3]. We then used a time-
92 calibrated Bayesian analysis to estimate divergence times on the ML topology with multiple fossil
93 calibration points (Table 2). The estimated median time to the most recent common ancestor
94 (tMRCA) for all Felidae was 20 million years ago (MYA; 95% credibility interval: 18.2 –22.0
95 MYA). This is in line with earlier estimates of 14.5 to 21.5 MYA [3]. The tMRCA for extant Felids
96 was found to be 14.2 million years ago, also similar to other estimates (e.g. 15.3 to 17.4 MYA [16]).
97 The calibrated phylogeny indicates a deep divergence between *Smilodon* and *Homotherium* (18.0
98 MYA; 95% credibility interval: 16.0 – 20.0 MYA, estimated sequence divergence ~11%),
99 supporting an Early Miocene separation into the tribes Smilodontini and Homotherini, respectively
100 (the latter is sometimes referred to as Machairodontini [17]). The oldest undisputed *Homotherium*
101 fossils from Early Pliocene assemblages in Ukraine and Kenya suggest either a Eurasian or African
102 origin of the genus, and a subsequent dispersal into America during the Late Pliocene (Blancan;
103 [18,19]). *Smilodon* remains have only been recovered on the American continents, from the
104 Miocene-Pliocene boundary to the Late Pleistocene, and have never been found in Eurasia [20]. The
105 deep divergence inferred from our mitogenome data between *Homotherium* and *Smilodon* is
106 congruent with the proposed evolution of these genera around the Miocene-Pliocene transition on
107 separate continents. Within *Smilodon* there are currently two recognised Late Pleistocene species: *S.*
108 *populator*, which has so far only been found in South America, and *S. fatalis*, the last surviving

109*Smilodon* species of North America [8]. The *Smilodon* specimen investigated in this study was
110recovered in Chile and is dated to 11,335 years before present (Table 1); it thus can be assigned to
111the South American Late Pleistocene species *S. populator*. The tMRCA of the three *Homotherium*
112individuals as inferred from the Bayesian analyses is 144,800 years (95% credibility interval:
11377,076 - 215,970 years, estimated sequence divergence 0.2 - 0.04%). This divergence date is
114relatively recent, and similar to that reported for other felid species (e.g. leopards from Asia [21]).
115

116**Late Pleistocene occurrence of *Homotherium* in Eurasia**

117Our genetic analyses corroborate published radiocarbon dates and morphological descriptions,
118which together provide conclusive evidence that the specimen recovered from the North Sea
119represents the first confirmed Late Pleistocene *Homotherium* from Eurasia, forcing a re-evaluation
120of the traditional view of the demographic processes that preceded extinction of this iconic
121megafaunal species. Very few other Late Pleistocene *Homotherium* fossils have been recovered in
122Europe [22,23], and their age, origin and species identification are subject to much discussion [24–
12327]. The *Homotherium* specimen investigated here was found on the Brown Bank region in the
124North Sea (approximately 80 km off the Dutch Coast), an area where Late Pleistocene and Early
125Holocene fossils are commonly found from species that existed in Western Eurasia [28].
126Furthermore, the fragile state of the North Sea mandible makes it unlikely to have been transported
127from remote regions, for example through taphonomic processes. Based on morphological
128characteristics, the specimen was identified as *Homotherium* rather than any other Late Pleistocene
129felid genus [4]. The Late Pleistocene age of this fossil has been confirmed through six independent
130radiocarbon dates (~28,000 years old [4]), which makes it the only firmly dated Late Pleistocene
131fossil in Europe assigned to the genus *Homotherium*. The occurrence of *Homotherium* in Europe
132during the Late Pleistocene could be the result of several different demographic scenarios. The Late
133Pleistocene *Homotherium* population in Eurasia may have existed at low population densities,
134effectively dropping under the "fossil detection threshold", with very few remains surviving in the
135fossil record, which has also been previously proposed as an explanation for the low abundance of
136*Homotherium* fossils in America [29,30]. This scenario would not be unique to *Homotherium*; for
137example, although there are currently only four fossils recovered from the Denisovan hominins
138from a single cave, genetic data indicates that they occupied large parts of Eurasia during the Late
139Pleistocene [31–34]. Despite its widespread Holarctic distribution during the Late Pleistocene,
140*Homotherium*, like other megafaunal species, proved vulnerable to environmental and/or ecological
141changes, which led to its eventual extinction. Alternatively, it is conceivable that the *Homotherium*
142found in the North Sea descends from a Late Pleistocene dispersal from a core population in Central

143Eurasia or Beringia, as has been suggested for other Pleistocene megafauna (e.g. mammoth [35] and
144wolves [36]). Similar to extant large felids [e.g. 37], *Homotherium* is likely to have been a highly
145mobile taxon, and may have re-colonised Europe during the Late Pleistocene after the resident
146population went extinct in the Middle Pleistocene. This scenario is consistent with the estimated
147coalescence timing of the European and American *Homotherium* mitochondrial lineages (95%
148Credibility Interval: 77 - 216 Ka).
149

150In order to identify which of the possible demographic scenarios is applicable to Late Pleistocene
151*Homotherium*, additional samples have to be recovered and analysed. However, all of these
152scenarios point to a situation where *Homotherium* roamed at least part of the Eurasian continent for
153hundreds of millennia later than was previously believed. This situation forces a re-assessment of
154the Late Pleistocene population dynamics and timing of extinction of this large felid species. Some
155of the general attributes which threaten extant large-bodied felids [38,39], such as large body-size,
156high trophic level (i.e. hypercarnivory [40–42]), and low population densities and/or fragmented
157populations may also have placed *Homotherium* at risk. However, our evidence of Late Pleistocene
158survival of *Homotherium* in Europe suggests that these factors may not have been the sole driving
159force behind its extinction, since it survived for over 200,000 years at low or fragmented population
160densities, as suggested by the scarcity of fossils. Thus, gathering additional insights into the
161population structure and extinction dynamics of *Homotherium* may also help explain why the
162extinction risks of extant felids are sometimes overestimated [43]. Ultimately, *Homotherium* was
163unable to survive the climatic and ecological changes that occurred the end of the Pleistocene, a
164time during which many other large-bodied mammals such as mammoths [35] and cave lions [44]
165also experienced severe population fluctuations and extinction. In order to gain a better
166understanding of the population dynamics of *Homotherium* during the Late Pleistocene and why it
167eventually went extinct, more samples will have to be recovered and analysed from Europe as well
168as Asia. In light of the morphological and genetic evidence for the Late Pleistocene occurrence of
169*Homotherium* in Europe, it is conceivable that some Late Pleistocene remains that are currently
170assigned to one of the more common large cat species (e.g. cave lion) could be re-identified as
171*Homotherium*.
172

173**Taxonomic revision of Holarctic *Homotherium***

174Species-level identification of sabre-toothed cats has been based on geographical and/or
175morphological data, which hold a number of inherent limitations [45]. The data presented here
176allow for a direct comparison at the mitochondrial DNA level between the commonly recognised

177*Homotherium* species that inhabited the North American and Eurasian continents: *H. serum* and *H.*
178*latidens*, respectively [8]. We found low mitogenome diversity among Late Pleistocene
179representatives of the genus, and a tMRCA of ~145,000 years. Previous studies based on short
180mitochondrial sequences from North American *Homotherium* have also found low levels of genetic
181diversity, despite considerable geographical (>2000 km) and temporal (>25,000 years) separation of
182the fossils [1]. We were unable to compare our North Sea and North American mitogenomes with
183previously published short mitochondrial sequences from other individuals [1,3], as we did not have
184complete sequence coverage for the relevant mitochondrial regions (e.g. 16S, cytB, ATP8).
185However, the very recent tMRCA (~145,000 years) for the three *Homotherium* mitogenomic
186sequences is also indicative of low diversity between the *Homotherium* sequences, particularly
187considering their geographical distance. Low intraspecific diversity in such a widespread species
188has been previously reported for other carnivores (e.g. ancient lion sequences [44]; modern wolf
189sequences [36]). We therefore compared the intraspecific diversity of the three *Homotherium*
190mitogenomes to the diversity between subspecies of other big cats (tiger, lion and leopard), and
191found the *Homotherium* sequence diversity to be lower than those for any extant felid subspecies
192(STAR methods). The low mitogenomic genetic diversity is further supported by the low genetic
193diversity measured between short ~~mtDNA~~mitochondrial DNA fragments from two North American
194*Homotherium* [1]. This degree of genetic similarity suggests that all three *Homotherium* individuals
195were representatives of a single Late Pleistocene species, thus casting doubt on the validity of the
196distinct American and Eurasian *Homotherium* species currently recognised (*H. serum* and *H.*
197*latidens*, respectively). Furthermore, the European *Homotherium* mitochondrial sequence is nested
198within the diversity of two American *Homotherium* sequences in the phylogeny (Figure 1), further
199supporting the monospecificity of all Late Pleistocene Holarctic *Homotherium* populations.
200

201Since the first *Homotherium* fossil discovery in 1824 [46], multiple *Homotherium* species have been
202proposed. However, these have typically been based on geographical or temporal separation of
203fossils, rather than distinguishable morphological characteristics [8]. In North America, older
204(Pliocene) fossils are considered morphologically distinct from younger Pleistocene finds, and are
205thus generally separated into two species; *H. ischyrus* and *H. serum*, respectively [7,29,47]. In the
206Eurasian fossil record such distinction between older and younger forms is controversial: while
207earlier studies recognised two [48], or even three distinct Eurasian species [49], recent finds from
208Spain suggest that all Pleistocene Eurasian *Homotherium* fossils are more accurately grouped into a
209single, morphologically variable species, *H. latidens* [7]. These authors also note that the variation

210within *H. latidens* is extensive enough to assign North American *H. serum* fossils – if they were
211found in Europe – to *H. latidens* [7].
212

213The morphological overlap between North American and Eurasian *Homotherium* fossils has been
214regarded as evidence that all Pleistocene *Homotherium* can be assigned to a single, morphologically
215variable species [5,6]. It has also previously been suggested, based on morphological similarities
216between two Early Pleistocene individuals from France [50] and Oregon, that these individuals
217should belong to the same species [30]. The high similarity found between ~~mtDNA~~mitochondrial
218DNA fragments recovered from two North American (Yukon and Great Lakes Region)
219*Homotherium* fossils also indicates a very close relationship between the individuals, despite their
220considerable geographical and temporal distance [1]. Although clearly limited due to small sample
221size, the mitochondrial DNA evidence we present here further supports the hypothesis, suggested
222previously based on morphological data, that at least Late Pleistocene North American and Eurasian
223*Homotherium* are monospecific, rather than two separate species. For reasons of priority, this taxon
224should be called *H. latidens* [51]; consequently, *H. serum* [52] is a junior synonym.
225

226**Conclusions**

227In this study, we present partial mitogenome sequences from two lineages of Machairodontinae,
228*Smilodon* and *Homotherium*, and confirm the phylogenetic relationships and evolutionary history of
229these iconic felids. Furthermore, the mitochondrial DNA we recovered from the North Sea
230*Homotherium* specimen confirms the Late Pleistocene survival of this enigmatic sabre-toothed cat
231in Eurasia. Much like the Denisovan hominins, the North Sea *Homotherium* represents another
232striking example of the major gaps in our knowledge of Pleistocene fauna composition on the
233Eurasian continent, and holds important clues about population and extinction dynamics of
234Pleistocene species. By applying DNA analysis on ancient samples, even a controversial find such
235as the North Sea *Homotherium* can be firmly identified. The *Homotherium* mitogenome sequences
236revealed low genetic diversity, which strongly supports the hypothesis based on morphology of a
237single, widespread Holarctic *Homotherium* species during the Late Pleistocene (*H. latidens*). This
238study highlights the importance of combining morphological and genetic information for species
239identification.

240

241**Author contributions**

242Conceptualisation, MH, JWFR, MTPG, RB, JLAP; Methodology, JLAP, MH, AB, MTPG, RB;
243Investigation, JLAP, RB; Formal Analysis, JLAP, RB, AB, MLZM, MW; Resources, JWFR, AL,
244NR, JAL, GB, JdV, MK, GZ, DN; Writing, JLAP, MH, AB, JWFR; Discussion, JLAP, MH, RB,
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246

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266**Figure legends**

267**Figure 1: Calibrated phylogeny for *Smilodon* and *Homotherium***

268 Time calibrated mitochondrial phylogeny of the Felidae, including the sabre-toothed cat *Smilodon*
269and scimitar-toothed *Homotherium*. Node support is indicated by Bayesian Posterior Probabilities
270(see Figure S2 for RAxML phylogeny and bootstrap values). Calibrated nodes are indicated with a
271star (see also Table 2). Blue node bars indicate the 95% credibility interval of divergence times. The
272lower axis shows millions of years. *Homotherium* artwork was provided by Binia De Cahsan. The
273image of the mandible is adapted from [4]. See also Figure S1-S2.

274**Table 1: Sample details of *Smilodon* and *Homotherium***

275Sample details for the *Smilodon* and *Homotherium* samples included in this study. In this table, only
276samples for which a (partial) mitogenome could be reconstructed are listed. All radiocarbon ages
277are given in uncalibrated years before present. See also Figure S1, Table S1-S4.

278**Table 2: Fossil constraint used for calibrated phylogeny**

279Fossil constraints and calibration priors used in the time-calibrated BEAST analysis [8,79–82].
280

281**person**

282 **STAR methods**

283

284 **CONTACT FOR REAGENT AND RESOURCE SHARING**

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

287

288 **METHOD DETAILS**

289

290 *Morphological description for sample YG 439.38 (North American* Homotherium*):* Specimen YG
291 439.38 from Dominion Creek, Yukon, consists of the distal three-quarters of a left humerus (Figure
292 S1). The specimen is generally well preserved, except some erosion on the posterior parts of both
293 epicondyles. *Homotherium* specimens are very rarely recovered in eastern Beringia (unglaciated
294 parts of Alaska and Yukon), but their humeri can be readily distinguished from much larger, and
295 more robust *Panthera leo spelaea*, the only other large Pleistocene felid that is also known from the
296 region (Table S2). Some of the key distinguishing characteristics are (1) general slenderness of the
297 humerus shaft; (2) the angle of intersection of the deltoid and medial ridges is relatively more acute;
298 (3) the lateral supracondylar ridge is relatively straight and sharp, while in *Panthera* it is slightly
299 convex and more obtuse crested; (4) the relatively small entepicondylar foramen; (5) the
300 entepicondylar bar is in a more anterior position; and (6) the relative prominence of the lateral
301 epicondyle and weaker development of muscle scar above. The specimen compares well with
302 descriptions and mensurational data from other *Homotherium* material from the Pleistocene of
303 Yukon [53] and areas in midcontinental North America [1,9]. Morphological characteristics for
304 remaining *Homotherium* specimens have been described elsewhere [3,4].
305
306
307 *Laboratory procedures:* All pre-PCR procedures were performed in dedicated ancient DNA
308 facilities with appropriate contamination precautions in place [e.g. 54]. Experiments for samples
309 YG 439.38 and ZMA20.042 were performed at the Centre for GeoGenetics, University of
310 Copenhagen. Samples SP1714 and SP1007 (Table 1) were processed in ancient DNA facilities of
311 the Evolutionary Adaptive Genomics group at Potsdam University. Preliminary PCR data
312 generation was performed in 2008 at the Max Plank Institute for Evolutionary Anthropology,
313 Leipzig (MPI EVA).

314

315*Preliminary PCR data:* DNA was extracted from sample SP1714 in dedicated cleanlab facilities at 316the MPI EVA using a silica spin column protocol and a vacuum manifold [55]. Primer pairs (Table 317S4) were split into two pools of non-overlapping fragments. A total of 4 multiplex PCRs were set up 318in 25µl reaction volumes using 5 µl template, containing: 1x AmpliTaq Gold buffer, 4 mM MgCl2, 3191 mg/ml BSA, 0.2mM each dNTP, 2U AmpliTaq Gold, and 1 µM of each primer in a pool of non-320overlapping primer pairs (even vs odd numbered primer pairs [56]). PCR cycling conditions were as 321follows: initial denaturation at 94°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds, 32255°C for 30 seconds and 72°C for 15 seconds, ending with a final extension for 5 minutes at 72°C. 323After multiplex PCR, a simplex PCR was carried out for each individual primer pair using the same 324conditions as described above and 5µl of a 40-fold dilution of the respective multiplex PCR. PCR 325products were tagged and built into NGS libraries [57], and sequenced on a 454-GS20. Raw data 326were demultiplexed using a custom script and aligned to make a final consensus sequence per PCR 327product. The resulting data were used for validating and extending the captured mitogenome 328sequences for sample SP1714 generated at the University of Potsdam (described below; Table S4).
329

330*Extractions:* All pre-PCR procedures (extraction, library building) were performed in dedicated 331ancient DNA facilities at the University of Potsdam and the Centre for GeoGenetics, University of 332Copenhagen, with contamination precautions in place. For specimens YG439.38 and ZMA20.042, 333samples of cortical bone were taken from long bone element (approx. 1 cm^3) using a Dremel 334powertool, reduced to powder in a Mikrodismembrator, and extracted according to the protocol 335described in Orlando et al. [58]. For the remaining *Homotherium* samples, DNA was extracted 336according to the protocol by Dabney et al. [59]. All procedures included negative controls that were 337processed in parallel with the samples.
338

339*Library preparation:* For specimens YG 439.38 and ZMA20.042, DNA extract and negative 340controls were built into genomic libraries using the NEB E6070 kit and a slightly modified version 341of the protocol as used by Vilstrup et al. [60]. Briefly, extract (30µl) was end-repaired and then 342passed through a MinElute column. The collected flow-through was then adapter-ligated and passed 343through a QiaQuick column. Adapter fill-in reaction was then performed on the flow-through, 344before final incubation at 37°C (30 minutes) followed by inactivation overnight at -20°C. For 345libraries of specimens YG 439.38 and ZMA20.042, we amplified in a 50µl reaction volume, using 34625µl of library for 12 cycles under the following reaction conditions. Final concentrations were 1.25 347U AccuPrime™ Pfx DNA Polymerase (Invitrogen), 1x AccuPrime™ Pfx reaction mix (Invitrogen),

3480.4mg/ml BSA, 120nM primer in TE, and 120nM of a multiplexing indexing primer containing a
349unique 6 nucleotide index code (Illumina). PCR cycling conditions consisted of an initial
350denaturation step at 95°C for 2 minutes, followed by 12 cycles of 95°C denaturation for 15 seconds,
35160°C annealing for 30 seconds, and 68°C extension for 30 seconds. A final extension step at 68°C
352for 7 minutes was also included. Library preparation success was checked on a 2% Agarose gel
353before purification using the QIAquick column system (Qiagen) and quantification was performed
354on an Agilent 2100 BioAnalyzer.
355
356For remaining *Homotherium* specimens, libraries were prepared according to the single-stranded
357library protocol as set out in Gansauge & Meyer [61]. The optimal cycle number for every library
358was estimated using qPCR [61]. Amplification was performed in 4 parallel reactions of 20 µl each.
359Final concentrations in the indexing PCR reaction: 0.5 U AccuPrime™ Pfx DNA Polymerase
360(Invitrogen), and 1x AccuPrime™ Pfx reaction mix (Invitrogen), 0.75 µM each of the Illumina
361indexing primers, with a unique 8 nucleotide index incorporated in the P7 primer. PCR cycling
362conditions were as follows: initial denaturation step at 95°C for 2 minutes, followed by the qPCR-
363estimated number of cycles of 95°C denaturation for 15 seconds, 60°C annealing for 30 seconds,
364and 68°C extension for 60 seconds, followed by a 3 min final extension at 68°C. Libraries were
365visualised on the Agilent Tapestation 2200 and measured using Qubit 2.0 Fluorometric
366quantification.
367
368*Capture:* For specimens YG 439.38 and ZMA20.042, two sets of capture experiments were
369performed. The first method used biotinylated RNA probes transcribed from fresh DNA extract
370derived from modern lion tissue by MYcroarray (Ann Arbor, MI, USA). The second method used
371previously published lion genome data [62] to identify exon coding regions and create biotinylated
372RNA baits that covered these regions. Both sets of baits were used in conjunction with MYbaits
373genome capture kit to enrich the ancient extracts for endogenous felid DNA. After capture and
374cleanup, enriched libraries were re-amplified for further sequencing using Phusion polymerase with
375primers IS5_reamp.P5 and IS6_reamp.P7 over 14 cycles [63]. The sequencing data resulted from a
376pooled product of both the whole-genome enrichment and exon capture. Thus, although the
377mitochondrial data is likely to have come from the whole-genome enrichment experiment as the
378exon capture bait set did not contain ~~mtDNA~~mitochondrial DNA baits, we could not distinguish
379between the two in the resulting data.
380For *Homotherium* samples SP1714 and SP1007, mitogenome MYbait capture baits were designed
381from preliminary mitogenome data from sample YG 439.38, using only regions with ≥5x coverage.
382Missing or ambiguous regions were replaced by a reconstructed ancestral felid mitogenome [64].

383Capture was performed according to the protocol described by Li et al. [65], at a hybridisation
384temperature of 65°C. Additional European *Homotherium* samples were screened for endogenous
385content using low-level shotgun sequencing, but due to the low estimated endogenous content, these
386samples were not used for sequence capture (Table S1).
387

388QUANTIFICATION AND STATISTICAL ANALYSES

389Bioinformatic procedures

390*Mitogenome assembly:* For samples YG 439.38 (*Homotherium*) and ZMA20.042 (*Smilodon*), an
391iterative mitogenome assembly method was used to reconstruct the mitogenome in the absence of a
392close reference. Raw sequences were trimmed using cutadapt v1.10 for single-end data (Martin,
3932011), using a length cut-off of 25bp. Before mitogenome assembly, duplicate reads were removed
394from the fastq data using PRINSEQ-lite v0.20.4 [66]. For both YG 439.38 (*Homotherium*) and
395ZMA20.042 (*Smilodon*), MitoBIM v1.8 [67] was used to reconstruct the partial mitogenomes.
396MITObim was implemented using three different references as starting bait sequences (*Felis catus*
397(Genbank: FCU20753), *Crocuta crocuta* (Genbank: JF894377.1) and *Prionodon pardicolor*
398(Genbank: NC_024569.1)) with default parameters apart from adjustments to the kmer value
399(kvalue = 25) and mismatch values [following 68]. We tried different mismatch values, ranging
400from 0-8%. For both the *Homotherium* and *Smilodon*, no additional mitogenomic information was
401recovered using a mismatch value of above 3%. We therefore decided upon 3% as our mismatch
402value. MITObim output mira files were converted to sam files and then visualised using Geneious.
403For each starting bait sequence, a reference consensus sequence was constructed using a minimum
404coverage value of 10x and a base call threshold of 75%. These three sequences were then aligned
405using Mafftv7.271 and a majority rule consensus base calling was implemented to generate the final
406*Homotherium* and *Smilodon* mitochondrial sequences.
407

408*Mitogenome mapping:* remaining *Homotherium* samples were aligned to the mitogenome assembly
409for YG439.38 (Table S1). Raw sequences were trimmed using SeqPrep (available from
410https://github.com/jstjohn/SeqPrep) for paired-end data, and cutadapt v1.10 for single-end data [69].
411All reads shorter than 30 bp were discarded: a more stringent length cut-off than for samples YG
412439.38 (*Homotherium*) and ZMA20.042 (*Smilodon*) to ensure reliable read alignment. The
413Burrows-Wheeler Aligner (BWA) v0.7.8 [70] was used for read mapping, with default values for
414seed length (32 bp) and mismatch values (0.04). Samtools v1.19 [71] was used to remove reads
415with a mapping quality <Q30. Duplicates were identified according to both the 5' and 3'-end
416mapping coordinates using MarkDuplicatesByStartEnd.jar

417((https://github.com/dariober/Java-cafe/tree/master/MarkDupsByStartEnd). The consensus
418sequence was generated using Geneious v7.0 [72], using a minimum sequence depth of 4x and a
41975% majority rule for base calling. For sample SP1714, short ~~mtDNA~~mitochondrial DNA
420sequences from earlier published work [3] and preliminary generated PCR data (Table S4) were
421compared to the mitogenome retrieved using capture, for an independent validation of parts of the
422mitogenome sequence (over 1,200 bp of the capture consensus sequence). Furthermore, regions
423where there was no coverage using the capture data could be supplemented using the PCR data
424(about 600 bp).
425

426**Phylogenetic analysis**

427*Alignment:* Mitogenome sequences were aligned using ClustalW v2 [73] as implemented in
428Geneious v7.0. The control region, as well as any positions in the alignment that contained missing
429data, were removed. The resulting alignment (6,649 bp in length) was manually annotated in
430Geneious using the domestic cat (Genbank~~ Acc. Nr.~~: FCU20753) as reference. All mitochondrial
431regions except for the control region were present in the alignment, although these were highly
432fragmented and partially incomplete due to the removal of missing data. For intraspecies
433comparison between *Homotherium* and other large-bodied felids, mitogenomes for tiger, lion and
434leopard subspecies were downloaded and aligned with the three *Homotherium* specimens using
435ClustalW v2. Alignment columns containing missing data were not considered to enable direct
436comparison of genetic distances within extant species with those estimated from partial
437*Homotherium* and *Smilodon* assemblies. The alignment contained four tiger subspecies (*Panthera*
438*tigris altaica* [GenBank: JF357973], *P. t. amoyensis* [GenBank: HM589215], *P. t. tigris* [GenBank:
439JF357968], and *P. t. sumatrae* [GenBank: JF357969]), two leopard subspecies (*Panthera pardus*
440*orientalis* [GenBank: KX655614], and *P. p. japonensis* [GenBank: KJ866876]) and two lion
441subspecies (*Panthera leo leo* [GenBank: KP001502] and *P. l. persica* [GenBank: KP001501]). The
442observed genetic distances (p-distance) was measured in MEGA v5.2 [74] to be 0.006, 0.007, 0.003
443and 0.001 for tiger, leopard, lion and *Homotherium*, respectively.
444

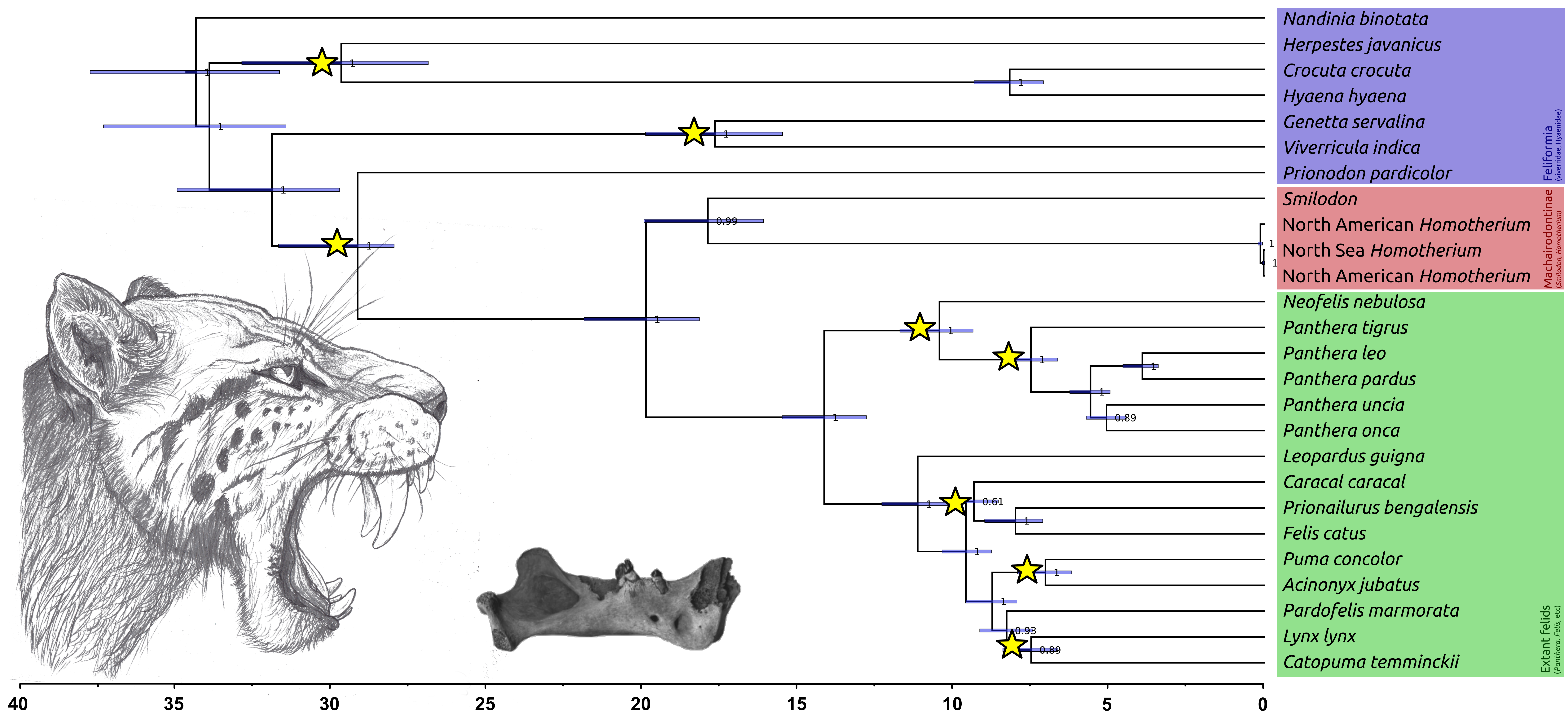
445*Partitionfinder:* An optimal set of partitions and substitution models was selected from all possible
446combinations of genes and tRNAs, considering all substitution models available in BEAST, under
447the Bayesian Information Criterion (BIC) in PartitionFinder v1.1.1 [75]. The partitionfinder analysis
448used the greedy search algorithm and linked branch lengths. PartitionFinder found best support for a
449five-partition scheme (BEAST xml input file available upon request).
450

451*RaxML:* The maximum likelihood tree was calculated using RaxML-HPC v8.2.4 [76] CIPRES
452black box version on the CIPRES Science Gateway [77], with default GTR+CAT substitution
453models for each partition. RAxML rapid bootstrapping was used with 1000 replicates. The African
454palm civet (*Nandinia binotata*, belonging to the monotypic family Nandiniidae) was used as
455outgroup.
456
457*BEAST*: Bayesian analyses were performed in BEAST v. 1.8.2 [78], with the 5 partitions selected by
458PartitionFinder. First, we tested for rate variation among lineages using a lognormal clock model on
459each partition (mean 0.05, standard deviation 0.05), with a uniform prior on the mean per-lineage
460substitution rate of 0 to 20% per million years, under a Birth-Death speciation tree prior. The
461MCMC chain was run for a sufficient number of generations to achieve convergence and adequate
462posterior sampling of all parameters (ESS >200), checked using Tracer v1.5 (available from
463~~http://www.beast.bio.ed.ac.uk/Tracer~~ http://beast.community/tracer). For some partitions, individual
464parameters of the GTR substitution model selected by PartitionFinder failed to converge, and so the
465simpler HKY model was used for these partitions in order to achieve convergence. The posterior
466sample of the ucld.stdev parameter, which describes substitution rate variation among lineages, was
467found to abut zero, thus not rejecting an absence of rate variation and justifying the use of a strict
468clock model. The analysis was rerun using a strict clock model with an uninformative uniform prior
469on the mean per-lineage substitution rate of 0 to 20% per million years, for molecular dating
470analyses with fossil calibration. The fossil calibrations that were used are listed in Table 2. The
471BEAUTI-generated XML input file is available upon request. TreeAnnotator v1.8.2 was then used
472to remove the first 25% of trees as burnin and extract the Maximum Clade Credibility (MCC) tree
473with nodes scaled to the median heights recovered by the posterior sample.
474

475DATA AND SOFTWARE AVAILABILITY

476*Homotherium* and *Smilodon* consensus sequences are available on GenBank (accession numbers:
477MF871700-MF871703).

478



Nandinia binotata
Herpestes javanicus
Crocuta crocuta
Hyaena hyaena
Genetta servalina
Viverricula indica
Prionodon pardicolor
Feliformia
(Viverridae, Hyaenidae)
Smilodon
North American Homotherium
North Sea Homotherium
North American Homotherium
Machairodontinae
(Smilodon, Homotherium)
Neofelis nebulosa
Panthera tigrus
Panthera leo
Panthera pardus
Panthera uncia
Panthera onca
Leopardus guigna
Caracal caracal
Prionailurus bengalensis
Felis catus
Puma concolor
Acinonyx jubatus
Pardofelis marmorata
Lynx lynx
Catopuma temminckii
Extant felids
(Panthera, Felis, etc)
0.99
0.89
0.61
0.93
0.89
40
35
30
25
20
15
10
5
0

Sample code	Species	Location	Age	Dating facility & number	Skeletal element	Collection	Reference
SP1007	*Homotherium latidens*	North Sea, The Netherlands	31,300±400	Utrecht University AMS facility-10456	Mandible	Rotterdam	[4]
			31,300±400	Utrecht University AMS facility-10999			
			26,900±400	Utrecht University AMS facility-10908			
			26,700±240	Utrecht University AMS facility-11064			
			28,100±220	Utrecht University AMS facility-11000			
			27,650±280	Utrecht University AMS facility-11065			
SP1714	*Homotherium latidens*	60- ile, Yukon Territory, Canada	>56,500	Oxford Radiocarbon Accelerator-10082	left humerus	Can, Mus Nat. Ottawa, CMN46442	[3]
YG439.38	*Homotherium latidens*	Quartz Creek, Dawson City, Yukon Territory	>47,500	Stafford LLC, UCIAMS-142835	left humerus	Yukon Government Collection, Whitehorse	Figure S1; Table S2
ZMA20.042	*Smilodon populator*	Ultima Esperanza, Chile	11,335±30	Stafford LLC, UCIAMS-142836	left tibia	Kruimel collection, Naturalis, Leiden	

Node	**Fossil**	**Fossil constraint**	**Calibration prior**	**References**
Genetta, Viverricula	*Genetta* fossil: 11.2M	Minimum 11.2M	Uniform: 50M – 11.2M	[79, 82]
Herpestes, Hyaena, Crocuta	hyaenid fossil: 16.4M	Minimum 16.4M	Uniform: 50M – 16.4M	[79, 82]
	herpestid fossil: 16.4M	Minimum 16.4M		[79, 82]
Felidae, *Prionodon*	Felidae stem fossils, *Prionodon* fossils	Minimum 28M	Uniform: 50M – 28M	[79, 82]
Lynx, Catopuma	*Lynx* fossil: 5.3M	Minimum 5.3M	Uniform: 10M – 5.3M	[79, 82]
Puma, Acinonyx	Acinonyx fossils: 3.8M	Minimum 3.8M	Uniform: 10M – 3.8M	[80, 81]
Caracal, Felis, Prionailurus	Caracal & Serval fossils: 3.8M	Minimum 3.8M	Uniform: 16M – 3.8M	[81]
Neofelis, Panthera	Oldest *Panthera* fossil: 3.8M	Minimum 3.8M	Uniform: 16M – 3.8M	[8, 81]
Panthera	Oldest *Panthera tigris* fossil: 1.5M	Minimum 3.5M	Uniform: 10M – 1.5M	[8]