Mitogenomics of the Extinct Cave Lion, *Panthera spelaea* (Goldfuss, 1810), Resolve its Position within the *Panthera* Cats

Ross Barnett*, Marie Lisandra Zepeda Mendoza*, André Elias Rodrigues Soares†, Simon Y. W. Ho‡, Grant Zazula§, Nobuyuki Yamaguchiǁ, Beth Shapiro†, Irina V. Kirillova¶, Greger Larson** and M. Thomas P. Gilbert*,††

The extinct cave lion (*Panthera spelaea*) was an apex predator of the Pleistocene, and one of the largest felid species ever to exist. We report the first mitochondrial genome sequences for this species, derived from two Beringian specimens, one of which has been radiocarbon dated to 29,860 ± 210 14C a BP. Phylogenetic analysis confirms the placement of the cave lion as the sister taxon to populations of the modern lion (*P. leo*). Using newly recovered stem pantherine fossils to calibrate a molecular clock, we estimate that *P. spelaea* and *P. leo* diverged about 1.89 million years ago (95% credibility interval: 1.23–2.93 million years), highlighting the likely position of this extinct carnivore as a distinct species.

Keywords: Cave lion; *Panthera leo spelaea*; Mitochondrial genome; Numt

Background
The extinct cave lion (*Panthera spelaea*) was an integral component of the late Pleistocene Holarctic ecosystem, occupying the position of apex predator (Barnett et al., 2009, Antón et al., 2005, Yamaguchi et al., 2004, Bocherens et al., 2011) alongside the scimitar cat (*Homotherium* sp.) (Barnett, 2014). The cave lion is known from plentiful remains preserved in the karstic cave systems of Europe and the permafrost sediments of Beringia (present day Siberia, Alaska and the Yukon). Despite this, *P. spelaea* has had an interesting history of taxonomic revision, having been variously considered a highly divergent population of the modern lion (Dawkins et al., 1866), a subspecies of the modern lion (Kurtén, 1985), and a full species in its own right (Sotnikova and Nikolskiy, 2006). There is currently no consensus on the taxonomic status of the cave lion.

Cave lions were significantly larger than their modern lion counterparts, and exhibit a unique cranial morphology that has a mosaic of characters present in the lion and tiger (Sotnikova and Nikolskiy, 2006, Hemmer, 2011, Vereshchagin, 1971, Gromova, 1932). Evidence from Pleistocene art demonstrates that the cave lion differed in external morphology from modern lions (Packer and Clotte, 2000). Male cave lions did not possess a mane, a notable secondary sexual character in the modern species, and this is likely to have had considerable effect on the ethology of the species (Yamaguchi et al., 2004, Nagel et al., 2003). Previous work has also demonstrated that the cave lion sensu stricto had an enormous range (Barnett et al., 2009), from western Europe to eastern Beringia, and were coeval with lion populations in southern North America (*Panthera atrox*; Montellano-Ballesteros and Carbot-Chanona, 2009) and Africa (*Panthera leo*; Bougariane et al., 2010). Despite dominating the Holarctic mammoth steppe for most of the late Pleistocene, the cave lion went extinct nearly simultaneously across its range, with terminal dates in Europe, Siberia, and Alaska all close to 14,000 cal BP. *Panthera atrox*...
disappeared only slightly later in southern North America (Stuart and Lister, 2010).

Ancient DNA (aDNA) studies based on partial fragments of the mitochondrial control region, ATP8, and cytochrome b genes from *P. spelaea* have not fully resolved the degree of separation between the various lineages of lion-like cats (Burger et al., 2004, Barnett et al., 2009, Ersmark et al., 2015). In particular, although prior analyses recovered *spelaea* and *leo* as sister taxa, these studies have relied on the Middle Pleistocene appearance of the ancestral cave lion (*Panthera [leo] fossilis*) to calibrate the age of the split for molecular dating analyses, without estimating the timing of the split directly. Here, we present the complete mitochondrial genomes of two cave lion specimens and, using a Bayesian phylogenetic approach, we analyse these sequences in combination with a strictly vetted set of published mitochondrial genomes. We use these to infer the evolutionary timeline of lion-like cats, calibrated using recently discovered pantherine fossils, and discuss the species status of *P. spelaea*.

**Materials and Methods**

**Sample Discovery**

Sample YG 401.410, a humerus (Figure 1, Table 1), was recovered from the Quartz Creek site in Yukon, Canada (Figure 2), on 24 July 2010. It has subsequently been stored in the Government of Yukon’s Palaeontology Program collection in Whitehorse. The specimen was subsampled in June 2013 by GZ and sent to the Centre for GeoGenetics (University of Copenhagen, Denmark) for processing. Hair sample F-2678/70 was found at the right bank of Malyi Anyui river (Chukotka, Russia) during the summer of 2008 (Kirillova et al., 2015). A small bundle of hair was subsampled by IVK and sent to the UCSC Paleogenomics Lab (Santa Cruz, USA) for processing.

**Radiocarbon Dating**

A section of bone from sample YG 401.410 was delivered to Stafford Research LLC (University of Aarhus, Denmark). Samples of crushed bone were decalcified and washed, treated with 0.05 N NaOH overnight to remove humics, soaked in 0.1 N HCl, gelatinized at 60°C at pH 2, and ultrafiltered at 30 kDa. The purified collagen was then graphitised and analysed at the W.M. Keck Carbon Cycle Laboratory of Earth, Atmospheric and Planetary Sciences at MIT. DNA extractions for ancient DNA were performed using standard protocols for ancient DNA (Cooper and Poinar, 2000). The extraction followed the protocol described by Orlando et al. (2013) in a dedicated ancient DNA laboratory at the Centre for GeoGenetics in Copenhagen, in parallel with negative extraction controls.

The DNA extract and negative control were then built into genomic libraries using the NEB E6070 kit (New England Biolabs), following a protocol slightly modified from that by Vilstrup et al. (2013). Briefly, extract (30 µL) was end-repaired and then passed through a MinElute column (Qiagen). The collected flow-through was then adapter-ligated and passed through a QiaQuick column (Qiagen). Adapter fill-in reaction was then performed on the flowthrough, before final incubation at 37°C (30 min) followed by inactivation overnight at −20°C.

We then amplified the DNA in a 50 µL reaction, using 25 µL of library for 12 cycles under the following reaction conditions. Final concentrations were 1.25 U AccuPrime™ PfX DNA Polymerase (Invitrogen), 1× AccuPrime™ PfX reaction mix (Invitrogen), 0.4 mg/mL BSA, 120 nM primer set in PE, and 120 nM 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. Amplified libraries were first checked for presence of DNA on a 2% agarose gel before purification using the QIAquick column system (Qiagen) and quantification on an Agilent 2100 BioAnalyzer. Quantified libraries were communally pooled in equimolar ratios and sequenced as single-end reads (100 bp) on an Illumina HiSeq2000 platform at the Danish National High-Throughput Sequencing Centre.

**Extraction and DNA Amplification**

Sample YG 401.410

Samples of cortical bone were taken (approx. 1 cm³) using a Dremel powertool and reduced to powder in a Mikrodissembrator. DNA extraction was performed as described by Orlando et al. (2013) in a dedicated ancient DNA laboratory at the Centre for GeoGenetics in Copenhagen, in parallel with negative extraction controls.

Sample F-2678/70

DNA extraction from hair sample F-2678/70 was performed in a dedicated, sterile, facility at UC Santa Cruz, using standard protocols for ancient DNA (Cooper and Poinar, 2000). The extraction followed the protocol described by Dabney et al. (2013), with the modifications suggested by Campos and Gilbert (2012). The Illumina DNA sequencing library was built following Meyer and Kircher (2010). Between each step, the libraries were cleaned using Sera-Mag SPRI SpeedBeads (ThermoScientific) in

| Specimen | Side | Minimum breadth of diaphysis (mm) | Maximum depth of diaphysis (mm) | Maximum breadth of distal end (mm) | Minimum anteroposterior diameter of articulating surface for ulna (mm) | Notes |
|----------|------|----------------------------------|---------------------------------|----------------------------------|-------------------------------------------------|-------|
| YG 401.410 | Left | 28.6 | 52.1 | 86.1 | 29.1 | Missing proximal end from pectoral ridge |

Table 1: Metric data from humerus specimen YG 401.410. Measurements to nearest 0.1 mm taken using digital calipers.
Figure 1: A) Four views of a humerus of cave lion (*Panthera spelaea*), specimen YG 401.410. This specimen was recovered from Quartz Creek in the Yukon Territory, Canada. The scale bar is 5 cm. B) Four views of cave lion (*Panthera spelaea*) hair bolus, sample F-2678/70, contains guard hair but is mainly represented by thick underfur of tightly packed, wavy fur hairs. This specimen was recovered from Malyi Anyui river in Chukotka, Russia.
Figure 2: A map of the approximate distributions of late Pleistocene lions and the provenance of samples used in this study. Red indicates the maximal range of Panthera spelaea; blue the maximal range of Panthera atrox; and green the maximal range of Panthera leo leo/Panthera leo persica. Stars show approximate locations of lion samples. The insets show details of the modern boundaries of Yukon Territory, Canada, and Chukotka, Russia, along with regional settlements. Sample YG 401.410 was found at Quartz Creek (Yukon Territory, Canada), 63.49N, 139.27W. Sample F-2678/70 was found at the right bank of Maly Anyui River (Chukotka, Russia), 68.18N, 161.44E.

18% PEG-8000. The resulting libraries were sequenced in a MiSeq Illumina sequencer with v3 kits at UCSC Paleogenomics Lab.

Sequence Processing
Sample YG401.410
The dataset consisted of 3,444,248 single-end sequences that were cleaned of adapter and low-quality sequences using AdapterRemoval v1.2-GG1 (Lindgreen, 2012). In order to obtain the mitochondrial sequence, we mapped the resulting 3,429,186 cleaned reads against a published mitochondrial genome from Panthera leo persica (GenBank accession JQ904290.1) using bwa (Li and Durbin, 2009). In order to take into account the circularity of the mitochondrial genome and to recover the reads mapping at the edges of the extremes of the reference sequence, we added the first 100 bases to the end. Clonality was removed with picard-tools v1.92 (Picard, 2013). The mapping was analysed for damage at the extremes with MapDamage v2.0 (Jónsson et al., 2013) and the quality of the damaged bases was rescaled with MapDamage. Realignment was performed with GenomeAnalysisTK (McKenna et al., 2010) on the rescaled mapping file in order to call SNPs with samtools v0.1.18 and bcftools (Li et al., 2009) with a minimum coverage of 8 and minimum quality of 30.

Nuclear copies of mitochondrial genes (numts) are known to occur in multiple members of the cat family Felidae. Some phylogenetic studies of felids have been compromised by the inclusion of numt sequences in alignment (Davis et al., 2010). Numts were anticipated to be a particular problem in genomes reassembled from high-throughput sequencing technologies, which involve short fragment lengths and are unable to preferentially target cytoplasmic copies (e.g. by PCR primer design).

In order to obtain a robust, non-chimaeric consensus sequence with GenomeAnalysisTK, we only used those SNPs supported by at least 2/3 of the reads mapping to that position. We used the resulting consensus sequence as a reference for a second mapping round, using the same mapping and consensus strategy as before. This was repeated three more times to make a total of four mapping rounds. The final consensus sequence has an average depth of 9.53× covering 89.5% of the mitogenome.

F-2678/70
The dataset consisted of 6,683,556 paired-end reads. Initial processing of these reads consisted of using AdapterRemoval v1.2-GG1 (Lindgreen, 2012) for the cleaning step, followed by merging reads including a minimum overlap of 10 base pairs between forward and reverse reads using SeqPrep (http://github.com/jstjohn/SeqPrep). A total of 6,123,696 merged reads were obtained. These merged reads were mapped against the Panthera leo persica mitochondrial genome (GenBank accession...
NC_018053.1) using MIA (http://github.com/udo-stenzel/mapping-iterative-assembler), a reference-based, iterative, short-fragment assembler that accepts circular genomes as reference sequences. A total of 9,582 reads mapped to the reference mitochondrial genome. The consensus sequence was called from the resulting output file, with each base having a minimum depth of coverage of 3x and 2/3 base agreement. The final assembly had an average depth of 28.16× covering 99.62% of the mitochondrial genome.

**Phylogenetic Analysis**

Complete mitochondrial genomes of lion (*Panthera leo*), leopard (*Panthera pardus*), jaguar (*Panthera onca*), snow leopard (*Panthera uncia*), tiger (*Panthera tigris*), clouded leopard (*Neofelis nebulosa*), and domestic cat (*Felis sylvestris catus*) were downloaded from GenBank (Table 2). Sequences of all 37 mitochondrial genes were extracted from these genomes.

We estimated the evolutionary relationships among pantherine cats using maximum likelihood in RAxML v8.0.14 (Stamatakis, 2014). The data set was partitioned into five subsets: the three codon positions of the 13 protein-coding genes; the 2 rRNA genes, and the 22 tRNA genes. A separate GTR+G model of nucleotide substitution was assigned to each subset of the data. The maximum-likelihood tree was estimated using 10 random starts. Node support was estimated using 1000 bootstrap replicates.

To co-estimate the phylogenetic relationships and evolutionary timescale, we analysed the mitochondrial genome sequences using a Bayesian phylogenetic approach in BEAST 1.8.2 (Drummond et al., 2012). The Bayesian information criterion was used to select the best-fitting model of nucleotide substitution for each data subset.

We compared two tree priors (Yule and birth-death) using Bayes factors, based on marginal likelihoods calculated using the stepping-stone estimator (Xie et al., 2011). To account for the potential presence of rate variation across lineages, we also used Bayes factors to compare the strict clock against the uncorrelated lognormal relaxed clock (Drummond et al., 2006). We included relative-rate parameters to allow each subset of the data to have a different evolutionary rate.

To calibrate our phylogenetic estimates of divergence times, we included age constraints based on the fossil record. Our calibrations were based on the stem snow leopard *Panthera blytheae* (Tseng et al., 2014), stem tiger *Panthera zdanskyi* (Mazak et al., 2011), and stem pantherine *Panthera paleosinensis* (Mazak, 2010). These fossils were used to specify uniform priors on the ages of corresponding nodes in the tree (Ho and Phillips, 2009). Previous estimates of the evolutionary timescale of cave lions were calibrated using fossil evidence from *P. fossilis* (Burger et al. 2004, Barnett et al. 2009), but the exact placement of this taxon is unclear. For example, incorrect assignment of a stem taxon to a crown clade can lead to overestimation of divergence times. Alternatively, divergence times can be underestimated when a taxon belonging to the crown group is erroneously assigned to the stem lineage. By using other fossil calibrations, we were able to test whether the split between cave lion and modern lion coincided with the existence of *P. fossilis*.

Posterior distributions of parameters, including the tree and divergence times, were estimated using Markov

| Taxon | Common name | Genbank accession | Reference |
|-------|-------------|-------------------|-----------|
| Panthera leo persica | Asian lion | NC018053 | (Bagatharia et al., 2013) |
| Panthera leo leo | African lion | KF776494 | (Ma and Wang, 2014) |
| Panthera leo | Lion | KP202262 | Unpublished, GenBank |
| Panthera pardus | Leopard | NC010641 | (Wei et al., 2011) |
| Panthera pardus japonensis | North Chinese leopard | KJ866876 | (Dou et al., 2014) |
| Panthera onca | Jaguar | NC022842 | Unpublished, GenBank |
| Panthera tigris sumatrae | Sumatran tiger | JF357970 | (Kitpipit and Linacre, 2012) |
| Panthera tigris tigris | Bengal tiger | JF357968 | (Kitpipit and Linacre, 2012) |
| Panthera uncia | Snow leopard | NC010638 | (Wei et al., 2011, Wei et al., 2009) |
| Neofelis nebulosa | Clouded leopard | NC008450 | (Wu et al., 2007) |
| Felis sylvestris catus | Domestic cat | FCU20753 | (Lopez et al., 1996) |
| Felis sylvestris catus NUMT | Nuclear pseudogene | FCU20754 | (Lopez et al., 1994, Lopez et al., 1996) |
| Panthera tigris NUMT | Nuclear pseudogene | DQ151551 | (Kim et al., 2006) |
| Panthera spelaeaYG401.410 | Cave lion | KX258451 | This study |
| Panthera spelaea F-2678/70 | Cave lion | KX258452 | This study |

Table 2: Mitochondrial genomes of pantherine cats analysed in this study.
chain Monte Carlo sampling. Samples were drawn every 2000 steps over a total of 20,000,000 steps. Convergence was checked using two independent chains, and the resulting samples were combined. Sufficient sampling was confirmed by inspection of effective sample sizes of parameters, which were all greater than 200.

Results

Radiocarbon dating

Sample YG401.410

We obtained two separate dating results for sample YG401.410. The dates were within three hundred $^{14}$C years of each other, with overlapping 95% confidence intervals. Collagen yield was substantial (Table 3), with $\delta^{13}$C and $\delta^{15}$N values appropriate for the trophic level of the species (Bocherens et al., 2011). Interestingly, the radiocarbon date for this specimen falls within a noticeable chronological gap for Eastern Beringian lions, suggesting that some of the absences observed by Stuart and Lister (2010) may be resolved with further sampling.

Sample F-2678/70

The hair sample has previously been radiocarbon dated by Kirillova et al. (2015). The reported uncalibrated AMS date of 28,690 ± 130 was much younger than dates associated with bone and claw from the same individual, which could have resulted from incomplete removal of modern carbon. For a full discussion see Kirillova et al. (2015).

Phylogeny and divergence times

Our initial estimates of the phylogeny gave unexpected placements for some of the taxa (data not shown). Inclusion of the mitochondrial genomes from the African lion (Ma and Wang, 2014) and Asian lion (Bagatharia et al., 2013) produced a tree with *P. spelaea* as the sister taxon of *P. l. persica*, in contradiction with previous studies (Barnett et al., 2009, Burger et al., 2004, Ersmark et al., 2015). Analysis of the ATP8 gene, which has been characterised for both cytoplasmic and nuclear origin (Barnett et al., 2009) in Panthera cats, revealed the presence of a numt sequence within the mitochondrial genome published by Ma and Wang (2014). Given that this mitochondrial genome sequence was assembled from published nuclear genome data (Cho et al., 2013), it is likely to include a significant proportion of nuclear-derived sequence. Therefore, this mitochondrial genome was excluded from further analyses.

The evolutionary relationships estimated using maximum-likelihood and Bayesian methods were congruent (Figure 3), with all nodes being strongly supported. The two samples of *P. spelaea* group together as the sister lineage to *P. leo*. Our estimates of divergence times largely overlap with previous estimates (Johnson et al., 2006, Barnett et al., 2005). However, the estimated split between *P. leo* and *P. spelaea* at 1.89 Ma (95% CI: 1.23–2.93 Ma) is considerably older than the first appearance of *Panthera fossilis*, which has been used as a calibrating node in previous studies (Burger et al., 2004, Barnett et al., 2009).

Discussion

Evolutionary history of the lion-like cats

Despite their global range and continued dominance of ecosystems in Africa, and until recently in Asia, the lion-like cats have left a confusing fossil trail. Remains of pantherine felids have been found in fossil beds dating to 3.46 million years ago at Laetoli in Tanzania (Barry, 1987), with recognisably leonine fossils known from Olduvai II at 1.4–1.2 million years ago (Hemmer, 2011). Lion fossils only become relatively abundant during the Middle Pleistocene, with the appearance of *Panthera fossilis*. This taxon is considered an ancestral form of *Panthera spelaea* and provides a minimum age for the separation between the *spelaea* and *leo* lineages. *P. fossilis* is known from MIS 17-15 (680-600 ka) from European sites such as Mosbach (Germany), Pakefield (UK) and Isemnia (Italy) (Hemmer, 2011, Turner and Antón, 1997, Lewis et al., 2010, Sabol, 2011). Given our divergence-time estimates (Figure 3), it would appear that *P. fossilis* must be already on the branch leading towards *P. spelaea* rather than close to the split. In

| UCIAMS   | UCIAMS-142833 | UCIAMS-143525 |
|----------|---------------|---------------|
| Fraction Modern | 0.0243 ± 0.0006  | 0.0234 ± 0.0006  |
| $D^{14}$C (%)       | -975.7 ± 0.6   | -976.6 ± 0.6   |
| $^{14}$C Age BP     | 29,860 ± 210   | 30,160 ± 220   |
| >30 kD Collagen yield(%) | 8.1          | N/A            |
| $\delta^{15}$N (%)  | 8              | N/A            |
| $\delta^{13}$C (%)  | -18.2          | N/A            |
| %N                  | 16.3           | N/A            |
| %C                  | 44.7           | N/A            |
| C/N (wt%/wt%)       | 2.75           | N/A            |
| C/N (atomic)        | 3.21           | N/A            |

Table 3: Results of two AMS radiocarbon analyses of sample YG401.410 with associated stable isotope and chemical analysis values.
view of this finding, we recommend that *P. fossilis* should only be used to provide a minimum age constraint for the split between the *leo* and *spelaea* lineages.

**Dating the divergence between spelaea and leo**

Much of the discussion of the taxonomic position of the cave lion has revolved around its degree of separation from modern lion populations. Although some authors have aligned the *spelaea* and *atrox* lineages with the tiger (Groiss, 1996, Herrington, 1986) and with the jaguar (Simpson, 1941, Christiansen and Harris, 2009), most have realised its close connection to the modern lion (Goldfuß, 1810, Dawkins et al., 1866, Vereshchagin, 1971, Turner, 1984, Kurtén, 1985, Sotnikova and Nikolskiy, 2006).

Previous genetic studies (Burger et al., 2004, Barnett et al., 2014) have used the first appearance of the ancestral cave lion (*Panthera fossilis*) (Sotnikova and Foronova, 2014, Peretto et al., 2015) to calibrate estimates of the pantherine evolutionary timescale. This study represents the first attempt to identify the divergence bounds between *spelaea* and *leo* without recourse to *P. fossilis* as a calibration. The identification of this split within the Early Pleistocene at 1.89 Ma, rather than the Middle Pleistocene, shows that the modern lion and cave lion lineages represent substantially distinct taxa. A caveat to this is that the estimates rely strongly on the fossil calibrations used. If reanalysis later shows that these fossils have been ascribed to the wrong lineages or are re-dated to different periods then their utility in the analysis will be compromised.

Comparison with other recent pantherines (Figure 3) demonstrates that the degree of mitochondrial divergence is considerably greater than that found between well-defined subspecies in modern lion (*P. leo*) (Barnett et al., 2014), leopard (*P. pardus*) (Uphyrkina et al., 2001), or tiger (*P. tigris*) (Luo et al., 2004). The estimated divergence time between *P. leo* and *P. spelaea* is also greater than that between the two newly recognised species of clouded leopard, *Neofelis nebulosa* and *N. diardi*, which has been estimated at 1.41 Ma (Buckley-Beason et al., 2006).

Mitochondrial data and the considerable morphological differences (Sotnikova and Nikolskiy, 2006) support the recognition of the cave lion as a full species: *Panthera spelaea*. Data from the nuclear genome will allow further testing of this proposal.

**Conclusions**

Our analyses of mitochondrial genome sequences reveal that the Middle Pleistocene *Panthera fossilis* is likely to represent a form already on the *spelaea* lineage. Its appearance in the fossil record demonstrates the initial spread of the ancestral cave lion form into Eurasia. Furthermore, our study has provided an estimate of 1.89 Ma (95% CI: 1.23–2.93 Ma) for the split between the lineages leading to the cave lion and modern lion. This molecular estimate appreciably antedates the appearance of *P. fossilis*, and provides further evidence that the cave lion was distinct enough to be considered a species in its own right.

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**Figure 3:** Bayesian estimate of the pantherine phylogeny based on mitochondrial genomes. The tree is drawn to a timescale, given in millions of years. Bars at nodes give 95% credibility intervals of node-age estimates. Values at nodes denote posterior probabilities and likelihood bootstrap support. The position of the root was inferred by using the domestic cat (*Felis sylvestris catus*) as the outgroup.
Availability of Supporting Data

Sequence data produced for this study have been uploaded to GenBank, with accession numbers KX258451 and KX258452. Raw data have been uploaded to the Sequence Read Archive at NCBI under study accession number SRP075782.

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Competing Interests

The authors declare that they have no competing interests.

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