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Short Communication

Molecular phylogeny of the extinct cave lion *Panthera leo spelaea*

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Abstract

To reconstruct the phylogenetic position of the extinct cave lion (*Panthera leo spelaea*), we sequenced 1 kb of the mitochondrial cytochrome *b* gene from two Pleistocene cave lion DNA samples (47 and 32 ky B.P.). Phylogenetic analysis shows that the ancient sequences form a clade that is most closely related to the extant lions from Africa and Asia; at the same time, cave lions appear to be highly distinct from their living relatives. Our data show that these cave lion sequences represent lineages that were isolated from lions in Africa and Asia since their dispersal over Europe about 600 ky B.P., as they are not found among our sample of extant populations. The cave lion lineages presented here went extinct without mitochondrial descendants on other continents. The high sequence divergence in the cytochrome *b* gene between cave and modern lions is notable.

1. Introduction

The cave lion [*Panthera leo spelaea* (Goldfuss, 1810)] was one of the most important carnivorous competitors of early man in Europe, from the early Middle Pleistocene onwards. It was an object of Palaeolithic art, such as the magnificent colour paintings in the Chauvet Cave (Ardèche, France) (Lorblanchet, 1995) or the impressive ivory sculptures from the Vogelherd cave (Swabian Alb, Germany) (Fig. 1). The first comprehensive morphological studies of cave lion remains, in the 19th and the beginning of the 20th century, showed a relationship to modern lions. Subsequent reinterpretations either linked cave lions to modern tigers or declared them a separate species. Osteological revisions have, however, always indicated a relationship to modern lions (Hemmer, 1974), although lately a new case has been made for a relationship to tigers based on brain endocasts (Groiss, 1996). Comparative morphological analysis of Pleistocene and Holocene lions on the level of geographic populations resulted in the description of two basic evolutionary lines: the *spelaea* group of the Holarctic Pleistocene and the *leo* group of Africa and southern Asia (Hemmer, 1974). Most authors favour the taxonomic combination of these groups within the species *Panthera leo* (Hemmer, 1974; Kurtén, 1968; Turner and Antón, 1997), but some prefer a taxonomic separation at the species level, into *Panthera spelaea* and *Panthera leo* (Baryshnikov and Boeskorov, 2001). Here, we report the mtDNA analysis of two Upper Pleistocene cave lions, one (Si) 47,180 + 1190/−1040 year B.P. and one

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Fig. 1. Cave lion ivory sculpture from Vogelherd cave, Swabian Alb, Germany (Aurignacian 32 kya).

(Ku) 31,890 ± 300 year B.P. old. Our results are consistent with the taxonomy of pantherine cats presented in Table 1.

2. Materials and methods

2.1. Fossil bone samples

One specimen (Si), an almost complete skeleton of a cave lion embedded in a greyish silty clay, was excavated in 1985 (Rosendahl and Darga, 2004) at Siegsdorf in southeastern Bavaria, Germany. For preservation the bone surfaces were treated with a silica gel resin. Samples were taken for radiocarbon dating and DNA analysis from the interior compact bone of the right femur. The high collagen yield (19.4 wt% bone) suggests that the bones were not significantly altered diagenetically and that the ^{14}C-AMS date of 47,180 + 1190/−1040 (KIA 14406) year B.P. is valid. The second, untreated sample (Ku) was recovered from layer 4 (a light-brown cave loam) of the Tischhofer cave, 2 km northwest of Kufstein, Tirol, Austria, 598 m a.s.l, in 1906 (Schlosser, 1910). A pelvis fragment of a juvenile cave lion was selected for dating and DNA analysis. The collagen yield was again high (12 wt% bone), suggesting that the ^{14}C-AMS date of 31,890 ± 300 (KIA 16510) year B.P. is also valid. Modern pantherine samples (*P. leo persica* and *P. pardus*) were from the Frankfurt/Main Zoo, Germany.

Table 1
Taxonomy of the pantherine cats (after Hemmer, 1974, 1978, in press, and this study)

Genus	Subgenus	Species	Subspecies
Neofelis		*Neofelis nebulosa* (clouded leopard) [AJ304497]	
Uncia		*Uncia uncia* (snow leopard)	
Panthera	*Tigris*	*Panthera tigris* (tiger) [AF053021, AF053039, AF053048, AF053051]	
	Panthera	*Panthera pardus* (leopard) [this study]	
		Panthera onca (jaguar)	
		Panthera leo (lion)	*spelaea* group (Pleistocene)
			Panthera leo fossilis (Early Middle Pleistocene European cave lion)
			Panthera leo vereshchagini (East Siberian and Beringian cave lion)
			Panthera leo atrox (North American cave lion)
			Panthera leo spelaea (Upper Pleistocene European cave lion) [this study]
			leo group (African and South-Asian lions)
			persica subgroup
			Panthera leo persica (South Asian lion) [this study]
			close to *persica* subgroup
			Panthera leo leo (Atlas lion)
			senegalensis subgroup:
			Panthera leo senegalensis (West African lion)
			Panthera leo azandica (North East Congo lion)
			Panthera leo nubica (East African lion) [AF384809, AF384817]
			Panthera leo bleyenberghi (Southwest African lion) [AF384811-AF384815]
			Panthera leo krugeri (Southeast African lion) [AF384816, AF384818]
			Panthera leo melanochaita (Cape lion)

Provided as (common name) [sequence origin]. Subspecies nomenclature is given for lions.

2.2. Diagenetic measurements

Fourier-transform infrared spectra were generated using KBr pellets. The spectra were used to generate a splitting factor (SF) as described in Termine and Posner (1966) and Weiner and Bar-Yosef (1990) as well as a carbonate:phosphate peak ratio (C/P) as shown in Wright and Schwarcz (1996). These measurements relate to the degree of mineral alteration in the bone sample. Powders were also subjected to elemental analysis, providing a percent value of whole bone nitrogen (% N) in each sample. This has been determined to relate to the remaining protein present in archaeological and fossil samples (Hedges et al., 1995). Finally, powders were subjected to small-angle X-ray scattering (SAXS) analysis on a Bruker AXS Nanostar (Karlsruhe) at the University of Stirling. This provided a detailed measurement of the bone crystallite size and shape in each sample.

2.3. Ancient DNA work

The DNA work was conducted in two laboratories, located in separate buildings: one ancient DNA laboratory devoted to pre-PCR procedures and free of other molecular work, and a second laboratory for the post-PCR analysis. The extractions were performed in a devoted ancient DNA laboratory where no felid DNA had previously been introduced. All rooms are regularly washed with bleach and UV-irradiated overnight. Every item entering these rooms is washed with bleach and subsequently UV-irradiated. Filtered water for cleaning is additionally UV-treated for at least 10 h. Two independent samples were taken from each specimen for the Mainz laboratory. A third sample from each lion was processed in Uppsala to where it was sent directly from the museums.

2.4. Extraction of ancient DNA samples

0.4–1 g powdered bone samples were incubated in 3–6 mL of extraction buffer (0.5 M EDTA, pH 8.5; 0.5% *N*-lauryl sarcosine; 19 mg/mL proteinase K) on a rotary shaker for 20 h at 37 °C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1); the supernatant was concentrated by Centricon 30 (Amicon) dialysis and finally washed several times with UV-treated HPLC-grade water.

2.5. PCR, cloning, and DNA-sequencing

Twelve PCR products in lengths of 87–209 bp were designed to cover 1051 bp of the cytochrome *b* gene of pantherines (Fig. 2). One additional primer pair amplifies a 474 bp amplicon and is used to test for the presence of contaminating undegraded DNA in the PCR. When possible, primers (Table 2) were designed so that they did not amplify either the human sequence or a known tiger nuclear insertion. The resulting PCR amplicons had a minimum of 2 bp and maximum of 59 bp overlap (without primers). Initially, primers were tested in a third laboratory so that no molecular work on modern pantherines was performed in the laboratories prior to ancient DNA analysis. After aDNA extraction, a series of PCR amplifications were performed until sequences from at least two DNA extracts and four independent PCR runs were available for each amplicon. Further, to detect possible heterogenous sequences and nuclear insertions, each PCR product was cloned at least once and 5–21 clones (average 9) were sequenced. In total, the

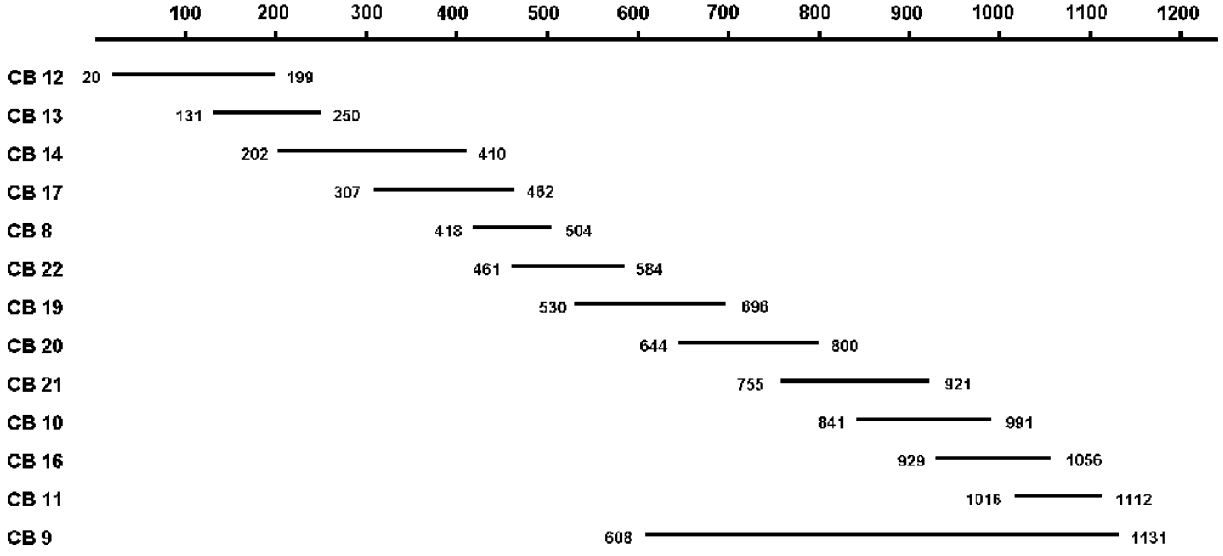

Fig. 2. Sequencing strategy. The lengths of the amplicons are shown including primers.The numbers refer to the position on the lion cytochrome *b* gene sequence.

Table 2
Primer sequences (5′–3′)

CB8u TTTTGAGGTGCAACTGTAATC
CB8l GAAGCCTCCTCAGATTCAC
CB9u TTCCATCCATACTATACAATCAA
CB9l GAGGAGGCGGTTTTCAA
CB10u CTCCGATCTATTCCCAACAAACT
CB10l CCGCTACTAGGAATCAGAATA
CB11u GTGGCCAACCTGTAGAAC
CB11l ATGCCTGAGATGGGTATTA
CB12u CAC ACC CCC TTA TCA AAA TTA TT
CB12l TAA CTG ATG AGA AAG CGG TTA T
CB13u AAATTCTCACCGGCCTCTTTCTA
CB13l TTGGCGTGTAGGTACCGGATAA
CB14u2 CAC ATT TGC CGC GAT GTA AAT
CB14l TGG CCC CAC GGT AAG ACA TAT
CB16u CCA AAC AGC GAG GAA TGA TG
CB16l TTG GCC AAT GGT GAT GAA G
CB17u TAC TAC GGC TCC TAC ACT TTC TCA
CB17l ATG GGA TTG CTG ATA GGA GGT TG
CB19u GATTCTTTGCCTTCCACTTCAT
CB19l2 AAGGCCTAGGATATCTTTGATTGTA
CB20u2 CAGATAAAATTCCATTTCATCCATA
CB20l TGGGGAGGGGTGCTTAGA
CB 21u4 ACC CCG ATA ACT ATA CCC C
CB21l GAGGGCAGGGATAATTGCTAAG
CB22u CATACATTGGGGCCGACCT
CB22l2 ACTACTGCTAGGGCTGAGATGATA

sequences were reproduced 14–37 times (average 19) so that 258 and 191 sequences, respectively, were produced to establish the 1051 bp sequence of both specimens.

The following sequences were acquired from GenBank: *Felis catus* (domestic cat): AB004238; *Neofelis nebulosa* (clouded leopard): AJ304497; *Panthera leo* (lion): AF384815, AF384811, AF384812, AF384813, AF384814, AF384818, AF384816, AF384817, and AF384809; *Panthera tigris* (tiger): AF053051, AF053039, AF053048, and AF053021.

The following sequences were produced:

P. leo persica (Asiatic lion) 1 (Zoo Frankfurt a. M., Germany); *P. leo persica* 2 (Zoo Frankfurt a. M., Germany); and *P. pardus* (leopard) (Zoo Frankfurt a. M., Germany).

Amplifications were carried out in a 50 μL reaction volume containing 50 mM KCl, 10 mM Tris–HCl (GeneAmp 10× Buffer II, PE Applied Biosystems); 2–2.5 mM $MgCl_2$, 200 μM each dNTPs, 1 μg T4 G32 protein, 0.2 μM of each primer, and 3.5 U of AmpliTaq Gold (PE Applied Biosystems). The PCR thermal cycling conditions were 94 °C for 5 min followed by 38–45 cycles at 94 °C for 30 s, at 54–60°C for 30 s, and at 72°C for 30 s. One extraction blank and two PCR negative controls were carried out for each PCR experiment.

The PCR product was purified using the QIAquick kit from Qiagen. For direct sequencing reactions of both strands the PRISM kit from Applied Biosystems was used. Additionally, the PCR products were cloned using a pUC18 (T-vector, in-house production) transformed to an *Escherichia coli* strain (RRI). DNA from selected clone medium was isolated using the CONCERT Rapid Plasmid Purification Kit (Gibco, Germany) following the manufacturer's protocol. Five to 21 (average 9) clones were sequenced using the universal reverse and forward primers. Sequencing reactions were run on an Applied Biosystems 310 automatic sequencer.

2.6. Phylogeny

The cytochrome *b* sequence data set consisted of 20 individuals and 1140 manually aligned positions. Maximum likelihood (ML) analysis and bootstrap analyses used the Linux version of PAUP* (Swofford, 2001) with the general time-reversible model and gamma distribution of rates (GTR + G) (Rodríguez et al., 1990; Yang, 1996). The model of evolution was selected by using MrModeltest, a simplified version of Modeltest 3.06 (Nylander, 2001; Posada and Crandall, 1998). Heuristic searches in PAUP* used TBR branch swapping on 100 random addition sequence trees, estimating all model parameters. The bootstrap analysis (Felsenstein, 1985) was set up to perform 100 replicates with simple addition sequence of taxa and model parameters set as estimated for the best tree found in the ML analysis. The Bayesian inference analyses used MrBayes (Huelsenbeck and Ronquist, 2001) and the same model as for ML. Three separate MrBayes analyses starting from random trees were performed. In each, 1,000,000 generations of Markov Chain Monte Carlo (MCMC) were run, sampling a tree every 10 generations. Majority-rule consensus trees were obtained by loading sampled trees into PAUP* after discarding trees sampled during chain "burnin" (1349, 1749, and 1599 trees were discarded, respectively). The trees from the three Bayesian analyses were identical and the posterior probabilities for clades were almost identical. Their means are used in Fig. 3.

2.7. Divergence time estimates

A likelihood ratio test for rate constancy (Felsenstein, 1988) was performed where the likelihood of our ML tree was compared to the likelihood of the same tree with the constraint of a strict molecular clock. The probability for rejecting the null hypothesis of rate constancy was $0.1 > p > 0.05$ (χ^2 10.2632; df = 18). Since the test did not reject rate constancy, estimates of divergence times for nodes were calculated using the clock-based Langley–Fitch method with the Powell algorithm available in Sanderson's r8s ("rates") program (Sanderson, 2002). The most distant outgroup, *Felis*, was pruned from the

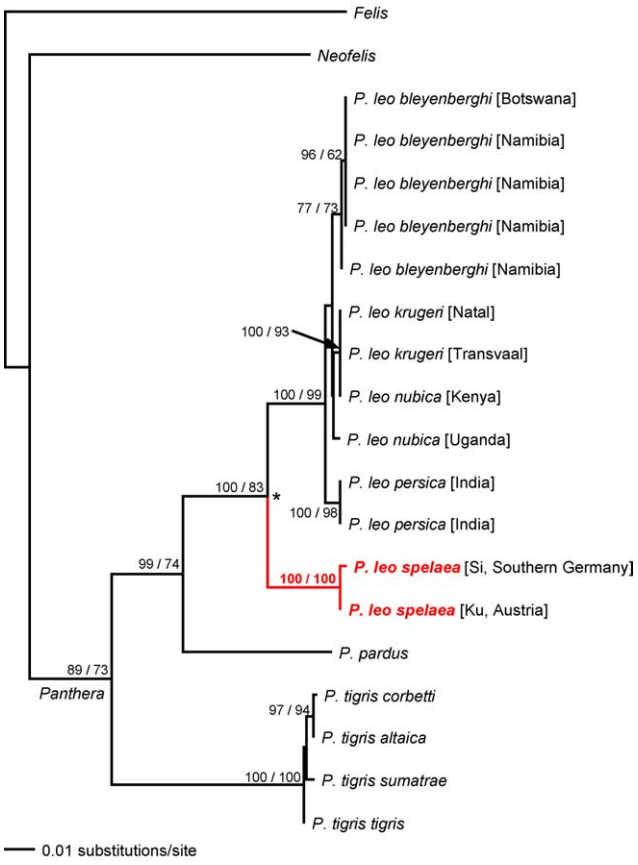

Fig. 3. Maximum likelihood phylogeny of the Panthera clade (GTR + G model; ln likelihood = −3038.43290). The cave lion clade is in red. Branch lengths are drawn proportional to estimated change; scale bar 0.01 substitutions per site. Node support values are attached by the nodes: clade probabilities (Bayesian posterior probabilities in percent) to the left and bootstrap percentages to the right. The node used to calibrate divergence time estimates is marked with an asterisk. Geographical origin of lions is noted within square brackets (*P. l. bleyenberghi*, *krugeri*, and *nubica* correspond to the *senegalensis* group; for subspecies nomenclature, see Table 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

tree prior to the analysis in order to obtain a non-zero length root branch. The program settings were as follows: gamma distribution of rates; five time guesses; five restarts. Confidence intervals (95%) for the estimated divergence times were also computed (Cutler, 2000) in r8s with the cutoff parameter set to 2.

An analysis of rate divergence times results in relative age estimates for all branch points (nodes) in the tree. In order to convert these to absolute times, it is necessary to fix one node as a calibration point; this point is therefore, in itself, not estimated by the analysis. We set the first split of the *Panthera leo* lineage in our ML tree to the date of the first appearance of *P. leo fossilis* in the European fossil record (marked by an asterisk in Fig. 3). The earliest date obtained for this appearance is 600 kya (Garcia Garcia, 2001).

3. Results and discussion

3.1. Diagenetic measurements (Table 3)

To test the general biomolecular state of preservation of the specimens before starting with the extensive ancient DNA analysis, bone samples were subjected to three separate diagenetic screening techniques: elemental analysis, Fourier-transform infrared spectroscopy, and SAXS. Very little mineral alteration is evident in either archaeological sample, as compared to the modern values for both SF and C/P. All crystallites measured using SAXS were determined to be plate-like (data not shown), which is also consistent with modern unaltered crystallites (Camacho et al., 1999). The crystallite sizes of the archaeological samples were significantly lower than those measured in the modern human sample, but this smaller size of 2.8–3 nm is consistent with the size seen in modern faunal samples (Wess and Hiller, unpublished data). In addition, very little nitrogen has been lost from the archaeological samples, indicating a high level of residual collagen. The lack of mineral alteration and high residual protein, indicate exceptional bone preservation, suggesting that these bones are suitable for recovery of ancient DNA (see Table 3).

3.2. Ancient DNA analysis and authenticity

From each specimen we obtained three separate bone samples, which were handled and analyzed by three workers independently in two separate labs (two in Mainz and one in Uppsala).

The mtDNA sequences derived from each bone underwent multiple verifications using independent samples, extractions, amplifications, cloning, and sequence determinations. In all cases, for all three samples the replicated mtDNA sequences were consistent across all

Table 3
Results of diagenetic screening procedures for cave lion samples Si and Ku, compared to three modern reference samples

Sample	SF	C/P	% N	SAXS thicknesses (nm)
Cave lion (Si)	2.76	0.469	3.83	2.99
Cave lion (Ku)	2.91	0.399	3.65	2.98
Modern bear reference	2.82	0.338	4.12	2.93
Modern human reference	2.72	0.445	4.17	3.75
Modern lion reference	2.85	0.375	4.1	2.55

experiments. Five nucleotide positions (one in Uppsala and four in Mainz), however, differed in more than one PCR from the established consensus sequence. All were C–T transitions and most likely due to decompositional deamination events (Gilbert et al., 2003; Hansen et al., 2001; Hofreiter et al., 2001a). In all cases a number of additional PCRs was performed, and a vast majority of sequences confirmed the original cytosine residue.

Contaminations, decompositional base modifications, nuclear insertions (Zischler et al., 1995), and, mainly, errors in the procedural design endanger the interpretation of ancient DNA results. Therefore, for each specimen the authenticity of the sequence has to be proven by various criteria (e.g., Hofreiter et al., 2001b). The authenticity of the sequences presented here is as ensured as possible, for the following reasons:

- Several different biomolecular screening methods showed the samples to be exceedingly well-preserved.
- Extraction and PCR blank controls were always negative.
- Sequences were reproduced various times from at least two independent extractions, and a total of at least four independent PCR amplifications.
- Overlapping PCR amplicons always produced the same sequence.
- In total 3 of the 12 PCR products (125 bp for Si and 163 bp for Ku) including 42 variable positions in Felidae were replicated in a second lab from a third bone sample.
- The obtained sequences can both be translated into an identifiable cytochrome *b* protein without nonsense mutations.
- Attempts to amplify a 474 bp amplicon using pantherine specific primers (CB9) failed, indicating that no modern DNA was involved in the enzymatic reaction.
- The sequences obtained from two specimens are different from each other, and reproducibly showed this individual difference.
- Both sequences make sense phylogenetically.

3.3. Cave lion phylogeny

The two 1051 bp cave lion sequences differ from each other at two base positions. Both are third codon position silent substitutions. The South African lion reference sequence (AF384818) differs from the fossil sequences by 40/38 silent substitutions and eight substitutions that result in an amino acid change. The cave lion sequences differ from nine undoubtedly sub-Saharan lions by 47–49 bp (45–47 bp), from two Asian lions (*P. leo persica*) by 50/48 bp, from a leopard (*P. pardus*) by 89/87 bp, and from four different subspecies of tiger (*P. tigris*) by 114–117 bp (112–115 bp). These results and the complete distance matrix in Table 4 agree well with the overview of pantherine taxonomy presented in Table 1.

We constructed a cladogram from the two cave lion sequences and extant species of the genus *Panthera* (tiger, leopard, and lion; Fig. 3). In accordance with morphological and behavioural studies of the phylogenetic relationships between the extant species of the genus *Panthera*, our cytochrome *b* tree shows that the tiger branch (subgenus *Tigris*) separated first from the branch of the jaguar (not shown), the leopard, and the lion (subgenus *Panthera*) (cf. Hemmer, 1978). An earlier analysis of 358 bp of the mitochondrial 12S RNA coding DNA and 289 bp of the cytochrome *b* gene is consistent with these relationships (Janczewski et al., 1995). The leopard and the lion represent the last species separation within the jaguar, leopard, and lion clade (Janczweski et al., 1995; Peters and Tonkin-Leyhausen, 1999). According to the palaeontological record, the first divergence in the subgenus *Panthera* took place in the late Middle Villafranchian at the end of the Pliocene, about 1900 kya, with the dispersal of the stem species out of Africa. This gave rise to the Holarctic base jaguar population (Hemmer et al., 2001). Therefore, the split between the subgeneric *Tigris* and *Panthera* clades cannot have been a later event, but rather an earlier one. Unfortunately, well-founded palaeontological dating is not yet possible for this point (Hemmer et al., 2001). Evolutionary rate constancy was not rejected for our data, and clock-based estimates of divergence times were therefore obtained. The age of the split between the subgeneric *Tigris* and *Panthera* clades was estimated to 1428–2295 kya (95% confidence interval), and the leopard–lion split to 1000–1559 kya. The latter split has not been unequivocally dated with palaeontological data, but our estimate is consistent with a likely Upper Villafranchian event at the beginning of the Lower Pleistocene.

Phylogenetic divergence within lions is marked by their dispersal over Europe in the early Middle Pleistocene, not before the Cromerian interglacial III or IV (Garcia Garcia, 2001), i.e., not before 600 kya. From this time on, the cave lion (*spelaea* group) developed in Europe, beginning with the early Middle Pleistocene *Panthera leo fossilis* and ending with the Upper Pleistocene *P. leo spelaea* (Hemmer, 1974). Our estimate for the more recent divergence within the *leo* group into the African and Asian extant lion subgroups, the *senegalensis* group in Africa (comprising all sub-Saharan African lions; Hemmer, 1974, in press) and *persica* in Asia (the north African Barbary lion, *leo*, is closer to the latter), is 74–203 kya. This split has not been dated before using palaeontological data.

In the context of this study, we have shown that a considerable mitochondrial genetic distance exists between these two cave lions and extant lions, one that is much larger than the range of genetic variation seen in living populations of lions. These results imply that our cave lion lineages were genetically isolated from the

Table 4
Distance matrix of sequences used in this study

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 *Felis*	—																			
2 *Neofelis*	0.3224	—																		
3 *P. leo* (Botswana)	0.30831	0.28851	—																	
4 *P. leo* (Namibia)	0.33078	0.28764	0.00104	—																
5 *P. leo* (Namibia)	0.30831	0.28851	0.00000	0.00104	—															
6 *P. leo* (Namibia)	0.30831	0.28851	0.00000	0.00104	0.00000	—														
7 *P. leo* (Namibia)	0.30831	0.28851	0.00000	0.00104	0.00000	0.00000	—													
8 *P. leo* (Natal)	0.32005	0.27911	0.00731	0.00430	0.00731	0.00731	0.00731	—												
9 *P. leo* (Transvaal)	0.32005	0.27911	0.00731	0.00430	0.00731	0.00731	0.00731	0.00000	—											
10 *P. leo* (Kenya)	0.32005	0.27911	0.00731	0.00430	0.00731	0.00731	0.00731	0.00000	0.00000	—										
11 *P. leo* (Uganda)	0.31044	0.27911	0.00635	0.00321	0.00635	0.00635	0.00635	0.00451	0.00451	0.00451	—									
12 *P. leo* persica	0.35607	0.28293	0.01220	0.01164	0.01220	0.01220	0.01220	0.01004	0.01004	0.01004	0.00900	—								
13 *P. leo* persica	0.35607	0.28293	0.01220	0.01164	0.01220	0.01220	0.01220	0.01004	0.01004	0.01004	0.00900	0.00000	—							
14 *P. leo* spelaea	0.36026	0.26236	0.05904	0.06103	0.05904	0.05904	0.05904	0.05896	0.05896	0.05896	0.06043	0.06159	0.06159	—						
15 *P. leo* spelaea	0.35443	0.26206	0.05620	0.05789	0.05620	0.05620	0.05620	0.05618	0.05618	0.05618	0.05760	0.05893	0.05893	0.00193	—					
16 *P. pardus*	0.26605	0.36516	0.13970	0.13844	0.13970	0.13970	0.13970	0.13945	0.13945	0.13945	0.13701	0.13818	0.13818	0.13696	0.13299	—				
17 *P. tigris* corbetti	0.29330	0.26427	0.19150	0.19435	0.19150	0.19150	0.19150	0.20602	0.20602	0.20602	0.20063	0.22154	0.22154	0.22123	0.21651	0.20798	—			
18 *P. tigris* altai	0.28807	0.27977	0.18806	0.19003	0.18806	0.18806	0.18806	0.20228	0.20228	0.20228	0.19704	0.21732	0.21732	0.21726	0.21256	0.20423	0.00088	—		
19 *P. tigris* sumatrae	0.27307	0.28704	0.18106	0.18410	0.18106	0.18106	0.18106	0.19481	0.19481	0.19481	0.18977	0.20889	0.20889	0.21445	0.20967	0.20164	0.00633	0.00541	—	
20 *P. tigris* tigris	0.27523	0.26569	0.18258	0.18598	0.18258	0.18258	0.18258	0.18938	0.18938	0.18938	0.18451	0.20268	0.20268	0.20851	0.20376	0.19601	0.00451	0.00358	0.00358	—

See Table 1 for GenBank accession numbers. Distances are corrected using the same model of evolution as in the maximum likelihood analysis. A general time-reversible model was used, with rates assumed to follow gamma distribution with a shape parameter = 0.244; this was estimated for the best maximum likelihood tree found. Numbers 3–7 are *Panthera leo bleyenberghi*, 8 and 9 are *P. leo krugeri*, and 10 and 11 are *P. leo nubica*.

ancestors of modern Asian and African lions from the early Middle Pleistocene onwards, and went extinct without contributing mitochondrial DNA to extant lineages. The question of whether the cave lion population of Europe and the extant lion populations of Africa and Asia should be recognized as different species may be a matter of convention. In our tree, the cave lion clade is a sister to the extant lions, which means that the cave lions may be excluded from or included within the species *P. leo*. However, the maximum possible number of lion generations since the 600 ky split in comparison with other pantherine species argues for the single species nomenclature (Hemmer, in press).

If it is assumed that the two cave lion sequences are representative for the European cave lions of that time, the very good support for both this clade (100% bootstrap as well as posterior probability) and for the clade of extant lions indicates that European cave lion populations may have left no mitochondrial descendants, whereas the mitochondrial genes of their contemporaneous African and Asian relatives survive in extant lion populations. It remains to be seen if the considerable sequence divergence between the clades (nearly 5%) will remain if the sample of fossil and extant specimens is increased. Further studies are needed to show if genetic changes or characteristics, as well as ecological factors, may have played a role in the extinction of cave lion populations in Europe at the end of the Pleistocene. This study represents another successful use of modern genetic and phylogenetic techniques to investigate long-standing palaeontological questions, and it is our hope that such studies continue to shed light on issues of evolutionary descent and speciation that cannot be as well elucidated by other means.

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References

Baryshnikov, G.F., Boeskorov, G., 2001. The Pleistocene cave lion, *Panthera spelaea* (Carnivora, Felidae) from Yakutia, Russia. Cranium 18, 7–24.

Camacho, N.P., Rinnerthaler, S., Paschalis, E.P., Mendelsohn, R., Boskey, A.L., Fratzl, P., 1999. Complementary information on bone ultrastructure from scanning small angle X-ray scattering and Fourier-transform infrared microspectroscopy. Bone 25, 287–293.

Cutler, D.J., 2000. Estimating divergence times in the presence of an overdispersed molecular clock. Mol. Biol. Evol. 17, 1647–1660.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using bootstrap. Evolution 39, 783–791.

Felsenstein, J., 1988. Phylogenies from molecular sequences: inference and reliability. Annu. Rev. Genet. 22, 521–565.

Garcia Garcia, N., 2001. Los Carnivorors de los Yacimientos Pleistocenos de la Sierra de Atapuerca. Thesis Doctoral.

Gilbert, M.T., Hansen, A.J., Willerslev, E., Rudbeck, L., Barnes, I., Lynnerup, N., Cooper, A., 2003. Characterization of genetic miscoding lesions caused by postmortem damage. Am. J. Hum. Genet. 72, 48–61.

Groiss, J.Th., 1996. Der Höhlentiger *Panthera tigris spelaea* (Goldfuss). Neves Jahrb. Geol. Paläont. Abh. 7, 399–414.

Hansen, A.J., Willerslev, E., Wiuf, C., Mourier, T., Arctander, P., 2001. Statistical evidence for miscoding lesions in ancient DNA templates. Mol. Biol. Evol. 18, 262–265.

Hedges, R.E.M., Millard, A.R., Pike, A.W.G., 1995. Measurements and relationships of diagenetic alteration of bone from three archaeological sites. J. Archaeol. Sci. 22, 201–209.

Hemmer, H., 1974. Untersuchungen zur Stammesgeschichte der Pantherkatzen (Pantherinae), Teil III: Zur Artgeschichte des Löwen Panthera (Panthera) leo (Linnaeus 1758). Veröffentlichungen der Zoologischen Staatssammlung München 17, 167–280.

Hemmer, H., 1978. The evolutionary systematics of living Felidae: present status and current problems. Carnivore 1, 71–79.

Hemmer, H., in press. Pleistozäne Katzen Europas—eine Übersicht. Cranium.

Hemmer, H., Kahlke, R.-D., Vekua, A.K., 2001. The Jaguar-Panthera onca gombaszoegensis (Kretzoi, 1938) (Carnivora: Felidae) in the late Lower Pleistocene of Akhalkalaki (South Georgie; Transcaucasia) and its evolutionary and ecological significance. Geobios 34, 475–486.

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

Hofreiter, M., Serre, D., Poinar, H.N., Kuch, M., Pääbo, S., 2001b. Ancient DNA. Nat. Rev. Genet. 2, 353–359.

Huelsenbeck, J.P., Ronquist, F., 2001. A program for the Bayesian inference phylogeny, version 2.01.

Janczewski, D.N., Modi, W.S., Stephens, J.C., O'Brien, S.J., 1995. Molecular evolution of mitochondrial 12S RNA and cytochrome *b* sequences in the pantherine lineage of Felidae. Mol. Biol. Evol. 12, 690–707.

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

Lorblanchet, M., 1995. Les Grottes Ornées de la Prehistoire. Errance, Paris.

Nylander, J.A.A., 2001. MrModeltest, Computer program, version 1.1b. Uppsala University.

Peters, G., Tonkin-Leyhausen, B.A., 1999. Evolution of acoustic communication signals of mammals: friendly close-range vocalizations in Felidae (Carnivora). J. Mamm. Evol. 6, 129–159.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Rodríguez, F., Oliver, J.L., Marin, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitution. J. Theor. Biol. 142, 485–501.

Rosendahl, W., Darga, R., 2004. Homo sapiens neanderthalensis et Panthera leo spelaea—du nouveau à propos du site de Siegsdorf (Chiemgau), Bavière/Allemagne. Rev. Palaeobiol. 3, 2.

Sanderson, M.J., 2002. Analysis of rates (r8s) of evolution (and other stuff), Computer program, version 1.5. University of California, Davis.

Schlosser, M., 1910. Die Bären-oder Tischoferhöhle im Kaisertal bei Kufstein. Abh. Math-physik. Königl. Bayr. Akad. Wiss. 24, 385–506.

Swofford, D.L., 2001. PAUP*. Phylogenetic analysis using parsimony and other methods, version 4.0.

Termine, J.D., Posner, A.S., 1966. Infra-red determination of the percentage of crystallinity in apatitic calcium phosphates. Nature 211, 268–270.

Turner, A., Antón, M., 1997. The Big Cats and their Fossil Relatives. Columbia University Press, New York.

Weiner, S., Bar-Yosef, O., 1990. States of preservation of bones from prehistoric sites in the Near East: a survey. J. Archaeol. Sci. 17, 196.

Wright, L.E., Schwarcz, H.P., 1996. Infrared and isotopic evidence for diagenesis of bone apatite at Dos Pilas Guatamala: palaeodietary implications. J. Archaeol. Sci. 23, 933–944.

Yang, Z., 1996. Among-site variation and its impact on phylogenetic analyses. TREE 11, 367–371.

Zischler, H., Höss, M., Handt, O., von Haeseler, A., Pääbo, S., Goudsimt, J., van der Kuyl, A.C., 1995. Detecting dinosaur DNA. Science 268, 1192–1193.