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First DNA sequences from Asian cave bear fossils reveal deep divergences and complex phylogeographic patterns

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Abstract

Until recently, cave bears were believed to have only inhabited Europe. However, recent morphological evidence suggests that cave bears' geographic range extended as far east as Transbaikalia, Eastern Siberia. These Asian cave bears were morphologically distinct from European cave bears. However, how they related to European lineages remains unclear, stressing the need to assess the phylogenetic and phylogeographic relationship between Asian cave bears and their European relatives. In this work, we address this issue using a 227 base-pair fragment of the mitochondrial control region obtained from nine fossil bone samples from eight sites from the Urals, Caucasus, Altai Mountains, Ukraine and Yana River region in Eastern Siberia. Results of the phylogenetic analyses indicate that (i) the cave bear from the Yana River is most closely related to cave bears from the Caucasus region; (ii) the Caucasus/Yana group of bears is genetically very distinct from both European cave bears and brown bears, suggesting that these bears could represent an independent species; and (iii) the Western European cave bear lineage reached at least temporarily to the Altai Mountains, 7000 km east of their known centre of distribution. These results suggest that the diversity of cave bears was greater than previously believed, and that they could survive in a much wider range of ecological conditions than previously assumed. They also agree with recent studies on other extinct and extant species, such as wolves, hyenas and steppe bison, which have also revealed higher genetic and ecological diversity in Pleistocene populations than previously known.

Keywords: ancient DNA, climate change, extinction, phylogeography, Pleistocene, speciation

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Introduction

Pleistocene cave bears have been extensively studied in terms of both classical palaeontology (Kurtén 1976; Musil 1980; Baryshnikov 1998, 2007; Rabeder 1999; Rabeder *et al*. 2000;) and ancient DNA research (Hänni *et al*. 1994; Loreille *et al*. 2001; Hofreiter *et al*. 2002, 2004a; Orlando *et al*. 2002). These studies have considerably improved our knowledge about cave bear biology and evolution. Based on morphological (Rabeder 1995) and genetic analyses (Hofreiter *et al*. 2004a), Rabeder *et al*. (2004) proposed that European cave bears comprise at least two species, *Ursus spelaeus* and *Ursus ingressus*, which were both derived from *U. deningeri*. An earlier study showed that a large cave bear,

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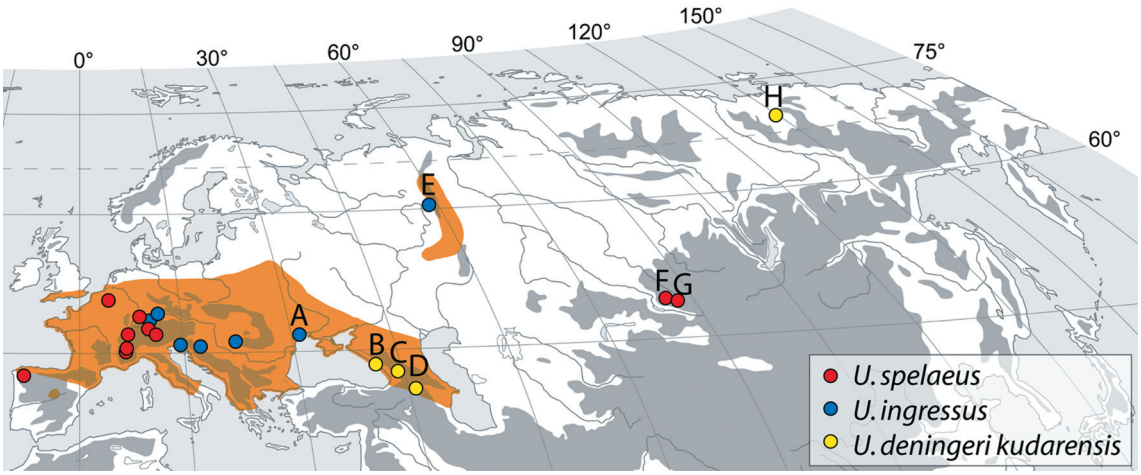

Fig. 1 Map of cave bear distribution: orange, distribution of *Ursus spelaeus sensu lato* including *U. deningeri* in Europe (after various sources). Locations where the newly sequenced specimens were found are given in capital letters. Coloured dots without denomination represent previously published sequences; the colours indicate their respective haplotype. Note that the location of specimen H is very distant from the currently known distribution of cave bears. The regions coloured in dark grey indicate an altitude greater than 500 m above sea level.

which was found at the Late Pleistocene Kudaro 3 cave site in the southern Caucasus and originally described as *U. spelaeus kudarensis*, differs considerably from the European *U. spelaeus* in its dental morphology. As a consequence, Baryshnikov (1998) assigned this cave bear to the more ancestral mid-Pleistocene *U. deningeri* group, and referred to it as *U. deningeri kudarensis*. Therefore, we use the term 'cave bear' as a summary term for a complex of distinct lineages which are all characterized by a 'spelaeoid' (i.e. cave-bear-like) rather than an 'arctoid' (i.e. brown-bear-like) morphology.

Until recently, cave bears were believed to be geographically confined to Europe (Kurtén 1976; Musil 1980; Kahlke 1994). However, palaeontological studies have shown that *Ursus rossicus*, a small cave bear that was initially found in the northern Caucasus (Borissiak 1930), occurred in both the Urals (Vereschagin & Baryshnikov 2000) and southeastern Siberia (Baryshnikov & Foronova 2001), and its geographic range extended as far east as Transbaikalia. Furthermore, Middle Pleistocene bear specimens resembling *U. deningeri* have now been discovered in many regions of Asia (Baryshnikov & Kalmykov 2005). These palaeontological findings stressed the need to assess the phylogenetic status of Asian cave bears and their relationship to the European lineages.

In this study, we evaluate the phylogenetic status of Asian cave bear fossils and provide insight into Eurasian cave bear systematics. We have sequenced part of the mitochondrial control region I extracted from cave bear fossils originating from the Urals, southern Ukraine (Black Sea region), the southern Caucasus and the Altai Mountains. Our sample also includes a unique specimen collected at the Yana River in northeastern Siberia (sample H), situated far to the northeast of cave bears' known distribution and habitat range.

Materials and methods

Samples

We obtained 21 samples from eight sites from the Urals, Caucasus, Altai Mountains, Ukraine and Yana River region in eastern Siberia (Fig. 1). The selection of bone specimens was based on their previous identification by palaeontologists as belonging to the cave bear lineage. The samples represent both classical cave bears (*Ursus spelaeus sensu lato: U. ingressus* and *U. spelaeus*) and *Ursus deningeri kudarensis*. For most of the specimens sampled (Table 1), the arguments supporting a 'spelaeoid' morphology have been published (Vereschagin & Baryshnikov 2000; Baryshnikov & Foronova 2001; Derevianko *et al*. 2003; Baryshnikov & Kalmykov 2005) or are contained in the respective institute's records. Exceptions are the samples from the Altai caves, where the associated faunal assemblages suggest that the bones originate from cave rather than brown bears, and the bone from the Siberian Arctic (sample H, Yana River region, Siberia). A more detailed description of this fossil and its geological provenance is provided in A. V. Sher, personal communication. Two of the samples were radiocarbon-dated: B, Ossenyaga cave, Caucasus, Russia (KIA25286, 34 550 + 580/–540), and G, Denisova cave, Altai, Russia (KIA25288, 49 430 + 2060/–1640).

Table 1 Cave bear samples of this study for which the complete ~285-bp sequence could be obtained. Listed are the sample's location designation in capitals (corresponding to Fig. 1), the collection number, species designation according to previous morphological studies as well as according to our mtDNA analyses, and the location and coordinates of the excavation site

Sample	Collection no.	Previous designation	Designation according to this study	Location	Coordinates
A	MPI-EVA 764	*U. spelaeus*	*U. ingressus*	Nerubajskoe 3, Odessa, Ukraine	46.53°N, 30.36°E
B	MPI-EVA 751	*U. spelaeus*	*U. deningeri kudarensis*	Ossenyaga cave, Caucasus, Russia	43.67°N, 39.90°E
C.1	MPI-EVA 1009	*U. deningeri kudarensis*	*U. deningeri kudarensis*	Kudaro 3 cave, Caucasus, Georgia (layer 2, horizon 5)	42.31°N, 43.41°E
C.2	MPI-EVA 1016	*U. deningeri kudarensis*	*U. deningeri kudarensis*	Kudaro 3 cave, Caucasus, Georgia (layer 3d)	42.31°N, 43.41°E
D	MPI-EVA 1636	*U. spelaeus*	*U. deningeri kudarensis*	Hovk 1, Caucasus, northern Armenia	40.88°N, 45°E
E	MPI-EVA 1012	*U. spelaeus*	*U. ingressus*	Medvezhiya cave, northern Ural, Russia (depth 4.4–4.6 m)	61.58°N, 58.02°E
F	MPI-EVA 1166	*Ursus*	*U. spelaeus*	Strashnaya cave, Altai, Russia	51.08°N, 83.03°E
G	MPI-EVA 553-D10	Animal remain	*U. spelaeus*	Denisova cave, Altai, Russia	51.24°N, 84.38°E
H	PIN 3723–496	*Ursus*	*U. deningeri kudarensis*	Oskhordokh, Adycha River (Yana River Region), Siberia, Russia	67.54°N, 135.67°E

MPI-EVA, Max Planck Institute for Evolutionary Anthropology.
PIN, Paleontological Institute of the Russian Academy of Sciences.

DNA sequencing

DNA was extracted from the sample material (295–520 mg) following the protocols described in Hofreiter *et al*. (2004a) and Rohland & Hofreiter (2007). For all samples, we attempted to amplify an approximately 285 base-pair (bp) long fragment of the mitochondrial control region I as in Hofreiter *et al*. (2002). We used primers from Hofreiter *et al*. (2002), primers that amplify shorter fragments (Hofreiter *et al*. 2004a), and primers specifically designed for this study (Table S1, Supporting Information). Amplifications were performed using either standard polymerase chain reaction (PCR) or multiplex PCR (Römpler *et al*. 2006). Conditions and annealing temperatures are provided in Table S1.

Amplification products were cloned into the pCR2.1-TOPO vector (Invitrogen) following the supplier's instructions. A minimum of three clones per sample was sequenced on an ABI 3730 sequencer using the BigDye Terminator version 1.1 Cycle Sequencing Kit and M13 universal primers. Complete sequences were obtained from nine samples and visually aligned using the BioEdit program (Hall 1999). Each sequence position was determined from at least two independent amplifications to avoid sequence errors due to changes in the sequences caused by template damage (Hofreiter *et al*. 2001). If consistent differences between two independent amplifications were found, we performed a third PCR to clarify which nucleotide represents the correct sequence.

For three of the specimens (specimens B, Ossenyaga cave, Caucasus, Russia; G, Denisova cave, Altai, Russia; H, Yana River region, Siberia, Russia; see Table 1), portions of the sequences were replicated in a second laboratory in Copenhagen (for detailed extraction and PCR protocols see Table S1).

Sequence data

Two different alignments were used for our analyses.

Alignment A (Table 2). To evaluate the phylogenetic relationships between Asian and European cave bears, we aligned all eight newly determined haplotypes (unique sequences, sample D, Hovk 1 cave, Caucasus, Armenia, shares a haplotype with sample C.2, Kudaro 3 cave, Caucasus, Russia) to 12 published cave bear haplotypes including sequences from all four previously described clades of cave bears (Orlando *et al*. 2002) (Table 2). Furthermore, 12 brown bear (*U. arctos*), 4 polar bear (*U. maritimus*), and 9 black bear (*U. americanus*) haplotypes were included in the alignment (Accession no. in Table 2). The black bears were used as outgroup to root the cave bear/brown bear phylogeny. Some brown bear haplotypes were lacking the last 30 bp of the alignment. A long pyrimidine stretch

Table 2 Specimens used for the phylogenetic reconstructions (Alignment A). Shown are GenBank Accession numbers (and identifiers for new samples used in this study), species designation given by the sequence, locations of excavation sites (not for the outgroup species)

Sample/(Accession no.)	Species designation	Location
A (EU352878)	*U. ingressus*	Nerubajskoe 3, Ukraine
B (EU352879)	*U. deningeri kudarensis*	Ossenyaga cave, Caucasus, Russia
C.1 (EU352880)	*U. deningeri kudarensis*	Kudaro 3 cave, Caucasus, Georgia
C.2 (EU352881)	*U. deningeri kudarensis*	Kudaro 3 cave, Caucasus, Georgia
D (EU352882)	*U. deningeri kudarensis*	Hovk 1 cave, Caucasus, Armenia
E (EU352883)	*U. ingressus*	Medvezhiya cave, Ural, Russia
F (EU352884)	*U. spelaeus*	Strashnaya cave, Altai, Russia
G (EU352885)	*U. spelaeus*	Denisova cave, Altai, Russia
H (EU352886)	*U. deningeri kudarensis*	Yana River region, Siberia, Russia
AH012172	*U. spelaeus*	Scladina, Belgium
AJ300166	*U. spelaeus*	Ramesch, Austria
AJ300168	*U. spelaeus*	Gailenreuth, Germany
AJ300169	*U. spelaeus*	Conturines, Italy
AJ300171	*U. ingressus*	Geissenklösterle, Germany
AJ300173	*U. ingressus*	Potocka Zijalka, Slovenia
AJ300174	*U. ingressus*	Vindija (1), Croatia
AJ300175	*U. ingressus*	Vindija (2), Croatia
AJ300176	*U. spelaeus*	Hohle Fels, Germany
AJ300177	*U. spelaeus*	Grotte Merveilleuse, France
AY149271	*U. spelaeus*	Cova Linares, Spain
AY149273	*U. spelaeus*	Balme à Collomb, France
DQ914411	*U. arctos*	
EF033734	*U. arctos*	
EF033896	*U. arctos*	
EF034022	*U. arctos*	
X75877	*U. arctos*	
AB010727	*U. arctos*	
AB013062	*U. arctos*	
EF033708	*U. arctos*	
EF033713	*U. arctos*	
EF033717	*U. arctos*	
EF033723	*U. arctos*	
EF033726	*U. arctos*	
EF033727	*U. maritimus*	
EF033728	*U. maritimus*	
EF033729	*U. maritimus*	
EF033730	*U. maritimus*	
AF012307	*U. americanus*	
AF012308	*U. americanus*	
AF012309	*U. americanus*	
AF012310	*U. americanus*	
AF012311	*U. americanus*	
AF012319	*U. americanus*	
AF012320	*U. americanus*	
AF012322	*U. americanus*	
AF012323	*U. americanus*	

(brown bears: 12 bp, cave bears: 25–28 bp) was removed from all sequences, since this region could not be aligned unambiguously. Thus, a 227-bp fragment of the control region was used for subsequent analyses. Sequences were aligned manually using the program package BioEdit (Hall 1999).

Alignment B (Table 3). For the molecular clock analyses, not only the amount of sequence data but also the amount of temporal information included in the data set influences the accuracy of the date estimates, in particular when only the age of the samples is used to calibrate the molecular clock. Thus, we aimed at including as many dated samples

Table 3 Specimens used for divergence time estimation (Alignment B). Shown are the GenBank Accession numbers and the identifiers for new samples used, as well as the age of the respective sample (*r, radiocarbon age; *s, age derived from stratigraphy). Numbers in brackets behind the sample location identify the sample if several accessions from the same location were used (see Fig. 2)

Accession no.	Species	Origin	Calibration age	Reference
AJ300166	*U. spelaeus*	Ramesch (1), Austria	47, 016*r	Hofreiter *et al*., 2001
AJ300167	*U. spelaeus*	Ramesch (2), Austria	52, 766*r	Hofreiter *et al*., 2001
AJ300168	*U. spelaeus*	Gailenreuth, Germany	40, 484*r	Hofreiter *et al*., 2001
AJ300169	*U. spelaeus*	Conturines, Italy	47, 594*r	Hofreiter *et al*., 2001
AJ300170	*U. ingressus*	Geissenklösterle (1), Germany	31, 069*r	Hofreiter *et al*., 2001
AJ300171	*U. ingressus*	Geissenklösterle (2), Germany	31, 166*r	Hofreiter *et al*., 2001
AJ300172	*U. ingressus*	Nixloch, Austria	31, 524*r	Hofreiter *et al*., 2001
AJ300173	*U. ingressus*	Potocka zijalka, Slovenia	31, 231*r	Hofreiter *et al*., 2001
AJ300174	*U. ingressus*	Vindija (1), Croatia	37, 402*r	Hofreiter *et al*., 2001
AJ300175	*U. ingressus*	Vindija (2), Croatia	37, 378*r	Hofreiter *et al*., 2001
AJ300176	*U. spelaeus*	Hohlefels, Germany	35, 550*r	Hofreiter *et al*., 2001
AJ300177	*U. spelaeus*	Grotte Merveilleuse, France	41, 277*r	Hofreiter *et al*., 2001
EU289397	*U. ingressus*	Pestera cu Oase, Romania (1)	42, 900*r	Richards *et al*., 2008
EU289397	*U. ingressus*	Pestera cu Oase, Romania (2)	46, 614*r	Richards *et al*., 2008
EU289397	*U. ingressus*	Pestera cu Oase, Romania (3)	46, 800*r	Richards *et al*., 2008
EU289401	*U. ingressus*	Pestera cu Oase, Romania (4)	45, 958*r	Richards *et al*., 2008
AY149239	*U. spelaeus*	Scladina, Belgium (1)	42, 500*s	Orlando *et al*., 2002
AY149243	*U. spelaeus*	Scladina, Belgium (2)	42, 500*s	Orlando *et al*., 2002
AY149255	*U. spelaeus*	Scladina, Belgium (3)	80, 000*s	Orlando *et al*., 2002
AY149266	*U. spelaeus*	Prélétang, France	40, 000*s	Orlando *et al*., 2002
AY149267	*U. spelaeus*	Scladina, Belgium (4)	42, 500*s	Loreille *et al*., 2001
AY149268	*U. spelaeus*	Scladina, Belgium (5)	42, 500*s	Loreille *et al*., 2001
AY149271	*U. spelaeus*	Cova-Linarès, Spain (1)	35, 000*s	Loreille *et al*., 2001
AY149272	*U. spelaeus*	Cova-Linarès, Spain (2)	35, 000*s	Loreille *et al*., 2001
AY149273	*U. spelaeus*	Balme à Collomb, France	30, 000*s	Loreille *et al*., 2001
A (EU352878)	*U. ingressus*	Nerubajskoe 3, Ukraine	56, 000*s	This study
B (EU352879)	*U. deningeri kudarensis*	Ossenyaga cave, Caucasus, Russia	40, 383*r	This study
C.1 (EU352880)	*U. deningeri kudarensis*	Kudaro 3 cave, Caucasus, Georgia	37, 000*s	This study
C.2 (EU352881)	*U. deningeri kudarensis*	Kudaro 3 cave, Caucasus, Georgia	38, 000*s	This study
D (EU352882)	*U. deningeri kudarensis*	Hovk 1 cave, Caucasus, Armenia	55, 000*s	This study
E (EU352883)	*U. ingressus*	Medvezhiya cave, Ural, Russia	Undated	This study
F (EU352884)	*U. spelaeus*	Strashnaya cave, Altai, Russia	Undated	This study
G (EU352885)	*U. spelaeus*	Denisova cave, Altai, Russia	52, 937*r	This study
H (EU352886)	*U. deningeri kudarensis*	Yana River region Siberia, Russia	Undated	This study
AJ809333	*U. arctos* (1)	Ramesch, Austria	47, 500*r	Hofreiter *et al*., 2004b
AJ809334	*U. arctos* (2)	Winden, Austria	40, 020*r	Hofreiter *et al*., 2004b
X75862	*U. arctos* (3)	Abruzzo, Italy	Recent	Taberlet & Bouvet, 1994

into our analyses as possible. A number of dated cave bear control region sequences are published but only a few cover the 227 bp we used for our phylogenetic analyses. In order to increase the amount of temporal information in our data set without losing too much sequence data, we included all currently available dated cave bear sequences (including duplicate haplotypes from samples of different ages) that overlap across at least 201 bp with the control region fragment we sequenced from the nine Asian cave bears. This resulted in a total of 34 cave bear sequences (Table 3). To calibrate the molecular clock using the age of the divergence of cave bears and brown bears, three brown bears of varying age from the two major European clades were added to this data set (Fig. 2).

Phylogenetic analyses (Alignment A)

To test the stability of phylogenetic reconstructions under different tree-building approaches, we performed analyses using neighbour-joining (NJ), maximum parsimony (MP), and three maximum likelihood (ML) search algorithms (heuristic search in PAUP*, IQPNNI algorithm, Quartet Puzzling), as well as a Markov chain Monte Carlo (MCMC)-based Bayesian approach.

For the model-based phylogenetic analyses, the best-fitting nucleotide substitution model was determined with ModelTest 3.7 (Posada & Crandall 1998). All three decision criteria (Posada & Buckley 2004) — hierarchical likelihood ratio tests (hLRT), the Akaike information criterion (AIC),

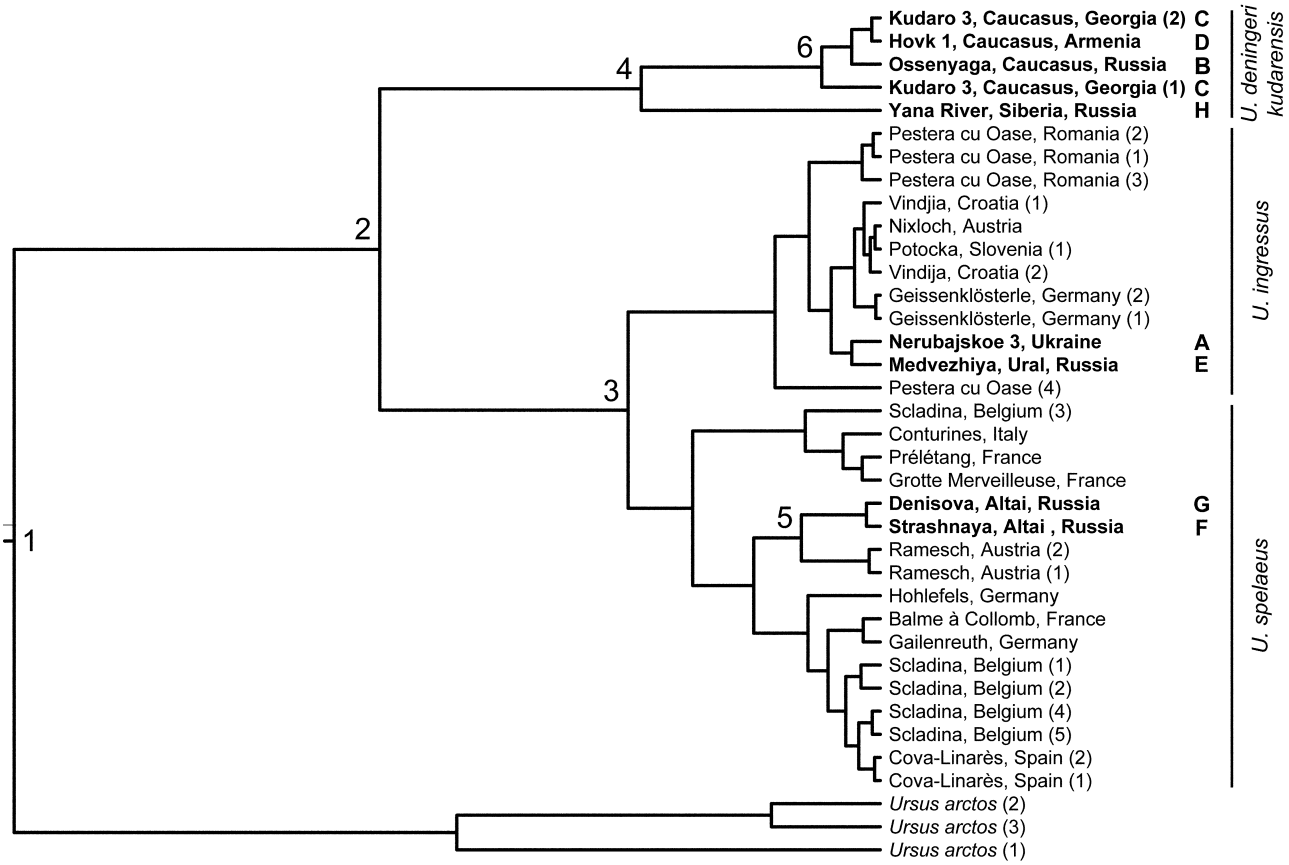

Fig. 2 Chronogram of Eurasian cave bears. Capital letters refer to samples found in locations as indicated in Fig. 1. Node labels show nodes for which divergence time estimates and/or bootstrap/posterior support are given in Tables 5 and 6, respectively. Numbers in brackets behind the sample location identify the sample if several accessions from the same location were used [i.e. Scladina (2) is sample number 2 from Scladina, cf. Table 3]. Tree reconstructed using BEAST 1.4.7, based on the 201-bp alignment (Table 3) as described in Materials and methods. Trees produced by other methods are available on request.

and the Bayesian information criterion (BIC) — supported HKY85 + Γ as the best-fitting model. Therefore, where applicable and if not stated differently, all analyses used HKY85 + Γ as the substitution model. The alignment was checked for phylogenetic signal using saturation plots (i.e. transitions and transversions vs. distance plots (Xia & Xie 2001)) and ungrouped likelihood mapping (Strimmer & von Haeseler 1997), both performed with Tree-Puzzle 5.3 (Schmidt *et al*. 2002). These methods indicated neither substitution saturation nor lack of phylogenetic signal. However, a lack of transversions was detected, consistent with ModelTest's preference for the HKY85 model.

Phylogenetic trees were reconstructed with PAUP* version 4.0b10 (Swofford 2003) using NJ, MP and one ML approach. For the MP tree, heuristic searches were performed with 10 replicates of random stepwise addition of taxa, followed by tree-bisection–reconnection (TBR). Branch lengths were evaluated using the ACCTRAN strategy. The PAUP-ML tree was reconstructed using the NJ tree as the starting tree, followed by TBR optimization using the parameters from the above ModelTest analyses. For all three analyses, bootstrap analyses were performed with PAUP* using the same settings as before, but using 1000 pseudosamples summarized with a 50%-majority-rule consensus (M_{50}). In addition, ML reconstruction was performed with IQPNNI (Vinh & von Haeseler 2004), using the HKY85 + Γ substitution model with four substitution rate categories running for a minimum of 200 iterations, with the stopping rule option switched on. A bootstrap tree was constructed from 100 pseudosamples generated with the SeqBoot program from PHYLIP 3.67 (Felsenstein 2005), summarized with the relative-majority consensus (M_{rel}) (Schmidt 2003) in Tree-Puzzle 5.3 5.3. The third ML-based approach was Quartet Puzzling with Tree-Puzzle 5.3 (Schmidt *et al*. 2002) applying the HKY85 + Γ substitution model with four Γ rate categories.

A Bayesian analysis was run as implemented in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Four independent runs were performed with one cold and one hot MCMC chain each. The chains were run for 50 million

generations sampling every 500th generation after discarding the first 2 million generations as burn-in. The results were checked for convergence using Tracer 1.4 (http://tree.bio.ed.ac.uk/software/tracer/).

Since all the methods described above resolved the phylogenetic relationship between the three main lineages (*U. spelaeus sensu lato*, *U. deningeri kudarensis* and *U. arctos*/*U. maritimus*) with only low to moderate support values, we also performed 4-cluster (i.e. the three lineages and the outgroup) likelihood mapping analyses (Strimmer & von Haeseler 1997) as implemented in Tree-Puzzle 5.3 (Schmidt *et al*. 2002). Using this approach, we tested for phylogenetic support for alternative topologies with the HKY85, TN93, HKY85 + Γ and TN93 + Γ nucleotide substitution models. For the latter two we used four substitution rate categories each.

To compare the maximum sequence divergence observed among the different Eurasian cave bear haplotypes to the maximum sequence divergence observed among brown bears, polar bears and black bears, respectively, we obtained all published control region sequences from these three species that covered the complete 201 bp fragment used in our molecular clock analyses and calculated the maximum sequence divergence within each taxon using BioEdit (Hall 1999).

Molecular clock analysis (alignment B)

Bayesian phylogenetic and molecular clock analyses were performed using BEAST 1.4.7 (Drummond *et al*. 2006; Drummond & Rambaut 2007) under the HKY85 + Γ nucleotide substitution model. The posterior distributions of the parameters in question (including root position) were estimated with three MCMC runs of 10 000 000 steps each. Trees were sampled from the posterior distribution every 1000th step after a discarded burn-in of 1 000 000 steps for each run, with one chain per run. Convergence of the chains and effective sample sizes (ESS) were verified using the program TRACER 1.4. This program was also used to analyse the combined results from all three chains.

For the molecular clock analyses, we constrained BEAST to estimate divergence times only on trees consistent with the topology reconstructed by all other methods. Molecular clock analyses were conducted under two different calibration systems.

First, we used the age of the dated samples for calibration of the molecular clock. Samples were either radiocarbon-dated (absolute dating) or assigned ages based on the stratigraphic context they were found in (relative dating, Table 3). Radiocarbon dates were calibrated using CalPal (http://www.calpal.de/, all dates were calibrated using the CalPal2007 HULU calibration curve). For three of the nine Asian cave bears (samples E, Medvezhiya cave, Ural, Russia; F, Strashnaya cave, Altai, Russia; H, Yana River region, Siberia, Russia), no age information was available. To reduce the noise introduced by these three undated sequences, we first estimated an average overall substitution rate for the remaining 31 sequences using the settings described above assuming a strict molecular clock with a constant-size coalescent prior on the entire tree. We also conducted one separate MCMC run with the same settings, sampling only from the prior distribution to evaluate potential biases introduced by the priors. As no biases could be identified, we used the estimated posterior distribution of the substitution rate as an additional normally distributed prior to estimate the age of different nodes in the complete tree of 34 samples. The latter analysis was conducted assuming both a constant substitution rate (strict clock) and a relaxed lognormal distribution of substitution rates. The different models were then compared using Bayes factors (BF) (Kass & Raftery 1995). BF were calculated as recommended by Suchard *et al*. (2001).

Second, we recalibrated the molecular clock, using the age of the divergence of cave bears and brown bears instead of the age of the samples to evaluate the effect of the time dependency of molecular rates (Ho *et al*. 2005). The analyses were conducted using the same MCMC settings as above. Recent studies (Rabeder & Withalm 2006) have shown that a bear discovered in Deutsch-Altenburg, that is at least 1.2 million years (Myr) old, was already part of the brown bear lineage. Hence, 1.2 million years ago (Ma) can be considered a minimum age for the divergence of cave bears and brown bears. The youngest known fossils of *Ursus etruscus*, which can be considered ancestral to both the cave bear and the brown bear lineages, were dated to about 1.6 Ma based on the stratigraphic context in which they were found (Rustioni & Mazza 1992; Rabeder & Withalm 2006; Sala & Masini 2007). Thus, 1.6 Ma can be used as an upper limit for the divergence date of cave bears and brown bears. These constraints are consistent with molecular estimates by Loreille *et al*. (2001). Therefore, we used a mean age of 1.4 Myr [SD 0.1 Myr, 95% highest posterior density (HPD) 1.2–1.6 Ma] as a normally distributed prior for the age of the root of the cave bear–brown bear phylogeny. Divergence time estimates were calculated with a constant-size coalescent prior on the cave bear clade and a uniform prior on the remaining branches (Gilbert *et al*. 2008), under the assumption of an uncorrelated log-normal distribution of substitution rates.

Results

We obtained the complete 285-bp sequence for nine specimens originating from eight different locations (Fig. 1, Table 1). One other sample was brown bear in origin and 11 samples either contained no amplifiable DNA or were so poorly preserved that only few amplification trials were successful. The partial sequences obtained from

three specimens in the Copenhagen laboratory (135 bp for samples B, Ossenyaga cave, Caucasus, Russia and G, Denisova cave, Altai, Russia and 79 bp for sample H, Yana River region, Siberia, Russia) perfectly match the sequences obtained in the MPI-EVA laboratory, except for a single G-to-A change in one sample (B) and one C-to-T change in another sample (G). Both changes are most likely due to cytosine deamination, which is common in ancient DNA (Hofreiter *et al*. 2001). Importantly, one of the replicated samples (H) is the unique specimen from the Yana River region.

An almost fully resolved phylogeny was reconstructed, using different tree-building methods but poor support values indicate uncertainty associated with several branches (Table 5, Fig. 2). Nevertheless several well-supported major clades independent of the tree-building method were recovered. The *Ursus deningeri kudarensis* specimens from the Caucasus (samples B, C.1, C.2 and D) and the Yana River region (sample H) form a separate clade, which is distinct from both the cave and brown/polar bear clades. Phylogenetic reconstructions group *U. deningeri kudarensis* with the cave bear clade, albeit with poor bootstrap/puzzle support (32–47% ML and 40% MP), and moderate posterior probability (MrBayes: 0.85). However, it is noteworthy that despite the low support values in these analyses, none of the methods found a contradicting clustering with greater support. Further analysis with four-cluster likelihood mapping suggests that the less complex models HKY85 and TN93 show greater support for grouping *U. deningeri kudarensis* with the cave bear clade than the more complex HKY85+G and TN93+G models (Fig. S1, Supporting Information). The Yana River specimen sequence (sample H) joins the branch of the Caucasus bears (B, C.1, C.2 and D), although it occupies a relatively basal position with mostly moderate support (55 to 89% bootstrap/puzzle support, posterior probability: 1; Table 5; Fig. 2). The Altai sequences (G, F) fall within the diversity of Western European cave bears, and the samples from the Urals and the Ukraine (A and E) form a monophyletic clade with accessions of *U. ingressus*.

The substitution rate estimated from the 201-bp alignment based on the age of 31 dated cave bears was 0.357 substitutions per site per Myr with a 95% HPD of 0.085–0.637. This corresponds to a normal distribution with a mean of 0.357 and a standard deviation of 0.17 substitution/site/Myr. The analysis was repeated three times. All three independent MCMC runs produced almost identical results, indicating convergence of the algorithm. ESS greatly exceeded 200 for all estimated ages and parameters, suggesting that the MCMC was mixing well and an appropriate amount of independent samples was collected from the posterior distribution (Drummond *et al*. 2007). When the substitution rate was only sampled from the prior distribution, the posterior resembled the uniform prior distribution assumed for the substitution rate (all values between 0 and 100 have the same probability). This suggests that our results are not influenced by biases introduced by the priors.

Population divergences within Eurasian cave bears were then dated using the complete 201-bp alignment of 34 cave bears (including the three undated samples; Table 3) and applying the same posterior distribution of the average substitution rate estimated in the previous step as a prior. A strict molecular clock model and a relaxed lognormal distribution of substitution rates were compared. The relaxed lognormal model fits the data decisively better ($\log_{10}$ BF: 9.9; Kass & Raftery 1995), although the age estimates provided by both models were similar. For both molecular clock models, three independent MCMC runs produced almost identical results, again indicating convergence of the algorithm. As in the previous analysis, ESS greatly exceeded 200 for all estimated ages (Table 6). Divergence time estimates based on the age of the cave bear–brown bear split were older than divergence times estimated using the age of radiocarbon-dated samples but the wide 95% HPD range of the former included the complete and much narrower 95% HPD range of the latter in all cases except for the root node. The highest posterior probabilities for shallow nodes [less than 100 000 years before present (BP) with external calibration] do not differ greatly from the respective values estimated with internal calibrations. However, external calibration produced far older estimates than internal calibration for more basal nodes (Table 6).

Discussion

Phylogenetic reconstructions and divergence times

We identified three distinct lineages of Eurasian cave bears. Each of the Asian cave bears could be assigned to one of these three lineages. With one exception, all phylogenetic approaches used produced consistent results. The phylogeny reconstructed by the Bayesian approach implemented in BEAST differed slightly from all other phylogenetic reconstructions (Fig. 2). BEAST uses a combination of a nucleotide substitution model and a molecular clock model to reconstruct phylogenies. The resulting tree topology might therefore differ from conventional approaches. In our case, we found that when only tip calibrations were used the 80 000-year-old *Ursus spelaeus* specimen from Scladina (Scladina, Belgium (3); Table 3) branched off basally to all other *U. spelaeus* in the unconstrained maximum clade-credibility tree. This position is inconsistent with all other phylogenies and also inconsistent with the BEAST phylogeny when only root calibration was used. Thus, this result could be an artefact caused by the age difference between the Scladina specimen and all other samples,

although both more samples and more sequence data would be necessary to test this assumption.

When evaluating the robustness of our phylogenetic reconstructions and the haplotype diversity identified, a number of factors have to be taken into account. The mitochondrial control region has been shown to evolve at very different rates in different taxa (Ho *et al*. 2007). If this was also the case for the taxa used in our analyses, it could introduce errors into the phylogenetic reconstructions. However, Ho *et al*. (2007) and Saarma *et al*. (2007) showed that the control regions of cave bears and brown bears evolve at similar rates and that even cave lions had roughly comparable rates (0.20–0.30 substitution/site/Myr for the fragment analysed by Ho *et al*. 2007). These findings suggest that the control region is suitable for estimating phylogenetic relationships of cave, brown, polar and black bears. A further limitation of our analyses is the amount of sequence data available. Ideally, a population level phylogeny would be reconstructed using sequence data from multiple independent loci (Carstens & Knowles 2007), but unfortunately, this is not possible for our study. The poor biochemical preservation of most samples limited the amplification length of mitochondrial DNA to about 100 bp including primers. We thus did not attempt amplification of nuclear DNA, which is generally much more fragmented than mitochondrial DNA (mtDNA). Furthermore, limited availability of previously sequenced samples precludes further sequencing of these accessions and limits our analyses to sequence fragments already published. Nevertheless, despite the short sequence length, the consistent support values throughout the tree produced by different phylogenetic approaches as well as the results from our likelihood mapping analyses (Fig. S1) suggest that our data set contains phylogenetic signal, and that the three major Eurasian cave bear lineages identified represent real evolutionary lineages. However, phylogenetic relationships within these lineages should be interpreted with caution as further knowledge regarding these evolutionary lineages has to be confirmed by analysing additional sequence data.

Another issue is that temporally spaced sampling can artificially increase the observed haplotype diversity. Consequently, diversity at any given time might have been lower than the total diversity observed over a period of thousands of years. Thus, part of the diversity observed in our data may be attributable to our samples being temporally spaced. However, the ages of representatives from all three major clades overlap. Thus, assuming that the ages assigned to the individual samples are correct, we are confident that the three major cave bear lineages identified do not represent artefacts of non-contemporary sampling.

In order to estimate divergence times, we sampled only those trees from the posterior distribution which were consistent with the consensus topology reconstructed by the different phylogenetic approaches (Fig. 2). The divergence time estimates (Table 6) should be considered preliminary, as there are several potential sources of error associated with these estimates. Whether substitution rates obtained merely from tip calibrations can be used to infer the age of deep divergences in a phylogeny is an issue currently under discussion (Ho *et al*. 2008). Ho *et al*. (2005) showed that substitution rates seem to decrease with increasing time depth until equilibrium is reached. This effect may cause the age of our deeper nodes to be underestimated when only the age of the tips is used for calibration. Alternatively, using an external fossil calibration may lead to an overestimation of the age of more shallow nodes. As the decrease in substitution rate over time is not linear (Ho *et al*. 2005), it might also be problematic to combine external and internal calibrations to estimate the age of internal nodes. Thus, we estimated divergence times with external and internal calibrations independent from each other in order to provide upper and lower limits for the age of particular nodes. The wide 95% HPD range associated with the externally calibrated date estimates reflects the uncertainty of the root age used for calibration (1.2–1.6 Ma). As expected, divergence time estimates for shallow nodes are fairly similar for both calibration systems due to the low genetic divergence (Table 6). If the substitution rate steadily decreases as the time depth increases, it could be expected that the true age of a node is somewhere between the highest posterior probabilities estimated under the two different calibration systems. Further potential sources of error are short sequence length and errors in the dating of the samples themselves. As discussed above, the former might be reduced with additional sequence data, although a recent study on woolly mammoth (Gilbert *et al*. 2008) showed that even complete mitochondrial genome sequences produced large confidence intervals for Pleistocene date estimates. As in the case of phylogenetic reconstructions, a potential approach to reduce the data-induced error in the divergence time estimates would be to sequence additional nuclear loci (Carstens & Knowles 2007). However, as described above, the poor biochemical preservation of several key samples most likely precludes such analyses. Furthermore, nuclear data, as well as the samples themselves, are not available for the published sequences which are vital for the comparative analyses.

Cave bear biogeography and evolution

Until recently, cave bears were believed to have been confined to Europe and adapted only to European Pleistocene environments. Several different haplotypes were identified within this range (Orlando *et al*. 2002) and can be assigned to two main lineages, *U. spelaeus* and *U. ingressus* (Hofreiter *et al*. 2004a). In this study, we identified a third, so far

unknown, lineage of bears that was genetically very distinct from both cave and brown bears. This group included bears from the Caucasus (samples B, C.1, C.2 and D) and northeastern Siberia (the Yana River region, sample H), the latter occurring more than 6000 km east of the previously known cave bear range. Based on their dental morphology, samples from the Caucasus had previously been identified as *U. deningeri kudarensis* (Baryshnikov 1998). Due to its genetic similarity to the Caucasus samples B, C.1, C.2 and D, we included sample H from the Yana River region into this lineage.

Phylogenetic reconstructions grouped *U. deningeri kudarensis* as a sister group to the remaining Eurasian cave bears, rather than as a sister group to brown bears (Fig. 2). This grouping often had less than 50% support, but no contradictory grouping with greater support could be identified. Likelihood mapping also suggested support for grouping *U. deningeri kudarensis* with *U. spelaeus sensu lato*. Furthermore, it provided evidence that the support decreases as the complexity of the implemented substitution model increases. This analysis indicates that, although ModelTest found a significantly higher likelihood for the HKY85 + Γ model than for less complex models of nucleotide substitution, the additional parameters might reduce the resolution power. This is potentially due to overfitting effects in conjunction with the absence of transversions in the alignment. Based on these analyses, it appears likely that *U. deningeri kudarensis* groups with Eurasian cave bears rather than with brown and polar bears and that *U. spelaeus sensu lato* and *U. deningeri kudarensis* (node 2) diverged sometime between 274 000 BP (95% HPD: 109 000–510 000 BP) and 814 000 BP (234 000 BP to 1.38 Ma). However, more sequence data would be needed to verify this conclusion.

Whether the three main lineages *U. deningeri kudarensis*, *U. spelaeus* and *U. ingressus* identified in this study represent independent species or can be considered a single, widespread and morphologically diverse species, as suggested by Baryshnikov (2006), remains unclear. The lack of mitochondrial gene flow between two alpine *U. spelaeus* and *U. ingressus* populations inhabiting the same alpine region in Austria, as well as the maintenance of morphological differences between these geographically overlapping populations for more than 15 000 years (Hofreiter *et al*. 2004a), argue against the single-species hypothesis for the two European lineages. The genetic distance between *U. deningeri kudarensis* and the European cave bear lineages is greater than the genetic distance between the two European lineages. Hence, an independent species status for the latter would suggest that *U. deningeri kudarensis* may also have been a separate species. However, comparisons of the maximum pairwise nucleotide differences of cave bears, brown bears, polar bears and black bears showed that the mitochondrial genetic distance is a poor indicator of the species status of a taxon. The maximum sequence divergence among the analysed Eurasian cave bears was observed between the 40 400-year-old-sample B from Ossenyaga cave in the Caucasus region and a 42 500-year-old sample from Scladina in Belgium (24 bp). The pairwise nucleotide difference between these samples was greater than any pairwise nucleotide difference among Eurasian brown bears and also greater than the maximum pairwise distance between brown bears and polar bears which are considered to be independent species (Table 4). Nevertheless it was smaller than the maximum pairwise nucleotide distance observed among North American brown bears. Thus, mitochondrial data alone is insufficient to evaluate the species status of the three identified cave bear lineages and additional nuclear DNA data would be necessary to test the multiple species hypothesis.

Aside from the overall phylogenetic position and divergence time of the Caucasus and Yana cave bears, the discovery of a cave bear fossil from the Yana River region with a sequence most closely related to — albeit still distinct from — the Caucasus bears, is quite remarkable. First, these regions are geographically situated about 6000 km apart. Second, the Yana River region lies beyond the Arctic Circle in an area from which no evidence of cave bears had previously been reported and much farther north than the most northerly record of cave bears to date (northern Urals, 62°N; see also A. V. Sher, personal communication). The cave bear populations from Hovk 1 (D), Ossenyaga (B) and Kudaro 3 (C.1, C.2), situated in the northern part of the southern Caucasus diverged from the Yana lineage (H) between 150 000 BP (60 000–323 000 BP) and 430 000 BP (22 000–973 000 BP) (Fig. 2, node 4) and were geographically separated from other Eurasian populations by the Greater Caucasus Range during glacial periods. While very distinct from other cave bear haplotypes, these specimens are genetically similar or even identical to one another. Based on our estimates, the Caucasus lineage diversified very recently, probably during the early Würm glaciation 68 000 BP (55 000–96 000 BP) — 95 000 BP (2000–238 000 BP) (node 6). Studies of mtDNA sequences obtained from house mouse subspecies (*Mus musculus*, Orth *et al*. 1996), wild goats (Manceau *et al*. 1999), and the white-breasted hedgehog (*Erinaceus concolor*, Seddon *et al*. 2002), show a similar differentiation between southern and northern Caucasian species and attest that the Greater Caucasus periodically acted as a biogeographic barrier. This provides additional evidence that the Caucasus acted as a refugium, which is also supported by the preservation of other Pleistocene relicts of large mammal fauna, e.g. rhinoceros species (Guerin & Barychnikov 1987).

Our results are consistent with previous studies (Rabeder 1995; Hofreiter *et al*. 2004a), which indicate that the European cave bears represent a monophyletic group with moderate to high support (60 to 85% bootstrap/puzzle support, posterior probability: 1; Table 5, node 3), and can

Table 4 Maximum pairwise nucleotide differences within and between different bear species based on a 201-bp fragment of the mitochondrial control region. The number of haplotypes on GenBank refers to the number of individual haplotypes characterized for the respective taxon. Duplicate haplotypes were not counted. The alignments are available on request

	No. of haplotypes on GenBank	Maximum pairwise sequence divergence
All brown and polar bears	75	25
North American brown and polar bears	50	25
North American brown bears	46	25
Eurasian brown bears	25	20
Black bears	17	15
Cave bears	25	24
Polar bears	4	4
Polar bears vs. brown bears	75	23

Table 5 Support values and posterior probabilities for the individual branches of the cave bear phylogeny (Fig. 2) based on a 227-bp control region alignment (Table 2). Support values below 50% are given in parentheses. n.a.*[1], branch not resolved using this method

	Bayesian	MP	NJ	ML	ML	ML
Tree-building method	(MrBayes)	(PAUP)	(PAUP)	(PAUP)	(IQPNNI)	(Tree Puzzle)
Node no.	Posterior probability, bootstrap or puzzle support for the various tree-building methods					
1	1.00	99	99	97	98	95
2	0.85	(40)	53	(32)	n.a.*[1]	(47)
3	1.00	70	85	60	73	76
4	1.00	55	61	64	63	89
5	0.98	(46)	(38)	(42)	(49)	54
6	1.00	99	100	98	100	60

Table 6 Divergence time (TMRCA, time to the most recent common ancestor) estimates for important events with regard to cave bear evolution and phylogeography. Estimates are based on the assumption of a relaxed log-normal distribution of substitution rates. Node no., node number as indicated in Fig. 2. ESS, effective sample size. TMRCA values, internal calibration/external calibration. 'Mean' is the mean age of the respective node, '95% lower' and '95% upper' refer to the lower and upper bounds of the 95% highest posterior density intervals

		TMRCA in thousand years ago		
Node no.	ESS	95% lower	Mean	95% upper
2	3014/1460	109/234	274/814	510/1378
3	3073/1372	88/73	173/414	310/809
4	3119/1900	60/22	150/430	323/973
5	2627/1502	59/7	88/144	130/328
6	4111/2427	55/2	68/95	96/238

be divided into a predominantly Eastern and Central European *U. ingressus* lineage and a predominantly Western European *U. spelaeus* lineage. Both lineages diverged between 173 000 BP (88 000–310 000 BP) and 414 000 BP (73 000–809 000 BP). The lower (internally calibrated) estimate is consistent with estimates (based on internal calibrations) for the divergence of Eastern and Western European brown bears (Saarma *et al*. 2007). It therefore suggests that both events might have been influenced by common environmental factors. However, the uncertainties and wide error ranges associated with these time estimates hinder the ability to correlate them unambiguously with palaeoclimatic events and other influences (Ho *et al*. 2008).

Ursus ingressus-like haplotypes were identified from as far east as the Urals (sample E) and the Ukraine (sample A). Haplotypes from these eastern populations fell within the diversity of Middle/Eastern European *U. ingressus* haplotypes. Interestingly, recent morphometric studies on teeth associated the Ukrainian cave bear with the cave bear subspecies *U. spelaeus odessanus* (von Nordmann 1858), raising the question of whether *U. ingressus* could be a junior synonym for this subspecies (Baryshnikov 2006, 2007). Future genetic and morphological studies on the type specimens for the respective groups are required in order to address this question.

In contrast to the distinct *U. deningeri kudarensis* samples from the Caucasus and Yana River regions, the two Asian

cave bears from the Altai Mountains (G and F) revealed haplotypes assigned to the western *U. spelaeus* lineage with a centre of distribution more than 7000 km west of the Altai. These results are consistent with the long-distance relationship observed in the *U. deningeri kudarensis* lineage, although Western European and Altai populations might have diverged even more recently, between 88 000 BP (55 000–142 000 BP) and 144 000 (7000–328 000 BP; node 5).

Cave bear ecology

The expansion of the cave bear distribution range to include several different climate zones even beyond the Arctic Circle also changes the previously accepted concept of cave bear ecology. Cave bears were clearly adapted to a much wider range of habitats than previously assumed. In addition, a recent study on a Romanian cave bear population revealed isotopic evidence for omnivory or possibly even carnivory. This finding is in contrast to all previous reports claiming that cave bears were strictly herbivorous (Richards *et al*. 2008). It suggests that cave bears might not only have been adapted to a wide range of environments, but have also occupied different ecological niches. Similar observations have recently been made for extant species. For example, a Pleistocene wolf population in Beringia seems to have been specifically adapted to prey on the now extinct megafauna (Leonard *et al*. 2007). Interestingly, the mitochondrial haplotypes carried by this wolf population have not been observed in extant wolves, indicating that it became extinct together with its prey. If this loss of ecological adaptations at the end of the Pleistocene and in the Holocene is a common pattern in large mammalian fauna, it appears likely that the ecological adaptability of mammalian species is substantially underestimated when only extant populations are studied. Our results, combined with several other recent studies, argue against the theory that climate changes alone drove cave bears and other highly adaptive species to extinction. Other causes, including human hunting, should be considered in order to understand their extinction (Barnosky *et al*. 2004).

Late Pleistocene and Holocene megafaunal diversity and extinction

While it is difficult to compare extinct and extant species, more and more data indicate that surviving species suffered heavy losses in genetic diversity at the end of the Pleistocene (Shapiro *et al*. 2004; Rohland *et al*. 2005; Chan *et al*. 2006; Leonard *et al*. 2007; Valdiosera *et al*. 2007, 2008; Calvignac *et al*. 2008). Extinct species, on the other hand, show substantial amounts of genetic diversity (Orlando *et al*. 2002; Weinstock *et al*. 2005; Barnes *et al*. 2007; this study). Thus, the loss of genetic diversity at the end of the Pleistocene was quite considerable, certainly for extinct species, but also for the surviving ones (Hofreiter 2007). Whether this is typical for Pleistocene cycles, as proposed for the steppe bison (Shapiro *et al*. 2004), or a unique event at the end of the last glacial cycle, is currently impossible to determine given the problems associated with molecular dating (Ho *et al*. 2005). Our results also emphasize the importance of studying a wide range of palaeontological specimens of a given species, especially those with unclear species affiliation outside the normal geographic range. Our report of genetically different specimens occurring well beyond the previously known geographic range of cave bears (the Yana River specimen, sample H), is in line with a recent study on Eurasian Neanderthals which further extended the previously known geographic distribution of this species (Teshik Tash in Uzbekistan) as far east as Okladnikow in the Altai Mountains (Krause *et al*. 2007). Similarly, a study of cave hyenas by Rohland *et al*. (2005) demonstrated that the most divergent haplotype originated from a single specimen found on the Pacific coast, while a recent study of mammoth biogeography illustrated that the most divergent haplotype observed so far originated from a single European specimen that yielded DNA sequences (Barnes *et al*. 2007). Moreover, our results demonstrate how easily our understanding of the ecology and geographic distribution of a species may be substantially changed by the discovery of a single specimen, as recently exemplified by the appearance of a unique Late Pleistocene *Homotherium* specimen from the North Sea, whereas all other European specimens of this genus date to the Middle Pleistocene (Reumer *et al*. 2003).

Conclusion

The results presented here alter our understanding of cave bear biogeography, evolution and ecology, and contribute to our understanding of Late Pleistocene megafaunal diversity and extinction. Our data show that the geographic range of cave bears extended well beyond Europe and included large parts of Northern Asia (Fig. 1). This extensive geographic range indicates that cave bears were adapted to a variety of habitats, and their populations probably occupied a diverse range of ecological niches. This finding is mirrored in several other recent studies that found that Pleistocene populations of both extinct and extant species display both large geographical and broad ecological ranges alongside with extensive genetic diversity. What was recognized as a single species of cave bears has been shown to consist of at least three widespread, morphologically and genetically distinct lineages. These lineages might represent three or even more distinct species with partly overlapping distributions, all of which became extinct at the end of the Pleistocene. Finally, our data show that before their extinction, cave bear evolutionary history involved a rather complex phylogeographic pattern and a

more substantial loss of biological diversity at the end of the Pleistocene than has been previously recognized.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Likelihood mapping plot analysing the branching order of the four groups *Ursus americanus* (as outgroup), *U. arctos* (with *U. maritimus*), *U. spelaeus sensu lato* (s.l.) (comprising *U. spelaeus* s.s. and *U. ingressus*), and *U. deningeri kudarensis*.

Table S1 Primers used in this study

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