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Mitogenetic structure of brown bears (*Ursus arctos* L.) in northeastern Europe and a new time frame for the formation of European brown bear lineages

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Abstract

We estimated the phylogenetic relationships of brown bear maternal haplotypes from countries of northeastern Europe (Estonia, Finland and European Russia), using sequences of mitochondrial DNA (mtDNA) control region of 231 bears. Twenty-five mtDNA haplotypes were identified. The brown bear population in northeastern Europe can be divided into three haplogroups: one with bears from all three countries, one with bears from Finland and Russia, and the third composed almost exclusively of bears from European Russia. Four haplotypes from Finland and European Russia matched exactly with haplotypes from Slovakia, suggesting the significance of the current territory of Slovakia in ancient demographic processes of brown bears. Based on the results of this study and those from the recent literature, we hypothesize that the West Carpathian Mountains have served either as one of the northernmost refuge areas or as an important movement corridor for brown bears of the Eastern lineage towards northern Europe during or after the last ice age. Bayesian analyses were performed to investigate the temporal framework of brown bear lineages in Europe. The molecular clock was calibrated using Beringian brown bear sequences derived from radiocarbon-dated ancient samples, and the estimated mutation rate was 29.8% (13.3%–47.6%) per million years. The whole European population and Western and Eastern lineages formed about 175 000, 70 000 and 25 000 years before present, respectively. Our approach to estimating the time frame of brown bear evolution demonstrates the importance of using an appropriate mutation rate, and this has implications for other studies of Pleistocene populations.

Keywords: Bayesian, ice age, molecular clock, phylogeography, refuge, TMRCA

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Introduction

Brown bear populations in northeastern Europe are currently among the largest and most viable in the continent, although in some places, such as Fenno-Scandia and the Baltic States, they passed through a severe bottleneck during the last two centuries (Swenson *et al*. 1994; Valdmann *et al*. 2001; Kojola *et al*. 2003). Timely management efforts have allowed populations to increase in number and expand. Viability of bear populations in Finland and Estonia was partially restored by immigrating bears from European Russia (Pulliainen 1990; Valdmann *et al*. 2001). However, brown bear populations in Norway, Latvia and Lithuania are still in the bottleneck phase (Zedrosser *et al*. 2001). The northeastern bear population in Europe is largely continuous, totalling about 40 000 bears. The majority of these bears inhabit the European part of Russia (> 35 000 bears), while populations of other countries are considerably smaller: Finland has 800–850 bears (Kojola & Määttä 2004), Estonia 250–500 (Valdmann *et al*. 2001) and Sweden about 2200 (J. Swenson, personal communication). There are also 20–40 bears in Latvia, none in Lithuania (only occasional wanderers from Belarus), about 250 individuals in Belarus and 8–21 in Norway (Zedrosser *et al*. 2001).

Climate fluctuations during the past three million years have been shaping the distribution and population structure of animal populations in the Northern Hemisphere (Taberlet *et al*. 1998; Hewitt 1999; Jaarola *et al*. 1999). Glaciations in the Pleistocene restricted most populations to refuge areas. At the end of the last glacial maximum (LGM), approximately 18 000 years before present (BP), many species began to expand their ranges in a northerly direction. The process of recolonization of northern Europe has been somewhat different between species, depending on factors such as population density in the refuge area, food resources and dispersal capability. Molecular techniques have substantially broadened possibilities for a deeper understanding of phylogeographical events of animal populations, especially those that took place at the Pleistocene–Holocene boundary and during the Holocene, as repeated cycles of restriction to refugia during glacial periods and outward expansion during interglacials have left distinctive marks on both mitochondrial and nuclear genomes of animals. The brown bear has been one of the model species for investigating locations of refuge areas of large mammals and their migrations from glacial refugia to current ranges (Taberlet *et al*. 1998; Hewitt 1999, 2000). In Europe, a remarkable degree of concordance was found between the geographical distribution of brown bears and their mtDNA haplotypes. Sequences of the mtDNA control region divide the European population of *U. arctos* into two major lineages, Eastern and Western, with the latter organized into two clades. The Eastern lineage is composed primarily of large and robust populations and the Western lineage of small, fragmented and threatened populations. The Eastern lineage includes bear populations from central, northern and eastern areas of Europe (Romania, Slovakia, Finland, northern Sweden, Estonia and Russia), while the Western lineage is represented by bears from central, southern and western Europe (Slovenia, Romania, Croatia, Italy, France, Greece and Bulgaria). Both lineages are present in Romania and Sweden (Taberlet & Bouvet 1994; Kohn *et al*. 1995). The western and eastern bears appear to have colonized most of Europe from Iberian and from Caucasian/Carpathian refuges, respectively (Taberlet *et al*. 1998; Hewitt 2000). These expansions met in central Sweden, after the melting of the Scandinavian ice-cap, and formed a hybrid zone (Taberlet *et al*. 1995). As stated by Hewitt (2000), however, the geographical origin of the eastern expansion remains unclear. Despite the brown bear being one of the model species for investigating Pleistocene and Holocene migrations, knowledge about the structure and history of the Eastern lineage is rather limited.

Accurate estimation of the timing of evolutionary events can be important in understanding the factors influencing Pleistocene population dynamics (Shapiro *et al*. 2004). This can be a difficult exercise, however, because a reliable calibration point is needed to translate genetic measurements onto an absolute time scale. In the past, studies of Pleistocene populations often used substitution rates obtained from comparisons between species (e.g. Menotti-Raymond & O'Brien 1993; Eizirik *et al*. 2001). The validity of this practice has recently been challenged on the grounds that observed mutation rates within populations tend to be much higher than the substitution rates measured between species (Ho *et al*. 2005; Ho & Larson 2006). Accordingly, it is more appropriate to obtain a mutation rate exclusively from individuals within the species of interest (or a closely related species), which can be done in a straightforward manner using dated ancient sequences (Drummond *et al*. 2002). If this is indeed the case, then many published date estimates for evolutionary events in the Pleistocene are actually overestimates (i.e. too old).

In this study, we examine the phylogenetic relationships of brown bears from northeastern Europe and investigate the postglacial phylogeography of the Eastern lineage. Using Bayesian analysis with a coalescent population model, we analyse the temporal pattern of formation of different brown bear lineages in Europe and estimate effective population sizes.

Materials and methods

Samples

Muscle, liver and hair samples of 231 brown bears were collected during 1996–2004. These samples cover large brown bear populations from Finland to the Ural Mountains, and include 43 samples from Estonia (from throughout the bear range in this country), 72 from Finland (from throughout

Table 1 Mitochondrial haplotypes (388 bp) and number of samples from Finland, Estonia and European Russia

Haplotype	GenBank accession	Finland*	Estonia	Russia	Total
1	DQ328803	—	—	8	8
2	AY331262	3/2	—	3	8
3	AY331263	1/—	—	—	1
4	AY331264	—/7	—	—	7
5	AY331265	23/27	—	20	70
6	AY331266	1/—	—	—	1
7	AY331267	—/1	—	28	29
8	AY331268	—	—	4	4
9	AY331269	2/—	—	—	2
10	AY331270	39/87	4	38	168
11	AY331271	1/3	—	3	7
12	AY331272	1/1	—	—	2
13	AY331273	—	34	—	34
14	AY331274	—	4	—	4
15	AY331275	—/1	—	—	1
16	AY331276	—/1	—	—	1
17	AY331277	—/1	—	—	1
18	AY331278	—/1	—	—	1
19	AY331279	—/1	—	—	1
20	AY331280	—/1	—	—	1
21	AY331281	1/1	—	—	2
22	DQ328804	—	1	1	2
23	DQ328805	—	—	1	1
24	DQ328814	—	—	1	1
25	DQ328806	—	—	1	1
26	DQ328807	—	—	1	1
27	DQ328808	—	—	1	1
28	DQ328809	—	—	2	2
29	DQ328810	—	—	1	1
30	DQ328811	—	—	1	1
31	DQ328812	—	—	1	1
32	DQ328813	—	—	1	1
		72/135	43	116	366

*this study/samples included from (Saarma & Kojola submitted).

the bear range) and 116 from nine oblasts of European Russia: Pskov, Novgorod, Leningrad, Tver, Arkhangelsk, Vologodsk, Kirov, Perm and Sverdlovsk, and from Komi Republic, Russia. Sample sizes and haplotype codes are given in Table 1 (haplotype table with their exact locations can be obtained from the authors by request).

Phylogenetic analysis

Total genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN) or the High Pure PCR Template Preparation Kit (Roche), following the manufacturer's protocol. A 631 bp fragment of the mitochondrial genome (covering the 3′ end of the cytochrome b sequence, pos. 16311–16445; tRNA-Thr, pos. 16446–16515; tRNA-Pro, pos. 16516–16580; and the 5′ portion of the control region, pos. 16581–16942; positions labelled in accordance with the mitochondrial genome sequence of a brown bear with GenBank accession number AF303110) was PCR-amplified with primers L15774 and H16498 (Shields & Kocher 1991). L15774 primer binds to mtDNA sequence 16311–16333 and H16498–16923–16942. 20–80 ng of purified genomic DNA and 4 pmol of primers were used for PCR (polymerase chain reaction). PCR was performed in a total volume of 20 μL. Cycling parameters were 5 min denaturing step at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C with 1 U Platinum® Taq DNA polymerase (Invitrogen), 0.2 mM dNTP and 1.5 mM $MgCl_2$. The PCR product was purified with shrimp alkaline phosphatase/exonuclease I treatment. One unit of each enzyme was added to 10 μL of PCR reaction and incubated for 30 min at 37 °C, followed by a 15 min inactivation at 80 °C.

DNA cycle sequencing was performed using the DYEnamic™ ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). Thirty-three cycles (15 s at 95 °C, 15 s at 50 °C and 60 s at 60 °C) were performed in a total volume of 10 μL. Sequences were resolved on an ABI PRISM® 377 automated DNA sequencer (Applied Biosystems). Both strands were sequenced using primers L15774 or H16498. Consensus sequences were created using the program CONSED (Gordon *et al*. 1998).

Sequences were aligned using CLUSTAL W (Thompson *et al*. 1994), and manually checked in the BIOEDIT sequence editing program (Hall 1999). For further analyses, sequences of 388 bp in length were used (corresponding to pos. 16407–16789 of AF30311, covering the 3′ end of the cytochrome b sequence, pos. 16407–16445; tRNA-Thr, pos. 16446–16515; tRNA-Pro, pos. 16516–16580; and 5′ portion of the control region, pos. 16581–16789). All mtDNA haplotypes were submitted to GenBank (Table 1). In addition, 135 sequences of brown bears (388 bp) of Finland were included from a previous study (Saarma & Kojola, submitted).

To evaluate the phylogenetic position of northern and eastern brown bears in the context of other European bear populations from both the Eastern and Western lineages, 15 mtDNA control region haplotypes of the Eastern and 18 haplotypes of the Western lineages were obtained from GenBank. Due to the shorter length of sequences from GenBank, this analysis is based on a 206 bp fragment of the mtDNA control region (covering 5′ portion of the mtDNA control region, pos. 16589–16789). Names of haplotypes and haplogroups derived from shorter (206 bp) sequences are shown with an inverted comma (′). Networks were calculated using a median-joining approach with default settings in the program NETWORK 4.111 (Bandelt *et al*. 1999).

Demographic analysis

Hypotheses of demographic expansion were tested using Fu and Li's *F* and Tajima's *D* statistics, and a mismatch

Table 2 Genetic polymorphism and hypotheses of demographic expansion for different subsets of the European brown bear population. H(n), number of haplotypes; I(n), number of individuals; *Hd*, haplotype diversity; π, nucleotide diversity; SD, standard deviation; *, without the pyrimidine tract (pos. 16663–16678 in AF303110)

Population subset	H(n)	I(n)	*Hd* ± SD	π ± SD	Fu and Li's *F*	Tajima's *D*
Whole population	32	366	0.74 ± 0.02	0.0049 ± 0.0002	–3.46 ($P < 0.02$)	–1.66 ($P > 0.05$)
Whole population*	22	366	0.35 ± 0.003	0.0011 ± 0.001	–4.22 ($P < 0.02$)	–2.29 ($P < 0.01$)
Haplogroup 5	11	100	0.50 ± 0.06	0.0021 ± 0.0003	–2.81 ($P < 0.05$)	–1.48 ($P > 0.1$)
Haplogroup 7	6	37	0.38 ± 0.10	0.0017 ± 0.0003	–2.65 ($P < 0.05$)	–1.69 ($P > 0.05$)
Haplogroup 10	15	229	0.44 ± 0.04	0.0015 ± 0.0002	–2.72 ($P < 0.05$)	–2.05 ($P < 0.05$)

distribution analysis was performed. Five alignments of brown bear haplotypes (Table 2) were statistically analysed using the DNASP 4.10.4 software package (Rozas *et al*. 2003).

Bayesian estimation of time to the most recent common ancestor and effective population size

Bayesian analyses were performed using the computer program BEAST version 1.3 (Drummond & Rambaut 2005) to investigate the history of brown bear lineages in Europe. The time to the most recent common ancestor (TMRCA) of the European population was estimated, along with the TMRCAs of the Eastern and Western lineages. In addition to 29 haplotypes (435 individuals) of the Eastern lineage, 18 haplotypes (107 individuals) of the Western lineage were included from GenBank. All analyses were performed using the HKY model of nucleotide substitution (Hasegawa *et al*. 1985). Rate variation among sites was modelled using a gamma distribution with six rate categories. A coalescent model with constant population size was used to estimate the effective population size in each clade of brown bears. Posterior distributions of parameters were approximated using two independent Markov chain Monte Carlo (MCMC) analyses of 20 000 000 steps each, following a discarded burn-in of 2 000 000 steps. Samples from the two chains, which yielded similar results, were combined. Convergence of the chains was checked using the program TRACER (Rambaut & Drummond 2004) and the effective sample size for each parameter was found to exceed 100, which suggests acceptable mixing and sufficient sampling (A. J. Drummond, personal communication).

To estimate TMRCAs, it is necessary to supply at least one calibration point or to specify an appropriate mutation rate for the analysis (Drummond *et al*. 2006). It is important to employ a suitable calibration that does not require excessive extrapolation of rates across different time scales (Ho *et al*. 2005; Penny 2005; Ho & Larson 2006). Consequently, in the analysis of population-level sequences of brown bears, it is not valid to use a substitution rate calibrated by divergences between species, or to extrapolate an ursid-wide substitution rate onto the intraspecific sequences. A mutation rate could not be directly estimated from the European brown bear sequences due to lack of a sound calibration point. Instead, mutation rates were estimated from an alignment of 35 radiocarbon-dated Beringian brown bear sequences obtained by Barnes *et al*. (2002), and from an alignment of 26 radiocarbon-dated cave bear sequences from Loreille *et al*. (2001), Hofreiter *et al*. (2002) and Orlando *et al*. 2001). All rates were estimated using BEAST (Drummond & Rambaut 2005), with 10 000 000 MCMC steps after a discarded burn-in of 1 000 000 steps. Using this rate information, a normal prior was placed on the mutation rate for the analysis of the European brown bear sequences.

Results

Phylogenetic analysis

The length of sequences varied from 382 to 388 bp (variation is attributable to indels at the pyrimidine tract, pos. 16663–16678). A total of 117 mutations were recorded at 30 positions, including seven parsimony-informative and 23 singleton sites. Among 231 brown bears, 25 mtDNA haplotypes were identified: nine from Finland, four from Estonia and 18 from European Russia (Table 1, Fig. 1). Finland shares five haplotypes with Russia and one with Estonia, while Estonia and Russia have two haplotypes in common. The majority of shared haplotypes are also the most abundant ones: haplotype 10 is the only one that Estonia, Finland and Russia all share, and it is by far the most numerous; haplotype 5, which is well-represented in Finland and Russia, is also numerous (Table 1). Altogether, 12 haplotypes were unique in Finland (most of them were recorded along the eastern border), two in Estonia and 12 in European Russia.

A mitochondrial DNA network was constructed from a 388 bp fragment of mtDNA of all 366 bears from Estonia, Finland and European Russia (Fig. 2). The brown bear population in northeastern Europe can be divided into three haplogroups, HG5, HG7 and HG10, named after the most abundant haplotypes that represent the respective cores of these haplogroups (Fig. 2). Haplogroup 10 is the largest, being composed of 14 haplotypes (229 individuals, 62.6% of all sequences), and is distributed throughout the territory under investigation. Within HG10, haplotype 10

Fig. 1 Schematic map of northeastern Europe (Finland, Estonia and European Russia), illustrating localities of different mitochondrial haplotypes of brown bears. For haplotype identity, see Table 1. Larger numbers in bold denote haplotypes that were found in more than one geographical entity.

is the most numerous (45.9%) and is distributed over a vast part of the bear range in Finland and European Russia. In Estonia, however, this haplotype is comparatively rare. Haplotypes 13 and 14 are characteristic only to Estonia, while haplotype 22 was found in Estonia and the neighbouring Leningrad oblast of Russia. Haplotype 13 is dominant (86.2% of bears from Estonia) and distributed all over the mainland of Estonia. Most of the haplotypes in HG10 were rare, represented often by a single individual (Table 1). Haplogroup 5 is also abundant (11 haplotypes, 100 individuals, 27.3% of all sequences) and widespread, but not as much as HG10. In contrast to HG10, it does not include brown bears from Estonia. Within HG5, haplotype 5 is the most frequent (70 individuals, 19.1%), with the rest of the haplotypes represented by single or few specimens. Both haplotype and nucleotide diversity are highest for HG5 (Table 2). Haplogroup 7 is the smallest (six haplotypes, 37 individuals and 10.1% of all sequences). It is almost entirely composed of bears from European Russia; only a single individual was from Finland and none from Estonia. The major component of HG7 is haplotype 7 (28 individuals, 7.9% of all sequences), and other haplotypes were rare, often represented by only a single individual (Table 1, Fig. 2).

Evaluation of the phylogenetic position of brown bears from northeastern Europe (24 haplotypes, 366 individuals) in the context of other European bear populations from both the Eastern (15 haplotypes, 69 individuals) and Western lineages (18 haplotypes, 107 individuals) has yielded a network with clear separation of these two lineages (data not shown). The network of bears from the Eastern lineage alone (29 haplotypes, 435 individuals) was composed of three haplogroups (Fig. 3a). Due to the shorter alignment used in this analysis, some haplotypes that were previously distinct in Fig. 2 are no longer distinguishable in this network. The core of all three haplogroups is formed by bears from Finland and Russia and the core of two haplogroups includes bears from Slovakia. Haplotypes from Romania are included in all haplogroups, but not in the core. Brown bears

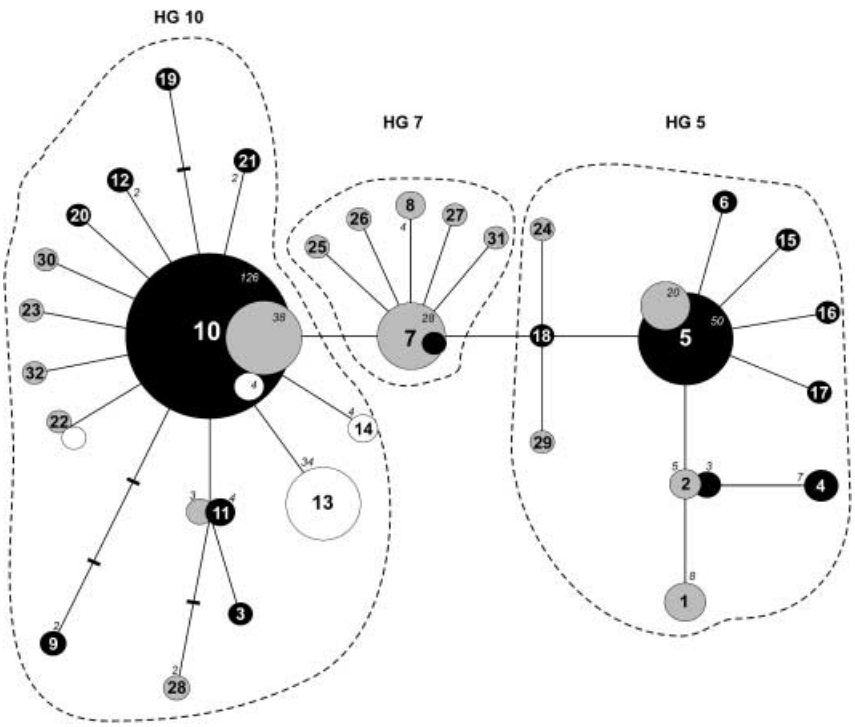

Fig. 2 Network showing the relationships among brown bear mtDNA haplotypes in northeastern Europe based on partial (388 bp) sequence of mtDNA, primarily the control region. For haplotype identity, see Table 1. Small letters denote number of animals analysed. HG — haplogroup. Haplotypes: Finland (black), Russia (grey), Estonia (white).

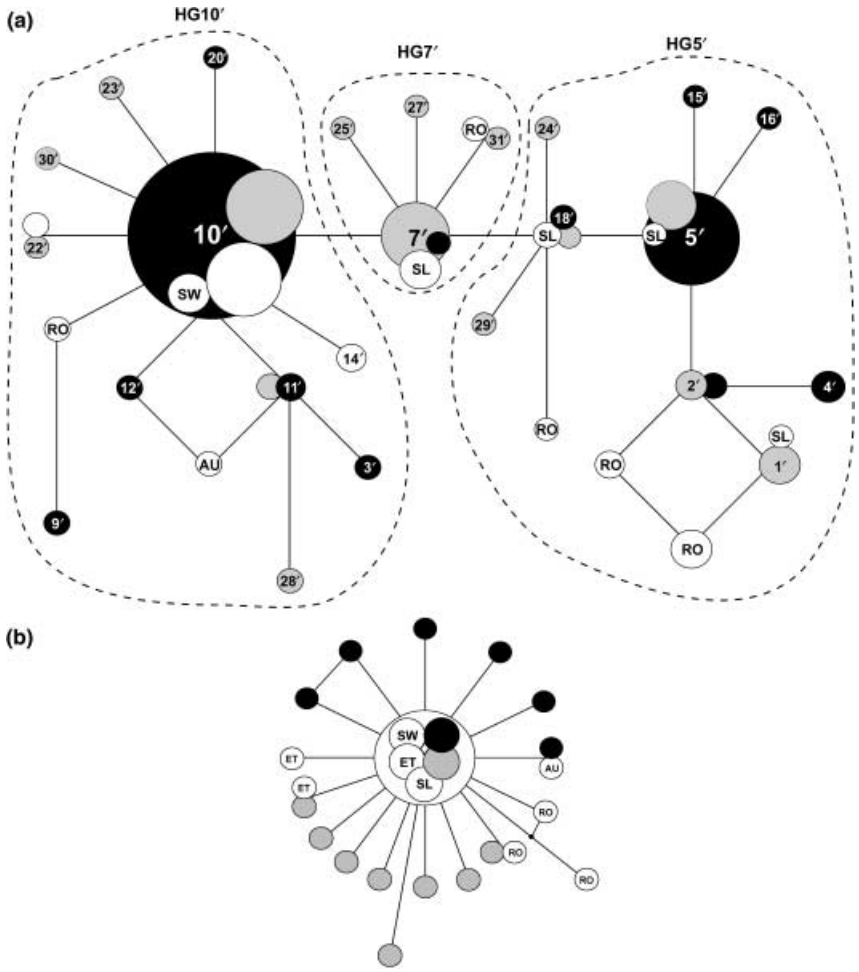

Fig. 3 (a) Network of brown bear mtDNA haplotypes belonging to the Eastern lineage: Estonia, Finland, European Russia, northern Sweden, Slovakia and Romania. Network is based on analysis of partial (206 bp) sequence of mtDNA control region. Names of haplotypes and — groups derived from 206 bp sequences are shown with an inverted comma (′). Haplotypes: Finland (black), Russia (grey), Estonia (white), Slovakia (SL), Romania (RO), Sweden (SW) and Austria (AU). (b) Network of brown bear mtDNA haplotypes belonging to the Eastern lineage, constructed without the pyrimidine tract. Haplotypes: Finland (black), Russia (grey); Estonia (ET), Slovakia (SL), Romania (RO), Sweden (SW) and Austria (AU).

from Finland, Estonia, European Russia and northern Sweden belong to the largest haplogroup 10′. The analysis also includes an ancient (~47 420 years old) representative from Austria that has been shown to belong to the Eastern lineage (Hofreiter *et al.* 2004). In the network, the ancient bear from Austria also belongs to HG10′. Four haplotypes in Finland and western European Russia are identical to those from Slovakia and a single individual from western Russia to a haplotype from Romania.

Demographic analysis

Hypotheses of demographic expansion were tested using Tajima's *D* and Fu and Li's *F* statistics (Table 2). Both tests yielded results that were consistent with population expansion of HG10 and for the whole population (without the pyrimidine tract). For HG5 and HG7, and for the whole population (with the pyrimidine tract), only Fu and Li's *F* achieved statistical significance. The unimodal distribution of pairwise differences among haplotypes (in the whole population and in different haplogroups) and nonsignificant raggedness index, obtained from mismatch distribution analysis (data not shown), are consistent with a model of sudden expansion (Harpending 1994).

Bayesian estimation of time to the most recent common ancestor and effective population size

The mutation rates estimated from Beringian brown bears and cave bears were, respectively, 29.8% per million years (Myr), with a 95% highest posterior density (HPD) of 13.3%–47.6% per Myr, and 26.2% per Myr (95% HPD 9.86%–53.2% per Myr). This suggests that the intraspecific mutation rates are similar for cave bears and brown bears, which are closely related species. In view of these results, the rate obtained from the Beringian brown bears was used in further analyses of the European brown bear sequences, in the form of a normal prior on the mutation rate (mean 29.8%, standard deviation 10.8%).

Bayesian analysis using MCMC integration was used to estimate TMRCA and effective population size (N_e) values for the Eastern and Western lineages and for the whole European population of brown bears. The most recent common ancestor (MRCA) of all bears included in this

Table 3 Results of Bayesian analyses: effective population size, N_e, and time to the most recent common ancestor (TMRCA, years) for three subsets of brown bear population in Europe

	95% highest posterior density		
	Mean	Lower	Upper
Population size			
All European	78 230	26 320	137 500
Eastern Lineage	26 770	9 218	49 430
Western Lineage	45 050	13 420	88 070
TMRCA			
All European	174 400	60 790	313 800
Eastern Lineage	24 420	5 790	49 500
Western Lineage	67 370	19 960	130 900

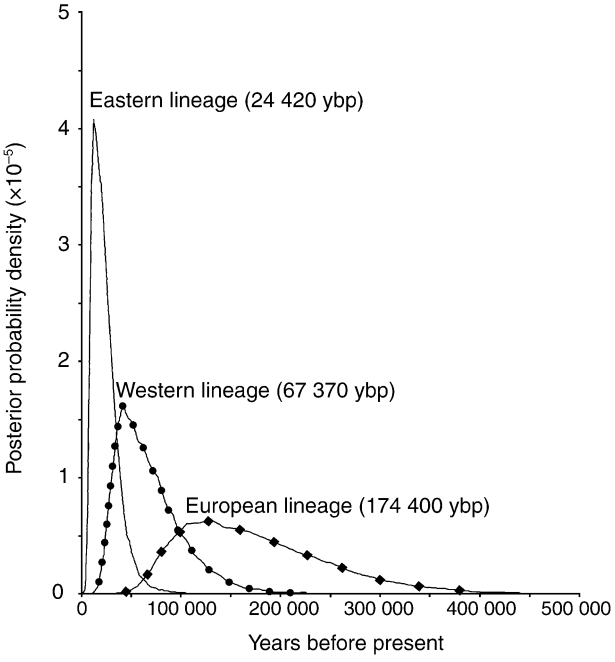

Fig. 4 Bayesian posterior probability densities of the TMRCA analysis for different brown bear lineages in Europe.

analysis lived around 175 000 years ago (95% HPD: 60 790–313 800 BP), while the European Western lineage was established approximately 70 000 BP (19 960–130 900 BP) and the Eastern lineage 25 000 BP (5790–49 500 BP) (Table 3; Fig. 4). Effective population sizes were estimated at 80 000 for the whole European population, 45 000 for the Western lineage, and 30 000 for the Eastern lineage (Table 3).

Discussion

Three haplogroups can be distinguished in the mtDNA network of northeastern brown bears: HG5, which comprises Finnish and Russian bears; HG7, comprising Russian bears; and HG10, which includes bears from Finland, Russia and Estonia (Fig. 2). Haplogroup 7 is formed almost exclusively by brown bears from Russia; the only Finnish bear that carried haplotype 7 was from northern Lapland, remote from all other bears analysed. The star-like mtDNA phylogeny with central and most abundant haplotypes 5, 7 and 10 suggests that these haplotypes are the ancestors of all analysed brown bear haplotypes in northeastern Europe. Haplotype 10 is by far the most numerous in Finland (60.9% of all Finnish bears) and is distributed over the entire bear range in the country, suggesting that it is a significant founder haplotype for the current Finnish brown bear population, having survived a dramatic bottleneck at the beginning of the last century. The largest portion of Russian bears also belongs to this haplotype (32.8% of all Russian bears). The Russian bear population may never have gone through a demographic bottleneck and so has a more even distribution of different haplotypes compared to Finland. Only four individuals analysed from Estonia carried haplotype 10 (9.3% of all Estonian bears); instead, Estonian bears tended to carry haplotype 13 (79.1% of all Estonian bears). Haplotypes 13 and 14 (9.3% of all Estonian bears) were unique to Estonia and are likely to be descendants of haplotype 10, being separated from it by a single mutation. These mutations were most probably introduced into the mitochondrial genome after the last ice age as the estimated mean value of the mutation rate for the 388 bp stretch of the mtDNA control region was one mutation per 8649 years. It is conceivable that bears with haplotype 10, so common in Finland and Russia today, were also numerous in Estonia after the ice age, but disappeared during a mitochondrial bottleneck at the beginning of the last century. This bottleneck is most likely responsible for the poor mitogenetic variability characteristic of the brown bear population in Estonia.

The bear populations of Finland and north-western Russia are continuous. The large and stable Russian bear population has been a reserve for its neighbouring countries and the Finnish population, for example, has received valuable additions during its recent decline (Pulliainen 1990). The fact that the bear population in Kareliya Republic (Russia) has not gone through a bottleneck in its recent history (Danilov *et al*. 1993), and that bears often cross the Finnish–Russian border (Pulliainen 1997; Kojola *et al*. 2003), explains why the Finnish population has retained relatively high matrilineal diversity compared, for example, to populations in Scandinavia and Estonia that have similar demographic histories with recent bottlenecks.

Haplotype 5 is also numerous in Finland (24.2% of all Finnish bears), and together with haplotype 10, form the basis of almost the whole population (85.1% of all Finnish bears). It was somewhat surprising that HG7 is composed almost entirely of Russian bears. Bears belonging to this haplogroup appear to be situated away from the Finnish border and only wandering males seem to reach Finland.

A founder event followed by demographic expansion would result in a unimodal distribution of pairwise differences between haplotypes. Populations that result from several colonization episodes generate a multimodal mismatch distribution due to the admixture of different lineages. This can be measured by the raggedness index (Harpending 1994). For brown bears of northern and eastern Europe, the star-like tree, absence of highly divergent lineages, statistically significant Fu and Li's *F*, unimodal mismatch distribution of haplotypes, and insignificant raggedness index support a model of sudden expansion.

The haplotypes represented in this study, together with those from GenBank, yielded a subdivided network in which the Western lineage was clearly distant from the Eastern one. This is in good agreement with previous findings (Randi *et al*. 1994; Taberlet & Bouvet 1994; Kohn *et al*. 1995). What was not so clear from previous analyses was the extent of the significance of the West Carpathian Mountains (Slovakia) in the history of the Eastern lineage. More extensive sampling, also including brown bears from the eastern part of European Russia, has provided additional information about the history of the Eastern lineage. We have identified four haplotypes from Finland and/or European Russia as being identical to haplotypes from Slovakia. Moreover, two perfectly matching haplotypes (5'- and 7') were found in Finland and over the studied area in European Russia (Fig. 5a), suggesting that one of the most significant recolonizations of northern Europe may have emanated from or passed through the West Carpathian Mountains. Furthermore, when the pyrimidine tract was excluded from the analysis, the phylogenetic network became very star-like, with all four haplotypes from Slovakia, together with haplotypes from Finland, Estonia, Russia and northern Sweden, forming a central haplotype from which all the other haplotypes have derived (Fig. 3b).

Based on these results, the following refuge/recolonization scenarios can be hypothesized:

1 West Carpathian Mountains as the northernmost refuge area for brown bears during the LGM

One of the major ice age refuges for brown bears, from which recolonization of northeastern Europe emanated, was located in the current territory of Slovakia, in the West Carpathian Mountains and perhaps also in the adjacent Great Hungarian Plain. The Carpathian mountain range is a reasonably good candidate as a refuge area for brown bears during the last ice age. At the LGM (23 000–18 000 BP) the European ice sheet extended south to 52°N and permafrost south to 47°N, leaving the West Carpathian Mountains at the forefront of habitable area, i.e. at the northernmost refuge. This interpretation is supported by recent studies indicating that the role of Quaternary refugia in Europe might be more complex in northern latitudes than previously thought. Increasing evidence suggests that the most extensively characterized southern European refugia for animal and plant taxa were supplemented by more northerly cryptic refugia in Europe during the Late Pleistocene. It has been proposed that cryptic refuge areas existed in Slovakia, northern Hungary, Belgium (Ardennes), southwestern Ireland, northwestern Scotland, the southern United Kingdom, and even the coastal region of Norway (Bilton *et al*. 1998; Stewart & Lister 2001; Jaarola & Searle 2002; Deffontaine *et al*. 2005). Bone remains of bank vole (*Clethrionomys glareolus*) dated to the period of the LGM have been found in the northern Polish Carpathians (Nadachowski *et al*. 2003). Moreover, the discovery of 18 000-year-old charcoal of yew (*Taxus baccata*) and Scots pine (*Pinus sylvestris*) in the Moravany region in western Slovakia (Litynska-Zajac 1995) implies that climatic conditions at the West Carpathian Mountains were most likely suitable for brown bears also. Based on evaluation of available brown bear data, it has been recently hypothesized that the Carpathian refuge can be considered the geographical origin of recolonization of northern Europe by brown bears of the Eastern lineage at the end of the last ice age (Sommer & Benecke 2005). The West Carpathian Mountain range is broken by numerous passes and these could have served as migratory corridors for brown bears. A territory with a low altitude between the West Carpathian Mountains and the eastern side of the Alps could also have functioned as a suitable corridor for recolonization. Our data, together with other similar findings, enhance the credibility of the West Carpathian refuge hypothesis.

2 Recolonization gradient in Carpathian Mountains

The recolonization was most effective from the West Carpathian Mountains and less effective from its eastern part, with a recolonization gradient declining eastwards (Fig. 5b). The East Carpathian Mountains (in the current territory of Romania) served also as a refuge area for brown bears during the last ice age. We have shown that Romanian haplotypes are members of all haplogroups, with one Romanian haplotype even being identical to a haplotype characterized from western Russia, and with the rest of the Romanian haplotypes being separated from the 'core' haplotype by just one or a few mutations (Fig. 3a, b). Nevertheless, compared to migration that began from the West Carpathian refuge, recolonization from the East Carpathian refuge appears to have been less effective (Fig. 5a, b). The recolonization gradient can be explained by a more northern position of the West Carpathian Mountains (leading edge) compared to its eastern range.

Kohn *et al*. (1995) have shown that the current Romanian population is a mix of haplotypes of Eastern and Western lineages. Considering that not a single western haplotype has been found in northeastern Europe, there had to be a forefront population in the Romanian part of the refuge,

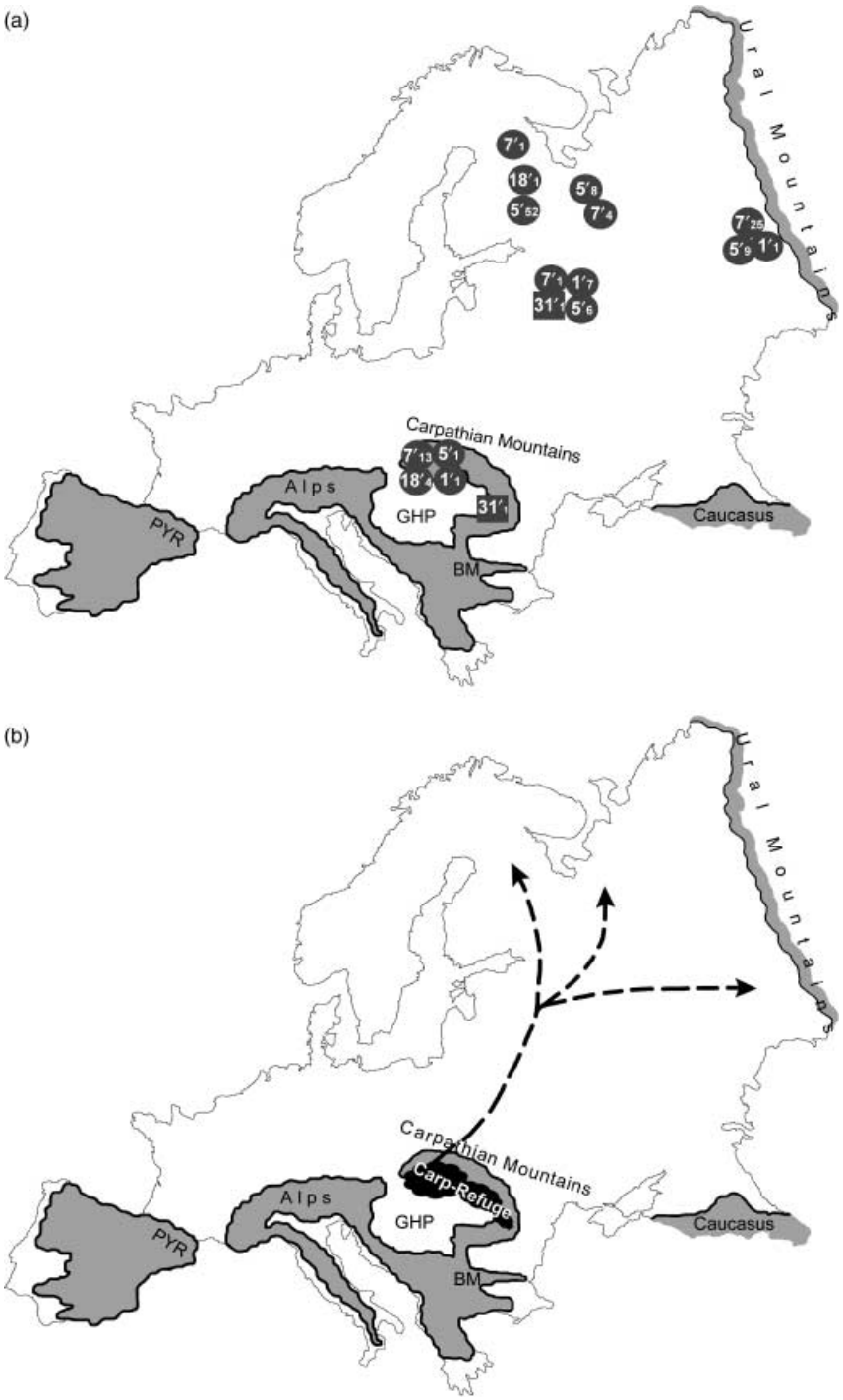

Fig. 5 (a) Schematic map of Europe representing localities of brown bear mtDNA haplotypes that coincide in Slovakia, Romania and in northeastern Europe. Major mountain areas are in grey. Great Hungarian Plain (GHP), Balkan Mountains (BM), Pyrenees (PYR). Large numbers in black circles designate haplotypes shared between Slovakia and northeastern Europe, and in black rectangles between Romania and northeastern Europe. Small numbers indicate the number of animals that have been analysed. (b) Schematic map of Europe showing the refuge area in Carpathian Mountains (black) and the direction (broken line) of recolonization. Major mountain areas in Europe are in grey. Carpathian Refuge (Carp-Ref) is coloured black area with a recolonization gradient declining eastwards; Great Hungarian Plain (GHP), Balkan Mountains (BM), Pyrenees (PYR).

formed entirely of eastern haplotypes (unless the migration of bears with western haplotypes into Romania occurred after the recolonizing bears had left).

The assumption that the ice age refuge was in the Balkans (Romania), instead of the West Carpathians (Slovakia), and that the migration went through Slovakia, requires that identical haplotypes are shared by both populations. However, not a single haplotype has been found that is shared between Slovakia and Romania (17 samples have been analysed from Slovakia and 15 from Romania), yet five haplotypes from Slovakia and Romania have been found identical to those of northeastern bears. Assuming that the migration wave still went through Slovakia, the prime suspect for a refuge is the Great Hungarian Plain.

However, there might be other refuge area(s) for northeastern brown bears as haplotype 10′ (the most abundant one

in northeastern Europe) was found neither in Slovakia nor in Romania. Territories of Hungary, Ukraine and Moldova are also likely candidates for refuge areas and remain to be investigated. On the other hand, haplotype 10′ appeared to be the major founder haplotype in Finland after the last bottleneck, explaining the abundance of haplotype 10′ in this country. In European Russia, where no bottleneck has been detected, haplotype 10′ is distributed almost evenly compared to other major haplotypes (Fig. 2), but its origin is still to be determined. Haplotype 10′ seems to belong to the youngest haplogroup. When brown bear genotypes were rooted with cave bear homologous sequences, the most ancient was the haplogroup 5′ (followed by HG7′, with HG10′ the most distant to cave bear), though it was not possible to determine the most ancient haplotype unequivocally as the closest haplotypes 5′, 6′ and 17′ (presumably haplotype 5′ is the most ancient as it is central in this haplogroup) were connected with cave bears through several median vectors (i.e. extinct or unsampled haplotypes; data not shown).

It appears that bears with haplotype 7′ did migrate primarily eastwards after the last ice age, as their concentration is highest near the Ural Mountains (Fig. 5a), with haplotype 1′ tending to follow the same pattern. Haplotype 5′ has a more even distribution and its high concentration in Finland can be explained by a founder effect.

The genetic studies of brown bear phylogeography in North America suggest invasion of bears to North America from a Eurasian population (Talbot & Shields 1996; Waits *et al*. 1998; Leonard *et al*. 2000; Barnes *et al*. 2002). Moreover, Leonard *et al*. (2000) demonstrated that brown bears of eastern Europe form a common clade with bears from Alaska and Siberia, suggesting that the invasion of North America from Eurasia was relatively recent. Their finding raises the following hypotheses: first, the Carpathian Mountains was just one of several refuge areas for brown bears that currently inhabit large parts of Europe; or secondly, repopulation after the last ice age emanated from a refuge that was situated in eastern Eurasia, presumably in Siberia. When we sequenced an additional portion of mtDNA control region and analysed sequences of brown bears from eastern and western Europe (Taberlet & Bouvet 1994; our unpublished data) with those of Siberia and northwestern America (Leonard *et al*. 2000; Barnes *et al*. 2002; Delisle & Strobeck 2002), all haplotypes of the Eastern lineage (i.e. Slovakia, Finland, Estonia and European Russia) formed a distinct clade separate from Siberian–American haplotypes, although both groups were closely positioned (unpublished data). This result is compatible with the first hypothesis that northern and eastern Europe were repopulated from the Carpathian Mountains (other refuge areas in Hungary, Ukraine and Moldova also warrant consideration), while the putative refuge in Siberia was probably acting as a refuge for eastern parts of Eurasia and Alaska and perhaps contributed to the population of eastern Europe to only a limited extent. The result also suggests that bears of Europe and Siberia/north-western America did share common ancestry before the last glacial maximum. As it is unlikely that bears from Carpathian Mountains colonized far east of Eurasia and America, the existence and role of more eastern refuges (such as a Siberian refuge) remains to be investigated.

In this study, the intraspecific mutation rate within Beringian brown bears could be estimated because the sequences were radiocarbon-dated. A similar rate was estimated from a closely related species, cave bears, offering some degree of confidence in the rate estimates. Both rates are substantially higher than those estimated from phylogenetic analyses of different bear species. This suggests that the hypothesized 'time dependency of molecular rates', which has previously been shown for birds and primates (Ho *et al*. 2005), may also hold for bears. Comparable mutation rates of around 30% per Myr have also been estimated from the control region of recently diverged species within the *Bison* (Shapiro *et al*. 2004) and *Bos* (Bradley *et al*. 1996; Troy *et al*. 2001) genera, but these are not as high as the 95% estimate obtained by an ancient DNA study of Adélie penguins (Lambert *et al*. 2002). These rate estimates imply that the phenomenon might extend across large endotherms in general. More empirical research is required in this area in order to confirm this suspicion. Indirect support for our estimate of the mutation rate for brown bears comes from observations of Estonian bears that carry two unique haplotypes (13 and 14). As mentioned above, these mutations were most probably introduced into the mtDNA control region after the last ice age when bears had already reached their current territory. The estimated mean value of the mutation rate, one mutation per 8649 years (95% HPD interval 5415–19 378) for the 388 bp stretch of the mtDNA control region, explains the observed data well. The estimates of population size (Table 3) are effective population sizes that reflect the combined effects of both geographical subdivision and population size on the maintenance of genetic diversity in a completely panmictic population. As the analysis is based on mtDNA sequences, all estimates of TMRCAs and effective population sizes represent events for the maternal lineage.

The time frame obtained in this study for the formation of brown bear lineages in Europe differs from those of previous studies. Taberlet & Bouvet (1994) estimated that Eastern and Western lineages diverged about 850 000 BP, based on the evolutionary rate of homologous human sequence. More recently, Hofreiter *et al*. (2002) obtained TMRCA estimates of about 640 000 BP for the Eastern lineage, 350 000 BP for the Western lineage, and 890 000 BP for the whole European population. These date estimates were calibrated using the split between cave and brown bears; in agreement with Ho & Larson (2006), we suggest that for intraspecific analyses, it is inappropriate to use a substitution rate calibrated by divergences between species

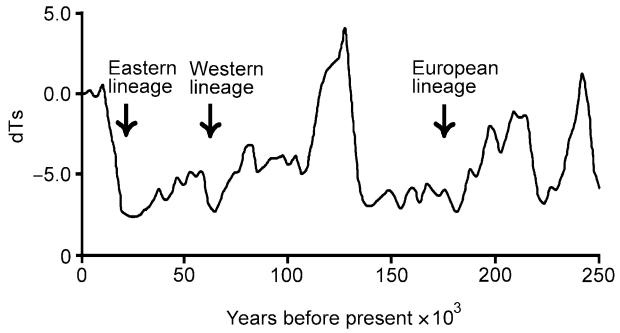

Fig. 6 Timescale of formation of different brown bear lineages in Europe in correlation with the surface temperature reconstructed from the ice core record of EPICA (EPICA Community Members 2004). Arrows indicate the mean TMRCA value for different lineages. Modified from Cheddadi *et al*. (2005).

because it does not take into account the large number of transient polymorphisms that are found within populations. If this practice is adopted on a large scale, it could reveal that many date estimates of evolutionary events in the Pleistocene could be incorrect. In particular, we would expect to find that time scales would become substantially compressed, and this could have a great impact on our understanding of Pleistocene population dynamics.

The uncertainty ranges on the TMRCA estimates are wide, making it difficult to correlate lineage formation events in the evolutionary history of brown bears to climatic fluctuations. All three of the TMRCA estimates are clearly unimodal (Fig. 4), however, indicating that mean estimates should provide a reasonable basis for inferences about the bears' demographic history. Provisional examination of the TMRCA estimates suggests that lineage formation occurred at a time when populations resided in refuge areas, i.e. during stadials (Fig. 6).

Conclusions

European brown bears of the Eastern lineage occupy a vast territory in the continent, with their habitats extending from central and northern Europe to the Ural Mountains. In this study, we have shown that the Eastern lineage is composed of three haplogroups, the core for two of them being formed by ancestral haplotypes from northeastern Europe and West Carpathian Mountains (Slovakia). This reflects their common history in the late Pleistocene and Holocene and suggests that one of the major ice age refuges for brown bears, from which recolonization of northeastern Europe emanated, was probably located in the West Carpathian Mountains. Based on current data we hypothesized that the recolonization was most effective from the West Carpathian Mountains and less effective from its eastern part, with a recolonization gradient declining eastwards.

We presented a new time frame for the formation of different lineages in Europe, based on Bayesian statistical analysis with a new mutation rate estimated for the mitochondrial control region of brown bears, suggesting that the evolution of modern brown bears occurred on a shorter timescale than previously proposed. This result is also relevant to other researchers studying the history of animal populations and who wish to incorporate a temporal framework into their analyses and interpretations. In order to elucidate further the spatial and temporal aspects of brown bears in Europe, ancient DNA analysis may be the most rewarding line of future research.

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