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Mitochondrial DNA haplogrouping of the brown bear, *Ursus arctos* (Carnivora: Ursidae) in Asia, based on a newly developed APLP analysis

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Sequence analyses of the complete brown bear, *Ursus arctos*, mitochondrial DNA (mtDNA) genome have detected scattered single nucleotide polymorphisms (SNPs) that define distinct mtDNA haplogroups in phylogeographical studies. The degraded DNA in historical samples, such as stuffed or excavated specimens, however, is often not suitable for sequence analyses. To address this problem, we developed an amplified product length polymorphism (APLP) analysis for mtDNA-haplogrouping *U. arctos* specimens by detecting haplogroup-specific SNPs. We verified the validity and utility of this method by analysing up to 170-year-old skin samples from *U. arctos* specimens collected widely across continental Eurasia. We detected some of the same haplogroups as those occurring in eastern Hokkaido (Japan) and eastern Alaska in continental Eurasia (the Altai and the Caucasus). Our results show that *U. arctos* in eastern Hokkaido and eastern Alaska descended from a common ancestor in continental Eurasia, and suggest that *U. arctos* occupied several refugia in southern Asia during the Last Glacial Maximum. *Biological Journal of the Linnean Society*, 2014, **111**, 627–635.

ADDITIONAL KEYWORDS: multiplex PCR – phylogeography – population structure – short-length sequence.

INTRODUCTION

The brown bear, *Ursus arctos*, is widely distributed around the Northern Hemisphere, in both the Palaearctic and Nearctic regions. Phylogeographical studies of *U. arctos* mitochondrial DNA (mtDNA) have revealed two major lineages (western and eastern lineages) that encompass several geographically distinct mtDNA clades (haplogroups), designated 1a, 1b, 2a, 2b, 3a1, 3a2, 3b, 4, 5, and 6 (Leonard, Wayne & Cooper, 2000; Barnes *et al*., 2002; Miller, Waits & Joyce, 2006; Calvignac *et al*., 2008; Calvignac, Hughes & Hänni, 2009; Davison *et al*., 2011; Hirata *et al*., 2013). *Ursus arctos* phylogeography has been studied in south-western Asia, the Middle East, and central Asia by using DNA from contemporary and ancient *U. arctos* specimens (Talbot & Shields, 1996; Matsuhashi *et al*., 2001; Miller *et al*., 2006; Galbreath, Groves & Waits, 2007; Calvignac *et al*., 2009; McCarthy, Waits & Mijiddorj, 2009). Although the distributional ranges and population sizes of *U. arctos* are larger in Asia and Russia than in North America or Western Europe (McLellan, Servheen & Huber, 2008), fewer genetic studies have been conducted in the former areas than in the latter.

Several recent studies have used complete mtDNA sequences to study *U. arctos* phylogeography. Keis *et al*. (2013) analysed complete mtDNA sequences from north-western Eurasian *U. arctos* specimens, and identified detailed phylogeographical structures, signatures of demographic history, and spatial processes that studies with short mtDNA sequences had previously failed to detect. Likewise, Hirata *et al*.

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(2013) used complete mtDNA sequences to reconstruct the maternal phylogeny of the north-eastern Eurasian *U. arctos* and to estimate divergence times. Consequently, the detected patterns were not evident from short mtDNA sequences.

Phylogeography based on mitochondrial genomes has best been studied in human (*Homo sapiens*) populations (e.g. Underhill & Kivisild, 2007), in which mtDNA haplogroups constitute major monophyletic groups (Torroni *et al*., 2000), and provide the basis for population structures. Human mtDNA haplogroups are based on single nucleotide polymorphisms (SNPs) scattered through the mitochondrial genome (Umetsu & Yuasa, 2005), but mtDNA haplogrouping by sequencing the whole genome is not always practical, particularly for aged or ancient samples, in which the DNA is degraded. Alternatively, amplified product length polymorphism (APLP) analysis permits mtDNA haplogrouping by detecting informative SNPs without having to sequence the entire mitochondrial genome (Watanabe *et al*., 1997; Umetsu *et al*., 2001, 2005).

In the present study, we developed an APLP method for haplogrouping *U. arctos* mtDNA, based on the complete mtDNA sequences reported by Hirata *et al*. (2013), as a means of determining the mtDNA haplogroups of degraded historical (aged or ancient) *U. arctos* samples, for which complete mitochondrial sequencing is impossible. This method uses several primer sets to detect haplogroup-specific SNPs by amplifying short-length sequences, and can determine mtDNA haplogroups. We tested this method by examining the phylogeography of *U. arctos* populations in Eurasia. Here we report our results and discuss the migration history of *U. arctos* in this region based on these results.

MATERIAL AND METHODS

APLP analysis

This method is based on the attachment of a non-complementary sequence to the 5′ end of one of two allele-specific primers, which then allows the two alleles to be distinguished as two different sizes of amplification products (Watanabe *et al*., 1997; Umetsu *et al*., 2001, 2005; Umetsu & Yuasa, 2005). Table 1 lists the sequences of APLP primers we used and their working concentrations, and Table 2 shows the haplogroup (clade)-specific SNP sites found in the alignment of the complete mtDNA sequences. The genomic positions of SNP sites were numbered based on the complete mtDNA sequence of *U. arctos* (GenBank accession no. AF303110), as reported by Delisle & Strobeck (2002). Our primer sets were based on previously reported complete mtDNA sequences of *U. arctos* in clades 1, 2b, 3a1, 3a2, 3b, 4, and 5, and detected five eastern lineages (clades 3a1, 3a2, 3b, 4, and 5) (Fig. 1A). We tested the specificity of the multiplex APLP reactions using 39 *Ursus maritimus* (polar bear) and *U. arctos* samples, as reported by Hirata *et al*. (2013). Polymerase chain reaction (PCR) amplifications for the APLP analysis were carried out in 10-μL reaction volumes, each containing 1 μL of template DNA solution, the optimum concentration of each primer (Table 1), and reagents from the Multiplex PCR Kit (Qiagen). Primer concentrations were adjusted to obtain balanced quantities of PCR products. Two separate multiplex PCR (sets A and B) reactions were performed. Multiplex PCR set A was designed to analyse four sites (7257C/T, 16259A/G, 7770T/C, and 10116G/A; Fig. 1B) and set B was designed to analyse five sites (11585G/A, 8392A/G, 8776C/T, 13180T/C, and 9271T/C; Fig. 1C). Thermal cycling conditions for both sets A and B were an incubation of 15 min at 95 °C, 30–40 cycles of 10 s at 94 °C, 10–20 s at 40–54 °C, and 5 s at 72 °C, with a final extension of 3 min at 72 °C. An aliquot (2 μL) of the PCR product was electrophoresed in a native polyacrylamide gel (10% T, 5% C) containing 375 mM Tris-HCl buffer (pH 8.9) in Tris (12.5 mM)-glycine (96 mM) running buffer. DNA bands were detected with an ultraviolet transilluminator after ethidium bromide staining.

mtDNA haplogrouping

We haplogrouped samples of skin from 67 *U. arctos* specimens widely collected from the Eurasian continent, from 1842 to 1998, and housed in the Zoological Institute, Russian Academy of Sciences, St Petersburg. Table 3 lists collection data and Figure 2 shows the locations of the collection sites. Total genomic DNA was extracted from each sample piece (about 5 × 5 mm) using the QIAamp DNA Micro Kit (Qiagen), following the manufacturer's protocols. PCR amplifications were carried out in 10-μL reaction volumes, each containing 1 μL of template DNA solution, which is the optimum concentration of each primer (Table 1), reagents from the Multiplex PCR Kit (Qiagen), and 0.5 μL of bovine serum albumin (BSA; 0.4 μg μL^{-1}). The PCR conditions were as described above. In cases when the multiplex PCR reactions produced no amplification products, single PCR reactions with the corresponding primer sets were performed separately.

RESULTS

Specificity of APLP primers for Eurasian *Ursus arctos*

Our primer sets amplify multiplex PCR products with molecular sizes ranging from 49 to 106 base pairs

Table 1. Profiles of oligonucleotide primers used for the amplified product length polymorphism (APLP) analysis

Set	Haplogroups	Primer	Sequence (5′ → 3′)	Concentration (pmol μl^{-1})	Product size (bp)
A	Clades 3a, 3b, 4, 5	7257T	CGGAATGATCTCTCACATaGTT	0.2	84
		7257C	cacaaCGGAATGATCTCTCACAaTGTC	0.05	89
		7257R3	GGATATTATCGCTCAGACTATTC	0.2	
	Clades 3a, 3b, 4	16259G	ACAACGAGGAATGATATTCtGG	0.15	71
		16259A	aggttACAACGAGGAATGATATTaCGA	0.05	76
		16259R2	AGTGTTAGTAGGTCTGCTACTAG	0.1	
	Clade 5	7770T	ATATGACATTCTTTCCTCAcCAT	0.4	60
		7770C	ccaatATATGACATTCTTTCCTCtGCAC	0.4	65
		7770R2	TCGGAATATCGCCGAGGTAT	0.05	
	Clade 4	10116A	AACTAGGAGCATGCTGtCCA	0.5	49
		10116G	agtgcAACTAGGAGCATGCTtACCG	0.15	54
		10116R2	TAGCGGGTTCAGAGGAGTAA	0.2	
B	Clades 3a, 3b	11585A	GTCAATTTCACCTGTCAAtGGAA	0.25	101
		11585G	ctgctGTCAATTTCACCTGTCAAAtGAG	0.1	106
		11585R	CTATAGCAGAAAAGGTTATGATCAG	0.25	
	Clade 3b	8392G	GCCAGCTATTATCTTGAaTCTG	0.1	86
		8392A	taggaGCCAGCTATTATCTTGtTTCTA	0.1	91
		8392R	ACAGTTAGTGAGGGATTATTGATC	0.05	
	Clade 3a	8776T	GCTCAGAAATTTGTGGCTCgAAT	0.15	75
		8776C	ggttaGCTCAGAAATTTGTGGCTgCAAC	0.05	80
		8776R	TCTTCAAAATAGGATAGTGGGAC	0.05	
	Clade 3a1	13180C	TAATTTTCATGCCAGTtGCCC	0.3	60
		13180T	agaccTAATTTTCATGCCAGaAGCCT	0.1	65
		13180R	TATCAAATGGAAAACTCCATGATCG	0.1	
	Clade 3a2	9271C	AAACAcATATTATCCATTCATAGC	0.45	50
		9271T	atgatAAACAAcTATTATCCATTCATAGT	0.35	55
		9271R	TATTAGTGCCCAGGTTTGTC	0.35	

The non-complementary nucleotides are written in small letters.

Table 2. Haplogroup (clade)-specific single nucleotide polymorphism (SNP) sites in the *Ursus arctos* mitochondrial genome

		SNP sites								
Haplogroup (clade)		7257	16259	7770	10116	11585	8392	8776	13180	9271
Western lineage	1	T	G	C	A	A	G	T	C	C
	2b	T	G	C	A	A	G	T	C	C
Eastern lineage	3a1	C	A	C	A	G	G	C	T	C
	3a2	C	A	C	A	G	G	C	C	T
	3b	C	A	C	A	G	A	T	C	C
	4	C	A	C	G	A	G	T	C	C
	5	C	G	T	A	A	G	T	C	C

(bp). Analyses of the 39 *U. arctos* and *U. maritimus* known mtDNA haplogroups confirmed the specificities of our primer sets. The APLP band patterns are shown in Figure 1B and C. The phylogeny of *U. arctos* and *U. maritimus*, consisting of eight mtDNA clades, and position numbers of the clade-specific SNPs and diagnostic nucleotides at those sites, are summarized in Figure 1A and Table 2.

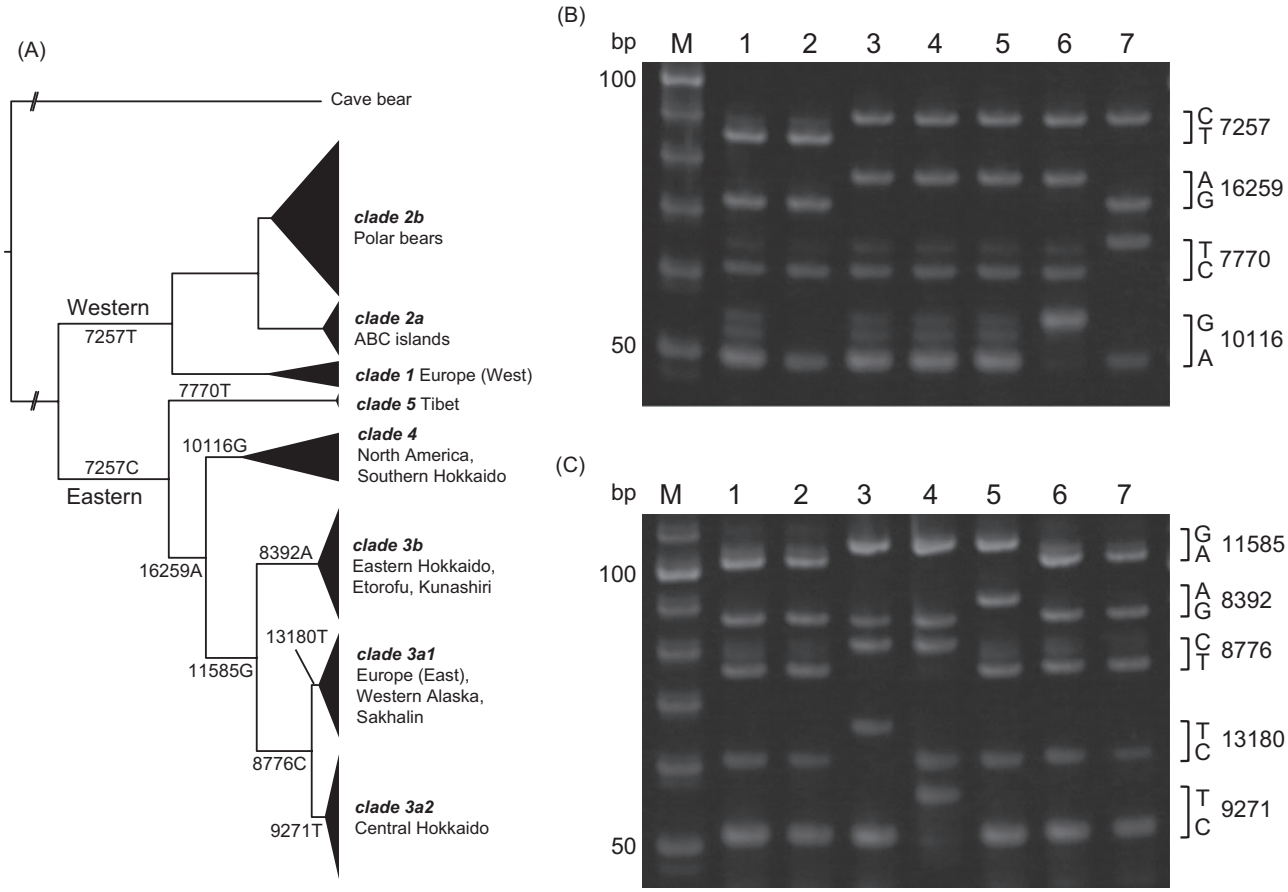

Figure 1. Phylogenetic relationships within *Ursus arctos* and *Ursus maritimus*, and amplified product length polymorphism (APLP) band patterns of *U. arctos* mtDNA haplogroups. A, phylogeny of *U. arctos* and *U. maritimus*, consisting of eight mtDNA clades. Numbers and nucleotides above branches show the positions of the clade-specific single nucleotide polymorphisms (SNPs) and diagnostic nucleotides at that site. The tree is modified from Hirata *et al.* (2013). B, APLP band patterns for primer set A. C, APLP band patterns for primer set B. B and C, lane 1, clade 1; lane 2, clade 2b; lane 3, clade 3a1; lane 4, clade 3a2; lane 5, clade 3b; lane 6, clade 4; lane 7, clade 5; M, marker, 10-bp DNA ladder. Reference samples representative of each of clades 1, 2b, 3a1, 3a2, 3b, 4, and 5 were Ua3, 1556, IPAE20, 835, 822 503, and 01, which were reported in Hirata *et al.* (2013). Numbers and nucleotides listed at the right are SNP position numbers and the specific nucleotides involved in the SNPs, as indicated in Table 1. The SNP at each site is distinguishable by band positions. *Ursus arctos* mtDNA haplogroups of museum skin samples (Table 3) are identified by referring to Table 2.

mtDNA haplogrouping of Eurasian *Ursus arctos*

Using the APLP analysis, we were able to determine the mtDNA haplogroup of 54 of the 67 Eurasian *U. arctos* samples examined (80.6%). There were no PCR products found in the negative control. Many of these bears fell into clades 3a1, 3b, and 5; the clade identities and locations of samples are indicated in Figures 2 and 3, and in Table 3. Clade 3a1 was the most broadly distributed group, with individuals detected from Leningrad Province south-eastwards through Bashkiria and the Ural, to Tien Shan, the Altai, and Lake Baikal in central Asia, and from the Amur River to north-eastern Siberia and Kamchatka in eastern Asia. Clade 3b was distributed in some local areas: the Caucasus, the Tannu-Ola Mountains, the Altai, and Krasnojarsk Province. Clade 5 was only distributed in the Qinghai-Tibet Plateau.

We detected three tentative novel mtDNA haplogroups that had SNPs that differed from those in our analytical scheme (Fig. 1A): we termed these the Western 1 (W1), Eastern 1 (E1), and Eastern 2 (E2) haplogroups (Table 3). Haplogroup W1 had T at site 7257, which is diagnostic for the western lineage; however, other SNP sites in haplogroup W1 differed from those of previously defined haplogroups in the western lineage (clades 1 and 2b). Three individuals from the Caucasus and western Iran belonged to this new haplogroup (Figs 2, 3; Table 3). Haplogroup E1 had C at site 7257, which is diagnostic for the eastern

Table 3. *Ursus arctos* skin samples used for mtDNA haplogrouping by the amplified product length polymorphism (APLP) analysis, single nucleotide polymorphisms (SNPs) detected in each sample, and haplogroup identity

Sample no.	Sampling year	Locality	SNP sites									Haplogroup (clade)
			7257	16259	7770	10116	11585	8392	8776	13180	9271	
U-3	1901	Tibetan plateau, Qinghai, Serg-tchu (upper Huang Ho)	C	G	T	A	A	G	T	C	C	5
U-4	1900	Tibetan plateau, Qinghai, Dzagyn-Gol (upper Huang Ho)	C	G	T	A	A	G	T	C	C	5
U-5	1901	Tibetan plateau, Qinghai, Serg-tchu (upper Huang Ho)	C	G	T	A	A	G	T	C	C	5
U-6	1901	Tibetan plateau, Qinghai, Magmutchu (upper Huang Ho)	C	G	T	A	A	G	T	C	C	5
U-8	1867	Caucasus, Georgia, Borzhomi	T	A	C	A	A	G	T	C	C	W1
U-10	1916	Kyrgizstan, west cost Issyk-Kyl Lake	C	A	C	A	A	G	T	C	C	E1
U-11	1878	Kyrgizstan, Tien-Shan	C	A	C	A	G	G	T	C	C	E2 (3a)
U-12	1914	Western Iran, Oshnoviyeh, SW from Urmia Lake	T	A	C	A	A	G	T	C	C	W1
U-13	1878	Kazakhstan, Karatau Mts.	C	A	C	A	A	G	T	C	C	E1
U-14	1878	Kyrgizstan, Tien-Shan	C	A	C	A	G	G	C	T	C	3a1
U-15	1912	Kyrgizstan, east cost Issyk-Kyl Lake	C	A	C	A	A	G	T	C	C	E1
U-16	1912	Kyrgizstan, east cost Issyk-Kyl Lake	C	A	C	A	A	G	T	C	C	E1
U-17	1914	Western Iran, Oshnoviyeh, SW from Urmia Lake	C	A	C	A	A	G	T	C	C	E1
U-18	1878	Kyrgizstan, Tien-Shan	C	A	C	A	A	G	T	C	C	E1
U-19	1878	Kyrgizstan, Tien-Shan	C	A	C	A	A	G	T	C	C	E1
U-20	1905	Western Caucasus	C	A	C	A	G	A	T	C	C	3b
U-22	1890	Caucasus, East Georgia, Lagodehi	T	A	C	A	A	G	T	C	C	W1
U-23	1906	North-Western Caucasus, 70 km to south Maikop	C	A	C	A	G	A	T	C	C	3b
U-24	1842	Kyrgizstan, Tien-Shan	C	A	C	A	A	G	T	C	C	E1
U-25	1915	Russia, Far East, Amur River	C	A	C	A	G	G	C	T	C	3a1
U-26	1930	North-East Siberia, mouth of Andyr River	C	A	C	A	G	G	C	T	C	3a1
U-27	1907	North-East Siberia, Andyr River, Markovo	C	A	C	A	G	G	C	T	C	3a1
U-28	1896	North-East Siberia, Andyr River, Markovo	C	A	C	A	G	G	C	T	C	3a1
U-29	1909	eastern coast of Kamchatka, Ust-Kamchatsk	C	A	C	A	G	G	C	T	C	3a1
U-30	1914	western coast of kamchatka, 50 km from Lopatka Cape	C	A	C	A	G	G	C	T	C	3a1
U-33	1884	Northern Tibet, China	C	G	T	A	A	G	T	C	C	5
U-34	1963	North-East Siberia, Kolymskyi Mts.	C	A	C	A	G	G	C	T	C	3a1
U-35	1915	eastern coast Baikal Lake, Kirbulik Bay	C	A	C	A	G	G	C	T	C	3a1
U-37	1915	eastern coast Baikal Lake, Chivyrkui River	C	A	C	A	G	G	C	T	C	3a1
U-38	1915	eastern coast Baikal Lake, Cheremshan River	C	A	C	A	G	G	C	T	C	3a1
U-39	1908	Western Caucasus, Sochi	C	A	C	A	G	G	T	C	C	E2 (3a)
U-40	1900	Tibetan plateau, mountains to west from Kukunor Lake	C	G	T	A	A	G	T	C	C	5
U-41	1900	Tibetan plateau, Orin-Nor Lake	C	G	T	A	A	G	T	C	C	5
U-42	1894	Nan-Shan, upper part Sulai-He River	C	G	T	A	A	G	T	C	C	5
U-44	1931	Russia, Bashkiria	C	A	C	A	G	G	C	T	C	3a1
U-46	1882	Russia, Leningrad Prov., Lodeinoe Pole	C	A	C	A	G	G	C	T	C	3a1
U-47	1908	Western Caucasus, Sochi	C	A	C	A	G	G	T	C	C	E2 (3a)
U-48	1908	Western Caucasus, Sochi	C	A	C	A	G	A	T	C	C	3b
U-49	1911	Western Caucasus, Sochi	C	A	C	A	G	A	T	C	C	3b
U-50	1915	Caucasus, East Georgia, Lagodehi	C	A	C	A	G	A	T	C	C	3b
U-51	1905	Western Caucasus	C	A	C	A	G	A	T	C	C	3b
U-52	1908	Western Caucasus, Sochi	C	A	C	A	G	G	T	C	C	E2 (3a)
U-55	1927	Northern Ural, Lyapin River	C	A	C	A	G	G	C	T	C	3a1
U-56	1928	Northern Ural, Lyapin River	C	A	C	A	G	G	C	T	C	3a1
U-57	1927	Northern Ural, Lyapin River	C	A	C	A	G	G	C	T	C	3a1
U-58	1927	Northern Ural, Lyapin River	C	A	C	A	G	G	C	T	C	3a1
U-59	1927	Northern Ural, Lyapin River	C	A	C	A	G	G	C	T	C	3a1
U-61	1914	Tuva, northern part Tannu-Ola Mts.	C	A	C	A	G	A	T	C	C	3b
U-62	1901	Altai, east coast of Teletskoe Lake	C	A	C	A	G	G	C	T	C	3a1
U-63	1925	Mongolia	C	A	C	A	G	G	T	C	C	E2 (3a)
U-64	1912	Altai, Chulyshman	C	A	C	A	G	A	T	C	C	3b
U-65	1908	Western Altai, Zmeinogorsk	C	A	C	A	G	A	T	C	C	3b
U-75	1998	Russia, Leningrad Prov.	C	A	C	A	G	G	C	T	C	3a1
U-76	1998	Russia, Krasnojarsk Prov.	C	A	C	A	G	A	T	C	C	3b

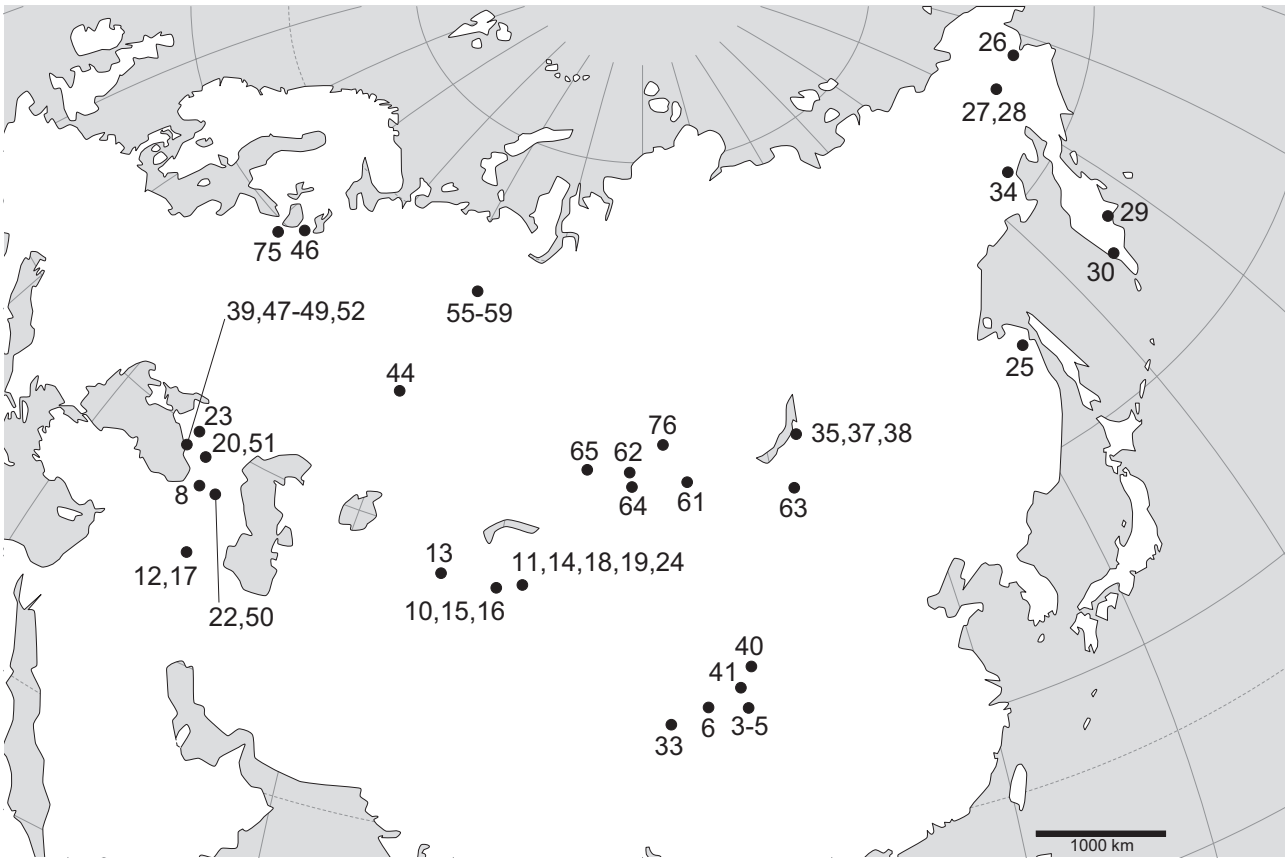

Figure 2. Map of Eurasia showing the localities of the *Ursus arctos* samples analysed. Filled circles indicate sample localities; sample ID numbers indicate individual *U. arctos* specimens at each locality. See Table 3 for a list of sample localities, collection dates, SNPs detected, and haplogroups (clades) identified.

lineage, but other SNP sites differed from previously defined haplogroups in the eastern lineage (clades 3a1, 3a2, 3b, 4, and 5). Novel haplogroup E1 was represented by eight individuals, from the Karatau, Kazakhstan, Lake Issy-Kul, Kyrgyzstan, Tien Shan, Kyrgyzstan, and western Iran (U-17; Figs 2, 3; Table 3). Haplogroup E2 grouped within clade 3a, but was distinct from both clades 3a1 and 3a2. Haplogroup E2 was represented by eight individuals from Tien Shan, Kyrgyzstan, the Caucasus, and Mongolia (Figs 2, 3; Table 3).

Multiple haplogroups were detected sympatrically in three areas (Figs 2, 3; Table 3): W1 and E2 in western Iran; 3b, W1, and E2 in the Caucasus; 3a1 and 3b in the Altai; 3a1, E1, and E2 in Kyrgzstan.

DISCUSSION

Haplogrouping of *Ursus arctos* mtDNA

The present study developed a method of APLP analysis for identifying *U. arctos* to mtDNA haplogroups by using only two multiplex PCRs followed by acrylamide gel electrophoresis. The APLP analyses of all samples, the complete mtDNA sequences of which were known, correctly identified individuals to haplogroups in all cases. Because the primer sets we designed yielded PCR fragments that were 49–106 bp in length, this analysis is applicable to old and ancient samples such as museum specimens and excavated remains, in which the DNA is often fragmented. As we had only one sample representing clade 1 and no samples representing clade 2a, we could not design and test primer sets capable of haplogrouping specimens in the western lineages (clades 1, 2a, and 2b). In addition, the primer sets we designed for clade 3b were based on complete mtDNA sequences from bears from eastern Hokkaido only, and not from other specimens in clade 3b, such as North American *U. arctos*. Further accumulation in DNA data banks of complete mtDNA sequences from *U. arctos* worldwide will allow the development of more comprehensive sets of mtDNA haplogroup-specific primers and facilitate phylogeographical studies of *U. arctos*.

The APLP analysis can be a much easier and more cost effective method for a large number of samples,

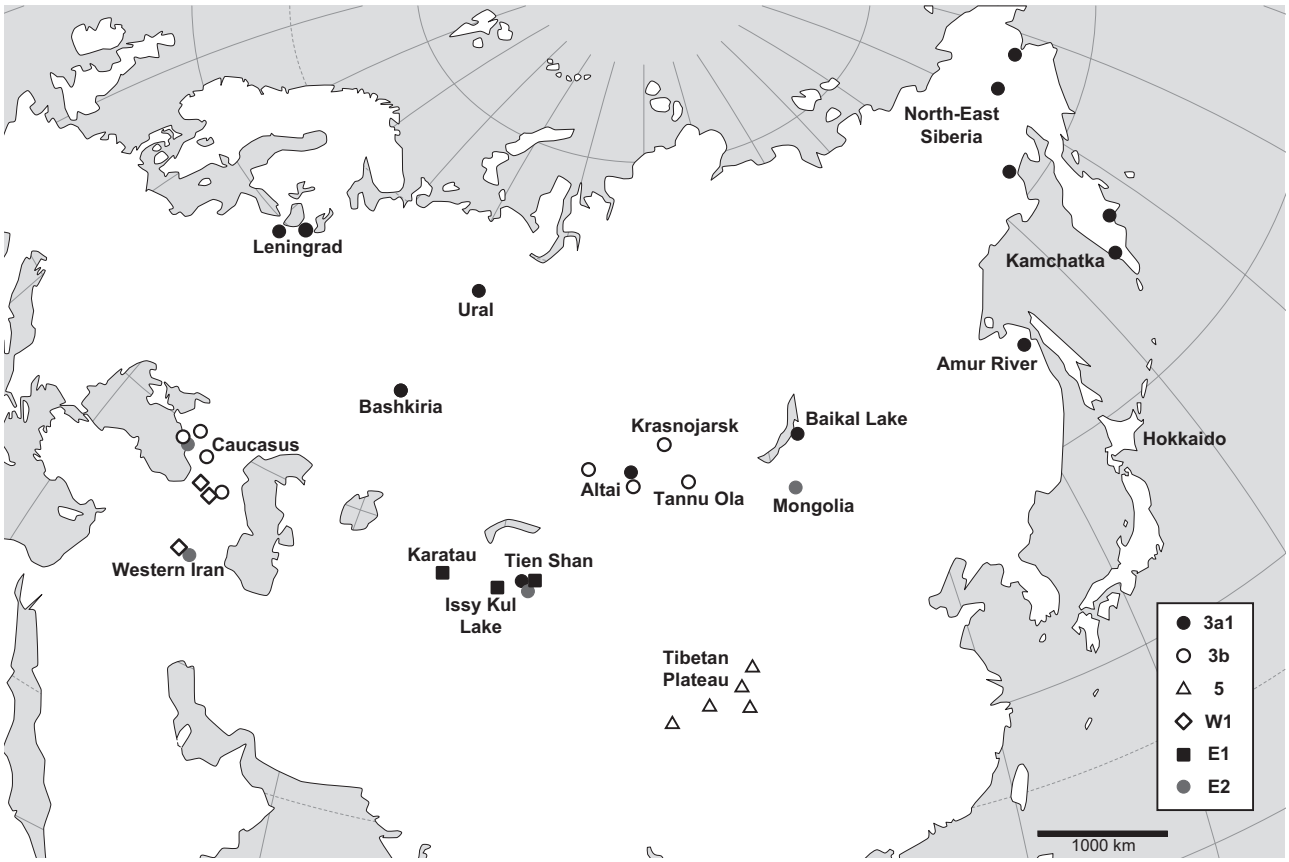

Figure 3. Geographical distribution of the *Ursus arctos* mtDNA haplogroups detected in continental Eurasia by the amplified product length polymorphism (APLP) analysis. Clades 3a1, 3b, and 5 are previously reported haplogroups, whereas W1, E1, and E2 are tentative novel haplogroups, defined in the present study. Key: ●, mtDNA clade 3a1; ○, clade 3b; △, clade 5; ◇, W1; ■, E1; ●, E2.

especially historical samples, and can efficiently detect the distribution of *U. arctos* mtDNA haplogroups. There is some limitation with this method, however, in that we cannot infer evolutionary history, such as the detection of signals of population demography (population reduction and expansion), from mtDNA haplogroup data.

We successfully determined the mtDNA haplogroup of 54 out of 67 (80.6%) old Eurasian samples, many of which came from relatively old museum skin samples, in which the DNA was often degraded, and generally not suitable for the extraction of long sequences. Our APLP method might also work with palaeontological and archaeological samples, and if so would be useful in studying the phylogeographical structure of *U. arctos* through time.

Phylogeography of northern and southern Eurasian *Ursus arctos*

We determined the mtDNA haplogroups of *U. arctos* across the Eurasian continent from European Russia to Kamchatka, and including south-western and central Asia (Figs 2, 3; Table 3). We detected three previously reported mtDNA haplogroups (clades 3a1, 3b, and 5). Korsten *et al.* (2009) found only one *U. arctos* mtDNA haplogroup (clade 3a) to be widely distributed in northern Eurasia, and concluded that this haplogroup underwent a severe bottleneck during the Last Glacial Maximum (LGM), followed by a demographic expansion. Corroborating this result, we found *U. arctos* of clade 3a1 to be widely distributed across Eurasia (Figs 2, 3; Table 3). We also identified one individual (U-14) in clade 3a1, collected from Tien Shan, Kyrgyzstan, in 1878. This suggests that clade 3a1 was previously more widely distributed in central Asia than it is now.

Only one individual representing clade 3b had previously been found on the Eurasian continent in the Russian Far East (Miller *et al.*, 2006), and the extent of the distribution of this clade was not clear. We found clade 3b from several areas in Eurasia, including the Altai and the Caucasus (Figs 2, 3; Table 3). In the Altai, *U. arctos* of clades 3a1 and 3b occurred

sympatrically. Because clade 3b was shared by both eastern Hokkaido and eastern Alaskan *U. arctos*, it appears that bears from this area descended from a most recent common ancestor on the Eurasian continent (Matsuhashi *et al.*, 2001; Korsten *et al.*, 2009). Our results show that representatives of clade 3b exist as a relict population in continental Eurasia, and indicate that clade 3b diverged in continental Eurasia prior to dispersal. The Caucasus and the Altai, where we detected clade 3b, are presently part of the southern distribution of the *U. arctos* in continental Eurasia (McLellan *et al.*, 2008), and these areas may have served as glacial refugia, with clade 3b representing a relict population in continental Eurasia. Clade 3b diverged earlier than clade 3a1 (Fig. 1A; Matsuhashi *et al.*, 2001; Korsten *et al.*, 2009; Hirata *et al.*, 2013). The younger mtDNA haplogroup (clade 3a1) probably also occupied these areas as refugia during the LGM, and then expanded across northern Eurasia. Several areas, such as the Altai and the Caucasus, could have served as refugia during the LGM in southern Eurasia.

Central and south-western Asia

All individuals from the Qinghai-Tibet Plateau belonged to clade 5 (Figs 2, 3; Table 3). This confirms that the Tibetan *U. arctos* (*Ursus arctos pruinosus*) are genetically distant from the rest of the eastern lineage, and form a geographically isolated population (Hirata *et al.*, 2013).

We detected three novel mtDNA haplogroups (W1, E1, and E2), which our APLP analysis did not classify in south-western and central Asia (Figs 2, 3; Table 3). Haplogroup W1 occurred in the Caucasus and western Iran, and clearly belongs to the western lineage. Haplogroup W1 bears from the Caucasus (U-8 and U-22) and western Iran (U-12) were collected in 1867, 1890, and 1914, respectively. These are the first western-lineage *U. arctos* detected from the Caucasus and Iran; all individuals previously analysed from the Caucasus belonged to the eastern lineage (Murtskhvaladze, Gavashelishvili & Tarkhnishvili, 2010), and those from Iran formed a distinct lineage within the eastern lineage (Talbot & Shields, 1996; Miller *et al.*, 2006). *Ursus arctos* were abundant and widespread throughout the Caucasus in the 18th and 19th centuries (Radde, 1899; Dinnik, 1914), but numbers have declined since the late 19th century, primarily as a result of habitat loss and unregulated hunting (Murtskhvaladze *et al.*, 2010). In the Middle East, *U. arctos* in the Lebanon (where they are now extinct) belonged to clade 1 in the western lineage (Calvignac *et al.*, 2009): the distribution of clade 1 in this region is thus discontinuous, interrupted by eastern lineage clade 3a in Turkey and Syria (Talbot & Shields, 1996; Calvignac *et al.*, 2009). This pattern suggests that the western lineage was historically more broadly distributed than now, ranging through the Caucasus and the Middle East, and that some populations have become locally extinct and have been replaced by the eastern lineage.

Individuals in haplogroup E1 in the eastern lineage were found from Kazakhstan, Kyrgyzstan, and western Iran (Figs 2, 3; Table 3). The *U. arctos* of Kazakhstan and Kyrgyzstan form a geographically restricted population, which may explain their distinct haplogroup within the eastern lineage.

Haplogroup E2 groups within clade 3a, but is distinct from both clades 3a1 and 3a2. Individuals in haplogroup E2 were found in southern Eurasia, from Kyrgyzstan, the Caucasus, and Mongolia (Figs 2, 3; Table 3). These records extend the known range of clade 3a *s.l.*, from south central Asia westwards to the Caucasus region.

As mentioned above, *U. arctos* bears representing multiple haplogroups were distributed sympatrically in the Middle East and central Asia (western Iran, the Caucasus, the Altai, and Kyrgyzstan). The higher genetic diversity in these areas compared with northern Eurasia (Figs 2, 3; Table 3) indicates that the Middle East and central Asia were refugia during the LGM. The Middle East was generally colonized by populations originating from the north.

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