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ORIGINAL PAPER

Phylogenetic relationships of ancient brown bears (*Ursus arctos*) on Sakhalin Island, revealed by APLP and PCR-direct sequencing analyses of mitochondrial DNA

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Abstract

To investigate the phylogenetic relationships of brown bears (*Ursus arctos*) on Sakhalin Island in the Far East, mitochondrial DNA (mtDNA) sequences were analyzed for 27 ancient and five contemporary specimens of brown bears obtained from Sakhalin. We successfully determined partial sequences of the mtDNA control region (254–394 base-pairs) and identified six novel haplotypes. All sequences from bears on Sakhalin grouped phylogenetically with clade 3a but were not clearly distinguishable as to subclades 3a1 and 3a2. However, by application of APLP method for detecting single-nucleotide polymorphisms (SNPs) specific to clades, the samples from Sakhalin were all identified to clade 3a1, which is currently widespread in eastern continental Eurasia. Although the ancestors of brown bears on Hokkaido Island, Japan, migrated from continental Eurasia through Sakhalin, none of the clades previously found on Hokkaido (clades 3a2, 3b, and 4) were detected on Sakhalin. Our results suggest that an ancestral bear population from clade 3a1 migrated from continental Eurasia to Sakhalin, independently and after the migration of clade 3a2 to Hokkaido. Alternatively, clade 3a2 could have evolved from phylogenetically closely related clade 3a1 due to geographical isolation on Hokkaido. Our data confirm that bear skull remains from an Okhotsk Cultural archaeological site on small Rebun Island off the northwest coast of Hokkaido were transported from Hokkaido, rather than from Sakhalin.

Keywords Ancient DNA · Bear-sending ceremony · Eurasian Continent · Hokkaido · Rebun Island

Introduction

Brown bears (*Ursus arctos*, Ursidae, Carnivora) are widely distributed from Eurasia to North America and can be grouped into 11 allopatric haplogroups (clades) by using mitochondrial DNA (mtDNA): 1a, 1b, 2a, 2b, 3a1, 3a2, 3b, 4, 5, 6, and 7 (Taberlet and Bouvet 1994; Leonard et al. 2000; Barnes et al. 2002; Miller et al. 2006; Calvignac et al. 2008; Calvignac

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et al. 2009; Davison et al. 2011; Hirata et al. 2013; Ashrafzadeh et al. 2016; Çilingir et al. 2016). To improve the success of mtDNA haplogrouping with fragmented DNA from historical bear specimens, Hirata et al. (2014) developed the amplified product length polymorphism (APLP) method. This method was originally developed in human to detect single-nucleotide polymorphisms (SNPs; scattered throughout the mitochondrial genome) that are specific to each clade (Umetsu et al. 2001). The APLP method has already been successfully used to identify clades in ancient brown bear bone remains excavated in Bulgaria (Mizumachi et al. 2020).

The brown bear also inhabits Hokkaido Island, Japan, where individuals belong to one of the three mtDNA clades (clades 3a2, 3b, and 4) that are allopatrically distributed on the island. Individuals in the three clades migrated from continental Eurasia via Sakhalin Island at different times; the first migration was to southern Hokkaido (clade 4), the second was to eastern Hokkaido (clade 3b), and the third was to central Hokkaido (clade 3a2) (Matsuhashi et al. 1999; Hirata et al. 2013). Sakhalin Island (76,400 km^2) is separated to the south from Hokkaido (83,450 km^2) by Soya Strait (La Pérouse Strait), and to the northwest from the Eurasian Continent by Mamiya Strait (Tatar Strait) (Fig. 1). One brown bear previously analyzed from Sakhalin (Hirata et al. 2013) belonged to mtDNA clade 3a1, which is largely a lineage of "eastern continental Eurasia" and not distributed on Hokkaido. While Sakhalin is thus a key locality for elucidating the migration history of brown bears, mtDNA data from the island are few. Gus'kov et al. (2013) reported from mitochondrial cytochrome *b* (cyt *b*) sequences that five modern brown bears on Sakhalin all belonged to clade 3a, but due to the low phylogenetic resolution of cyt *b*, could not further distinguish whether the bears were in subclade 3a1 or 3a2.

Brown bear remains occur in paleontological and archaeological sites on Sakhalin (Gorbunov 2002; Alekseeva et al. 2004; Kuzmin et al. 2005; Kirillova and Tesakov 2008; Kirillova et al. 2009). Vasilevski (2008) summarized changes in the mammoth fauna on Sakhalin from the Middle Pleistocene to the Holocene. Kuzmin et al. (2005) reported a ^{14}C date of 12,685 ± 140 radio carbon years before present (RCYBP; SOAN-5522) for brown bear bones excavated from Ostantsevaya Cave on Sakhalin, indicating that this species had migrated there by the end of the Pleistocene, but mitochondrial phylogeny remains unclear for ancient brown bears on Sakhalin.

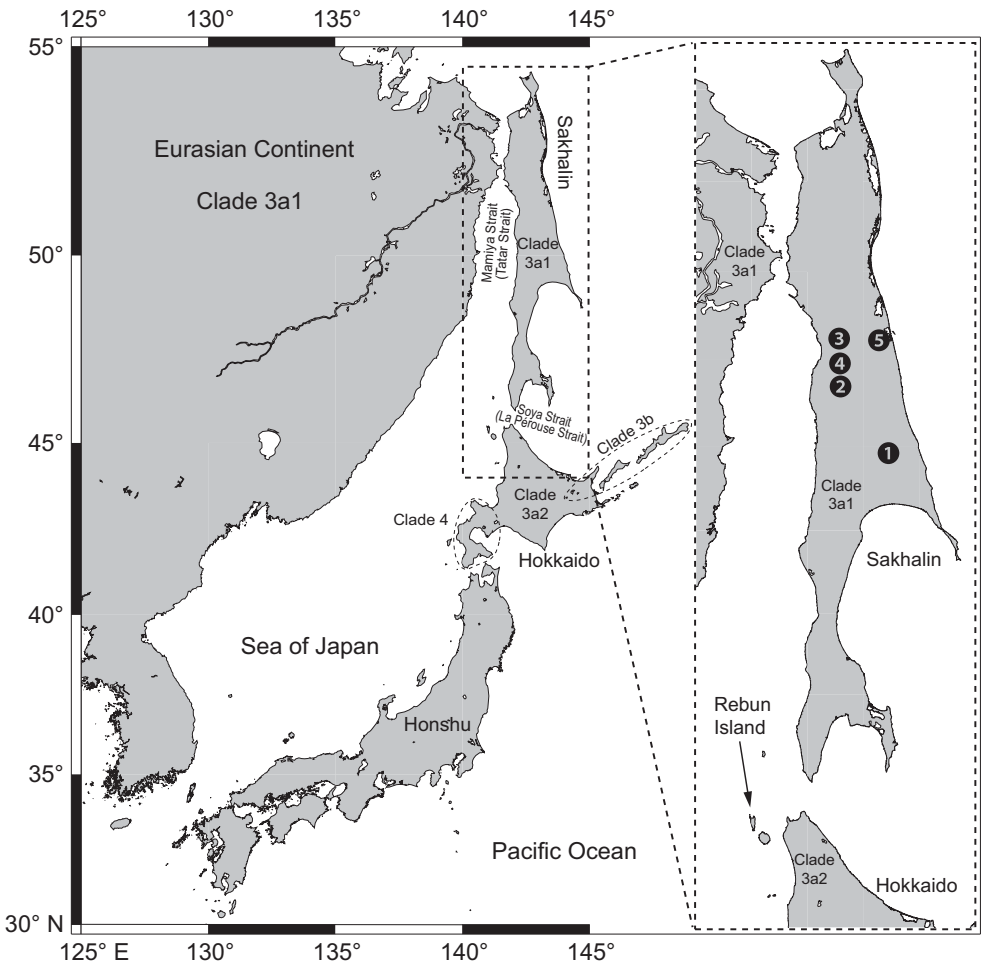

Fig. 1 Locations of archaeological sites where brown bear remains were excavated, and collecting localities for modern brown bears on Sakhalin Island. Black circles indicate collecting localities, with the numbers corresponding to locality numbers in column four (Table S1): (1) Smirnykh region (modern) and Ostantseyava Cave (archaeological), Vaida Mountain; (2) Beloye Village, Tymovsky region (modern); (3) Puzi-1 site, Ado-Tymovo (archaeological); (4) Ado-Tymovo (modern); (5) Ygvo-village, Nogliksky region (modern). The map also shows the geographical distributions of previously reported clades

As well as contributing to knowledge of the migration history of brown bears, genetic information on Sakhalin bears can help to further understand the archaeology of the bear-sending ceremony of the prehistoric people on Sakhalin and Hokkaido. Hallowell (1926) mentioned that brown bears have been involved in northern hemispheric cultures such as ceremonies and rituals since prehistoric times. The bear-sending ceremony in Japan is known as "Iyomante" in the Ainu Culture (13–20th centuries, A.D.) of Hokkaido, where this animal is God of mountains (reviewed by Fitzhugh and Dubreuil 1999; Masuda et al. 2001). Many brown bear remains involved in this ritual activity have been found (Oba and Ohyi 1976, 1981) in archaeological sites of the Okhotsk Culture on Rebun Island (81.33 km^2), located off the northwest coast of Hokkaido, but not within the native distribution of the brown bear (see Fig. 1). Masuda et al. (2001) analyzed mtDNA sequences from bear skulls from the Kafukai-1 site on Rebun and concluded that ancient people likely transported the skulls there from several regions on Hokkaido for use in the bear-sending ceremony. It remained unclear, however, whether the bear remains originated from Hokkaido or Sakhalin, because of a paucity of information from Sakhalin.

Our study used the APLP method to identify haplogroups among ancient and modern brown bears from Sakhalin, in conjunction with PCR amplification and direct sequencing of the mtDNA control region for phylogeny reconstruction. Here we present our results and discuss the origin of archaeological brown bear remains found on Rebun Island.

Material and methods

Samples, dating, and DNA extraction

Bone fragments and a tooth from 27 ancient brown bears excavated from archaeological sites (Holocene strata) on Sakhalin, and bones from five modern brown bears from the island, were collected and stored by S. V. Gorbunov at the Tymovsk Museum, and are currently at the Museum of Institute of Plant and Animal Ecology, Ural Branch, Russian Academy of Sciences (Fig. 1; Table S1).

Bear bone samples were selected from the deepest and the uppermost strata of the Puzi-1 site (Gorbunov 2002). The ^{14}C dates for two samples (753–187 yBP: SPb-3077 and SPb-3098; Table S1) were determined with the conventional method by using a Quanthus 1220 liquid scintillation counter at the Department of Geology and Geoecology, Herzen State Pedagogical University. Previous studies provided dates from Ostantsevaya Cave (9620–6455 yBP: Kuzmin et al. 2005) and the Ado-Tymovo-6 site (550–180 yBP: Alekseeva et al. 2004; Alekseeva and Gorbunov 2012) (Table S1).

Total DNA was extracted from samples as described by Masuda et al. (2001) and Mizumachi et al. (2020). Bone fragments were powdered by using sandpaper or an electric drill in a clean plastic box placed in a laminar flow cabinet (biological safety cabinet) sterilized with ultraviolet illumination. In all laboratory work, disposable plastic gloves, tubes, and tips were used to prevent contamination with extraneous DNA. Negative control reactions lacking DNA template were performed to confirm the absence of contaminating external DNA in reaction mixtures.

PCR amplification and nucleotide sequencing

To determine partial mtDNA control region sequences from the ancient specimens, three overlapping fragments were amplified with PCR using three primer-sets: UR6/UR3 (Masuda et al. 2001), mtF/mtR (Hänni et al. 1994), and UR4/UR7 (Masuda et al. 2001). When no fragment between the UR6 and UR3 primer positions was amplified, possibly due to the occurrence of T/C repeat regions, the other two sets of primers (UR-F1/UR-R1 and UR-F2/UR-R2; Mizumachi et al. 2020) were used to avoid amplification of the T/C repeat region. Reactions were performed in 20 μl volumes, each containing 10 μl of Multiplex PCR Master Mix (Qiagen), 0.2 μl each of the forward and reverse primers (25 pmol/μl), 0.4 μl of bovine serum albumin (BSA, 0.4 μg/μl), 2 or 3 μl of DNA extract, and 6.2 or 7.2 μl of distilled water. Thermal cycling conditions were 95 °C for 15 min; 35 cycles of 94 °C for 30 s, [50 °C (UR6/UR3), 55 °C (mtF/mtR), 65 °C (UR4/UR7), 55 °C (UR-F1/UR-R1), or 55 °C (UR-F2/UR-R2)] for 2 min, and 72 °C for 1 min; and 72 °C for 10 min. To check for PCR amplification, 5 μl of each PCR product and 1 μl of loading dye (Toyobo) were electrophoresed on a 2% agarose gel; the gel was stained with ethidium bromide, and bands were visualized with ultraviolet illumination. PCR products for direct sequencing were purified with a QIAquick PCR Purification Kit (Qiagen).

Cycle sequencing was done using the same primers as for the initial amplification. PCR was performed with a BigDye v1.1 Cycle Sequencing Kit (Applied Biosystems, ABI) in 10 μl volumes, each containing 1 μl of the purified PCR product, 1.75 μl of 5×BigDye sequencing Buffer (ABI), 0.5 μl of BigDye Terminator Reaction Mix (ABI), 1.6 μl of the primer (1 pmol/μl), and 5.15 μl of distilled water. Cycle sequencing conditions were 96 °C for 1 min, and 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 2 min. Cycle sequencing products were purified by isopropanol precipitation and a 70% ethanol wash, and then dissolved in 10 μl of formamide for sequence determination with an ABI 3730 DNA Analyzer. Since ancient DNA is usually deteriorated and fragmented, PCR amplification and sequencing were performed twice to three times for each fragment to confirm the reproducibility of the sequence data. Sequences obtained in this study were deposited in the DNA Data Bank of Japan (DDBJ) under accession nos. LC548407–LC548414 (Table 1).

Table 1 Mitochondrial DNA haplogrouping by the APLP analysis and direct sequencing on ancient and modern brown bear samples from Sakhalin. Sample nos. refer to those in Table S1

Sample no.	SNP sites									APLP haplogroup (clade)	mtDNA control region haplotype sequence (sequence length, bp) Accession number
	7257	16259	7770	10116	11585	8392	8776	13180	9271		
1	C	A	T	A	G	G	C	T	C	3a1	SH01-4 (393) LC548410
2	C	A	T	A	G	G	C	T	C	3a1	SH01-1 (390) LC548407
3	C	A	T	A	G	G	C	T	C	3a1	SH01-4 (393) LC548410
4	C	A	T	A	G	G	C	T	C	3a1	SH01-3 (391) LC548409
5	C	A	T	A	G	G	C	T	C	3a1	SHS01 (254) LC548414
6	C	A	T	A	G	G	C	T	C	3a1	SH01-1 (390) LC548407
7	C	A	T	A	G	G	C	T	C	3a1	SH01-3 (391) LC548409
8	C	A	T	A	G	G	C	T	C	3a1	SHS01 (254) LC548414
9	C	A	T	A	G	G	C	T	C	3a1	SH01-1 (390) LC548407
10	C	A	T	A	G	G	C	T	C	3a1	SH01-2 (391) LC548408
11	C	A	T	A	G	G	C	T	C	3a1	SH01-4 (393) LC548410
12	C	A	T	A	G	G	C	T	C	3a1	SH01-5 (394) LC548411
13	C	A	T	A	G	G	C	T	C	3a1	SH01-3 (391) LC548409
14	C	A	T	A	G	G	C	T	C	3a1	SH01-1 (390) LC548407
15	C	A	T	A	G	G	C	T	C	3a1	SH01-2 (391) LC548408
16	C	A	T	A	G	G	C	T	C	3a1	SH01-3 (391) LC548409
17	C	A	T	A	G	G	C	T	C	3a1	SHS01 (254) LC548414
18	C	A	T	A	G	G	C	T	C	3a1	SH01-1 (390) LC548407
19	C	A	T	A	G	G	C	T	C	3a1	SH01-1 (390) LC548407
20	C	A	T	A	G	G	C	T	C	3a1	SH01-3 (391) LC548409
21	C	A	T	A	G	G	C	T	C	3a1	SH01-1 (390) LC548407
22	C	A	T	A	G	G	C	T	C	3a1	SH01-3 (391) LC548409
23	C	A	T	A	G	G	C	T	C	3a1	SH01-3 (391) LC548409
24	C	A	T	A	G	G	C	T	C	3a1	SH01-3 (392) LC548409
25	C	A	T	A	G	G	C	T	C	3a1	SH02 (391) LC548412
26	C	A	T	A	G	G	C	T	C	3a1	SH01-1 (390) LC548407
27	C	A	T	A	G	G	C	T	C	3a1	SHS01 (254) LC548414
28	C	A	T	A	G	G	C	T	C	3a1	SH01-1 (390) LC548407
30	C	A	T	A	G	G	C	T	C	3a1	SH03 (390) LC548413
31	C	A	T	A	G	G	C	T	C	3a1	SH01-1 (390) LC548407
32	C	A	T	A	G	G	C	T	C	3a1	SH01-3 (391) LC548409
34	C	A	T	A	G	G	C	T	C	3a1	SHS01 (254) LC548414

Sequence analysis

Our phylogenetic analysis included mtDNA control-region sequences from our present study, sequences from modern individuals from other areas obtained from DDBJ, and sequences from ancient brown bears excavated from Rebun Island, northern Japan (Masuda et al. 2001). Sequences were aligned by using MEGA ver. 6.0 (Tamura et al. 2013). The optimal substitution model for the phylogenetic analysis determined by MEGA was K2P + G (gamma) (Kimura 1980). A neighbor-joining tree was constructed with MEGA, with nodal support assessed by analysis of 1000 bootstrap pseudoreplicates. Haplotype networks were constructed by using TCS software (Clement et al. 2000) and PopART version 1.7.2 (Leigh and Bryant 2015).

APLP method for mtDNA haplogrouping

The APLP analysis was conducted as described by Hirata et al. (2014) and Mizumachi et al. (2020). PCR amplifications were performed in 10 μl volumes, each containing 5.0 μl of 2×Qiagen Multiplex PCR Master Mix (Qiagen), 1.5 μl of primer mixture, 0.2 μl of BSA (20 mg/ml), 1–2 μl of DNA extract, and 1.3–2.3 μl of distilled water. Two separate multiplex PCR

mixtures (sets A and B) were prepared to detect several different SNPs simultaneously. Multiplex PCR set A detects SNP polymorphism 7257C/T, 16259A/G, 7770T/C, and 10116G/A; set B detects 11585G/A, 8392A/G, 8776C/T, 13180T/C, and 9271T/C. Primer sequences and combinations for each multiplex PCR are given in Hirata et al. (2014). The PCR conditions for both sets were 95 °C for 15 min; 35–45 cycles of 94 °C for 10 s, 54 °C for 10 s, and 72 °C for 5 s; and 72 °C for 3 min. To check amplifications, 3 μl from each PCR reaction were mixed with 1 μl of loading dye (Toyobo) and electrophoresed on a 10% native polyacrylamide gel (19:1 acrylamide:bisacrylamide) in Tris (12.5 mM)-glycine (96 mM) running buffer; gels were stained with ethidium bromide and bands visualized under ultraviolet illumination. For DNA samples where multiplex PCR did not provide results, we performed singleplex PCR in 10 μl volumes, each containing 5.0 μl of 2×Qiagen Multiplex PCR Master Mix (Qiagen), 0.5 μl of a mixture of two forward primers and one reverse primer (25 pmol/μl each primer), 0.2 μl of BSA (20 mg/ml), 1–2 μl of DNA extract, and 2.3–3.3 μl of distilled water. The PCR conditions were 95 °C for 15 min; 35–45 cycles of 94 °C for 10 s, 54–60 °C for 10 s, and 72 °C for 5 s; and 72 °C for 3 min. To confirm the reproducibility of DNA bands, each multiplex or singleplex PCR amplification was conducted two to three times.

Results

Phylogenetic analysis

We obtained partial sequences of the mtDNA control region from 32 brown bear samples from Sakhalin. Because fragments were not always amplified and successfully sequenced from each sample with primer-set UR6/UR3, the sequence length obtained was about 390 base-pairs (bp) for 27 bears and 254 bp for five bears (Table 1). The 390-bp sequences from 27 individuals comprised seven haplotypes: SH01-1 (10 bears), SH01-2 (2), SH01-3 (9), SH01-4 (3), SH01-5 (1), SH02 (1), and SH03 (1). Haplotype SH01 separated into five subtypes according to the number of T/C repeats, positioned at nucleotide sites 82–95. Haplotype SH01-4 was shared by three bears (IPAE1, IPAE20, and NOV2), as previously reported by Hirata et al. (2013); the other six haplotypes were novel. The 254-bp sequences (without the T/C repeat) from five individuals were identified as a single haplotype (SH-S1), which was identical to the corresponding 254-bp region in haplotypes SH01-1, 01-2, 01-3, 01-4, 01-5, and SH03.

Our neighbor-joining tree (Fig. 2) for control-region haplotypes (390 bp) shows three clades (3a, 3b, and 4). In clade 3a, haplotypes from Hokkaido comprise subclade 3a2 with a bootstrap value of 29%. While the Sakhalin samples identifiable with clade 3a1 do not clearly form a clade, probably because of insufficient sequence length, the APLP analysis (below) indicates they represent canonical clade 3a1.

In the haplotype network in Fig. 3, all three haplotypes (SH01, SH02, and SH03) from Sakhalin are grouped with clade 3a1. Clades 3b and 4, containing individuals from eastern and southern Hokkaido, respectively, are genetically distant from groups 3a1 and 3a2, which is consistent with the results in Fig. 2.

APLP haplogrouping of brown bears on Sakhalin

We also analyzed all samples from Sakhalin by the APLP method. All 32 haplotypes obtained from ancient and contemporary specimens grouped in clade 3a1 (lineage of eastern continental Eurasia), regardless of the sequence length obtained by direct sequencing (Table 1; Figs. 2 and 3). The 32 brown bear individuals from Sakhalin shared nucleotides at all SNP sites detected by APLP (Table 1). Together, the APLP and direct sequencing data clearly show that the mtDNA sequences from brown bears on Sakhalin belong to clade 3a1.

Discussion

Phylogenetic position of brown bears on Sakhalin

Brown bears representing three mtDNA clades (3a2, 3b, and 4) are thought to have migrated from continental Eurasia to Hokkaido via Sakhalin Island, though at different times (Matsuhashi et al. 1999; Hirata et al. 2013). Based on the complete mitochondrial genome from one individual, however, Hirata et al. (2013) reported that the Sakhalin brown bear groups in clade 3a1 (lineage of eastern continental Eurasia). This suggested the possibility that no clades previously found on Hokkaido occur on Sakhalin. Brown bear remains occur among most of the mammalian remains excavated from late Pleistocene and Holocene strata on Sakhalin (Kuzmin et al. 2005; Kirillova and Tesakov 2008; Kirillova et al. 2009). To determine whether mtDNA clades 3a2, 3b, and 4 are represented on Sakhalin, we analyzed samples from 32 ancient and modern brown bears from the island. All mtDNA sequences from these samples grouped in clade 3a1, which is the sister clade to 3a2 currently distributed on Hokkaido. This result suggests that no migrants from other mtDNA clades may have arrived on Sakhalin within the last thousand years. In the present study, however, because the sampling area was biased at the centre of the island, the possibility that the results were affected by it cannot be ruled out. Future study using samples throughout the island is required.

Sequences determined by direct sequencing showed genetic variation within clade 3a1 on Sakhalin, indicating that some microevolution has occurred there. The APLP data allow the assignment of haplogroups, but more precise genetic information was obtained in combination with direct sequencing. Haplotype SH01-4 in clade 3a1 had already been detected in

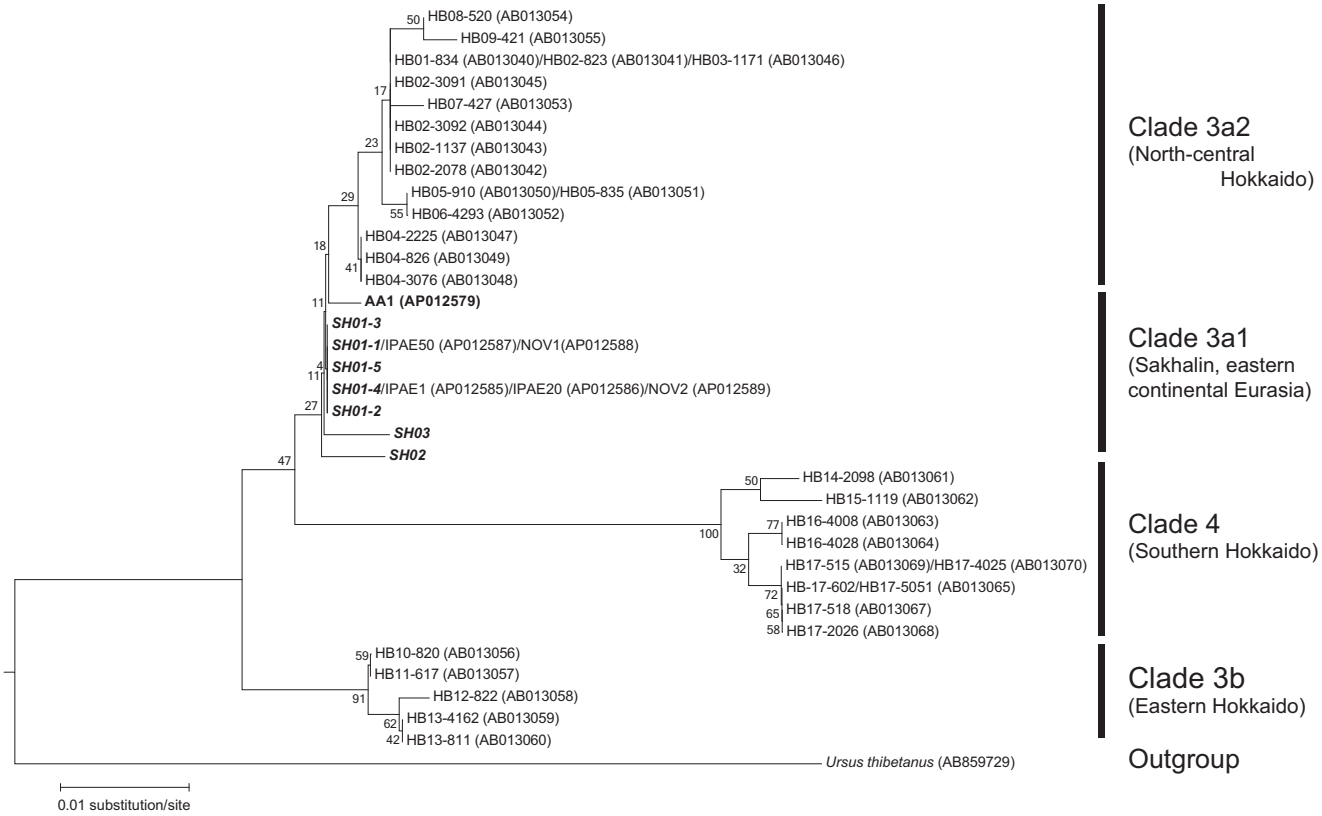

Fig. 2 Neighbor-joining tree for mitochondrial control region sequences (about 390 bp) from ancient and modern brown bears on Sakhalin and other areas. One sequence from the Asiatic black bear (*Ursus thibetanus*) is included as an outgroup. Numbers near nodes are bootstrap values in percent. Haplotypes in bold italic font are those from ancient bears on Sakhalin, sequenced in this study. Haplotype AA1 is from a modern brown bear on Sakhalin, reported by Hirata et al. (2013). Haplotypes reported by Matsuhashi et al. (1999) and Hirata et al. (2013) are followed by accession numbers in parentheses

three bears (IPAE1, IPAE20, and NOV2) from western Russia (Hirata et al. 2013).

During the last glacial period, a land bridge extended from continental Asia via Sakhalin to Hokkaido. As sea levels rose

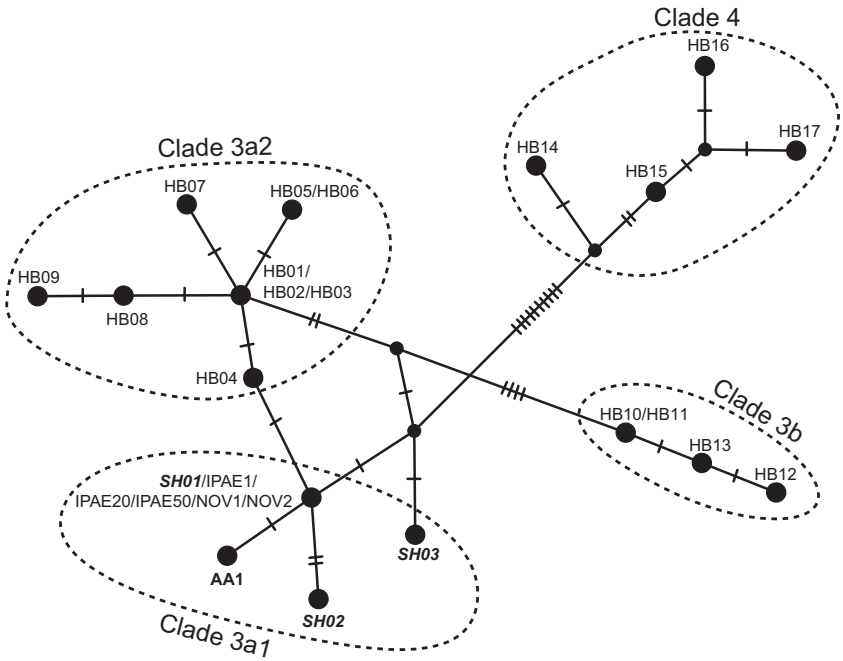

Fig. 3 Haplotype network for mitochondrial control region sequences (about 390 bp) from ancient and modern brown bears from Sakhalin and other areas. Larger black circles indicate haplotypes; smaller unlabeled black circles indicate intermediate haplotypes not detected. Haplotypes in bold italic font are from Sakhalin, sequenced in this study. Haplotype AA1 is from a modern bear Sakhalin, reported by Hirata et al. (2013). Each hatch mark on a line between circles indicates one nucleotide substitution. Haplotypes reported by Matsuhashi et al. (1999) and Hirata et al. (2013) are labeled in plain bold font

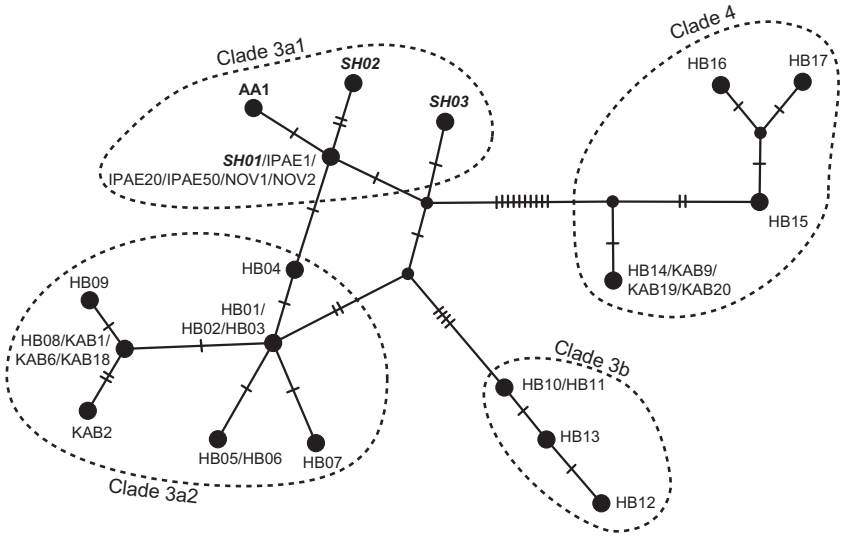

Fig. 4 Haplotype network for mitochondrial control region sequences (about 360 bp) from brown bears from Rebun Island, Sakhalin, and Hokkaido. Sequences from Rebun Island (KAB1, KAB2, KAB6, KAB9, KAB18–20) are from Masuda et al. (2001), and those from Hokkaido (HB01–17) are from Matsuhashi et al. (1999). Each hatch mark on a line between circles indicates one nucleotide substitution

after the Last Glacial Maximum, Soya Strait between Sakhalin and Hokkaido (about 55 m in depth; Igarashi and Zharov 2011) opened earlier than Mamiya Strait (about 12 m in depth) between Sakhalin and the continent. In other words, the land connection that allowed the migration of mammals between continental Asia and Sakhalin remained in place more recently than the connection between Sakhalin and Hokkaido. Our data indicate that the ancestors of the Sakhalin brown bear population shared clade 3a1, which had become differentiated from clade 3a on the Eurasian continent and that they migrated independently to Sakhalin after migration of the clade 3a2 population to Hokkaido, in agreement with Hirata et al. (2013). The haplotype sequences in clade 3a1 from Sakhalin and the Eurasian continent comprise a multifurcate group (Fig. 2), whereas clade 3a2 in the Hokkaido brown bears is well resolved. This indicates that clade 3a1 spread relatively rapidly in eastern continental Eurasia (Hirata et al. 2014) and then to Sakhalin. By the time clade 3a1 reached Sakhalin across the land bridge, Soya Strait had already opened between Sakhalin and Hokkaido. The phylogenetic data obtained in our study is thus consistent with the zoogeographical history of brown bears on the continent, Sakhalin, and Hokkaido during the Pleistocene and Holocene.

Archaeological implications of phylogenetic data on Sakhalin bears

Many brown bear remains with evidence of having been used in rituals were excavated from the Okhotsk Cultural archaeological site (Kafukai-1) on Rebun Island (Oba and Ohyi 1976, 1981), although this small island is not within the native distribution of brown bears. A phylogenetic analysis by Masuda et al. (2001) indicated that brown bear skulls from this site used for the bear-sending ceremony originated on Hokkaido; no data on Sakhalin bears were available at that time.

To further investigate whether the bear remains from the Kafukai-1 site originated on Sakhalin, we reconstructed a network of haplotypes obtained from remains from Kafukai-1 (Masuda et al. 2001), contemporary Hokkaido bears (Matsuhashi et al. 1999), and the Sakhalin bears sequenced in our study (Fig. 4). The network shows clearly that among seven haplotypes from Kafukai-1, four (KAB1, KAB2, KAB6, and KAB18 in Fig. 4) belong to clade 3a2 (north-central Hokkaido lineage) and three (KAB9, KAB19, and KAB20) to clade 4 (southern Hokkaido lineage). Thus, the bears detected as remains on Rebun Island appear to have been transported there from Hokkaido by the ancient people of the Okhotsk Culture (4th–13th centuries C.E.) (Amano 2016), as proposed by Masuda et al. (2001). These results suggest that the Okhotsk Culture on Rebun was culturally more closely related to Hokkaido than to Sakhalin, at least with regard to the bear-sending ceremony.

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