Molecular phylogeography of the brown bear (*Ursus arctos*) in eastern Eurasia

(ユーラシア東部におけるヒグマの分子系統地理学的研究)

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Doctoral Dissertation

March 2017

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Acknowledgments

I would like to express my sincere gratitude to Professor Ryuichi Masuda (Hokkaido University) for instruction through this study and giving me an opportunity to start my scientific research. I am grateful to Professor Takeo Horiguchi (Hokkaido University), Professor Kazuhiro Kogame (Hokkaido University), Professor Masaoki Takagi (Hokkaido University), and Associate Professor Toru Katoh (Hokkaido University) for reviewing the manuscript and helpful suggestions. I also thank Professor Matthew H. Dick (Hokkaido University) for invaluable comments and editing the manuscript. I am very grateful to Dr. Tsutomu Mano (Hokkaido Research Organization), Dr. Alexei V. Abramov (Russian Academy of Sciences), Dr. Gennady F. Baryshnikov (Russian Academy of Sciences), Dr. Pavel A. Kosintsev (Russian Academy of Sciences), Dr. Alexandr A. Vorobiev (Russian Academy of Sciences), Dr. Evgeny G. Raichev (Trakia University), Dr. Hiroshi Tsunoda (Saitama Institute of Environmental Science), Dr. Yayoi Kaneko (Tokyo University of Agriculture and Technology), Dr. Koichi Murata (Nihon University), Mr. Daisuke Fukui (Asahikawa Municipal Asahiyama Zoological Park) and Kobe Municipal Oji Zoo, Himeji City Zoo, Asahikawa Municipal Asahiyama Zoological Park for providing invaluable specimens and helpful suggestions in this study. I gratefully acknowledge the past and present members of our laboratory for constructive and fruitful discussion. I would like to thank the Japan Student Services Organization (JASSO) and Japan Society of the Promotion of Science (JSPS) for financial supports during the graduate course, and without their financial supports I could not continue my study. A part of the study was supported by a Grant-in-Aid for JSPS Fellows (Research Project Number:14J02026) from the Japan Society of the Promotion of Science. Finally, I sincerely express my great gratitude to my parents and family for understanding my study and continuous encouragement.

Abstract

The mammal fauna of the Japanese islands, especially Hokkaido had experienced the repeated colonization of the animals from the Eurasian Continent during the Pleistocene glacial periods. The brown bear (*Ursus arctos*) is widely distributed on the Holarctic region, and occurs only on Hokkaido and adjacent southern Kuril Islands (Kunashiri and Etorofu) in Japan. To clarify the migration history of the brown bears in Hokkaido and adjacent insular populations, resolving detailed phylogenetic relationships and estimating the precise timings of the population immigrations to Hokkaido are needed. In addition, it is necessary to reveal the detailed genetic structure of the brown bears across the Eurasian Continent, which could be the evolutionary source of the Hokkaido brown bears. Furthermore, the brown bear shows male-biased dispersal and female philopatry. Thus, most of the previous studies using maternally inherited mitochondrial DNA (mtDNA) markers might not represent the comprehensive phylogeographic history of the brown bears. Considering the sex-biased migration for the evolutionary history of the brown bear is essential, and it is needed to uncover the paternal phylogeographic structuring of the brown bear.

In Chapter I, complete mtDNA sequences of 35 brown bears from Hokkaido, the southern Kuril Islands (Kunashiri and Etorofu), Sakhalin Island, and the Eurasian Continent (Ural Mountains, European Russia, Bulgaria, and Tibet), and those of four polar bears were analyzed. Based on these sequences, I reconstructed the maternal phylogeny of the brown bear and estimated divergence times of brown bear lineages. The brown bear on Hokkaido was divided into distinct maternal lineages (central, eastern, and southern Hokkaido), and three independent migrations to the island could have occurred. Although Sakhalin Island is considered to be a transient area for ancestors of the Hokkaido brown bears, the Sakhalin brown bear grouped with eastern European and western Alaskan brown bears. Thus, Sakhalin brown bear did not originate at the same time as the dispersal of brown bears into Hokkaido. Brown bears in southern Kuril Islands (Kunashiri and Etorofu) adjacent to Hokkaido Island were closely related to eastern Hokkaido brown bears, and could have diverged from the eastern Hokkaido lineage after formation of the channel between Hokkaido and the southern Kuril Islands.

In Chapter II, I developed an amplified product length polymorphism (APLP) analysis for mtDNA-haplogrouping of brown bear specimens by detecting haplogroup-specific SNPs. Using this newly developed method, I successfully analyzed 54 historical skin specimens up to 170-year-old collected widely across continental Eurasia, and verified the validity of this method. Some of the same brown bear mtDNA haplogroups as those occurring in eastern Hokkaido and eastern Alaska were found within the Eurasian Continent (Altai Mountains and Caucasus Mountains). This result shows that brown bears in eastern Hokkaido and eastern Alaska descended from a common ancestor on the Eurasian Continent, and that brown bears occupied several refugia in southern Asia during the Last Glacial Maximum.

In Chapter III, to elucidate the paternal genetic structure around northeastern Asia and assess the influence of male-mediated gene flow for the genetic connectivity among and within intraspecific populations, paternally inherited Y-chromosomal DNA sequences and Y-linked microsatellites of 124 brown bears from Hokkaido, southern Kuril Islands (Kunashiri and Etorofu), Sakhalin, and the Eurasian Continent (Kamchatka Peninsula, Ural Mountains, European Russia, and Tibet) were analyzed. The paternal lineage of Hokkaido brown bears was differentiated from those of the continental Eurasian/North American populations, and showed the lack of recent genetic connectivity from the continental populations. Within Hokkaido Island, weak spatial genetic structuring of the paternal lineages was found, and this is supposed to be formed through male-mediated gene flow among natal populations within Hokkaido Island. Different dispersal patterns between males and females of the brown bear combined with the founder effect and subsequent genetic drift could have contributed to the makeup of the brown bears on Etorofu Island, which is composed of the inconsistent origins of the maternal and paternal lineages.

The results demonstrate that brown bear phylogeography around Hokkaido is considerably complicated than previously expected only from the mtDNA studies. The present study demonstrated the importance of using sex-specific uni-parentally inherited markers, both maternally (mtDNA) and paternally (Y-chromosomal DNA) inherited markers to study the phylogeography of the animals, especially exhibiting the extensive sexual differences in behavior.

General Introduction

The mammal fauna of the Japanese islands involves high species diversity and endemic elements, and it can be divided into three biogeographic regions; Hokkaido, Hondo (Honshu-Shikoku-Kyushu), and Ryukyu (Kawamura 1991; Dobson 1994). The level of endemism is high in Hondo and Ryukyu, whereas that is low in Hokkaido due to the influence of continuous connection to the Eurasian Continent through Sakhalin via land bridges during the Pleistocene glacial periods, and experienced the repeated colonization of the animals from the Eurasian Continent (Dobson 1994; Millien-Parra and Jaeger 1999).

One of the large terrestrial mammals, the brown bear (*Ursus arctos*) is only distributed on Hokkaido and adjacent southern Kuril Islands (Kunashiri and Etorofu) in Japan (Ohdachi et al. 2009); however, it was once distributed on Honshu in mid- to late Pleistocene evidenced by the presence of some fossils (Takakuwa et al. 2007). After the reduction and extinction of the brown bear in Honshu, the ecological niche of brown bears is considered to be replaced by the Japanese black bears (*Ursus thibetanus*), which is currently distributed widely on Honshu (Wu et al. 2015). The brown bear has omnivorous diet and highly adapted to the various range of habitats from the northern arctic tundra to dry desert (Servheen et al. 1999). The distribution of the brown bear covers broad range of the Holarctic region in Europe, Asia, and North America, and shows the most widespread distribution among eight extant Ursidae species.

Phylogeographical analyses are a discipline that deals with the spatial arrangements of genetic lineages, especially within and among closely related species (Avise et al. 1987; Avise 2000, 2009). Because of wide range of distribution, geographical variability of ecological and morphological traits besides the availability of subfossil specimens for genetic analyses of historical populations, phylogeography of the brown bears has been most intensively studied and becoming a useful model for Pleistocene and Holocene phylogeography among wild mammals (Davison et al. 2011). Furthermore, brown bears show the remarkable behavioral characteristics, male-biased dispersal (males move and disperse from its birthplace to where it reproduces) and female philopatry (females stay and breed at or near their places of origins) (McLellan and Hovey 2001; Proctor et al. 2004; Støen et al. 2006; Zedrosser et al. 2007). Due to this behavioral trait, pronounced geographic genetic structuring can be detected by using

maternally inherited mitochondrial DNA (mtDNA) of the brown bears, and this species is useful for studying broad range of intraspecific phylogeographic patterns. Previous phylogeographic studies of the brown bear based on mtDNA control region sequences having high mutation rate showed intraspecific genetic structuring worldwide in Europe, Asia, and North America (Taberlet and Bouvet 1994; Kohn et al. 1995; Talbot and Shields 1996; Masuda et al. 1998; Waits et al. 1998; Matsuhashi et al. 1999, 2001; Shields et al. 2000; Miller et al. 2006; Galbreath et al. 2007; Saarma et al. 2007; Saarma and Kojola 2007; Korsten et al. 2009; McCarthy et al. 2009; Murtskhvaladze et al. 2010). In addition, historical and exterminated populations of the brown bears is elucidated by ancient DNA analyses (Barnes et al. 2002; Matheus et al. 2004; Valdiosera et al. 2007, 2008; Calvignac et al. 2008, 2009).

Due to the complex geological background that the continuous connection between Hokkaido and the Eurasian Continent through Sakhalin via land bridges during the Pleistocene glacial periods, the brown bears in northeastern Asia, especially on Hokkaido Island, exhibit unique genetic population structuring (Masuda et al. 1998; Matsuhashi et al. 1999, 2001). The brown bears on Hokkaido constisted of three distinct mtDNA lineages (central, eastern, and southern Hokkaido), and each population on Hokkaido is more closely related to the other populations on the Eurasian or North American Continent than to neighboring Hokkaido populations. Three lineages are thought to have diverged on the Eurasian Continent prior to dispersal onto Hokkaido via land bridges in different three periods: the southern lineage colonized first, followed by the eastern and then the central lineages. To further clarify the migration history of the brown bears in Hokkaido and adjacent insular populations, therefore more detailed studies on the phylogenetic relationships and precise timings of the population immigrations to Hokkaido are required. In addition, it is necessary to reveal the detailed genetic structure of the brown bears across the Eurasian Continent, which could be the evolutionary source of the Hokkaido brown bears.

Although male-biased dispersal was also found in Hokkaido brown bears from the home range size and habitat use, these sizes were smaller than those reported from North America and Europe (Sato et al. 2008). The sex-biased dispersal and reproductive system of ursine bears is presumed to have extensively contributed to the molecular evolutionary history of the ursids,

including the brown bear (Nakagome et al. 2008). Thus, most of the previous studies using maternally inherited mtDNA markers might not represent the comprehensive phylogeographic history of the brown bears since it lacks the effects of the male-mediated gene flow.

In addition to the mtDNA genetic studies, brown bears in northeastern Asia, especially in Hokkaido and southern Kuril Islands show morphological unique features compared to those in the Eurasian Continents. The skulls of Hokkaido brown bears are generally smaller than those of other northeastern Asian brown bears. Baryshnikov et al. (2004) reported that the brown bears from Hokkaido and southern Kuril Islands are morphometrically well distinguished from other brown bears inhabiting the regions around the Okhotsk Sea, and can be classified an identical subspecies *Ursus arctos ferox*. The Hokkaido brown bear population shows morphometrically geographical heterogeneity: clinal increase of the cranium from southwest to northeast on Hokkaido (Yoneda and Abe 1976; Ohdachi et al. 1992). It is intriguing that morphometrical test of the phylogeographical patterns of the modern population demonstrates that females were remarkably differentiated among the three Hokkaido populations; however, males belonging to central Hokkaido and southern Hokkaido populations showed similarity to each other, whereas males in eastern Hokkaido (including southern Kuril Islands) population was distinct (Baryshnikov et al. 2004). This suggests the possibility that the male-biased dispersal might have extensively contributed to the sexual morphometric differences between males and females in the brown bears. Considering the sex-biased migration for the evolutionary history of the brown bear is essential, and it is needed to uncover the paternal genetic relationships within and among insular (Hokkaido, southern Kuril Islands, and Sakhalin) and continental (Eurasian and North American) populations.

In Chapter I, to further elucidate the migration history of the brown bear on Hokkaido Island, I analyzed the complete mtDNA sequences of 35 brown bears from Hokkaido, the southern Kuril Islands (Etorofu and Kunashiri), Sakhalin Island, and the Eurasian Continent (Ural Mountains, European Russia, Bulgaria, and Tibet), and those of four polar bears. Based on these sequences, I reconstructed the maternal phylogeny of the brown bear and estimated divergence times to investigate the timings of brown bear migrations, especially in northeastern Eurasia.

In Chapter II, using complete sequences of brown bear mtDNA in Chapter I, I have detected scattered single nucleotide polymorphisms (SNPs) that define distinct mtDNA haplogroups in phylogeographical studies. The degraded DNA in historical samples such as stuffed and excavated specimens, however, is often not suitable for sequence analyses. To address this problem, I developed an amplified product length polymorphism (APLP) analysis for mtDNA-haplogrouping brown bears using the haplogroup-specific SNPs. I verified the validity and utility of this method by analyzing 67 skin samples, up to 170 years old, from brown bears collected widely across the Eurasian Continent.

In Chapter III, to elucidate the paternal genetic structure around northeastern Asia and assess the influence of male-mediated gene flow for the genetic connectivity among and within intraspecific populations, paternally inherited Y-chromosomal DNA sequences and Y-linked microsatellites of 124 brown bears from Hokkaido, southern Kuril Islands (Kunashiri and Etorofu), Sakhalin, and the Eurasian Continent (Kamchatka Peninsula, Ural Mountains, European Russia, and Tibet) were analyzed.

Chapter I

Molecular phylogeography of the brown bear *Ursus arctos* in northeastern Asia based on analyses of complete mitochondrial DNA sequences

Introduction

The brown bear (*Ursus arctos*) is widely distributed in the Holarctic region, from Europe to North America. Among the continental islands of northeastern Asia, brown bears occur on Hokkaido Island and the southern Kuril Islands. The phylogeography of the brown bear has been studied in Europe, North America, and Asia. In addition, nomenclature for geographically distinct mitochondrial DNA (mtDNA) clades was proposed by Leonard et al. (2000) and extended by Barnes et al. (2002), Miller et al. (2006), and Calvignac et al. (2008, 2009).

European brown bears are divided into two major lineages, eastern (clade 3a) and western (clade 1), based on mtDNA control region sequences (Taberlet and Bouvet 1994). The eastern lineage is widely distributed on the Eurasian Continent, from northeastern Europe to Far Eastern Russia (Saarma et al. 2007; Korsten et al. 2009; Murtskhvaladze et al. 2010; Tammeleht et al. 2010). The western lineage comprises two groups: the Iberian lineage (clade 1a) and the Balkan/Italian lineage (clade 1b) (Taberlet and Bouvet 1994; Kohn et al. 1995). In North America, four clades have been identified among extant brown bears: clade 2a is distributed in the Admiralty, Baranof, and Chichagof (ABC) island group in southeastern Alaska, clade 3a in western Alaska, clade 3b in eastern Alaska, and clade 4 in continental North America (Talbot and Shields 1996; Waits et al. 1998). MtDNA analyses show the polar bear (*Ursus maritimus*) to be embedded within the brown bear clade and to be most closely related to the ABC islands brown bear (clade 2a) (Cronin et al. 1991; Shields and Kocher 1991; Talbot and Shields 1996; Shields et al. 2000; Lindqvist et al. 2010; Miller et al. 2012).

Phylogenetic analyses of the mtDNA control region and cytochrome *b* (cyt *b*) showed that the brown bear population on Hokkaido Island, Japan, comprises three lineages (central, eastern, and southern: Matsuhashi et al. 1999): these lineages differ in the proportions of the skull (Baryshnikov et al. 2004; Baryshnikov and Puzachenko 2009). Matsuhashi et al. (1999) suggested that the three lineages diverged on the Eurasian Continent prior to dispersal onto Hokkaido via land bridges in different three periods: the southern lineage colonized first, followed by the eastern and then the central lineages. Matsuhashi et al. (2001) found the central Hokkaido lineage to be most closely related to clade 3a containing eastern European and western Alaskan bears, and the eastern Hokkaido lineage to be most closely related to the

eastern Alaskan lineage (clade 3b). While the mtDNA control region phylogeny showed the southern Hokkaido lineage to be most closely related to the Tibetan brown bear, the cyt *b* phylogeny did not support this relationship. In addition, Miller et al. (2006) found the southern Hokkaido lineage, which they labeled clade 3d, to group with the Tibetan brown bear (clade 5). In contrast, Korsten et al. (2009), Davison et al. (2011), and Edwards et al. (2011) found that the southern Hokkaido lineage groups with the North American lineage, labeled as clade 4. In summary, there is a possibility that the southern Hokkaido lineage groups with North American lineage, and that is Tibetan brown bears are a distinct lineage.

Interspecific phylogenies of bears have recently been reconstructed using the complete mitochondrial genome (Yu et al. 2007; Bon et al. 2008; Krause et al. 2008; Lindqvist et al. 2010; Miller et al. 2012). When this approach is applied to intraspecific analyses of bears, complete mtDNA sequences have the potential to greatly facilitate phylogeographic interpretation (Davison et al. 2011). Shorter mtDNA regions (e.g., control region and cyt *b*) used for phylogenetic analyses may bias time estimates due to homoplasy and are generally associated with considerable uncertainty (Davison et al. 2011).

To further elucidate the migration history of the three lineages of brown bears on Hokkaido Island, it is important to clarify the phylogenetic relationships between the southern Hokkaido and Tibetan brown bears, and among brown bears from Hokkaido and neighboring areas such as the southern Kuril Islands (Etorofu and Kunashiri) and Sakhalin. In the present study, I analyzed complete mtDNA sequences from 35 brown bears from Hokkaido, the southern Kuril Islands (Etorofu and Kunashiri), Sakhalin, and the Eurasian Continent (Russia, Bulgaria, and Tibet) to reconstruct maternal phylogeny. In addition, I determined the complete mtDNA sequences from four polar bears. Using Bayesian analysis, I inferred times of divergence of these bear populations to shed light on their migration history.

Materials and Methods

Samples and DNA Extraction

Muscle or liver samples from 20 brown bears collected on Hokkaido Island were obtained from the Environmental and Geological Research Department, Hokkaido Research Organization.

Bear tissue samples were also obtained from the following regions and sources: four samples from Etorofu Island, one from Kunashiri Island, one from southern Sakhalin, and two from Novgorod (Zoological Institute, Russian Academy of Sciences, St. Petersburg); three samples from the Ural Mountains (Institute of Plant and Animal Ecology, Russian Academy of Sciences, Ekaterinburg); two samples from the Balkan Mountains (Trakia University, Bulgaria); hairs from two Tibetan brown bears (Kobe Municipal Oji Zoo, Japan); and blood or muscle samples from four polar bears (Asahikawa Municipal Asahiyama Zoo, Japan). Sample numbers and geographical locations are shown in Fig. 1. Total genomic DNA was extracted using the DNeasy Tissue & Blood Kit (QIAGEN) or the QIAamp DNA Micro Kit (QIAGEN), following the manufacturer's protocols.

DNA Amplification

Complete mtDNA sequences were amplified with 11 primer sets reported in Delisle and Strobeck (2002) (Table 1). In the case of poor PCR performance, 13 newly designed primer sets were used (Table 1). PCR amplifications were performed in 20 μl reaction volumes each containing 2.0 μl of 10× PCR buffer (Takara), 1.6 μl of dNTP mixture (2.5 mM each dNTP; Takara), 0.1 μl of *Taq* DNA polymerase (5 U/μl; Takara), 0.1 μl each of forward and reverse primers (25 pmol/μl), 1.0 μl of DNA extract, and 14.9 μl of distilled water. Thermal cycling conditions were 3 min at 94˚C; 30–40 cycles of 1 min at 94˚C, 1 min at 50–60˚C, and 1.5 min at 72˚C; and a final extension for 10 min at 72˚C. For expected amplicon sizes more than 2 kilo-base pairs (kbp) or for samples that showed poor PCR performance with *Taq* DNA polymerase (Takara), PCR amplifications were conducted in 20 μl reaction volumes containing 4.0 μl of 5× PrimeSTAR GXL DNA Buffer (Takara), 1.6 μl of dNTP mixture (2.5 mM each dNTP; Takara), 0.4 μl of PrimeSTAR GXL DNA Polymerase (1.25 U/μl, Takara), 0.2 μl each of forward and reverse primers (25 pmol/μl), 0.4 μl of bovine serum albumin (BSA; 0.4 μg/μl), 1.0–4.0 μl of DNA extract, and 9.2–12.2 μl of distilled water. Thermal cycling conditions were 30–40 cycles of 10 s at 98˚C, 15 s at 50–60˚C, and 2 min at 68˚C. PCR products were purified with the QIAquick Purification Kit (QIAGEN), following the manufacturer's protocol.

Sequencing

DNA cycle sequencing was performed by using the BigDye v3.1 Cycle Sequencing Kit (Applied Biosystems, ABI) with the sequencing primers reported in Delisle and Strobeck (2002) or newly designed primers (Tables 1 and 2). PCR for sequencing was performed in 10 µl volumes containing 1.75 µl of 5× BigDye Sequencing Buffer (ABI), 0.5 µl of Ready Reaction Premix (ABI), 1.0 µl of DNA template, and 5.15 µl of distilled water. Thirty cycles of (10 s at 96˚C, 5 s at 50˚C, and 4 min at 60˚C) were performed. Amplified DNA fragments were purified with isopropanol, and then formamide was added. Sequences were determined on an ABI 3730 DNA Analyzer. The exact length of the mtDNA control region was not determined due to the presence of variable-number tandem repeats (VNTRs). The genomic positions of two rRNAs, 22 tRNAs, 13 coding genes, and the control region were determined in relation to the complete mtDNA sequence of the brown bear (GenBank accession no. AF303110; Delisle and Strobeck 2002). DNA sequences generated in the present study have been deposited in the DDBJ/NCBI/EMBL databases under accession nos. AP012559−012597.

Phylogenetic Analyses

Complete mtDNA sequences for nine brown bears (AF303110, Delisle and Strobeck 2002; EU497665, Bon et al. 2008; GU573486, GU573487, GU573489, and GU573491, Lindqvist et al. 2010; JX196367, JX196368, and JX196369, Miller et al. 2012), 27 extant polar bears (AF303111, Delisle and Strobeck 2002; AJ428577, Arnason et al. 2002; GU573485 and GU573490, Lindqvist et al. 2010; JX196370, JX196371, JX196372, JX196373, JX196374, JX196375, JX196376, JX196377, JX196378, JX196379, JX196380, JX196381, JX196382, JX196383, JX196384, JX196385, JX196386, JX196387, JX196388, JX196389, JX196390, JX196391, and JX196392, Miller et al. 2012), and one ancient polar bear (GU573488, Lindqvist et al. 2010) were obtained from GenBank and included in the alignment of sequences I obtained. The complete mtDNA sequence from one cave bear (*Ursus spelaeus*) (EU327344, Bon et al. 2008) was used as an outgroup. The sequence alignment was performed using Clustal W (Clustal et al. 1994) in MEGA 5.05 (Tamura et al. 2011). Insertions and deletions (indels), VNTRs consisting of 10-bp units in the control region, and ambiguous sites were

excluded from analyses. NADH dehydrogenase subunit 6 (ND6) sequences were also excluded from the analyses because this gene is encoded on the strand (light strand) opposite in direction from the other 12 coding genes, and because its nucleotide composition is heterogeneous. Consequently, the corrected alignment used for phylogenetic inference was 15,863 bp long and included two rRNA genes, 22 tRNA genes, 12 coding genes, and the control region (excluding VNTRs).

Phylogenetic trees were reconstructed by maximum-likelihood (ML) and Bayesian inference (BI). In both the ML and BI analyses, a partition model was used in which different substitution models were applied to different gene partitions. The best-fit substitution model was determined for each partition by using the Akaike information criterion (AIC) for ML and the Bayesian information criterion (BIC) for BI, implemented in Kakusan 4 (Tanabe 2011). The ML tree was reconstructed using Treefinder version March 2011 (Jobb et al. 2004) and Phylogears 2 (Tanabe 2008) with 100 trials of the likelihood ratchet method (Vos 2003). Branching support for the ML tree was assessed by bootstrap analysis with 1000 pseudoreplicates. BI was performed with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) in two simultaneous runs of 50,000,000 Markov chain Monte Carlo (MCMC) generations, with trees for estimation of the posterior probability distribution sampled every 100 generations; the first 5,000,000 trees were discarded as burn-in.

Estimation of divergence times

Divergence times were estimated with BEAST v1.6.2 (Drummond and Rambaut 2007), with different substitution models applied to different gene partitions. The best-fit substitution model for each partition was estimated with the BIC implemented in Kakusan 4 (Tanabe 2011). The uncorrelated lognormal model was used to describe the relaxed clock. The constant size coalescent prior on the tree was used. Radiocarbon dates for ancient sequences were used to calibrate divergence ages for terminal nodes (internal calibration), including a mean age of 120 kilo years before present (kyBP) for the ancient polar bear (Lindqvist et al. 2010) and 31.87 kyBP for the cave bear (Bon et al. 2008). Posterior probability distributions of parameters including the trees were obtained by MCMC sampling. Trees were sampled every 10,000

generations from a total of 500,000,000 generations, with the first 50,000,000 generations discarded as burn-in. Acceptable mixing and convergence to the stationary distribution were checked by using Tracer v1.4 (Rambaut and Drummond 2007). The maximum clade credibility tree was produced with TreeAnnotator v1.6.2.

Results

Phylogenetic relationships within the brown bears

Complete mtDNA sequences for 35 brown bears and four polar bears were successfully determined. The ML and BI phylogenies were inferred using the newly obtained sequences and 38 previously published complete mtDNA sequences from brown bears and polar bears, with the cave bear as an outgroup. The ML and BI analyses yielded identical tree topologies showing the 73 brown and polar bears divided into eight clades (1, 2a, 2b, 3a1, 3a2, 3b, 4, and 5) (Figs. 2 and 3). Brown bears were divided into a western lineage (clades 1, 2a, and 2b) and an eastern lineage (clades 3a1, 3a2, 3b, 4, and 5), with clade 3a further subdivided into clades 3a1 and 3a2 based on five parsimony-informative sites. The polar bears formed clade 2b embedded within the western lineage, which otherwise contained brown bears, and which was the sister clade to the ABC islands group of brown bears (clade 2a).

The brown bears on Hokkaido fell into three clades: 3a2 (central Hokkaido), 3b (eastern Hokkaido), and 4 (southern Hokkaido) (Figs. 2 and 3). Clade 3a2 was the sister clade to 3a1, which contained eastern European, western Alaskan, and Sakhalin brown bears. Clade 3b (bootstrap value, 100%; posterior probability, 1.0) (Fig. 3) contained eastern Hokkaido, Etorofu, and Kunashiri brown bears (Figs. 2 and 3), with three of four individuals from Etorofu (Etorofu 2, 3, and 4) sharing the same haplotype. Southern Hokkaido bears grouped with North American bears in clade 4 (bootstrap value, 92.8%; posterior probability, 1.0), although the divergence was deep (Figs. 2 and 3). Tibetan brown bears, comprising clade 5, were basal in the eastern lineage. Two individuals from the Balkan Mountains in Bulgaria fell into different clades, sample Ua3 into clade 1 in the western lineage and sample Ua1 into clade 3a1 in the eastern lineage.

Divergence times

Divergence times were estimated by the BI method using internal calibration based on two radiocarbon-dated ancient DNA samples. This permitted the time to the most recent common ancestor (TMRCA) to be estimated for brown bear clades (Fig. 2, Table 3). TMRCA estimates are summarized in Table 3.

Discussion

Phylogeography

The estimated TMRCA between the eastern and western lineages at approximately 566 kyBP (95%HPD: 251–944 kyBP) in the present study is consistent with the oldest fossil of the brown bear reported, from the early Pleistocene (Early Biharian, approximately 1.20 million years before present: MyBP) of Europe (Rabeder et al. 2010; Wagner and Čermák 2012). The three phylogenetically distinct maternal lineages of brown bears on Hokkaido are currently allopatrically distributed (Fig. 1), as Matsuhashi et al. (1999) also found. This pattern suggests that three independent migrations to the island occurred. Hokkaido Island was connected with Sakhalin and the Eurasian Continent by land bridges until the end of the last glacial period at approximately 12 kyBP (Ohshima 1990). After the splits of the three ancestral lineages of Hokkaido brown bears on the Eurasian Continent, each lineage could have migrated to Hokkaido at different times before the disappearance of the land bridges. Tsugaru Strait between Hokkaido Island and Honshu Island of Japan was formed in the late Pleistocene approximately 100–150 kyBP (Ohshima 1990). Brown bears do not occur on Honshu Island at the present time, but brown bear fossils from mid- to late Pleistocene deposits have been reported from Honshu (Takakuwa et al. 2007). The results of the present study (Fig. 2) show that the ancestors of southern Hokkaido brown bears diverged from those of North American brown bears at approximately 194 kyBP (95%HPD: 67–399 kyBP) (Fig. 2). After dispersal to Hokkaido, bears from the southern Hokkaido population could then have dispersed southward to Honshu before Tsugaru Strait formed (approximately 100–150 kyBP); the Honshu population later went extinct.

In the present study, to discuss the migration history of the brown bear, I analyzed complete mtDNA sequences, from which the matrilineal lineages of brown bears were inferred. If such complete mtDNA datasets are applied to population modeling (e.g. Approximate Bayesian Computation: ABC approaches), the analysis would reveal details of the migration history of the brown bear population. In the present dataset, however, the specimen sampling per geographical region was biased (i.e., brown bears from Hokkaido were broadly collected, whereas brown bears from the Eurasian continent were collected from limited areas). This bias may mean that the migration history of the population was incorrectly estimated. Therefore, I did not carry out this analysis, and only discuss molecular phylogeny and divergence time estimates. Further sampling of specimens from the Eurasian continent is needed.

Relationship between brown bears in southern Hokkaido and Tibet

The southern Hokkaido brown bears grouped with North American brown bears (clade 4) rather than Tibetan brown bears (Figs. 2 and 3), a result that strongly supported the relationship previously reported by Korsten et al. (2009), Davison et al. (2011), and Edwards et al. (2011). In contrast, this resutlt was inconsistent with that of Matsuhashi et al. (2001) based on 266-bp mtDNA control region sequences. Matsuhashi et al. (2001) speculated that Tibetan brown bears might have been a relict population of the Eurasian brown bear, which in their results shared a common ancestor with the bears that colonized southern Hokkaido. The Tibetan brown bears were genetically distant from the rest of the eastern lineage, with an estimated divergence at approximately 343 kyBP (95%HPD: 139–582 kyBP) (Figs. 2 and 3; Table 3). This result suggests that Eurasian brown bears dispersed early into Tibet, and that the brown bears on the Tibetan Plateau subsequently remained geographically isolated from the source population.

The TMRCA between southern Hokkaido and North American brown bears (clade 4) at approximately 194 kyBP (95%HPD: 67–399 kyBP) indicates that the former could have moved onto Hokkaido first among the three Hokkaido lineages. Because the southern Hokkaido brown bears are closely related to the North American brown bears (clade 4) and distant from Tibetan brown bears, they possibly migrated onto Hokkaido via the land bridge connecting with Sakhalin and the Eurasian Continent. Other brown bears in clade 4 originating from the

Eurasian Continent could have colonized North America via Beringia, because ancient brown bears at 36 kyBP in Alaska group into clade 4 (Leonard et al. 2000; Barnes et al. 2002).

In addition, my study shows that among southern Hokkaido brown bears, genetically more closely related mtDNA haplotypes are found in geographically closer locations (Figs. 1 and 3), compared with eastern and central Hokkaido brown bears, supporting the results of Matsuhashi et al. (1999) based on mtDNA control region data. This distribution pattern indicates that the phylogenetically more related haplotypes in clade 4 appeared in southern Hokkaido and became fixed after immigration, and that the ancestor of the southern Hokkaido lineage was the first brown bear lineage to immigrate to the island.

Relationships between brown bears in eastern Hokkaido and the southern Kuril Islands

After the divergence of clades 3a and 3b approximately 165 kyBP (95%HPD: 63–292 kyBP) (Fig. 2, Table 3), representatives of clade 3b could have colonized Hokkaido via Sakhalin. Based on mtDNA cyt *b* and control region sequences, clade 3b includes brown bears from both eastern Hokkaido and eastern Alaska, suggesting that they descended from a MRCA on the Eurasian Continent (Matsuhashi et al. 2001; Korsten et al. 2009). The appearance of clade 3b brown bears at approximately 21 kyBP in the North American fossil record suggests that representatives of this clade colonized North America via Beringia (Barnes et al. 2002). Because no complete mtDNA sequences were available from eastern Alaskan brown bears in clade 3b, however, I did not estimate the overall TMRCA for clade 3b nor calculate the timing of the divergence between the eastern Hokkaido and eastern Alaskan lineages.

Individuals from Kunashiri and Etorofu Islands grouped with the eastern Hokkaido lineage (clade 3b) (Figs. 2 and 3), with the former populations more closely related to one another than either is to the Hokkaido population. This suggests that the Kunashiri and Etorofu populations originated through dispersal from eastern Hokkaido, a conclusion confirmed by the results of a craniometrical analysis (Baryshnikov and Puzachenko 2009). Kunashiri Island was connected to Hokkaido by a land bridge 8–110 kyBP, whereas Etorofu Island remained separate during that period (Igarashi 2000). The brown bears on Kunashiri likely became isolated from the eastern Hokkaido lineage when Notsuke Channel formed a sea barrier, and

subsequently dispersed to Etorofu. Kunashiri Channel between Kunashiri and Etorofu is now approximately 25 km wide, though it could have been narrower during glacial maxima.

Relationships between brown bears in central Hokkaido and Sakhalin

In my study, clade 3a was subdivided into two geographically distinct clades: clade 3a1 comprising continentally widespread bears from eastern Europe, western Alaska, and Sakhalin; and clade 3a2 comprising local bears from central Hokkaido (Figs. 2 and 3). Clades 3a1 and 3a2 could have diverged on the Eurasian Continent, with a TMRCA of approximately 53 kyBP (95%HPD: 21–95 kyBP) for clade 3a. Clades 3a1 and 3a2 are differentiated by only five parsimony-informative nucleotide sites. The Kodiak brown bear (western Alaskan lineage) was more closely related to eastern European than to central Hokkaido bears, suggesting that this lineage dispersed to North America after divergence from the lineage leading to the central Hokkaido bears. Since the divergence time between clades 3a1 and 3a2 is approximately 53 kyBP (95%HPD: 21–95 kyBP), the ancestors of the central Hokkaido bears (clade 3a2) could have diverged earlier within clade 3a and dispersed into Hokkaido from the Eurasian Continent via Sakhalin before the appearance of Soya Strait between Hokkaido and Sakhalin/the Eurasian Continent approximately 12 kyBP (Ohshima 1990). On the other hand, western Alaskan brown bears grouped with eastern European brown bears, which means that representatives of clade 3a1 had migrated into North America after separation from the Eurasian population approximately 40 kyBP (95%HPD: 15–72 kyBP) but before formation of Bering Strait 11–13 kyBP (Elias et al. 1996). This is consistent with the lack of clade 3a brown bears among earlier Alaskan fossils (Leonard et al. 2000; Barnes et al. 2002). Two different colonization waves for clade 3a1 and 3a2 thus occurred at different times.

Although Sakhalin Island is considered to be a transient area for ancestors of the Hokkaido brown bears, the Sakhalin brown bear grouped with western Alaskan and eastern European brown bears in clade 3a1. This implies that the Sakhalin brown bear population did not originate at the same time as the dispersal of brown bears (clade 3a2) into central Hokkaido, but later during the geographical expansion of clade 3a1. Korsten et al. (2009) reported that brown bears in northern Eurasia (clade 3a) recently underwent a sudden expansion. Thus,

representatives of clade 3a1 could have migrated onto Sakhalin separately from the other continental population on the island. One Sakhalin individual grouped with clade 3a1 in the present study, but it is possible that Sakhalin brown bears are composed of not only clade 3a1 brown bears but also other relict brown bears (i.e. clades 3a2, 3b, and 4) like the Hokkaido brown bears. Further analyses of Sahkahin brown bears are needed, and analyses of ancient DNA will also provide insights into the migration history of clades 3a1 and 3a2, but unfortunately no bear remains have yet been detected in Pleistocene strata on Sakhalin or Hokkaido.

Phylogenetic relationships between brown bears and polar bears

My study showed the clade containing polar bears to be the sister group to the ABC islands brown bears; brown bears thus comprise a paraphyletic group. The TMRCAs for extant polar bears, all polar bears, and clade 2 (ABC islands brown bears and polar bears) were 41 kyBP (95%HPD: 18–68 kyBP), 136 kyBP (95%HPD: 120–164 kyBP), and 161 kyBP (95%HPD: 125–216 kyBP), respectively, indicating that the polar bear diverged from the ABC brown bear in the late Pleistocene. These results are remarkably similar to those of two studies based on the complete mtDNA genomes. Lindqvist et al. (2010) estimated corresponding TMRCAs at approximately 44 kyBP, 134 kyBP, and 152 kyBP, respectively, and Miller et al. (2012) also estimated corresponding TMRCAs at approximately 52 kyBP, 136 kyBP, and 162 kyBP, respectively. In contrast, in a study based on multiple, biparentally inherited nuclear loci, Hailer et al. (2012) estimated that polar bears diverged much earlier from brown bears, in the middle Pleistocene, approximately 600 kyBP, and that polar bears comprised the sister group to brown bears. Miller et al. (2012) highlighted the incongruence between the matrilineal and nuclear relationships among brown bears and polar bears. The discordant position of the ABC brown bears suggests an interspecific admixture as well as contrasting estimates for timing of divergence in the polar-brown bear lineage. The pronounced similarity in mtDNA characters between polar bears and brown bears may be explained by hybridization events in the Pleistocene (Edwards et al. 2011). To fully understand the evolutionary relationships between the two closely related species, it will be necessary to also analyze paternally inherited markers.

Chapter II

Mitochondrial DNA haplogrouping of the brown bear *Ursus arctos* in Asia based on a newly developed APLP analysis

Introduction

As shown in Chapter I, analyses of complete mtDNA sequences revealed the detailed phylogeographical structures, signatures of demographic history, and spatial processes that short mtDNA sequences had previously failed to detect. Phylogeography based on the mitochondrial genomes has been best studied in human (*Homo sapiens*) populations (e.g. Underhill and 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 and 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. 1996; Umetsu et al. 2001; Umetsu et al. 2005).

In the present study, I developed an APLP method for haplogrouping brown bear mtDNA, based on the complete mtDNA sequences reported in Chapter I, as a means of determining the mtDNA haplogroups of degraded historical (aged or ancient) brown bear 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. I tested this method by examining the phylogeography of brown bear populations in Eurasia. Here I report my results and discuss the migration history of brown bears in this region based on these results.

Materials 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. 1996; Umetsu et al. 2001; Umetsu et al. 2005; Umetsu and Yuasa 2005). Table 4 lists the sequences of APLP primers used and their working concentrations, and Table 5 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 the brown bear (GenBank accession no. AF303110) reported by Delisle and Strobeck (2002). Primer sets in my study were based on previously reported brown bear complete mtDNA sequences in clades 1, 2b, 3a1, 3a2, 3b, 4, and 5, and detected five eastern lineages (clades 3a1, 3a2, 3b, 4, and 5) (Fig. 4A). I tested the specificity of the multiplex APLP reactions using 39 brown and polar bear samples reported in Chapter 1. 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 4), and reagents from the Multiplex PCR Kit (QIAGEN). Primer concentrations were adjusted to obtain balanced amounts of PCR products. Two separate multiplex PCR (sets A and B) reactions were performed. Multiplex PCR set A was designed to analyze four sites (7257C/T, 16259A/G, 7770T/C, and 10116G/A) (Fig. 4B) and set B, five sites (11585G/A, 8392A/G, 8776C/T, 13180T/C, and 9271T/C) (Fig. 4C). 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; and 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

I haplogrouped samples of skin from 67 brown bears widely collected from the Eurasian Continent between 1842 and 1998 and housed in the Zoological Institute, Russian Academy of Sciences, St. Petersburg. Table 6 lists collection data and Fig. 5 shows the locations of 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 protocol. PCR amplifications were carried out in 10 μl reaction volumes each containing 1 μl of template DNA solution, the optimum concentration of each primer (Table 4), reagents from the Multiplex PCR Kit (QIAGEN), and 0.5 μl of bovine serum albumin (BSA; 0.4 μg/μl). 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 brown bears

The primer sets in my study amplified multiplex PCR products with molecular sizes ranging from 49 to 106 bp. Analyses of the 39 brown bears and polar bears of known mtDNA haplogroups confirmed the specificity of my primer sets. APLP band patterns are shown in Figs. 4B and C. The phylogeny of brown and polar bears, consisting of eight mtDNA clades, the position numbers of the clade-specific SNPs, and diagnostic nucleotides at those sites are summarized in Fig. 4A and Table 5.

MtDNA haplogrouping of the Eurasian brown bears

Using the APLP analysis I developed, I determined the mtDNA haplogroups for 54 of 67 Eurasian brown bear samples (80.6%). I observed no PCR products in negative controls. Many of these bears fell into clades 3a1, 3b, and 5; the clade identity and locations of samples are indicated in Figs. 5 and 6, and Table 6. Clade 3a1 was the most broadly distributed group, with individuals detected from Leningrad Province southeastward through Bashkiria and the Urals; to Tien Shan, Altai, and Lake Baikal in central Asia; and from the Amur River to northeastern Siberia and Kamchatka in eastern Asia. Clade 3b representative occurred in some local areas: Caucasus, Tannu-Ola Mts., Altai, and Krasnojarsk Province. Clade 5 was detected only on the Qinghai-Tibet Plateau.

I detected three tentative novel mtDNA haplogroups that had SNPs different from those in the current analytical scheme (Fig. 4A); these were termed the Western 1 (W1), Eastern 1 (E1), and Eastern 2 (E2) haplogroups (Table 6). 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. 5 and 6; Table 6). Haplogroup E1 had C at site 7257, which is diagnostic for the eastern 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 Karatau, Kazakhstan, Lake Issy-Kul, Kyrgyzstan, Tien Shan, Kyrgyzstan, and western Iran (U-17) (Figs. 5 and 6; Table 6). 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, Caucasus, and Mongolia (Figs. 5 and 6; Table 6).

Multiple haplogroups were detected sympatrically in three areas (Figs. 5 and 6; Table 6): W1 and E2 in western Iran; 3b, W1, and E2 in Caucasus; 3a1 and 3b in Altai; 3a1, E1, and E2 in Kyrgzstan.

Discussion

Haplogrouping of brown bear mtDNA

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

The APLP analysis is a much easier and more cost effective method for a large number of samples, especially historical samples, and can efficiently detect the distribution of brown bear mtDNA haplogroups. However, a limitation of this method is that mtDNA haplogroup

data alone cannot infer evolutionary history, such as the detection of signals of population demography (population reduction and expansion).

I 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. The APLP method might also work with palaeontological and archaeological samples, and if so, it would be useful in studying the phylogeographical structure of brown bears through time.

Phylogeography of northern and southern Eurasian brown bears

I determined the mtDNA haplogroups of brown bear across the Eurasian Continent from European Russia to Kamchatka, and including southwestern and central Asia (Figs. 5 and 6; Table 6). These haplogroups included three previously reported mtDNA haplogroups (clades 3a1, 3b, and 5). Korsten et al. (2009) found only one brown bear mtDNA haplogroup (clade 3a) to be widely distributed in northern Eurasia, and concluded that this haplogroup underwent a severe bottleneck during the LGM, followed by a demographic expansion. Corroborating this result, I found brown bears of clade 3a1 to be widely distributed across Eurasia (Figs. 5 and 6; Table 6). I 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 been found in the Russian Far East on the Eurasian Continent (Miller et al. 2006), and the extent of the distribution of this clade was not clear. I found clade 3b from several areas in Eurasia, including Altai and Caucasus (Figs. 5 and 6; Table 6). In the Altai, brown bears of clades 3a1 and 3b occurred sympatrically. Because clade 3b was shared by both eastern Hokkaido and eastern Alaskan brown bears, it appeared that bears from this area descended from a most recent common ancestor on the Eurasian Continent (Matsuhashi et al. 2001; Korsten et al. 2009). My results show that representatives of clade 3b exist as a relict population on the Eurasian Continent, and that clade 3b diverged on the Eurasian Continent prior to dispersal. The Caucasus and Altai, where clade 3b was found, are presently part of the southern distribution of the brown bear on the Eurasian Continent (McLellan et al. 2008), and these areas may have served as glacial refugia, with clade 3b

representing a relict population. Because clade 3b diverged earlier than clade 3a1, as shown in Chapter 1 (Fig. 4A), 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 Altai and Caucasus could have served as refugia during the LGM in southern Eurasia.

Central and southwestern Asia

All individuals from the Qinghai-Tibet Plateau belonged to clade 5 (Figs. 5 and 6; Table 6). This confirms that the Tibetan brown bears (*U. a. pruinosus*) are genetically distant from the rest of the eastern lineage and form a geographically isolated population (Chapter 1).

I detected three novel mtDNA haplogroups (W1, E1, and E2), which my APLP analysis did not classify in southwestern and central Asia (Figs. 5 and 6; Table 6). 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 brown bears detected from the Caucasus and Iran; all individuals previously analyzed from the Caucasus belonged to the eastern lineage (Murtskhvaladze et al. 2010), and those from Iran formed a distinct lineage within the eastern lineage (Talbot and Shields 1996; Miller et al. 2006). Brown bears 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 due to habitat loss and unregulated hunting (Murtskhvaladze et al. 2010). In the Middle East, brown bears in 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 and 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 been replaced by the eastern lineage, perhaps even in historical time.

I found individuals in haplogroup E1 in the eastern lineage from Kazakhstan, Kyrgyzstan, and western Iran (Figs. 5 and 6; Table 6). The brown bears 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. I found individuals in haplogroup E2 in southern Eurasia, from Kyrgyzstan, the Caucasus, and Mongolia (Figs. 5 and 6; Table 6). These records extend the known range of clade 3a sensu lato from south central Asia westward to the Caucasus region.

As mentioned above, brown bears representing multiple haplogroups were distributed sympatrically in the Middle East and central Asia (western Iran, Caucasus, Altai, and Kyrgyzstan). The higher genetic diversity in these areas compared to northern Eurasia (Figs. 5 and 6; Table 6) indicates that the Middle East and central Asia were refugia during the LGM. The Middle East was generally otherwise colonized by populations originating from the north.

Chapter III

Paternal phylogeographic structure of the brown bear *Ursus arctos* in northeastern Asia and the effect of male-mediated gene flow for the insular populations

Introduction

Sex-biased dispersal is the consequences of the mating systems and dispersal strategies of the individuals, and most of the mammals show male-biased dispersal (males disperse from the natal areas) and female philopatry (females stay in the natal areas) (Greenwood 1980; Dobson 1982; Pusey 1987). Empirical patterns, that sex-biased dispersal is widespread among mammals and varies widely in direction and intensity, have been shown using molecular information of bi-parentally and uni-parentally inherited sex-linked genetic markers (Petit et al. 2002; Prugnolle and de Meeus 2002; Lawson Handley and Perrin 2007). In practice, sexually inconsistent genetic differentiation patterns caused by sex-biased migration is observed in diverse taxonomic groups of large mammals; humans (Hughes and Rozen 2012; Wei et al. 2013), bonobos (Eriksson et al. 2006), chimpanzees (Langergraber et al. 2007), orang-utatns (Nater et al. 2011; Nietlisbach et al. 2012), hamadryas baboon (Hammond et al. 2006), canids (Brown et al. 2011; Sacks et al. 2013), and domesticated animals such as sheep and horses (Meadows et al. 2006; Lippold et al. 2011). One of the large terrestrial mammal, brown bear (*Ursus arctos*), which is widely distributed throughout the Holarctic region, shows the remarkable male-biased dispersal (McLellan and Hovey 2001; Proctor et al. 2004; Støen et al. 2006; Zedrosser et al. 2007). The sex-biased dispersal and reproductive system of ursine bears is presumed to have extensively contributed to the molecular evolutionary history of the ursids (Nakagome et al. 2008).

Previous phylogeographic studies of the brown bear based on the maternally inherited mitochondrial DNA (mtDNA) showed extensive intraspecific geographical genetic structuring in maternal lineages within the species (Davison et al. 2011; Hirata et al. 2013; Keis et al. 2013; Hirata et al. 2014). Especially, the brown bear population on Hokkaido Island, northern Japan, a peripheral insular population of the Eurasian Continent is composed of three distinct maternal lineages allopatrically distributed (Matsuhashi et al. 1999; Hirata et al. 2013). It is suggested that the three mtDNA lineages diverged on the Eurasian Continent prior to migration onto Hokkaido via land bridges in different three periods, and the southern Hokkaido lineage colonized first followed by the eastern Hokkaido and then the central Hokkaido lineages. Besides the female philopatric nature might have caused this explicit pattern on the small island. Hokkaido island is extremely smaller, compared to the adjacent Eurasian Continent, although higher maternal genetic diversity has been maintained than the northern continental Eurasia, where a single mtDNA lineage of the brown bear is predominantly distributed (Saarma et al. 2007; Korsten et al. 2009; Murtskhvaladze et al. 2010; Hirata et al. 2014). This is presumably the consequence of the past demographic history

of the brown bear on Hokkaido, where multiple immigrations of the different maternal lineages from the continent occurred during the glacial period when the ocean level regressed. Hokkaido Island must have served as the glacial refugia of the brown bear.

In addition to the maternally inherited mtDNA, the influence of sex-biased gene flow can be measured using the complement sex-linked marker, the male-specific non-recombining region of the Y-chromosomal DNA (Kutschera et al. 2014, 2016; Bidon et al. 2014, 2015; Schregel et al. 2015). In contrast to the mtDNA pronouncing matrilineal genetic structuring, male-specific Y-chromosomal DNA polymorphisms of the brown bear showed low intraspecific variability and did not show any clear phylogeographic structure throughout the Eurasian and the North American Continents (Bidon et al. 2014). Thus, extensive male-biased dispersal was thought to have caused gene flow across large geographical distances through the continents and the force for homogenizing the genetic divergent variation in the brown bears. Besides, the male-mediated gene flow could have connected the bear populations between the Alaskan ABC islands and the North American mainland, and played substantial roles for maintaining high genetic variability of the insular populations.

On the other hands, assessments of male gene flow during the recovery of the brown bears from near extinction clarified that male gene flow probably had little or no impact on the demographic recovery process in Scandinavia (Schregel et al. 2015). Lack of wide range male gene flow during a short time of the recovery process, reduced the Y-chromosomal DNA haplotype diversity in the post bottlenecked populations. Such a low degree of Y-chromosomal DNA haplotype admixture suggests that both males and females have contributed to large-scale genetic connectivity in this case. Thus, the role of male-mediated gene flow for the evolutionary history of the brown bears would depend on the population conditions such as time depths, distribution areas, the existence or absence of previously occupied populations.

Therefore, to consider the sex-biased migration is essential for understanding the comprehensive evolutionary history of the brown bear around northeastern Asia: how their population around Hokkaido and adjacent insular populations were formed, and how paternal lineages of the brown bears contributed to the genetic variation of Hokkaido and adjacent insular populations.

In the present study, to reveal their paternal genetic variation and genetic structure around northeastern Asia, I analyzed polymorphisms of paternally inherited Y-chromosomal DNA sequences and Y-linked microsatellites on the brown bear population of Hokkaido, southern Kuril Islands (Kunashiri and Etorofu Islands), Sakhalin, and the Eurasian Continent (Kamchatka Peninsula, Ural Mountains, European Russia, and Tibet). Then, I discuss the

effects of the sex-biased migration of the brown bear to the evolutionary history of each insular and continental populations, and differences of genetic contribution for population makeup. In addition, I validate the existence of the male-mediated gene flow among geographically adjacent insular populations around northeastern Asia.

Materials and Methods

Samples and DNA Extraction

Muscle or liver samples from 55 male brown bears collected on Hokkaido Island, Japan, were obtained from Environmental and Geological Research Department, Hokkaido Research Organization (Fig. 7). Male bear tissue samples were also obtained from the following regions and sources: 10 samples from Etorofu (Itrup) Island, one from Kunashiri (Kunashir) Island, one from southern Sakhalin, and one from Novgorod (Zoological Institute, Russian Academy of Sciences, St. Petersburg); 53 samples from the Ural Mountains, two samples from the Kamchatka Peninsula (Institute of Plant and Animal Ecology, Russian Academy of Sciences, Ekaterinburg); hairs of one male individual from Tibet (Kobe Municipal Oji Zoo, Japan) (Figs. 7, 8, and 9). Male brown bear individuals were confirmed by sex determination methods following Bidon et al. (2013). Total genomic DNA was extracted using the DNeasy Tissue & Blood Kit (QIAGEN) or the QIAamp DNA Micro Kit (QIAGEN), following the manufacturer's protocols. PCR amplifications were conducted in 5 µl reaction volumes containing 2.5 µl of 2× Multiplex PCR Master Mix (QIAGEN), 0.5 µl of primer mixture, 0.25 µl of bovine serum albumin (BSA; 0.4 µg/µl), 0.75 µl of distilled water, and 1.0 µl of DNA extract.

PCR amplification, sequencing, and microsatellite genotypeing

Female brown bears were included for all PCR amplification as a control to confirm the male specificity. No amplification was observed from female samples in each amplification experiment.

Same sequencing primer sets and touchdown thermal cycling conditions for amplifying seven fragments of Y-linked sequences (318.2C, 318.3C, 318.7C, 318.10B, 318.11C, 579.1B,

and 579.3C) were used as shown in Bidon et al. (2014). Touchdown PCR amplifications were conducted in 10 µl reaction volumes containing 2.0 µl of 5× PrimeSTAR GXL DNA Buffer (Takara), 0.8 µl of dNTP mixture (2.5 mM each dNTP; Takara), 0.2 µl of PrimeSTAR GXL DNA Polymerase (1.25 U/µl, Takara), 0.1 µl each of forward and reverse primers (25 pmol/µl), 0.2 µl of bovine serum albumin (BSA; 0.4 µg/µl), 5.2–6.2 µl of distilled water, and 1.0–2.0 µl of DNA extract. Touchdown thermal cycling conditions were 3 min at 95˚C; 10 cycles of 30 s at 94˚C, 25 s at 69˚C, 66˚C (decreasing by 0.5 ˚C per cycle), or 68˚C (decreasing by 1 ˚C per cycle) and 75 s at 72˚C; 25 cycles of 30 s at 94˚C, 25 s at 64˚C, 61˚C, or 58˚C, and 75 s at 72˚C and a final extension for 10 min at 72˚C. PCR products were purified with the QIAquick Purification Kit (QIAGEN), following the manufacturer's protocol. DNA cycle sequencing was performed by using the BigDye v3.1 Cycle Sequencing Kit (Applied Biosystems, ABI) with the same primers used for PCR amplifications. PCR for sequencing was performed in 10 µl volumes containing 1.75 µl of 5× BigDye Sequencing Buffer (ABI), 0.5 µl of Ready Reaction Premix (ABI), 1.6 µl of primer (1 pmol/µl), 5.15 µl of distilled water, and 1.0 µl of DNA template. Twenty-five cycles of 10 s at 96˚C, 5 s at 50˚C, and 4 min at 60˚C were performed. Amplified DNA fragments were purified with isopropanol, and then formamide was added. Sequences were determined on an ABI 3730 DNA Analyzer. The sequences were assembled and edited in phred/phrap/chromaseq (Ewing et al. 1998; Ewing and Green 1998; Maddison and Maddison 2015). The sequence alignment was performed using the MUSCLE (Edgar 2004) in MEGA7 (Kumar et al. 2016).

Nine Y-linked microsatellite alleles were determined by two sets of multiplex PCRs, and same primer sets were used as shown in Bidon et al. (2014). Each multiplex PCR was performed in 5 µl reaction volumes each containing 2.5 µl of 2× Multiplex PCR Master Mix (QIAGEN), 1.0 µl of primer mixture, 0.25µl of bovine serum albumin (BSA; 0.4 µg/µl), 0.25 µl of distilled water, and 1.0 µl of DNA extract. Touchdown thermal cycling conditions were 3 min at 95˚C; 20 cycles of 30 s at 94˚C, 25 s at 68˚C (decreasing by 0.5 ˚C per cycle), and 75 s at 72˚C; 15 cycles of 30 s at 94˚C, 25 s at 58˚C, and 75 s at 72˚C and a final extension for 10 min at 72˚C. Y-linked microsatellites were determined on an ABI 3730 DNA Analyzer and a GeneScan 600 LIZ Size Standard (ABI). Allele sizes of the Y-linked microsatellites were

determined using GeneMapper v4.1 (ABI). Nine Y-chromosome microsatellites was genotyped, and allele size data is shown in Table 7. Three Y-chromosome microsatellite markers were excluded because some individuals had pseudoheterozygous genotypes. Six out of nine Y-chromosome microsatellite markers were used for further analysis.

Data Analyses

Summary statistics

Brown bears, polar bears, and American black bears data in Bidon et al. (2014) was added to the dataset, and subsequent analysis was implemented. Insertions and deletions (indels) were excluded from the sequence alignment dataset. Summary statistics: number of haplotypes (H), the frequency of the dominant haplotype (fH), number of segregating sites (S), nucleotide diversity (π), Watterson's θ_W (per site), Tajima's *D*, Fu and Li's *D* and *F*, and Fu's F_S based on 5,287 bp (5.3 kb data set) Y-chromosomal DNA linked sequences of 168 brown bears were calculated in DnaSP version 5.10.1 (Librado and Rozas 2009) and Arlequin ver 3.5.2.2 (Excoffier and Lischer 2010). Y-chromosomal DNA compound haplotypes were determined based on the combination of the Y-linked SNPs on 3,071 bp (3.1 kb data set) Y-chromosomal DNA linked sequences and six Y-linked microsatellites alleles. Genetic diversity of Y-chromosomal DNA compound haplotypes of 214 brown bears in terms of number of haplotypes (H), haplotype diversity (HD ± SE), and mean number of pairwise differences (MPD ± SE) within each population were calculated.

Haplotype network analysis

Median-joining (MJ) network (Bandelt et al. 1999) using Y-chromosomal DNA compound haplotypes combined with Y-linked SNPs of 3.1 kb Y-linked sequences and Y-linked microsatellites was reconstructed using Network 5.0.0.0 (http://www.fluxus-engineering.com). For network calculations, more quickly evolving Y-linked microsatellite loci were weighted inversely to their variance in repeat length (318.9 = 8, 318.4 = 9, 318.2 = 8, 369.1 = 2, 318.1 = 9, and 318.6 =1) and SNP loci were weighted 10 times the highest Y-linked microsatellites weight (Y-linked SNPs = 90). MJ network of 3.1 kb and 5.3 kb Y-chromosomal DNA

sequences were reconstructed using POPART (Leigh and Bryant 2015). Haplotype nomenclature based on 5.3 kb and 3.1 kb Y-chromosomal DNA linked sequences corresponds to that of Bidon et al. (2014). Nomenclature of Y-chromosomal DNA compound haplotypes was assigned according to the order in combination of 3.1 kb Y-chromosomal DNA linked sequences and genotypes from six microsatellite loci. Three sub-populations within Hokkaido Island, central Hokkaido, eastern Hokkaido, and southern Hokkaido, were delimited based on the three distinct maternal lineages, clade 3a1, 3b, and 4, respectively (Matsuhashi et al. 1999; Hirata et al. 2013).

Population differentiation analysis

Pairwise population differentiation values (R_{ST}) for Y-chromosomal polymorphisms among populations (Slatkin 1995) of the brown bear were calculated with 1,000 permutations using Arlequin ver 3.5.2.2 (Excoffier and Lischer 2010). Hierarchical analysis of molecular variance (AMOVA) was implemented in Arlequin ver 3.5.2.2 (Excoffier and Lischer 2010). AMOVA testing for Y-chromosomal polymorphisms of each geographical partition was implemented. Geographical partitions of the groups were defined as North American group, European group, Hokkaido group, and Etorofu group. Based on the AMOVA analysis, the Etorofu population was included in the Eurasian group and implemented the remaining analyses. Three populations within the Hokkaido group, central Hokkaido, eastern Hokkaido, and southern Hokkaido, were defined based on the three distinct maternal lineages (Matsuhashi et al. 1999; Hirata et al. 2013). Brown bear populations composed of one individual, Tibet, Sakhalin, and Kunashiri were excluded from AMOVA analysis.

TMRCA estimations by Bayesian Analysis

Time to the most recent common ancestors (TMRCAs) and the times of population splitting were estimated using the Bayesian-based coalescent approach implemented in the software BATWING (Wilson et al. 2003). The coalescences of all the individuals from all brown bears was investigated, both Hokkaido and Etorofu brown bears, only Hokkaido brown bears, and only Etorofu brown bears. Demographic model of constant population size was assumed. Prior

distributions were applied as a mean mutation rate priors of human Y-chromosomal DNA microsatellites, 6.9 x 10^{-4} mutations per locus per generation with a gamma distribution (Zhivotovsky et al. 2004) according to Wei et al. (2013) and as a mean effective population size 10,000 with a gamma distribution. For each BATWING run, a total of one million MCMC cycles were performed and 10% of each run was discarded as burn-in. A generation time of ten years for brown bears was assumed (Cahill et al. 2013) to convert the number of generations into years. In BATWING analyses, all brown bear individuals were included as a dataset. Geographical population subdivisions were defined as Eurasia, North America, Hokkaido, and Etorofu. The Hokkaido population was treated as one population. One individual from Kunashiri was included in the Hokkaido population and one individual from Sakhalin was included in the Eurasia population.

Results

Y-chromosomal DNA polymorphisms and genetic diversity of the brown bear

No haplotypes of Y-chromosomal DNA were shared among brown bears, polar bears (*U. maritimus*), and American black bears (*U. americanus*), and each of these three bear species showed a monophyletic relationship for each other in both 5.3 kb and 3.1 kb Y-linked sequence data sets (Figs. 10 and 11). In the 3.1 kb Y-linked sequence data set, including the data set by Bidon et al. (2014), all six haplotypes (BR1, BR2, BR3, BR4, BR5, and BR6) were found in 214 brown bears (Fig. 10). Haplotype BR6 was newly found in four individuals from Ural Mountains. Except 11 individuals having minor haplotypes (BR2, BR3, BR4, BR5, and BR6), 207 individuals examined in the present study had haplotype BR1. All individuals from Hokkaido retained haplotype BR1. In 5.3 kb Y-linked sequence data set, including the data set of Bidon et al. (2014), nine haplotypes (BR1.1, BR1.2, BR1.4, BR1.5, BR2, BR3, BR5, BR6.1, and BR6.2) discriminated by nine segregating sites were found in 168 brown bears (Fig. 11). Four haplotypes (BR1.4, BR1.5, BR6.1, and BR6.2) were newly detected in the present study. The maximum number of nucleotide difference between haplotypes was five. All individuals from Hokkaido shared haplotype BR1.4, and 58% of brown bears in the present study had haplotype BR1.4. A total of 98 individuals from Hokkaido, Kunashiri, Etorofu, Sakhalin, Tibet,

Ural Mountains, Kamchatka, and European Russia had haplotype BR1.4. Three haplotypes (BR1.5, BR6.1, and BR6.2) were found only in brown bears from Ural Mountains. Nucleotide diversity (π) and Watterson's θ_W per site of all brown bears were $1.5\pm0.1\times10^{-4}$ and $3.0\pm1.2\times10^{-4}$, respectively. The indices of all of four neutrality tests in 5.3 kb data set did not show any significant deviations from neutral expectation, and paternal brown bears did not experience recent population expansion or contraction: Tajima's D = -1.19 ($P > 0.10$), Fu and Li's D = -1.95 ($P > 0.05$), Fu and Li's F = -2 ($P > 0.05$), Fu's Fs = -3.548 ($P > 0.05$).

Of the Y-chromosomal DNA compound haplotypes of Y-linked SNPs of 3.1 kb Y-linked sequences and six Y-linked microsatellites alleles, 80 haplotypes were found in 214 individuals (Table 7) and 39 haplotypes were new ones. Of the brown bears of Western Asia including Ural Mountains, 32 haplotypes were found, and both the haplotype diversity (HD = 0.96±0.01) and mean number of pairwise differences (MPD = 3.59±1.85) were the highest (Table 8). MPD of Hokkaido was nearly one-third of that of Western Asia, which was the highest among all populations. A total of eight haplotypes were found in 55 individuals of Hokkaido. Both of genetic diversity indices of Hokkaido showed lower values (HD = 0.73 and MPD = 1.23), compared with those in the North American Continent and the Eurasian Continent populations except Canada. Within Hokkaido, both HD and MPD were the highest in central Hokkaido (HD = 0.76±0.07, MPD = 1.26±0.82) followed by those in eastern Hokkaido (HD = 0.60±0.15, MPD = 0.85±0.65) and then those in southern Hokkaido (HD = 0.38±0.15, MPD = 0.41±0.40). All of the eight haplotypes found on Hokkaido was identified from the central Hokkaido population. On Etorofu Island, only two haplotypes with one microsatellite mutational step difference were found from ten individuals, and MPD was the lowest among all brown bear populations, where multiple individuals were investigated.

Y-chromosomal DNA haplotype networks of the brown bear

Only haplotypes found in Hokkaido showed clear geographic clustering in the Y-chromosomal DNA haplotype network among all brown bear populations, and no haplotypes were shared with brown bears of the Eurasian Continent and the North American Continent (Fig. 12). Except the Hokkaido populations, the Y-chromosomal DNA network did not show any explicit

geographic structure. Within Hokkaido Island, Y-chromosomal DNA haplotypes were shared among populations, and their geographical distribution of haplotypes within Hokkaido Island did not show any separation distinct from three maternal population structure (Figs. 7 and 13). Three male individuals (ID nos. 602, 6071, and 6081) with discrepant maternal lineages (mtDNA clades 4, 3b, and 3a2, respectively) were exceptionally found on the inconsistent maternal populations (mtDNA clades 3a2, 3a2, and 3b, respectively) across the maternal border (Fig. 7). These individuals having the common paternal haplotypes were found in both presumed natal and dispersed populations across these maternal borders.

Within Hokkaido Island, the haplotype distribution did not show any allopatric separation, but biased haplotype distribution tendency was observed (Figs. 7 and 13). The central Hokkaido population had all of the haplotypes observed through Hokkaido, and showed the highest haplotype diversity, compared with the eastern Hokkaido and southern Hokkaido populations. The frequency of haplotype BR1_05 was the highest in Hokkaido and this haplotype was found in all of three Hokkaido populations. Haplotype BR1_06, which was one microsatellite mutational step from major haplotype BR1_05, was also identified from all of three Hokkaido populations. Minor haplotypes (BR1_02, BR1_07, and BR1_08), which were derived from 1–3 microsatellite mutation steps, were found only in the central Hokkaido population.

Among populations of southern Kuril Islands, geographically neighboring to Hokkaido Island, one brown bear shared a haplotype (BR1_04) with Hokkaido brown bears (Figs. 7 and 13). Although the brown bears of the eastern Hokkaido population had the highest frequency of this haplotype, two haplotypes found on Etorofu Island were more closely related to those of the Eurasian Continent than those of Hokkaido (Figs. 7 and 12). One haplotype (BR1_12) was shared with brown bears from Ural Mountains, and the other (BR1_13) was a haplotype specific to the Etorofu Island brown bears. One Sakhalin brown bear had another haplotype (BR1_18), which was closely related to those of the Eurasian Continental brown bears. One Tibetan brown bear had a distinct haplotype (BR1_10), which differed from that of Ural Mountains brown bear by six microsatellite mutation steps. Brown bears from Ural Mountains and Kamchatka had highly variable haplotypes within the same populations, compared with the

other populations, but both populations exhibited no clear relationships between genetic relatedness and geographical locations.

Population differentiation analysis among brown bear populations

Hierarchical analyses by AMOVA were implemented in different geographical partitions of brown bear populations and groups (Tables 9, 10, 11, and 12). When the Etorofu population was included within the Eurasian Continent group, the percentage of variance among groups became highest (44.43%) among those of partitions; the Etorofu population was defined within the North American group (41.35%) or Hokkaido group (23.51%). When the Etorofu population was included within the Hokkaido group, a high proportion of variance explained within-populations, not among-groups. These AMOVA results supported that the Etorofu population clustered with the Eurasian Continent better than North American or Hokkaido. In the case that continents (Eurasia and North America) and the island (Hokkaido) were separated to two groups, the proportion of variance explaining among groups was highest, and the differentiation was more pronounced. The AMOVA results supported that the Hokkaido group was highly differentiated from the Eurasian Continent group (including Etorofu) and North American group. There was no genetic connectivity between the Hokkaido group and the other continental groups. When the geographical partition was defined as Hokkaido plus Etorofu, the AMOVA revealed a high proportion of variance explaining among populations. If only the Hokkaido populations were defined as one group, a high proportion of variance explained within populations. Thus, the substantial amount of genetic variation of the Hokkaido plus Etorofu groups was attributed to differences between the Hokkaido and Etorofu populations. In addition, there were no substantial population differentiations among three Hokkaido populations (central, eastern, and southern Hokkaido).

Pairwise population differentiations among three Hokkaido brown bear populations were low and non-significant only for population pairs between central and southern Hokkaido (Table 13). Whereas Etorofu Island geographically faces Kunashiri Island and Hokkaido Island, the pairwise genetic differentiation (R_{ST}) among brown bear populations showed that the Etorofu population was significantly differentiated from the three Hokkaido populations

(central, eastern, and southern Hokkaido) (R_{ST} = 0.92, 0.89, and 0.98, respectively). The population differentiation comparison with the Etorofu population, indicated that the pairwise population differentiation between Etorofu and East Asia populations was the lowest (R_{ST} = 0.18). The three Hokkaido populations were significantly differentiated from all of three North American and five Eurasian Continental populations. No paternal lineage structuring within Hokkaido was found, showing that the Hokkaido populations were a panmictic paternal population. Among the Hokkaido populations, however, biased and different degree of population differentiations were observed (Table 13). Between populations of central and southern Hokkaido, the R_{ST} value was extremely low, and no significant differentiations were shown. On the other hands, significant population differentiations were found between central Hokkaido and eastern Hokkaido, and also between eastern Hokkaido and southern Hokkaido. Excluding the Etorofu population from the Eurasian Continental group, R_{ST} values between North American and Eurasian Continental populations were lower than those between North American and Hokkaido populations, and between the Eurasian Continental and Hokkaido populations.

Bayesian analyses of divergence time estimation of the brown bear populations

The time to the most recent common ancestors (TMRCAs) and effective population sizes (N_e) of the paternal lineages based on Y-linked SNPs of 3.1 kb Y-linked sequences and six Y-linked microsatellites were estimated with BATWING (Table 14). TMRCA of all males was 472.655 kyBP (95% CI: 186.797–1048.846). That of both the Hokkaido and Etorofu populations was 127.779 kyBP (40.336–332.066), and slightly older than the splitting time between the Hokkaido brown bears and the other populations, 124.566 kyBP (16.537–645.604 kyBP). TMRCA of the Hokkaido populations was 55.316 kyBP (15.659–153.922 kyBP) with an effective population size of 1,723 (569–4,380). The splitting time between the Etorofu brown bears and the other populations was 36.948 (1.038–277.595 kyBP). TMRCA of the Etorofu brown bears was 4.366 kyBP (0.539–15.752 kyBP) with an effective population size of 295 (31–1,125), which was less than one-fifth of the Hokkaido populations.

Discussion

The paternal phylogeographic structuring of the brown bears around northeastern Asia was uncovered by using Y-chromosomal DNA polymorphisms, and found contrasting patterns to the maternal phylogeographic structuring not only on the Eurasian and the North American Continents but also around Hokkaido and adjacent insular regions. Sex-biased dispersal behavior of the brown bear could have been markedly effective to the insular brown bear populations, and caused the discrepancy for the evolutionary history between insular and continental populations of the brown bear.

Paternal phylogeography of the brown bear on Hokkaido Island

Paternal DNA analysis revealed that the Hokkaido brown bears were highly differentiated from those of the continental Eurasian and North American populations, and showed the lack of genetic connectivity from the continental populations (Fig. 12; Tables 9 and 13). All of the Hokkaido brown bears was suggested to have split off from the other continental brown bears approximately 124.566 kyBP (16.537–645.604 kyBP) (Table 14). In contrast, weak phylogeographic structures of the paternal haplotypes were observed throughout the Eurasian and the North American Continents, probably resulting from male-mediated gene flow across the continent as shown in Bidon et al. (2014). Therefore, paternal lineages of Hokkaido and the continents might have experienced the independently different evolutionary history.

In case of the brown bears on the North America, male-mediated gene flow connects the populations between the Alaskan ABC islands and the North American mainland, and plays an important role for maintaining the high genetic variability of the insular population (Bidon et al. 2014). In contrast, there was no direct evidence of male-mediated gene flow across the sea straits between Hokkaido Island and the Eurasian Continent. It is supposed that male-mediated gene flow from the continent might have scarcely played for patrilineal genetic variability of the brown bears in Hokkaido after the separation of Hokkaido Island in the last glacial period when the ocean level regressed, rather substantial genetic drift within Hokkaido Island might have contributed to their paternal genetic diversity and their population differentiation from the

continent. Thus, the influence of the male-mediated gene flow to the insular genetic diversity is different from area to area.

All Y-chromosomal DNA haplotypes found in Hokkaido showed clear geographic clustering to each other, whereas there was little geographic structuring for distribution of the paternal haplotypes within Hokkaido Island (Figs. 7, 12, and 13). This pattern is inconsistent with the result from the mtDNA analyses that the brown bears on Hokkaido Island is composed of three distinct maternal lineages allopatrically distributed (Matsuhashi et al. 1999; Hirata et al. 2013). This inconsistent patterns between the maternal and paternal genetic structures on Hokkaido suggest that strong male-biased and long distance dispersal of males played a significant role in the genetic makeup of brown bear populations. Moreover, the paternal genetic diversity of the Hokkaido brown bears was much lower than those of the continental North American and Eurasian brown bears. Thus, male-biased dispersal could have served more strongly for homogenizing the genetic diversity on the restricted small island than the broad continent.

The paternal lineages of Hokkaido brown bears showed an extremely recent coalescence than their maternal lineages. TMRCA by mtDNA data of the Hokkaido brown bears was previously estimated approximately 268 kyBP (109–457 kyBP), and those of each maternal lineage was approximately 27 kyBP (10–49 kyBP), 42 kyBP (14–80 kyBP), and 36 kyBP (12–67 kyBP) in central, eastern, and southern Hokkaido lineage, respectively (Hirata et al. 2013). In contrast, TMRCA of Hokkaido brown bears by paternal data was estimated approximately 55.316 kyBP (15.659–153.922 kyBP) in the present study (Table 14), and this timing is roughly five times recent, compared with mtDNA data. TMRCA by Y-chromosomal DNA better coincided with that of each maternal Hokkaido lineages than those of all maternal Hokkaido lineages. This discrepancy of the coalescence might be explained by pronounced sexual differences in dispersal behavior of the brown bears. The brown bear populations on Hokkaido was assumed to be formed through the immigration of the different lineages from the Eurasian Continent in three different periods (Hirata et al. 2013). In that case, highly diverged paternal haplotypes descended from the past multiple immigration events should be discovered from Hokkaido. Against to the expectation, all of the Y-chromosomal DNA haplotypes found

in the extant Hokkaido brown bears had recently diverged haplotypes with only a few microsatellite mutational step differences to each other (Figs. 7, 12, and 13; Table 7). In the 3.1 kb and 5.3 kb Y-linked sequence data set, all individuals from Hokkaido had haplotype BR1 and BR1.4, respectively. No relatively old haplotypes discriminated by more slowly evolving SNPs were found in the Hokkaido brown bears, which was presumed to be the relict of the past multiple immigrations from the Eurasian Continent. Relatively old haplotypes might have been swept out or become extinct on Hokkaido. Thus, all paternal haplotypes shared by the Hokkaido brown bears are specific to Hokkaido.

In the present study, one major haplotype (BR1_05) was found through Hokkaido Island (Figs. 7 and 13; Table 7). Besides, direct evidence that three male individuals (ID nos. 602, 6071, and 6081) with discrepant maternal lineages (mtDNA clades 4, 3b, and 3a2, respectively) was exceptionally found on the inconsistent maternal clades (mtDNA clades 3a2, 3a2, and 3b, respectively) beyond the matrilineal separation border (Fig. 7). Because these individuals having the common paternal haplotypes observed on both presumed natal and dispersed populations across these borders and male mtDNA does not inherit to the offspring, these are probably the consequence of the dispersals of males during one generation. In addition, effective dispersal of extant males and male-mediated gene flow between the two natal areas discriminated by two discrepant mtDNA lineages (between central and eastern Hokkaido populations) in the southern Akan-Shiranuka region of Hokkaido had been detected using combination of maternal mtDNA and autosomal microsatellite markers (Sato et al. 2011; Itoh et al. 2012). These signs of male-mediated gene flow among populations illustrate that distinct maternal phylogeographic structures within Hokkaido Island had been maintained by strong female philopatric behaviors even though frequent crossing by males among these maternal populations occurred from the past to the present.

It is conceivable that distribution patterns of Y-chromosomal haplotypes were relatively panmictic since paternal population structuring was weak: however, the biased and different degree of pairwise population differentiation among three Hokkaido populations was observed (Table 13). This result might have been the trace of biased dispersal tendency and mixing of males from past to the present. Between central and southern Hokkaido populations, there was

no paternal population differentiation, and this suggests that frequent movement between these populations caused interexchange of paternal haplotypes. On the other hands, significant population differentiations were observed between central and eastern Hokkaido, and between eastern and southern Hokkaido, and this suggests that some restriction for the brown bear migration or inactive movement between these populations. The tendency corresponds to the insight obtained from morphometrical test of the phylogeographical patterns of Hokkaido brown bears based on mtDNA: females were markedly differentiated based on three Hokkaido populations, but males belonging to central and southern Hokkaido populations showed a similarity, whereas males in eastern Hokkaido was distinct (Baryshnikov et al. 2004).

Paternal phylogeography of the brown bear in southern Kuril Islands

Only one male of Kunashiri Island analyzed in the present study had haplotype BR1_04, which was frequently found in eastern Hokkaido (Figs. 7 and 13). The paternal lineage of the Kunashiri brown bear population might be comprised of the relict paternal haplotypes widely distributed from eastern Hokkaido to Kunashiri before the formation of the channel between them. This paternal genetic relationship between Kunashiri and eastern Hokkaido populations is consistent with that maternal lineage of the southern Kuril Islands (Kunashiri and Etorofu) could have originated from eastern Hokkaido (Hirata et al. 2013).

It is expected from the previous mtDNA result that paternal lineages of the Etorofu population have experienced the same demographic history as the Kunashiri population. However, it is intriguing that the paternal lineage differentiation between Hokkaido and the Etorofu was confirmed. Two haplotypes found on Etorofu Island were more closely related to the haplotypes from the Eurasian Continent than those from Hokkaido, even though a large number of individuals (n = 55) sampled broadly across the Hokkaido was genotyped (Fig. 12; Tables 9 and 13). TMRCA of both Hokkaido and Etorofu brown bears comes relatively recent in matrilineal than in patrilineal. TMRCA of both Hokkaido and Etorofu paternal lineages was traced back to before the splitting of the Hokkaido lineage from the other continental lineages 127.779 kyBP (40.336–332.066 kyBP) (Table 14). In the maternal lineage, brown bears from both southern Kuril Islands is derived from the part of the eastern Hokkaido lineage less than 42

kyBP (14–80 kyBP) (Hirata et al. 2013). The estimated male effective population size on Etorofu Island was particularly smaller than that on Hokkaido Island. A small number of individuals might have contributed to the makeup of the population on Etorofu.

In the present study, Y-chromosomal DNA typing was done on ten individuals of Etorofu, of which four were analyzed for the previous analysis of the complete mtDNA sequences; three of the four individuals had an identical mtDNA haplotype excluding the fast evolving variable number of tandem repeats on the control region (Hirata et al. 2013). Brown bears on Etorofu Island likely consisted of the maternally related individuals. In addition, only two Y-chromosomal DNA haplotypes differing by one microsatellite mutation step were found from ten individuals in Etorofu Island and showed a lowest genetic diversity (Fig. 12; Table 8). Etorofu Island brown bears are likely composed of the paternally related individuals. Geologically, Kunashiri Island was connected to Hokkaido by a land bridge 8–110 kyBP, whereas Etorofu Island remained separate during that period (Igarashi 2000). The brown bears on Etorofu Island might be maintained by relatively inbred brown bears for a long time, thus founder effect and subsequent genetic drift on the isolated insular population might have played extensively to the genetic diversity of the Etorofu Island brown bear population. In contrast to the maternal lineages, the basal paternal population of the Etorofu brown bears possibly originated by the dispersed male individuals having these paternal haplotypes from the Eurasian Continent. Thus, different dispersal patterns of males and females of the brown bears suggested to have contributed to the makeup of the brown bears on Etorofu Island, which is composed of the inconsistent origins of the maternal and paternal lineages. Furthermore, it is conceivable that there was little recent male-mediated gene flow between Hokkaido/Kunashiri and Etorofu Islands.

In summary, brown bears on Hokkaido and adjacent southern Kuril Islands (Kunashiri and Etorofu Islands) experienced the different paternal evolutionary history, and it demonstrates that phylogeography around these regions is considerably more complicated than expected from the mtDNA studies. Exceptionally in southern Kuril Islands (both Kunashiri and Etorofu Islands), brown bears with white pelage have been observed (Sato et al. 2011). Bears having white pelage is similarly found in American black bears inhabiting on the Kermode

Islands (Ritland et al. 2001). Their insular population could have been established and maintained in population by a combination of genetic isolation and small population sizes in insular habitats, with possible contribution of selective pressure and/or nonrandom mating (Marshall and Ritland 2002). In the case of brown bears within southern Kuril Islands, as the consequence of founder effect and subsequent strong genetic drift process might have led the nonrandom mating, populations having these unique genetic and morphological characters might have been fixed and maintained uniquely on these insular populations. Further studies will be expected to uncover the detailed demographic history and local adaptation of the southern Kuril Islands brown bears by analyzing bi-parentally inherited nuclear genomes.

Paternal phylogeography of the brown bear on the Eurasian Continent and Sakhalin

Brown bears from Ural Mountains and Kamchatka Peninsula had highly variable haplotypes on the same populations, compared with the other populations. But both populations did not exhibit any clear relationships between genetic relatedness and geographical locations. All of the paternal genetic diversity indices of the brown bear populations were the highest in Western Asia including Ural Mountains in the present study, whereas only one mtDNA lineage of the brown bear (clade 3a1) was found and relatively low maternal genetic diversity of the brown bear was observed in this region (Saarma et al. 2007; Korsten et al. 2009; Murtskhvaladze et al. 2010; Hirata et al. 2013, 2014). The high genetic variation within populations compared to the low genetic differentiation among populations in this region supports that male-mediated gene flow highly contributed to the brown bear population history of the Eurasian Continent, especially around the Ural Mountains.

The maternal lineage of the Tibetan brown bears was genetically distant from the rest of the lineages in the other Eurasian Continental brown bears, and the Tibetan population had been suggested to be geographically isolated from the other Eurasian populations for a long time and there was no genetic connectivity via female brown bears (Hirata et al. 2013). In the present study, only one Tibetan male individual was genotyped and found to have a haplotype differed from haplotypes of the Ural Mountains brown bear, by only fast evolving six microsatellite mutation steps. The paternal lineage of the Tibetan brown bear had not so genetically distant

from the other continental Eurasian brown bears as expected from the maternal genetic relationships. Therefore, it is difficult to conclude whether male-mediated gene flow from the other continental populations influenced the connectivity and makeup of the Tibetan brown bear population.

Sakhalin Island is located on the intermediate region between Hokkaido Island and the Eurasian Continent, and these regions were connected via land bridges until the appearance of Soya Strait approximately 12 kyBP (Ohshima 1990). One Sakhalin brown bear had a distinct paternal haplotype, which was more closely related to haplotypes of the Eurasian Continental brown bears than those of the Hokkaido brown bears. This genetic connectivity and close relationship with brown bears on the Eurasian Continent than those on Hokkaido is consistent with the result of the maternally inherited mtDNA sequences (Hirata et al. 2013). It is conceivable that genetic exchange of both paternal and maternal lineages between the Eurasian Continent and the Sakhalin were sustained until relatively recent time after the separation of Hokkaido Island.

Conclusions

A weak spatial structure of paternal lineages in the extant Hokkaido brown bears could have been formed through continual extensive gene flow via male dispersals and the exchange of paternal haplotypes among natal populations after the last population immigration into Hokkaido from eastern Siberia via land bridges during last glacial period when the ocean level regressed. Consequently, distinct allopatric genetic structure observed on the maternal lineages was homogenized in the paternal genetic structure; however, the heterogeneous male-mediated gene flow among populations within Hokkaido Island was indicated. Further study will be needed to further understand how much the male-mediated gene flow among natal populations within Hokkaido Island have influenced the connectivity and the maintenance of each local population. Brown bears on Hokkaido and adjacent southern Kuril Islands (Kunashiri and Etorofu) experienced the different paternal evolutionary history, and it demonstrates that phylogeography around these regions is more considerably complicated than expected from the mtDNA studies. I demonstrated that sex-biased dispersal had played a significant role in the

evolutionary history of the brown bear not only on the continental but also on the peripheral insular populations; Hokkaido Island, southern Kuril Islands, and Sakhalin. Phylogeography around these regions is also more complicated than previously assumed. Therefore, bi-parentally inherited autosomal DNA and whole genome data of Asian brown bears will be required to clarify the detailed demographic history and local adaptation.

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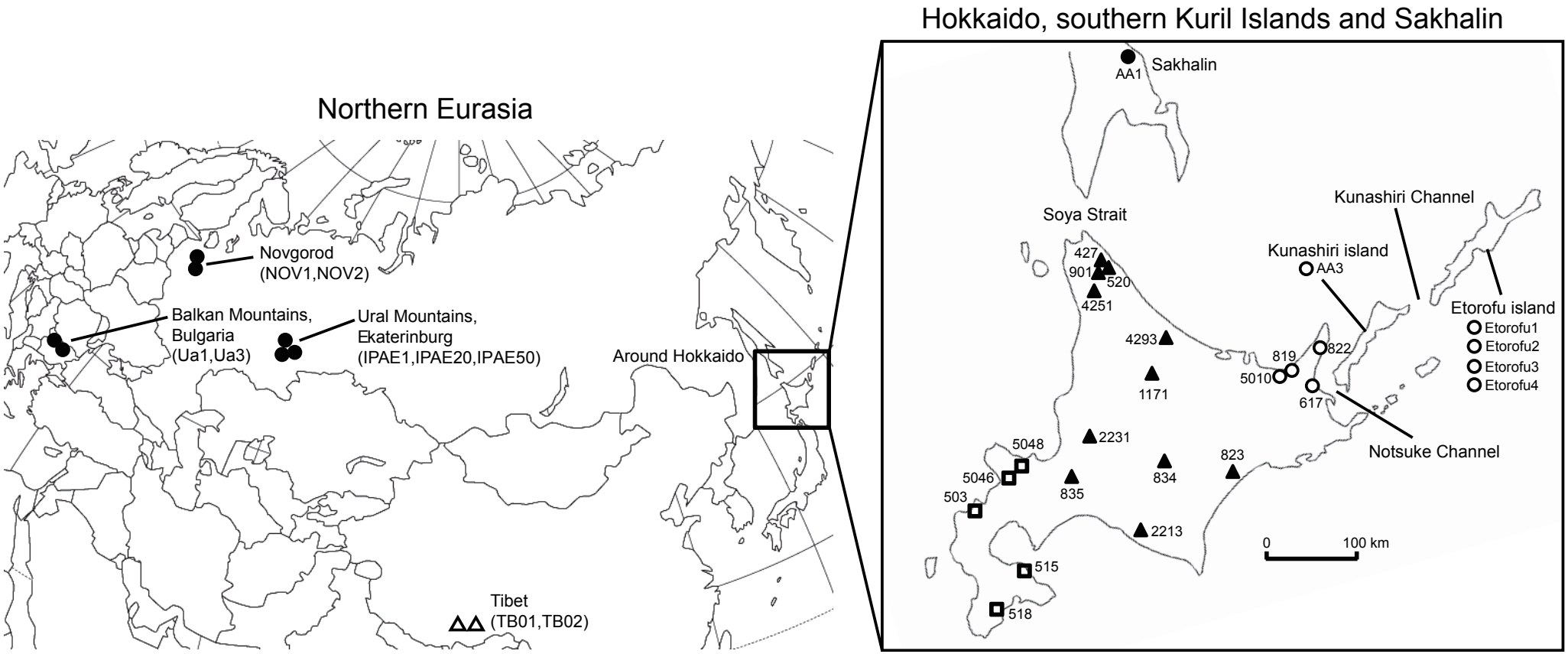

Fig. 1. Geographical distribution of brown bear mtDNA clades on the Eurasian Continent and Hokkaido Island, Japan.
Filled circles, mtDNA clade 3a1; filled triangles, clade 3a2; open circles, clade 3b; open squares, clade 4; open triangles, clade 5. Numbers next to the symbols are sample numbers.

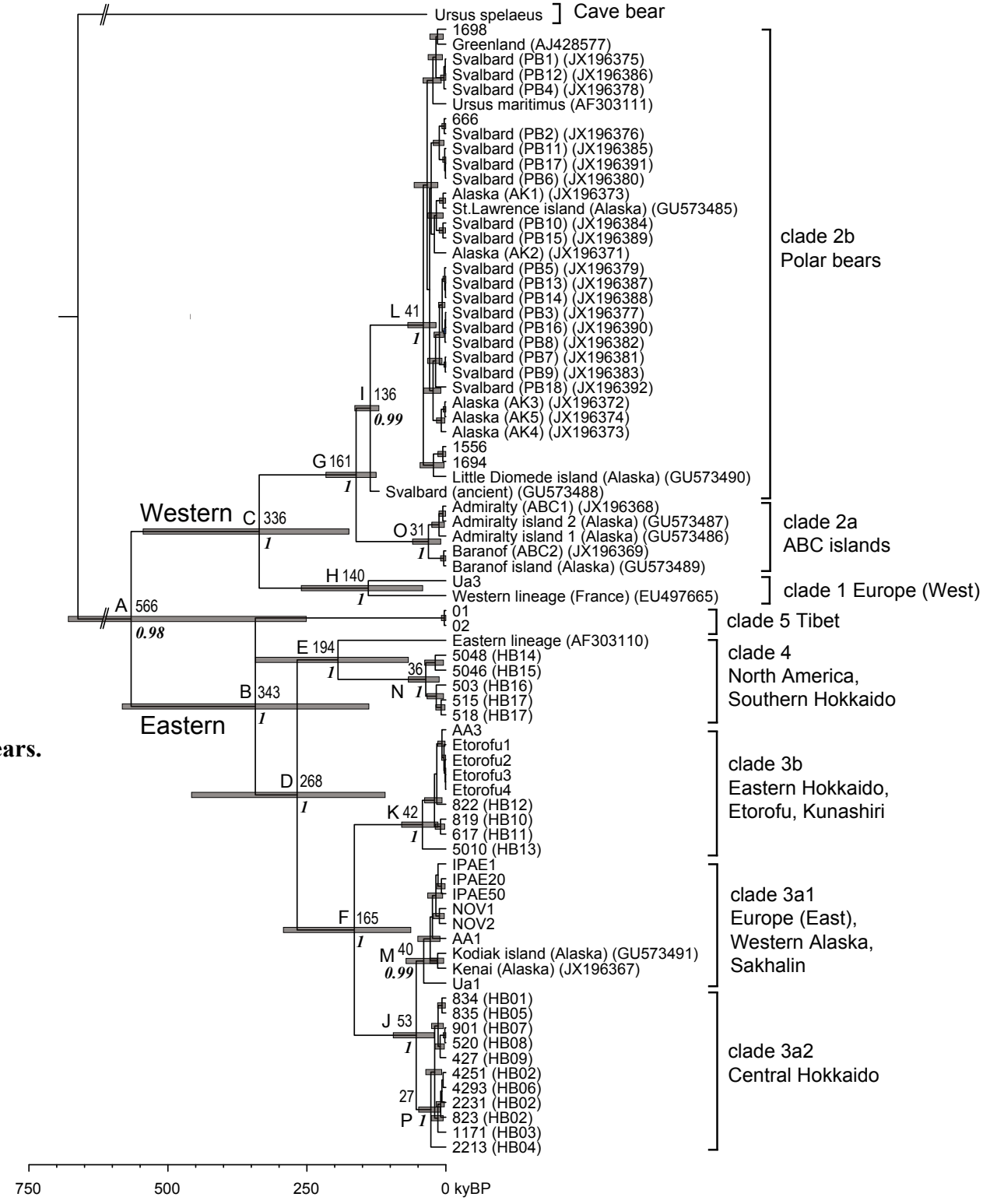

Fig. 2. Maximum clade credibility tree from a BEAST. Bayesian analysis of complete mtDNA sequences from brown bears. The time scale was calibrated using radiocarbon dates of ancient bear sequences previously reported (see the text). Major mtDNA clades and their geographic regions are labeled. Nodes of interest are those with a posterior probability of 0.98-1.00 (in italics).The numbers at nodes A-P indicate mean ages in kyBP. Node bars represent the 95% highest posterior density of nodal age estimates.Terminal taxa are indicated by sample numbers or by accession numbers previously reported. Haplotype numbers in the parentheses for Hokkaido brown bear samples correspond to the mtDNA control region haplotypes of Matsuhashi et al. (1999). Slanted double lines near the root indicate that portions of lines or node bars have been omitted due to space constraints. Detailed information on nodal ages is given in Table 3.

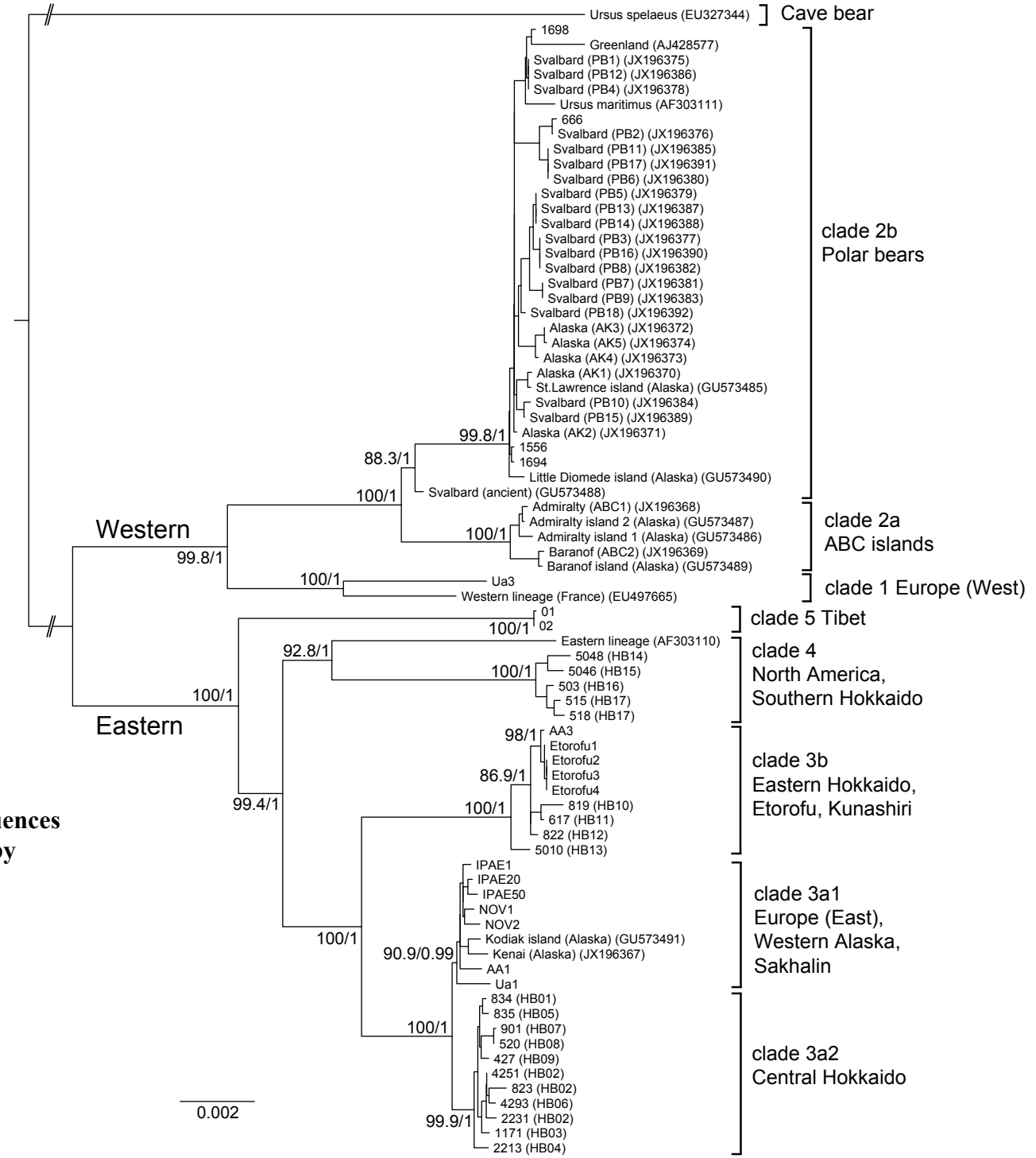

Fig. 3. Maximum likelihood (ML) tree for complete mtDNA sequences from brown bears and polar bears.Terminal taxa are indicated by sample numbers or by accession numbers previously reported. Haplotype numbers in parentheses for Hokkaido brown bear samples correspond to the mtDNA control region haplotypes of Matsuhashi et al. (1999). The tree from Bayesian inference (BI) was identical in topology to the ML tree. Bootstrap values greater than 50% (ML) and posterior probability values (BI) are indicated above major branches. Major mtDNA clades and their geographic regions are also indicated. Slanted double lines near the tree root indicate that portions of lines were omitted due to space constraints. The scale bar indicates the estimated number of nucleotide substitutions per site.

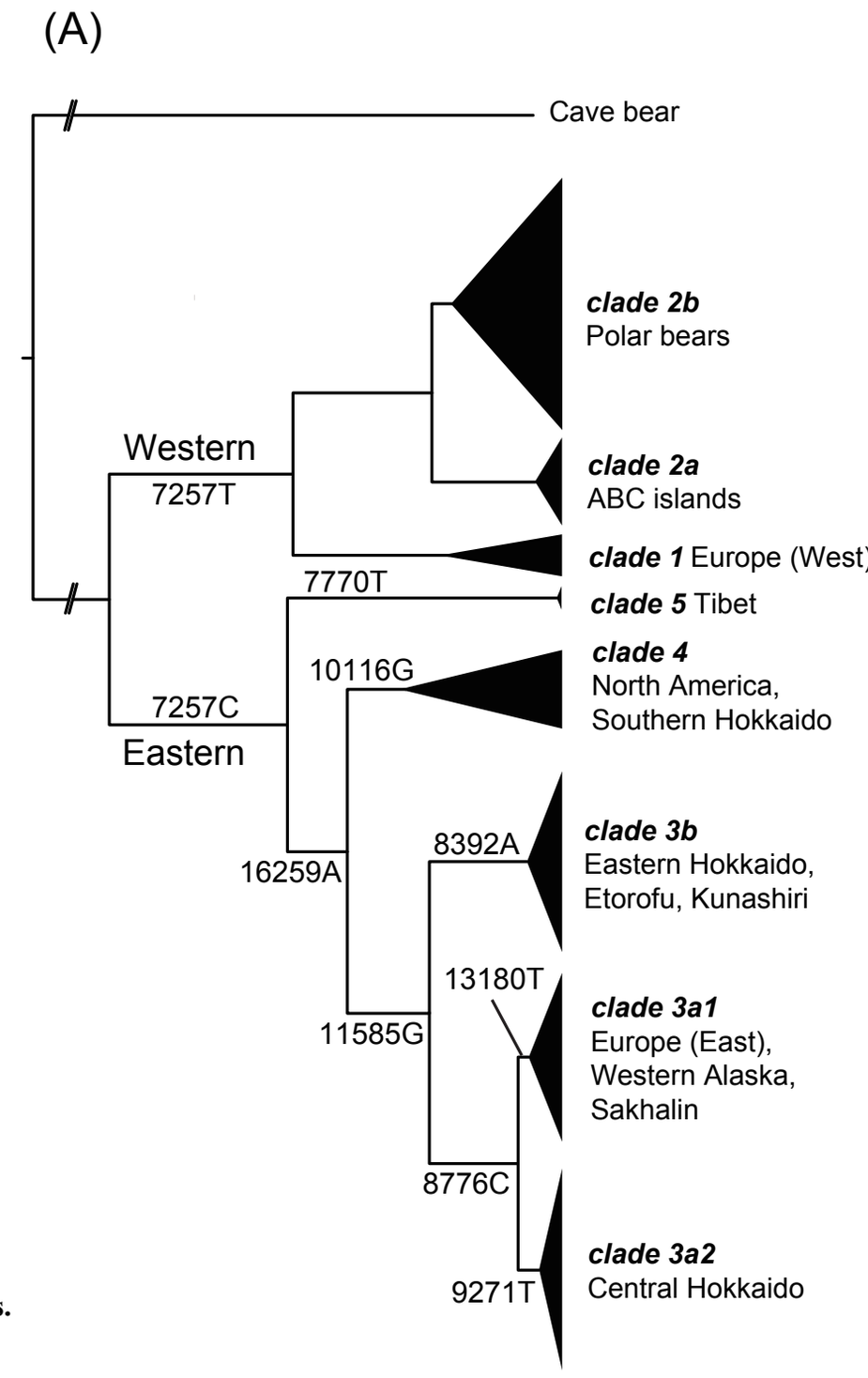

Fig. 4A. Phylogeny of brown and polar bears, consisting of eight mtDNA clades. Numbers and nucleotides above branches show the positions of each clade-specific SNP and the diagnostic nucleotide at that site. The tree is modified from Figure 2.

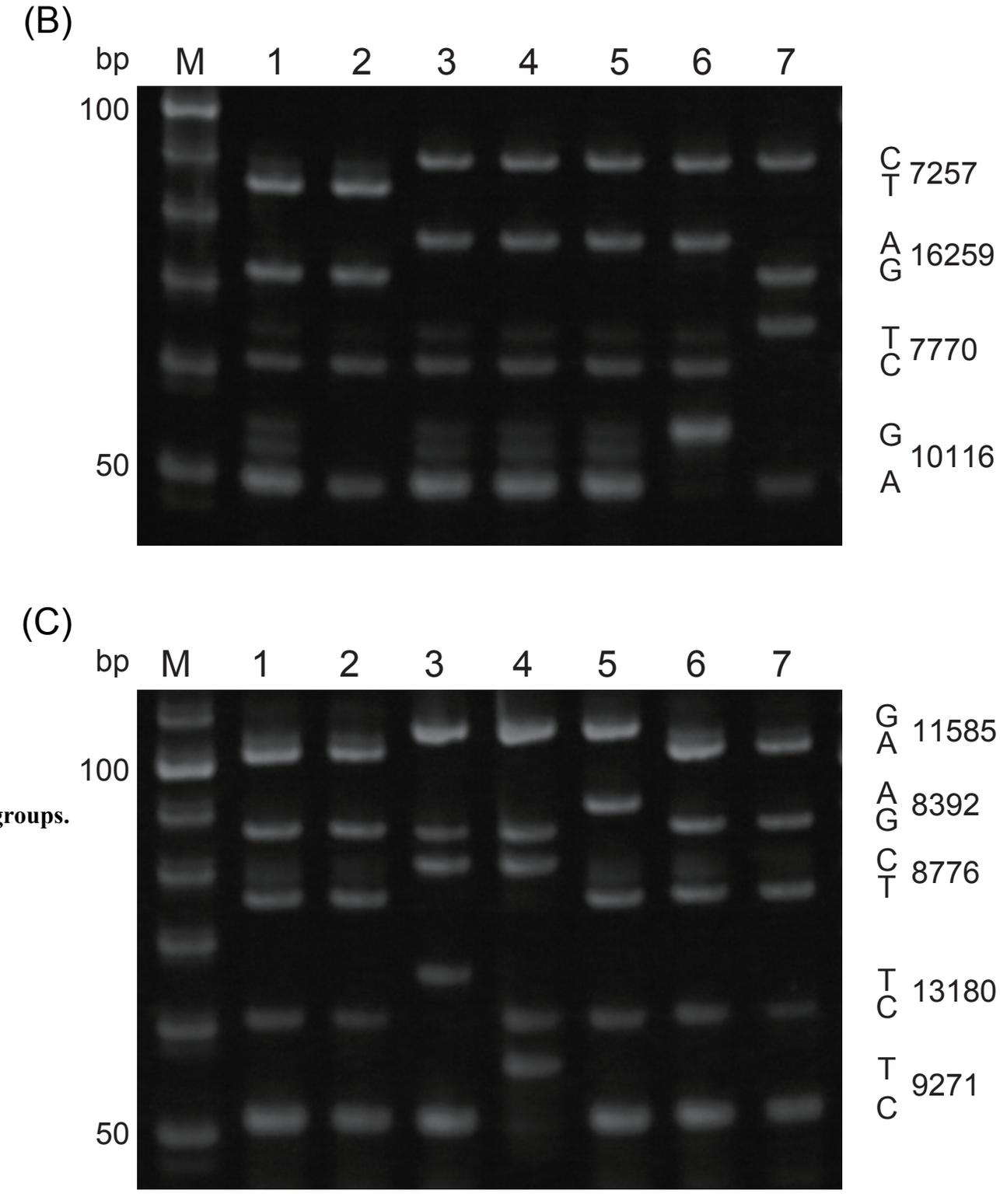

Fig. 4B, C. APLP band patterns for brown bear mtDNA haplogroups. from primer sets A and B, respectively.
Lane 1, clade 1; Lane 2, clade 2b; Lane 3, clade 3a1;Lane 4, clade 3a2; Lane 5, clade 3b; Lane 6, clade 4; Lane 7, clade 5; M, marker,10-bp DNA ladder. Reference samples representative of each of clades 1, 2b, 3a1, 3a2, 3b, 4, and 5 were Ua3, 1556, IPAE20, 835, 822 503, and 01, which were reported in Chapter 1. Numbers and nucleotides listed at the right are SNP position numbers and the specific nucleotides involved in the SNPs, as indicated in Table 4. The SNP at each site is distinguishable by band positions. Brown bear mtDNA haplogroups for museum skin samples (Table 6) can be identified by referring to Table 5.

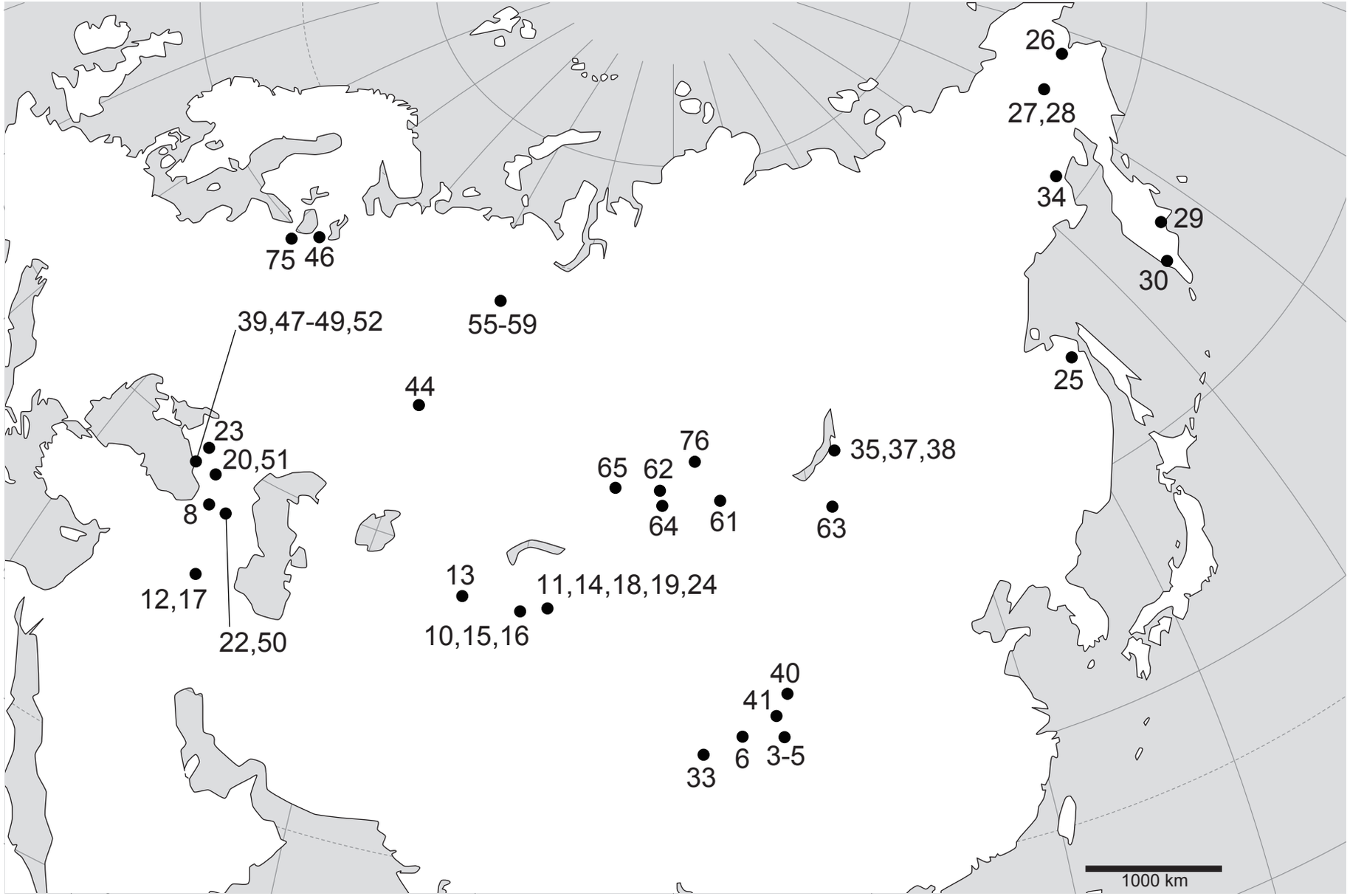

Fig. 5. Localities of brown bear samples analyzed.
Filled circles indicate sample localities; sample ID numbers indicate individual brown bear specimens at each locality.
See Table 6 for a list of sample localities, collection dates, SNPs detected, and haplogroups (clades) identified.

Fig. 6. Geographical distribution of the brown bear mtDNA haplogroups detected in continental Eurasia by 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. Black filled circles, mtDNA clade 3a1; open circles, clade 3b; open triangles, clade 5; open diamonds, W1; black filled squares, E1; gray filled circles, E2.

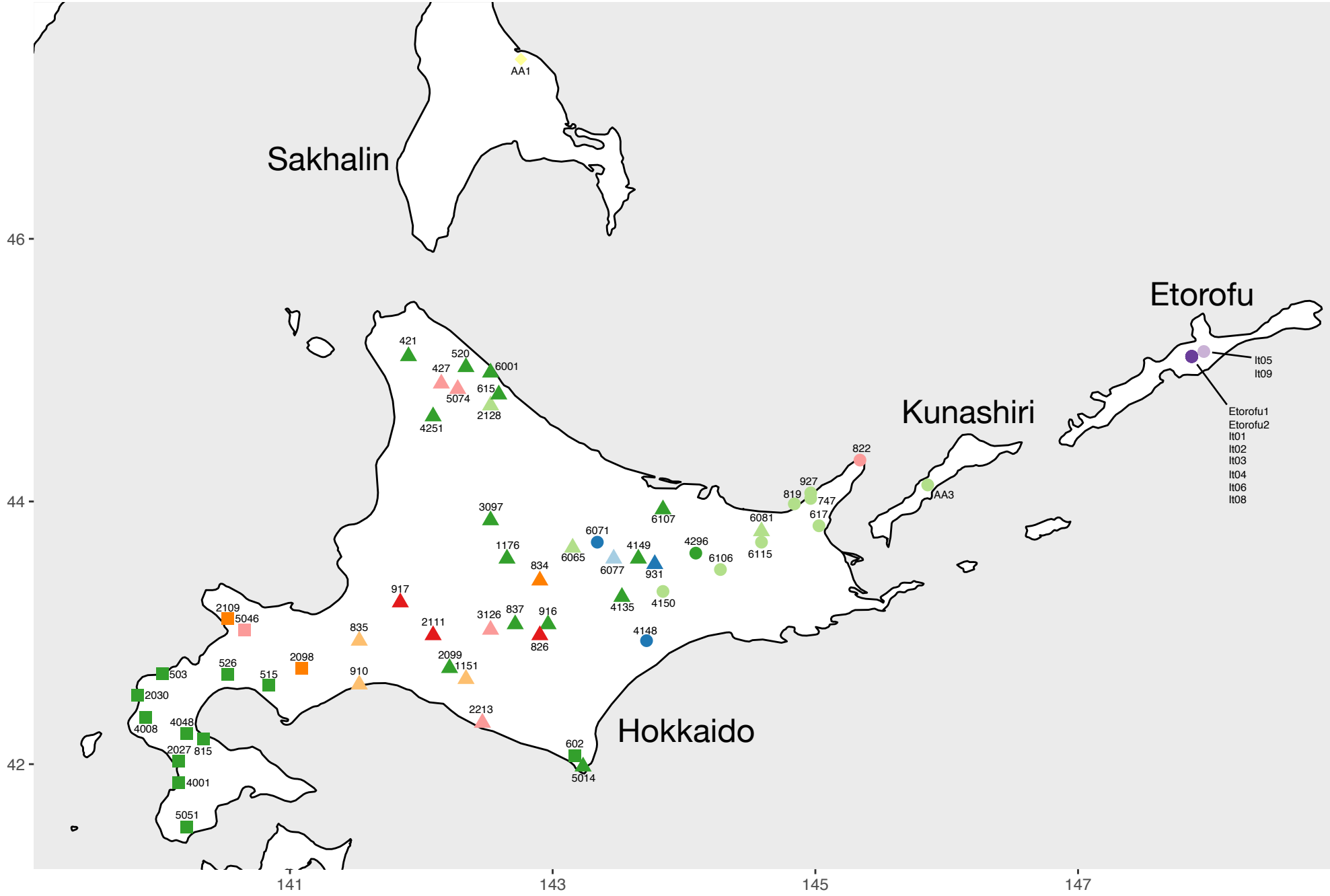

Fig. 7. Geographical distribution of the brown bear Y-chromosomal DNA compound haplotypes around Hokkaido.
The characters on the plots denote sample IDs of brown bears. The shape of the plot indicates mtDNA haplogroups (lineages) based on the complete mtDNA sequences (Hirata et al. 2013). The colors of plots show Y-chromosomal DNA compound haplotypes genotyped in the present study.

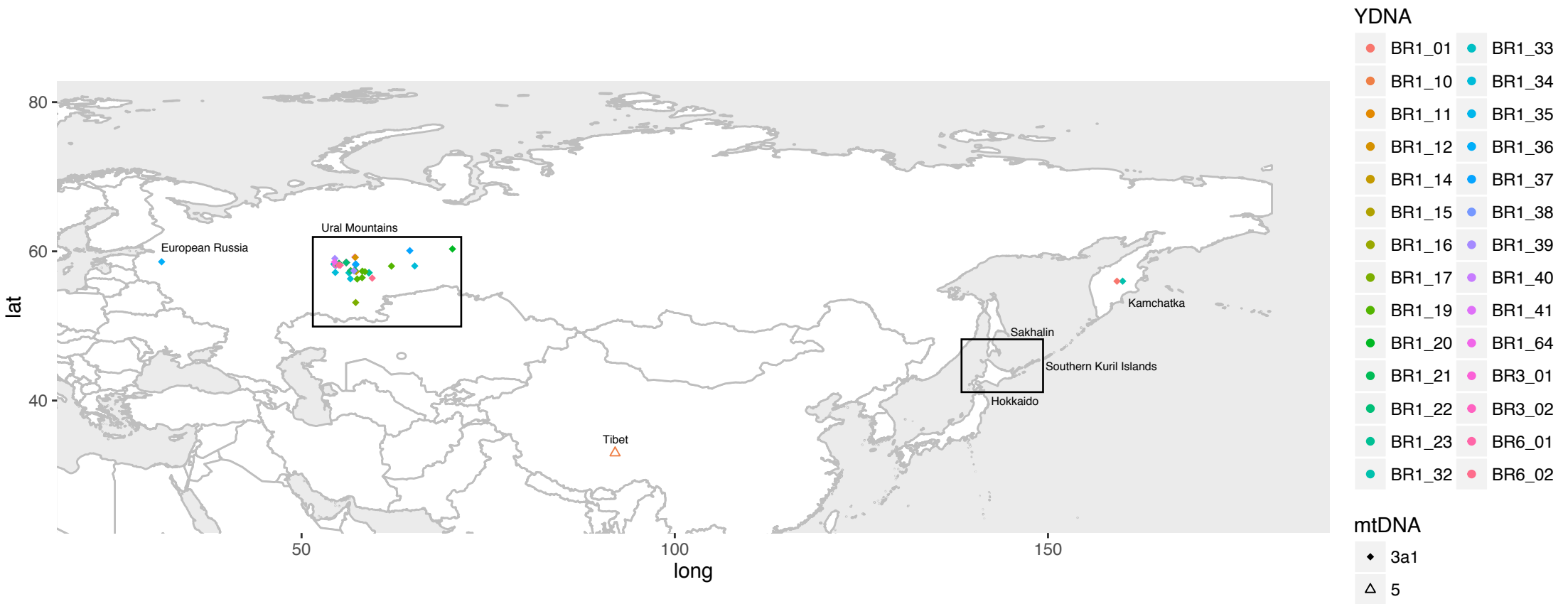

Fig. 8. Geographical distribution of the brown bear Y-chromosomal DNA compound haplotypes on the Eurasian Continent.
The shape of the plot indicates the number of the brown bears with mtDNA haplogroups based on the complete mtDNA sequences (Hirata et al. 2013).
The color of the plot shows the number of the brown bears with Y-chromosomal DNA compound haplotypes genotyped in the present study.

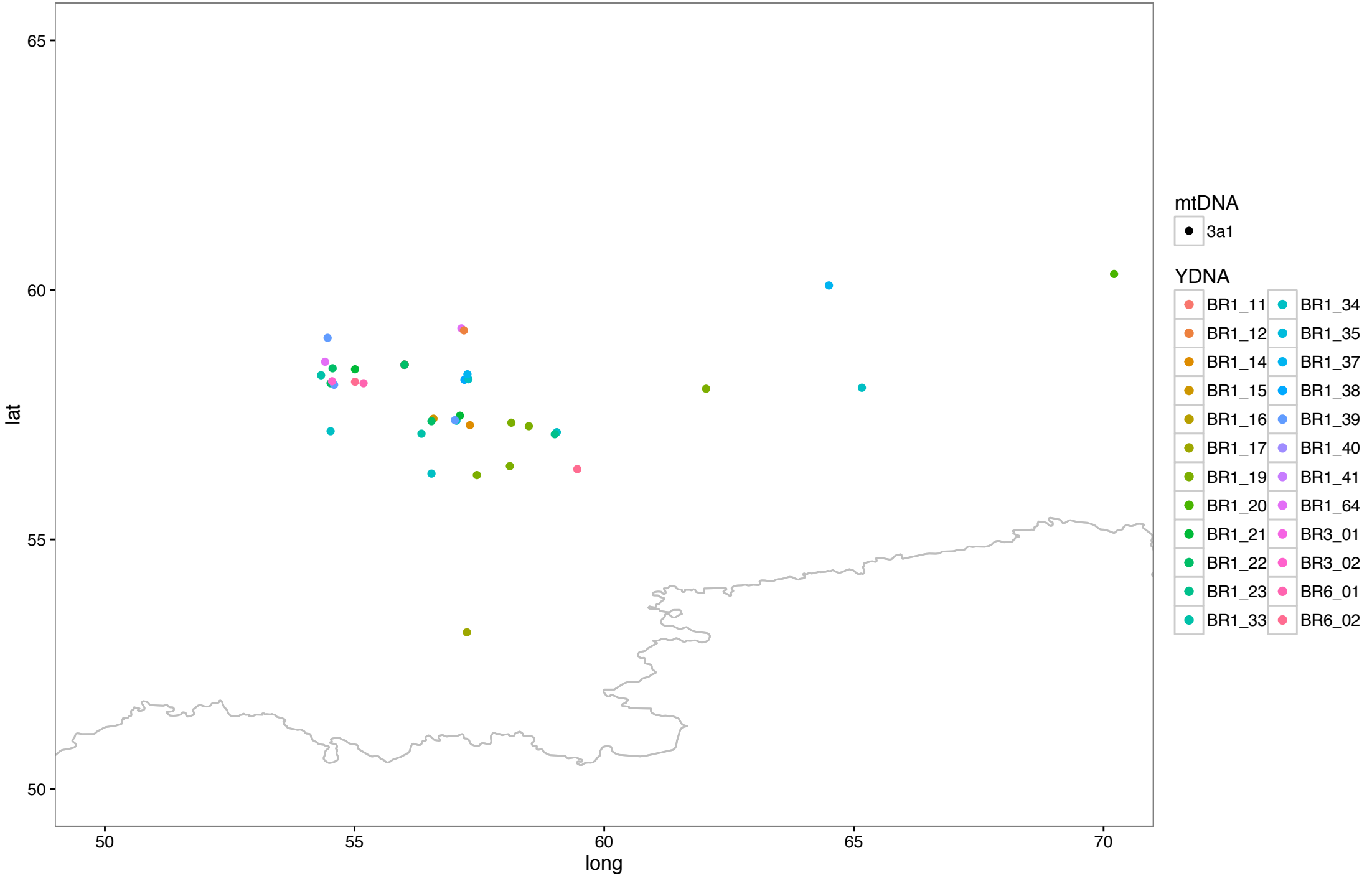

Fig. 9. Geographical distribution of the brown bear Y-chromosomal DNA compound haplotypes around the Ural Mountains. Enlarged map of the Fig. 8 around the Ural Mountains. The shape of the plot indicates the number of the brown bear individual with mtDNA haplogroups based on the complete mtDNA sequences (Hirata et al. 2013). The color of the plot indicates the brown bears with Y-chromosomal DNA compound haplotypes genotyped in the present study.

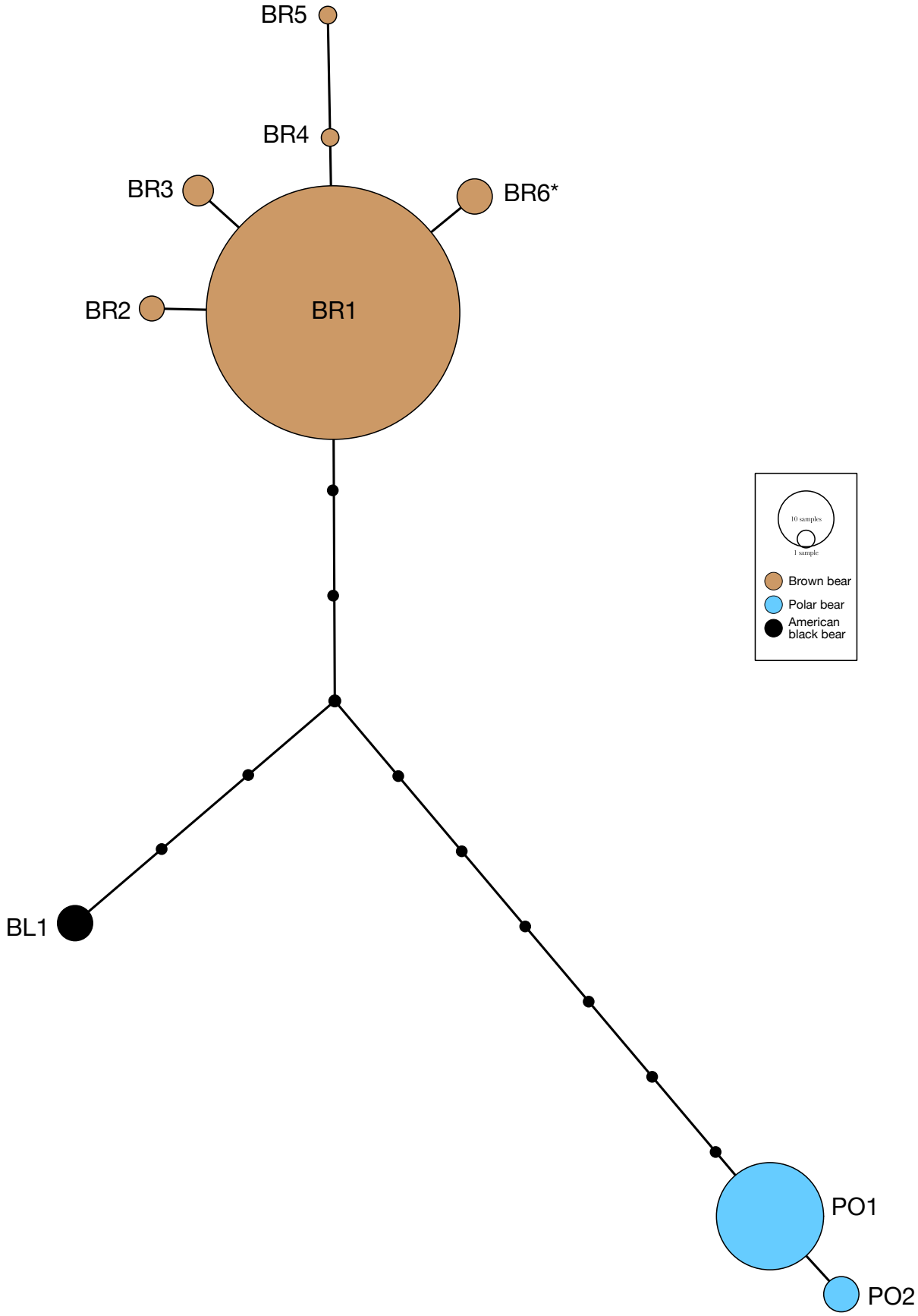

Fig. 10. Median-joining (MJ) network reconstructed using 3.1 kb Y-linked sequence data set. The difference of the haplotype colors denotes the difference of the bear species: brown, brown bear; blue, polar bear; black, American black bear. Small and closed circles indicate estimated intermediate and non-observed haplotypes, and lines between haplotype circles represent single mutational steps by single nucleotide polymorphisms (SNPs). The size of haplotype circles is proportional to the number of individuals observed. Haplotypes with asterisks denote the newly found haplotypes in the present study. Haplotype nomenclature based on 3.1 kb Y-chromosomal DNA linked sequences corresponds to that of Bidon et al. (2014).

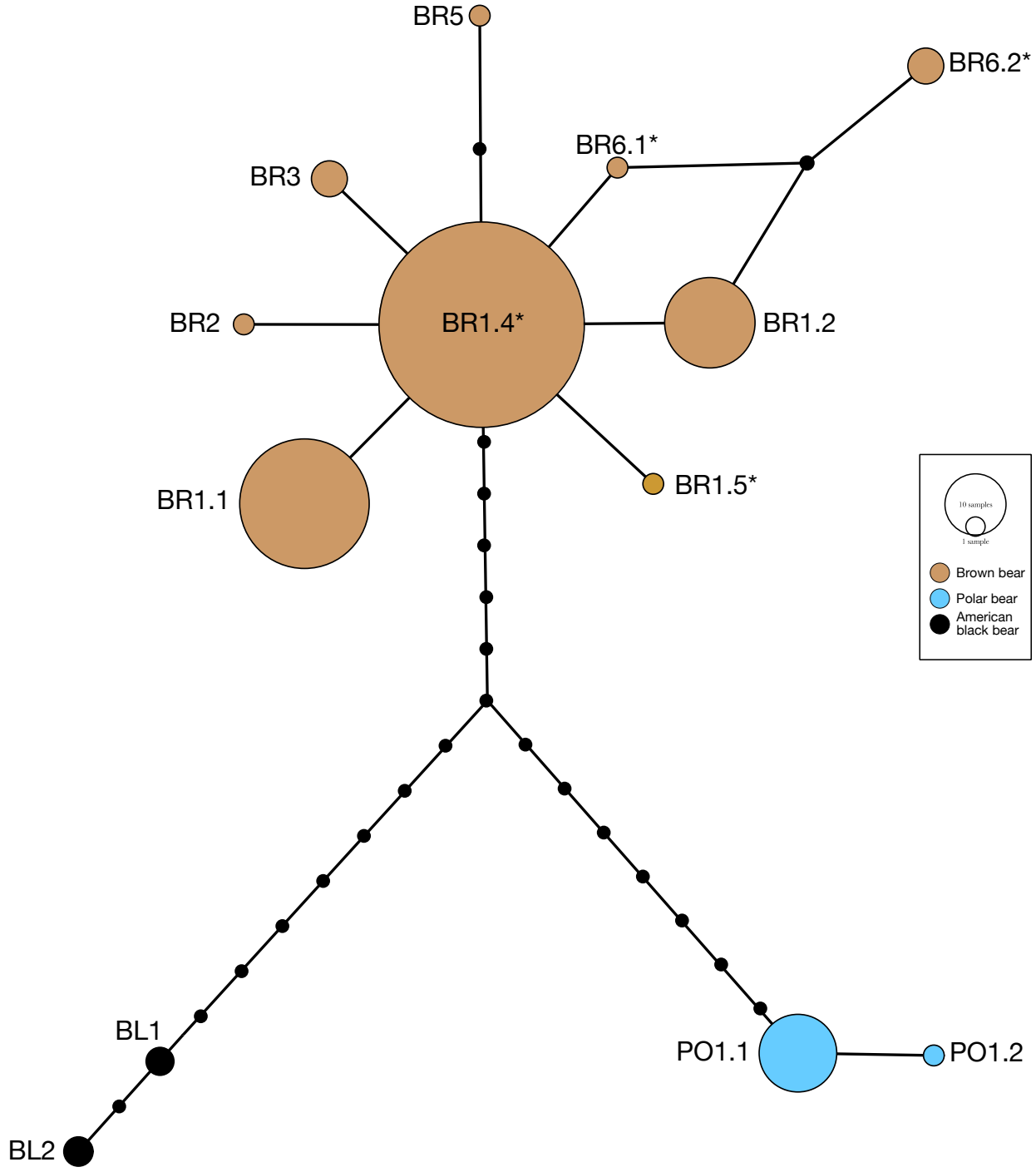

Fig. 11. Median-joining (MJ) network reconstructed using 5.3 kb Y-linked sequence data set. The difference of the haplotype colors denotes the difference of the bear species: brown, brown bear; blue, polar bear; black, American black bear. Small and closed circles indicated estimated intermediate and non-observed haplotypes, and lines between haplotype circles represent single mutational steps by single nucleotide polymorphisms (SNPs). The size of haplotype circles is proportional to the number of individuals observed. Haplotypes with asterisks denote the newly found haplotypes in the present study. Haplotype nomenclature based on 5.3 kb Y-chromosomal DNA linked sequences corresponds to that of Bidon et al. (2014).

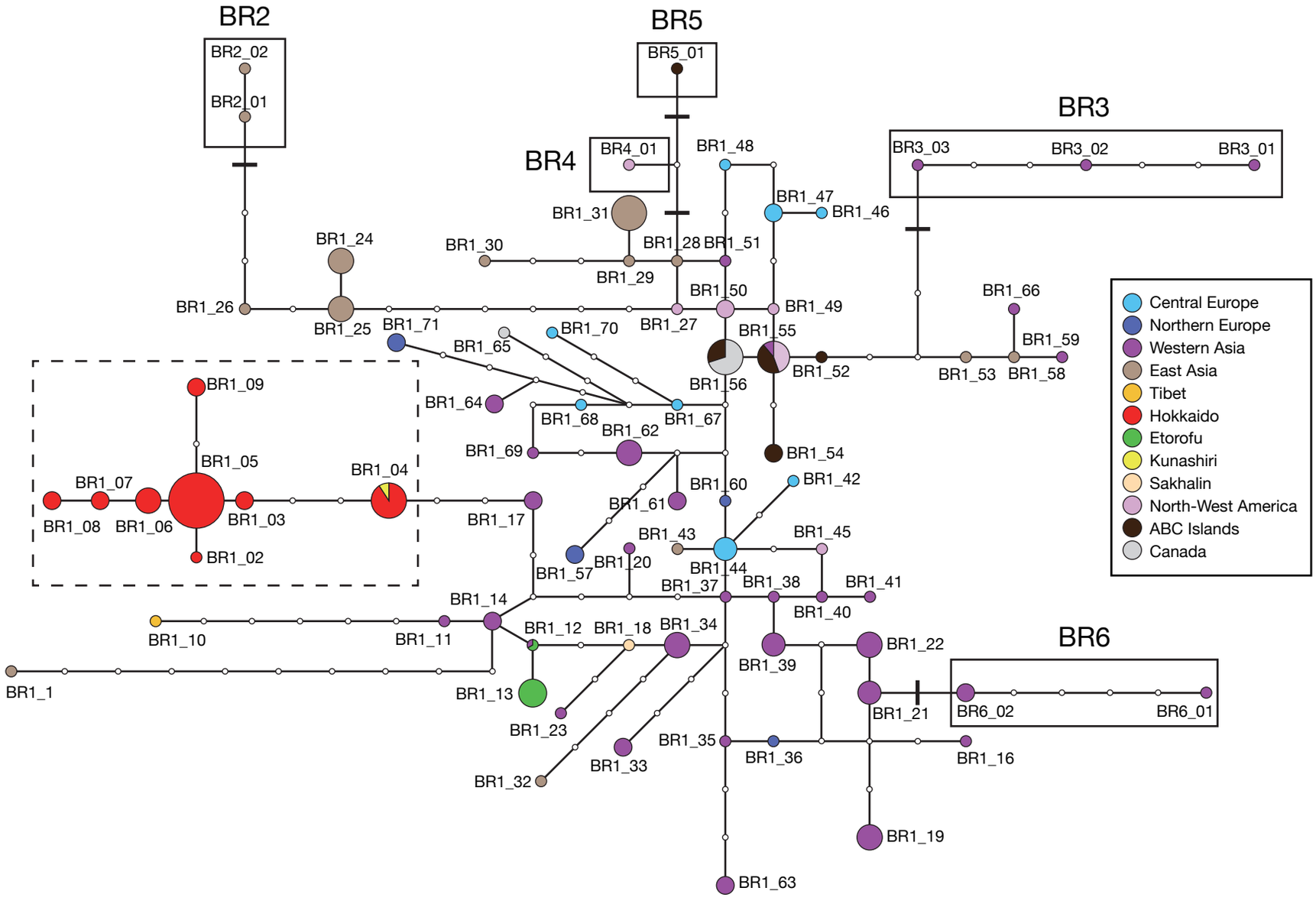

Fig. 12. Median-joining (MJ) network of the brown bears reconstructed using Y-chromosomal DNA compound haplotypes combined with Y-linked SNPs of 3.1 kb sequences and Y-linked microsatellites.

All haplotypes closed by the broken line indicates those from Hokkaido (including one Kunashiri brown bear).The haplotypes closed by solid lines denote the same haplotypes (BR2–BR6) as those distinguished by only 3.1 kb Y-linked sequences. The remaining haplotypes has the same haplotypes (BR1) as those distinguished by only 3.1 kb Y-linked sequences. One color corresponds to a different brown bear population. One slash on the network line denote a single mutational step difference distinguished by single nucleotide polymorphisms (SNPs). Small and open circles indicate estimated intermediate and non-observed haplotypes, and lines between haplotype circles represent single mutational steps by microsatellites. The size of haplotype circles is proportional to the number of individuals with those haplotypes. Haplotype names were added to the median-joining network of the brown bears in Fig. 12.

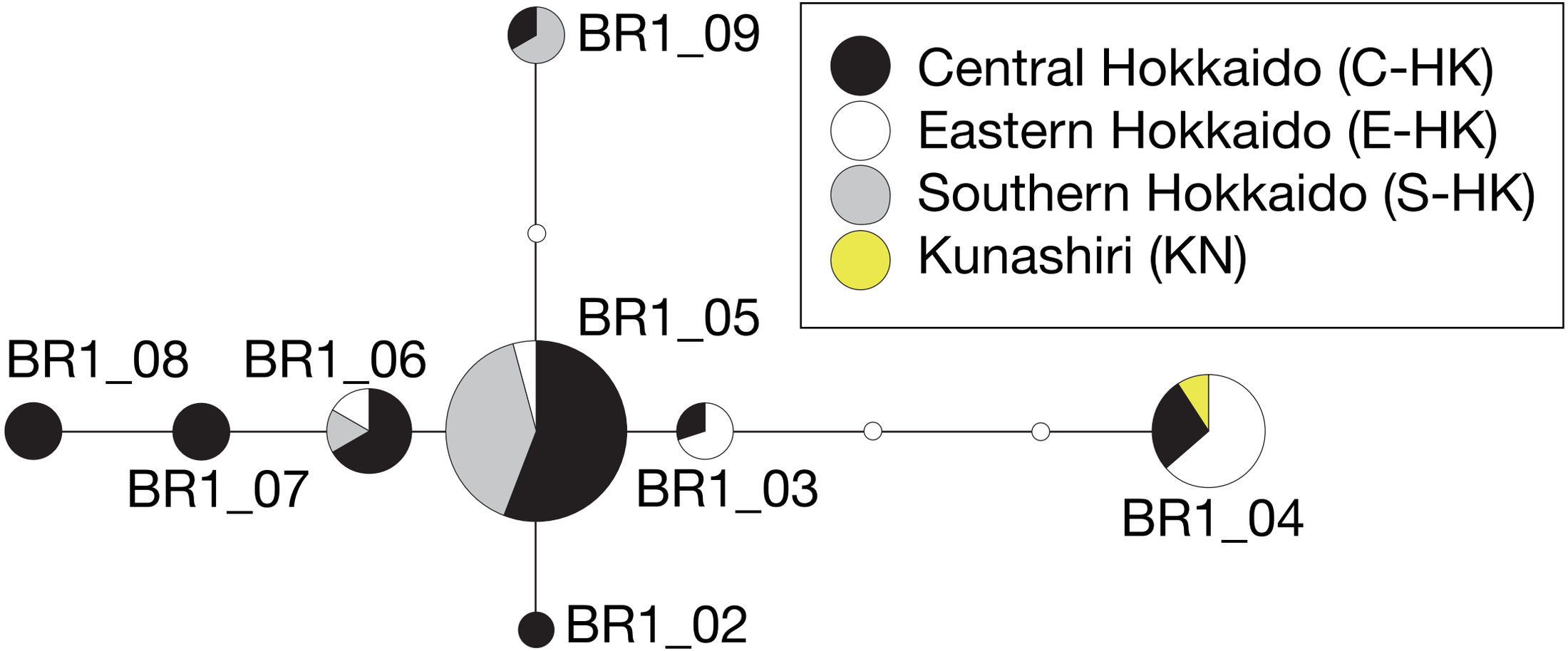

Fig. 13. Median-joining (MJ) network of the brown bears reconstructed using Y-chromosomal DNA compound haplotypes combined with Y-linked SNPs of 3.1 kb sequences and Y-linked microsatellites.
Brown bears from Hokkaido (including one Kunashiri brown bear). One color corresponds to the different maternal population on Hokkaido. Small and open circles indicated estimated intermediate non observed haplotypes, and lines between haplotype circles represent single mutational steps by microsatellites. The size of haplotype circles is proportional to the number of individuals.

Table 1. Oligonucleotide primers used to amplify fragments for the complete brown bear mtDNA genome.

Fragment	Forward primer (5'-3')		Reverse primer (5'-3')		Amplified size (bp)	Reference
1	mtDNA1H	5’-CAAATGGGACATCTCGATGGACTA	mtDNA1L	5’-CAGCTATCACCAGGCTCGTTAG-3’	2595	Delisle and Strobeck (2002)
2	mtDNA2H	5’-GGAGATAAGTCGTAACAAGGTAAGCA-3’	mtDNA2L	5’-TCCTACGATGTTGGGTCCTTT-3’	1887	Delisle and Strobeck (2002)
3	mtDNA3H	5’-AATCCAGGTCGGTTTCTATCTA-3’	mtDNA3L	5’-ATCCTATATGGGCGATTGATGAGT-3’	1959	Delisle and Strobeck (2002)
4	mtDNA4H	5’-CGAAAATGTTGGTTTATACCCTTCC-3’	mtDNA4L	5’-TGCCAAGCTCTGTGGTGAAT-3’	1397	Delisle and Strobeck (2002)
5	mtDNA5H	5’-GGACTGCAAGAACATATCTCACATCAA-3’	mtDNA5L	5’-GGAGGAGGACATCCATGTAGTCATTC-3’	1809	Delisle and Strobeck (2002)
6	mtDNA6H	5’-GCTCATTTATTTCACTAACAGCAGT-3’	mtDNA6L	5’-GGGCTACAGCAAATTCAAGGAT-3’	1852	Delisle and Strobeck (2002)
7	mtDNA7H	5’-TTGGCTCACTTTCTACCTCAAGG-3’	mtDNA7L	5’-GTGGGGATGATGATTTTTAGCATTGTA-3’	1888	Delisle and Strobeck (2002)
8	mtDNA8H	5’-CCAAAACAAATGATTTCGACTCA-3’	mtDNA8L	5’-GGTTCCTAAGACCAATGGATTACTTCT-3’	1888	Delisle and Strobeck (2002)
9	mtDNA9H	5’-AAACCATCATTCACACGAGAAAAC-3’	mtDNA9L	5’-GAGTTAGTAATAGGGCTCAGGCGTT-3’	1534	Delisle and Strobeck (2002)
10	mtDNA10H	5’-TACTCCTGTTTCAGCCCTACTCCA-3’	mtDNA10L	5’-GCTGGTTTCTCGAAGCCTGGTGATT-3’	3263	Delisle and Strobeck (2002)
11	mtDNA11H	5’-CTAACATGAATCGGAGGACAACCAG-3’	mtDNA11L	5’-GGCTCATCTAGGCATTTTCAGTG-3’	1917	Delisle and Strobeck (2002)
2A	mtDNA2AH	5’-GCTTACACCCAGAGGATTTCAC-3’	mtDNA2AL	5’-TGGGAATGCTGGAGGTGATG-3’	904	This study
2B	mtDNA2BH	5’-CAGCAACGGATAACCACTGATAG-3’	mtDNA2BL	5’-GGAGAGGATTTGAACCTCTGAG-3’	964	This study
3A	mtDNA3AH	5’-AGCAATCCAGGTCGGTTTCTATC-3’	mtDNA3AL	5’-GATTCATAGGAAAGAAGCTGTTAGGAG-3’	1053	This study
3B	mtDNA3BH	5’-CTAGCAGAAGGAGAGTCAGAATTAG-3’	mtDNA3BL	5’-GTGAGCGATTGAAGAGTATGCTAG-3’	1125	This study
6A	mtDNA6AH	5’-CTCGGCGATATTCCGACTATC-3’	mtDNA6AL	5’-GTTGTGGCATCTTCATTAAGGAGAAG-3’	1141	This study
6B	mtDNA6BH	5’-CAATCCCAGGACGGCTAAAC-3’	mtDNA6BL	5’-TAGGCCTGAATGAGGGCTAC-3’	1032	This study
7A	mtDNA7AH	5’-TGGCTCACTTTCTACCTCAAG-3’	mtDNA7AL	5’-GTGTCAATATCATGCTGCTGCTTC-3’	1035	This study
7B	mtDNA7BH	5’-GGATTTCACGGACTTCATGTAATC-3’	mtDNA7BL	5’-GCTGATAGGGAGTCGGTAAAG-3’	1143	This study
9A	mtDNA9AH	5’-ACACGAGAAAACGCCCTGATAG-3’	mtDNA9AL	5’-TGTAGGCAGCGGTTATGGATG-3’	1552	This study
10A	mtDNA10AH	5’-GCCTCACCTAGCATTCCTTCATATC-3’	mtDNA10AL	5’-TTCGGATGTTGGTCATTAAGGTTC-3’	1458	This study
10B	mtDNA10BH	5’-CTTCCTCACTAAAAAACCCCGAATC-3’	mtDNA10BL	5’-TTGTCTGAGTCAGATGGGATTC-3’	1140	This study
10C	mtDNA10CH	5’-CCAACACCATCAAACATCTCAGCATG-3’	mtDNA10CL	5’-GCTCCCGGACTAAGTGAAATACATG-3’	1448	This study
11A	mtDNA11AH	5’-CATGAATTGGAGGACAACCAGTAG-3’	mtDNA11AL	5’-AGGCATTTTCAGTGCCTTGCTTTAC-3’	1888	This study

Table 2. Internal oligonucleotide primers used to sequence the complete mtDNA genomes of brown bears.

Internal primer (5'-3')		Reference
mtDNA1L.intA	5’-GATGATTGTGCTTACTCTTG-3’	This study
mtDNA1L.intB	5’-GTAGCTAATGGGAAGGCTA-3’	This study
mtDNA2H.intA	5’-CAACGGATAACCACTGATAG-3’	This study
mtDNA3H.int2	5’-CTATCAGTCCTACTAAT-3’	Delisle and Strobeck (2002)
mtDNA5H.intA	5’-TAGCAGGCATCTCTTCTATTC-3’	This study
mtDNA6H.int2	5’-CAAGAACTAAAGCCTGGA-3’	Delisle and Strobeck (2002)
mtDNA7H.intA	5’-GTACCACTTCTCAATACTTC-3’	This study
mtDNA8H.intA	5’-CTGGTTATTATCACTCGATG-3’	This study
mtDNA10H.int2	5’-GGAAGTATTTTCGCAG-3’	Delisle and Strobeck (2002)
mtDNA10H.intA	5’-CAACTTCATCATCTTTCAAG-3’	This study
mtDNA10H.intB	5’-CAAATGTCCTTCTGAGGAG-3’	This study

Table 3. Bayesian age estimates (kyBP) for the most recent common ancestor (MRCA) of brown bear clades. Nodes refer to those in Fig. 2. Divergence times were estimated based on (a) complete mtDNA sequences and (b) mtDNA control region sequences. –, indicates that no divergence time estimations are available. Node ages without parentheses have no information on 95%HPD.

Node	MRCA	Node age (95%HPD)			
		This study[a]	Lindqvist et al. (2010)[a]	Davison et al. (2011)[b]	Miller et al. (2012)[a]
A	All brown bears	566 (251– 944)	490 (360–620)	263 (162–400)	509
B	Clades 3a, 3b, 4, and 5	343 (139–582)	–	–	–
C	Clades 1 and 2	336 (174–545)	310 (240–400)	–	314
D	Clades 3 and 4	268 (109–457)	–	140 (87–213)	–
E	Clade 4	194 (67–399)	–	87 (42–147)	–
F	Clades 3a and 3b	165 (63–292)	–	92 (51–133)	–
G	Clade 2	161 (125–216)	152 (131–177)	160 (124–210)	162
H	Clade 1	140 (42–260)	–	100 (49–164)	–
I	Clade 2b; All polar bears	136 (120–164)	134 (122–149)	146 (120–179)	136
J	Clade 3a	53 (21–95)	–	49 (18–86)	–
K	Clade 3b	42 (14–80)	–	75 (43–113)	–
L	Clade 2b; Extant polar bears	41 (18–68)	44	–	52
M	Clade3a1	40 (15–72)	–	–	–
N	Clade 4; Southern Hokkaido	36 (12–67)	–	–	–
O	Clade 2a	31 (9–60)	28	45 (10–91)	–
P	Clade 3a2	27 (10–49)	–	–	–

Table 4. Profiles of oligonucleotide primers used in the APLP analyses.

Set	Haplogroups	Primer	Sequence (5'-3')	Concentration (pmol/μl)	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 noncomplementary nucleotides are written in small letters.

Table 5. Haplogroup (clade)-specific SNP sites in the brown bear mitochondrial genome.

Haplogroup (clade)		SNP sites								
		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

Table 6. Brown bear skin samples used for mtDNA haplogrouping by APLP analysis, 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

Table 7. List of Y-chromosomal DNA compound haplotypes (YDNA haplotype), Y-chromosomal DNA haplotypes based on only 3.1 kb Y-chromosomal DNA linked sequences (YSNP_haplotype), Y-linked SNPs on 3.1 kb Y-chromosomal DNA linked sequences, fragment sizes of six Y-linked microsatellites ma

YDNA haplotype	YSNP_haplotype	YSNP41	YSNP228	YSNP234	YSNP773	YSNP1302	Y318.9	Y318.4	Y318.2	Y369.1	Y318.1	Y318.6	Central_Europe	Northern_Europe	Western_Asia	East_Asia	Tibet	Hokkaido	Etorofu	Kunashiri	Sakhalin	North-West_America	ABC_Islands	Canada
BR1_01	BR1	C	A	C	G	C	124	212	234	247	277	404	-	-	-	1	-	-	-	-	-	-	-	-
BR1_02	BR1	C	A	C	G	C	124	212	234	265	285	394	-	-	-	-	-	1	-	-	-	-	-	-
BR1_03	BR1	C	A	C	G	C	124	212	234	267	281	394	-	-	-	-	-	3	-	-	-	-	-	-
BR1_04	BR1	C	A	C	G	C	124	212	234	267	281	400	-	-	-	-	-	10	-	1	-	-	-	-
BR1_05	BR1	C	A	C	G	C	124	212	234	267	285	394	-	-	-	-	-	26	-	-	-	-	-	-
BR1_06	BR1	C	A	C	G	C	124	212	234	267	289	394	-	-	-	-	-	6	-	-	-	-	-	-
BR1_07	BR1	C	A	C	G	C	124	212	234	267	289	396	-	-	-	-	-	3	-	-	-	-	-	-
BR1_08	BR1	C	A	C	G	C	124	212	234	267	293	396	-	-	-	-	-	3	-	-	-	-	-	-
BR1_09	BR1	C	A	C	G	C	124	212	234	271	285	394	-	-	-	-	-	3	-	-	-	-	-	-
BR1_10	BR1	C	A	C	G	C	126	212	234	261	281	394	-	-	-	-	1	-	-	-	-	-	-	-
BR1_11	BR1	C	A	C	G	C	126	212	234	261	281	406	-	-	1	-	-	-	-	-	-	-	-	-
BR1_12	BR1	C	A	C	G	C	126	212	234	263	277	406	-	-	1	-	-	-	2	-	-	-	-	-
BR1_13	BR1	C	A	C	G	C	126	212	234	263	277	408	-	-	-	-	-	-	8	-	-	-	-	-
BR1_14	BR1	C	A	C	G	C	126	212	234	263	281	406	-	-	2	-	-	-	-	-	-	-	-	-
BR1_15	BR1	C	A	C	G	C	126	212	234	263	285	404	-	-	2	-	-	-	-	-	-	-	-	-
BR1_16	BR1	C	A	C	G	C	126	212	234	263	285	404	-	-	1	-	-	-	-	-	-	-	-	-
BR1_17	BR1	C	A	C	G	C	126	212	234	265	281	402	-	-	2	-	-	-	-	-	-	-	-	-
BR1_18	BR1	C	A	C	G	C	126	212	234	267	277	406	-	-	-	-	-	-	-	-	1	-	-	-
BR1_19	BR1	C	A	C	G	C	126	212	234	267	285	408	-	-	5	-	-	-	-	-	-	-	-	-
BR1_20	BR1	C	A	C	G	C	126	212	234	269	281	408	-	-	1	-	-	-	-	-	-	-	-	-
BR1_21	BR1	C	A	C	G	C	126	212	234	269	285	404	-	-	4	-	-	-	-	-	-	-	-	-
BR1_22	BR1	C	A	C	G	C	126	212	234	271	285	404	-	-	5	-	-	-	-	-	-	-	-	-
BR1_23	BR1	C	A	C	G	C	126	212	236	267	277	408	-	-	1	-	-	-	-	-	-	-	-	-
BR1_24	BR1	C	A	C	G	C	126	214	236	249	277	404	-	-	-	5	-	-	-	-	-	-	-	-
BR1_25	BR1	C	A	C	G	C	126	214	236	249	281	404	-	-	-	5	-	-	-	-	-	-	-	-
BR1_26	BR1	C	A	C	G	C	126	214	236	249	281	408	-	-	-	1	-	-	-	-	-	-	-	-
BR1_27	BR1	C	A	C	G	C	126	214	236	263	281	404	-	-	-	-	-	-	-	-	-	1	-	-
BR1_28	BR1	C	A	C	G	C	126	214	236	263	281	406	-	-	-	1	-	-	-	-	-	-	-	-
BR1_29	BR1	C	A	C	G	C	126	214	236	265	281	406	-	-	-	1	-	-	-	-	-	-	-	-
BR1_30	BR1	C	A	C	G	C	126	214	236	265	281	412	-	-	-	1	-	-	-	-	-	-	-	-
BR1_31	BR1	C	A	C	G	C	126	214	236	267	281	406	-	-	-	10	-	-	-	-	-	-	-	-
BR1_32	BR1	C	A	C	G	C	128	212	234	263	277	402	-	-	-	1	-	-	-	-	-	-	-	-
BR1_33	BR1	C	A	C	G	C	128	212	234	265	281	410	-	-	2	-	-	-	-	-	-	-	-	-
BR1_34	BR1	C	A	C	G	C	128	212	234	267	277	406	-	-	8	-	-	-	-	-	-	-	-	-
BR1_35	BR1	C	A	C	G	C	128	212	234	267	281	402	-	-	1	-	-	-	-	-	-	-	-	-
BR1_36	BR1	C	A	C	G	C	128	212	234	267	285	402	-	1	-	-	-	-	-	-	-	-	-	-
BR1_37	BR1	C	A	C	G	C	128	212	234	269	281	406	-	-	1	-	-	-	-	-	-	-	-	-
BR1_38	BR1	C	A	C	G	C	128	212	234	271	281	406	-	-	1	-	-	-	-	-	-	-	-	-
BR1_39	BR1	C	A	C	G	C	128	212	234	271	285	406	-	-	4	-	-	-	-	-	-	-	-	-
BR1_40	BR1	C	A	C	G	C	128	212	234	273	281	406	-	-	1	-	-	-	-	-	-	-	-	-
BR1_41	BR1	C	A	C	G	C	128	212	234	273	281	408	-	-	1	-	-	-	-	-	-	-	-	-
BR1_42	BR1	C	A	C	G	C	128	214	234	265	281	406	1	-	-	-	-	-	-	-	-	-	-	-
BR1_43	BR1	C	A	C	G	C	128	214	234	269	277	406	-	-	-	1	-	-	-	-	-	-	-	-
BR1_44	BR1	C	A	C	G	C	128	214	234	269	281	406	4	-	-	-	-	-	-	-	-	-	-	-
BR1_45	BR1	C	A	C	G	C	128	214	234	273	281	406	-	-	-	-	-	-	-	-	-	1	-	-
BR1_46	BR1	C	A	C	G	C	128	214	236	259	281	400	1	-	-	-	-	-	-	-	-	-	-	-
BR1_47	BR1	C	A	C	G	C	128	214	236	259	281	402	2	-	-	-	-	-	-	-	-	-	-	-
BR1_48	BR1	C	A	C	G	C	128	214	236	259	281	406	1	-	-	-	-	-	-	-	-	-	-	-
BR1_49	BR1	C	A	C	G	C	128	214	236	263	281	402	-	-	-	-	-	-	-	-	-	1	-	-
BR1_50	BR1	C	A	C	G	C	128	214	236	263	281	404	-	-	-	-	-	-	-	-	-	2	-	-
BR1_51	BR1	C	A	C	G	C	128	214	236	263	281	406	-	-	1	-	-	-	-	-	-	-	-	-
BR1_52	BR1	C	A	C	G	C	128	214	236	265	277	402	-	-	-	-	-	-	-	-	-	-	1	-
BR1_53	BR1	C	A	C	G	C	128	214	236	265	277	406	-	-	-	1	-	-	-	-	-	-	-	-
BR1_54	BR1	C	A	C	G	C	128	214	236	265	281	398	-	-	-	-	-	-	-	-	-	-	2	-
BR1_55	BR1	C	A	C	G	C	128	214	236	265	281	402	-	-	1	-	-	-	-	-	-	4	4	-
BR1_56	BR1	C	A	C	G	C	128	214	236	265	281	404	-	-	-	-	-	-	-	-	-	-	3	7
BR1_57	BR1	C	A	C	G	C	128	214	236	265	285	406	-	2	-	-	-	-	-	-	-	-	-	-
BR1_58	BR1	C	A	C	G	C	128	214	236	267	277	406	-	-	-	1	-	-	-	-	-	-	-	-
BR1_59	BR1	C	A	C	G	C	128	214	236	267	277	408	-	-	-	-	-	-	-	-	-	-	-	-
BR1_60	BR1	C	A	C	G	C	128	214	236	269	281	406	-	1	-	-	-	-	-	-	-	-	-	-
BR1_61	BR1	C	A	C	G	C	128	214	236	269	285	402	-	-	2	-	-	-	-	-	-	-	-	-
BR1_62	BR1	C	A	C	G	C	128	214	236	271	285	404	-	5	-	-	-	-	-	-	-	-	-	-
BR1_63	BR1	C	A	C	G	C	130	212	234	263	281	402	-	-	2	-	-	-	-	-	-	-	-	-
BR1_64	BR1	C	A	C	G	C	130	214	234	267	285	400	-	-	2	-	-	-	-	-	-	-	-	-
BR1_65	BR1	C	A	C	G	C	130	214	236	263	289	404	-	-	-	-	-	-	-	-	-	-	-	1
BR1_66	BR1	C	A	C	G	C	130	214	236	267	277	406	-	-	1	-	-	-	-	-	-	-	-	-
BR1_67	BR1	C	A	C	G	C	130	214	236	267	281	404	1	-	-	-	-	-	-	-	-	-	-	-
BR1_68	BR1	C	A	C	G	C	130	214	236	267	285	406	3	-	-	-	-	-	-	-	-	-	-	-
BR1_69	BR1	C	A	C	G	C	130	214	236	271	285	406	-	-	1	-	-	-	-	-	-	-	-	-
BR1_70	BR1	C	A	C	G	C	132	214	236	265	281	406	1	-	-	-	-	-	-	-	-	-	-	-
BR1_71	BR1	C	A	C	G	C	132	216	236	265	285	400	-	2	-	-	-	-	-	-	-	-	-	-
BR2_01	BR2	C	C	C	G	C	126	214	236	251	289	408	-	-	-	1	-	-	-	-	-	-	-	-
BR2_02	BR2	C	C	C	G	C	126	214	236	251	289	410	-	-	-	1	-	-	-	-	-	-	-	-
BR3_01	BR3	T	A	C	G	C	126	212	234	265	277	404	-	-	1	-	-	-	-	-	-	-	-	-
BR3_02	BR3	T	A	C	G	C	126	212	234	265	277	410	-	-	1	-	-	-	-	-	-	-	-	-
BR3_03	BR3	T	A	C	G	C	128	214	236	265	277	410	-	-	1	-	-	-	-	-	-	-	-	-
BR4_01	BR4	C	A	T	G	C	126	214	236	263	285	408	-	-	-	-	-	-	-	-	-	1	-	-
BR5_01	BR5	C	A	T	C	C	126	214	236	263	289	406	-	-	-	-	-	-	-	-	-	-	1	-
BR6_01	BR6	C	A	C	G	G	126	212	234	269	285	394	-	-	1	-	-	-	-	-	-	-	-	-
BR6_02	BR6	C	A	C	G	G	126	212	234	269	285	404	-	-	3	-	-	-	-	-	-	-	-	-

Table 8. Genetic diversity of Y-chromosomal DNA of the brown bear geographical populations based on the combination of 3.1-kb Y-chromosomal DNA sequences and six Y-linked microsatellite loci.

Population	n	H	HD±SD	MPD±SD
All brown bears	214	79	0.97±0.01	4.08±2.04
Northwest America (NW-A)*	10	6	0.84±0.10	2.31±1.38
ABC Islands (ABC)*	11	5	0.82±0.08	1.82±1.13
Canada (CAN)*	8	2	0.25±0.18	0.75±0.61
Central Europe (C-EU)*	14	8	0.89±0.06	2.68±1.52
Northern Europe (N-EU)*	11	5	0.78±0.11	2.62±1.51
Western Asia (W-AS)	61	32	0.96±0.01	3.59±1.85
East Asia (E-AS)	31	14	0.86±0.05	2.60±1.43
Sakhalin (SH)	1	1	1.00	0.00
Tibet (TB)	1	1	1.00	0.00
Etorofu (ET)	10	2	0.36±0.16	0.36±0.38
Kunashiri (KN)	1	1	1.00	0.00
Hokkaido (HK)	55	8	0.73±0.05	1.23±0.79
Central Hokkaido (C-HK)	30	8	0.76±0.07	1.26±0.82
Eastern Hokkaido (E-HK)	11	4	0.6±0.15	0.85±0.65
Southern Hokkaido (S-HK)	14	3	0.38±0.15	0.41±0.40

Asterisks show citations from Bidon et al. (2014)

n: Sample size, H: Number of haplotypes, HD: Haplotype Diversity,
MPD: Mean number of pairwise differences within population,
SD: Standard deviation

Table 9. Analysis of molecular variance (AMOVA) for Y-chromosomal DNA polymorphisms of each geographical partition.

Geographical partitions	Sum of squares	Variance components	Percentage of variance (%)
(EU+ET), NA, and HK ($N_{Groups} = 3$)			
Among groups	5290.74	38.71*	44.43
Among population, within groups	2729.17	17.89*	20.54
Within populations	6104.21	30.52*	35.03
EU, (NA+ET), and HK ($N_{Groups} = 3$)			
Among groups	5212.04	34.79*	41.35
Among population, within groups	2807.87	18.81*	22.36
Within populations	6104.21	30.52*	36.28
EU, NA, and (HK+ET) ($N_{Groups} = 3$)			
Among groups	3597.58	18.68	23.51
Among population, within groups	4422.33	30.24*	38.06
Within populations	6104.21	30.52*	38.42
[(EU+ET), NA], HK ($N_{Groups} = 2$)			
Among groups	5122.78	57.32*	54.68
Among population, within groups	2897.13	16.99*	16.21
Within populations	6104.21	30.52*	29.12
Within (HK+ET) ($N_{Groups} = 1$)			
Among populations	2256.75	49.72*	82.27
Within populations	653.90	10.72	17.73
Within HK ($N_{Groups} = 1$)			
Among populations	235.27	6.40*	33.95
Within populations	647.50	12.45	66.05

*$P < 0.001$

EU, Eurasian Continent (C-EU, Central Europe; N-EU, Northern Europe; W-AS, Western Asia; E-AS, Eastern Asia),
NA, North American Continent (C-EU, Central Europe; N-EU, Northern Europe; W-AS, Western Asia; E-AS, Eastern Asia; CAN, Canada),
HK, Hokkaido (C-HK, Central Hokkaido; E-HK, Eastern Hokkaido; S-HK, Southern Hokkaido),
ET, Etorofu Island

Table 10. Analysis of molecular variance (AMOVA) for Y-chromosomal DNA polymorphisms of each geographical partition.

Geographical partitions	Sum of squares	Variance components	Percentage of variance (%)
EU, NA+ET, and HK (N_{Groups} = 3)			
Among groups	5212.04	34.79**	41.35
Among population, within groups	2807.87	18.81**	22.36
Within populations	6104.21	30.52**	36.28
(EU, NA+ET), HK (N_{Groups} = 2)			
Among groups	5122.78	57.32**	54.68
Among population, within groups	2897.13	16.99**	16.21
Within populations	6104.21	30.52**	29.12
(EU, HK), NA+ET (N_{Groups} = 2)			
Among groups	489.02	-2.98	-4.16
Among population, within groups	7530.89	43.98**	61.49
Within populations	6104.21	30.52**	42.67
(NA+ET, HK), EU (N_{Groups} = 2)			
Among groups	2302.94	11.97	15.25
Among population, within groups	5716.97	35.99**	45.86
Within populations	6104.21	30.52**	38.89
EU, NA+ET (N_{Groups} = 2)			
Among groups	89.26	-6.13	-11.47
Among population, within groups	2572.60	22.74**	42.52
Within populations	5456.72	36.87**	68.95
EU, HK (N_{Groups} = 2)			
Among groups	4723.03	54.76*	49.73
Among population, within groups	2378.15	20.57**	18.68
Within populations	5740.24	34.79**	31.59
NA+ET, HK (N_{Groups} = 2)			
Among groups	2909.11	60.28*	73.79
Among population, within groups	664.99	9.79**	11.98
Within populations	1011.47	11.63**	14.23
Within EU (N_{Groups} = 1)			
Among populations	2142.88	27.03**	37.49
Within populations	5092.74	45.07	62.51
Within NA+ET (N_{Groups} = 1)			
Among populations	429.72	13.68**	56.82
Within populations	363.97	10.40	43.18
Within HK (N_{Groups} = 1)			
Among populations	235.27	6.40**	33.95
Within populations	647.50	12.45	66.05

*P < 0.05; **P < 0.01

EU, Eurasian Continent; NA, North American Continent; HK, Hokkaido; ET, Etorofu Island.

Table 11. Analysis of molecular variance (AMOVA) for Y-chromosomal DNA polymorphisms of each geographical partition.

Geographical partitions	Sum of squares	Variance components	Percentage of variance (%)
EU+ET, NA, and HK (N_{Groups} = 3)			
Among groups	5290.74	38.71**	44.43
Among population, within groups	2729.17	17.89**	20.54
Within populations	6104.21	30.52**	35.03
(EU+ET, NA), HK (N_{Groups} = 2)			
Among groups	5122.78	57.32**	54.68
Among population, within groups	2897.13	16.99**	16.21
Within populations	6104.21	30.52**	29.12
(EU+ET, HK), NA (N_{Groups} = 2)			
Among groups	204.48	-8.52	-12.65
Among population, within groups	7815.43	45.30**	67.31
Within populations	6104.21	30.52**	45.35
(NA, HK), EU+ET (N_{Groups} = 2)			
Among groups	3333.05	25.03*	29.66
Among population, within groups	4686.86	28.84**	34.18
Within populations	6104.21	30.52**	36.16
EU+ET, NA (N_{Groups} = 2)			
Among groups	167.96	-4.24	-7.85
Among population, within groups	2493.91	21.44**	39.65
Within populations	5456.72	36.87**	68.20
EU+ET, HK (N_{Groups} = 2)			
Among groups	5086.26	58.31*	51.93
Among population, within groups	2706.47	20.95**	18.66
Within populations	5746.64	33.03**	29.41
NA, HK (N_{Groups} = 2)			
Among groups	1957.69	49.74	74.69
Among population, within groups	257.97	3.97**	5.96
Within populations	1005.07	12.89**	19.35
Within EU+ET (N_{Groups} = 1)			
Among populations	2471.20	26.53**	38.83
Within populations	5099.14	41.80	61.17
Within NA (N_{Groups} = 1)			
Among populations	22.70	-0.25	-1.86
Within populations	357.57	13.75	101.86
Within HK (N_{Groups} = 1)			
Among populations	235.27	6.40**	33.95
Within populations	647.50	12.45	66.05

*P < 0.05; **P < 0.01

EU, Eurasian Continent; NA, North American Continent; HK, Hokkaido; ET, Etorofu Island.

Table 12. Analysis of molecular variance (AMOVA) for Y-chromosomal DNA polymorphisms of each geographical partition.

Geographical partitions	Sum of squares	Variance components	Percentage of variance (%)
EU, NA, and HK+ET (N_{Groups} = 3)			
Among groups	3597.58	18.68	23.51
Among population, within groups	4422.33	30.24**	38.06
Within populations	6104.21	30.52**	38.42
(EU, NA), HK+ET (N_{Groups} = 2)			
Among groups	3461.63	30.39*	34.29
Among population, within groups	4558.28	27.72**	31.27
Within populations	6104.21	30.52**	34.44
(EU, HK+ET), NA (N_{Groups} = 2)			
Among groups	204.48	-8.52	-12.65
Among population, within groups	7815.43	45.3**	67.31
Within populations	6104.21	30.52**	45.35
(NA, HK+ET), EU (N_{Groups} = 2)			
Among groups	2302.94	11.97	15.25
Among population, within groups	5716.97	35.99**	45.86
Within populations	6104.21	30.52**	38.89
EU, NA (N_{Groups} = 2)			
Among groups	135.95	-5.34	-9.72
Among population, within groups	2165.58	21.08**	38.36
Within populations	5450.32	39.21**	71.36
EU, HK+ET (N_{Groups} = 2)			
Among groups	3393.10	28.26	29.26
Among population, within groups	4399.63	35.30**	36.55
Within populations	5746.64	33.03**	34.19
NA, HK+ET (N_{Groups} = 2)			
Among groups	1294.64	20.68	30.84
Among population, within groups	2279.45	34.76**	51.82
Within populations	1011.47	11.63**	17.34
Within EU (N_{Groups} = 1)			
Among populations	2142.88	27.03**	37.49
Within populations	5092.74	45.07	62.51
Within NA (N_{Groups} = 1)			
Among populations	22.70	-0.25	-1.86
Within populations	357.57	13.75	101.86
Within HK+ET (N_{Groups} = 1)			
Among populations	2256.75	49.72**	82.27
Within populations	653.90	10.72	17.73

*$P < 0.05$; **$P < 0.01$

EU, Eurasian Continent; NA, North American Continent; HK, Hokkaido; ET, Etorofu Island.

Table 13. Pairwise population differentiations (R_{ST}) for Y-chromosomal DNA markers among brown bear populations

	NW-A	ABC	CAN	C-EU	N-EU	W-AS	E-AS	ET	C-HK	E-HK	S-HK
NW-A											
ABC	-0.03										
CAN	-0.08	0.05									
C-EU	-0.004	0.11	-0.06								
N-EU	0.37*	0.41*	0.36*	0.26*							
W-AS	0.169*	0.25*	0.17*	0.14*	0.22*						
E-AS	0.19*	0.24*	0.18*	0.23*	0.43*	0.45*					
ET	0.73*	0.79*	0.86*	0.59*	0.85*	0.48*	0.18*				
C-HK	0.79*	0.77*	0.81*	0.80*	0.77*	0.72*	0.68*	0.92*			
E-HK	0.63*	0.59*	0.72*	0.64*	0.67*	0.53*	0.48*	0.89*	0.42*		
S-HK	0.89*	0.87*	0.93*	0.84*	0.86*	0.74*	0.65*	0.98*	0.04	0.58*	

*$P < 0.05$

NA group, North American Continent (NW-A, Northwest America; ABC, ABC-islands; CAN, Canada),
EU group, Eurasian Continent (C-EU, Central Europe; N-EU, Northern Europe; W-AS, Western Asia; E-AS, Eastern Asia; ET, Etorofu Island),
HK group, Hokkaido (C-HK, Central Hokkaido; E-HK, Eastern Hokkaido; S-HK, Southern Hokkaido),
Comparison between NA and EU populations are shaded in light gray, between NA and HK populations are in medium gray,
between EU and HK populations are shaded in dark gray. Comparison within EU, NA, and HK populations are in white.

Table 14. BATWING analysis of TMRCA time estimates based on the Y-chromosomal DNA markers.

	TMRCA (mean)	Splitting time (mean)	95% Credible Interval
All brown bears	472655	-	186797–1048846
HK – (NA+EU+ET)	-	124566	16537–645604
ET – (NA+EU+HK)	-	36948	1038–277595
HK+ET	127779	-	40336–332066
HK	55316	-	15659–153922
ET	4366	-	539–15752

TMRCA (scaled using effective population size, N_e)

EU, Eurasian Continent (C-EU, Central Europe; N-EU, Northern Europe; W-AS, Western Asia; E-AS, Eastern Asia; SK, Sakhalin),
NA, North American Continent (C-EU, Central Europe; N-EU, Northern Europe; W-AS, Western Asia; E-AS, Eastern Asia; CAN, Canada),
HK, Hokkaido (C-HK, Central Hokkaido; E-HK, Eastern Hokkaido; S-HK, Southern Hokkaido; KN, Kunashiri Island),
ET, Etorofu Island