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Ancient DNA Analysis of Brown Bear (*Ursus arctos*) Remains from the Archeological Site of Rebun Island, Hokkaido, Japan

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ABSTRACT—Ancient DNA was analyzed from skull remains of 12 brown bears (*Ursus arctos*) excavated from the archeological site of the Okhotsk Culture on Rebun Island of Hokkaido, where no natural populations of brown bears currently occur, in order to trace their original habitats. The Okhotsk Culture developed around southern coastal regions of the Okhotsk Sea (southern Sakhalin, Rebun and Rishiri Islands, northern and eastern Hokkaido, and southern Kuril Islands) during 6–11th centuries, A.D. The ancient people of those days are considered to have involved brown bears for traditional ceremonies and rituals. From the skull remains, partial fragments (approximately 250–360 base pairs) of the mitochondrial DNA (mtDNA) control region were successfully sequenced. Compared with sequence data of modern brown bears of the Hokkaido main land, ancient mtDNAs of Rebun Island were phylogenetically classified into either of two lineages of modern mtDNA: the north-central Hokkaido lineage and southern Hokkaido lineage. The southern Hokkaido lineage was identified from three juvenile (less than one year old) ancient bears, while the north-central Hokkaido lineage was mainly from adults (more than three years old). Our findings demonstrated that juvenile ancient bears of Rebun Island were originated from southern Hokkaido, which was an outside area of the Okhotsk Culture and belonged to the Epi-Jomon Culture with a close relation to a northern part of the Tohoku district. The molecular phylogeographic study on ancient and modern brown bears provides an insight to further understanding zooarcheology and ancient people's cultures around Hokkaido.

INTRODUCTION

The brown bear (*Ursus arctos*) is the largest terrestrial mammal in northern Eurasia and North America. Since prehistoric times, this animal has been involved in northern hemispheric people's cultures such as ceremonies and rituals (Hallowell, 1926). In Japan, the bear-sending ceremony is known as 'Iyomante' in the Ainu Culture (13–20th centries, A.D.) of Hokkaido, where the brown bear was the god of mountains (reviewed by Fitzhugh and Dubreuil, 1999). Although the origin of this bear ceremony is still unclear, it might be traced to brown bear remains of bones and artifacts excavated from archeological sites of the Okhotsk Culture, which developed around southern coastal areas of the Okhotsk Sea (southern Sakhalin, Rebun and Rishiri Islands, northern and eastern Hokkaido, and southern Kuril Islands) during 6–11th centuries, A.D.

The Kafukai site on Rebun Island (Fig. 1) is one of the most famous archeological sites of the Okhotsk Culture, because this site included a lot of faunal remains such as brown bears (Oba and Ohyi, 1976; 1981). Meanwhile, currently on Rebun Island, no natural populations of brown bears occur, and there are no records of their fossils naturally formed before the Okhotsk Cultural Period. Therefore, brown bear remains of the Kafukai site are thought to be originated from the outside of Rebun Island. Although their ages, dead seasons, and sex were morphologically investigated by Ohyi *et al.* (1980), their original habitats are still unknown. It is quite difficult to obtain enough information of cranial features from fragmented parts of archeological bone remains for estimating their original habitats, compared with geographic cranial variations of modern Hokkaido brown bears as reported by Ohdachi *et al.* (1992).

On the other hand, the Hokkaido main island is currently a brown bear's habitat: one of the southernmost borders in Northern Hemisphere. Matsuhashi *et al.* (1999) investigated phylogeographic patterns of the modern population of Hokkaido brown bears based on mitochondrial DNA (mtDNA) control region sequences. They found that there are three genetic lineages which are located separately in north-central Hokkaido (Cluster A), eastern Hokkaido (Cluster B), and south-

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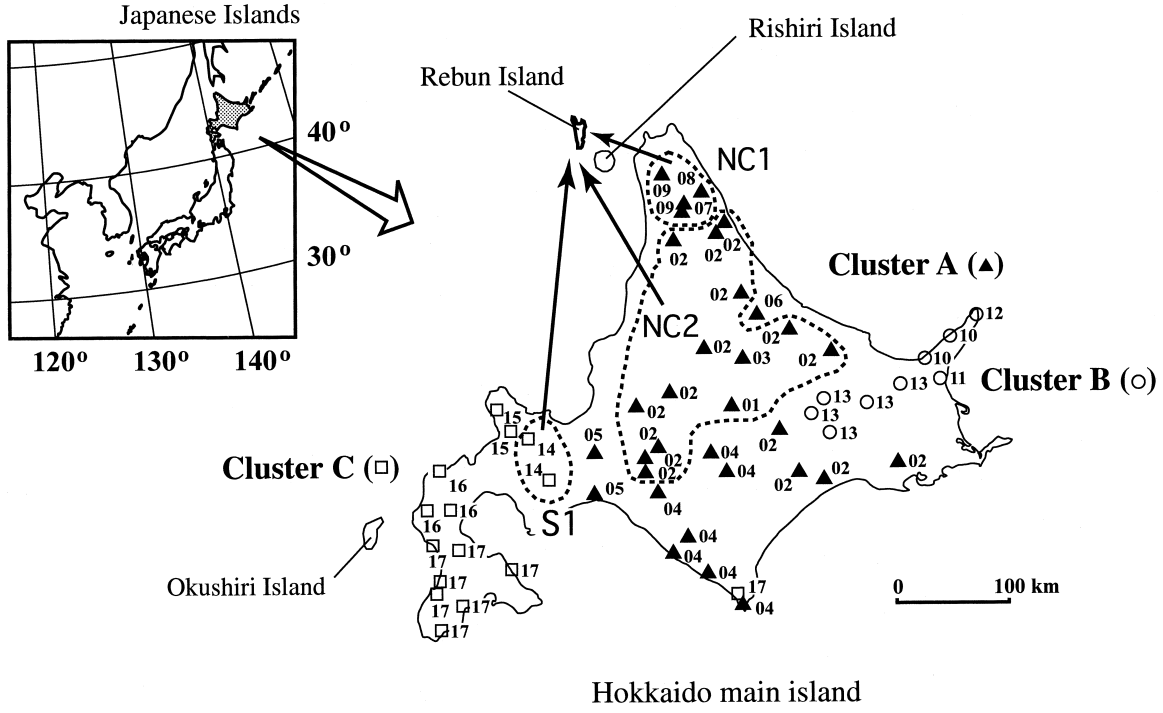


Fig. 1. Estimation of origins (Areas NC1, NC2, and S1) of ancient brown bears of Rebun Island. Triangles, circles, and squares with numbers indicate the localization on the Hokkaido main island of mtDNA control region haplotypes (HB01-17) which belong to Clusters A, B, and C, respectively (Matsuhashi *et al.*, 1999). On the left map, the gray area is the Hokkaido main island.

ern Hokkaido (Cluster C). These three lineages could have diverged in the continent and then immigrated into Hokkaido by the last glacial period (approximately 12,000 years ago) (Matsuhashi *et al.*, 1999). This 'triple population structure' of modern brown bears is specific to Hokkaido, compared with other populations of Europe (Taberlet and Bouvet, 1994) and North America (Waits *et al.*, 1998). Because, in the Okhotsk Cultural Period, Rebun Island had already been a small island separated from the Hokkaido main island, it is likely that brown bear remains of Rebun Island were originated within the Hokkaido main island. If mtDNA sequences are obtained from ancient brown bears of Rebun Island, the molecular phylogenetic study could provide invaluable information for tracing their original habitats and understanding zooarcheology of the Okhotsk Culture.

In the present study, we successfully sequenced ancient DNAs for 12 skull remains of brown bears excavated from the Kafukai archeological site on Rebun Island. Compared with mtDNA sequence data of modern Hokkaido brown bears, we here estimate original habitats of ancient brown bears of Rebun Island and then discuss zooarcheology and intercultural association around Hokkaido.

MATERIALS AND METHODS

Skull remains and ancient DNA extraction

Skull remains of brown bears were excavated from the Kafukai site on Rebun Island, 1968–1972 (Oba and Ohyi, 1976; 1981)(Fig. 2), and currently preserved at the Hokkaido University Museum. Periods of the brown bear remains were estimated as approximately 1,000–1,400 years ago due to the ^{14}C method of ashes found in the site (Oba and Ohyi, 1981). To guarantee that all specimens were from different individuals, we used parts of skulls or canines with which we could identify individuals (Table 1). Ages and death seasons were cited from the data of Ohyi *et al.* (1980), where they were estimated from width conditions of cementum layers of teeth.

Ancient DNA was extracted using the method of Hagelberg (1994) with some modifications. Parts of skull remains were powdered with sand papers or electric drills. Roots of canines were also powdered using electric drills. Approximately 0.2–0.5 g of powders per specimen were treated with 5 ml of 0.5M EDTA in a 15 ml-plastic tube with rotating at room temperature. The EDTA solution was changed every 30 min until the color (firstly brownish or yellowish) of supernatant became clear, after centrifugation at 3,000 rpm for 15 min. The pellets were then suspended with 5 ml of 0.5M EDTA and 1 mg of proteinase K, and incubated at 37°C overnight. The suspension was extracted using the phenol-chloroform extraction method (Sambrook *et al.*, 1989): twice with phenol:chloroform (1:1) and once with chloroform. The extracts were then concentrated into approximately 50 μl of TE buffer using Centricon-30 microconcentrators (Amicon). The aliquot was applied to the following polymerase chain reaction (PCR). To eliminate contamination of external DNAs, we autoclaved all solutions and used disposable sterile plastic apparatuses with plastic gloves. It was confirmed that negative controls of reaction mixtures generated no PCR-products.

PCR amplification and sequencing

Three different fragments (neighboring and partially overlapping each other) per specimen were PCR-amplified using three sets of primers (forward/reverse), UR6/UR3, mtF/mtR, and UR4/UR7: UR6, 5'-GGTACATACTACTATTTTACCCC-3'; UR3, 5'-TCCTTATGTAAGACCAAGCGTAATATG-3'; mtF, 5'-GCCCCATGCATATAAGCATG-3'; mtR, 5'-GGAGCGAGAAGAGGTACACGT-3'; UR4, 5'-CCAGGCCTCGAGAAACCAGC-3'; UR7, 5'-ATCGAGATGTCCCATTTGAAG-3'. These primers were newly designated in the present study,

Fig. 2. Two ancient brown bear skulls excavated from the Kafukai archeological site on Rebun Island (photographed in 1972). The bar on the right side indicates 30 cm.

Table 1. Profiles of ancient brown bear remains from Rebun Island.

Sample Code No.[4]	Remain No.[1]	Sex[2]	Part of skull[2]	Age[2]	Dead season[2]	Sequence length/lineage[3]/accession No.
KAB1	3689	Female	Canine (R, Man.)	10	Spring	360 bp/NC/ AB055135
KAB2	3690	Male(?)	Part of Max.	<1	Autumn	360 bp/NC/ AB055136
KAB6	4665	Male	Canine (L, Man.)	8	Spring	360 bp/NC/same as KAB1
KAB9	20293	Male(?)	R, Man.	<1	Autumn	363 bp/S/ AB055137
KAB11	20292	Male	L, Man.	2	Spring(?)	254 bp/NC/same sequence as HB01-03[5]
KAB12	20241	Female	Part of Max.	1(?)	Summer-Autumn(?)	254 bp/NC/same sequence as HB01-03[5]
KAB13	4660	Male	Canine (R, Max.)	>20	Spring	254 bp/NC/ AB055138
KAB14	4655	Female	Canine (R, Max.)	7	Spring	362 bp/NC/ AB055139
KAB16	20290	Male	Canine (R, Max.)	>20	Spring	255 bp/NC/ AB055140
KAB18	14110	Female	Canine (R, Man.)	8	Spring	360 bp/NC/same as KAB1
KAB19	3282	Male	R, Man.	<1	Autumn	363 bp/S/ AB055141
KAB20	21108	Male(?)	R, Max.	<1	Autumn	363 bp/S/same as KAB9

[1] Remain numbers registered at the Hokkaido University Museum.
[2] Data cited from Ohyi *et al.* (1980). R, right; L, left; Max., maxilla; Man., mandible. Ages (years old) and dead seasons were analyzed by the cementum-layer method. But ages of over 20 years old were not measured precisely using this method.
[3] NC, north-central Hokkaido mtDNA lineage (Cluster A); S, southern Hokkaido mtDNA lineage (Cluster C).
[4] Sequence data will appear in the DDBJ nucleotide sequence databases with the following accession numbers: AB055135-055141.
[5] Haplotypes HB01-03 were reported in Matsuhashi *et al.* (1999).

except the forward (mtF) and reverse (mtR) reported by Hänni *et al.* (1994). PCR was performed with 35–40 cycles (94°C for 1 min, 50–60°C for 30 sec, and 72°C for 1 min), using *rTaq* DNA polymerase (Takara) with 5–10 μl of DNA extracts in 50 μl of reaction mixture. Bovine serum albumin of 20 μg was added into the reaction mixture to eliminate some effects of PCR inhibitors which were often contained in aged specimens. PCR products were then purified using the centrifugal dialysis kit QIAquick (Qiagen), and used as template for PCR-product direct sequencing. Sequencing reaction was done with the Thermo Sequenase pre-mixed cycle sequencing kit (Amersham). Sequencing was performed using an automated sequencer (Hitachi SQ-5500). The following sequencing primers labeled with the Texas Red were newly designed in the present study: UR6in, 5'-TTTTACCCCATGTCCTATTC-3'; UR3in, 5'-CCAAGCGTAATATGTACATGC-3'; mtFin, 5'-GCATGTACATATTACGCTTGG-3'; mtRin 5'-CACGTACTCGCAAGGGTTGC-3'; UR4in 5'-CCAGCAACCCTTGCGAGTAC-3'; UR7in 5'-CCATTTGAAGGGTTAGTAGGATT-3'. Combination of the three fragments yielded approximately 360 base-bairs (bp) at maximum per specimen. Because, on some specimens, the fragment between UR6/UR3 was not amplified due to DNA degradation or some effects of PCR inhibitors, their final sequences were 254 bp. The 17 haplotypes, HB01-17 (Accession Nos. AB013040, AB013045-47, AB013050, AB013052-59, AB013061-63, AB013065), of the mtDNA control region from modern Hokkaido brown bears were used from Matsuhashi *et al.* (1999).

The homologous mtDNA sequence (Accession No. AB013071) of the Asiatic black bear (*Selenarctos thibetanus*) as outgroup was cited from Matsuhashi *et al.* (1999).

Sequence analysis

Sequence alignment was done using the GeneWorks computer software (Intelligenetics). Insertions or deletions (indels) were compensated due to observation by eyes. Phylogenetic trees were constructed by the neighbor-joining (Saitou and Nei, 1987) and UPGMA using the Mega computer software (Kumar *et al.*, 1993). Genetic distances were calculated using the Kimura's (1980) two-parameter method. Bootstrap values (Felsenstein, 1985) were derived from 1,000 replications. Parsimonious relationships between sequences were presented by hand-written networks.

RESULTS AND DISCUSSION

Sequence variations of ancient mtDNA

From 12 of 20 brown bear remains of Rebun Islands, we successfully PCR-amplified and determined partial sequences (approximately 250–360 bp) of the mtDNA control region (see Appendix). Because the sequence fragment between primers UR6 and UR3 was not always PCR-amplified from all specimens due to DNA degradation or some effects of PCR inhibitors, the analyzed sequence length was approximately 360 bp from eight bears, and 254 bp from 12 bears (Appendix). Eight other specimens failed to yield any DNA fragment to be PCR-amplified. The sequence alignment showed that most of nucleotide substitutions were transitions. Between nucleotide site numbers 51–62, there were indels involving T and C repeats (Appendix).

Three ancient bears (KAB1, KAB6, and KAB18) shared identical 254-bp sequences with the haplotype HB08 (Appendix). The 360-bp sequences of these three remains had one indel site with HB08.

The 254-bp sequences from three ancient bears (KAB11, KAB12, and KAB14) were identical with those of HB01, HB02, and HB03: the original sequences (approximately 700 bp reported by Matsuhashi *et al.* (1999)) of HB01, HB02, and HB03 were different from one another, but their 254-bp sequences were identical between them. Sequence differences of 360-bp between KAB14, HB01/03, and HB02 were 1–4 indels (Appendix).

Three ancient bears (KAB9, KAB19, and KAB20) shared 254-bp sequences identical with the haplotype HB14. At the 360-bp region, KAB9 and KAB20 shared identical sequences, while there was an indel site among these two specimens, KAB19, and HB14 (Appendix).

Sequences of KAB2 (360 bp), KAB13 (254 bp), and KAB16 (254 bp) were all individual (Appendix).

Phylogenetic relationships between ancient and modern mtDNA sequences and estimation of original habitats of ancient bears

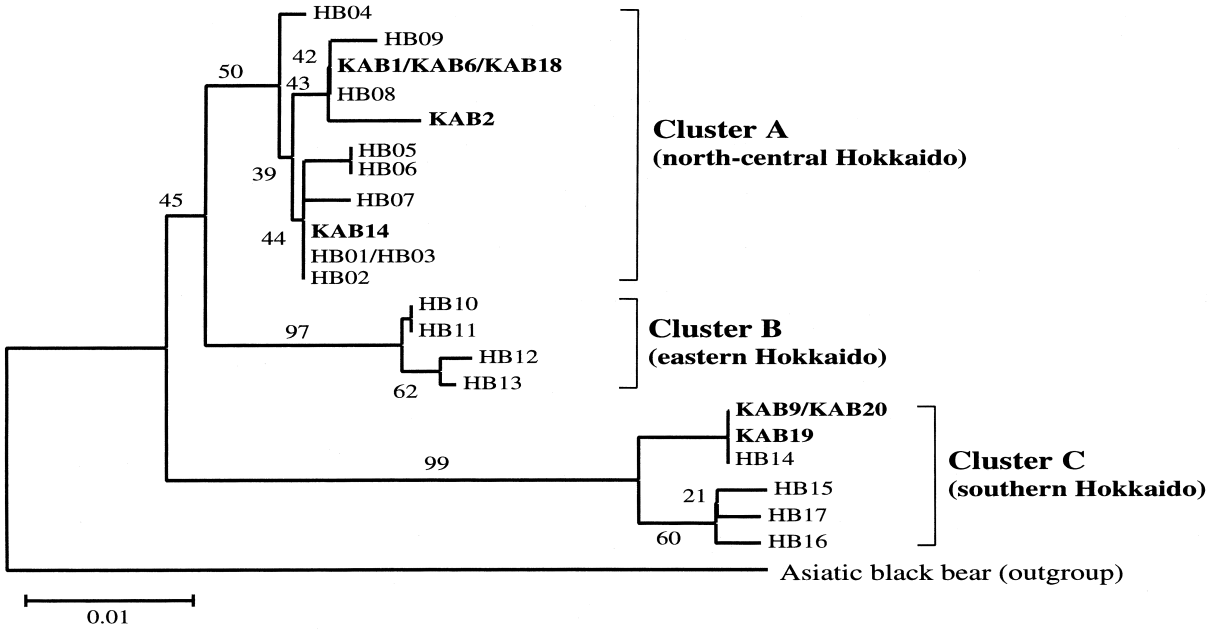
Neighbor-joining relationships between mtDNA sequences (Fig. 3A: 360 bp; Fig. 3B: 254 bp) commonly showed that sequences examined were grouped into three clusters (A–C), and that ancient brown bears of Rebun Island were included in either of two mtDNA lineages of modern Hokkaido brown bears: three ancient bears (KAB9, KAB19, and KAB20) in the southern Hokkaido lineage (Cluster C) and the others in the north-central Hokkaido lineage (Cluster A) (Cluster names followed Matsuhashi *et al.* (1999)). None of the 12 ancient bears shared the eastern Hokkaido mtDNA lineage (Cluster B), supported with 87–97% bootstrap values (Fig. 3). The UPGMA tree demonstrated the similar relationships between sequences (data not shown) to those by the neighbor-joining tree. Parsimonious networks (Fig. 4) also showed the relationships of clusters basically same as those of the neighbor-joining (Fig. 3) and UPGMA trees. Since the phylogenetic relationships among the 254-bp sequences (Figs. 3B and 4B) were quite similar to those of the 360-bp sequences (Figs. 3A and 4A), we considered that the relationships based on the 254-bp sequences was reliable and utilized this data set in the following discussion. Consequently, the mtDNA phylogeny revealed that the three ancient bears (KAB9, KAB19, and KAB20) were originated from southern Hokkaido, and the others from north-central Hokkaido.

Considering the phylogenetic relationships (Figs. 3 and 4) as well as the localization (Fig. 1) of mtDNA haplotypes (HB01-17) in Hokkaido (Matsuhashi *et al.*, 1999), it was clear that the phylogenetically closer haplotypes of modern brown bears were located at geographically closer regions (Fig. 1). The three clusters (A–C) are considered to have immigrated into Hokkaido in different times after divergence in the continent, while the minor sequence variations within the clusters could have caused at those localities in Hokkaido after the immigration (Matsuhashi *et al.*, 1999). The two haplotypes, HB08 and HB09, phylogenetically closer to sequences of six ancient bears (KAB1, KAB2, KAB6, KAB13, KAB16, and KAB18), were located at the northern area (named Area NC1) in the Cluster A (north-central Hokkaido lineage) region (Fig. 1). This means that those six ancient bears are originated from Area NC1, although branching patterns in Cluster A are not so clear with relatively lower bootstrap values (<50% in Fig.2). Sequences of KAB11, KAB12, and KAB14 were identical with HB01, HB02, and HB03, all of which are located at a wide area (named Area NC2 in Fig. 1) of the Cluster A region. This indicates that these three ancient bears (KAB11, KAB12, and KAB14) come from the inside of Area NC2, although it is difficult to determine the precise location in Area NC2. It is reasonable to consider that ancient bears of Rebun Island are originated from at least some northern part of Hokkaido, which is geographically close to Rebun Island (Fig. 1).

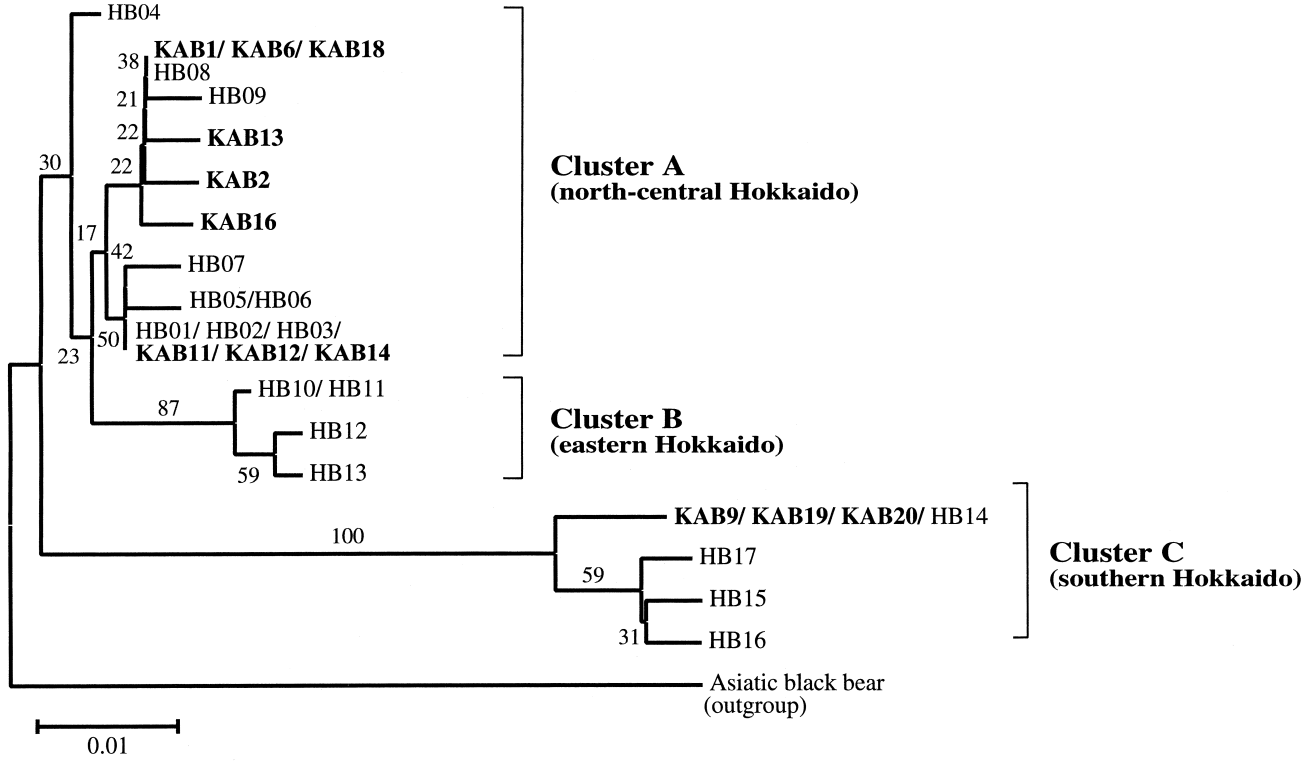
Meanwhile, sequences of three ancient bears (KAB9, KAB19, and KAB20) were phylogenetically closer to the haplotype HB14, which was located at the northern area (named Area S1 in Fig. 1) of the Cluster C (southern Hokkaido lineage) region. This means that the three ancient bears are originated from Area S1. Cluster C was strongly supported with 99-100% bootstrap values (Fig. 3).

Age-related sequences of ancient brown bears from Rebun Island

Fig. 5, based on the data of Table 1, demonstrated that most of ancient bears having the north-central Hokkaido mtDNA lineage were from adults of more than three years old. By contrast, the three ancient bears sharing the souhtern



(A)



(B)

Fig. 3. Neighbor-joining relationships among mtDNA control region sequences from ancient brown bears of Rebun Island and those from modern brown bears of the Hokkaido main island. The outgroup is the homologous sequence of the Asitatic black bear (*Selearctos thibetanus*). Numbers near internal branches are bootstrap values derived from 1,000 replications. The bars indicate the Kimuraís two-parameter distance. Indel sites were eliminated for distance estimation. A: constructed due to approximately 360-bp sequences (including eight ancient bears). B: constructed due to 254-bp sequences (including 12 ancient bears).

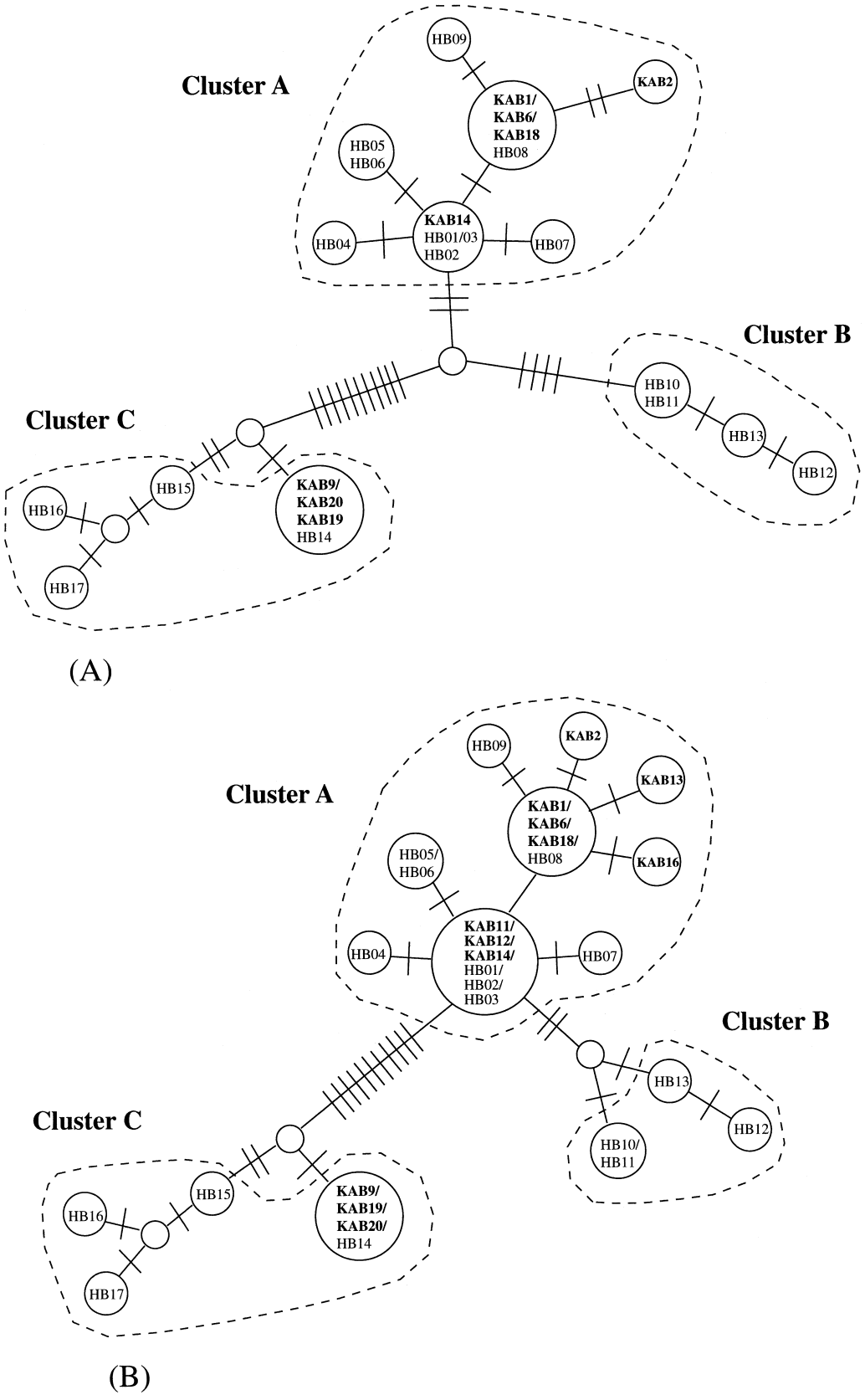


Fig. 4. Parsimonious networks among mtDNA control region sequences from ancient brown bears of Rebun Island and those from modern brown bears of the Hokkaido main island. Indels sites were eliminated for construction of networks. Open circles show presumed sequences. One slash means a nucleotide substitution shown in Appendix. A: constructed due to approximately 360-bp sequences (including eight ancient bears). B: constructed due to 254-bp sequences (including 12 ancient bears).

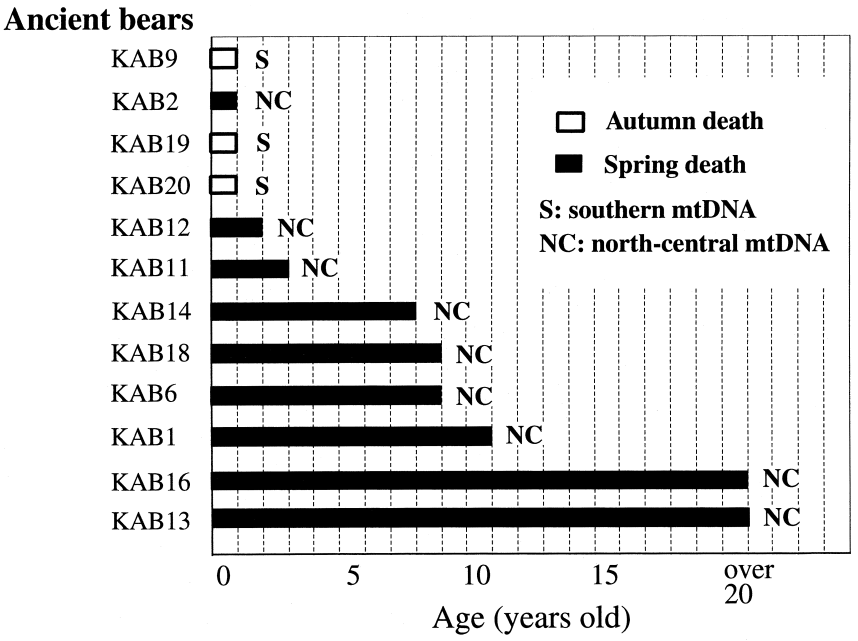


Fig. 5. The relationships between ages, dead seasons, and mtDNA lineages of ancient brown bears of Rebun Island which were constructed using data of Table 1. The southern Hokkaido mtDNA was identified from juvenile ancient bears which died in the autumn, while the north-central Hokkaido mtDNA was from most of adults which died in the spring.

Hokkaido mtDNA lineage were from juveniles of less than one year old. In addition, adults were killed in the spring while juveniles were done in the autumn (Fig. 5). Only skulls and limb bones for body parts were found from the Kafukai archeological site (Ohyi *et al.*, 1980). This indicates that adult bears were hunted by ancient people around Areas NC1 and NC2 of northern Hokkaido in the spring just after their hibernation, and that selected parts of their bodies such as heads and limbs were brought to Rebun Island. On the other hand, juvenile bears were likely captured around Area S1 of southern Hokkaido, kept living there from spring to autumn or directly brought to Rebun Island, and then used for the ceremony (Ohyi *et al.*, 1980). Archeological findings indicate that this ceremonial system lasted to the end of the Okhotsk Culture. There is no trace that the ancient people treated adult bears differentially from juveniles through the period. Of course, this is on the assumption that ancient people of the Okhotsk Culture did not propagate brown bears for the ceremony (Ohyi, 1997). In that time, southern Hokkaido was an outside area of the Okhotsk Culture and belonged to the Epi-Jomon Culture with a close relation to a northen part of the Tohoku district. Our findings strongly show the occurrence of intercultural association between the ancient people of Rebun Island and those of southern Hokkaido during the Okhotsk Cultural Period. Recently, Inui (1997) reported the finding of an archeological site of the Okhotsk Culture on Okushiri Island, which adjoins southern Hokkaido (for the location, see Fig. 1). In addition, Matsumura *et al.* (2001) examined cranial characters and tooth ablation customs of ancient people of the Jomon Period (before the Okhotsk Cultural Period), and reported that there were biological and cultural contacts between the Jomon people of Rebun Island and those of southern Hokkaido.

On the other hand, the origin of the bear ceremony 'Iyomante' of the Ainu Culture is still controversial. In this ceremony, people used adult bears which were hunted in the field as well as juvenile bears which were kept by themselves for one or two years. Ohyi (1997) reported that the ceremony form using the kept juvenile bears could have been transferred from the continental people of the Amur area to Ainu people of Sakhalin and Hokkaido in 16–17th centuries, A.D. By contrast, the origin of Iyomante is reported to be traced to the Okhotsk Culture (Watanabe, 1974; Amano, 1990) or the Satsumon Culture (8–12th centuries, A.D., just prior to the Ainu Culture) in Hokkaido (Nishimoto, 1989; Nishimoto and Sato, 1991; Sato, 1993). Our results of the present study indicate the development of the ceremony using the kept juvenile bears in the Okhotsk Culture, supporting the hypothesis (Watanabe, 1974; Amano, 1990) that Iyomante is originated from the Okhotsk Culture.

Advantage of DNA analysis for zooarcheology

In the present study, we revealed genetic characteristics of ancient brown bears excavated from the Kafukai site of Rebun Island, and estimated their original habitats in Hokkaido. In addition, our results demonstrated the intercultural communications during the Okhotsk Cultural Period. Thus, the ancient DNA study makes a great advance in interdisciplinary researches in cooperation with genetics of modern populations, archeology, and paleoenvironmental science.

For estimating original habitats of ancient brown bears of Rebun Island, we supposed that mtDNA phylogeography (triple

population structure) of brown bears on the Hokkaido main island in the Okhotsk Cultural Period was the same as, or very similar to that of the present time. Considering genetic structures of modern brown bear populations of the continent together with paleoenvironmental changes around Hokkaido, it is likely that mtDNA distribution in the Hokkaido brown bear population has finally been established just after the last glacial period (approximately 12,000 years ago) and then it has not been changed so largely (Masuda *et al.*, in preparation). At present, it could be reasonable to directly apply genetic information of the modern bear populations to estimating original habitats of ancient brown bears of Rebun Island.

This is the first report of ancient DNAs on archeological remains of brown bears. Up to date, ancient DNA information of zoological remains in Japan has been reported only from ancient dogs (Okumura *et al.*, 1999). Further studies of ancient brown bears involving other archeological sites could provide invaluable information for further understanding not only anthropological events but also zoogeography, paleoecology, and paleoenvironmental changes around northern Japanese islands including Hokkaido.

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Appendix. Alignment of mitochondrial DNA control region sequences from ancient brown bears (KAB1-20) from Rebun Island as well as from modem brown bears (HB01–17) of our previous study (Matsuhashi *et al.*, 1999). Dots indicates identities with nucleotides of the sequence of KAB1/6/18. Dashes show the blank of data (5' end) or indels (internal sites of sequences).

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                      10         20         30         40         50         60         70
KAB1/6/18     TTCATATATA CCGTCTTATG TACTGTACCA TCGCAGTATG  TCCTCGAATA CCTT------ -CCCCCCCTA
KAB2          .......... .G........ .......... ..........  .......... .G..T----- --........
KAB14         .......... .......... .......... ..........  .......... ....TT---- -.........
KAB9/20       .......... ..A....... .G........ ..........  .......... .-..TTTTT- --........
KAB19         .......... ..A....... .G........ ..........  .......... .-..TTTT-- -.........
KAB11/12      ---------- ---------- ---------- ----------  ---------- ---------- ----------
KAB13         ---------- ---------- ---------- ----------  ---------- ---------- ----------
KAB16         ---------- ---------- ---------- ----------  ---------- ---------- ----------
HB01/HB03     .......... .......... .......... ..........  .......... ....T----- -.........
HB02          .......... .......... .......... ..........  .......... ....TTTTT- -.........
HB04          .......... .......... .......... ..........  .......... ....TT---- -.........
HB05          .......... .......... .......... ..........  .......... ....TT---- -.........
HB06          .......... .......... .......... ..........  .......... ....T----- C.........
HB07          .......... .......... .......... ..........  .......... ....T----- -.........
HB08          .......... .......... .......... ..........  .......... ....------ C.........
HB09          .......... .......... .......... ..........  .......... ....------ -.........
HB10          .......... ..A....... ......G... ..........  .......... ....T----- C.........
HB11          .......... ..A....... ......G... ..........  .......... ....T----- -.........
HB12          .......... ..A....... ......G... ..........  .......... ....T----- -.........
HB13          .......... ..A....... ......G... ..........  .......... ....T----- -.........
HB14          .......... ..A....... .G........ ..........  .......... .-..TTTTT- -.........
HB15          .......... ..A....... .G........ ..........  .......... .-..TTT--- -.........
HB16          .......... ..A....... .G........ ..........  .......... .-..TTTT-- -.........
HB17          .......... ..A....... .G........ ..........  .......... .-..TTTTTT T.........

                     80         90        100        110        120        130        140
KAB1/6/18     TGTATATCGT GCATTAATGG CGTGCCCCAT GCATATAAGC ATGTACATAT TACGCTTGGT CTTACATAAG
KAB2          .......... .......... .......... ..........  .......... .......... ..........
KAB14         .......... .......... .......... ..........  .......... .......... ..........
KAB9/20       .......... .......... .......... ..........  ........G. .......... ..........
KAB19         .......... .......... .......... ..........  ........G. .......... ..........
KAB11/12      ---------- ---------- ---------- ----------  --........ .......... ..........
KAB13         ---------- ---------- ---------- ----------  --........ .......... ..........
KAB16         ---------- ---------- ---------- ----------  --........ .......... ..........
HB01/HB03     .......... .......... .......... ..........  .......... .......... ..........
HB02          .......... .......... .......... ..........  .......... .......... ..........
HB04          .......... .......... .......... ..........  .......... .......... ..........
HB05          .......... .......... .......... ..........  .......... .......... ..........
HB06          .......... .......... .......... ..........  .......... .......... ..........
HB07          .......... .......... .......... ..........  .......... .......... ..........
HB08          .......... .......... .......... ..........  .......... .......... ..........
HB09          .......... .......... .......... ..........  .......... .......... ........G.
HB10          .......... .......... T......... ..........  .......... .......... ...G......
HB11          .......... .......... T......... ..........  .......... .......... ...G......
HB12          .......... .......... T......... ..........  .......... .......... ...G......
HB13          .......... .......... T......... ..........  .......... .......... ...G......
HB14          .......... .......... .......... ..........  ........G. .......... ..........
HB15          .......... .......... .......... ..........  ........G. .......... ..........
HB16          .......... .......... .......... ..........  ........G. .......... ..........
HB17          .......... .......... .......... ..........  ........G. .......... ..........
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Appendix. continued.

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                    150        160        170        180        190        200        210
KAB1/6/18     GACCTACGTT CCGAAAGCTT ATTTCAGGCG TATGGTCTGT AAGCATGTAT TTCACTTAGT CCGGGAGCTT
KAB2          .......... .......... .......... ...A...... .......... .......... ..........
KAB14         ...T...... .......... .......... .......... .......... .......... ..........
KAB9/20       ...T...... .......... .......... .........C .......... .......... ..........
KAB19         ...T...... .......... .......... .........C .......... .......... ..........
KAB11/12      ...T...... .......... .......... .......... .......... .......... ..........
KAB13         .......... .......... .......... .......... .......... .......... ..........
KAB16         .......... .......... .......... .......... .......... .......... ..........
HB01/HB03     ...T...... .......... .......... .......... .......... .......... ..........
HB02          ...T...... .......... .......... .......... .......... .......... ..........
HB04          ...T...... .......... .......... .......... .......... .......... ..........
HB05          ...T...... .......... ........T. .......... .......... .......... ..........
HB06          ...T...... .......... ........T. .......... .......... .......... ..........
HB07          ...T...... .......... .......... .......... .......... .......... ..........
HB08          .......... .......... .......... .......... .......... .......... ..........
HB09          .......... .......... .......... .......... .......... .......... ..........
HB10          ...T...... .......... .......... .......... .......... .......... ..........
HB11          ...T...... .......... .......... .......... .......... .......... ..........
HB12          .......... .......... .......... ....A..... .......... .......... ..........
HB13          ...T...... .......... .......... ....A..... .......... .......... ..........
HB14          ...T...... .......... .......... .........C .......... .......... ..........
HB15          ...T...... ..........C. ......... .........C .......... .......... ..........
HB16          ...T...... ..........C. ......... ....A....C .......... .......... ..........
HB17          ...T...... ..........C. ......... ....A....C .......... .......... ..........

                    220        230        240        250        260        270        280
KAB1/6/18     GATCACCAGG CCTCGAGAAA CCAGCAACCC TTGCGAGTAC GTGTACCTCT TCTCGCTCCG GGCCCATGG-
KAB2          .......... .......... .......... .......... .......... .......... .........-
KAB14         .......... .......... .......... .......... .......... .......... .........-
KAB9/20       A......... .......... .......T.. .......... .....T.... .......... .......A.-
KAB19         A......... .......... .......T.. .......... .....T.... .......... .......A.-
KAB11/12      .......... .......... .......... .......... .......... .......... .........-
KAB13         .......... .......... .......... .......... .......... .......... .........-
KAB16         .......... .......... .......... .......... .......... .......... ........AG
HB01/HB03     .......... .......... .......... .......... .......... .......... .........-
HB02          .......... .......... .......... .......... .......... .......... .........-
HB04          .......... .......... .......... .......... .......... .......... .........-
HB05          .......... .......... .......... .......... .......... .......... .........-
HB06          .......... .......... .......... .......... .......... .......... .........-
HB07          .......... .......... .......... .......... .......... .......... .........-
HB08          .......... .......... .......... .......... .......... .......... .........-
HB09          .......... .......... .......... .......... .......... .......... .........-
HB10          A......... .......... .......... .......... .......... .......... .........-
HB11          A......... .......... .......... .......... .......... .......... .........-
HB12          A......... .......... .......... .......... .......... .......... .........-
HB13          A......... .......... .......... .......... .......... .......... .........-
HB14          A......... .......... .......T.. .......... .....T.... .......... .......A.-
HB15          A......... .......... .......T.. .......... .....T.... .......... ........A-
HB16          A......... .......... .......T.. .......... .....T.... .......... ........A-
HB17          A......... .......... .......T.. .......... .......... .......... ........A-
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Appendix. continued.

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                    290        300        310        320        330        340        350
KAB1/6/18      GGTGTGGGGG TTTCTATGTT GAAACTATAC CTGGCATCTG GTTCTTACTT CAGGGCCATG ATAGCTCTAG
KAB2           .......... .......... .......... .......... .......... .......... ..........
KAB14          .......... .......... .......... .......... .......... .......... ..........
KAB9/20        AA........ .......... .........T .......... .......... .......... ..........
KAB19          AA........ .......... .........T .......... .......... .......... ..........
KAB11/12       .......... .......... .......... .......... .......... .......... ..........
KAB13          .......... .......... .........T .......... .......... .......... ..........
KAB16          .......... .......... .......... .......... .......... .......... ..........
HB01/HB03      .......... .......... .......... .......... .......... .......... ..........
HB02           .......... .......... .......... .......... .......... .......... ..........
HB04           .A........ .......... .......... .......... .......... .......... ..........
HB05           .......... .......... .......... .......... .......... .......... ..........
HB06           .......... .......... .......... .......... .......... .......... ..........
HB07           .......... ........C. .......... .......... .......... .......... ..........
HB08           .......... .......... .......... .......... .......... .......... ..........
HB09           .......... .......... .......... .......... .......... .......... ..........
HB10           .......... .......... .......... .......... ........C. .......... ..........
HB11           .......... .......... .......... .......... ........C. .......... ..........
HB12           .......... .......... .......... .......... ........C. .......... ..........
HB13           .......... .......... .......... .......... ........C. .......... ..........
HB14           AA........ .......... .........T .......... .......... .......... ..........
HB15           AA........ .......... .........T .......... .......... .......... ..........
HB16           AA........ .......... .......... .......... .......... .......... ..........
HB17           AA........ .......... .........T .......... .......... .......... ..........

                    360      368
KAB1/6/18      ATTCCAATCC TACTAACC
KAB2           .......... ........
KAB14          .......... ........
KAB9/20        .C........ ....G..T
KAB19          .C........ ....G..T
KAB11/12       .......... ........
KAB13          .......... ........
KAB16          .......... ........
HB01/HB03      .......... ........
HB02           .......... ........
HB04           .......... ........
HB05           .......... ........
HB06           .......... ........
HB07           .......... ........
HB08           .......... ........
HB09           .......... ........
HB10           .......... ........
HB11           .......... ........
HB12           .......... ........
HB13           .......... ........
HB14           .C........ ....G..T
HB15           .C........ ....G..T
HB16           .C........ ....G..T
HB17           .C........ ....G..T
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