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Mitochondrial DNA control region variations in the sable *Martes zibellina* of Hokkaido Island and the Eurasian Continent, compared with the Japanese marten *M. melampus*

Tomo Inoue[1], Takahiro Murakami[2], Alexei V. Abramov[3] and Ryuichi Masuda[1,*]

[1] *Department of Natural History Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan*
[2] *Shiretoko Museum, Shari 099-4113, Japan*
[3] *Zoological Institute, Russian Academy of Sciences, Saint-Petersburg 199034, Russia*

Abstract. To reveal phylogeographical features of the sable *Martes zibellina* on Hokkaido Island, northern Japan, we analyzed the 5'-portion sequences of the mitochondrial DNA control region (535–537 base pairs), and compared their sequence variations with those in Russia. Genetic differences between the Hokkaido and Russian individuals, revealed by the present study, indicated that the Hokkaido population has not been well-differentiated after the immigration into Hokkaido from the Eurasian Continent. The intraspecific variations of *M. zibellina* showed interpopulation-differentiations due to 'isolation by distance' within Hokkaido, and they were smaller than those of the Japanese marten *M. melampus*, endemic to Honshu, Kyushu and Sikoku Islands of Japan. The genetic diversity in *M. melampus* populations introduced to Hokkaido was lower than that of *M. melampus* native on Honshu and Kyushu, and the two mitochondrial DNA haplotypes were distributed at restricted areas in southern Hokkaido. These findings suggest founder effects in introduced populations of *M. melampus* on Hokkaido. In addition, the clear phylogenetic separation between *M. zibellina* and *M. melampus* indicates that no hybridization between them have occurred on Hokkaido so far, although further studies using paternally and biparentally inherited genetic markers are necessary.

Key words: control region, Japanese marten, *Martes melampus*, *Martes zibellina*, sable.

There are two species of *Martes* on the Japanese archipelago: one is the sable *Martes zibellina* occurring on Hokkaido Island (Murakami 2009), and the other is the Japanese marten *M. melampus* that is endemic to Honshu, Shikoku and Kyushu Islands (Masuda 2009). Out of Japan, *M. zibellina* is distributed widely across northern parts of the Eurasian Continent (Wozencraft 2005), and the population of Hokkaido is regarded as subspecies *M. z. brachyura* (Murakami 2009). On the other hand, *M. melampus* was introduced to Hokkaido from Honshu in 1940's, and currently it is expanding the distribution in southern and central Hokkaido (Inukai 1975; Murakami and Ohtaishi 2000). The morphological and genetical studies showed that the two species are closely related to each other among *Martes* (Anderson 1970; Masuda and Yoshida 1994; Koepfli et al. 2008).

Since 1920 the sable hunting has been prohibited on Hokkaido, and little information on their distribution and behavior on Hokkaido has been available. Recent studies revealed that *M. zibellina* is distributed widely in central, eastern and northern Hokkaido (Murakami and Ohtaishi 2000). The mitochondrial DNA (mtDNA) phylogenetic studies on the Japanese *M. zibellina* have also been done by some researchers (Hosoda et al. 1999; Kurose et al. 1999; Murakami et al. 2004). Kurose et al. (1999) analyzed the mtDNA cytochrome *b* gene, and reported that the genetic variation of *M. zibellina* on Hokkaido is relatively smaller, and it has no clear geographic structures. However, because this result may reflect the small sample size (Kurose et al. 1999), further studies using more samples have been needed to reveal the genetic characters of *M. zibellina* populations on Hokkaido. Hosoda et al. (1999) analyzed phylogeny of the mtDNA cytochrome *b* gene of *M. zibellina* in

*To whom correspondence should be addressed. E-mail: masudary@ees.hokudai.ac.jp

Hokkaido and Russia, and reported that there were no substantial genetic differences between the two populations. However, because they examined the samples collected from only two locations, further study using more samples from various locations have been needed to reveal the relationships between *M. zibellina* of Hokkaido and Russia.

Meanwhile, it was reported that the closely related *Martes* species, the pine marten *M. martes*, naturally hybridize with *M. zibellina* in eastern Europe where they sympatrically occur (Heptner et al. 1967; Bakeyev and Sinitsyn 1994). By contrast, on the Japanese archipelago, the natural distribution of *M. zibellina* and *M. melampus* has been separated by the Tsugaru strait (Blakiston's line) (Masuda 2009; Murakami 2009). Recently, however, they have an opportunity to co-exist on Hokkaido due to introduction of *M. melampus* from Honshu to Hokkaido (Inukai 1975; Murakami and Ohtaishi 2000). Therefore, hybridization between the two *Martes* species on Hokkaido has been threatened, but it has not been reported so far. For conservation of *M. zibellina* native to Hokkaido, it is needed to clarify whether or not the hybridization between the two species has occurred.

In the present study, we sequenced the mtDNA control regions of natural populations of *M. zibellina* on Hokkaido and Russia, natural populations of *M. melampus* on Honshu and Kyushu, and introduced populations of *M. melampus* on Hokkaido, and then investigated the phylogeographic relationships among them. The possibility of hybridization between the two *Martes* species is also discussed.

Materials and methods

Samples and DNA extraction

Specimens for *M. zibellina* and *M. melampus* from various locations (Fig. 1) of Hokkaido, Honshu, Kyushu and Russia were listed in Table 1. Total DNA was extracted from liver, muscle or blood using the DNeasy Blood & Tissue Kit (QIAGEN).

PCR amplification and direct sequencing

The 5'-portion of mtDNA control region (535–537 base-pairs, bp) was amplified using two PCR primers, UR-1 (5'-CTCCACTATCAGCACCCAAAG-3': Taberlet and Bouvet 1994) and MZCRP-R1 (5'-TGTCCTGTC ACCATTGACTG-3': this newly designed sequence was almost identical with DLOOP-MelR reported by Sato et al. 2009, showing one-base shorter than the latter and one-base different between them). For some samples which could not have any amplification using above primers, we divided the 5'-portion of the control region into segments 1 and 2. Segment 1 was amplified using UR-1 and H169498M (Statham et al. 2005), and segment

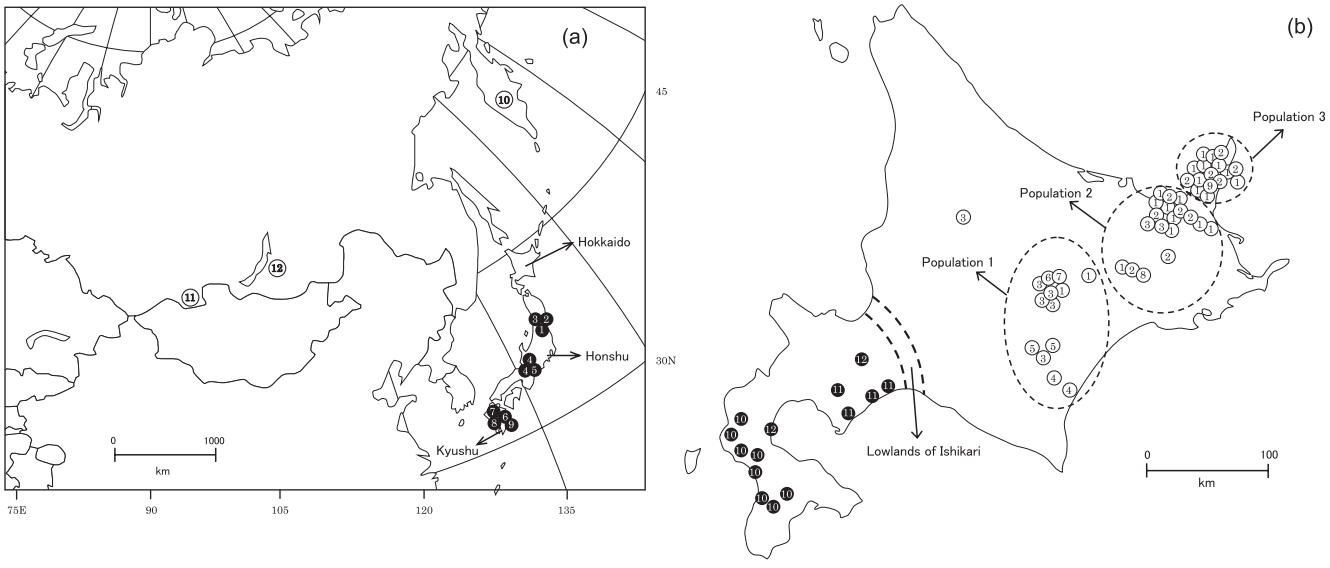

Fig. 1. (a) Sampling locations of the sable *Martes zibellina* (open circles) in the Eurasian Continent and the Japanese marten *M. melampus* (closed circles) on Honshu and Kyushu Islands of Japan. Numbers in circles show haplotypes identified from those locations. (b) Sampling locations of *M. zibellina* native (open circles) and *M. melampus* introduced (closed circles) to Hokkaido. Numbers in circles show haplotypes identified from those locations. One circle indicates one individual. Individuals of *M. zibellina* from the three areas circled by brokenlines were grouped into three populations 1, 2 and 3, respectively. "Lowlands of Ishikari" is a current boundary of the distribution of the two *Martes* species (Murakami and Ohtaishi 2000; Murakami 2009).

Table 1. Profiles of samples examined in the present study

Sample name	Sampling location	Year of collection	Haplotype	Accession no.#
Martes zibellina				
MZ-1	Shari-town, Hokkaido	1991	MZ2	AB525716
MZ-3	Kiyosato-town, Hokkaido	1992	MZ1	AB525715
MZ-4	Shari-town, Hokkaido	1992	MZ2	AB525716
MZ-5	Shari-town, Hokkaido	1993	MZ1	AB525715
MZ-7	Kiyosato-town, Hokkaido	1997	MZ2	AB525716
MZ-8	Shari-town, Hokkaido	1997	MZ2	AB525716
MZ-10	Shari-town, Hokkaido	1997	MZ1	AB525715
MZ-11	Ashoro-town, Hokkaido	1987	MZ3	AB455691*
MZ-12	Kamishihoro-town, Hokkaido	1995	MZ1	AB525715
MZ-13	Kamishihoro-town, Hokkaido	1996	MZ3	AB455691*
MZ-14	Ashoro-town, Hokkaido	1997	MZ6	AB525719
MZ-16	Hokkaido	unknown	MZ1	AB525715
MZ-20	Ashoro-town, Hokkaido	1999	MZ7	AB525720
MZ-22	Shari-town, Hokkaido	1998	MZ2	AB525716
MZ-23	Memuro-town, Hokkaido	1999	MZ3	AB455691*
MZ-24	Ashoro-town, Hokkaido	1999	MZ1	AB525715
MZ-25	Shari-town, Hokkaido	1999	MZ1	AB525715
MZ-26	Rausu-town, Hokkaido	1999	MZ1	AB525715
MZ-27	Shari-town, Hokkaido	1999	MZ1	AB525715
MZ-28	Shari-town, Hokkaido	1999	MZ2	AB525716
MZ-29	Shari-town , Hokkaido	1999	MZ1	AB525715
MZ-34	Shari-town, Hokkaido	2000	MZ1	AB525715
MZ-36	Shari-town, Hokkaido	2000	MZ1	AB525715
MZ-37	Shari-town, Hokkaido	2000	MZ1	AB525715
MZ-38	Shari-town, Hokkaido	1999	MZ1	AB525715
MZ-39	Teshikaga-town, Hokkaido	2000	MZ2	AB525716
MZ-41	Shari-town, Hokkaido	2001	MZ1	AB525715
MZ-42	Shari-town, Hokkaido	2001	MZ1	AB525715
MZ-44	Kushiro-city, Hokkaido	2001	MZ1	AB525715
MZ-45	Teshikaga-town, Hokkaido	2001	MZ3	AB455691*
MZ-46	Shibetsu-town	2001	MZ1	AB525715
MZ-47	Rausu-town, Hokkaido	2001	MZ2	AB525716
MZ-48	Kiyosato-town, Hokkaido	2002	MZ3	AB455691*
MZ-49	Kushiro-city, Hokkaido	2002	MZ2	AB525716
MZ-50	Shari-town, Hokkaido	2002	MZ2	AB525716
MZ-51	Rausu-town, Hokkaido	2002	MZ1	AB525715
MZ-55	Kushiro-city, Hokkaido	2003	MZ8	AB525721
MZ-58	Shari-town, Hokkaido	2002	MZ9	AB525722
MZ-59	Shari-town, Hokkaido	2003	MZ1	AB525715
MZ-65	Shari-town, Hokkaido	2005	MZ2	AB525716
MZ-75	Shari-town, Hokkaido	2001	MZ1	AB525715
MZI-1	Shari-town, Hokkaido	1991	MZ1	AB525715
MZI-HIG1	Kamishihoro-town, Hokkaido	1997	MZ3	AB455691*
MZI-HIG2	Kamishihoro-town, Hokkaido	1997	MZ3	AB455691*
MZI-CH2	Taiki-town, Hokkaido	1992	MZ4	AB525717
MZI-CH4	Memuro-town, Hokkaido	1996	MZ5	AB525718
MZI-CH5	Nakasatsunai-village, Hokkaido	1997	MZ4	AB525717
MZI-CH6	Obihiro-city, Hokkaido	1997	MZ5	AB525718
MZI-A2	Asahikawa-city, Hokkaido	1994	MZ3	AB455691*
MZI-76285	Barguzin Nature Reserve, Russia	1915	MZ10	AB525723
MZI-97078	Sayany Mts., Central Siberia, Russia	1923	MZ11	AB525724
MZI-97079	Kamchatka, Russia	1918	MZ12	AB525725

Table 1. Profiles of samples examined in the present study (continued)

Sample name	Sampling location	Year of collection	Haplotype	Accession no.#
Martes melampus				
MME-1	Iwate	1993	MM1	AB525726
MME-MR1	Morioka-city, Iwate	1998	MM2	AB525727
MME-MR2	Morioka-city, Iwate	1995	MM3	AB525728
MME-G1	Gifu	1994	MM4	AB525729
MME-G2	Gifu	1994	MM4	AB525729
MME-G4	Gifu	1994	MM5	AB525730
MME-K1	Fukuoka	1992	MM6	AB525731
MME-K2	Fukuoka	1993	MM7	AB525732
MME-K16	Fukuoka	1998	MM8	AB525733
MME-K17	Oita	1999	MM9	AB525734
MM-3	Hiyama, Hokkaido	1998	MM10	AB525735
MM-4	Hiyama, Hokkaido	1998	MM10	AB525735
MM-5	Imakane-town, Hokkaido	1999	MM10	AB525735
MM-6	Setana-town, Hokkaido	1999	MM10	AB525735
MM-7	Noboribetsu-city, Hokkaido	1999	MM11	AB525736
MM-8	Shiraoi-town, Hokkaido	1999	MM11	AB525736
MM-13	Assabu-town, Hokkaido	1999	MM10	AB525735
MM-14	Yakumo-town, Hokkaido	1999	MM12	AB455699**
MM-15	Yakumo-town, Hokkaido	1999	MM10	AB525735
MM-16	Assabu-town, Hokkaido	1999	MM10	AB525735
MM-17	Eniwa-city, Hokkaido	1999	MM12	AB455699**
MM-19	Tomakomai-city, Hokkaido	2000	MM11	AB525736
MM-21	Assabu-town, Hokkaido	2000	MM10	AB525735
MM-22	Date-city, Hokkaido	2001	MM11	AB525736

Sequences have been registered to DDBJ/GenBank/EMBL data base with accession numbers.
* Reported by Sato et al. (2009).
** Reported by Sato et al. (2009).

2 was amplified using MZCRP-F1 (5'-ATGTGTACC TCTTCTCGCTC-3': this newly designed sequence was partly identical with DLOOP-MelF reported by Sato et al. 2009) and MZCRP-R1. The PCR amplifications were performed in 50 μl of reaction mixture: 5.0 μl of 10 × Reaction buffer (Takara), 4.0 μl of dNTP, 0.5 μl of each primer (25 pmol/μl), 0.25 μl of r*Taq* DNA polymerase (5 units/μl, Takara) and 1.0 μl of DNA extracts. After denaturing at 94°C for 3 min, 40 cycles were performed using a thermal cycler (Takara TP600) with the following program: denaturing at 94°C for 1 min; annealing at 50°C–60°C for 1 min; extension at 72°C for 1 min, and then the reaction was completed at 72°C for 10 min. To check PCR amplification, 9 μl of each PCR product was electrophoresed on a 2% agarose gel, stained by ethidium bromide, and visualized under an ultraviolet illuminator. The remaining 41 μl of each PCR product was purified using QIAquick PCR Purification Kit (Qiagen).

Purified PCR products were used as templates for cycle PCR. For sequencing primers, UR-1, MZCR-SF1 (5'-CATCTCGATGGACTAATGAC-3'), MZCRS-F2 (5'-ATTTCCTCTCCCCATGTCTT-3'), MZCRS-R1 (5'-GATACCAAATGCATGACACC-3'), MZCRS-R2 (5'-GAGCGAGAAGAGGTACACAT-3') and MZCRS-R3 (5'-GACCATTGACTGAATAGCACC-3') labeled with Texas Red were used. These sequencing primers except for UR-1 were newly designed in the present study. After denaturing at 95°C for 4.5 min, 30 cycles were performed with the following programs: denaturing at 95°C for 30 sec; annealing at 55°C for 30 sec; extension at 72°C for 1 min, and then the reaction was completed at 72°C for 7 min. Cycle PCR products were electrophoresed by an automated sequencer (Hitach SQ5500). All sequencing primers were able to be used for analysis of *M. melampus*.

Data analysis

A neighbor-joining tree (Saitou and Nei 1987) using Kimura's two parameter distances (Kimura 1980) was constructed by MEGA4 (Tamura et al. 2007). As an outgroup, the homologous sequence of the wolverine *Gulo gulo* (Accession number EF581374: Meschersky 2007) was used. Parsimony networks among obtained mtDNA haplotypes were produced by TCS (Clement et al. 2000). Genetic differences (p-distance) were calculated by MEGA4. The haplotype diversity (h) and nucleotide diversity (π) were calculated by ARLEQUIN Ver. 2.000 (Schneider et al. 2000). To evaluate gene flow within *M. zibellina* of Hokkaido, we divided the samples into three groups (populations 1, 2 and 3) arbitrarily based on sampling points (see Fig. 1b), and calculated pairwise *Fst* values using ARLEQUIN Ver. 2.000.

Results

Sequence variations in M. zibellina

Including insertion and/or deletion (indel) sites, 14 sites in the 5' portion of the control region were variable among 52 individuals of *M. zibellina* (Table 2). Of them, a total of 12 haplotypes were identified: nine haplotypes from Hokkaido (MZ1–MZ9) and three from Russia (MZ10–MZ12). Sequence differences among the haplotypes ranged from 0.19% to 1.31%. Haplotype MZ1 was the most frequent on Hokkaido (44.9%, 22/49 individuals). Haplotypes identified from Hokkaido formed a star-like parsimony network, and each of them possessed 1–3 base substitutions, compared with MZ1 (Fig. 2a). The smallest difference between haplotypes from Hokkaido and those from Russia was 2-base substitutions observed between MZ3 and MZ12. Table 3 shows the haplotype diversity and nucleotide diversity in *M. zibellina*.

The pairwise *Fst* value between populations 1 and 3 (0.17285) was larger than those between other populations, and the differentiation was statistically significant ($P = 0.011$). The pairwise *Fst* value between populations 1 and 2 and that between populations 2 and 3 were 0.0931 and 0.0501, respectively. The differentiations of the latter were not significant ($P = 0.065$ and 0.153, respectively).

Sequence variations in M. melampus

Including indels, 27 sites in the 5' portion of the control region were variable among 24 individuals of *M. melampus* (Table 2). Of them, a total of 12 haplotypes were identified: nine haplotypes from Honshu and Kyushu (MM1–MM9) and three haplotypes from Hokkaido (MM10–MM12). Sequence differences among haplotypes ranged from 0.19% to 2.99%. Table 3 shows the haplotype diversity and nucleotide diversity in all *M. melampus*. The values of the Hokkaido-introduced population were smaller than those of the Honshu- and Kyushu-native populations. In haplotypes from Honshu, no correlations between haplotype-clustering and sampling localities were seen in both the parsimony network (Fig. 2b) and the neighbor-joining tree (Fig. 3). For example, three haplotypes (MM1, MM2 and MM3) from Iwate Prefecture, northern Honshu, were not closely related to each other. Haplotypes MM3 showed a close affinity with MM4 from Gifu Prefecture, central Honshu, rather than MM1 and MM2. On the other hand, four haplotypes from Kyushu (MM6, MM7, MM8 and MM9) formed a cluster with 1–2 base substitutions among them (Figs. 2b and 3). None of haplotypes identified from Hokkaido (MM10, MM11 and MM12) were shared by individuals of Honshu and Kyushu. Furthermore, each of the three haplotypes from Hokkaido was included in clusters different from each other (Figs. 2b and 3).

Phylogenetic relationships between M. zibellina and M. melampus

The neighbor-joining tree (Fig. 3) showed that haplotypes of *M. zibellina* were clustered into a single cluster with a 97% bootstrap value and that those of *M. melampus* formed another single cluster with a 95% bootstrap value (Fig. 3). Sequence differences between the two species were 4.39% on average. The nucleotide diversity of *M. zibellina* was much lower than that of *M. melampus* (Table 3). Both the parsimony networks (Fig. 2) and neighbor-joining tree (Fig. 3) also showed that intraspecific variations of *M. zibellina* were smaller than that of *M. melampus*. The distributions of pairwise distances (Fig. 4) indicated that *M. melampus* on Honshu and Kyushu had wider substitution ranges (0–16 bases), whereas *M. zibellina* on Hokkaido had smaller substitution ranges (0–4 bases), indicating a lower intraspecific variation in *M. zibellina* of Hokkaido than *M. melampus* of Honshu and Kyushu.

Discussion

Genetic diversity in M. zibellina

The present study revealed that the intraspecific variation of *M. zibellina* of Hokkaido was smaller than that of

Table 2. Sequence variations among the mtDNA control region haplotypes (535–537 bp) of *Martes zibellina* (MZ1–MZ12) and *M. melampus* (MM1–MM12)

Haplotype number	Variable site																																							
	17	43	70	71	72	77	83	90	91	102	123	134	135	143	145	146	149	153	155	160	161	166	173	208	216	219	222	248	415	426	427	434	436	437	439	482	487	488	498	530	532
MZ1	T	T	T	T	–	–	–	C	A	C	A	A	C	G	C	T	A	C	G	C	C	C	A	C	C	T	G	A	C	T	A	G	G	G	C	G	T	C	G	G	C
MZ2	.	.	.	.	–	–	–	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MZ3	.	.	.	.	–	–	–	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MZ4	.	.	.	.	–	–	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
MZ5	.	.	.	.	–	–	–	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MZ6	.	.	.	.	T	–	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
MZ7	.	.	.	.	–	–	–	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MZ8	.	.	.	.	–	–	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MZ9	.	.	.	.	–	–	–	.	G	.	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MZ10	.	.	.	.	T	–	–	.	.	.	.	.	.	.	.	.	G	.	.	T	T	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MZ11	C	.	–	–	–	–	C	.	.	.	.	.	.	.	T	.	G	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MZ12	.	.	.	.	–	–	–	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MM1	A	C	.	–	–	–	–	.	G	G	.	.	T	.	T	.	G	.	.	.	.	T	G	.	T	.	.	.	T	G	G	A	A	A	T	A	C	T	.	A	.
MM2	A	.	.	–	–	–	–	T	.	G	.	.	.	.	T	C	G	.	.	.	.	.	.	.	T	C	.	.	T	G	G	A	A	A	T	.	C	T	A	A	T
MM3	A	.	.	–	–	C	–	.	.	A	.	G	.	A	T	C	G	T	.	.	.	T	.	.	T	C	A	.	.	G	G	A	A	A	T	.	.	.	A	A	.
MM4	A	.	.	.	–	C	–	.	.	G	.	G	.	A	T	C	G	T	.	.	.	T	.	.	T	C	A	.	T	G	G	A	A	A	T	.	.	.	A	A	.
MM5	A	.	.	–	–	–	–	.	.	G	.	.	T	.	T	C	G	T	.	.	.	.	.	.	T	C	.	.	T	G	G	A	A	A	T	A	C	T	A	A	.
MM6	A	.	.	–	–	–	–	T	.	A	.	.	T	.	T	C	.	T	.	.	T	.	.	.	T	C	.	.	T	G	G	A	A	A	T	A	C	T	A	A	.
MM7	A	.	.	–	–	–	–	T	.	A	.	.	.	.	T	C	G	T	.	T	T	.	.	.	T	C	.	.	T	G	G	A	A	A	T	A	C	T	A	A	.
MM8	A	.	.	–	–	–	–	T	.	A	.	.	.	.	T	C	.	T	.	.	T	.	.	.	T	C	.	.	T	G	G	A	A	A	T	A	C	T	A	A	.
MM9	A	.	.	–	–	–	–	T	.	A	.	.	T	.	T	C	G	T	.	T	T	.	.	.	T	C	.	.	T	G	G	A	A	A	T	A	C	T	A	A	.
MM10	A	.	.	–	–	–	–	T	.	A	.	.	T	.	T	C	G	T	A	T	.	T	.	.	T	C	.	G	T	G	G	A	A	A	T	A	C	T	A	A	.
MM11	A	.	.	–	–	C	–	.	G	G	.	G	.	A	T	C	G	T	.	.	.	T	.	.	T	C	A	.	T	G	G	A	A	A	T	.	.	.	A	A	.
MM12	A	.	.	–	–	–	–	T	.	G	G	.	T	.	T	C	G	T	.	.	.	.	.	.	T	C	.	.	T	G	G	A	A	A	T	.	C	T	A	A	.

Dots indicate identical nucleotides with sequences of MZ1, and dashes show indel sites.

Table 3. Molecular diversities of *Martes zibellina* and *M. melampus* examined in the present study

Species	Locality	Number of samples	Number of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)
Martes zibellina	Hokkaido	49	9	0.73	0.00242
	Russia	3	3	1.00	0.00874
Martes melampus	Honshu and Kyushu	10	9	0.98	0.01541
	Hokkaido	14	3	0.62	0.01249

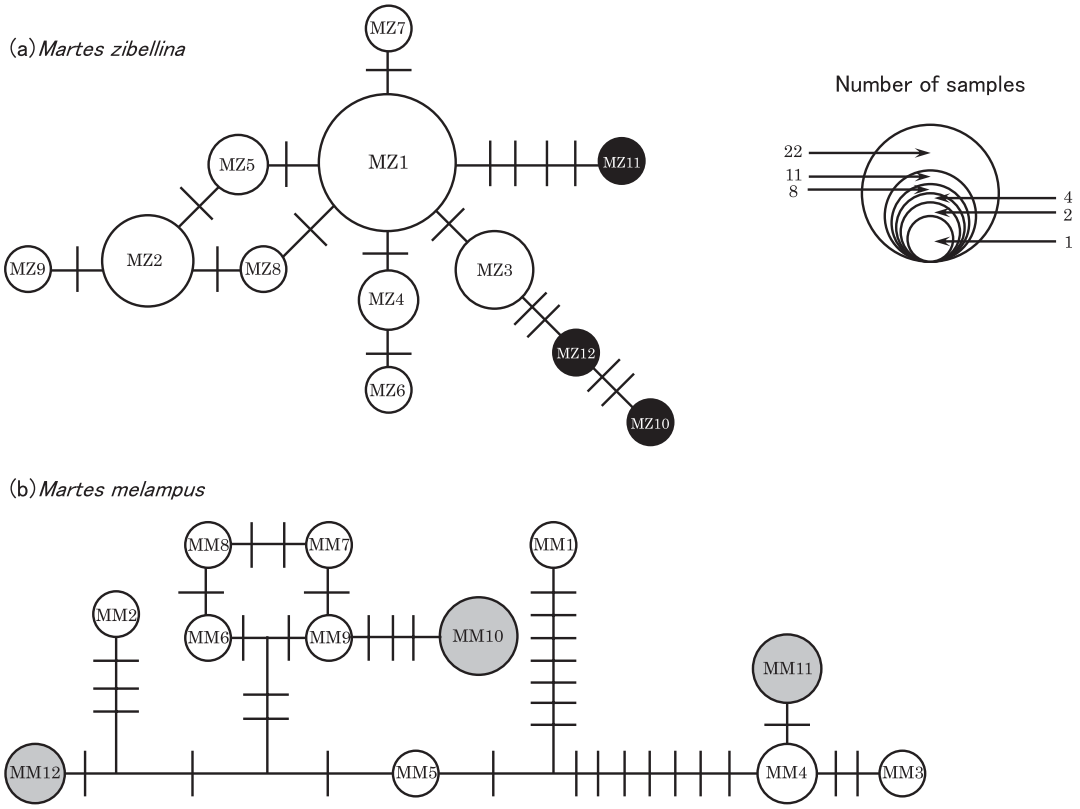

Fig. 2. Parsimony networks of haplotypes for *Martes zibellina* (a) and *M. melampus* (b). The sizes of circles show numbers of individuals having those haplotypes, and one slash indicates one-base substitution. (a) MZ1–MZ9 indicated by open circles are haplotypes from *M. zibellina* of Hokkaido, and MZ10–MZ12 indicated by closed circles are those from *M. zibellina* of the Eurasian Continent. (b) MM1–MM9 indicated by open circles are haplotypes from *M. melampus* (natural populations) of Honshu and Kyushu, and MM10–MM12 indicated by gray circles are those from *M. melumpus* (introduced populations) of Hokkaido.

M. melampus. The result supports the previous study on mtDNA cytochrome *b* phylogeny of the two species in Japan (Kurose et al. 1999). Although *M. zibellina* is distributed widely in northern Eurasia, such small intraspecific variations suggest that the Hokkaido population has not been genetically well-differentiated from continental populations, after immigration into Hokkaido in the last glacial period. The haplotype most frequently identified from *M. zibellina* of Hokkaido was MZ1, and the other haplotypes from Hokkaido had 1–3 base substitutions from MZ1 (Figs. 2a and 4). This suggests that those haplotypes were derived from MZ1 within the Hokkaido populations and/or their direct ancestors that immigrated into Hokkaido from the Eurasian Continent through land-bridge of the last glacial period. Another explanation of the low genetic diversity in *M. zibellina* of Hokkaido is possible occurrence of past bottleneck event in the Eurasian Continent. After bottleneck in the Eurasian Continent, a part of the populations might have immigrated into Hokkaido, still showing the bottleneck effects such as a low genetic diversity. Otherwise, the bottleneck event on Hokkaido caused by the sable hunting for fur in the early 20th century (Inukai 1957) might have affected the current genetic diversity of the Hokkaido populations.

In addition, the present study revealed that sequence

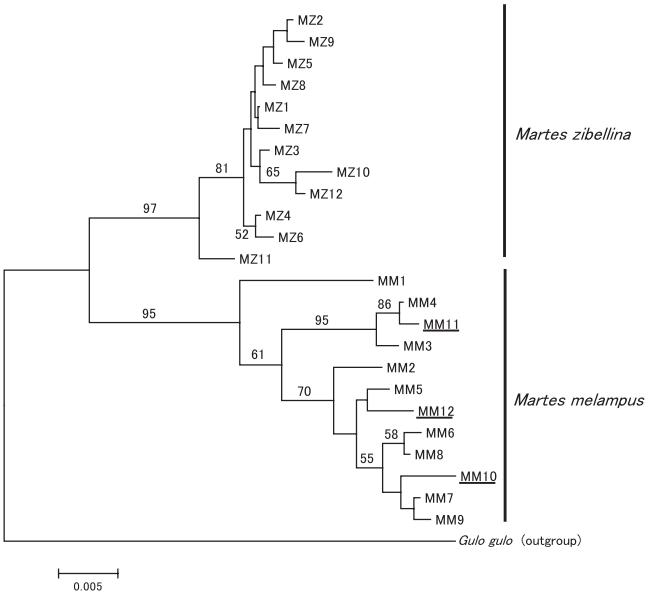

Fig. 3. A neighbor joining tree constructed for *Martes zibellina* and *M. melampus* on the basis of the 5' portion of the mtDNA control region sequences (535–537 bp). The scale indicates genetic distances estimated with Kimura's (1980) two parameter method. Numbers (%) near internal branches show bootstrap values (>50%) derived from 1,000 replications. Haplotypes MZ1–MZ12 are of *M. zibellina*, and MM1–MM12 are of *M. melampus*. Three haplotypes (MM10, MM11 and MM12) identified from introduced *M. melampus* populations of Hokkaido are underlined. The sequence of *Gulo gulo* was used as an outgroup.

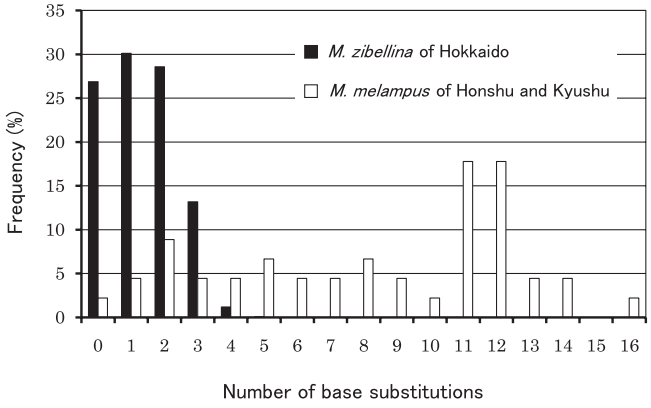

Fig. 4. Distributions of pairwise distances (numbers of base substitutions) in 49 individuals of *Martes zibellina* on Hokkaido and in 10 individuals of *M. melampus* on Honshu and Kyushu. Closed bars show frequencies of pairwise sequence distances of *M. zibellina* of Hokkaido and open bars show those of *M. melampus* of Honshu and Kyushu. Frequencies in *M. melampus* ranged from 0 to 16 base substitutions, whereas those of *M. zibellina* were between 0 and 4 base substitutions.

differences between haplotypes of *M. zibellina* from Hokkaido and Russia were not considerable. The previous study using mtDNA cytochrome *b* (Hosoda et al. 1999) also reported that intraspecific variations between Hokkaido and continental Russia were not so high. The small difference (only two-base substitutions) between haplotypes from Hokkaido (MZ3) and Russia (MZ12) is allowed to suggest that the *M. zibellina* populations in Hokkaido and Eurasia could have genetic communications until recent time. Land-bridges between the Eurasian Continent and Hokkaido are considered to have disappeared completely in the last glacial age, about 12000 years ago (Ohshima 1991). Because until the end of the last glacial age, the Eurasian Continent, Sakhalin and Hokkaido were periodically connected through land-bridges, *M. zibellina* populations distributed in these areas could exchange genetic elements between each other.

To reveal migration history and the bottleneck effects of *M. zibellina* on Hokkaido, further studies using samples from the overall distribution in Eurasia including other parts of Hokkaido are needed.

Genetic differentiations among populations of M. zibellina in Hokkaido

We divided *M. zibellina* in Hokkaido into three populations based on sampling locations, and calculated pairwise *Fst* values among them (Fig. 1b). The results showed a statistically significant divergence between populations 1 and 3. Although pairwise *Fst* values among the three populations were not so high, frequencies of mtDNA haplotypes were different from population to population (Fig. 1b and Table 4). These findings indicate that populations have been differentiated due to isolation by distance, although degrees of divergence among individuals in Hokkaido were not so dominant. Because mtDNA is maternally inherited, the results could reflect female migration patterns. To further understand geographic variations and population structures of *M. zibellina* on Hokkaido, it is needed to examine more samples especially from northern and central Hokkaido and to analyze Y chromosome-linked genes which are paternally inherited as well as autosome genes which are biparentally inherited.

Genetic diversity and phylogeography of M. melampus on Honshu and Kyushu

The present study revealed that mtDNA diversities of *M. melampus* on Honshu and Kyusyu were higher than those of *M. zibellina* on Hokkaido (Table 3, Figs. 2 and 4). *Martes melampus* is a Japanese endemic species known only Honshu, Shikoku and Kyushu (Masuda

Table 4. Haplotypes identified from three populations of *M. zibellina* in Hokkaido and the frequencies

Population	Number of individuals in each population	Haplotype frequency								
		MZ1	MZ2	MZ3	MZ4	MZ5	MZ6	MZ7	MZ8	MZ9
Population 1	13	2 (15.4)	0	5 (38.5)	2 (15.4)	2 (15.4)	1 (7.7)	1 (7.7)	0	0
Population 2	18	9 (50.0)	6 (33.3)	2 (11.1)	0	0	0	0	1 (5.6)	0
Population 3	16	10 (62.5)	5 (31.3)	0	0	0	0	0	0	1 (6.3)
Total number of individuals	47	21	11	7	2	2	1	1	1	1

Numbers in parentheses are parcentage frequencies. MZ-16, whose sampling location was unknown, and MZI-A2 from Asahikawa were excluded for grouping of the three populations (see Fig. 1b).

2009). On the other hand, *M. zibellina* is distributed widely across the northern parts of Eurasia (Wozencraft 2005; Murakami 2009). The differences of mtDNA variations between the two *Martes* species could have resulted from differences of their population- and migration-histories.

In *M. melampus* from Honshu, the correlations between genetic distances of haplotypes and geographic distances of sampling locations were absent. Kurose et al. (1999) reported the similar result based on phylogenetic analysis of the mtDNA cytochrome *b* gene. As one of causes for formation of such phylogeographic structures, the incomplete geographic isolation among the local populations on Honshu can be considered. In Japan, fossils of *Martes* have been recorded from layers of the Late Pleistocene (Masuda 2009), when the Japanese archipelago experienced the glacial and interglacial epochs many times (Machida 2003). Changes of environmental conditions such as temperature and vegetation could have reduced and expanded their distribution areas repeatedly, resulting in formation of such phylogeographic structures. On the other hand, Sato et al. (2009) analyzed combined sequences of some mtDNA genes (cytochrome *b*, control region, and NADH dehydrogenase subunit 2 genes) of *M. melampus* mainly from western parts of Japan, and reported that the relationships between their genetic distances and geographic distances of sampling locations are mostly congruent. By contrast, in the present study, we examined *M. melampus* from eastern and central parts of Honshu and Kyushu. The result of Sato et al. (2009) that individuals from Kyushu formed a monophyletic group is consistent with that of the present study. There might be some differences of zoogeographical histories between populations of western Japan except for Kyushu and those of eastern Japan. Another possible explanation of unclear phylogeographic structures of *M. melampus* in Honshu is transportation and naturalization of *M. melampus* from one area to another area within Honshu. Because *M. melampus* had been introduced into Hokkaido and Sado Island (Hosoda et al. 1999), artificial introductions among multiple areas of Honshu might have occurred, and affected such current phylogeographic structures of *M. melampus*.

Founder effects in M. melampus populations introduced into Hokkaido

The Hokkaido population of *M. melampus* originated from introduced animals from Honshu (Inukai 1975). In the present study, we clarified that the genetic diversity of *M. melampus* on Hokkaido was lower than that of *M. melampus* populations native to Honshu and Kyushu. In addition, each of the two haplotypes (MM10 and MM11) was shared by eight and four individuals in restricted areas of southern Hokkaido, respectively (Fig. 1b). The findings indicate founder effects in the Hokkaido population of *M. melampus* after the introduction from Honshu. Although Inukai (1975) reported that *M. melampus* on Hokkaido originated from those captured in the Tohoku region of northern Honshu, three haplotypes (MM1, MM2 and MM3) from Iwate Prefecture of this region were not shared by any individuals from Hokkaido examined in the present study. Of the three haplotypes from Hokkaido (MM10, MM11 and MM12), MM10 was relatively closely related to MM6-MM9 from Kyushu, and MM11 was closely related to MM4 identified from Gifu Prefecture, central Honshu (Figs. 2b and 3). Furthermore, MM12 was identical with one haplotype (accession no. AB455699) of *M. melampus* from Okayama Prefecture, western Honshu, reported by Sato et al. (2009). Sato et al. (2009) also examined one individual of *M. melampus* from Hokkaido, and reported that the haplotype from Hokkaido is closely related to AB455699 and another haplotype from Okayama Prefec-

ture. These findings show that *M. melampus* might have been introduced to Hokkaido from various areas of Honshu and Kyushu. To conclude the origins and pathways of introduction of *M. melampus* currently occurring in Hokkaido, more data of genetics on Hokkaido, Honshu, Kyushu and Shikoku populations are required.

Genetic relationships between M. zibellina and M. melampus on Hokkaido

It had been reported that natural hybridization between *M. zibellina* and the pine marten *M. martes* could occur (Heptner et al. 1967; Bakeyev and Sinitsyn 1994). Although *M. zibellina* had been distributed in overall Hokkaido until the Meiji period, the real distribution of *M. zibellina* at the time when *M. melampus* was introduced into Hokkaido (1940's) is unclear (Murakami 2001). Murakami and Ohtaishi (2000) reported that the current distribution of *M. zibellina* and *M. melampus* on Hokkaido are divided along the lowlands of Ishikari (see Fig. 1b): native *M. zibellina* occurs in northern and introduced *M. melampus* occurs in southern Hokkaido. Sugimoto et al. (2009) also reported that there were no places where the two species co-exist based on fecal DNA analysis. No evidence on the hybridization between the two *Martes* species has been reported so far. Both the present and previous studies show that the two species are clearly separated into different clades in phylogenic trees (Fig. 3 in the present study; Hosoda et al. 1999; Kurose et al. 1999). In addition, individuals showing morphological features such as coat colors, the ratio of the tail length to head and body length, etc. (Murakami 2001) of *M. zibellina* and the mtDNA types of *M. melampus*, or those showing reciprocal features have not been found in the present study. It suggests that no hybridization between *M. zibellina* and *M. melampus* on Hokkaido has occurred. Based on only the data of mtDNA which is maternally inherited, however, it cannot be concluded. To clarify the problem on the hybridization between the two *Martes* species, further studies using more samples from all the distribution areas on Hokkaido and autosomal DNA markers are needed.

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