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Phylogeographic Variation in Two Mustelines, the Least Weasel *Mustela nivalis* and the Ermine *M. erminea* of Japan, Based on Mitochondrial DNA Control Region Sequences

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ABSTRACT—The least weasel *Mustela nivalis* and the ermine *M. erminea* of Japan are considered relicts of the last glacial period. To study phylogeographic variation in these mustelines, fragments of the mitochondrial DNA control region were sequenced. In both species, the control region included tandem repeats of 10-base motifs at the 3' portion specific to the genus *Mustela*. Phylogenetic trees of the 5' portion (581–584 bases) of *M. nivalis* indicated that haplotypes in the Hokkaido population clearly diverged from those in the Honshu population. Geographic pattern of intraspecific variations illustrated by this result is concordant with that by the result of a previous cytogenetic study, which revealed the karyotypic differentiation between the two populations. These findings might suggest that the Hokkaido and Honshu populations of *M. nivalis* were geographically isolated in advance of the formation of the Tsugaru strait between Hokkaido and Honshu. Distribution pattern of the mitochondrial DNA haplotypes within Hokkaido suggests that *M. nivalis* experienced the repeated changes of environments during glacial and inter-glacial periods of the Quaternary, or that this species immigrated from Sakhalin/Siberia into Hokkaido so recently. On the other hand, *M. erminea* exhibited a smaller sequence divergence between the Hokkaido and Honshu populations, suggesting a shorter geographic isolation than in *M. nivalis*.

INTRODUCTION

The least weasel *Mustela nivalis* is the smallest species among the genus, distributed only in cold regions such as northern Eurasia, northwestern Africa, and North America (Nowak, 1991). In Japan, distribution of *M. nivalis* is restricted to Hokkaido (the northernmost island of Japan) and some higher mountains in the northern part of Honshu (Kawamichi, 1996)(see Fig. 1). It is hypothesized that the Tsugaru strait between Hokkaido and Honshu was formed around 0.1–0.15 million years (My) ago, whereas the Soya strait, isolating Hokkaido from Sakhalin/Siberia, was completed much more recently, around 12,000 years ago (Ohshima, 1991). It is likely that *M. nivalis* immigrated from the continent into the Japanese islands through land bridges, and then was isolated there by straits. A fossil of *M. nivalis* was reported from the Quaternary remains of Gifu Prefecture (Kawamura *et al.*, 1989) (see Fig. 1), which is located far south of the current range of the species. Therefore, *M. nivalis* is considered one of relicts of the last glacial period in Japan (Iziri, 1979).

Obara (1991) reported the occurrence of Robertsonian chromosomal divergence between populations of *M. nivalis* from Hokkaido (2n=42) and northern Honshu (2n=38). On the other hand, the Siberian continent population has a 2n=42 karyotype, apparently identical with that of the Hokkaido population (Mandahl and Fredga, 1980). A previous study (Masuda and Yoshida, 1994a) reported the mitochondrial DNA (mtDNA) cytochrome *b* sequence divergence between the Hokkaido and Honshu populations, which was parallel to intraspecific difference of other mustelid species. These karyotypic and mtDNA differentiation could have been fixed to the Honshu population after its geographic isolation.

The ermine *Mustela erminea* is considered another musteline adapted to cold regions of the Northern Hemisphere (Iziri, 1979; Nowak, 1991). In Japan, this species is distributed in Hokkaido and high altitude areas of the northern and central Honshu (Kawamichi, 1996)(Fig. 1). Fossils of *M. erminea* were found from the Quaternary remains in the more western areas, i.e., Yamaguchi Prefecture of western Honshu (Kawamura *et al.*, 1989) (see Fig. 1) and northern Kyushu (Nakagawa *et al.*, 1997).

Their common distribution pattern in cold regions of Japan suggests that these two species have the similar migration history to each other and adapted themselves to the cold environment. Among all mustelid species of Japan,

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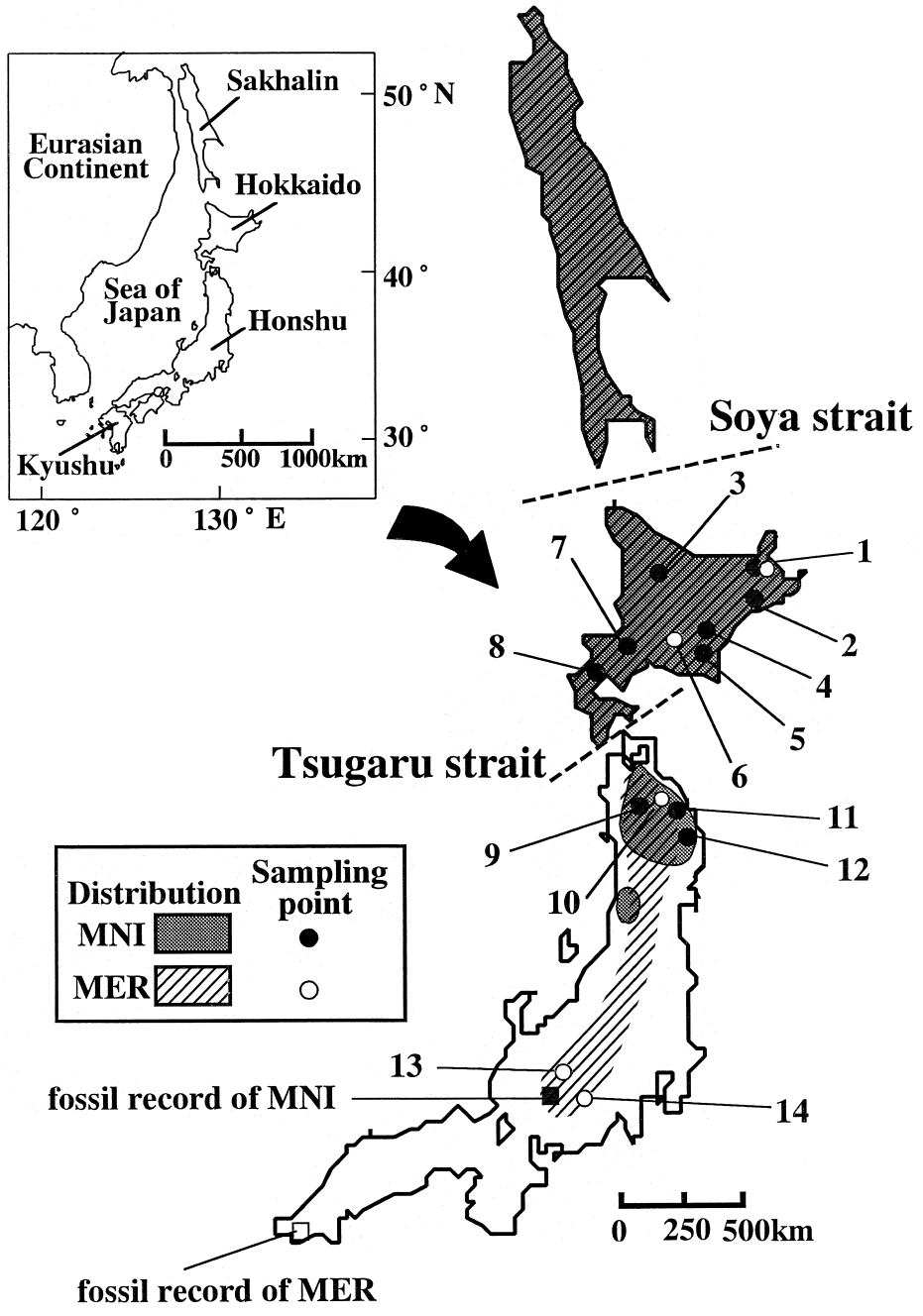

Fig. 1. Sampling localities of the least weasels *Mustela nivalis* (MNI) and the ermines *M. erminea* (MER). Locality numbers on the Japanese islands correspond to those in Table 1. Distribution areas of the two species (dots and slashes, respectively) are derived from those in Kawamichi (1996). Fossil records of these species were shown by squares.

only *M. nivalis* and *M. erminea* share some characteristics such as changing of the coat color from dark to white in the winter.

In this paper, we present geographic sequence variations in the mtDNA control regions of these two mustelines in Japan and discuss their bearings on the migration history.

MATERIALS AND METHODS

Animals and DNA extraction

Animals examined in the present study and their localities were described in Table 1 and Fig. 1, respectively. Muscle tissues from animals were frozen at –80°C or preserved in 70% ethanol until use. Total DNAs were extracted according to the phenol/proteinase K/sodium dodecyl sulfate (SDS) method of Sambrook *et al.* (1989) with some modifications (Masuda and Yoshida, 1994b). A small piece (approximately 1×1×1 mm) of tissue was excised with a scalpel and washed once with 500 µl STE buffer (0.1M NaCl/10 mM Tris/1 mM

Table 1. Profiles of the least weasels *Mustela nivalis* (MNI) and the ermines *M. erminea* (MER) examined in this study[1)]

Species	Sample name	Sex	Sampling locality/supplier	Locality No.[2)]	Accession Number or sequence identity
Mustela nivalis	MNI-AKI1	♂	Kazuno-shi, Akita/M. Hisasue	9	AB006728
	MNI-IWA1	♂	Kunohe-gun, Iwate/F. Sekiyama	11	same as MNI-AKI1
	MNI-IWA2	♂	Iwaizumi-cho, Iwate/F. Sekiyama	12	same as MNI-AKI1
	MNI-S12	♂	Shari-cho/Y. Masuda	1	AB006720
	MNI-S13	♀	Shari-cho/Y. Masuda	1	AB006721
	MNI-S14	♂	Shari-cho/Y. Masuda	1	same as MNI-S13
	MNI-5	♂	Horokanai-cho/H. Abe	3	AB006717
	MNI-YOT1	♀	Mt.Youtei/M. Takahashi	8	AB006719
	MNI-OB1	♂	Makubetu-cho/H. Yanagawa	5	AB006718
	MNI-OB2	♀	Shihoro-cho/H. Yanagawa	4	same as MNI-S13
	MNI-HIT1	♀	Sapporo-shi/T. Saito	7	AB006722
	MNI-HIT2	♂	Sapporo-shi/T. Saito	7	AB006723
	MNI-N26	♂	Unknown/T. Saito	–	AB006724
	MNI-N27	♂	Sapporo-shi/T. Saito	7	AB006725
	MNI-N28	♀	Sapporo-shi/T. Saito	7	same as MNI-N27
	MNI-N29	♀	Sapporo-shi/T. Saito	7	same as MNI-N27
	MNI-N30	♀	Sapporo-shi/T. Saito	7	AB006726
	MNI-N31	♂	Sapporo-shi/T. Saito	7	same as MNI-YOT1
	MNI-N32	♂	Shibecha-cho/T. Saito	2	AB006727
	MNI-N33	♂	Sapporo-shi/T. Saito	7	same as MNI-N30
	MNI-N34	♀	Sapporo-shi/T. Saito	7	same as MNI-HIT1
	MNI-N35	♀	Shibecha-cho/T. Saito	2	same as MNI-N32
	MNI-N37	♀	Sapporo-shi/T. Saito	7	same as MNI-HIT1
Mustela erminea	MER-IWA1	♀	Ninohe-gun, Iwate/F. Sekiyama	10	AB006732
	MER-YAM1	♀	Fujimi-cho, Nagano/Y. Yamamoto	14	AB006729
	MER-TOG1	♀	Togakushi-mura, Nagano/T. Tanabe	13	AB006733
	MER-1	♀	Shari-cho/Y. Masuda	1	AB006730
	MER-CH1	♂	Hidaka Mountains/H. Yanagawa	6	AB006731
Mustela putorius furo	MFU-1	♂	Domestic	–	AB010379

[1)] All tissues used were muscle.
[2)] Locality numbers correspond to those in Fig. 1.

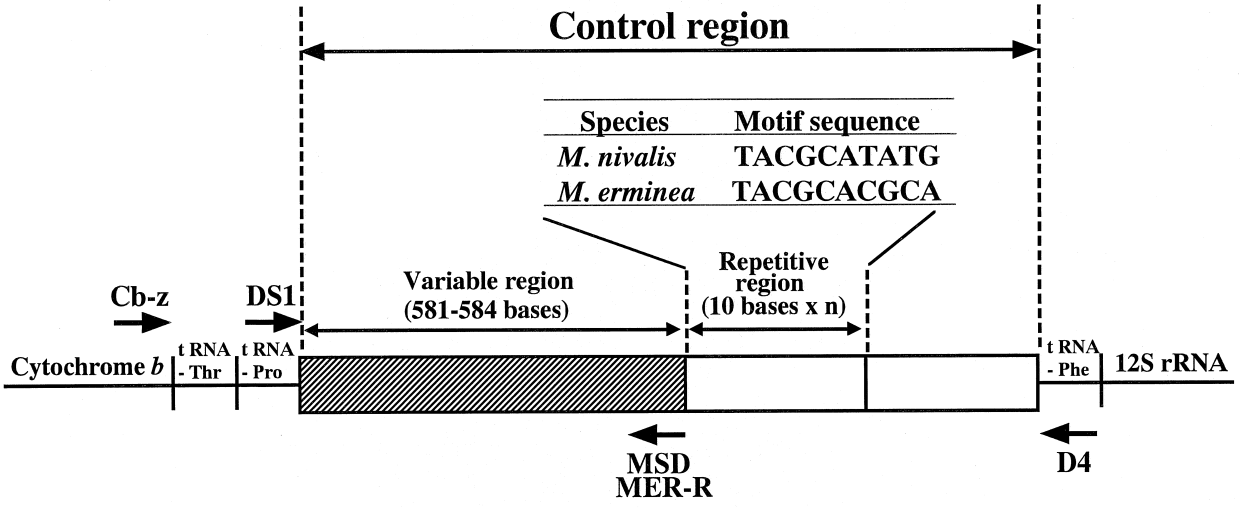

Fig. 2. Schematic diagram of the mitochondrial DNA control region of *Mustela nivalis* and *M. erminea*. Large arrows (Cb-Z, DS1, MSD, MER-R, and D4) show primers used for PCR amplification and/or sequencing. The variable region (slashed), which is more variable between individuals, was used for phylogenetic analysis.

EDTA). Using a small glass homogenizer, tissues were homogenized with 500 μl STE buffer containing a final concentration of 0.5% SDS and 5 μg/ml proteinase K. After incubation at 37°C overnight, the homogenate was extracted 2–3 times with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). STE buffer extracted by the same procedure was used as a negative control in subsequent polymerase chain reaction (PCR) amplification.

After washing with 70% ethanol, hair roots of the ferret *Mustela putorius furo* as an outgroup were incubated in 5% Chelex-100 (Bio-

Rad) at 56°C overnight, vortexed for 10 sec, and then boiled for 8 min (Walsh *et al.*, 1991). The supernatant of 10 μl was used for template of subsequent PCR amplification.

PCR, sequencing, and sequence analysis

The whole control region was amplified using two primers, Cb-z 5'-ATGAATTGGAGGACAACCAGT-3'and D4 5'-AGGCATTTTCAG-TGCCTTGCTTTG-3' designed from sequences reported by Gerald and Thomas (1991) and Árnason and Johnsson (1992), respectively (Fig. 2). As a result of direct sequencing of PCR products, a repetitive region was found in the 3' portion, but it was difficult to sequence the entire repetitive region because of possible heteroplasmy and/or DNA replication slippage of PCR. On the other hand, since the first 5' site region (581-584 base) was found to be readable and variable between individuals, this 5' portion was used for phylogenetic analysis (Fig. 2).

Symmetric PCR amplification to generate double-stranded DNAs was done in a total volume of 50 μl of the reaction mixture with 20 μg of the bovine serum albumin (Boehringer). PCR reactions were repeated 35 times using a DNA thermal cycler (PJ2000, Perkin-Elmer Cetus) as follows: 94°C, 1 min denaturing; 57°C, 1 min annealing; 72°C, 2 min extension, and then the reaction was completed at 72°C for 10 min. To check PCR amplification, an aliquot of 10 μl from the PCR products was electrophoresed on a 2% agarose gel, stained by ethidium bromide, and visualized under an ultraviolet illuminator. The remaining PCR product of 40 μl was purified with a PCR product purification kit QIAquick (QIAGEN).

Purified PCR products were labeled using Catalyst (Perkin-Elmer) and sequenced using the ABI Prism™ 377 DNA sequencing system (Perkin-Elmer). Based on sequences determined with the primer Cb-z, three sequencing primers, DS1

5'-CAACAGCCCCACCATCAGCAC-3', MSD 5'-GGGGGTGGG-TATATGTGTAT-3', and MER-R 5'-CGCGGGTGGTGTATAAATAT-3' were newly designed (Fig. 2).

Sequence alignment was done using GeneWorks (Intelligenetics). Insertions and/or deletions (indels) were compensated through observations by eye, and eliminated for reconstruction of the neighbor-joining tree (Saitou and Nei, 1987) using MEGA (Kumar *et al.*, 1993), based on the distance matrix estimated with Kimura's (1989) two parameter method, and hand-written parsimonious networks.

RESULTS

Repetitive sequences in mtDNA control regions

The mtDNA control regions of *M. nivalis* and *M. erminea* were successfully amplified with primers Cb-z and D4 (Fig. 2). In order to determine the whole sequence of the control region, PCR products were directly sequenced using these PCR primers. As a result of sequencing, tandem repeats of 10-base motifs were found in the 3' portion. A basic motif of the array in *M. nivalis* was 5'-TACGCATATG-3', while that in *M. erminea* was 5'-TACGCACGCA-3' (Fig. 2). Based on molecular size obtained by electrophoresis of PCR products, the repetitive region was estimated as approximately 350 bases, suggesting that more than 30 copies of motifs comprise this region (data not shown). However, the complete sequence of the repetitive region could not been determined because of mixed sequences due to possible heteroplasmy in the cell and/or artificial DNA slippage of PCR. Therefore, the 5' portion (variable region of 581-584 bases, see Fig. 2) was used for phylogenetic analysis (Fig. 2). The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank nucleotide sequence database with the following accession numbers: AB006717-AB006733, and AB010379 (Table 1).

Sequence variation in *M. nivalis*

A sequence alignment of the control region of *M. nivalis* (Table 2) showed 19 sites to be variable among 582 bases (excluding two indels) and transitional bias in nucleotide substitutions. Intraspecific sequence differences varied from 0.17% to 2.43%. Sequence differences within Hokkaido (N=20) ranged from 0.17% to 1.39%, whereas all three animals from Honshu showed no variation. Sequence differences between the Hokkaido population and the Honshu population were from

Table 2. Sequence variation of the control region (581-584 bases) in the least weasels *Mustela nivalis* (MNI) and the ermines *M. erminea* (MER). Dots indicate identical nucleotides or indels with those of MNI-S12. Dashes show indel sites.

	Variable site
	1111111112222222233333444444455555555555555
	1113444666777777899003445666111445660013302566880222333346777 7
Sample name	3413663583571234561893470372383578993468045244031633790123090157
MNI-S12	CCC–TC–ATTCCTTCCTCTAATTAGCCCTTTACTCATCAAATCAATGGATTTTGTTACTCACTC
MNI-S13,S14,OB2	·····T·····A·····T·····
MNI-OB1	·····G·····T·····A·····T·····
MNI-YOT1,N31	·····–·CT··G·····A·····T·····
MNI-5	·····–·CT·····A·····T·····
MNI-HIT1,N34,N37	·····C·····A·····T·····
MNI-HIT2	·····A·····T·····
MNI-N26	·····C·····AA·····T·····
MNI-N27,N28,N29	·····G·····A·····T·····
MNI-N30,N33	·····–·CTC··C·····AA·C·····T·····
MNI-N32,N35	·····–·CTC··C·····AA·····T·····
MNI-AKI1,IWA1,IWA2	T·····T·C·····C·G·····T·····T····C·C····GTC·····
MER-1	TTTTCTCC·TTT····C··CCCC·ATTGC·C–·ATTCTGCGCTGG–··TG·CCCACC·T·TTTC–
MER-CH1	TTTTCTCC·–TT·C··C··CCCC·ATTGC·C–·ATTCTGCGCTGG–··TG·CCCACC·T·TTTC–
MER-IWA1	TTTTCTCC·–TT·C·TC··CCCC·ATTGC·C–TATTCTGCGCTGG–··TG·CCCACC·T·TTTC–
MER-YAM1	TTTTCTCC·–TT····C··CCCC·ATTGCCC–··ATTCTGCGCTGG–··TG·CCCACC·TCTTTC–
MER-TOG1	TTTTCTCC·TTTC···C··CCCC·ATTGCCC–··ATTCTGCGCTGG–··TG·CCCACC·TCTTTC–

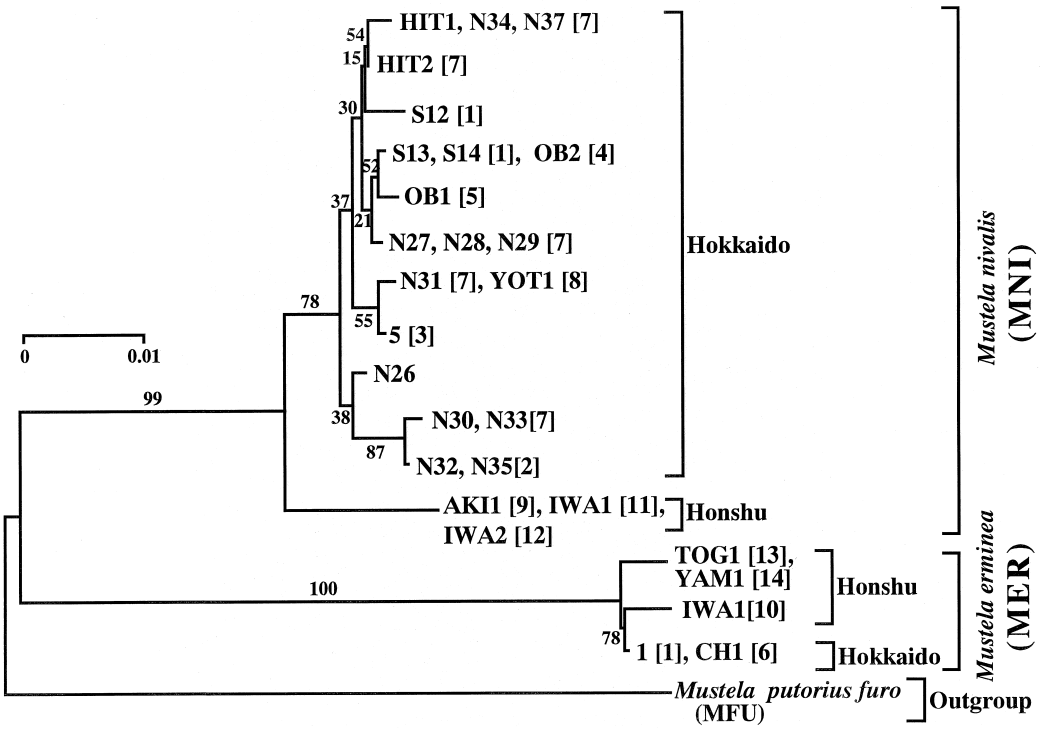

Fig. 3. A neighbor-joining tree reconstructed for *Mustela nivalis* (MNI), *M. erminea* (MER), and *M. putorius furo* (MFU, outgroup) on the basis of the 5' variable portion of the control region sequences (581-584 bases). The scale indicates genetic distance estimated with Kimura's (1980) two parameter method, eliminating indel sites. Numbers (%) on internal branches are bootstrap values derived from 1,000 replications. Sample names with locality numbers in brackets refer to those in Table 1.

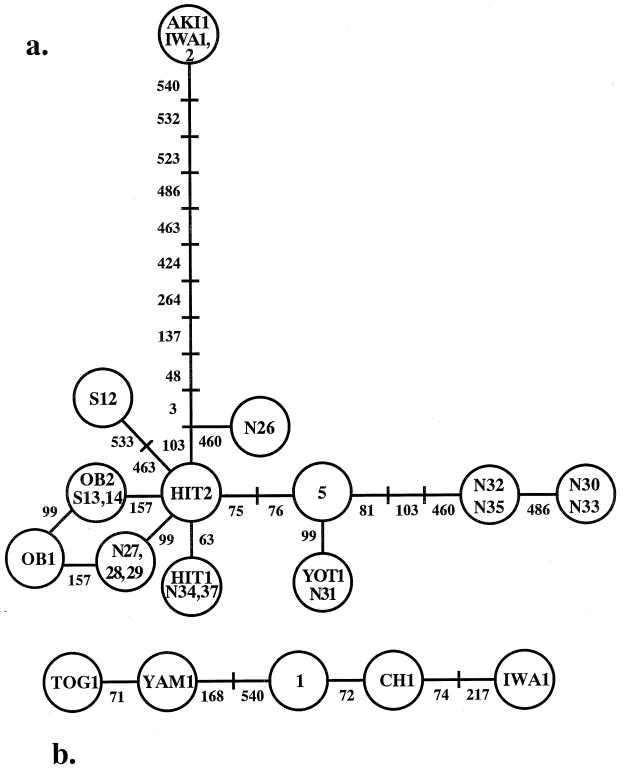

Fig. 4. Hand-drawn parsimonious networks of haplotypes for *Mustela nivalis* (a) and *M. erminea* (b). One slash indicates a presumed sequence. One number above the line shows a site of nucleotide substitution.

1.91% to 2.43% (2.14% in average). From 20 individuals in the Hokkaido population, 11 haplotypes were identified. The neighbor-joining tree (Fig. 3) indicated that these 11 haplotypes formed one cluster with a 78% bootstrap value, while one common haplotype obtained from the Honshu population split basally from the cluster of the Hokkaido population. The parsimonious network (Fig. 4a) also exhibited a relatively large, discontinuous divergence between the Hokkaido and Honshu populations.

In Hokkaido, five of the 11 haplotypes were located at one sampling locality No. 7 (Sapporo) (Fig. 1). Meanwhile, some haplotypes were specific to restricted areas: for example, MNI-S13 (same as MNI-S14 and -OB2), -S12, -OB1, and -N32 (same as MNI-N35) were specific to eastern parts of Hokkaido. However, the phylogenetically closer haplotypes were not always located at geographically closer areas: for instance, one haplotype from Sapporo (MNI-N30 and -N33, locality No. 7 in Fig. 1) was closely related (87% bootstrap value) to that from Shibecha (MNI-N32 and -N35, locality No. 2 in Fig. 1), which is far away from Sapporo (Figs. 3 and 4a).

Sequence variation in *M. erminea*

Five animals examined here had sequences different from each other (Table 2). Among 581 bases (excluding one indel), six sites were variable and all substitutions were transitions (Table 2). In the neighbor-joining tree (Fig. 3) eliminating the single indel site, only two lowest branches were expressed for three haplotypes of Honshu and one for two haplotypes of

Hokkaido because of the occurrence of additional indels between *M. erminea* and *M. putorius furo* as outgroup. Intraspecific sequence differences of *M. erminea* varied from 0.35% to 0.69%. Both the neighbor-joining tree (Fig. 3) and the parsimonious network (Fig. 4b) clearly showed the smaller genetic differentiation of *M. erminea* between Hokkaido and Honshu, compared with that of *M. nivalis*. Especially, the parsimonious network (Fig. 4b) demonstrated the linear relationships, showing the intermediate position of the two Hokkaido haplotypes (MER-1 and -CH1) between MER-IWA1 and the other Honshu haplotypes (MER-TOG1 and -YAM1).

DISCUSSION

The present study revealed that *Mustela nivalis* and *M. erminea* share a repetitive sequence portion comprising 10-base motifs in the mtDNA control region (Fig. 2). In homologous regions of two other *Mustela* species, *M. itatsi* and *M. sibirica*, we identified the similar repetitive sequences (unpublished data). These findings suggest that such repetitive sequences commonly occur in the control region of the genus *Mustela*. Hoelzel *et al*. (1994) reported that other carnivores, such as the American mink *Mustela vison*, the domestic dog *Canis familialis*, the lion *Panthera leo* and the brown bear *Ursus arctos*, also share similar heteroplasmic repetitive sequences in the control region. The repetitive sequences of the control region could have occurred prior to divergence of Carnivora. The difficulty in sequencing the whole repetitive region suggests the heteroplasmic situation in the cell and/or DNA replication slippage of PCR caused in this repeated region.

The 5' sequence adjoining to the repetitive region was more informative for phylogenetic analysis (Fig. 2). Interestingly, the genetic variation between the Hokkaido and Honshu populations was much larger in *M. nivalis* than in *M. erminea* (Figs. 3 and 4). Obara (1991) reported a karyotypic differentiation between the Hokkaido (2n=42) and the Honshu populations (2n=38) of *M. nivalis*. In addition, Masuda and Yoshida (1994a, b) reported that the difference of the mitochondrial cytochrome *b* sequence between these two populations of *M. nivalis* largely corresponds to those between different subspecies in other Japanese mustelids. The present results further confirm the occurrence of the Hokkaido-Honshu divergence in this species.

Vigilant *et al*. (1989) estimated the evolutionary rate of the homologous human control region as approximately 8.4% per My. With the application of this coefficient to our results, the divergence time between the Honshu and Hokkaido populations of *M. nivalis* could be estimated as around 0.3 My before present. The cytochrome *b* sequence difference (0.8%) between the two populations, reported by Masuda and Yoshida (1994a), also refers to about 0.3 My on the basis of the temporary rate of sequence divergence generally assumed for the mammalian cytochrome *b* (Mayer *et al*., 1990). The two estimates are identical to each other. The Tsugaru strait, which currently separates Honshu from Hokkaido (see Fig. 1), is considered to have been formed around 0.15 My before present (Ohshima, 1991). Thus, both the present results and the result of Masuda and Yoshida (1994a) suggest that the two populations of *M. nivalis* were isolated prior to formation of the Tsugaru strait.

Within the Hokkaido population of *M. nivalis*, 11 haplotypes were identified. Five of them were found at Sapporo (locality No. 7), and the others (except MNI-N26) were out of Sapporo (locality Nos. 1–5, and 8) (Tables 1, 2). Relative phylogenetic affinities between the haplotypes (Figs. 3 and 4a) did not correlate with geographic distances between sampling localities. Our investigation by the maximum likelihood method also did not show clear geographic structure (data not shown), yielding results similar to those by the neighbor-joining and parsimonious analyses in the present study. By contrast, Hokkaido populations of other mammals including the sika deer *Cervus nippon* (Nagata *et al*., 1998) and the brown bear *Ursus arctos* (Matsuhashi *et al*., 1999) exhibited some clear phylogeographic pattern of mtDNA control region haplotypes. Such a haplotype distribution in the Hokkaido population of *M. nivalis* might be explained as follows. The Japanese islands experienced several glacial and inter-gracial periods in the Quaternary, and the Soya strait (see Fig. 1), isolating Hokkaido from Sakhalin and Siberia, was formed around 12,000 years ago (Ohshima, 1991). By the last glacial age, *M. nivalis* in Hokkaido could have suffered from substantial changes of environments such as temperature and vegetation, and consequently their distribution areas might have been reduced and expanded repeatedly. Such repetitious habitat changes could impede fixation of mtDNA haplotypes into particular local populations under the operation of bottleneck effects. Another explanation is that *M. nivalis* could have immigrated from the continent into Hokkaido so recently as to let the current Hokkaido population retain the genetic variation possessed by the original immigrant population. This might be applicable to the genetic diversity found in animals from Sapporo (locality No. 7). Based on our data of the control region, the divergence time among haplotypes identified within the Hokkaido population was estimated as less than 0.2 My.

By contrast, genetic distance between the Hokkaido and Honshu populations of *M. erminea* was parallel with the variation within the Hokkaido population of *M. nivalis* (Fig. 3). The divergence between the two populations of *M. erminea* could have occurred more recently compared with that of *M. nivalis*, probably when the Tsugaru strait was formed. For more convincing explanations, however, further studies of variations in *M. erminea* on the basis of local samples are required. Also, future comparative study together with the continental populations may illustrate the migration history of the two dwarf mustelids into the Japanese islands, as well as the geographic relationships between Japan and the continent in glacial periods.

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