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ZOOLOGICAL SCIENCE **16**: 693–700 (1999) © 1999 Zoological Society of Japan

Intraspecific Variation of Mitochondrial Cytochrome *b* Gene Sequences of the Japanese Marten *Martes melampus* and the Sable *Martes zibellina* (Mustelidae, Carnivora, Mammalia) in Japan

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ABSTRACT—To assess genetic variations of two Japanese species of the genus *Martes*, the Japanese marten *M. melampus* and the sable *M. zibellina*, the whole regions (1,140 base pairs) of the mitochondrial cytochrome *b* gene were sequenced. Intraspecific variable sites were different between these two species, and most substitutions were transitions resulting in synonymous mutations. Molecular phylogenetic trees exhibited genetic differentiation between the two species. Genetic variations among *M. melampus* from Honshu, Shikoku, and Kyushu were larger than those among *M. zibellina* from Hokkaido. Genetic distance between cytochrome *b* haplotypes did not correlate to geographic distance between sampling localities. This result suggests the introgression of mitochondrial DNA haplotypes between local populations, probably resulting from incomplete geographic isolation, and/or their recent expansion on each island during a short period.

INTRODUCTION

The Japanese marten *Martes melampus*, occurring in Honshu, Kyushu, and Shikoku, is an indigenous mammalian species, which is thought to have speciated after geographic isolation on the Japanese islands from the Asiatic continent. On the other hand, the congeneric species, the sable *M. zibellina*, is widely distributed in Siberia, Sakhalin, and Hokkaido. Distribution of these two species on the Japanese islands is divided by the Tsugaru strait located between Hokkaido and Honshu (Fig. 1). The same distributional pattern (Hokkaido – Honshu) over the Tsugaru strait is known on some related mammalian species: the brown bear *Ursus arctos* — the Asiatic black bear *Selenarctos thibetanus*; the Eurasian red squirrel *Sciurus vulgaris* — the Japanese squirrel *S. lis*; the Eurasian flying squirrel *Pteromys volans* — the Japanese small flying squirrel *P. momonga* (Abe *et al*., 1994). Therefore, this strait is considered as an important demarcation, the Blakiston's line. Phylogenetic relationships among these animals provide a deep insight to understand the origin of Japanese mammalian fauna. However, phylogenetics of these animals including the two *Martes* species is not revealed, with some exceptions (Masuda and Yoshida, 1994a; Oshida *et al*., 1996; Ohdachi *et al*., 1997).

The first appearance of the two *Martes* species was recorded from the late Pleistocene deposits (Anderson, 1970). Anderson (1970; 1994) proposed that *M. melampus* have descended from *M. zibellina* on southern parts of the Japanese islands, based on morphological data. Masuda and Yoshida (1994a) investigated partial sequences of the mitochondrial DNA (mtDNA) cytochrome *b* on all Japanese species of the family Mustelidae, and they found a close genetic relationship between *M. melampus* and *M. zibellina*. Genetic distance between the two species almost corresponded to interspecific differences among other mustelid species (Masuda and Yoshida, 1994a).

The Tsushima Island population of *M. melampus* is classified as an endemic subspecies *M. m. tsuensis* Thomas, 1897, based on morphological characters. Meanwhile, the Honshu population is sometimes divided into two subspecies: *M. m. melampus* with a yellowish coat color in the winter and *M. m. bedfordi* with a relatively dark brown coat color in the winter (Thomas, 1905; Imaizumi, 1960). The former is mainly dis-

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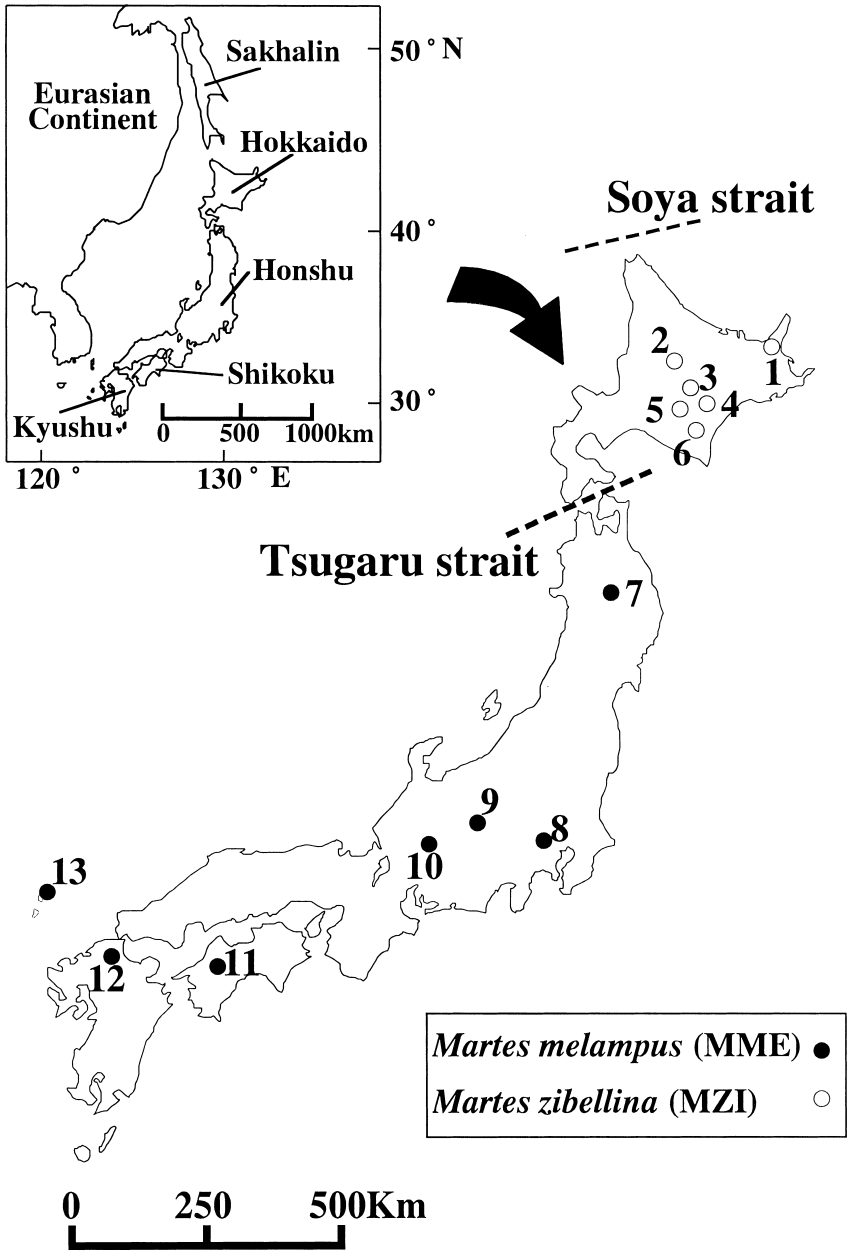

Fig. 1. Sampling localities of *M. melampus* (MME) and *M. zibellina* (MZI). Numbers on the Japanese islands refer to those in Table 1.

tributed in northern Honshu, while the latter lives in southern Honshu and Shikoku (Thomas, 1905).

In the present study, to further investigate the genetic characteristics of the two species of *Martes*, we sequenced the whole region (1,140 base pairs:bp) of the cytochrome *b* gene. Based on the mtDNA data, we discuss phylogeographic variations and population structure of these species of the Japanese islands.

MATERIALS AND METHODS

Samples and DNA extraction

Martes melampus and *M. zibellina* examined in the present study were listed in Table 1 and Fig. 1. The yellow-throated marten *M. flavigula* distributed widely in eastern and southern Asia was used as outgroup. Muscle tissue was frozen at –80°C or preserved in 70% ethanol at room temperature until use. Fibroblasts were obtained from a primary skin culture. Total DNAs were extracted from muscles and fibroblasts due to the phenol/proteinase K/sodium dodecyl sulfate (SDS) method of Sambrook *et al.* (1989) with some simplified modifications as indicated by Masuda and Yoshida (1994a; 1994b). DNA from hair samples were extracted by the following method. Hair roots (approximately 5 mm) were washed with 70% ethanol, incubated in 5% Chelex-100 (Bio-Rad) at 56°C overnight, and then boiled for 8 min (Walsh *et al.*, 1991). The supernatant of 10 μl was used as template of subsequent polymerase chain reaction (PCR) amplification.

PCR amplification and direct sequencing

The whole cytochrome *b* region (1,140 bp) was amplified using the two primers: Cb-1N 5'-GATATGAAAAACCATCGTTG-3' (Masuda

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Table. 1. Profiles of Japanese martens *Martes melampus*, sables *M. zibellina*, and yellow-throated martens *M. flavigula* (outgroup) examined in the present study

species	Code	Tissue	Sampling locality if known	Locality Number[1)]	Accession Number[2)]
Martes melampus	MME-1	muscle	Morioka-city, Iwate	7	AB012351
	MME-MR1	skin	Morioka-city, Iwate	7	AB012343
	MME-TKY1	hair	Hinode-town, Tokyo	8	AB012341
	MME-TKY2	hair	Hinode-town, Tokyo	8	same as MME-TKY1
	MME-TKY3	hair	Hinode-town, Tokyo	8	AB012346
	MME-TKY4	muscle	Hinode-town, Tokyo	8	AB012348
	MME-C1	muscle	Nagano	9	AB012345
	MME-OS1	muscle	Kitaazumi-gun, Nagano	9	AB012347
	MME-TOG1	muscle	Togakushi-village, Nagano	9	AB012352
	MME-G1	muscle	Kamioka-town, Gifu	10	AB012355
	MME-G5	muscle	Ohno-gun, Gifu	10	AB012354
	MME-TOB1	hair	Hirota-village, Ehime	11	AB012353
	MME-K1	muscle	Kitakyushu-city, Fukuoka [920502][3)]	12	AB012342
	MME-K2	muscle	Kitakyushu-city, Fukuoka [930911][3)]	12	AB012344
	MME-K3	muscle	Ohita [931018–4][3)]	12	AB012349
	MME-TSU1	muscle	Tsushima Island, Nagasaki	13	AB012350
	MME-TSU2	muscle	Tsushima Island, Nagasaki	13	same as MME-TSU1
	MME-TSU3	muscle	Tsushima Island, Nagasaki	13	same as MME-TSU1
Martes zibellina	MZI-1	muscle	Shari-town, Hokkaido	1	AB012360
	MZI-2	muscle	Shari-town, Hokkaido	1	same as MZI-1
	MZI-A2	fibroblast	Ahahikawa-city, Hokkaido	2	AB012359
	MZI-HIG1	muscle	Hokkaido	–	same as MZI-A2
	MZI-HIG2	muscle	Kamishihoro-town, Hokkaido	3	same as MZI-A2
	MZI-CH1	muscle	Taiki-town, Hokkaido	6	AB012361
	MZI-CH2	muscle	Taiki-town, Hokkaido	6	same as MZI-1
	MZI-CH3	muscle	Taiki-town, Hokkaido	6	AB012357
	MZI-CH4	muscle	Memuro-town, Hokkaido	5	AB012358
	MZI-CH6	muscle	Obihiro-city, Hokkaido	4	AB012356
Martes flavigula	MFL-DUZ1	Hair	Duzit Zoo, Thailand	–	AB012362
	MFL-CHI1	Hair	Chiang Mai Zoo, Thailand	–	AB012363

[1)] Each locality number refers to that in Fig. 1.
[2)] The nucleotide sequence data reported in the present study will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with these accession numbers.
[3)] Specimen Nos. of the Kitakyushu Museum of Natural History.

and Yoshida, 1994a); Cb-Y3 5'-ACCTCTTCCTTGAGTCTTAGG-3' which was newly designed in the present study (Fig. 2). PCR amplifications were performed in 50 μl reaction volumes. In cases where PCR was inhibited for some reason, 20 μg of bovine serum albumin (Boehringer) was added into the reaction mixture. 35 cycles were performed with the following programs using a DNA thermal cycler (PJ2000, Perkin-Elmer Cetus): denaturing 94°C for 1 min; annealing 50°C for 1 min; extension 72°C for 2 min, and then the reaction was completed at 72°C for 10 min. To check PCR amplification, 10 μl of the PCR product was electrophoresed on a 2% agarose gel, stained by ethidium bromide, and visualized under an ultraviolet illuminator. The remaining 40 μl of each PCR product was purified with QIAquick (QIAGEN).

Purified PCR products were labeled using the cycle labeling system Catalyst (Perkin-Elmer Cetus) and sequenced using the ABI Prism™ 377 automated sequencer. Sequencing primers were the same as PCR primers and an internal primer Cb-NY 5'-GGTGCAACCGTAATTACCAAC-3', which was newly designed in the present study (Fig. 2).

Sequence analysis

Sequence alignment was done using GeneWorks (Intelligenetics). The neighbor-joining tree (Saitou and Nei, 1987) using Kimura's two-parameter distance (Kimura, 1980) were constructed by Mega (Kumar *et al.*, 1993). The minimum path networks were summarized to construct a parsimonious network of phylogenetic relationships between the haplotypes.

RESULTS

Interspecific variation of the cytochrome *b* sequences

The neighbor-joining tree (Fig. 3) showed that *M. melampus* and *M. zibellina* were clearly separated into two different groups (85% and 100% bootstrap values, respectively). We then investigated substitution frequencies in every 100 bp segment of the whole region between *M. melampus* and *M. zibellina*, as well as within each species (Fig. 2). Variable regions, where there were more than 10 substitutions between the two species, appeared at three segments: the nucleotide numbers (nt) 400 to 500, nt 500 to 600, and nt 900 to 1,000 (Fig. 2). *M. melampus* shared one to five substitutions throughout the whole region, while one or two substitutions within *M. zibellina* occurred at some limited segments: nt 1 to 300; nt 400 to 500; and nt 900 to 1,000 (Fig. 2).

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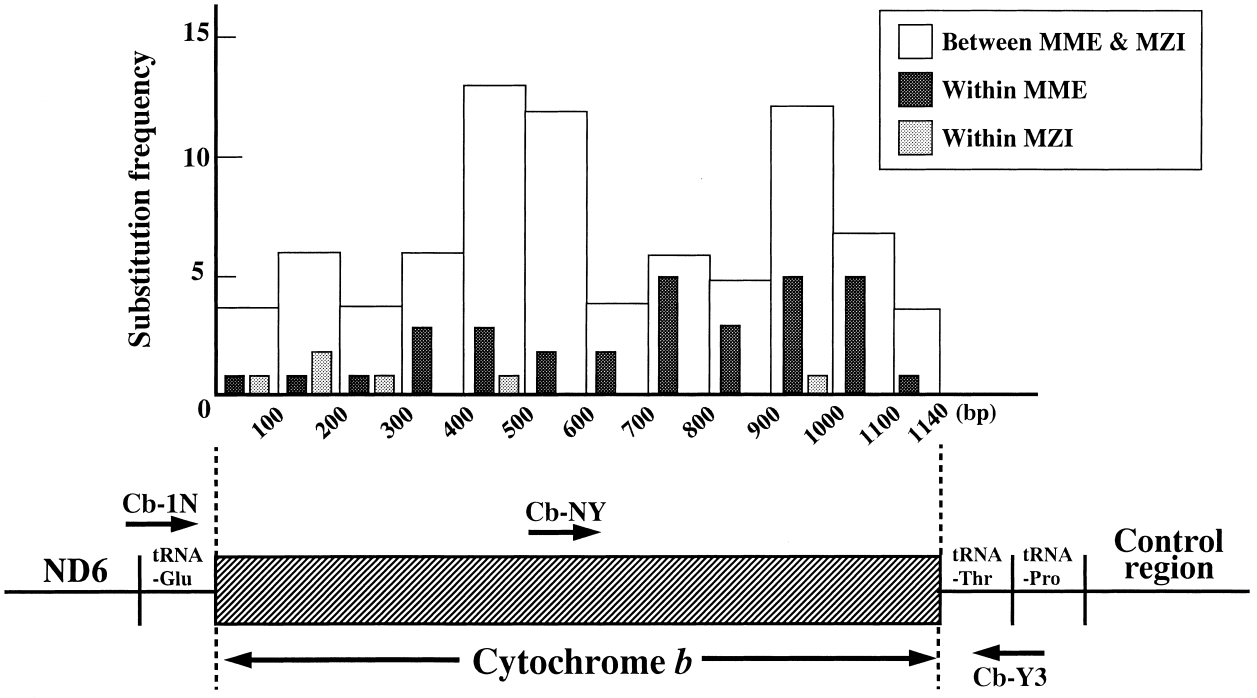

Fig. 2. Schematic diagram of cytochrome *b* gene of *M. melampus* (MME) and *M. zibellina* (MZI). Above: the histogram indicates variable site numbers in every 100 bp fragment of inter- and intra-species. Below: large arrows (Cb-1N, Cb-NY, and Cb-Y3) show the primer positions used for PCR amplification and sequencing.

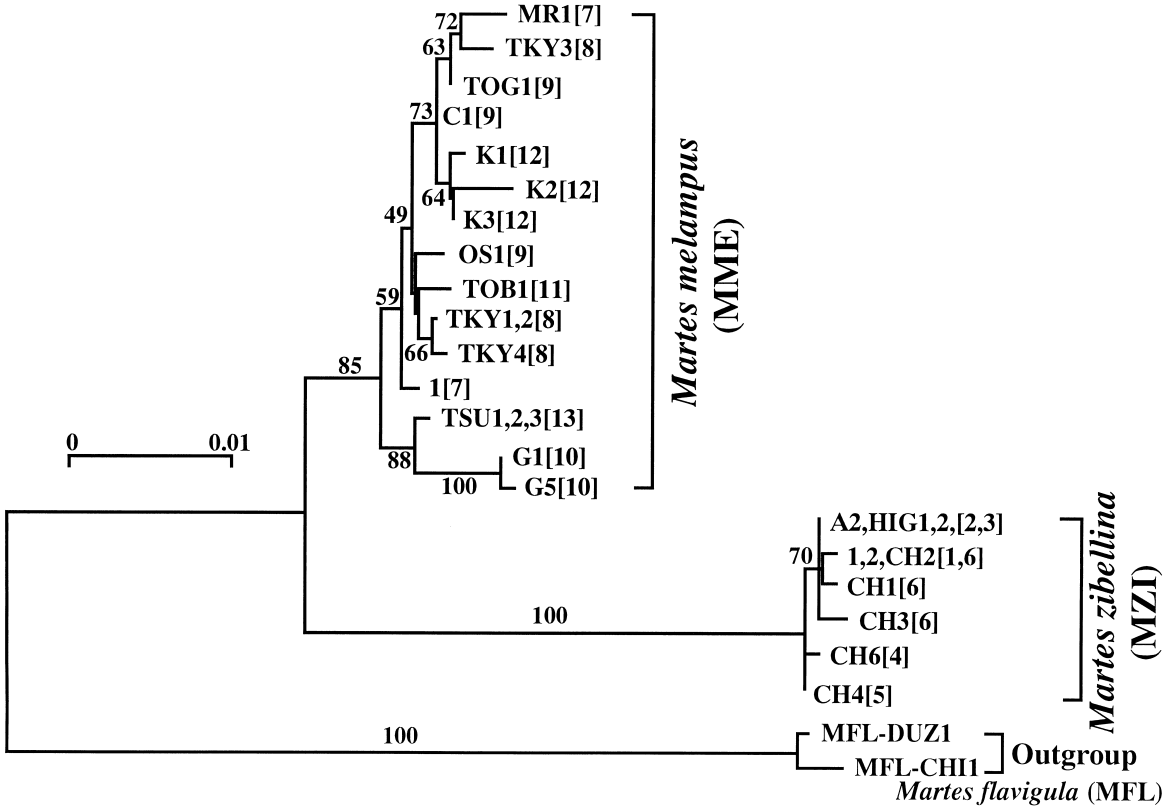

Fig. 3. A neighbor-joining tree reconstructed by the cytochrome *b* nucleotide sequences (1,140 bp) for *M. melampus* (MME), *M. zibellina* (MZI), and *M. flavigula* (MFL, outgroup). The bar indicates genetic distance estimated with Kimura's two parameter method (Kimura, 1980). Numbers (%) on internal branches are bootstrap values derived from 1,000 replications. Haplotype names with locality numbers in brackets refer to those in Table 1.

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Table 2. Sequence variation of the whole cytochrome *b* gene (1,140 bp) of Japanese martens *Martes melampus* (MME) and sables *M. zibellina* (MZI)

	Variable site
Haplotype*	1111111111111111112222333333344444444455555555666677777788888999999999990000000011113699122569233400467900445777455556681348145689112230111226778893345689012232361037558173348939287831479258495508844098239584305647605240187350826 26
MME-MR1	TTACTCAATA AACCCCCTGA AAACAGTTTT GCCCACACCG CGAGAGCGCC AAGCTTACCT GCAATGGAAA TCT
MME-1	·····G·····C·····T·T·A···A····A·····A····C·····
MME-TKY1, TKY2	·····T···G·····C·····T·T·····A·····A····C·····
MME-TKY3	·····G·····C·····T·····G··C·····
MME-TKY4	·····T···G·····C·····T·T·····A·····A·····
MME-C1	·····G·····C·····A···A····C·····
MME-OS1	·····G·····CA·····T·T·····A·····AA···C·····
MME-TOG1	·····G·····C·····A·····C·····
MME-G1	··G·C·····C·····G·G·····CC·····TAT··AG·····GA··C···A····C·····
MME-G5	··G·C·····C·····G·G····C·CC·····TAT··AG·····GA··C···A····C·····
MME-TOB1	·····G·····C·····TT·T·····A·····A····C····C··
MME-K1	·····G·····C·····A·····A··G·CA·····
MME-K2	·····AGG·····C·····G···A·····A··G·C·G·····
MME-K3	·····G·····C·····A·····A··G·C·····
MME-TSU1,TSU2, TSU3	·····C·····G·G·····C·····T·T··A·T····GA·····A····C·····
MZI-1, 2, CH2, CH5	C··T·TG·CGCGTTTTTC·G·GG·GAC·CC·ATTGTG·T··A·A·A···TGGATCGTTCA···CCA·GG·TC
MZI-A2, HIG1, HIG2	C··T·TG·CGCGTTTTTC·G·GG·GAC·CC·ATTGTG·T··A·A·A···TGGATC··TTCA···CCA·GG·TC
MZ1-CH1	CG·T·TG·CGCGTTTTTC·G·GG·GAC·CC·ATTGTG·T··A·A·A···TGGATC··TTCA···CCA·GG·TC
MZ1-CH3	C··TCTGTCGCGTTTTTC·G·GG·GAC·CC·ATTGTG·T··A·A·A···TGGATC··TTCA···CCA·GG·TC
MZI-CH4	C··T·TG·CGCGTTTTTC·G·GG·GAC·CC·ATTGTG·T··A·A·A···TGGATC··TTCA···CCA·GG·TC
MZI-CH6	C··T·TG·CGCGTTTTTC·G·GG·GAC·CC·ATTGTG·T··A·A·A···TGGATC··TTCA···CCA·GG·TC

Dots indicate identical nucleotides with those of MME-MR1. Sequences for yellow-throated martens *M. flavigula* as outgroup are not included in this alignment.

*Sample numbers refer to those in Table 1.

Sequence variation of *M. melampus*

Thirty-two sites among 18 individuals of *M. melampus* were variable in the cytochrome *b* sequences (Table 2). Most nucleotide substitutions were transitions resulting in synonymous mutations. Transversions were observed at six sites (Table 2). Intraspecific differences were less than 1.58%, and 15 haplotypes were identified. Three individuals (MME-TSU1, -TSU2, and -TSU3) from Tsushima Island shared an identical sequence (Table 2).

The neighbor-joining tree (Fig. 3) did not show tight relationships between haplotype clustering and sampling localities. For example, four individuals (TKY1-4) from Tokyo, two (1, MR1) from Iwate, three (TOG1, C1, OS1) from Nagano did not form any cluster showing each local specificity, but some animals from those localities were more closely related to animals from northern Kyushu or Shikoku (Fig. 3). In addition, the Tsushima Island population (MME-TSU1, -TSU2, and -TSU3) was clustered with the Gifu population (MME-G1 and -G5) of Honshu with an 88% bootstrap value. The parsimonious network (Fig. 4a) also exhibited a very similar clustering.

Sequence variation of *M. zibellina*

Among 11 individuals of *M. zibellina*, six nucleotide sites were variable, where three sites were transitions and the others were transversions, and six haplotypes were identified (Table 2). Intraspecific differences were less than 0.35%. These values were smaller than those (less than 1.58%) of *M. melampus* as described above.

Phylogenetic trees (Figs. 3 and 4b) showed that no clear geographic structure of haplotype distribution nor clustering of local specific haplotypes was inferred in Hokkaido.

DISCUSSION

Genetic differentiation in *Martes* in Japan

The present study revealed intraspecific variations of the cytochrome *b* sequences of two *Martes* species on the Japanese islands. Nucleotide substitutions in *M. melampus* occurred throughout the whole cytochrome *b* region, whereas *M. zibellina* shared substitutions at some confined regions (Fig. 2). Three peaks of relatively higher substitution frequencies (more than 10) observed between the two species almost referred to variable sites along mammalian cytochrome *b* genes previously reported by Irwin *et al.* (1991): nt 400–500 and nt 500–600 to one part of the Redox center and nt 900–1000 to one transmembrane domain (Fig. 2). These results generally support nonrandom distribution of synonymous substitutions throughout this gene region (Irwin *et al.*, 1991). However, some substitutions are likely selected at some limited sites although the selection mechanism is still unknown.

Evolutionary history of *M. melampus*

Anderson (1970, 1994) postulated that the genus *Martes* reached the Japanese islands during the Pleistocene through the Kuril Islands from Kamtchatka and also from Sakhalin. Then, it spread to Honshu, Shikoku, and Kyushu, evolving to

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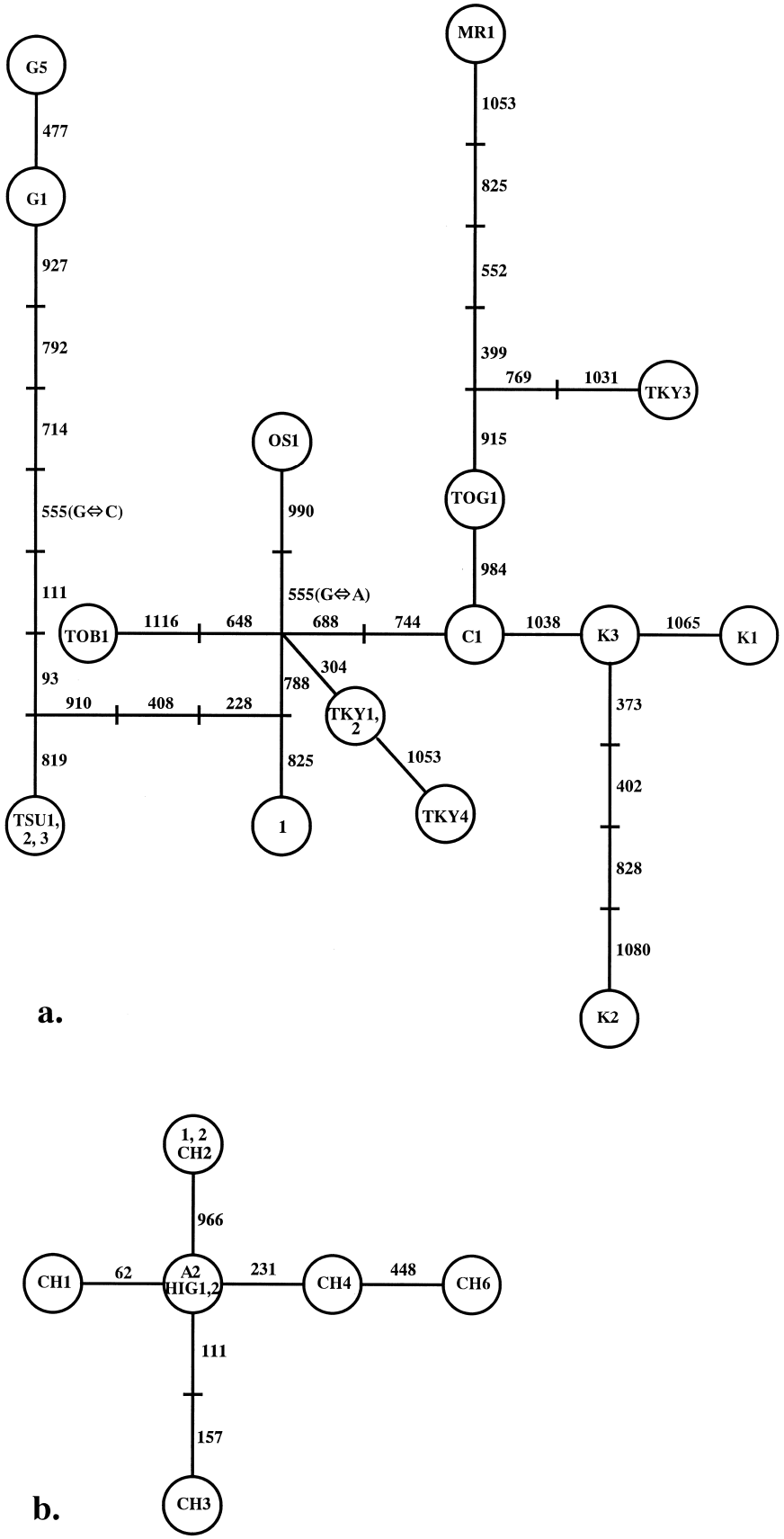

Fig. 4. Hand-drawn parsimonious networks of haplotypes for *M. melampus* (**a**) and *M. zibellina* (**b**). One slash indicates a presumed sequence. One number above the line shows a site of nucleotide substitution.

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M. melampus, and finally reached the Korean Peninsula, although distribution in Korea is uncertain so far. At present, distribution of these two species on the Japanese islands is separated by the Tsugaru strait (the Blakiston's line) between Hokkaido and Honshu. This geographic barrier could have contributed to the evolution of *M. melampus* and *M. zibellina*, if the Anderson's postulate (1970) is correct.

Intraspecific percentage differences in the cytochrome *b* genes of other carnivores were reported as follows: 0.8% for *Mustela nivalis* (Masuda and Yoshida, 1994a), 0.8% for *Mustela itatsi* (Masuda and Yoshida, 1994b), and 0.3–0.5% for *Canis lupus* (Wayne and Jenks, 1991). These values are similar to our data of intraspecific difference (less than 1.58%) of *M. melampus*.

Meanwhile, the clustering of haplotypes in phylogenetic trees (Figs. 3 and 4a) did not correspond with the geographically expected relationships between populations. Our results suggest that mtDNA introgression between local populations might have resulted from the incomplete geographic isolation within each island, and/or that they might have recently expanded to the Japanese islands during a short period. Another possibility is that the sequences might be of pseudogenes in nuclear genome. MtDNA-like sequences integrated into nuclear genome generally show different evolutionary rates from cytoplasmic mtDNA (Lopez *et al*., 1997; Zischler *et al*., 1998). All cytochrome *b* sequences determined in the present study, however, shared the stop codon [AGA] at the 3' terminal and no nucleotide deletions nor insertions, compared with the same genes (1,140 bp) of other mammals reported by Irwin *et al.* (1991). Moreover, coexistence of different nucleotides at each site was not observed in the sequencing of the present study. Thus, the discordance between sampling localities and phylogenetic relationships is not due to detection of nuclear copies of mtDNA. Our sequences are guaranteed to be for the cytoplasmic mtDNA. In the past, the fur industry used *M. melampus* and some animals were likely translocated to different areas in Japan (Inukai, 1975), although detailed information has not been recorded. In fact, *M. melampus* has recently been captured in Hokkaido (Dr. I. Ogawa, personal communication). Thus, they could have escaped from farms and been naturalized in introduced localities. This problem might cause the genetic results found in the present study. We need a further survey on history and genetic variation of this species.

The Honshu populations of *M. melampus* are sometimes divided into two forms: *M. m. melampus* and *M. m. bedfordi* based on winter coat colors (Thomas, 1905; Imaizumi, 1960). But there are no differences of restriction fragment length polymorphisms of the 28S ribosomal DNA between these two forms (Hosoda and Oshima, 1993). Although animals examined in the present study were not distinguished by the coat colors, the sequence variations from the three main islands (Honshu, Kyushu, and Shikoku) revealed no clear geographic structure between islands and within the island.

Interestingly, the Tsushima Island population, classified as *M. m. tsuensis*, was closer to the Gifu population (Fig. 4a), supported by 88% bootstrap value (Fig. 3). Moreover, our investigation by the maximum likelihood method (data not shown) on these sequences showed a topology similar to the neighbor-joining (Fig. 3) and the parsimonious network (Fig. 4a). By contrast, Nagata *et al*. (1995) investigated the cytochrome *b* gene of the Japanese sika deer *Cervus nippon*, and found that the Tsushima Island population was distantly related to the Hokkaido and Honshu populations. These findings tell us that *M. melampus* might have an immigration time different from that of the sika deer.

Evolutionary history of *M. zibellina*

Genetic variation among the Hokkaido population, which is classified as one separate subspecies *M. zibellina brachyura* Temminck, 1844, was much smaller than that within *M. melampus*. The smaller sample size of *M. zibellina* might be responsible for this result. No clear geographic structure in Hokkaido was inferred from the mtDNA variations obtained in the present study. Currently, we investigated mtDNA control region sequences of the Hokkaido population of the least weasel *Mustela nivalis*, and found no clear geographic structure also in that population (Kurose *et al*., 1999). Hokkaido is thought to be a refugium for *Martes zibellina* and *Mustela nivalis* in the last glacial age of the Quaternary. Our results suggest that populations of these mustelid species could have expanded in Hokkaido recently during a short term. Otherwise, the repeat of spread and reduction of their habitats through the glacial and interglacial ages might have impeded the fixation of haplotypes to local populations. To examine genetic variation within each local population, further study is required by using more specimens from comprehensive areas including the continent and polymorphic nuclear DNA markers such as microsatellites.

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