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Authors: Kurose, Naoko, Masuda, Ryuichi, Siriaroonrat, Boripat, and Yoshida, Michihiro C.

Source: Zoological Science, 16(4) : 693-700

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.16.693
Intraspecific Variation of Mitochondrial Cytochrome b Gene Sequences of the Japanese Marten *Martes melampus* and the Sable *Martes zibellina* (Mustelidae, Carnivora, Mammalia) in Japan

Naoko Kurose¹, Ryuichi Masuda¹,²*, Boripat Siriaroonrat³, and Michihiro C. Yoshida¹,²

1Cytogenetics Laboratory, Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University,
2Chromosome Research Unit, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan and
3Institute of Science and Technology for Research and Development, Mahidol University Salaya, Nakornpathom 73170, Thailand

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-
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...
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′-GGTGCAACGTAATTCACAC-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).
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.
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) from Tsushima Island shared an identical sequence (Table 2). The present study revealed intraspecific variations of the cytochrome *b* gene (1,140 bp) of Japanese martens *Martes melampus* (MME) and sables *M. zibellina* (MZI).

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...
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.
In fact, although detailed information has not been recorded, if the Anderson's postulate (1970) is correct, integrated into nuclear genome generally show different evolutionary rates from mtDNA-like sequences in nuclear genome. MtDNA-like sequences are guaranteed to be for the cytoplasmic mtDNA. MtDNA-like sequences are not due to detection of nuclear copies of mtDNA. 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; Imaiizumi, 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.

Acknowledgments

We thank V. Arsaithamkul (Duzit Zoo, Thailand), M. Baba (Kitakyushu Natural Museum of History), S. Dakemoto, E. Kanda (Tokyo Wildlife Research Center), Y. Kaneko (Tokyo University of Agriculture and Technology), Y. Masuda (Shiretoko Museum), Y. Miyaura (Tobe Zoo), Y. Mouri (Tobe Zoo), Y. Okada (Tsushima Branch Office of Nagasaki Prefectural Government), K. Saenwong (Chiang Mai Zoo, Thailand), F. Sekiyama (Iwate Prefectural Museum), H. Shimizu (Ohmachi Alpine Museum), T. Tsujimoto (Morioka Zoo), H. Yanagawa (Obihiro University of Agriculture and Veterinary Medicine), for supplying specimens. We are grateful to the Institute of Low Temperature Science of Hokkaido University for technical support. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan, and by the 2nd Toyota High-tech Research Grant Program to R. M.

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(Received June 1, 1998 / Accepted April 28, 1999)