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FULL PAPER

Mitochondrial DNA variation in the Japanese marten *Martes melampus* and Japanese sable, *Martes zibellina*

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Abstract

Genetic relationship among Japanese sables, *Martes zibellina* and the introduced Japanese martens, *Martes melampus* in northern Japan was revealed by analyzing a 521-524bp DNA sequence from the cytochrome *b* (112bp) /transfer RNA-threonine (67bp)/tRNA-proline (65bp) and control region (277-280bp) of the mitochondrial genome. Intraspecific differences in sequences of *M. zibellina* and *M. melampus* (3.8-15.0% and 1.9-16.4%, respectively) were similar to interspecific differences between these two species (5.8-16.6%). Comparison of sequence data exhibited five haplotypes of *M. melampus* and four haplotypes of *M. zibellina*, which clustered into two groups (clusters-A and-B). Cluster-A included two haplotypes of *M. melampus* and two haplotypes of *M. zibellina* , whereas cluster-B included three haplotypes of *M. melampus* and two haplotypes of *M. zibellina*. Results of this study lead three possible explanations. Firstly, past hybridization between *M. zibellina* and *M. melampus* might have occurred. Secondary, these two species might have similar heteroplasmy of mt DNA. Thirdly, these haplotypes might have come from nuclear genome. Although further intensive studies are needed to make a conclusion, detection of hybridization with the Japanese marten are occurred or not is quite important to conserve the Japanese sable.

Keywords : *Martes zibellina, Martes melampus,* genetic variation, mitochondrial DNA, hybridization, heteroplasmy

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Introduction

On Hokkaido, in northernmost Japan, there are two species of the genus *Martes, M. melampus* and *M. zibellina. Martes melampus* is thought to have been introduced in Hokkaido island in the 1940s[8] and the current distribution includes southwestern and central Hokkaido[13]. *M. zibellina* currently is distributed on central, eastern, and northern Hokkaido[13], although originally they were widespread over Hokkaido[7]. Currently, these two species coexists around contact zone shown on Fig. 1.

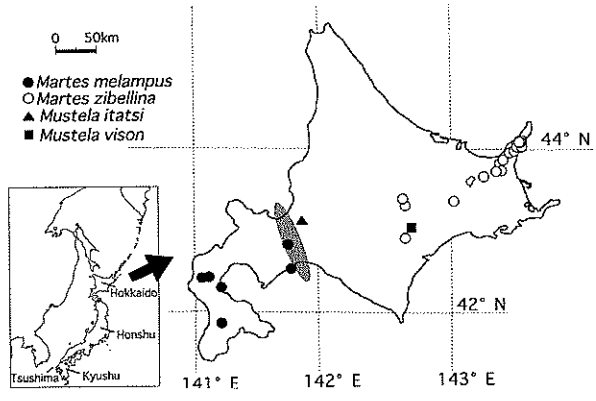


Fig. 1. Map showing sample localities of the Japanese sable *Marts zibellina*, the Japanese marten *M. melampus*, and outgroups (the Japanese weasel *Mustela itatsi* and the mink *Mustela vison*). Shaded area indicates the contact zone of *Marts zibellina and M. melampus* (estimated from Murakami and Ohtaishi[13]).

Anderson[2] reported that *M. melampus* and *M. zibellina* were closely related based on their morphological similarities. Although several studies using genetic data, supported Anderson[2]'s conclusion, they are considered to be distinct species[12]. In a comprehensive analysis of several species of mustelids based on 375bp of the cytochrome *b* gene, Masuda and Yoshida[12] reported that the sequence divergence between *M. melampus* and *M. zibellina* was smaller than interspecific differences within the genus *Mustela*, but was larger than intraspecific differences within each species. Kurose *et al*.[10], sequenced the entire cyt *b* gene (1140bp) of the two species and reported that *M. melampus* and *M. zibellina* were clearly separable into two different groups. Hosoda *et al*.[6] conducted an RFLP (Restriction fragment length polymorphism) analysis of the ribosomal DNA of *M. melampus* and *M. zibellina* and reported that among 24 restriction sites of the nuclear r DNA spacer, there were no differences within each species, however one site differed between the two species.

As mitochondrial DNA diverges earlier than nucleic DNA, it is useful for analysis of genetic relationship of closely related species. Furthermore, *M. melampus* was thought to have speciated from *M. zibellina* recently (approximately 1-2 million years ago[6]), variable regions than used in previous analyses are needed to examine genetic relationships. Therefore, in this study a portion of the cytochrome *b*/transfer RNA/control region was examined to test the genetic relationships of the two *Martes* species.

Materials and Methods

Species identification We distinguished *M. zibellina* and *M. melampus* by their fur colors which had mentioned by Kadosaki[8]. Fig. 2 shows that the method to identify each species.

Samples and DNA extraction Liver tissues were obtained from 17 specimens of *M. zibellina* and seven specimens of *M. melampus* collected at various localities on Hokkaido (Fig. 1). Liver tissue of a Japanese weasel, *Mustela itachi*, and an American mink, *Mustela vison* were used as outgroups. Tissue samples were preserved in 80% ethanol. Total DNA was extracted from approximately 8mm^3

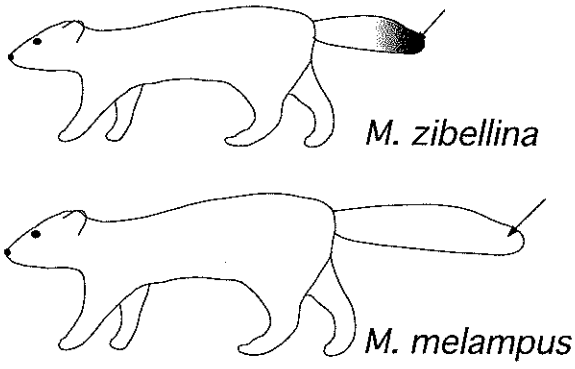
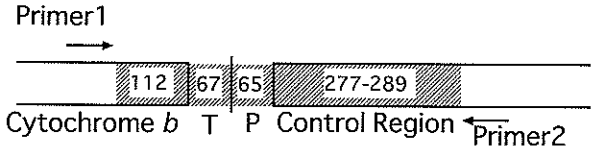


Fig. 2. A method for the identification of the Japanese sable (*M. zibellina*) and the Japanese marten (*M. melampus*). The color of the tip of the Japanese sable's tail is darker than its body color, while the color of the tip of the Japanese marten's tail is the same to or lighter than its body color.



Primer1:TGAATTGGAGGACAACCAGT
Primer2:CCTGAAGTAGGAACCAGATG

Fig. 3. The schematic diagram of the mitochondrial DNA cytochrome *b*, threonine-tRNA (T), proline tRNA (P) and control region. Arrows indicate positions of primers for PCR. A shaded area shows the portion analyzed in this study.

of tissue using DNeasy tissue kits by QIAGEN (Hilden, Germany).

Nucleotide sequence analysis A part of the mitochondrial cyt *b* / transfer RNA-threonine / tRNA-proline/ control region was PCR-amplified using 20-bp primers (5'-TGA ATTGGAGGACAACCAGT-3' and 5'-CCTGA AGTAGGAACCAGATG-3';[4,15], Fig. 3). PCR was performed with 35 cycles (94℃ for 30 sec, 55℃ for 60 sec, 72℃ for 90 sec, and 72℃ for 10 min), using *rTaq* DNA polymerase (TAKARA BIO Inc., Ootsu, Japan) with 10-100ng DNA extracts in a total volume of 50 μl. PCR products (40μl volume) was purified by Qia quick purification kit (QIAGEN). Purified PCR products (30-90 ng) were used as templates for direct sequencing by fluorescence-labeled dideoxynucleotides, Big Dye Terminator (Perkin Elmer, Massachusetts, USA) and GeneAmp PCR systems 9700 (Perkin Elmer). Sequencing was conducted using an ABI Prism™ 377 (Perkin Elmer) and a power mac computer (Apple Computer Inc., California, USA).

Data analysis Sequences were aligned by a multiple alignment program, Clustal W ver. 1.7[16]. Kimura-2 parameter genetic distances[9] were calculated using the "Phylip" developed by Dr. Felsenstein in 1995. Phylogenetic trees were reconstructed by the neighbor-joining method[14] using the "Neighbor" program in the Phylip. One thousand bootstrapping pseudo replications were conducted by using the program "Seqboot" in the Phylip. A parsimonous network was hand-drawn from the parsimonous tree calculated by the program "Dnapar" in the Phylip.

Analysis of sequences

DNA sequences were compared with the sequences reported by Kurose et al.[10], who had studied intraspecific variation of cyt *b* sequences in *M. zibellina* and *M. melampus*. The samples of *M. melampus* used in their study were obtained from Honshu island, where *M. zibellina* is not known to occur[10]. A 112 bp sequence region of the 3' end of the cyt *b*, common to our sequences and those presented in Kurose *et al.*[10] were used for constructing phylogenetic trees using the neighbor joining method by "Neighbor" in the Phylip.

Sequences of the haplotypes in the present study can be accessed in the DDBJ nucleotide databases under accession numbers AB 052718-AB 052726.

Results

A 521-533base pair (*Martes species*, 521-524bp; outgroup, 527-533 bp) region, consisting of the 3' end of the cyt *b* (112bp), tRNA-thr (67bp), tRNA-pro (65bp) and first 5' sequences of the control region (277-289bp) was obtained. Four haplotypes (MZ-1to MZ-4) of *M. zibellina*, five haplotypes (MM-1to MM-5) of *M. melampus*, and two haplotypes (MI-1; *Mustela itatsi* and MV-1; *Mustela vison*) of outgroups were detected.

The neighbor-joining tree indicated that haplotypes MM-1, MM-2, MZ-2and MZ-3 composed cluster-A, whereas MM-3, MM-4, MM-5, MZ-1, and MZ-4composed cluster-B (Fig. 4). Haplotypes of cluster-A had 523-524 base pairs, whereas those of cluster-B had only 521 bp. The node dividing the two clusters was confirmed by a high bootstrap value (100%, Fig. 4). The parsimonous networks (Fig. 5) showed that nucleotide substitution numbers among cluster-A haplotypes was much larger than that of cluster-B displayed a 2-3bp deletion (521bp). Intraspecific differences in sequences of *M. zibellina* and *M. melampus* were 3.8-15.0% and 1.9-16.4%, respectively. These values were similar to interspecific differences between these two species (5.8-16.6%). Sequence divergences within cluster-B were 0.58% to 1.36%while those of cluster-A were 4.32% to 4.37%. Comparison in the common sequence (112bp of 3' end of cytochrome *b*) between Kurose *et al.*[10]'s study and the current study, Kurose *et al.*[10] did not report cluster-B haplotypes

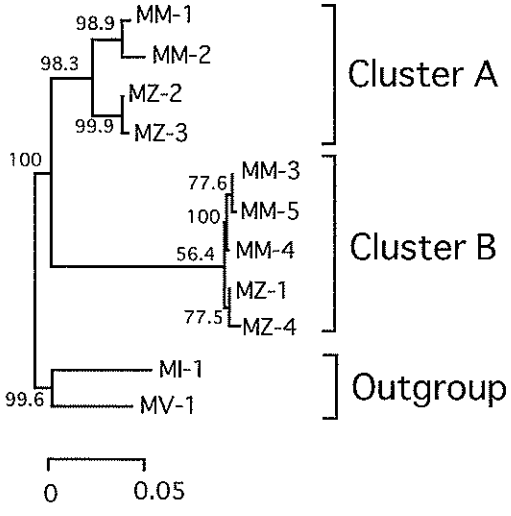


Fig. 4. Neighbor-joining tree of the mt DNA sequences of the two *Martes* species (MZ: sable, MM: Japanese marten) and two outgroups (MI: Japanese weasel, MV: mink). Numbers on internal branches are bootstrap values from 1,000replications. Numbers of nucleotide substitution per site indicated by the scale are Kimura's[9] 2-parameter distances.

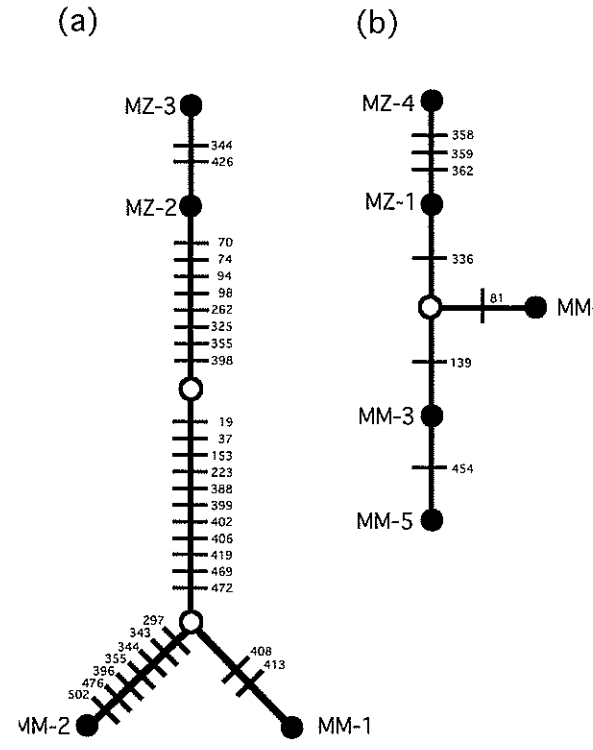


Fig. 5. Hand-drawn parsimonous networks of haplotypes of cluster-A (a) and cluster-B (b). Each cluster includes sable (MZ) and Japanese marten (MM) haplotypes. Solid circles indicate identified haplotypes, and open circles indicate hypothetical haplotypes. Each bar on the branch means one nucleotide substitution at the position of that number.

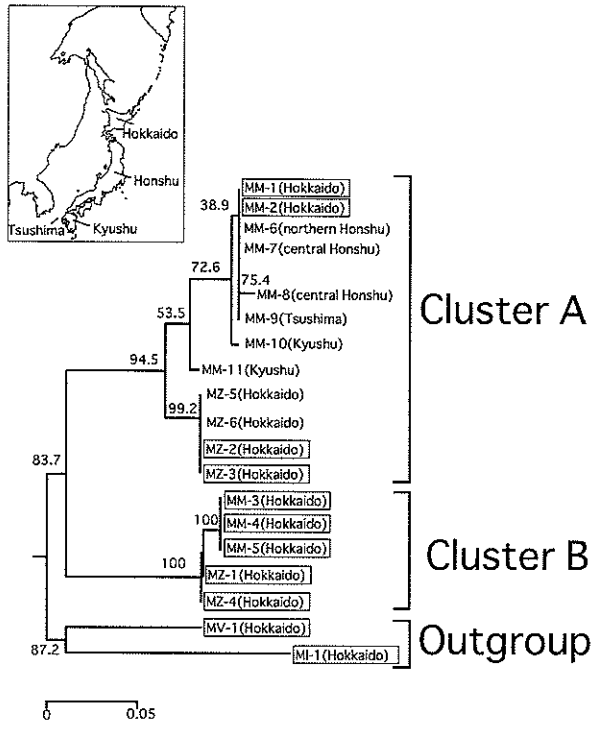


Fig. 6. Neighbour-joining tree of the 3' end of cytb sequences (112bp) of the two *Martes* species includes haplotypes detected by Kurose et al.[10]'s study and those detected by the current study. Haplotypes of Kurose et al.[10]'s study are indicated by MM-6 ~11and MZ-5 ~6, while those of the current study are indicated by MM-1 ~5, MZ-1 ~4, MV-1 and MI-1 (MZ: sable, MM: Japanese marten, MI: Japanese weasel, MV: mink). Letters within the parenthesis after each haplotype name represents sampling localities. Numbers along internal branches are bootstrap values from 1,000 replications. Numbers of nucleotide substitution per site indicated by the scale are Kimura[9] 2-parameter distances.

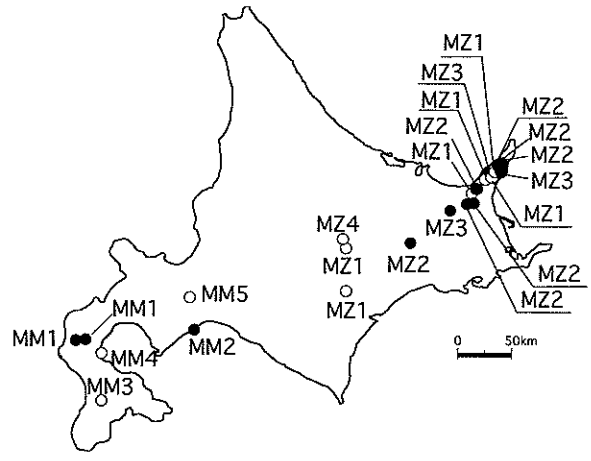


Fig. 7. Geographic distribution of haplotypes of the Japanese sable (MZ) and the Japanese marten (MM). Open circles are haplotypes of cluster-A in the phylogenetic tree showed on Fig. 2, while closed circles are those of cluster-B. Haplotype numbers are correspond to Fig. 2.

(Fig. 6). Geographic distributions of cluster-A haplotypes and of cluster-B haplotypes were overlapped (Fig. 7).

Discussion

Results indicate that the two *Martes* species on Hokkaido were closely related, in contrast to previous studies[6,10,12]. The 375 bp sequence of cytochrome *b* gene[12], the complete sequence of cytochrome *b*[10], the 402 bp sequence of cytochrome *b* and the nuclear ribosomal DNA-RFLP[6] indicated that the sequence divergence between *M. zibellina* and *M. melampus* was greater than intraspecific sequence divergence. However, the neighbor-joining tree of the two *Martes* in our study indicated two distinct clusters, each containing both haplotypes of *M. zibellina* and *M. melampus*.

First possible explanation of this discrepancy is past hybridization between the two species. Anderson[2] suggested that *M. martes*, *M. zibellina*, *M. melampus* and *M. americana* showed many similarities in morphology and ecology, and he stated that the four species could be considered a "superspecies". In fact, as natural hybridization between *M. zibellina* and *M. martes* had been reported[3], hybridization between *M. zibellina* and *M. melampus* also is likely. However, *M. zibellina* and *M. melampus* can be easily distinguished by fur colors[8] and skull morphology[1] and no intermediate status was found among samples ex-

amined in this study. Thus, these results indicate that hybridization is not occurred currently, but it might have occurred in the past. After that, each species might have evolved distinctively and might have maintained both haplotypes of mt DNA.

Previous studies did not detect cluster-B haplotypes among *M. zibellina* and *M. melampus* in Japan, while we could detect it in this study, although we could compare only 112 bp of cytochrome *b* gene. If the genus *Martes* had come from Russia to Japan and diverged into *M. melampus*[10], they should share the two haplotypes. Small sample size of the two species on Hokkaido in previous reports might be one reason for the lack of cluster-B haplotypes. Why the *M. melampus* outside Hokkaido did not have cluster-B haplotypes is still unclear. If cluster-B haplotypes exist only in Hokkaido, the origin of this haplotype might be after the formation of the Tsugaru straits dividing Hokkaido and Honshu. At that time, *M. melampus* and *M. zibellina* in Japan had only cluster-A. After that, *M. zibellina* population, which had cluster-B haplotypes, might come into Hokkaido. In fact, sequence divergences within cluster-B were lower than those of cluster-A and it supports our estimation. However, if this is true, hybridization occurred after introduction of *M. melampus* into Hokkaido, and it has spread over entire Hokkaido. As mentioned above, we believe that the two species are currently distinct in Hokkaido. However, comparison in variable nuclear DNA markers, such as microsatellite loci, among the two *Martes* on Hokkaido, Honshu (only *M. melampus* is distributed) and Russia (only *M. zibellina* is distributed) is necessary to evaluate whether hybridization is currently occurred or not.

Second possible explanation of our result is similar mitochondrial heteroplasmy of the two *Martes* species in Hokkaido. As *M. melampus* was thought to have speciated from *M. zibellina*[12], they could have similar mitochondrial heteroplasmy. Although we do not have enough data to evaluate whether this estimation is true or not, it should be investigated to form the conclusion.

Only four haplotypes were found among 17 individuals of *M. zibellina*. Kurose *et al.*[11], sequencing control region of 20 least weasels, *Mustela nivalis*, on Hokkaido, reported that there were 11 haplotypes. Thus the diversity of haplotypes in *Martes zibellina* was lower than that of *Mustela nivalis*. Kurose *et al.*[10] and Hosoda *et al.*[6] also reported low genetic variation of *M. zibellina* on Hokkaido. This result indicates that the population of *M. zibellina* may have gone through a bottleneck. It might be caused by heavy hunting pressure for fur in the early 20th[7].

Third possible explanation of our result is that the cluster-B might have originated from partial copy of mitochondrial DNA that had introduced into nuclear DNA. As mammal chromosomes contain sequences originated from mitochondrial DNA, we might have read them in the nuclear DNA. In order to eliminate this possibility, further analysis using DNA sample prepared by gradient centrifugation method may be needed.

M. zibellina has been considered as an endemic subspecies, Japanese sable (*M. zibellina brachyura*) and categorized as Data Deficient (DD) in the IUCN Red List[5]. Further intensive studies on detecting threatens of hybridization with Japanese marten for this sub-species are pressing needs.

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