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A Molecular Phylogeny of the Family Mustelidae (Mammalia, Carnivora), Based on Comparison of Mitochondrial Cytochrome b Nucleotide Sequences

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ABSTRACT—To study the phylogenetic relationships between the species of the family Mustelidae, by using the improved polymerase chain reaction-product direct sequencing technique, nucleotide sequences (375 bases) of the mitochondrial cytochrome b gene were determined on ten species from five genera of the Mustelidae and three species of other carnivore families, all of which are distributed in or around Japan. The molecular phylogenetic tree indicated a clear separation of five genera: *Mustela* and *Martes* from the subfamily Mustelinae, *Lutra* and *Enhydra* from the subfamily Lutrinae, and *Meles* from the subfamily Melinae. This clustering agreed with the previously reported morphological and karyological taxonomy. Furthermore, the relationships between the intrageneric species were discussed in more detail. This is the first report on the molecular phylogeny throughout the Japanese species of the Mustelidae, inferred from the mitochondrial DNA sequences.

INTRODUCTION

The family Mustelidae, which includes 64 species, has been most diversified in the order Carnivora which consists of more than 230 species [10]. The distribution of the mustelid species is widespread on a global scale. However, detailed relationships between the species in this family have not been fully examined.

In Japan, ten species of the Mustelidae are known: *Mustela itatsi* Temminck, 1844; *Mustela sibirica* Pallas, 1773; *Mustela nivalis* Linnaeus, 1766; *Mustela erminea* Linnaeus, 1758; *Mustela vison* Schreber, 1777; *Martes melampus* (Wagner, 1840); *Martes zibellina* (Linnaeus, 1758); *Meles meles* (Linnaeus, 1758); *Lutra lutra* (Linnaeus, 1758); *Enhydra lutris* (Linnaeus, 1758). At present, the latter two aquatic species have become endangered in Japan. *Mustela sibirica*, which is native on Tsushima Islands, and *Mustela vison* were introduced to Japan from Korea and North America, respectively. Obara [32] suggested that *Mustela erminea* is an ancestral type with karyological characters similar to *Mustela itatsi, Mustela nivalis*, and *Martes melampus*. *Meles meles* was karyologically separated from the other species of the Mustelidae [32]. However, the phylogenetic relationships throughout Japanese mustelid species have yet to be resolved.

A molecular genetic approach provides important information on the relationships between closely related species. Since the mitochondrial DNA (mtDNA) evolves more rapidly than the nuclear DNA [8], the mtDNA analysis is a useful method for the phylogenetic study [5, 7, 16]. Recently, the advent of polymerase chain reaction (PCR) led the advance in nucleotide sequence analysis from small amounts of DNA [29, 30, 33, 34]. Moreover, the PCR technology also developed the mtDNA analysis for the evolutionary study of many animal species including carnivores [23, 25, 39, 40, 42]. Previously, we reported the improved PCR product-direct sequencing technique and characterization of nucleotide sequence variations of the mitochondrial cytochrome b genes on three weasel species: *M. itatsi, M. sibirica*, and *M. nivalis* [27]. In that report, we showed that the analysis of this gene is useful for examination of the genetic difference between closely related species.

In this study, we determined partial nucleotide sequences of the cytochrome b genes for ten species of the Mustelidae and other carnivores using the improved PCR product-direct sequencing technique. Based on sequence comparison, we presented the molecular phylogeny of intra- and intergeneric species in the Mustelidae and discussed the relationships between them, as compared with the previously reported karyological and morphological data.

MATERIALS AND METHODS

Animals and DNA extraction

Animals examined in this study were listed in Table 1. Muscle tissues were dissected from each animal and frozen at −80°C or preserved in 70% ethanol. Total DNAs were extracted according to the phenol/proteinase K/sodium dodecyl sulfate (SDS) method of Sambrook *et al.* [36] with simplified modifications [27]. In brief, a small piece (about 2×2×2 mm) of tissue was excised with a scalpel and washed several times with 1 ml of STE buffer (0.1 M NaCl/10 mM Tris/1 mM EDTA). Using a small glass homogenizer, the tissue was homogenized with 500 μl of STE buffer containing a final concentration of 0.5% SDS and 5 μg/ml of proteinase K. After incubation at 37°C overnight, the homogenate was extracted twice

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TABLE 1. Species of the family Mustelidae and other carnivore families analyzed in this study

Species	Code	Common name	No. examined	Source (Collecting locality if known)
Family Mustelidae				
Mustela itatsi	MIT	Japanese weasel	2*	Rishiri Town Museum and Shiretoko Museum (Rishiri Island and Abashiri-gun, Hokkaido; ref. [26]**)
Mustela sibirica	MSI	Siberian weasel	2*	Kitakyushu Museum of Natural History (Onga-gun, Fukuoka Pref.; ref. [26]**)
Mustela erminea	MER	Stoat or ermine	1	Shiretoko Museum (Shari-cho, Hokkaido)
Mustela nivalis	MNI1	Least weasel	1	Iwate Prefectural Museum (Kunohe-gun, Iwate Pref.)
Mustela nivalis	MNI2	Least weasel	1	Shiretoko Museum (Shari-gun, Hokkaido; ref. [26]**)
Mustela vison	MVI	American mink	2*	Shiretoko Museum (Shari-gun, Hokkaido)
Martes melampus	MME	Japanese marten	1	Iwate Prefectural Museum (Morioka-shi, Iwate Pref.)
Martes zibellina	MZI	Sable	2*	Shiretoko Museum (Shari-cho and Kiyosato-cho, Hokkaido)
Meles meles	MEL	Japanese badger	1	(Ohno-gun, Gifu Pref.)
Lutra lutra	LLU	River otter	2*	Kobe Oji Zoo (originated from Russia)
Enhydra lutris	ELU	Sea otter	1	Suma Aqualife Park (originated from North Pacific)
Family Ursidae				
Selenarctos thibetanus	STH	Asiatic black bear	1	(Ohno-gun, Gifu Pref.)
Family Otariidae				
Zalophus californianus	ZCA	California sea lion	1	Kobe Oji Zoo (originated from North America)
Family Phocidae				
Phoca vitulina	PVI	Harbor seal	—	ref. [4]***

* Two individuals showed no intraspecific sequence variation.
** The cytochrome b sequences were cited from our previous paper [27].
*** The cytochrome b sequence was cited from the previously published paper of Arnason and Johnsson [4].

with an equal volume of phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). All procedures were done in 1.5 ml microcentrifuge tubes. STE buffer extracted by the same procedure was used as a negative control in the following PCR amplification.

Symmetric and asymmetric PCR amplification

Partial regions of the mitochondrial cytochrome b genes were PCR-amplified using the method of Kocher *et al.* [25] with some modifications [27]. Referring to the published information of Irwin *et al.* [23], primer sequences were designed as L14724 (5′-GATATG-AAAAACCATCGTTG-3′) and H15149 (5′-CTCAGAATGATA-TTTGTCCTCA-3′) by the consensus between the mtDNA sequences of the human [2], the bovine [3], and the mouse [6]. Primer names identify the light (L) or heavy (H) strand and the 3' end-position of the primer in the human mtDNA sequence [2]. Primers were synthesized on an Applied Biosystems 391 DNA synthesizer.

Symmetric PCR was done for the amplification of double-stranded DNAs with a GeneAmp PCR reagent kit (Perkin-Elmer/Cetus) according to the manufacturer's instruction. A total volume of 50 μl of the reaction mixture included 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, each dNTP at 200 μM, 1.25 U of *Taq* DNA polymerase, each primer at 250 nM, and 1–5 μl of the extracted DNA solution as a template. Each cycle was programed by denaturing at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. After 35 cycles, the extension reaction was completed by incubation at 72°C for 10 min.

Asymmetric PCR was performed to generate single-stranded DNAs according to the method of Gyllensten and Erlich [14]. The PCR procedure was basically the same as symmetric PCR, except for 30 cycles of reaction, a 1:100 (2.5 nM:250 nM) ratio of two primers, and 1 μl of the symmetric PCR product as a DNA template in 100 μl of the reaction mixture.

Direct sequencing of PCR products and sequence analysis

Single-stranded DNAs produced by asymmetric PCR were concentrated using a Centricon-30 microconcentrator (Amicon), and 7 μl of the concentrated DNA was sequenced with 10 ng of the PCR primer for the L or H strand, [^{32}P] dCTP (Amersham), and T7 DNA polymerase (United States Biochemical), according to the dideoxynucleotide chain reaction method [37]. Reaction products were electrophoresed on 6% polyacrylamide gels containing 7 M urea. Gels were dried and exposed to Fuji RX X-ray films for 1–5 days.

Sequence analysis and phylogenetic tree construction by the neighbor-joining method [35] were performed with the Clustal V computer software [18]. Numbers of nucleotide substitutions per site were estimated for multiple substitutions using Kimura's two-parameter method [24]. Bootstrap methods [13] were used in the Clustal V to assess the degree of support for internal branches of the tree. Cytochrome b sequences of MIT (Accession No. D26130), MSI (Accession No. D26132), and MNI2 (Accession No. D26133) were cited from our previous report [27]. PVI sequence was obtained from the published paper of Arnason and Johnsson [4].

RESULTS

From a small piece of each animal tissue, total DNAs were extracted with the simplified method, and the partial

(a)

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          10         20         30         40         50         60         70         80
MIT   ATGACCAACA TTCGCAAAAC CCACCCACTA ACCAAAATCA TCAACAACTC ATTCATTGAC TTACCCGCCC CATCAAACAT
MSI   .......... .......... .......T.. .......... .......... ....G..... C.T....... ..........
MER   .......... ....T..... T......T.. .......... .......... ...T..C... C.C.....T. ..........
MNI1  .......... ....T..... T........G .......... .......... .......... C.C.....T. ..........
MNI2  .......... ....T..... T........G .......... .......... .......... C.C.....T. ..........
MVI   .......... ....T..... .......T.. ........T. .......... .........T C....T..T. ..........
MME   .......... ....T..... T......... G.T....... .......T.. ......C... .....T..T. ..........
MZI   .......... ....T..... T......... G......... .......T.. ......C... .....T..T. ..........
MEL   .......... ....T...T. G......... .........C .......T.. ...T...... C.......A. ....C..T..
LLU   .......... .......... T......T.. G......... .......... GC....C..T C.......G. ....G..T..
ELU   .......... ....T..G.. ...T..C... .....G.... .......... ......C... C.......A. ..........
PVI   .......... .C..A..... ...T..... .TA.....T. .......... ......C... C.....A.A. .......T..
ZCA   .......... ....A...GT A..T.....G G.......T. ......GT.. .C.T..C... C.G...A.A. ....T.....
STH   .......... .CT.A..... ...T...T.. G......... .T........ .C.......T C.C..A..A. .......T..

          90         100        110        120        130        140        150        160
MIT   TTCAGCGTGA TGAAACTTCG GCTCCCTTCT CGGAATCTGC CTAATTATTC AGATTCTTAC AGGTTTATTT TTAGCTATAC
MSI   C.....A... .......... .......... .......... .......... .......... .......... .....C....
MER   C.....A... .......... .......C.. .......... .....C.... .......... .......... .....C..G.
MNI1  C.....A... .......... .T.....C.. .......... ..T....... .......... .......... .....C..G.
MNI2  C.....A... .......... .......... .......... ..T....... .......... .......... .....C..G.
MVI   C.....A... .......... .G.....A.. .......... ......C... .A........ ...C.....C .....C....
MME   ...C..A... .......... .......C.. T......... .....CC.A. .......... .......... C....C....
MZI   ...C..A... .....T.... .......C.. T........T ..G..CC.A. .......... .......... C....C....
MEL   C.....A... ........T. .A.....C.. T......... .....CC.A. .A..C..... .........C C....C....
LLU   ......A... .....T..T. .A.....A.. A....CT... T....CC... .......... .......... .....C....
ELU   C.....A... .......... .A.....A.. ......T... T....CC... .......... .......... ..........
PVI   C..G..A... ........T. .A..T..... .......... .....CC.G. ....CT.A.. ...C.....C C....C....
ZCA   C.....A... ........T. .A.....C.. ..C.GCA... T..GCCT.A. .A..C..A.. ...CC.T..C C.........
STH   C.....A... ........T. .A........ ....G.A... ...G..C.A. .......A.. ...CC..... C....C....

          170        180        190        200        210        220        230        240
MIT   ACTATACATC GGACACAGCC ACAGCCTTTT CATCAGTCAC CCATATCTGT CGAGACGTCA ACTATGGCTG AATCATCCGA
MSI   .......... A......... .......... .......... ......T... .......... .......... ..........
MER   .T..C..... A......... ........C. .......... ...C.....C .......... ....C..... ..........
MNI1  .......... A..T...... .......... .......... ...C...... .......... ....C..... ...T......
MNI2  .......... A..T...... .......... .......... ...C...... .......... ....C..... ...T......
MVI   ....C..... A........T .......... .......... T.....T..C ........T. .T.....T.. ......T...
MME   ....C..... A......... ........C. .......... ...C..T..C .......... ....C..A.. ...T......
MZI   ....C..... A......... ........C. ....G..... ...C..T..C .......... ....C..... G..T..T...
MEL   .T..C...C. A.....CA.T ..C.....C. .......T.. A..C...... ........T. .......... ......T...
LLU   ....C..... A......A.. ........C. ........G. A..C.....C .......... ....C..... G..T......
ELU   .T........ A......A.. ........C. ........G. .........C .......... ....C..... ...T.....G
PVI   ....C..C.. A......A.. ........C. .......A.. ...C.....C ........A. ....C..... .........T
ZCA   .......C.. A.....CA.. .......... .......... ...C..T..C .......... ....C..... ..........
STH   .......... A...G..A.T .......... ....G...G. ...C..T..C .........C .T..C..A.. ...T......

          250        260        270        280        290        300        310        320
MIT   TATATACATG CAAACGGAGC CTCCATATTC TTTATCTGCC TGTTCCTACA CGTAGGGCGA GGCTTATATT ACGGATCCTA
MSI   ..C.....C. .......... T......... ..C....... .......... .......... ..T....... .......T..
MER   ..C.....C. .......... T......... ..C....... .A........ ......A... .......... ....T..T..
MNI1  ..C....... .......... T.....G... .....T.... .A........ ......A... .......... .T..T..T..
MNI2  ..C....... .......... T......... .....T.... .A........ ......A... .......... .T..T..T..
MVI   ........C. ....T..... T......... ...G.T...T .......... T.....A... .......... .T.....T..
MME   ..C....... .C.....G.. T......... ..C....... .......G.. ...C..A..G ...C....C. .T.....T..
MZI   .......... .C.....G.. T......... ..C....... .......G.. ...C..A..G ..T.....C. .T.....T..
MEL   .....G..C. .T..T..... .......... .......... .A...T.... T.....A... ...C...... ..........
LLU   ..C.....C. .......... .......... ..C....... .......... T.....A..C ...C.G..C. .......T..
ELU   ..C....... .......... A......... .....T.... .A........ T.....A... ..A.....C. .T.....T..
PVI   ...C.T..C. ....T..... T........T ..C....... .A.A.A.G.. T.....A... ..AC.G.... ....C.....
ZCA   ..C..G..C. ....T..... .......... .......... .C.A.A.G.. ......A... ..AC.G..C. ..........
STH   .......... .......... ......G... ..C....... .A...A.... ......A..G .....G.... .T..C..T..
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                330        340        350        360        370   375
MIT   TATATTCACC GAAACATGAA ACATCGGCAT TATCTTATTA TTCGCAGTCA TAGCA
MSI   .......... .......... .......... .........G .......... .....
MER   .......C.. .......... ....T..... .......... .......... .....
MNI1  .......T.. .......... .......... .......... .......... .....
MNI2  .......T.. .......... .......... .......... .......... .....
MVI   ......TC.T .......... .T..T..T.. C......C.. ...A....A. .....
MME   .....A.C.. ........G. ....T..... C...C..... ........T. .....
MZI   .....A.C.. ........G. ....T..T.. C...C..... .......... .....
MEL   .......C.T .......... .T.....T.. C.....G... ...A....A. .....
LLU   .......C.T .......... ...CT..T.. ...TC..C.. ......AC.. .....
ELU   C.....TT.. .......... ....T..T.. .G.....C.. ...A....T. .....
PVI   C.C......A ..G....... .......... ....C.C... ...A.C.... ....T
ZCA   ..C.C.A..A .......... ....T..... C...C.CC.. ..TA..A... ....T
STH   CC..C..T.A .......... .T..T...TA CG..C.CC.. ..TA....T. ....C
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(b)

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                10         20         30         40         50         60         70         80
MIT   MTNIRKTHPL TKIINNSFID LPAPSNISAW WNFGSLLGIC LIIQILTGLF LAMHYTSDTA TAFSSVTHIC RDVNYGWIIR
MSI   .......... ........V. .......... .......... .......... .......... .......... ..........
MER   .......... .......... .......... .......... .......... .......... .......... ..........
MNI1  .......... .......... .......... .......... .......... .......... .......... ..........
MNI2  .......... .......... .......... .......... .......... .......... .......... ..........
MVI   .......... .......... .......... .......... ..L....... .......... .......... ..........
MME   .......... A......... .......... .......... ..L....... .......... .......... ..........
MZI   .......... A......... .......... .......... ..L....... .......... .......... ..........
MEL   ......S... ...L...... .......... .......... ..L....... ......P..T .......... ..........
LLU   .......... A......L.. .......... ........T. ..L....... .........T ......A... ..........
ELU   .......... .......... .......... .......... ..L....... .........T ......A... ..........
PVI   .......... M......... ..T....... .......... ..L....... .........T .......... ..........
ZCA   ......V... A....S.L.. ..T....... .......AA. .AL....... .........T .......... ..........
STH   ....W..... A......L.. .......... ........V. .VL....... ........AT ......A... ...H......

                90         100        110        120   125
MIT   YMHANGASMF FICLFLHVGR GLYYGSYMFT ETWNIGIILL FAVMA
MSI   .......... .......... .......... .......... .....
MER   .......... .......... .........P .......... .....
MNI1  .......... .......... .........S .......... .....
MNI2  .......... .......... .........S .......... .....
MVI   .......... .V........ .........P .......... .T...
MME   .......... .......... ........YP .......... .....
MZI   .......... .......... ........YP .......... .....
MEL   .......... .......... .........P .......... .T...
LLU   .......... .......... .........P ....T..... ..T..
ELU   .......... .......... .........S .......V.. .T...
PVI   .L........ ....YM.... .......T.. .......... .T...
ZCA   .......... ....YM.... .......TL. .......... .TI..
STH   .......... .....M.... .......LLS ......YV.. .T...
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FIG. 1. Cytochrome b sequences in species of the family Mustelidae. a: nucleotide sequences (375 bases) obtained by the PCR product-direct sequencing technique. b: amino acid sequences (125 amino acids) deduced from nucleotide sequences (a). Characters are nucleotide or amino acid codes recommended by IUPAC-IUB. Dots denote identity with nucleotides or amino acids of MIT.

region of the mitochondrial cytochrome b gene was successfully amplified by symmetric and asymmetric PCRs. Using the direct sequencing technique of single-stranded DNAs, nucleotide sequences (375 bases) were determined without any gap or insertion in all species (Fig. 1a).

Two individuals from each of the five mustelid species (*Mustela itatsi, M. sibirica, M. vison, Martes zibellina*, and *Lutra lutra*) showed no intraspecific sequence variation, indicating the possible close relationships between the two individuals: samples of *M. itatsi, M. sibirica,* and *M. vison* were originated from the introduced small populations, and two specimens of *M. zibellina* were collected from the close localities in Hokkaido, and two individuals of *L. lutra* were brought from Russia and kept in the zoo (Table 1). *Mustela*

TABLE 2. Percentage differences (above diagonal) and numbers of transitions/transversions (below diagonal) for cytochrome b nucleotide sequences (375 bases) of the Mustelidae and other carnivore species

Code	MIT	MSI	MER	MNI1	MNI2	MVI	MME	MZI	MEL	LLU	ELU	PVI	ZCA	STH
MIT	—	4.3	8.3	7.5	6.7	12.3	12.3	13.1	14.7	14.4	11.7	17.1	20.0	18.7
MSI	15/1	—	6.4	7.2	6.4	11.2	12.3	13.1	15.7	13.9	12.3	16.8	20.0	17.9
MER	28/3	22/2	—	5.3	5.1	11.7	9.3	10.7	13.3	11.5	10.4	15.5	18.9	17.9
MNI1	24/4	24/3	19/1	—	0.8	12.5	11.5	12.5	15.7	14.9	10.7	18.1	20.5	17.1
MNI2	21/4	21/3	18/1	3/0	—	12.3	11.2	12.3	15.7	14.7	10.4	17.6	20.5	17.1
MVI	41/5	36/6	38/6	40/7	39/7	—	14.7	14.9	13.1	15.7	13.1	18.9	21.3	17.6
MME	38/8	37/9	26/9	33/10	32/10	46/9	—	3.5	16.3	13.3	13.9	18.4	20.0	18.1
MZI	42/7	41/8	32/8	38/9	37/9	48/8	12/1	—	16.8	13.6	14.9	19.7	21.6	19.5
MEL	41/14	44/15	35/15	43/16	43/16	38/11	47/14	50/13	—	16.5	16.5	16.3	18.4	21.1
LLU	46/8	43/9	34/9	46/10	45/10	54/5	38/12	40/11	50/12	—	12.8	18.7	19.2	18.4
ELU	36/8	37/9	30/9	30/10	29/10	44/5	40/12	45/11	48/14	42/6	—	17.3	19.5	17.1
PVI	43/21	41/22	36/22	45/23	43/23	49/22	44/25	50/24	34/27	47/23	44/21	—	15.7	18.7
ZCA	54/21	53/22	47/24	52/25	52/25	58/22	50/25	57/24	48/21	49/23	52/21	43/16	—	18.4
STH	50/20	48/19	50/17	46/18	46/18	47/19	48/20	52/21	55/24	49/20	44/20	51/19	50/19	—

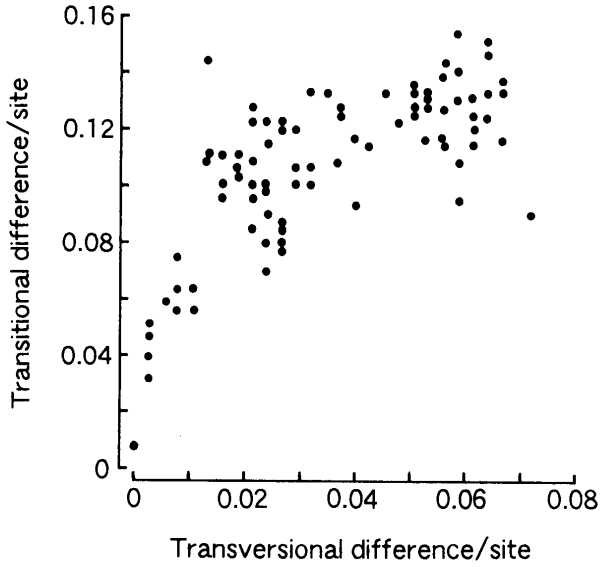

FIG. 2. The transitional difference per site was plotted against the transversional difference per site in pairwise comparisons of cytochrome b nucleotide sequences.

nivalis from Iwate Prefecture (MNI1) showed 0.8% (3/375 bases) sequence difference from that from Hokkaido (MNI2) (Tables 1 and 2). From each of the other five mustelid species, one sample was examined (Table 1). Percentage sequence differences and numbers of transitions/transversions obtained from all pairwise comparisons were shown in Table 2.

In Figure 2, the transitional difference per site was plotted against the transversional difference per site. These differences were positively related among the mustelid species and the high transition bias was observed (Fig. 2).

Among the mustelid species, percentage differences were between 4.3 and 16.8% (3–63/375 bases) (Table 2). Outgroup species including *Phoca vitulina* (PVI), *Zalophus californianus* (ZCA), and *Selenarctos thibetanus* (STH) showed 15.5–21.6% (58–81/375 bases) sequence differences from the mustelid species (Table 2).

Among the *Mustela* species except for *M. vison* (MVI), sequence differences were between 4.3 and 8.3% (3–31/375

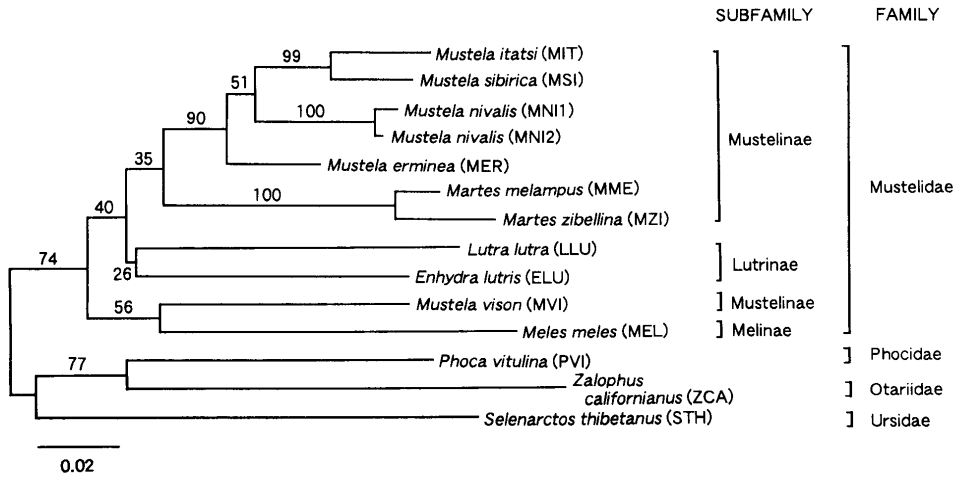

FIG. 3. Phylogenetic tree of cytochrome b nucleotide sequences constructed by the neighbor-joining method and Kimura's two-parameter method. The scale bar indicates an evolutionary distance of 0.02 substitution per site. Numbers above branches are bootstrap probability values based on 500 replications.

bases) (Table 2). MVI showed higher differences (11.2–12.5%, 42–47/375 bases) from the other *Mustela* species. Two species of the genus *Martes, M. melampus* (MME) and *M. zibellina* (MZI), showed 3.5% (13/375 bases) difference between them. These two *Martes* species differed by 9.3–14.9% (35–56/375 bases) of sequences from the *Mustela* species. *Meles meles* (MEL), *Lutra lutra* (LLU), and *Enhydra lutris* (ELU) showed relatively higher differences (10.4–16.8%, 39–63/375 bases) from those of the *Mustela* and *Martes* species (Table 2). Amino acid sequences deduced from nucleotide sequences were shown in Figure 1b.

The phylogenetic tree of cytochrome b nucleotide sequences was constructed using the neighbor-joining method, based on the matrix of the numbers of nucleotide substitutions per site expected by Kimura's two-parameter method (Fig. 3). The species of the genus *Mustela* in the subfamily Mustelinae, except for MVI, were separated from the other mustelid species with 90% bootstrap value. Two species of the genus *Martes*, MME and MZI, were clustered with 100% bootstrap value. MVI was distantly related to the cluster of the subfamily Mustelinae. LLU and ELU from the subfamily Lutrinae were grouped into the same lineage, although the bootstrap value was low (26%). MEL, the subfamily Melinae, was positioned distantly from the other mustelid species. Among outgroup species, ZCA was closer to PVI than STH.

DISCUSSION

In this study, we examined the molecular phylogenetic relationships between the species of the family Mustelidae which are distributed in or around Japan. Using the DNA extraction and the PCR product-direct sequencing techniques improved by us, the cytochrome b nucleotide sequences (375 bases) were successfully determined (Fig. 1a). The amino acid sequences (Fig. 1b) deduced from the nucleotide sequences indicated that synonymous mutations were more numerous than nonsynonymous ones. This pattern of nucleotide substitution is similar to that reported in previous works [7, 9, 17, 28, 38]. The high transition bias was observed, and the transitional difference was positively related to the transversional one among the mustelid species (Fig. 2). Our findings agree with the previous reports and reflect the initial high transition bias of mtDNA, which gradually decreases over time as transversions are accumulated [7, 9, 11, 17, 19]. Since the high transition bias was found among the intrafamilial species which showed relatively higher sequence homology, Kimura's two-parameter method [24] was used for estimating numbers of nucleotide substitutions per site in the phylogenetic tree construction.

The molecular phylogenetic tree of the cytochrome b nucleotide sequences (Fig. 3) revealed that the species of the Mustelidae were divided into three major clusters: the first which includes four species (MIT, MSI, MNI, and MER) of the genus *Mustela*, the second which includes two species (MME and MZI) of the genus *Martes*, and the third which includes LLU and ELU. MEL and MVI were distantly related to the other mustelid species. The results suggest a monophyletic grouping of each genus, except for MVI in the *Mustela*. According to the Carnivora classifications by Ewer [12] and Wozencraft [44], the family Mustelidae was divided into at least four subfamilies: Mustelinae, Lutrinae, Melinae, and Mephitinae. The classification of subfamilies was shown on the phylogenetic tree in Figure 3. Our results support the classification of subfamilies, except for the position of MVI (Fig. 3).

In the previous study [27], we reported a clear distinction of the cytochrome b sequences between *M. itatsi* (MIT) and *M. sibirica* (MSI). The present study throughout the Japanese mustelid species indicated that MIT and MSI, though genetically distinct, were clustered and separated from the other *Mustela* species with the high bootstrap value (99%) (Fig. 3). By contrast, Wozencraft [45] proposed that MIT is a synonym of MSI, based on the morphological similarity. Ewer [12] and Nowak [31] classified the Japanese weasel as MSI, but they did not recognize MIT in the classification of the *Mustela* species. On the one hand, Imaizumi [22] classified MIT and MSI as two subspecies of *M. sibirica, M. s. itatsi* and *M. s. coreana*, respectively, based on the difference of a ratio of the tail length to the head and body lengths (the T-HB ratio): about 0.4 in *M. s. itasti* and greater than 0.5 in *M. s. coreana*. In the previous study [27], using the T-HB ratio we unambiguously identified MIT (0.39–0.41 of five specimens) and MSI (0.53–0.56 of two specimens). Between specimens identified as MIT and MSI, sequence difference was 4.3% [27]. Although this value is somewhat smaller than those among the *Mustela* species revealed in the present study (5.1–8.3% excluding MVI, Table 2), its magnitude apparently exceeds the intraspecific level. The intraspecific differences of the cytochrome b sequences were 0.8% in MIT [27] and 0.8% in *M. nivalis* (Table 2). In addition, Wayne and Jenks [41] reported the intraspecific differences of the canine cytochrome b sequences: 0.3–1.3% in the coyote *Canis latrans* and 0.3–0.5% in the gray wolf *C. lupus*. Therefore, we concluded that it is reasonable for MIT and MSI to be classified as distinct species.

Two specimens of *M. nivalis* collected from Iwate Prefecture (MNI1) and Hokkaido (MNI2) showed 0.8% (3/375 bases) nucleotide difference (Table 2), all of which were synonymous (Fig. 1b). Obara [32] reported the karyotype differences between M. nivalis from Aomori Prefecture (2n=38) and that from Hokkaido (2n=42), and he proposed that these two animals are distinct species, *M. namiyei* and *M. nivalis nivalis*, respectively. *M. nivalis* from the Eurasian Continent, which has the karyotype (2n=42) [26] similar to the Hokkaido type, was proposed as an ancestral type of *M. nivalis* occurring in Japan [32]. Although, in this study, a karyotype could not be analyzed on MNI1 and MNI2, their collecting localities (Iwate, close to Aomori, and Hokkaido, respectively) suggest that MNI1 and MNI2 may have different karyotypes as shown by Obara's data [32]. However, the sequence difference (0.8%) between MNI1 and MNI2 was much smaller than the interspecific difference within the

genus *Mustela* (Table 2), indicating that they are not distinct species. Since the two MNI types are karyologically different and geographically isolated, it seems to be reasonable that they are classified as subspecies. Further studies on more specimens from Honshu and Hokkaido are, however, still necessary to conclude whether the two types are subspecies or distinct species.

It is interesting that *M. vison* (MVI) was outside of the monophyletic grouping of the genus *Mustela* in the phylogenetic tree (Fig. 3). Holmes [20], by a cladistic analysis, divided the genus *Mustela* into four monophyletic groups: the first which consisted of only MVI, the second which included *M. sibirica* and other species, the third which included *M. nivalis, M. erminea,* and other species, and the last which consisted of different species from our study. Our results support Holmes' classification of the genus *Mustela* and the position of MVI.

Two species of the genus *Martes, M. melampus* (MME) and *M. zibellina* (MZI), were grouped with 100% bootstrap value and they were closer to the genus *Mustela* (Fig. 3), supporting the morphological classification in that both the *Martes* and the *Mustela* are classified into the subfamily Mustelinae [12, 44]. The cytochrome b sequence difference between MME and MZI was 3.5% (13/375 bases) (Table 2), all of which were synonymous mutations (Fig. 1b). Previous morphological reports indicate that MME and MZI may be conspecific [1, 15]. In fact, the sequence difference (3.5%) between MME and MZI is smaller than the interspecific differences (4.3–8.3%) within the genus *Mustela* including MIT, MSI, MER, and MNI (Table 2), but it is larger than the intraspecific differences such as: 0.8% for MNI (Table 2), 0.8% for MIT [27], 0.3–0.5% for *C. lupus* [42]. As compared with these sequence differences, it is reasonable for MME and MZI to be classified as distinct species.

Lutra lutra (LLU) was grouped with *Enhydra lutris* (ELU) (Fig. 3). This concurs with morphological classifications of Ewer [12] and Wozencraft [44] that these two aquatic species were classified into the subsfamily Lutrinae.

Meles meles (MEL) was distantly related to the other mustelid species (Fig. 3), in agreement with the karyological taxonomy by Obara [32]. Morphologically, MEL was classified into the subfamily Melinae by Ewer [12] and Wozencraft [44]. Hosoda *et al.* [21] reported the phylogenetic relationship among carnivore species including MIT, MNI, MME, and MEL, based on the restriction fragment length polymorphisms of the nuclear ribosomal DNA. Our finding basically agree with the result of Hosoda *et al.* [21] on the four species.

Among outgroup species, *Zalophus californianus* (ZCA) was closer to *Phoca vitulina* (PVI) than *Selenarctos thibetanus* (STH), supporting the previous findings from the DNA hybridization analysis of Wayne *et al.* [41]. Our results also agree mostly with the karyological phylogeny on these species reported by Wurster and Benirschke [46] and Wozencraft [43].

This is the first report on the molecular phylogeny throughout the Japanese species of the family Mustelidae, inferred from the mtDNA sequences. The relationships between the intergeneric species and between the intrageneric species were examined in detail, based on the cytochrome b sequence comparison. As a whole, the molecular phylogenetic tree was in agreement with the previously reported karyological and morphological taxonomy.

SEQUENCE AVAILABILITY

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence data bases with the following accession numbers; D26515 for MER; D26516 for MNI1; D26517 for MVI; D26518 for MME; D26519 for MZI; D26520 for MEL; D26521 for LLU; D26522 for ELU; D26523 for STH; D26524 for ZCA.

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REFERENCES

1 Anderson E (1970) Quaternary evolution of the genus *Martes* (Carnivora, Mustelidae). Acta Zool Fennica 130: 1–132

2 Anderson S, Bankier AT, Barrell BG, De Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290: 457–465

3 Anderson S, De Bruijn MHL, Coulson AR, Eperon IC, Sanger F, Young IG (1982) Complete sequence of bovine mitochondrial DNA: Conserved features of the mammalian mitochondrial genome. J Mol Biol 156: 683–717

4 Arnason U, Johnsson E (1992) The complete mitochondrial DNA sequence of the harbor seal, *Phoca vitulina*. J Mol Evol 34: 493–505

5 Avice JC (1991) Ten unorthodox perspectives on evolution prompted by comparative population genetic findings on mitochondrial DNA. Annu Rev Genet 25: 45–69

6 Bibb MJ, Van Etten RA, Wright CT, Walberg MW, Clayton DA (1981) Sequence and gene organization of mouse mitochondrial DNA. Cell 26: 167–180

7 Brown WM (1985) The mitochondrial genome of animals. In "Molecular Evolutionary Genetics" Ed by RJ MacIntyre, Plenum Press, New York, pp 95–130

8 Brown WM, George MJr, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA 76: 1967–1971

9 Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: Tempo and mode of evolution. J Mol Evol 18: 225–239

10 Corbet GB, Hill JE (1991) A World List of Mammalian Species. 3rd ed, Oxford Univ Press, Oxford

11 DeSalle R, Freedman T, Pragner EM, Wilson AC (1987) Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. J Mol Evol 26: 157–164

12 Ewer RF (1973) The Carnivores. Cornell Univ Press, New

York

13 Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783–791

14 Gyllensten UB, Erlich HA (1988) Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc Natl Acad Sci USA 85: 7652–7656

15 Hagmeier EM (1961) Variation and relationships in North American marten. Canadian Field-Naturalist 75: 122–138

16 Harrison RG (1989) Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. Trends Ecol Evol 4: 6–11

17 Hedges SB, Bezy RL, Maxson LR (1991) Phylogenetic relationships and biogeography of xantusiid lizards, inferred from mitochondrial DNA sequences. Mol Biol Evol 8: 767–780

18 Higgins DG, Bleasby AJ, Fuchs R (1992) Clustal V: Improved software for multiple sequence alignment. Comput Appl Biosci 8: 189–191

19 Hixon JE, Brown WM (1986) A comparison of the small ribosomal RNA genes from the mitochondrial DNA of the great apes and humans: Sequence divergence, structure, evolution, and phylogenetic implications. Mol Biol Evol 3: 1–18

20 Holmes T (1985) A phylogeny of the Mustelinae (abstract). Am Soc Mammal, 65th Annu Meeting Abstr p 60

21 Hosoda T, Suzuki H, Yamada T, Tsuchiya K (1993) Restriction site polymorphism in the ribosomal DNA of eight species of Canidae and Mustelidae. Cytologia 58: 223–230

22 Imaizumi Y (1960) Coloured Illustrations of the Mammals of Japan. Hoikusha, Osaka (in Japanese)

23 Irwin DM, Kocher TD, Wilson AC (1991) Evolution of the cytochrome *b* gene of mammals. J Mol Evol 32: 128–144

24 Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111–120

25 Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proc Natl Acad Sci USA 86: 6196–6200

26 Mandahl N, Fredga K (1980) A comparative chromosome study by means of G-, C-, and NOR-bandings of the weasel, the pygmy weasel and stoat (*Mustela*, Carnivora, Mammalia). Hereditas 93: 75–83

27 Masuda R, Yoshida MC (1994) Nucleotide sequence variation of cytochrome b genes in three species of weasels, *Mustela itatsi, Mustela sibirica,* and *Mustela nivalis*, detected by improved PCR product-direct sequencing technique. J Mamm Soc Japan 19: 33–43

28 Meyer A, Wilson AC (1990) Origin of tetrapods inferred from their mitochondrial DNA affiliation to lungfish. J Mol Evol 31: 359–364

29 Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H (1986) Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. Cold Spring Harbor Symp Quant Biol 51: 263–273

30 Mullis KB, Faloona FA (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol 155: 335–350

31 Nowak RM (1991) Walker's Mammals of the World, 5th. Johns Hopkins Univ Press, Baltimore

32 Obara Y (1991) Karyosystematics of the mustelid carnivores of Japan. Honyurui Kagaku [Mammalian Science] 30: 197–220 (in Japanese with English abstract)

33 Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickel cell anemia. Science 230: 1350–1354

34 Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487–491

35 Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425

36 Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. 2nd ed, Cold Spring Harbor Laboratory, New York

37 Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

38 Smith MF, Patton JL (1991) Variation in mitochondrial cytochrome *b* sequence in natural populations of South American akodontine rodents (Muridae: Sigmodontinae). Mol Biol Evol 8: 85–103

39 Thomas RH, Schaffner W, Wilson AC, Pääbo S (1989) DNA phylogeny of the extinct marsupial wolf. Nature 340: 465–467

40 Thomas WK, Pääbo S, Villablanca FX, Wilson AC (1990) Spatial and temporal continuity of kangaroo rat populations shown by sequencing mitochondrial DNA from museum specimens. J Mol Evol 31: 101–112

41 Wayne RK, Benveniste RE, Janczewski DN, O'Brien SJ (1989) Molecular and biochemical evolution of the Carnivora. In "Carnivore Behavior, Ecology, and Evolution" Ed by JL Gittleman, Cornell Univ Press, New York, pp 465–494

42 Wayne RK, Jenks SM (1991) Mitochondrial DNA analysis implying extensive hybridization of the endangered red wolf *Canis rufus*. Nature 351: 565–568

43 Wozencraft WC (1989) The phylogeny of the recent Carnivora. In "Carnivore Behavior, Ecology, and Evolution" Ed by JL Gittleman, Cornell Univ Press, New York, pp 495–535

44 Wozencraft WC (1989) Classification of the recent Carnivora. In "Carnivore Behavior, Ecology, and Evolution" Ed by JL Gittleman, Cornell Univ Press, New York, pp 569–593

45 Wozencraft WC (1993) Order Carnivora. In "Mammal Species of the World: A Taxonomic and Geographic Reference, 2nd ed" Ed by DE Wilson and DM Reeder, Smithsonian Inst Press, Washington, pp 279–348

46 Wurster DH, Benirschke K (1968) Comparative cytogenetic studies in the Order Carnivora. Chromosoma 24: 336–382