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Low Genetic Diversity in Japanese Populations of the Eurasian Badger *Meles meles* (Mustelidae, Carnivora) Revealed by Mitochondrial Cytochrome *b* Gene Sequences

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ABSTRACT—To assess the level of genetic variations of the Eurasian badger *Meles meles* in Japan, the entire sequences (1,140 base pairs) of the mitochondrial cytochrome *b* gene were phylogenetically examined. Most of substitutions between haplotypes were transitions resulting in synonymous mutations. A phylogenetic tree reconstructed by sequence differences clearly showed that Japanese populations of *Meles meles* were differentiated from continental populations (from the Baikal area and eastern Europe) of *M. meles*. By contrast, genetic distances among Japanese populations were much smaller, and their geographic structures did not reflect geographic distances between sampling localities. The results indicate that polymorphisms of the ancestral populations still remain via loss of haplotypes by population size changes. In addition, *M. meles* could have occupied the present habitats in Japanese main islands (Honshu, Shikoku, and Kyushu) in a short period, possibly after the last glacial age.

Key words: Eurasian badger, *Males meles*, cytochrome *b*, phylogeny, Japanese islands

INTRODUCTION

The Eurasian badger *Meles meles* (Linnaeus, 1758) currently occupies most of woodlands and steppe zones of the Palearctic region, from Asia through Central Asia to Europe (Corbet, 1978; Nowak, 1991). Geographic variations in cranial and external characters are known to be considerable. Ognev (1931) recognized six species of *Meles* in the Palearctic region including *Meles meles* (Linnaeus, 1758) (distributed in continental Europe); *M. leptorhynchus* Milne-Edwards, 1867 (distributed from Ural Mountains to Asia, including the form *amurensis* from Far East); and the Japanese badger *M. anakuma* Temminck, 1844. Most taxonomists, however, considered *M. meles* as a single species of the genus *Meles* (Ellerman and Morrison-Scott, 1951; Heptner *et al*., 1967; Corbet, 1978; Wozencraft, 1993). Heptner *et al*. (1967) examined cranial characters and coloration of *M. meles*, and recognized three groups of subspecies: "*meles*" (all Europe to east up to Volga River, Caucasus, and southern parts of Middle Asia), "*arenarius-leptorhynchus*" (to east from Volga River, Ural Mountains, and Siberia), and "*amurensis-anakuma*" (Prymorie, Korea, and Japan). Later, Baryshnikov and Potapova (1990) examined external characters of Palearctic badgers more thoroughly and supposed the existence of two allopatric badger species – the European badger *M. meles* and the Asian badger *M. anakuma* (including most of badger populations of the Asian continent and Japan). In addition, based on paleontological materials, they also suggested that European and Asian badgers evolved separately since the Middle Pleistocene. Lynch (1994) performed a multivariate analysis for craniometric variations among Eurasian badgers. He showed an east-west clinal variation across Eurasia and a

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sole cranially distinct form in Japan, suggesting that *Meles* is represented by two subspecies of a single species *Meles meles*: the nominotypical form, *M. meles meles*, which occurs throughout Eurasia and *M. m. anakuma* in Japan. Thus, taxonomy of the Eurasian badger is still controversial, while the Japanese badger populations are commonly considered to be morphologically distinct from the continental populations.

Kawamura *et al*. (1989) reported that fossils of *M. m. anakuma* were excavated from the layer of the Late Middle Pleistocene in southern Japan. Kaneko *et al*. (1996) reported that the body weight and size of the Japanese badgers are smaller than those of the British population. Neal and Cheeseman (1996) pointed out that the Japanese population has the smaller body size and lighter coat color when compared with continental populations. However, very little information has been known on phylogeny and genetics of the Japanese badger.

To estimate levels of genetic diversity in Japanese and some continental populations of *M. meles*, we sequenced the entire region (1,140 base-pairs, bp) of mitochondrial DNA (mtDNA) cytochrome *b* gene and constructed a molecular phylogeny. On the basis of the resultant tree, we discuss geographic variations and population structures of *M. meles* in Japanese islands.

MATERIALS AND METHODS

Samples and DNA extraction

Specimens of *Meles meles* examined in the present study are listed in Table 1 and Fig. 1. Muscles or hairs were obtained from cases of traffic accidents, animals captured at ecological surveys, or animals kept in zoos. The hog badger *Arctonyx collaris* from Thailand was used as an outgroup. Muscle tissues were frozen at –80°C or preserved in 70% ethanol at room temperature until use. Total DNAs were extracted from muscles by the phenol/proteinase K/sodium dodecyl sulfate 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 method of Walsh *et al*. (1991) as follows: 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. The supernatant of 10 μl was used as template of subsequent polymerase chain reaction (PCR) amplification.

PCR amplification and direct sequencing

The entire cytochrome *b* region (1,140 bp) was amplified using the two primers: Cb-M1 5'-CTCACATGGAATCTAACCATGAC-3'; Cb-MR1 5'-TCTTCCTTGAGTCTTAGGGAG-3' (Kurose *et al*., 2000) (Fig. 2). PCR amplification was performed in 50 μl of the reaction mixture. When PCR was inhibited for some reason, 20 μg of bovine serum albumin (Boehringer) was added into the reaction mixture. Thirty-five cycles were performed with the following programs using a DNA thermal cycler (PJ2000, Perkin-Elmer Cetus): denaturing at 94°C for 1 min; annealing at 50°C for 1 min; extension at 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 a DNA thermal cycler (PCR cycler 9700, Perkin-Elmer) and sequenced using the ABI Prism™ 377 automated sequencer. Sequencing primers were the same as the PCR primers, and the following internal primers Cb-L3 5'-CTTACATGTAGGACGAGGCCT-3'; Cb-L4 5'TCCCATTCCA-TCCATATTACAC-3'; Cb-LR3 5'GATTGCGTATGCGAATAAGAA-3'; Cb-LR4 5'-CGGTTGCACCTCAAAAAGACA-3'; Cb-LR5 5'-AGG-GGATACCAGAGGGGTT-3'; Cb-LR6 5'-GTAAGATTGCGTATGC-GAATAAG-3', were newly designed in the present study.

Sequence analysis

Sequence alignment was done using GeneWorks (Intelligenetics).

Table 1. Profile of the Eurasian badger *Meles meles* examined in the present study

Sample	Smaple name	Sampling locality	Locality No. in Fig. 1	Accession No. **
Meles meles	MR1	Japan: Morioka-shi, Iwate Pref.	1	AB049791
	TKY1	Japan: Hinode-cho, Tokyo	2	AB049803
	TKY12	Japan: Chichibu-shi, Saitama Pref.	3	AB049805
	MAT1	Japan: Machida-shi, Tokyo	4	AB049798
	CHI2	Japan: Chiba Pref.	5	AB049793
	JPN2(2)	Japan: Takayama-shi, Gifu Pref.	6	AB049794
	JPN3(3)	Japan: Wakayama Pref.	7	AB049796
	JPN4(4)	Japan: Gifu Pref.	6	AB049804
	JPN5(5)	Japan: Gifu Pref.	6	AB049792
	YMG1	Japan: Yamaguchi-shi, Yamaguchi Pref.	8	AB049795
	YMG3	Japan: Yamaguchi-shi, Yamaguchi Pref.	8	AB049797
	YMG4	Japan: Yamaguchi-shi, Yamaguchi Pref.	8	AB049790
	TOB1	Japan: Kawauchi-cho, Ehime Pref.	9	AB049801
	K1	Japan: Beppu-shi, Oita Pref.	11	AB049806
	K6	Japan: Oita Pref.	11	AB049800
	K7	Japan: Takeda-shi, Oita Pref.	11	AB049802
	K8	Japan: Kitakyushu-shi, Fukuoka Pref.	10	AB049799
	ZIS33	Russia: Transbaikalia, west coast of Goose Lake (ZIN O.35081*)	12	AB049807
	ZIS35	Russia: Leningrad Province (ZIN O.35056*)	13	AB049809
	ZIS36	Russia: Leningrad Province (ZIN O.35057*)	13	AB049808
Arctonyx collaris	CHI1	Thailand: Chiang Mai Zoo		AB049810

* Specimen number of Zoological Institute, Russian Academy of Sciences.
** Sequence data will appear in the DDBJ nucleotide sequence database with accession numbers.

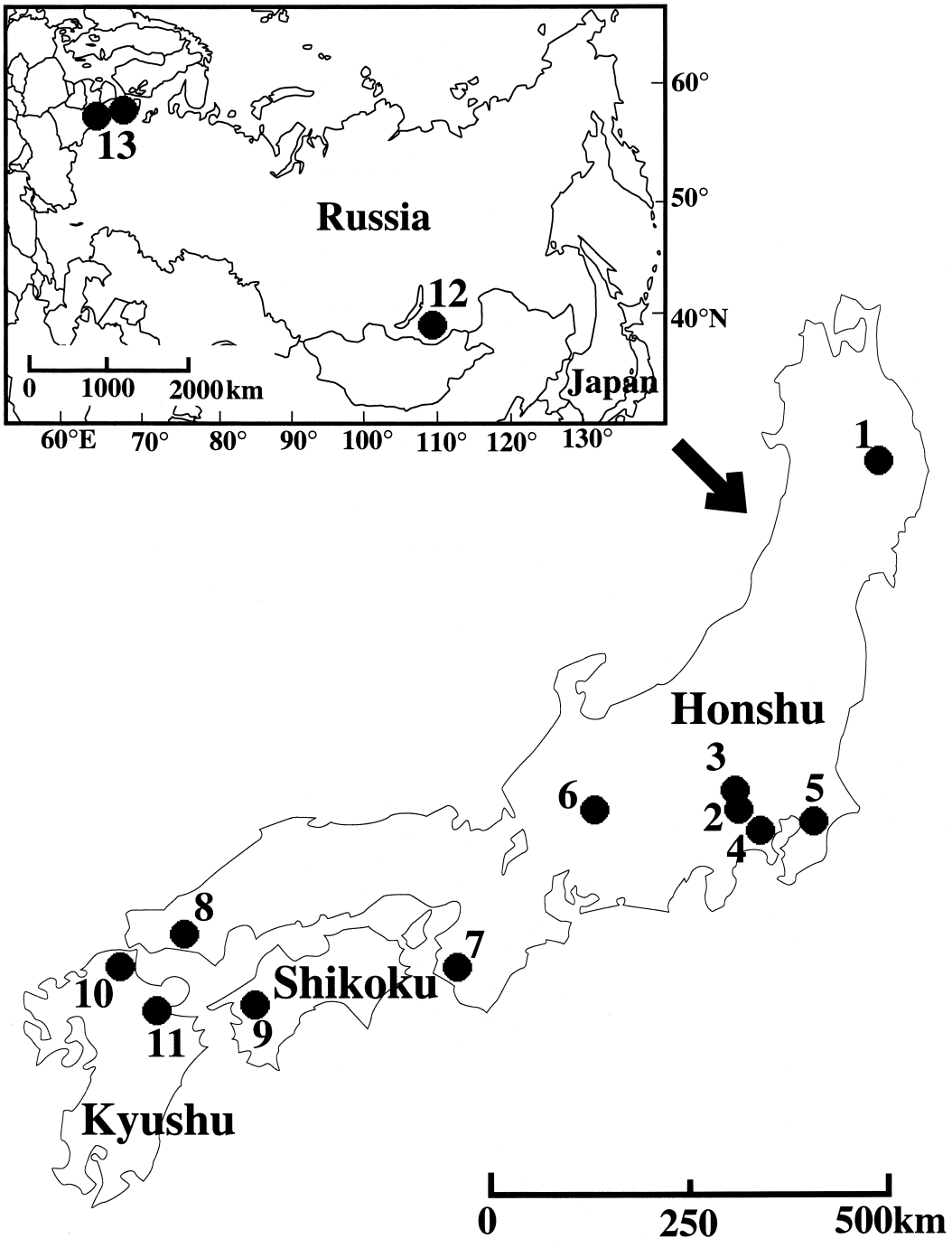

Fig. 1. Sampling localities of *Meles meles*. Numbers on the map refer to those in Table 1.

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

RESULTS

All the haplotypes of cytochrome *b* sequenced from 20 badgers were different from each other. The sequence alignment (Table 2) showed that 97 sites of 1,140 bp were variable among all of the 20 badgers (excluding outgroup). Transversions were observed at 13 sites (Table 2). Most nucleotide substitutions within the Japanese populations were transitions resulting in synonymous mutations. Percentage sequence difference among all badgers varied from 0.09% to 8.95% (2.31% in average). The average sequence difference within the Japanese populations (0.49%) was much smaller than that (6.95%) between the Japanese and continental populations.

The neighbor-joining phylogenetic tree (Fig. 2) indicated that the badgers can be divided into three groups. Two bad-

Table 2. Sequence alignment of the cytchrome *b* gene (1,140 bp) of the Eurasian badger *Meles meles*. Dots indicate identity with those of MR1.

	Variation
	1111111111222222222222222233333333334444444455555555555556666666
	1234446680011368889000011223344588802234556691124569901123456668990012337
Sample	5120581912514520368034709181436502794738573961464080810987634891272326098
MR1	TGCCCTCACACTACTTCCTCACCTTACACTGTCATTACATTGCAAAGTCACGAAATATTCTTCCTCACACTTT
TKY1	.....C.....G.....
TKY12	.....C.....C.....G.....
MAT1	.....A.....
CHI2	.....
JPN2	.....
JPN3	.....
JPN4	.....T.....C.....G....
JPN5	.....T.....
YMG1	.....T.....
YMG3	.....T.....
YMG4	.....
TOB1	.....C....G.....C.....
K1	.....
K6	.....G.....
K7	.....
K8	.....T.....
ZIS33	CA..T..GT.....CCT.C...T.....CA....CGT....T.C.....AG...TC...G.....A..
ZIS35	.ATA.CT..T.CGT.C.TC..TTCC.T...A.TG.C.TGCCATGT..A.GTAGG.C..CTCCTTCT.T..ACC
ZIS36	.ATA.CT..T.CGT.C.TC..TTCC.TG..A.TG.C.TGCCATGT..A.GTAGG.C..CTCCTTCT.T.TAC.

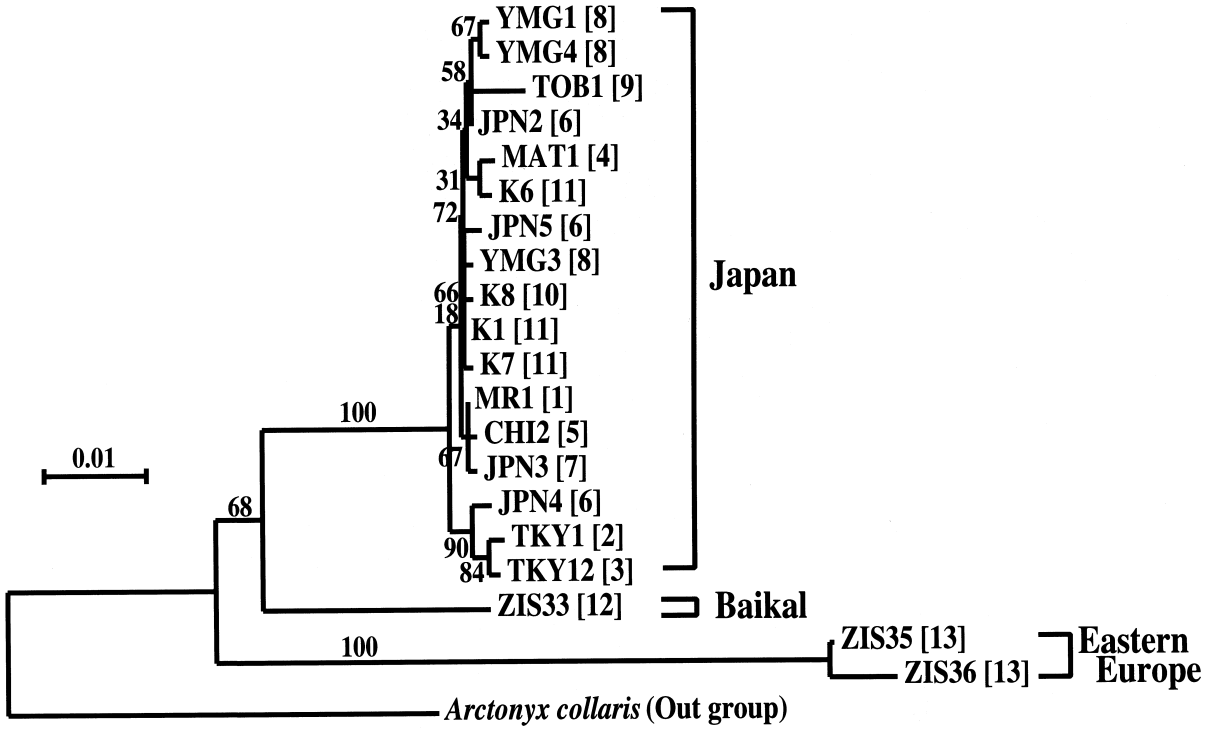

Fig. 2. Neighbor-joining relationships reconstructed by the cytochrome *b* nucleotide sequences (1,140 bp) for *Meles meles* and *Arctonyx collaris* (outgroup). The schale indicates genetic distance estimated with Kimura's two parameter method. Numbers (%) on internal branches are bootstrap values derived from 1,000 replications. Sample names with locality numbers in brackes refer to those in Table 1.

gers (ZIS35 and ZIS6) from a population in the eastern Europe were grouped together with a 100% bootstrape value. As the second group, a badger from the Baikal area (ZIS33) was split from the others. All Japanese badgers were clustered as the third group with a 100% bootstrap value (Fig. 2).

The parsimonious network (Fig. 3) exhibited a very simi-

site
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666666677777777777777888888888888888889999999999999000000000000111111
889999900112333455668001134445567778990001111245679012345566789001122
473456769340025503586476951392520346143695679428027389326958902353929
TCAACCTGCCGGTACCAGCTCTAACGTATTCCAATCCTACTATTCCCTAGCCGGCGGGTTTGCCATACT
....T..........T.AT...............G...C..............................T.
....T............A................G...C..............................T.
..........A....T..T...............G...................................
....A................
..........A.......................G...................................
............................A..
.................A................G...C..............................T.
.................................G..........T........................
..........A.......................G.............................T....
.................................G...................................
..........A.....T.................G.............................T.....
..........A....................T.G.............................T...G..
.................................G...................................
...............T..T..............G...................................
.............G...................G...................................
.................................G...................................
CT.G..C.T.....T..A.C.......G....G.C......GC.....GA...A.AA..C.A..GC...
..GGTT.ATT.AC.T..A.CTCGGT.C..CA.GG.TTC.TC....TTC.ATT.ATAAACC.A.....TC
..GGTT.ATT.AC.T..A.CTCGGTAC..CA.GG.TTCGTC..CTTTC.ATT.ATAAACCCA.....TC

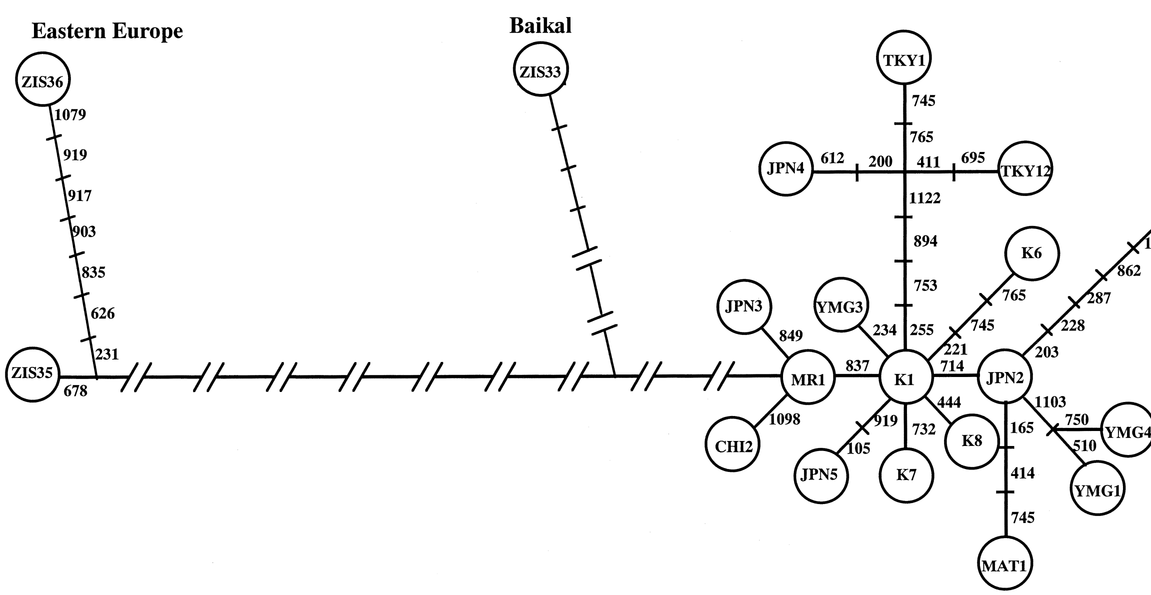

Fig. 3. Hand-drawn parsimonious networks of haplotypes for *Meles meles*. One slash indicates a presumed haplotype. One number above the line shows a site of nucleotide substitution.

lar relationship among haplotypes, where the eastern European and Baikal groups were again remote to the Japanese populations.

In the Japanese populations, the phylogenetic relationships between haplotypes were not always parallel with geographic distances between sampling localities (Figs. 2 and 3). For example, four individuals (TKY1, TKY12, MAT1, and CHI2) from eastern Japan 'Kanto District' did not form a common cluster. Three individuals from Gifu Prefecture (JPN2, JPN4, and JPN5), three from Yamaguchi Prefecture (YMG1, YMG3, and YMG4) and three from Oita Prefecture (K1, K6, and K7) were not also grouped at every prefecture.

DISCUSSION

The present study examined cytochrome *b* sequence

variations of *Meles meles* from Japanese islands and the Eurasian continent. The sequence differences between the Japanese and continental populations were remarkably large (Figs. 2 and 3). Based on morphological differences, the Japanese population was often classified as a distinct subspecies *M. m. anakuma* (Ellerman and Morrison-Scott, 1951; Heptner *et al*., 1967; Lynch, 1994; Abe, 1994). The large genetic differences between the Japanese and continental badgers obtained in the present study support the subspecies classification. Our preliminary study (A. V. Abramov *et al*., in preparation) also suggests that the Japanese population of *M. meles* has essentially smaller skulls and weaker dentition than continental populations, while it has some craniological similarities (reduction of first premolars and one-rooted second lower premolar) to the Siberian population.

On the other hand, genetic variations within the Japanese populations were relatively low (<1.32%; 0.49% in average) (Fig. 2). The phylogenetic relationships (genetic distances) between haplotypes did not always correspond with geographic proximity (geographic distances) between sampling localities. This indicates that polymorphisms of ancestral populations still remain via loss of haplotypes by population size changes. Furthermore, *M. meles* could have occupied the present habitats in Japanese islands (Honshu, Shikoku and Kyushu) in a short period after the last glacial age.

Similarly, we found the lack of genetic diversity between populations in the Japanese marten *Martes melampus*, a Japanese endemic species (except for Hokkaido) in Mustelidae, based on the study of cytochrome *b* gene sequences (Kurose *et al*., 1999). Intraspecific differences of *Martes melampus* were less than 1.58%, which is close to the value (1.32%) of the *Meles meles* variation obtained in the present study. Recently, Kaneko (2001) confirmed the occurrence of the delayed implantation in the Japanese population of *Meles meles*. In addition, Tatara *et al*. (1994) reported that *Martes melampus* has the similar mechanism of delayed implantation to *Meles meles*. These two species of Mustelidae might have experienced the similar history of migration and expansion of their habitats in Japanese islands.

The individuals from the Eurasian continent were significantly differentiated between Siberian (Baikal area) and eastern European populations in *M. meles*. These populations might have been segregated by a geographic barrier such as Ural Mountains. Ognev (1931) and Heptner *et al*. (1967) supposed that the geographic barriers such as Ural Mountains or Volga River might have segregated the populations. Otherwise, geographic isolation by distance likely have genetically differentiated the continental populations. In order to further elucidate the migration history of *M. meles* in Eurasia, it is necessary to investigate genetic structures of continental populations using specimens from other comprehensive localities.

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