Phylogeographic Sympatry and Isolation of the Eurasian Badgers (Meles, Mustelidae, Carnivora): Implications for an Alternative Analysis using Maternally as Well as Paternally Inherited Genes

Authors: Sara Tashima, Yayoi Kaneko, Tomoko Anezaki, Minoru Baba, Shuuji Yachimori, et. al.

Source: Zoological Science, 28(4) : 293-303

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.28.293
Phylogeographic Sympatry and Isolation of the Eurasian Badgers (*Meles*, Mustelidae, Carnivora): Implications for an Alternative Analysis Using Maternally as well as Paternally Inherited Genes

Sara Tashima¹, Yayoi Kaneko², Tomoko Anezaki³, Minoru Baba⁴, Shuuji Yachimori⁵, Alexei V. Abramov⁶, Alexander P. Saveljev⁷ and Ryuichi Masuda¹*

¹Department of Natural History Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan
²Tokyo University of Agriculture and Technology, Fuchu 183-8538, Japan
³Gunma Museum Natural History, Tomioka 370-2345, Japan
⁴Kitakyushu Museum of Natural History and Human History, Kitakyushu 805-0071, Japan
⁵Shikoku Institute of Natural History, Susaki 785-0023, Japan
⁶Zoological Institute, Russian Academy of Sciences, Saint-Petersburg 199034, Russia
⁷Russian Research Institute of Game Management and Fur Farming, Russian Academy of Agricultural Sciences, Kirov 610000, Russia

In the present study, to further understand the phylogenetic relationships among the Eurasian badgers (*Meles*, Mustelidae, Carnivora), which are distributed widely in the Palearctic, partial sequences of the mitochondrial DNA (mtDNA) control region (539–545 base-pairs) as a maternal genetic marker, and the sex-determining region on the Y-chromosome gene (*SRY*: 1052–1058 base-pairs), as a paternal genetic marker, were examined. The present study revealed ten *SRY* haplotypes from 47 males of 112 individuals of the Eurasian Continent and Japan. In addition, 39 mtDNA haplotypes were identified from those animals. From the phylogeography of both the uniparentally inherited genes, four lineages were recognized as Japanese, eastern Eurasian, Caucasian, and western Eurasian. The distribution patterns of the mtDNA lineages showed the existence of a sympatric zone between the eastern and western Eurasian lineages around the Volga River in western Russia. Furthermore, the present study suggested that in the Japanese badgers, the larger genetic differentiation of the Shikoku population was attributable to geographic history in the Japanese islands.

Key words: Eurasian badger, *Meles*, mitochondrial DNA, phylogeography, *SRY* gene, contact zone

INTRODUCTION

The Eurasian badgers (*Meles*, Mustelidae, Carnivora) are medium-sized mustelids widely distributed in the Palearctic, from the Japanese Islands in the East to the British Islands in the West across the Eurasian Continent, and have adapted themselves to various environments, such as deciduous woodlands, pastures, arable lands, sand dunes, steppes, semi-deserts, and mountainous districts (Neal and Cheeseman, 1996). The distribution range of the extant badgers probably arises from that of its ancestor, the genus *Melodon*, which originated from south China, where fossils with similar morphologies were found in the late Pliocene layer of China (Neal and Cheeseman, 1996). The distribution range of the extant badgers probably arises from that of its ancestor, the genus *Melodon*, which originated from south China, where fossils with similar morphologies were found in the late Pliocene layer of China (Neal and Cheeseman, 1996). The *Meles* lineage must have spread to Europe between the late Pliocene and the early Pleistocene, based on evidence that the oldest fossils of the badger in Europe, *Meles thorali*, were excavated from a layer representing the late Pliocene in France (Guérin et al., 2004) and Greece (Lyras and van der Geer, 2007). Morphological study of molars indicated that the *Meles* lineage diverged into European and Asian lineages after the westward spread (Baryshnikov et al., 2003). The divergence is thought to be caused by glaciations of mountains, transgression of the Caspian Sea, restructing of landscape, and other paleontological factors (Baryshnikov et al., 2003; Abramov and Puzachenko, 2005). Ellerman and Morrison-Scott (1951) recognized at least 23 subspecies or geographical groups in *Meles meles*. By contrast, Heptner et al. (1967) reported that the genus *Meles* consists of only one species, *Meles meles*, and that three groups of subspecies are recognized: “meles” in Europe and the west of the Volga River, “arenarius-leptorhynchus” in the east of the Volga...
River to Siberia, and “amurensis-anakuma” in eastern Asia including Japan. Recently, however, several researchers proposed that the genus Meles consists of three species, the European badger *M. meles*, the Asian badger *M. leucurus*, and the Japanese badger *M. anakuma*, based on cranial characters (Lynch, 1994; Abramov, 2001; Abramov and Puzachenko, 2006), pattern of mask coloration (Abramov, 2003), molars (Baryshnikov et al., 2003), baculum (Abramov, 2002), and external parasites (Abramov and Medvedev, 2003).

In addition to morphological studies, molecular phylogenetic analyses using mitochondrial DNA (mtDNA) revealed variations and characteristics among regional populations of the Eurasian badgers. Kurose et al. (2001) investigated the molecular phylogenetics of the mtDNA cytochrome b gene, and reported a large differentiation among badgers of Japan, Siberia (Transbaikalia), and Europe (western Russia). Furthermore, based on mtDNA control region sequences, Marmi et al. (2006) showed that the Eurasian badgers are divided into four groups: Europe, Southwest Asia, North and East Asia, and Japan.

Among badgers in the Eurasian Continent, the boundary of the European and Asian groups is thought to be located around the Volga River or the Ural Mountains (Ognev, 1931; Heptner et al., 1967). Abramov et al. (2003) reported, based on skull morphologies and fur colorations, that the area around the Volga River or the Ural Mountains (Ognev, 1931; Abramov et al., 2003) is a sympatric zone for the European and Asian groups. Abramov et al. (2003) also reported that the area around the Volga River or the Ural Mountains (Ognev, 1931; Abramov et al., 2003) is a sympatric zone for the European and Asian groups. Abramov et al. (2003) reported, based on skull morphologies and fur colorations, that the area around the Volga River or the Ural Mountains (Ognev, 1931; Abramov et al., 2003) is a sympatric zone for the European and Asian groups.
The Kanto population consists of badgers from Tokyo, Gunma, and Chiba Prefectures. The Shikoku population consists of badgers from Kochi Prefecture. The Kyushu population consists of badgers from Oita, Fukuoka, Kumamoto, and Nagasaki Prefectures. Because only one or four samples were available from Iwate, Gifu, and Yamaguchi Prefectures, they were not treated as populations.

### Amplification and sequencing of the mtDNA control region

For Ta-cloning of PCR products, the mtDNA control region was amplified using two primers: UR1 (Taberlet and Bouvet, 1994) and Car125-R (Shimatani et al., 2008). A total of 20 μl of the PCR reaction mixture containing 2 μl of 10×PCR buffer (Takara), 1.6 μl of 2.5 mM dNTP mixture, 0.2 μl of rTaq DNA polymerase (5 units/μl, Takara), 0.5 μl of 20 mg/ml bovine serum albumin (Roche), 0.2 μl of each of the above two primers (25 pmol/μl) and 2.5 μl of the DNA extract from hair of one badger (sample code MEL-MM2, Table 1). The PCR condition was one cycle of 94°C for 3 min; 30 cycles of 94°C for 1 min; 55°C for 1 min; 72°C for 1 min; and one cycle of 72°C for 10 min. The PCR amplifications were carried out in a PCR thermal cycler TP600 (Takara).

The PCR products were cloned using the Original TA Cloning Kit (Invitrogen) and following the manufacturer’s instructions. Plasmid DNA obtained by the TA cloning was extracted into 50 μl TE buffer with the QiAprep Miniprep Kit (Qiagen) and preserved at 4°C. The PCR conditions were one cycle of 94°C for 3 min; 30 cycles of 94°C for 30 sec; 55°C for 30 sec; 72°C for 1 min; 1 cycle of 72°C, 7 min, using the Thermo Sequence pre-mixed cycle sequencing kit (Amersham). The PCR products were run using an automated DNA sequencer (Hitachi SQ5500). Universal primers T7 and M13 primers (sequences shown in the Original TA Cloning Kit, Invitrogen) labeled at the 3’end with Texas-Red were used for sequencing primers.

From obtained nucleotide sequences, the following three primers for PCR and sequencing were newly designed in the present study: ANK-R1 (5'-ATGGTCTGTAAACATTGATGCT-3'), MeIDS-F (5'-ACATACTGTTGTCATGC-3') and MeIDS-R2 (5'-GGCCTTTATGGCCATAATG-3').

For direct sequencing, the 5’ portion of the mtDNA control region was amplified using primers UR1 and ANK-R1. A total volume of 50 μl of the PCR reaction solution consisted of 5 μl of 10×PCR buffer (Takara), 4 μl of 2.5 mM dNTP mixture, 0.25 μl of rTaq DNA polymerase (5 units/μl, Takara), 0.5 μl of each of the above two primers (25 pmol/μl), and 1–5 μl of extracted DNA (about 1–100 ng/μl). The PCR reactions were carried out in a PCR thermal cycler TP600 (Takara), and the PCR cycle condition was one cycle of 94°C for 3 min; 40–45 cycles of 94°C for 1 min; 50°C for 2 min; 72°C for 1 min; and one cycle of 72°C for 10 min. The PCR products were purified with the QiAquick PCR Purification Kit (Qiagen) and subjected to the cycle PCR Sequencing reaction. The reaction condition was one cycle of 94°C for 3 min; 30 cycles of 94°C for 30 sec; 55°C for 30 sec; 72°C for 1 min; and one cycle of 72°C for 7 min, using a Thermo Sequence pre-mixed cycle sequencing kit (Amersham). The PCR products were run using an automated DNA sequencer (Hitachi SQ5500). Cycle PCR using UR1, ANK-R1, MeIDS-F and MeIDS-R2 labeled at the 3’ end with Texas-Red and nucleotide sequencing were the same as mentioned above.

### Amplification and sequencing of the SRY gene

To amplify the SRY coding region and the adjacent non-coding region, two PCR primers, MSRy-R2 and MSry-R2 (Yamada and Masuda, 2010) were used. A total of 50 μl of the PCR reaction solution contained 5 μl of 10×PCR buffer (Takara), 4 μl of 2.5 mM dNTP mixture, 0.25 μl of rTaq DNA polymerase (5 units/μl, Takara), 0.5 μl of each of the above two primers and 1–5 μl of the DNA extract. The PCR reactions were carried out in a PCR thermal cycler TP600 (Takara) and the reaction condition was one cycle of 94°C for 3 min; 45 cycles of 94°C for 1 min; 50°C for 1.5 min; 72°C for 1 min; and one cycle of 72°C for 10 min.

The DNA samples, from which the above amplification failed with rTaq polymerase, were applied to the Multiplex PCR Kit (Qiagen): the PCR reaction solution contained 25 μl of Multiplex master mix, 0.5 μl of each of the above two primers (25 pmol/μl).
and 5–10 μl of the DNA extract, and was totalized with 5 μl of
DNase free water. The PCR reactions were carried out in a PCR
thermal cycler TP600 (Takara), and the PCR cycle condition was
one cycle of 95°C for 15 min; 40 cycles of 94°C for 30 sec; 55°C
for 30 sec; 72°C for 1 min; and one cycle of 72°C for 10 min.

The PCR product purification and cycle PCR using MSRY-
SQF1, MSRY-SQF3, MSRY-SQR1 and MSRY-SQR3 (Yamada and
Masuda, 2010) 3’-labeled with Texas-Red were the same as men-
tioned above.

Phylogenetic analysis of nucleotide sequences

A sequence alignment was performed by CLUSTAL W (Thompson et al., 1994).
Sequence differences were calculated by MEGA 4 (Tamura et al., 2007) and the hap-
loftype diversity and nucleotide diversity were calculated by ARLEQUIN 3.1 (Excoffier et al., 2005). A phylogenetic tree by the neighbor-
joining method (Saitou and Nei, 1987) was constructed using MEGA 4. The mtDNA
control region sequence of the hog badger Arctonyx collaris (536 base-pairs, bp:
accession number AJ563704: Marmi et al., 2006), and the SRY sequences of the
Japanese marten Martes melampus (accession number AB491590: Yamada and
Masuda, 2010) and the sable Martes zibellina (accession number AB491569: Yamada and
Masuda, 2010) were used as an outgroup.

Moreover, for the mtDNA analysis, val-
ues of Tajima’s D (Tajima, 1989) and Fu’s
F may be calculated to test the
recent population expansion of the Japanese
badgers. In addition, to estimate the genetic
structures of the Japanese badgers, the
hierarchical analyses of molecular variance
(AMOVA) (Excoffier et al., 1992) were per-
formed in ARLEQUIN 3.1. This analysis
evaluates genetic variations of three parti-
tions: among groups, among populations
within groups, and within populations. Here,
each regional population such as Tohoku
and Kanto was defined as a “population.”
Two “groups” were set: Group 1 including
one population (Kanto, Shikoku or Kyushu)
and Group 2 including the other populations.

RESULTS

Sequence variations of the mtDNA
control region of the Eurasian bad-
gers

An alignment of the mtDNA control
region (539–545 bp) of the Eurasian
badgers showed that nucleotide substi-
tutions and indels occurred at 74 sites
(Table 2). Sequence differences of
Kimura’s two-parameter distances
(Kimura, 1980) among all Eurasian
badgers were 0.0–6.2% (2.3% on aver-
age). From the continental badgers
having 59 polymorphic sites, 12 haplo-
types (E-1 to E-6, C-1, C2, and W-1 to
W-4) were identified (Table 3), and the
sequence differences were 0.2–5.8%
(3.6% on average). From the Japanese badgers having 28
polymorphic sites, 27 haplotypes (J-1 to J-27) were identi-
cified (Table 3), and the sequence differences were 0.2–1.7%
(0.6% on average).

The neighbor-joining phylogenetic tree (Fig. 2) showed that
mtDNA haplotypes were grouped into four clades with
70–100% bootstrap values, all of which were distributed all-
opatrically: clade 1 consisting of J-1 to J-27 identified from

Fig. 1. (A) The sampling locations in Eurasia. Numerals in circles are the numbers of indi-
viduals from those locations. (B) The sampling locations on the Japanese islands. Numerals
in parentheses are the numbers of individuals from those locations.
Table 2. Alignment of the mtDNA control region haplotypes (539–545 bp) identified from the Eurasian badgers. Dots represent nucleotides identical with those of haplotype J-1. Dashes indicate deletions.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Haplotype</th>
<th>Geographic distribution of the mtDNA control region haplotypes in the Eurasian badgers.</th>
<th>Japanese islands</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade 1</td>
<td>J-1</td>
<td>Iwate 1, Kanto 1, Gifu 2, Yamanaguchi 3, Shikoku 4, Kyushu 8, Chelyabinsk 1, Amur 1, Kirov 1, Leningrad 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clade 2</td>
<td>E-1</td>
<td>Iwate 1, Kanto 1, Gifu 2, Yamanaguchi 3, Shikoku 4, Kyushu 8, Chelyabinsk 1, Amur 1, Kirov 1, Leningrad 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clade 3</td>
<td>C-1</td>
<td>Iwate 1, Kanto 1, Gifu 2, Yamanaguchi 3, Shikoku 4, Kyushu 8, Chelyabinsk 1, Amur 1, Kirov 1, Leningrad 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Geographic distribution of the mtDNA control region haplotypes in the Eurasian badgers.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Haplotype</th>
<th>Japanese islands</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade 1</td>
<td>J-1</td>
<td>Iwate 1, Kanto 1, Gifu 2, Yamanaguchi 3, Shikoku 4, Kyushu 8, Chelyabinsk 1, Amur 1, Kirov 1, Leningrad 1</td>
<td></td>
</tr>
<tr>
<td>Clade 2</td>
<td>E-1</td>
<td>Iwate 1, Kanto 1, Gifu 2, Yamanaguchi 3, Shikoku 4, Kyushu 8, Chelyabinsk 1, Amur 1, Kirov 1, Leningrad 1</td>
<td></td>
</tr>
<tr>
<td>Clade 3</td>
<td>C-1</td>
<td>Iwate 1, Kanto 1, Gifu 2, Yamanaguchi 3, Shikoku 4, Kyushu 8, Chelyabinsk 1, Amur 1, Kirov 1, Leningrad 1</td>
<td></td>
</tr>
</tbody>
</table>

Clade 4

<table>
<thead>
<tr>
<th>Clade 4</th>
<th>Haplotype</th>
<th>Japanese islands</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade 1</td>
<td>J-1</td>
<td>Iwate 1, Kanto 1, Gifu 2, Yamanaguchi 3, Shikoku 4, Kyushu 8, Chelyabinsk 1, Amur 1, Kirov 1, Leningrad 1</td>
<td></td>
</tr>
<tr>
<td>Clade 2</td>
<td>E-1</td>
<td>Iwate 1, Kanto 1, Gifu 2, Yamanaguchi 3, Shikoku 4, Kyushu 8, Chelyabinsk 1, Amur 1, Kirov 1, Leningrad 1</td>
<td></td>
</tr>
<tr>
<td>Clade 3</td>
<td>C-1</td>
<td>Iwate 1, Kanto 1, Gifu 2, Yamanaguchi 3, Shikoku 4, Kyushu 8, Chelyabinsk 1, Amur 1, Kirov 1, Leningrad 1</td>
<td></td>
</tr>
</tbody>
</table>
the Japanese badgers, clade 2 consisting of E-1 to E-6 from the eastern Eurasian badgers, clade 3 consisting of C-1 and C-2 from the Caucasian badgers, and clade 4 consisting of W-1 to W-4 from the western Eurasian badgers. In addition, Fig. 2 showed the close relatedness between clades 1 and 2, with a 99% bootstrap value, and between clades 3 and 4 with an 84% bootstrap value.

The mtDNA control region sequences determined in the present study were deposited to DDBJ/GenBank/EMBL databases with the following accession numbers: AB538970–AB539001 and AB551122–AB551128.

Population structures among regional populations, based on mtDNA haplotype distribution

For the Eurasian Continent, both haplotype E-4 of clade 2 (eastern Eurasia) from one badger and haplotypes W-1 and W-4 of clade 4 (western Eurasia) from one and three badgers, respectively, were found in Kirov Province, indicating that the area of Kirov located between the Volga and the Kama Rivers is a contact zone of distributions of clades 2 and 4 (see the sampling location in Fig. 1). There were no haplotypes shared between Kirov and Chelyabinsk Province in Russia, except for E-4 (Table 3).

On the other hand, on the Japanese Islands, three out of the 27 haplotypes were shared by different island-populations, whereas the other 24 haplotypes were not shared by any regional populations and specific to each region: for example, three haplotypes J-18, J-19 and J-20 were found from only the badgers from the Shikoku Island. To test the hypothesis of a recent population expansion, which was suggested by mtDNA cytochrome b data in the study of Kurose et al. (2001), values of Tajima’s D and Fu’s F_S were calculated. If a population has experienced a recent population expansion and not reached an equilibrium, due to a bottleneck, the D value and F_S value would be expected to be negative. In the present study, the Tajima’s D value was negative (D = −1.445), and statistically significant (P value = 0.049). In addition, Fu’s F_S value was significantly negative (F_S = −15.544, P = 0.000). The results indicate their experience of the recent population expansion in the Japanese islands.

For the Japanese badgers, the percentages of variations in each partition by ANOVA are shown in Table 4. The percentage of variation ‘between groups’ was the highest (34.99%) in a comparison of the Shikoku population versus the other populations, whereas the lowest value (−12.30%) was obtained in a comparison of the Kyushu population versus the other populations. This shows that the Shikoku population has been genetically differentiated from the other populations.

Sequence variations and phylogeny of the SRY gene

The SRY sequences (1052–1058 bp), including the coding region (660 bp) and a part of non-coding region (392–398 bp), were determined for 48 male badgers, and the sequence alignment showed 29 polymorphic sites, including indel sites (Table 5). The SRY coding region spread between nucleotide sites 234–893, including four non-synonymous substitutions (nucleotide sites 643, 675, 682 and 697). Sequence differences were 0.1–1.7% (0.7% on average).

Among the continental badgers showing 15 polymorphic sites were found and four haplotypes (Me1, Me2, Mc1 and Mw1) were identified. Within the continental badgers, eight males from Chelyabinsk showed either of two haplotypes Me1 and Me2, but two males from Armenia in the Caucasus shared one haplotype (Mc1), and two males from Kirov and two from Leningrad Province in western Russia commonly

| Table 4. Percentages of variations of mtDNA control region haplotypes in the Japanese badgers by AMOVA. Values in parentheses are variance components. The Kanto population consists of badgers from Tokyo, Gunma, and Chiba. The Shikoku population consists of badgers from Kochi. The Kyushu population consists of badgers from Oita, Fukuoka, Kumamoto, and Nagasaki. |
|---|---|---|---|---|
| Group 1 | Group 2 | Between groups | Among populations within groups | Within populations |
| Kanto | Iwate, Gifu, Yamaguchi, Shikoku, Kyushu | −7.12 (−0.12) | 30.84 (0.51) | 76.27 (1.25) |
| Shikoku | Iwate, Kanto, Gifu, Yamaguchi, Kyushu | 34.99 (0.64) | 1.20 (0.02) | 63.81 (1.17) |
| Kyushu | Iwate, Kanto, Gifu, Yamaguchi, Shikoku | −12.30 (−0.10) | 32.22 (0.50) | 80.08 (1.25) |

Fig. 2. A neighbor-joining tree of the mtDNA control region haplotypes. Numbers near internal branches are bootstrap values (>70%) derived from 1,000 replications. Clade names refer to those in Table 2. The control region sequence from the hog badger Arctonyx collaris (accession no. AJ563704) was used as an outgroup. The scale below the tree shows Kimura’s two-parameter distances.
possessed another haplotype Mw1 (Table 6). Two haplotypes Me1 and Me2 from eastern Eurasia shared a specific insertional sequence TCCCCC at nucleotide sites 164–169 (Table 5). A haplotype Mj1 was predominantly found, and shared by ten males from the Kanto region, two males from Shikoku, and five males from Kyushu.

A neighbor-joining phylogenetic tree (Fig. 3) showed that SRY haplotypes were divided into four lineages: lineage 1 consisting of Mj1–Mj6 from the Japanese badgers, lineage 2 consisting of Me1 and Me2 from the eastern Eurasian badgers, lineage 3 for Mc1 of the Caucasian badgers, and lineage 4 for Mw1 of the western Eurasian badgers. The close relationship between lineage 1 (Japanese badgers) and lineage 2 (Eastern Eurasian badgers), and that between lineage 3 (Caucasian badgers) and lineage 4 (Western Eurasian badgers) were supported by 90% and 56% bootstrap values, respectively (Fig. 3). The branching topologies of the SRY haplotype lineages (Fig. 3) were in concordance with those in the phylogenetic tree of the mtDNA control region haplotypes (Fig. 2).

The haplotype diversities in lineage 1 (Japanese badgers) and lineage 2 (eastern Eurasian badgers) were 0.3708 and 0.5357, respectively. Because there was only one haplotype identified from the Caucasian badgers (lineage 3) and western Eurasian badgers (lineage 4), respectively, their haplotype diversities were zero.

As we analyzed the SRY gene of Meles species for the first time, the SRY sequences determined in the present study were deposited to DDBJ/GenBank/EMBL databases with the following accession numbers: AB539127–AB539136.

**DISCUSSION**

**Evolutionary features of the SRY gene as paternal genetic marker in the Eurasian badgers**

Nucleotide sequences of the SRY gene obtained in the present study consisted of the coding region (660 bp), of which protein comprises 52 N-terminal amino acid residues, the HMG-box region, and 90 C-terminal amino acid residues, and its 5′- and 3′-flanking regions (233 bp and 154 bp, respectively). In the Japanese and the eastern Eurasian lineages, there were no substitutions within the coding region in each lineage, whereas the 3′- and 5′-flanking regions were variable. Of the 29 polymorphic sites, 12 and nine sites were found in the 3′- and 5′-flanking regions, respectively. All nonsynonymous substitutions obtained from the Eurasian badgers occurred in the N-terminal region and the C-terminal region, where the sequence of the HMG-box region was highly conserved among the badgers. The previous studies also reported that the HMG-box sequences are highly conserved among species in cetaceans (Nishida et al., 2003) and felids (King et al., 2007).

In contrast, as the SRY protein outside of HMG-box was reported to have no functions (Whitfield et al., 1993), non HMG-box sequences are poorly conserved in primates (Whitfield et al., 1993), rodents (Tucker and Lundrigan, 1993) and felids (King et al., 2007). The evolutionary features of the SRY gene of the Eurasian badgers were in concordance with other mammalian species. In addition, the present study revealed that the molecular phylogeny of the SRY gene reconstructed zoogeographical history within the Eurasian badgers, in agreement with data of mtDNA, which is maternally inherited. Thus, the speciation among Eurasian badgers was inferred.
badgers have been well advanced as shown by differentiations of the SRY sequences with a slower mutation rate.

**Phylogenetic features revealed by the mtDNA control region as well as SRY gene sequences**

The present study demonstrated that the Eurasian badgers (genus *Meles*) are divided into at least four groups as shown not only by the use of the mtDNA control region as a maternal genetic marker, but also of the SRY gene as a paternal genetic marker. Paternal phylogenetic data of the Eurasian badgers are presented for the first time in the present study. The results of the present study were not discordant with a previous study (Marmi et al., 2006) that showed that the mtDNA phylogeography of the Eurasian badgers can be separated into four groups: Europe, South and West Asia, East Asia, and Japan. Therefore, the present study and Marmi et al. (2006) support the taxonomical view that the genus *Meles* should be classified as *M. meles* (European badger), *M. leucurus* (Asian badger), and *M. anakuma* (Japanese badger) (Abramov, 2003; Abramov and Puzachenko, 2006; Wozencraft, 2005; Larivière and Jennings, 2009), and also the view that *M. meles* in western Eurasia can be further separated morphologically into the European badger and the Transcaucasian badger (Baryshnikov et al., 2003; Abramov and Puzachenko, 2005, 2006).

The fossils of the genus *Meles* including extinct and extant species were excavated from layers of the late Pliocene to the middle Pleistocene in China and Europe (Madurell-Malapeira et al., 2009), and there is no doubt that the genus *Meles* has been distributed widely over the Eurasian Continent since that time. Indeed, the neighboring trees of both mtDNA and the SRY gene indicated that *Meles* first diverged into the Asian lineage including the Japanese and eastern Eurasian clades, and the European lineage including western Eurasian and Caucasian clades, supporting the scenario proposed by Baryshnikov et al. (2003). The European lineage then split into western Eurasian and Caucasian clades, while the Asian lineage diverged into eastern Eurasian and Japanese clades. The calculated divergence time between western Eurasian and Caucasian clades, and that between Japanese and eastern Eurasian clades, were about 1.1 million years ago (Mya) and 0.5 Mya, respectively, by using the divergence rate of $1.92 \times 10^{-8}$ substitutions/nucleotide/year for the mtDNA control region estimated by Marmi et al. (2006). The causes of divergence among these lineages in the Eurasian Continent were thought to be geographic isolations due to mountain glaciation, transgression of the Caspian Sea, landscape rearrangements in the glacial epoch, and other paleogeographic factors (Baryshnikov et al., 2003; Abramov and Puzachenko, 2005). Actually, in the divergence time between western Eurasian and Caucasian clades, the Caspian Sea transgressed and connected with the present Black Sea (Dumont, 1998). The transgression might be one of the causes of the split, and lasted until 0.7 Mya (Dumont, 1998). After the regression, the Caucasus Mountains and the Black Sea would have functioned as geographic barriers that obstructed gene flow (e.g. Seddon et al., 2002). Thus, the genetic differentiations among the three groups of *Meles* in the continent could have been conducted due to such geographical isolations.

On the other hand, for the divergence of the Asian lineage, the fossil records in Japan indicate that the badgers had migrated to the Japanese Islands around 0.43 Mya (Ogino et al., 2009). In present Japan, the badgers occur on the three main islands consisting of Honshu, Shikoku and Kyushu Islands (see Fig. 1B), except Hokkaido, indicating that the badgers migrated to the Japanese islands through the land bridge(s), which was formed in the Korea Strait (Kawamura et al., 1989; Kawamura, 1991). The land bridge(s), which had connected the Japanese islands with the Eurasian Continent, were thought to have reformed and disappeared several times following eustatic changes of sea level, and the Japanese islands have been disconnected from the continent since about 0.1 Mya, when the Korean Strait was formed (Ohshima, 1990). Considering the palaeoenvironmental changes around the Japanese islands, the divergence between the Japanese and eastern Eurasian clades may have already started prior to the final formation of the Korea Strait.

**Sympatric zone of two types of badgers in western Russia**

One (sample code: MEL-KR/V5 in Table 1) of the five individuals from Kirov Province, which is located in the area between the Volga River and the Kama River in the Vyatka River watershed (see Fig. 1A), possessed mtDNA haplotype E-4 belonging to the eastern Eurasian clade, that was the predominant haplotype in Chelyabinsk Province (Table 1 and Table 3). The distribution border between eastern and western Eurasian badgers has been thought to be the Volga River or the Ural Mountains (Ognev, 1931; Heptner et al., 1967). Based on morphological characters of skulls and furs, Abramov et al. (2003) reported that the boundary of the range between the European and the Asian badgers is located along the Volga River, and that the sympatric zone between the two forms is a country between the Volga River and the Kama River (see Fig. 1A). The present study of mtDNA phylogeography also strongly showed that the boundary and contact zone of the two forms are located around the Volga River, unfortunately, however, morphological information on samples of Kirov Province was not available. The current range revealed by the present genetic study suggests that the Ural Mountains do not act as geographic barrier to the Eurasian badgers. The Ural Mountains were formed in the late Carboniferous period and are presently the oldest mountains in the world. The highest mountain in the northern Urals is Mt. Narodnaya at 1,894 meters high located at 65°02'N, and Mt. Yamantau at 1,638 meters high located at 54°26'N is the highest in the southern area (Hara, 2007). But the area between the two highest mountains is covered with smaller mountain ranges (Hara, 2007). Since the Eurasian badgers are found at a sea level of 2,500–3,000 meters in the Tien Shen Mountains (Novikov, 1956), the Ural Mountains could not have been geographic barriers for migration of badgers. It is known that geographical barriers such as the Ural Mountains may have blocked east- or westward migrations of some mammals. This fairly widespread phylogeographic model has been identified among a number of mammal species, such as the root vole (*Microtus oeconomus*, Brunhoff et al., 2003), the collared lemmings (*Dicrostonyx* spp., Fedorov et al., 1999) and the common vole (*Microtus arvalis*, Haynes et al., 2003).
Among these species, the suture zones between genetically distinct lineages are likely to lie near the range of the Ural Mountains that may have represented a barrier for dispersal. The same model was suggested for the badgers (Marmi et al., 2006). By contrast, works on other mammal species, namely, the field voles (Microtus arvalis, Jaarola and Searle, 2002), the flying squirrels (Pteromys volans, Oshida et al., 2005), both the pygmy and common shrews (Sorex minutus and S. araneus, Blinton et al., 1998) and the brown bear (Ursus arctos, Korsten et al., 2009) have presented a different model for the mammalian phylogeography, suggesting that current populations of these species exhibit closely related maternal haplotypes throughout northern continental Eurasia. Such is likely to be the case of the European and Asian badgers discussed in the present study. Our data do not allow us to identify the Ural Mountains as the modern suture zone between both species. On the basis of the findings of craniological subfossils of Meles, it is evident that until the Late Holocene epoch the eastern limit of the European badger’s range used to reach the Ural but later shifted westwards to the current location around the Volga River (Gasilin and Kosintsev, 2010). At the same time, the range of Asian badger began shifting to regions west of the Ural.

However, no evidence was obtained for occurrence of both lineages of the paternally inherited SRY gene. In addition, within the area of Kirov, males having the western Eurasian mtDNA haplotype did not share any eastern Eurasian SRY haplotype, and males having the eastern mtDNA haplotype also did not share any western Eurasian SRY haplotype. Further analysis of paternal genes such as SRY using more samples from a sympatric zone from the Volga-Kama area could contribute more to the understanding of contact and possible hybridization between western and eastern lineages of the Eurasian badgers.

Geographic isolations of the Japanese badger with islands separation history

The present study showed that the phylogenetic relationships among mtDNA control region haplotypes do not correspond with geographical structures of the Japanese badger populations. This coincided with the previous data of Kurose et al. (2001) using the mtDNA cytochrome b gene. Three mtDNA haplotypes (J-2, J-5 and J-8) of the present study were shared among different regional populations in Japan (see Table 3). Especially, J-2 was found both from Honshu and Kyushu, and J-5 was obtained both from Honshu and Shikoku, which are geographically isolated by the Seto Inland Sea (Fig. 1A). In addition, SRY haplotype M1 was shared by individuals from Honshu, Kyushu and Shikoku. It is likely that the regional populations of the Japanese badgers have not been well differentiated from each other. Similarly, the Japanese marten Martes melampus, endemic to Japan, is not well-differentiated genetically among the Japanese islands (Kurose et al., 1999). In contrast, other Japanese endemic mammals, such as the Japanese macaque Macaca fuscata (Kawamoto et al., 2007) and the giant squirrel Petarista leucogenys (Oshida et al., 2009), have major mtDNA lineages and clear phylogeographic structures. Such phylogeographic features specific to the Japanese badgers might have been derived from recent population expansion after the last glacial period, as shown by branching of the mtDNA tree (Fig. 2), as well as the values of Tajima’s D and Fu’s Fs. The mtDNA haplotype diversification of the Japanese badgers occurred between 0.23–0.11 Mya when using the divergence rate of 1.92 x 10^-8 substitutions/nucleotide/year for the mtDNA control region estimated by Marmi et al. (2006). This age includes the Mindel-Riss and Riss-Würm interglacial periods. In addition, from the palaeoecological investigation of Lake Biwa on Honshu, Miyoshi et al. (1999) reported that during that time the Japanese islands at least once underwent the interglacial period. This suggests that the mtDNA haplotypes of the Japanese badgers diverged during the interglacial periods.

Moreover, the AMOVA analysis (Table 4) showed that the Shikoku population is genetically differentiated from the other populations in Japan. Tashima et al. (2010) also reported the relatively large genetic differentiation of the Shikoku population from the Kyushu and Honshu populations, based on the data of biparentally inherited microsatellite polymorphisms. The Seto Inland Sea, separating Honshu, Shikoku and Kyushu Islands, was formed 7,000–5,000 years ago (Ohshima, 1990), and the formation was completed with the formation of the Kannon Strait locating between Honshu and Kyushu Islands. The earlier geological separation of the Shikoku Island could have differentiated the badger population of Shikoku to a greater extent from those of Honshu and Kyushu.

ACKNOWLEDGMENTS

We thank T. Tsujimoto, H. Tanaka, S. Dakemoto, V. Sotnikov, O. Kruglova, I. Khorozyan, and V. A. Solovyev for supplying badger samples. This study was supported in part by a Grant-in-Aid for Scientific Research (Nos. 17405012 and 22405003) from the Japan Society for the Promotion of Science and the Russian Foundation for the Basic Research (No. 09-09-00073).

REFERENCES


Abramov AV (2002) Variation of the baculum structure of the Palearctic badger (Carnivora, Mustelidae, Meles). Russ J Theriol 1: 57–60


Abramov AV, Puzachenko FY (2005) Sexual dimorphism of cranio-logical characters in Eurasian badgers, Meles spp. (Carnivora, Mustelidae), Zool Anzeiger 244: 11–29


Bliton DT, Mirol PM, Mascheretti S, Fredga K, Zima J, Searle JB
(2010) Genetic diversity within the Japanese badgers (Meles anakuma), as revealed by microsatellite analysis. Mammal Study 35: 221–226

(Received May 6, 2010 / Accepted October 12, 2010)