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

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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 *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 thoralis*, 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, restructuring 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

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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 phylogeny 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 between the main stream of the Volga River and the Kama River (left tributary of the Volga River) is a sympatric zone of the European badger *M. meles* and the Asian badger *M. leucurus*. However, until now, the precise location of the boundary between them has not been clarified.

Meanwhile, on the Japanese islands, the Japanese badger occurs on Honshu, Shikoku, and Kyushu, in various environments: deciduous larch plantation, deciduous forests, cedar/cypress plantation, and agricultural area (Kaneko, 2009). Kurose et al. (2001) examined the phylogeny of the mtDNA cytochrome *b* gene, and reported that there is a low genetic diversity among the Japanese badgers and that no clear relationships between genetic distances and geographic distances of sampling locations were found. In contrast, Tashima et al. (2010) studied polymorphisms of nuclear DNA microsatellite loci in the Japanese badgers, and found some genetic differentiations among the Honshu, Shikoku, and Kyushu populations, as well as within the Honshu population.

In addition to maternally inherited mtDNA genes and biparentally inherited microsatellites, analysis of Y chromosomal genes can provide information on the phylogenetical relationships among the Eurasian badger species and populations from the perspective of paternal inheritance. As the sex-determining region on the Y-chromosome gene (*SRY*) is located on the non-recombining region of the Y chromosome (NRY) (Nagai, 2001), the molecular phylogenetic analyses of *SRY* have demonstrated evolutionary history also on carnivores, such as the Felidae (King et al., 2007), Ursidae (Nakagome et al., 2008), and Mustelidae (Yamada and Masuda, 2010). Given the merits of using the *SRY* gene in various carnivores, it seems reasonable to tackle it also in Eurasian badger species. Indeed, in recent ecological studies, the badgers are known to do extra-group mating with male-biased movement, which was reported both in an

English high density population (Macdonald et al., 2008) and a Japanese low density population (Kaneko et al., 2009).

In the present study, to further understand the population structure of the Eurasian badgers, two genes were investigated: the mtDNA control region as a maternally inherited gene, and *SRY* as a paternally inherited gene. Based on the genetic data obtained, the phylogeographic relationships among the Eurasian badger populations and the zoogeographical history are discussed.

## MATERIALS AND METHODS

### Samples and DNA extraction

Tissue samples (muscle, hair, skin, liver and blood) were obtained from 112 individuals of the Eurasian badgers. Sample profiles and locations are shown in Table 1 and Fig. 1, respectively. Total DNA was extracted from hairs using the QIAamp DNA Micro Kit (Qiagen) and from the other tissues using the DNeasy Blood & Tissue Kit (Qiagen). The extracted DNA in 200 µl of TE buffer (about 1–100 ng/µl) was preserved at 4°C until used.

**Table 1.** Sample profiles of the Eurasian badgers examined in the present study. <sup>a</sup>M, male; F, female. Determined from the external morphology or the *SRY* gene amplification by PCR.

Sample code	Sex <sup>a</sup>	Tissue	Sampling locality (island on contry)	Haplotypes	
				mt DNA control region	<i>SRY</i>
MEL-MR1	F	muscle	Iwate (Honshu, Japan)	J-8 (AB538977)	–
9301M	M	blood	Tokyo (Honshu, Japan)	–	Mj5 (AB539131)
9442M	M	blood	Tokyo (Honshu, Japan)	J-7 (AB538976)	Mj2 (AB539128)
0008F	F	blood	Tokyo (Honshu, Japan)	J-2 (AB538971)	–
0009M	M	blood	Tokyo (Honshu, Japan)	J-13 (AB538982)	Mj2 (AB539128)
HB99000	M	skin	Tokyo (Honshu, Japan)	J-8 (AB538977)	–
HB99001	M	skin	Tokyo (Honshu, Japan)	J-8 (AB538977)	–
HB99002	F	skin	Tokyo (Honshu, Japan)	J-2 (AB538971)	–
HB99299	F	skin	Tokyo (Honshu, Japan)	J-10 (AB538979)	–
HB05630F	F	skin	Tokyo (Honshu, Japan)	J-2 (AB538971)	–
HB05725M	M	skin	Tokyo (Honshu, Japan)	J-8 (AB538977)	Mj1 (AB5389127)
HB050708F	F	hair	Tokyo (Honshu, Japan)	J-3 (AB538972)	–
HB060331	M	hair	Tokyo (Honshu, Japan)	J-3 (AB538972)	Mj3 (AB5389129)
HB0607281F	F	hair	Tokyo (Honshu, Japan)	J-6 (AB538975)	–
HB0607282M	M	hair	Tokyo (Honshu, Japan)	J-8 (AB538977)	Mj1 (AB5389127)
HB0607283M	M	hair	Tokyo (Honshu, Japan)	J-6 (AB538975)	Mj1 (AB5389127)
HB0607284M	M	hair	Tokyo (Honshu, Japan)	J-2 (AB538971)	Mj4 (AB5389130)
HB0607285F	F	hair	Tokyo (Honshu, Japan)	J-8 (AB538977)	–
HB0607286F	F	hair	Tokyo (Honshu, Japan)	J-5 (AB538974)	–
HB0607287M	M	hair	Tokyo (Honshu, Japan)	J-9 (AB538978)	Mj1 (AB5389127)
HB0607288M	M	hair	Tokyo (Honshu, Japan)	J-2 (AB538971)	–
HB000915M	M	hair	Tokyo (Honshu, Japan)	J-12 (AB538981)	Mj1 (AB5389127)
MEL-MF2	F	blood	Tokyo (Honshu, Japan)	J-8 (AB538977)	–
MEL-MM2	M	blood	Tokyo (Honshu, Japan)	J-6 (AB538975)	Mj1 (AB5389127)
MEL-TKY1	M	skin	Tokyo (Honshu, Japan)	J-6 (AB538975)	Mj1 (AB5389127)
MEL-TKY2	M	skin	Tokyo (Honshu, Japan)	J-12 (AB538981)	Mj1 (AB5389127)
MEL-TKY3	F	muscle	Tokyo (Honshu, Japan)	J-8 (AB538977)	–
MEL-TKY24	M	muscle	Tokyo (Honshu, Japan)	J-1 (AB538970)	Mj1 (AB5389127)
MEL-TKY26	M	muscle	Tokyo (Honshu, Japan)	J-6 (AB538975)	–
TB06720F	F	skin	Tokyo (Honshu, Japan)	J-11 (AB538980)	–
UB06720M	M	skin	Tokyo (Honshu, Japan)	J-10 (AB538979)	–
UB06720U	M	skin	Tokyo (Honshu, Japan)	J-2 (AB538971)	Mj1 (AB5389127)
MEL-TKY25	M	muscle	Chiba (Honshu, Japan)	J-4 (AB538973)	–
VM07-73	M	muscle	Gunma (Honshu, Japan)	J-21 (AB551122)	–
VM07-145	M	muscle	Gunma (Honshu, Japan)	J-13 (AB538982)	Mj1 (AB5389127)
VM07-148	M	muscle	Gunma (Honshu, Japan)	J-22 (AB551123)	Mj1 (AB5389127)
VM-07-149	F	muscle	Gunma (Honshu, Japan)	J-21 (AB551122)	–
VM07-153	M	muscle	Gunma (Honshu, Japan)	J-6 (AB538975)	Mj1 (AB5389127)
VM08-48	M	muscle	Gunma (Honshu, Japan)	J-23 (AB551124)	Mj1 (AB5389127)
VM08-69	M	muscle	Gunma (Honshu, Japan)	J-8 (AB538977)	–
VM08-98a	M	muscle	Gunma (Honshu, Japan)	J-24 (AB551125)	–
VM08-98b	M	muscle	Gunma (Honshu, Japan)	J-6 (AB538975)	–
VM09-2	M	muscle	Gunma (Honshu, Japan)	J-8 (AB538977)	Mj1 (AB5389127)
VM09-9	F	muscle	Gunma (Honshu, Japan)	J-25 (AB551126)	–
VM09-89	M	muscle	Gunma (Honshu, Japan)	J-25 (AB551126)	–
VM09-90	M	muscle	Gunma (Honshu, Japan)	J-25 (AB551126)	Mj1 (AB5389127)

Table 1. Continued.

Sample code	Sex <sup>a</sup>	Tissue	Sampling locality	Haplotypes	
				Control region	SRY
VM09-95	M	muscle	Gunma (Honshu, Japan)	J-2 (AB538971)	Mj1 (AB5389127)
VM09-97	F	muscle	Gunma (Honshu, Japan)	J-2 (AB538971)	–
VM09-119	F	muscle	Gunma (Honshu, Japan)	J-2 (AB538971)	–
VM09-227	M	muscle	Gunma (Honshu, Japan)	J-25 (AB551126)	Mj1 (AB5389127)
MEL-2	F	muscle	Gifu (Honshu, Japan)	J-8 (AB538977)	–
MEL-3	F	muscle	Gifu (Honshu, Japan)	J-16 (AB538985)	–
MEL-4	M	muscle	Gifu (Honshu, Japan)	J-15 (AB538984)	–
MEL-5	F	muscle	Gifu (Honshu, Japan)	J-8 (AB538977)	–
MEL-YMG1	F	liver	Yamaguchi (Honshu, Japan)	J-14 (AB538983)	–
H13-009	F	muscle	Kochi (Shikoku, Japan)	J-19 (AB538988)	–
H13-029	F	muscle	Kochi (Shikoku, Japan)	J-18 (AB538987)	–
H13-056	F	muscle	Kochi (Shikoku, Japan)	J-18 (AB538987)	–
H13-057	F	muscle	Kochi (Shikoku, Japan)	J-18 (AB538987)	–
H13-075	F	muscle	Kochi (Shikoku, Japan)	J-19 (AB538988)	–
H16-016	F	muscle	Kochi (Shikoku, Japan)	J-5 (AB538974)	–
H16-017	M	muscle	Kochi (Shikoku, Japan)	J-20 (AB538989)	Mj1 (AB5389127)
H16-059	F	muscle	Kochi (Shikoku, Japan)	J-18 (AB538987)	–
H16-064	F	muscle	Kochi (Shikoku, Japan)	J-19 (AB538988)	–
H16-077	F	muscle	Kochi (Shikoku, Japan)	J-18 (AB538987)	–
H16-104	F	muscle	Kochi (Shikoku, Japan)	J-5 (AB538974)	–
H17-004	F	muscle	Kochi (Shikoku, Japan)	J-18 (AB538987)	–
H17-044	M	muscle	Kochi (Shikoku, Japan)	J-18 (AB538987)	Mj1 (AB5389127)
H17-114	F	muscle	Kochi (Shikoku, Japan)	J-5 (AB538974)	–
H18-010	M	muscle	Kochi (Shikoku, Japan)	J-18 (AB538987)	Mj6 (AB5389132)
H18-178	M	muscle	Kochi (Shikoku, Japan)	J-19 (AB538988)	Mj6 (AB5389132)
MEL-K1	M	muscle	Oita (Kyushu, Japan)	J-2 (AB538971)	Mj1 (AB5389127)
MEL-K2	M	muscle	Oita (Kyushu, Japan)	J-2 (AB538971)	Mj1 (AB5389127)
MEL-K3	M	muscle	Oita (Kyushu, Japan)	J-17 (AB538986)	–
MEL-K4	F	muscle	Oita (Kyushu, Japan)	J-2 (AB538971)	–
MEL-K5	F	muscle	Oita (Kyushu, Japan)	J-2 (AB538971)	–
MEL-K6	F	muscle	Oita (Kyushu, Japan)	J-2 (AB538971)	–
MEL-K7	M	muscle	Oita (Kyushu, Japan)	J-2 (AB538971)	Mj1 (AB5389127)
MEL-K8	M	muscle	Fukuoka (Kyushu, Japan)	J-2 (AB538971)	Mj1 (AB5389127)
MEL-K9	M	muscle	Kumamoto (Kyushu, Japan)	J-17 (AB538986)	Mj1 (AB5389127)
MEL-K10	M	muscle	Oita (Kyushu, Japan)	J-26 (AB551127)	–
MEL-K11	F	muscle	Fukuoka (Kyushu, Japan)	J-17 (AB538986)	–
MEL-K12	F	muscle	Nagasaki (Kyushu, Japan)	J-2 (AB538971)	–
MEL-K13	M	muscle	Oita (Kyushu, Japan)	J-2 (AB538971)	Mj1 (AB5389127)
MEL-K14	F	muscle	Fukuoka (Kyushu, Japan)	J-27 (AB551128)	–
MEL-K15	F	muscle	Fukuoka (Kyushu, Japan)	J-17 (AB538986)	–
MEL-FE1	F	skin	Far East (Russia)	E-5 (AB538994)	–
MEL-KRV4	M	skin	Far East (Russia)	E-2 (AB538991)	–
MEL-ZIS33	M	muscle	Transbaikalia (Russia)	E-6 (AB538995)	Me1 (AB5389133)
MEL-ZIM52	M	muscle	Chelyabinsk Prov. (Russia)	E-4 (AB538993)	–
MEL-ZIM53	M	muscle	Chelyabinsk Prov. (Russia)	E-1 (AB538990)	Me1 (AB5389133)
MEL-ZIM54	M	muscle	Chelyabinsk Prov. (Russia)	E-4 (AB538993)	Me1 (AB5389133)
MEL-ZIM55	M	muscle	Chelyabinsk Prov. (Russia)	E-4 (AB538993)	–
MEL-ZIM56	M	muscle	Chelyabinsk Prov. (Russia)	E-4 (AB538993)	Me2 (AB5389134)
MEL-ZIM58	M	muscle	Chelyabinsk Prov. (Russia)	E-4 (AB538993)	Me2 (AB5389134)
MEL-ZIM59	M	muscle	Chelyabinsk Prov. (Russia)	E-4 (AB538993)	Me2 (AB5389134)
MEL-ZIM60	F	muscle	Chelyabinsk Prov. (Russia)	E-4 (AB538993)	–
MEL-ZIM61	F	muscle	Chelyabinsk Prov. (Russia)	E-1 (AB538990)	–
MEL-ZIM62	F	muscle	Chelyabinsk Prov. (Russia)	E-3 (AB538992)	–
MEL-ZIM63	F	muscle	Chelyabinsk Prov. (Russia)	E-1 (AB538990)	–
MEL-ZIM64	M	muscle	Chelyabinsk Prov. (Russia)	E-3 (AB538992)	Me1 (AB5389133)
MEL-ZIM65	F	muscle	Chelyabinsk Prov. (Russia)	E-4 (AB538993)	–
MEL-ZIM66	M	muscle	Chelyabinsk Prov. (Russia)	E-1 (AB538990)	Me1 (AB5389133)
MEL-KRV1	M	muscle	Kirov Prov. (Russia)	W-4 (AB538999)	Mw1 (AB5389136)
MEL-KRV2	M	muscle	Kirov Prov. (Russia)	W-4 (AB538999)	Mw1 (AB5389136)
MEL-KRV3	M	muscle	Kirov Prov. (Russia)	W-1 (AB538996)	–
MEL-KRV5	F	skin	Kirov Prov. (Russia)	E-4 (AB538993)	–
MEL-KRV6	F	skin	Kirov Prov. (Russia)	W-4 (AB538999)	–
MEL-ZIS35	M	muscle	Leningrad Prov. (Russia)	W-3 (AB538998)	Mw1 (AB5389136)
MEL-ZIS36	M	muscle	Leningrad Prov. (Russia)	W-2 (AB538997)	Mw1 (AB5389136)
MEL-Ar1	M	muscle	Armenia	C-2 (AB539001)	Mc1 (AB5389135)
MEL-Ar2	M	muscle	Armenia	C-1 (AB539000)	Mc1 (AB5389135)

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 Car12S-R (Shimatani et al., 2008). A total of 20 µl of the PCR reaction mixture containing 2 µl of 10xPCR 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; 40 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.

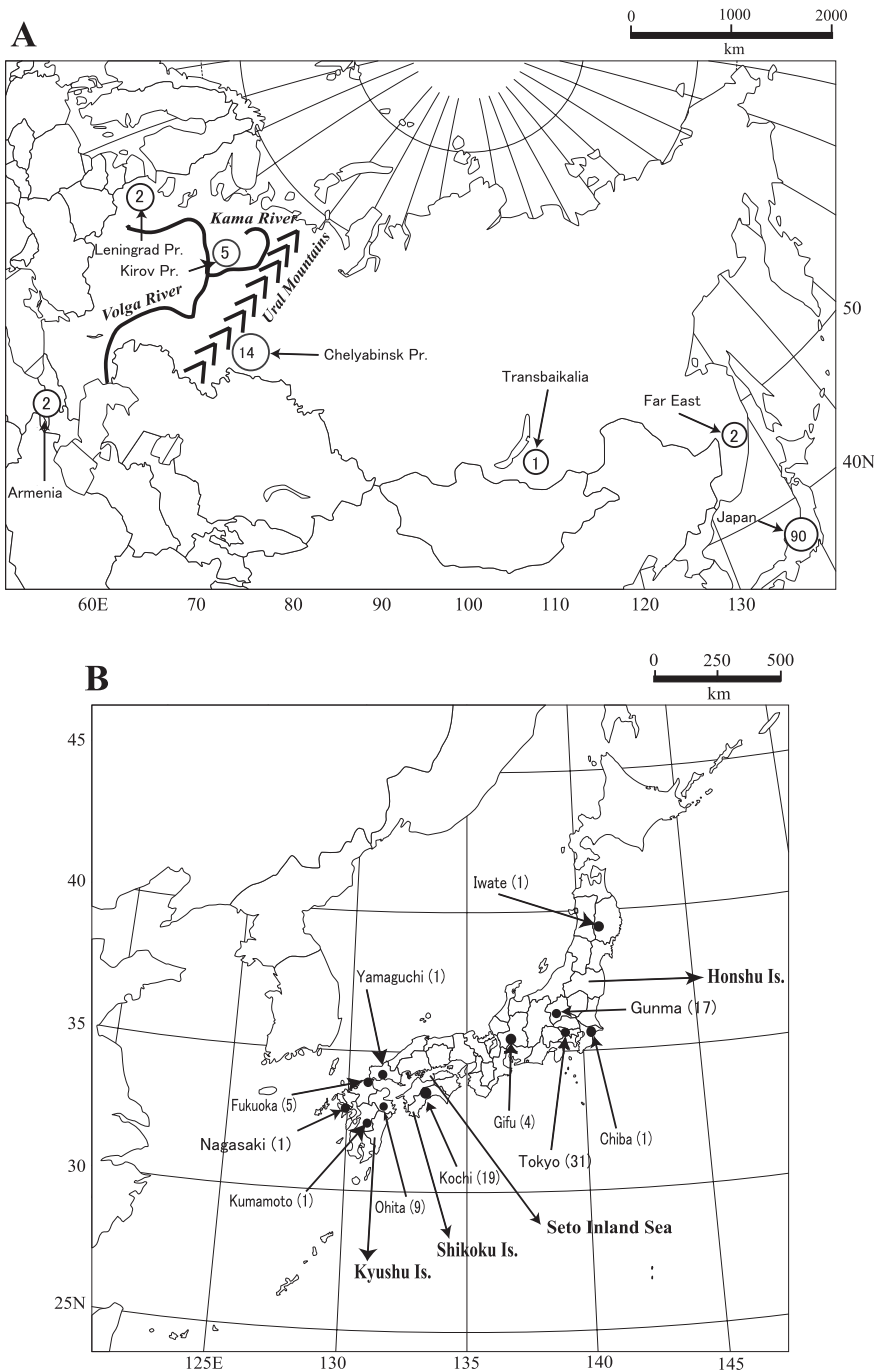
From obtained nucleotide sequences, the following three primers for PCR and sequencing were newly designed in the present study: ANK-R1 (5'-ATGTCCTGTAACCATGACTG-3'), MeIDS-F (5'-ACATAACTGTGGTGCATGC-3') and MeIDS-R2 (5'-GGCCTT-TATGGCCATAGCTG-3').

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 10xPCR 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 PCRsequencing 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-F2 and MSRY-R2 (Yamada and Masuda, 2010) were used. A total of 50 µl of the PCR reaction solution contained 5 µl of 10xPCR 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)



**Fig. 1.** (A) The sampling locations in Eurasia. Numerals in circles are the numbers of individuals from those locations. (B) The sampling locations on the Japanese islands. Numerals in parentheses are the numbers of individuals from those locations.

and 5–10  $\mu$ l of the DNA extract, and was totalized with 5  $\mu$ l 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 mentioned 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 haplotype 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 AB491589; Yamada and Masuda, 2010) were used as an outgroup.

Moreover, for the mtDNA analysis, values of Tajima's  $D$  (Tajima, 1989) and Fu's  $F_S$  (Fu, 1997) were 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 performed in ARLEQUIN 3.1. This analysis evaluates genetic variations of three partitions: 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) of more than ten animals, and Group 2 including the other populations.

## RESULTS

### Sequence variations of the mtDNA control region of the Eurasian badgers

An alignment of the mtDNA control region (539–545 bp) of the Eurasian badgers showed that nucleotide substitutions 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 average). From the continental badgers having 59 polymorphic sites, 12 haplotypes (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 identified (Table 3), and the sequence differences were 0.0–1.7% (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 allopatrically: clade 1 consisting of J-1 to J-27 identified from



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).

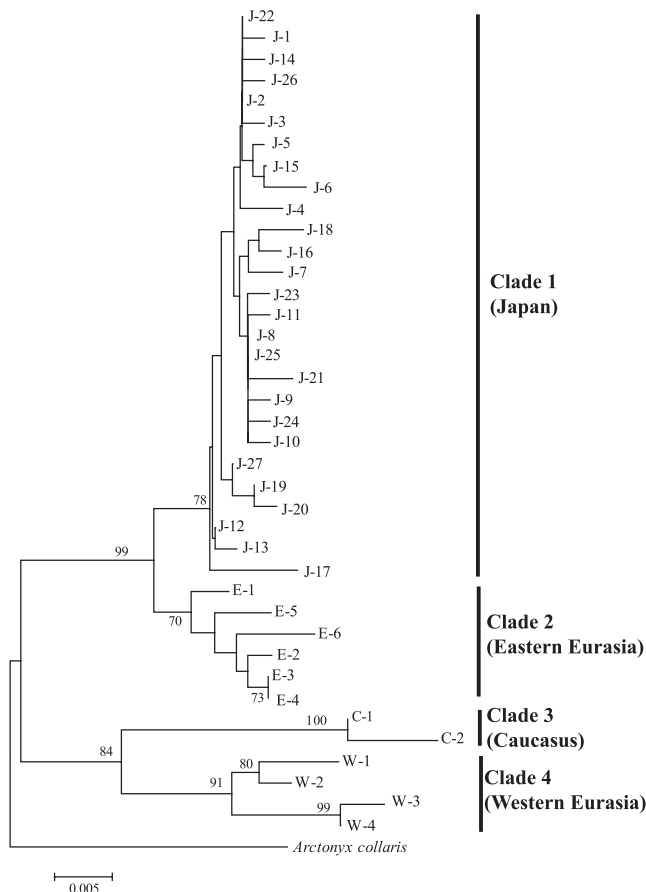
On the other hand, on the Japanese Islands, three out of the 27 haplotypes were shared by different island-populations, whereas the other 24 haplotypes were not shared by any regional populations and specific to each region: for example, three haplotypes J-18, J-19 and J-20 were found from only the badgers 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 of '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



**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.

**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
<b>Kanto</b>	Iwate, Gifu, Yamaguchi, Shikoku, Kyushu	-7.12 (-0.12)	30.84 (0.51)	76.27 (1.25)
<b>Shikoku</b>	Iwate, Kanto, Gifu, Yamaguchi, Kyushu	34.99 (0.64)	1.20 (0.02)	63.81 (1.17)
<b>Kyushu</b>	Iwate, Kanto, Gifu, Yamaguchi, Shikoku	-12.30 (-0.19)	32.22 (0.50)	80.08 (1.25)

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).

Among the Japanese badger sequences, ten sites were polymorphic including indels, and six haplotypes (Mj1 to Mj6) were identified (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.

**Table 5.** Polymorphic sites of *SRY* haplotypes (1052–1058 bp) identified from the Eurasian badgers. <sup>a</sup>Dots represent nucleotides identical with those of haplotype Mj1. Dashes represent deletions.

Clade	Haplotype	Polymorphic sites <sup>a</sup>																									
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Clade 1	Mj1	A	A	---	---	---	G	T	C	G	G	G	A	C	C	C	A	C	T	A	A	T	T	C	A	---	C
	Mj2	.	.	---	---	---	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Mj3	.	.	---	---	---	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Mj4	.	.	---	---	---	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Mj5	.	.	---	---	---	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Mj6	.	.	---	---	---	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Clade 2	Me1	G	G	T	C	C	C	C	C	.	C	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Me2	G	G	T	C	C	C	C	C	.	C	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Clade 3	Mc1	G	G	---	---	---	A	C	T	A	.	A	G	G	A	.	.	.	.	.	.	.	.	.	.	.	.
Clade 4	Mw1	G	G	---	---	---	.	C	T	A	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.

**Table 6.** Geographical distribution of *SRY* haplotypes in the Eurasian badgers.

Clade	Haplotype	Japan	Chelyabinsk	Armenia	Kirov	Leningrad
Clade 1 (Japan)	Mj1	26				
	Mj2	2				
	Mj3	1				
	Mj4	1				
	Mj5	1				
	Mj6	2				
Clade 2 (Eastern Eurasia)	Me1		5			
	Me2		3			
Clade 3 (Caucasus)	Mc1			2		
Clade 4 (Western Eurasia)	Mw1				2	2
Total		33	8	2	2	2

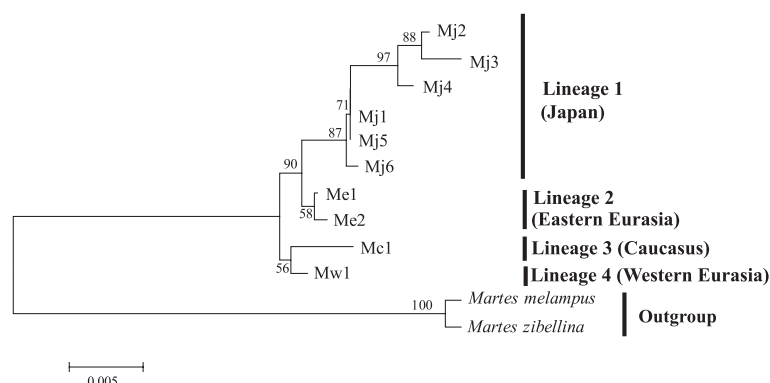
**DISCUSSION**

**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* 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



**Fig. 3.** A neighbor-joining tree of *SRY* haplotypes from male badgers. Numbers near internal branches are bootstrap values (> 50%) derived by 1,000 replications. The *SRY* sequences from the Japanese marten *Martes melampus* (accession no. AB491590), and the sable *M. zibellina* (accession no. AB491589), were used as outgroup. Clade names refer to those of Table 5. The scale shows Kimura's two-parameter distances.



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 neighbor-joining 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-KRV5 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 (Fig. 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 agrestis*, Jaarola and Searle, 2002), the flying squirrels (*Pteromys volans*, Oshida et al., 2005), both the pygmy and common shrews (*Sorex minutus* and *S. araneus*, Bilton 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 Urals 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 Urals.

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 Mj1 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 *Petaurista 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 *F<sub>s</sub>*. The mtDNA haplotype diversification of the Japanese badgers occurred between 0.23–0.11 Mya when using the divergence rate of  $1.92 \times 10^{-8}$  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 palaeolimnological 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.

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