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## Molecular phylogeny of Eurasian badgers (*Meles*) around the distribution boundaries, revealed by analyses of mitochondrial DNA and Y-chromosomal genes



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### ABSTRACT

Based on previous molecular and morphological analyses, Eurasian badgers are currently classified into *Meles meles* distributed in Europe, *M. canescens* in the Caucasus and Middle East, *M. leucurus* in continental Asia and *M. anakuma* in Japan. The precise locations of their distribution boundaries are still unclear. Therefore, in the present study, we clarified the phylogenetic relationships in the genus *Meles* around its distribution boundaries, based on mitochondrial DNA (mtDNA) and Y-chromosomal genes. From 71 badgers examined, 29 mtDNA haplotypes were identified, including new 26 types. Multiple haplotypes of SRY and CAN-SINES were identified from 23 males, including two new haplotypes of SRY. The mtDNA phylogeny showed that the Ural Mountains were not a current distribution boundary between *M. meles* and *M. leucurus*. In addition, our results supported the hypothesis that the Bosphorus Strait is a geographical barrier between *M. meles* and *M. canescens*. The badgers from Bulgaria north of the Bosphorus Strait shared haplotypes with *M. meles*. On the other hand, badgers from Far Eastern Russia distributed in the eastern peripheral region of the Asian Continent had haplotypes of *M. leucurus*. The badgers from Norway and Finland shared haplotypes with *M. meles*.

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### 1. Introduction

The Eurasian badgers (genus *Meles*; family Mustelidae) are distributed widely in the Palaearctic from Ireland to Japan. They have adapted to various environments, such as deciduous forests, steppes and mountainous areas (Neal and Cheeseman,

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1996). The genus *Meles* was earlier classified into one species, which was divided into multiple subspecies, based on morphological and ecological differences (Heptner et al., 1967). However, recent studies have suggested that the Eurasian badgers should be divided into four species: the European badger (*Meles meles*) distributed in Europe, the Caucasian badger (*M. canescens*) in the Caucasus and Middle East, the Asian badger (*M. leucurus*) in the Asian Continent, and the Japanese badger (*M. anakuma*) on the Japanese islands, based on multiple analyses of mitochondrial DNA (mtDNA) (Del Cerro et al., 2010; Marmi et al., 2006; Tashima et al., 2011a), nuclear DNA (Del Cerro et al., 2010) and skull morphometrics (Abramov and Puzachenko, 2006, 2013).

The presumed boundary between *M. meles* and *M. leucurus* is situated somewhere around the Volga and Kama Rivers in western Russia (Abramov and Puzachenko, 2006; Tashima et al., 2011a) or the Ural Mountains (Heptner et al., 1967). Based on analysis of skull morphometrics, Abramov and Puzachenko (2005, 2006) suggested that there is an overlapping zone between the two species in a narrow region between the Volga and Kama Rivers. However, Gasilin and Kosintsev (2010) analyzed morphology of subfossils excavated from layers of the Middle Holocene, and reported that the boundary between *M. meles* and *M. leucurus* at that time was located 1500 km east of the current boundary. Therefore, further investigations of the badgers distributed around the Ural Mountains would be important to reveal the phylogeographical history of the Eurasian badgers.

The geographical barrier between *M. meles* and *M. canescens* was reported to be the Black Sea, Caucasus Mountains and Caspian Sea (Marmi et al., 2006; Abramov and Puzachenko, 2013). In addition, mtDNA cytochrome *b* analyses revealed that the Bosphorus Strait, connecting the Black Sea and Mediterranean Sea, is one of the geographical barriers between the two species (İbiş et al., 2015). Frantz et al. (2014) reported that the last glacial refugium of *M. meles* in Europe was located in the Balkan Peninsula, using a mtDNA control region sequence from one specimen from Bulgaria. Therefore, it is necessary to investigate more badgers from Bulgaria and reveal their phylogenetic characteristics.

The distribution boundary between *M. anakuma* and *M. leucurus* is thought to be the Sea of Japan (Wozencraft, 2005). Kurose et al. (2001) analyzed the phylogeny of mtDNA cytochrome *b*, and reported a large difference between *M. anakuma* and the other *Meles* species. Previous studies reported that *M. leucurus* is also found on Jeju Island and on the Korean Peninsula based on analysis of cytochrome *b* gene (Koh et al., 2014) and the control region (Lee et al., 2016).

Previous ecological studies of badgers showed certain sexual differences in behaviour patterns; male badgers have higher dispersal rates than females (Kruuk and Parish, 1987; Rogers et al., 1998; Roper et al., 2003). Therefore, it would be more informative to analyze the phylogeny using not only data from mtDNA as a maternally inherited gene, but also data from Y-chromosomal DNA as a paternally inherited gene for revealing the population structure and migration history of badgers. Previous phylogenetic analyses of Y-chromosomal genes also indicated that badgers can be divided into four species, using the *SRY* gene (sex-determining region of Y) (Tashima et al., 2011a) and CAN-SINES (short interspersed nuclear elements, unique to genomes of the order Carnivora) within Y-chromosomal DNA (Tashima et al., 2011b).

Thus, there have been several studies on the phylogeography of Eurasian badgers. However, badgers inhabiting the boundary areas between the proposed species have not been sufficiently studied to establish where those boundaries are. In the present study, we re-examine the phylogeny of populations distributed close to the boundaries between the different badger species using sequence data of mtDNA control region, *SRY* gene and CAN-SINES, and then further discuss the phylogeographical relationships among genus *Meles*.

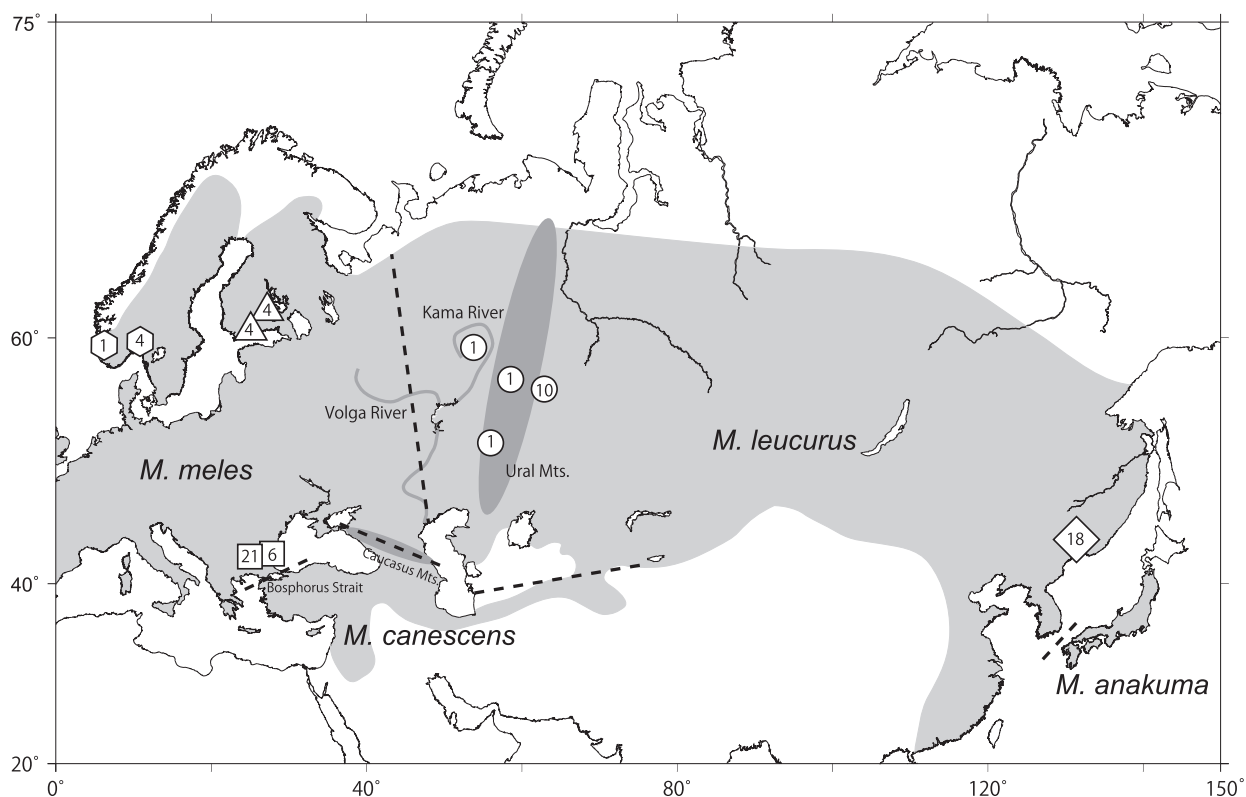
## 2. Material and methods

### 2.1. Samples and DNA extraction

Samples of Eurasian badgers were obtained from 71 individuals: 27 from Bulgaria, five from Norway, eight from Finland, 13 from the Ural area and 18 from Far Eastern Russia (Fig. 1, Table 1). Total DNA was extracted from muscle tissues preserved in ethanol using the DNeasy Blood and Tissue Kit (Qiagen) and from hairs and skins using the QIAamp DNA Investigator (Qiagen), according to the manufacturer's protocol. Total DNA was eluted in TE buffer and stored at 4 °C.

### 2.2. Amplification and sequencing of mtDNA control region

Polymerase chain reaction (PCR) of the mtDNA control region was performed using two primers, UR1 (Taberlet and Bouvet, 1994) and ANKR1 (Tashima et al., 2011a). In addition, we newly designed two PCR primers for the skin samples: AND-R1(5'-GTTAGTAGGATTGGATTGAGGAC-3') and AND-F1(5'-CCAAGCCTCGAGAAACCATC-3'). The PCR amplification was performed in 20 µl of a reaction volume containing 2 µl of 10 × buffer (Takara), 1.6 µl of dNTP mixture, 0.1 µl of *rTaq* DNA polymerase (5 U/µl, Takara), 0.2 µl of each of the two primers (25 pmol/µl), 1–2 µl of the DNA extract and 13.9–14.9 µl of distilled water. The PCR cycle conditions were one initial cycle of 94 °C for 1 min and 30 cycles of 94 °C for 1 min; 58.3 °C for 1 min; 72 °C for 1 min; and one cycle of 72 °C for 4 min. The PCR amplification was carried out in a thermal cycler TP350 (Takara). Unsuccessfully amplified samples using the above procedure were applied to another PCR using PrimeSTAR GXL (Takara) or Multiplex PCR kit (Qiagen), according to the manufacturer's protocol. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). The cycle PCR condition consisted of one cycle of 96 °C for 1 min and 25 cycles of 96 °C for 10 s; 50 °C for 5 s; 60 °C for 4 min, using the Big Dye Terminator v1.1 or v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequence determination was made on an ABI3730 DNA Analyzer (Applied Biosystems).



**Fig. 1.** Sampling locations in the distribution (grey area) of Eurasian badgers, *Meles*. The symbols and numerals correspond to sampling locations (Bulgaria, within squares; Finland, within triangles; Norway, within hexagons; Ural, within circles; Far East Russia, within a diamond) and numbers of examined samples, respectively. Broken lines indicate the hypothetical geographical boundaries. The Ural Mountains are shown in dark grey.

### 2.3. Amplification and sequencing of Y-chromosome genes

The PCR amplification and sequence determination of the *SRY* gene and the CAN-SINES in the final intron of *ZFY* (zinc-finger protein on Y-chromosome) were performed using the basically same method as the mtDNA control region. To amplify and sequence the CAN-SINES, we used the two primers, MELSN-F2 and MELSN-R2, in accordance with Tashima et al. (2011b). On the other hand, to amplify the *SRY* gene, we used two primers, MSRY-F2 and MSRY-R2 (Yamada and Masuda, 2010). For sequencing *SRY* gene, we newly designed primers: MELSR-R1 (5'-GTCTTCCGACGAGGTGCGATA-3'), MELSR-f2 (5'-TCAAA-GACGCAAGGTGGC-3') and MELSR-r2 (5'-CACTGTCTGTGGCAGGTC-3'). An additional primer (MELSR-F1: 5'-GAGAATCCC-CAAATGCAAAA-3', Mizumachi et al., 2017) was used for the sequencing.

### 2.4. Sequence data analyses

Sequence alignment was performed using MEGA ver. 6 (Tamura et al., 2013). Phylogenetic trees of the mtDNA control region by the neighbour-joining method (Saitou and Nei, 1987), using Kimura's two-parameter (Kimura, 1980), were constructed using the MEGA. Bootstrap values were derived from 1,000 replications. The mtDNA control region of the hog badger *Arctonyx collaris* (accession no. AJ563704: Marmi et al., 2006) was used as an outgroup. The minimum spanning networks among *SRY* haplotypes were constructed using TCS 1.21 software (Clement et al., 2000).

Numbers of polymorphic sites, haplotype diversity ( $h$ ) and nucleotide diversity ( $\pi$ ) were calculated using ARLEQUIN ver. 3.5 (Excoffier and Lischer, 2010). The pairwise sequence differences among sampling locations were calculated by Kimura two-parameter using MEGA.

## 3. Results

### 3.1. MtDNA diversity and geographic variation in Eurasian badgers

Sequences of the mtDNA control region (541–544 base-pairs, bp) were obtained from 71 Eurasian badgers, which included 29 haplotypes of which 26 were new, compared to data in the DNA Data Bank of Japan (DDBJ) (Table 1).

**Table 1**  
Profiles of Eurasian badgers (*Meles spp.*) examined in the present study.

Sample code	Sex <sup>a</sup>	Location	Haplotypes			Supplier
			mtDNA control region	SRY gene	CAN-SINEs	
MEL-2010-Mm1	F	Stara Zagora, Bulgaria	BL2 (LC185432)			E. G. Raichev, Trakia Univ.
MEL-2010-Mm2	M	Stara Zagora, Bulgaria	BL5 (LC185435)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2010-Mm3	F	Stara Zagora, Bulgaria	BL2 (LC185432)			E. G. Raichev, Trakia Univ.
MEL-2010-Mm4	M	Stara Zagora, Bulgaria	BL2 (LC185432)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2010-Mm5	M	Stara Zagora, Bulgaria	BL5 (LC185435)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2010-Mm6	M	Stara Zagora, Bulgaria	BL3 (LC185433)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2011-14	M	Sredna gora, Bulgaria	BL2 (LC185432)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2011-49	M	Plovdiv, Bulgaria	BL1 (LC185431)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2011-68	M	Stara plamina, Bulgaria	BL2 (LC185432)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2011-101	M	Sredna gora, Bulgaria	BL4 (LC185434)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2011-110	F	Stara plamina, Bulgaria	BL2 (LC185432)			E. G. Raichev, Trakia Univ.
MEL-2011-112	F	Sredna gora, Bulgaria	BL2 (LC185432)			E. G. Raichev, Trakia Univ.
MEL-2011-124	M	Varna, Bulgaria	BL5 (LC185435)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2011-131	M	Varna, Bulgaria	BL5 (LC185435)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2011-137	M	Varna, Bulgaria	BL2 (LC185432)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2011-138	F	Varna, Bulgaria	BL5 (LC185435)			E. G. Raichev, Trakia Univ.
MEL-2012-7	F	Stara Zagora, Bulgaria	BL2 (LC185432)			E. G. Raichev, Trakia Univ.
MEL-2012-15	M	Kazanlak, Bulgaria	BL2 (LC185432)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2012-28	F	Kazanlak, Bulgaria	BL6 (LC185436)			E. G. Raichev, Trakia Univ.
MEL-2012-35	F	Pleven, Bulgaria	BL1 (LC185431)			E. G. Raichev, Trakia Univ.
MEL-2012-37	M	Dolen Chiflik, Bulgaria	BL1 (LC185431)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	E. G. Raichev, Trakia Univ.
MEL-2012-39	F	Stara Zagora, Bulgaria	BL4 (LC185434)			E. G. Raichev, Trakia Univ.
MEL-2012-40	F	Dolen Chiflik, Bulgaria	BL5 (LC185435)			E. G. Raichev, Trakia Univ.
MEL-2012-53	F	Kolena, Bulgaria	BL5 (LC185435)			E. G. Raichev, Trakia Univ.
MEL-2013-6	F	Varna, Bulgaria	BL5 (LC185435)			E. G. Raichev, Trakia Univ.
MEL-14-01	F	Plovdiv, Bulgaria	BL2 (LC185432)			E. G. Raichev, Trakia Univ.
Bul bad No1	F	Stara Zagora, Bulgaria	BL4 (LC185434)			E. G. Raichev, Trakia Univ.
2(KN46943)	F	Llaukansaari, Finland	FN2 (LC185438)			V. Haukisalml, Finnish Museum of Natural History
12(KN 48375)	F	Saksala, Finland	FN3 (LC185439)			V. Haukisalml, Finnish Museum of Natural History
20(KN 48982)	M	Vessolandet. londbole, Finland	FN1 (LC185437)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	V. Haukisalml, Finnish Museum of Natural History
21(KN 48983)	F	Vessolandet. londbole, Finland	FN1 (LC185437)			V. Haukisalml, Finnish Museum of Natural History
27(KN 49014)	M	Saksala, Finland	FN4 (LC185440)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	V. Haukisalml, Finnish Museum of Natural History
28(KN 49015)	M	Saksala, Finland	FN1 (LC185437)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	V. Haukisalml, Finnish Museum of Natural History
29(KN 49016)	F	Vessolandet. londbole, Finland	FN1 (LC185437)			V. Haukisalml, Finnish Museum of Natural History
32(KN 49050)	M	Vessolandet. londbole, Finland	FN1 (LC185437)	Mw1(AB539136) <sup>b</sup>	SNW1(AB551120) <sup>b</sup>	V. Haukisalml, Finnish Museum of Natural History
Nm1	?	Vestfold, Norway	NR1 (LC185441)			Ø. Wiig, Zoological Museum, Univ. of Oslo
Nm4	?	Akeshus, Norway	NR1 (LC185441)			Ø. Wiig, Zoological Museum, Univ. of Oslo
Nm6	?	Vestfold, Norway	NR1 (LC185441)			Ø. Wiig, Zoological Museum, Univ. of Oslo
Nm10	?	Oslo, Norway	W-4 (AB538999) <sup>b</sup>			Ø. Wiig, Zoological Museum, Univ. of Oslo
Nm14	?	Fjaere, Norway	BL1 (LC185431)			Ø. Wiig, Zoological Museum, Univ. of Oslo
#355132	F	Middle Urals, Russia	RS1 (LC185442)			P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#364616	F	Middle Urals, Russia	E-5 (AB538994) <sup>b</sup>			

Table 1 (continued)

Sample code	Sex <sup>a</sup>	Location	Haplotypes			Supplier
			mtDNA control region	<i>SRY</i> gene	CAN-SINEs	
#364617	F	Middle Urals, Russia	RS2 (LC185443)			P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#364618	F	Middle Urals, Russia	RS4 (LC185445)			P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#364619	F	Middle Urals, Russia	RS3 (LC185444)			P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#364620	F	Middle Urals, Russia	RS2 (LC185443)			P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#364621	F	Middle Urals, Russia	RS1 (LC185442)			P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#416829	F	Middle Urals, Russia	E–5 (AB538994) <sup>b</sup>			P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#452478	M	Middle Urals, Russia	RS5 (LC185446)	Me1(AB539133) <sup>b</sup>	SNE1(AB551119) <sup>b</sup>	P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#452479	F	Middle Urals, Russia	RS1 (LC185442)			P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#452634	M	Middle Urals, Russia	E–1 (AB538990) <sup>b</sup>	Me1(AB539133) <sup>b</sup>	SNE1(AB551119) <sup>b</sup>	P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#491951	F	South Urals, Russia	E–5 (AB538994) <sup>b</sup>			P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
IPAE-54	F	Middle Urals, Russia	RS6 (LC185447)			P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#491952	M	Far East, Russia	RS4 (LC185445)	Me4 (LC185458)	SNE1(AB551119) <sup>b</sup>	P. Kosintsev, Inst. of Plant and Animal Ecology, RAS
#1265	F	Primorsky Krai, Russia	ERS1 (LC185448)			A. Kryukov, Inst. of Biology and Soil Science, RAS
#1652	M	Primorsky Krai, Russia	ERS2 (LC185449)	Me3 (LC185457)	SNE1(AB551119) <sup>b</sup>	A. Kryukov, Inst. of Biology and Soil Science, RAS
#1696	F	Primorsky Krai, Russia	ERS3 (LC185450)			A. Kryukov, Inst. of Biology and Soil Science, RAS
#1738	F	Primorsky Krai, Russia	RS4 (LC185445)			A. Kryukov, Inst. of Biology and Soil Science, RAS
#1741	F	Primorsky Krai, Russia	ERS4 (LC185451)			A. Kryukov, Inst. of Biology and Soil Science, RAS
#1742	M	Primorsky Krai, Russia	ERS6 (LC185453)	Me3 (LC185457)	SNE1(AB551119) <sup>b</sup>	A. Kryukov, Inst. of Biology and Soil Science, RAS
#1743	M	Primorsky Krai, Russia	ERS9 (LC185456)	Me3 (LC185457)	SNE1(AB551119) <sup>b</sup>	A. Kryukov, Inst. of Biology and Soil Science, RAS
#1746	F	Primorsky Krai, Russia	E–5 (AB538994) <sup>b</sup>			A. Kryukov, Inst. of Biology and Soil Science, RAS
#1747	F	Primorsky Krai, Russia	ERS2 (LC185449)			A. Kryukov, Inst. of Biology and Soil Science, RAS
#1748	F	Primorsky Krai, Russia	ERS7 (LC185454)			A. Kryukov, Inst. of Biology and Soil Science, RAS
#1749	M	Primorsky Krai, Russia	ERS4 (LC185451)	Me3 (LC185457)	SNE1(AB551119) <sup>b</sup>	A. Kryukov, Inst. of Biology and Soil Science, RAS
#1833	M	Primorsky Krai, Russia	ERS4 (LC185451)	Me3 (LC185457)	SNE1(AB551119) <sup>b</sup>	A. Kryukov, Inst. of Biology and Soil Science, RAS
#2029	F	Primorsky Krai, Russia	ERS5 (LC185452)			A. Kryukov, Inst. of Biology and Soil Science, RAS
#2030	F	Primorsky Krai, Russia	ERS8 (LC185455)			A. Kryukov, Inst. of Biology and Soil Science, RAS
#2031	M	Primorsky Krai, Russia	ERS5 (LC185452)	Me3 (LC185457)	SNE1(AB551119) <sup>b</sup>	A. Kryukov, Inst. of Biology and Soil Science, RAS
#2188	F	Primorsky Krai, Russia	ERS4 (LC185451)			A. Kryukov, Inst. of Biology and Soil Science, RAS
#2222	M	Primorsky Krai, Russia	ERS6 (LC185453)	Me3 (LC185457)	SNE1(AB551119) <sup>b</sup>	A. Kryukov, Inst. of Biology and Soil Science, RAS

<sup>a</sup> M, male; F, female.<sup>b</sup> Previously reported haplotypes.

Table 2 indicates the results of molecular diversities of the badger mtDNA control region. The haplotype and nucleotide diversities were relatively high in each population from the five regions (Bulgaria, Norway, Finland, Ural and Far East Russia).

Table 3 indicates the genetic distances between populations of the different sampling locations using Kimura's two-parameter model. The values between Russia (Ural and Far East) and Europe (Bulgaria, Norway and Finland) were higher (0.0405–0.0442), whereas that between Ural and Far East Russia was lower (0.0086). The values across Europe were relatively low (0.00483–0.0106).

We constructed a neighbour-joining phylogenetic tree for the mtDNA control region with sequence data obtained in the present study and also obtained from DDBJ (accession nos. AB538970–AB538974, AB538990–539000; haplotype names are J-1-5, E-1-6, C-1, 2 and W-1-4) (Fig. 2). Eurasian badgers were divided into four groups: Europe, Caucasus, continental Asia and Japan. Badgers from the Urals and Far Eastern Russia were grouped in the Asian badger (*M. leucurus*) (but with low bootstrap support). In contrast, badgers from Bulgaria, Finland and Norway were unambiguously clustered into the European badger (*M. meles*). Sequences of the mtDNA control region obtained in the present study were deposited to the DDBJ/Genbank/EMBL databases with accession nos.: LC185431–LC185456.

### 3.2. Variation of Y-chromosomal genes

We determined sequences of the *SRY* gene (1052–1060 bp) and CAN-SINEs (208–210 bp) on the Y chromosome from 23 males badgers (Table 1). It was impossible to amplify any fragments of Y-chromosomal DNA from some old skins of the Norwegian badgers, probably due to severe fragmentation of DNA. Among the results of the *SRY* sequence alignment, we found one haplotype from the Urals, two haplotypes from Far Eastern Russia, and one haplotype from Europe. The two haplotypes from Far Eastern Russia were newly identified (haplotypes Me3 and Me4). One *SRY* haplotype from the Urals and that from Europe were identical with the previously reported haplotypes, Me1 (AB539133) and Mw1 (AB539136), respectively. Sequences of the *SRY* obtained in the present study were deposited to the DDBJ/Genbank/EMBL databases with accession nos.: LC185457 and LC185458.

On the other hand, one haplotype of CAN-SINEs from Russia and that from Europe were identical with the previously reported haplotypes, SNE1 (AB551119) and SNW1 (AB551120), respectively.

The network tree of the *SRY* gene, with sequence data obtained from DDBJ (accession nos. AB539127–AB539136; haplotypes were named as Mj1-6, Me1, 2, Mc1 and Mw1) (Fig. 3), showed clustering of four clades, in congruence with the result of the mtDNA control region. The result of the phylogenetic trees of CAN-SINEs corresponds with those of previous studies (Tashima et al., 2011b), because all haplotypes of CAN-SINEs were identical with the previously reported ones. The badgers from Russia (the Urals and Far East) were included in *M. leucurus*, and those from Europe were in *M. meles*.

## 4. Discussion

### 4.1. Molecular phylogenetic relationships among Eurasian badgers

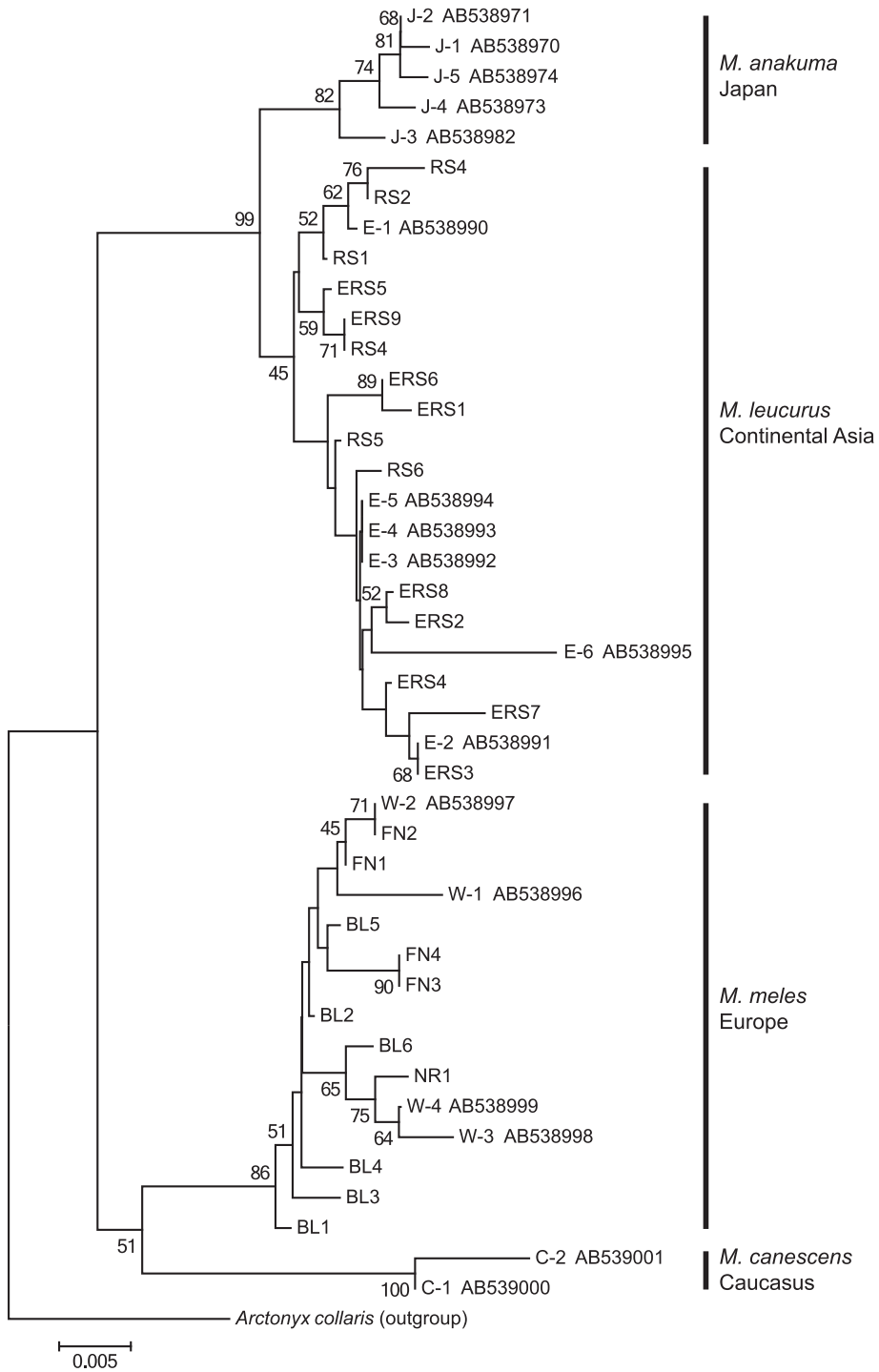
The mtDNA phylogenetic analyses in the present study clearly showed that the badgers from Bulgaria, Norway and Finland are included in *M. meles*, and that those from the Urals and Far Eastern Russia are in *M. leucurus*. The genetic differentiation

**Table 2**  
Molecular diversity on the mtDNA control region of Eurasian badgers examined in the present study.

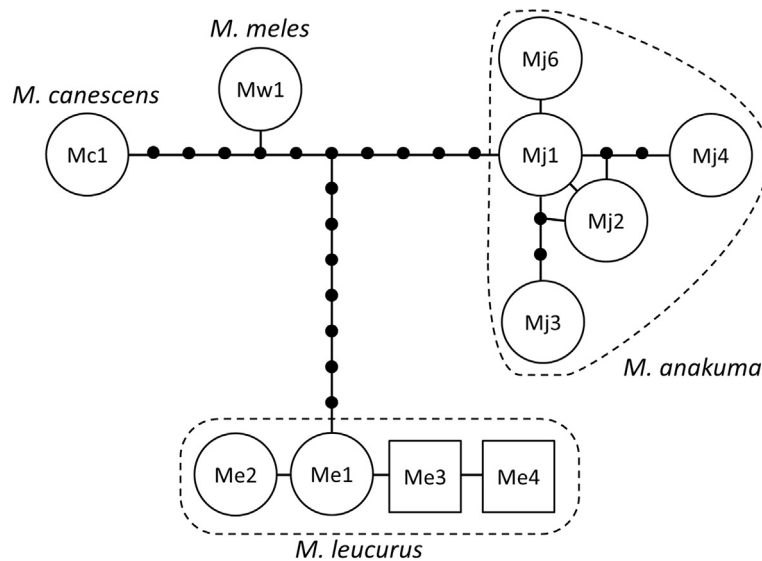
Population	n	Number of haplotypes	Number of polymorphic sites	Haplotype diversity (Average $\pm$ SE)	Nucleotide diversity (Average $\pm$ SE)
All	71	29	46	0.95 ( $\pm$ 0.0124)	0.024 ( $\pm$ 0.0120)
Bulgaria	27	6	8	0.75 ( $\pm$ 0.0553)	0.0025 ( $\pm$ 0.00177)
Norway	5	3	7	0.70 ( $\pm$ 0.218)	0.0056 ( $\pm$ 0.00406)
Finland	8	4	5	0.64 ( $\pm$ 0.184)	0.0033 ( $\pm$ 0.00242)
Ural	13	8	9	0.91 ( $\pm$ 0.0559)	0.0062 ( $\pm$ 0.00384)
Far East Russia	18	11	15	0.93 ( $\pm$ 0.0382)	0.0088 ( $\pm$ 0.00505)

**Table 3**  
Genetic distances between populations estimated using Kimura's two-parameter model.

Population	1	2	3	4	5
1. Bulgaria					
2. Norway	0.0083				
3. Finland	0.0048	0.0106			
4. Ural	0.0405	0.0440	0.0429		
5. Far East Russia	0.0416	0.0439	0.0442	0.0086	



**Fig. 2.** A neighbour-joining phylogenetic tree of mtDNA haplotypes. Haplotypes with accession numbers show those cited from the DDBJ. Haplotypes without accession numbers indicate new sequences obtained in the present study. Numbers near internal branches are bootstrap values (>40%) derived from 2000 replications. The scale below the tree shows Kimura's two-parameter distances.



**Fig. 3.** Parsimony network of *SRY* haplotypes (open circles and squares) from male badgers. Closed small circles show presumed haplotypes. Two types (squares) were newly found in the present study. One bar between haplotypes corresponds to one nucleotide substitution.

between *M. meles* (Bulgaria, Norway and Finland) and *M. leucurus* (Ural and Far East Russia) was supported by large genetic distances (0.0405–0.0442) (Table 3).

In addition, the genetic distance (0.0106) showed some differentiation between Norwegian and Finish badgers, even though the two areas are located in Scandinavia. Abramov et al. (2009) reported that the crania of Scandinavian badgers are smaller than those from other European countries, especially, those from southwestern Norway which have the smallest skulls. The genetic difference detected in the present study is consistent with the reports of Abramov et al. (2009).

Frantz et al. (2014) reported that refugia occurred in the Balkan and Iberia Peninsulas in the Last Glacial Maximum, and that badgers in Scandinavia consist of descendants from these two refugia. Populations from Bulgaria and Finland shared haplotype BL1. Haplotypes BL5 and BL6 are similar to those found in Scandinavia. The genetic similarity between Bulgarian and Scandinavian haplotypes supports the assumption about origin of Scandinavian populations from southern refugia.

#### 4.2. Distribution boundaries of Eurasian badgers

We revealed that badgers distributed around the Ural Mountains are included in the Asian badger (*M. leucurus*). The results suggest that at present the Ural Mountains are not a geographical barrier between *M. leucurus* and *M. meles*, and that badgers could have migrated through this range of mountains. Previous studies proposed that the contact zone between the two badger species is located between the Volga and Kama Rivers, which run west of the Urals (Abramov and Puzachenko, 2006; Tashima et al., 2011a). In the present study, the haplotypes found around the Kama River proved to be from *M. leucurus*. Gasilin and Kosintsev (2010) analyzed subfossils of badgers excavated from the Middle Holocene epoch layer, and reported the boundary between the two badger species was located 1500 km east of the current boundary. To reveal the migration history of badgers around the Urals, it is important to carefully investigate ancient DNA from those Holocene subfossils.

Focusing on South Europe, Frantz et al. (2014) reported that a refugium of Eurasian badgers had been located in the Balkan Peninsula during the last glacial period, based on the mtDNA control region sequence of only one badger specimen from Bulgaria. All 27 badgers from Bulgaria examined in the present study were included in *M. meles*, based on phylogenetic features of the mtDNA control region, *SRY* gene and CAN-SINES. İbiş et al. (2015) reported that the Bosphorus Strait located south of Bulgaria is a distribution boundary between *M. meles* and *M. canescens*, based on mtDNA cytochrome *b* sequences. This is not incongruent with our results. The similar phylogeographical pattern based on mtDNA has also been reported in other mammals, such as the white-breasted hedgehog (*Erinaceus concolor*) (Seddon et al., 2002) and the European ground squirrel (*Spermophilus citellus*) (Gündüz et al., 2007).

On the other hand, badgers from Far Eastern Russia shared some haplotypes of *M. leucurus*. Kurose et al. (2001) and Tashima et al. (2011a,b) reported that *M. leucurus* is distributed around Lake Baikal, based on analysis of mtDNA cytochrome *b* and the control region, respectively. These data indicated that *M. leucurus* is widespread from the Volga River to Far Eastern Russia. Koh et al. (2014) showed that badgers from Jeju Island also shared *M. leucurus* cytochrome *b* haplotypes. Lee et al. (2016) also reported *M. leucurus* control region haplotypes in badgers from the Korean Peninsula. Now it is certain that the Sea of Japan separates the distribution between *M. leucurus* and *M. anakuma*.



#### 4.3. Differences of behavior patterns

Previous studies reported that male badgers disperse more widely than females (Kruuk and Parish, 1987; Rogers et al., 1998; Roper et al., 2003). In the present study, the phylogeographical results of paternally inherited *SRY* and *CAN-SINES* were both in congruence with those of the mtDNA control region as a maternally inherited gene. This suggests that male badgers do not migrate across the distribution boundaries between *Meles* species, although they have a higher dispersal ability than females.

In the present study, no genetic evidence of hybrids between *M. leucurus* and *M. meles* was found. Because the region around the Volga and Kama Rivers is their contact zone, one may expect to find hybrids having both *M. meles* type of Y-chromosomal DNA and *M. leucurus* type of mtDNA, or the other way round (*M. leucurus* type of Y-chromosomal DNA and *M. meles* type of mtDNA). However, in addition to the present study, Tashima et al. (2011a) also found no hybrids between these two species. It is necessary to examine precisely genotypes of badgers distributed in the delta region.

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