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Insights into the Turkish and Iranian badgers (the genus *Meles*) based on the mitochondrial cytochrome *b* gene sequences

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Abstract

To investigate the genetic diversity and phylogenetic status of Eurasian badgers (genus *Meles*) from Turkey and Iran, we used sequence data from the complete mitochondrial cytochrome *b* gene (1140 bp) and examined 11 samples from Turkey and one sample from Iran. Relying on Bayesian, Maximum Likelihood, Neighbor-Joining and Network methods, significant genetic differences corresponding to *Meles meles* from the European part and *Meles canescens* from the Asian part of Turkey and from Iran confirmed previous genetic and morphometric results and provide another example for the barrier effect of the Bosphorus and Dardanelle Straits for mammalian species. Additionally, this study suggested that the Asian samples of Turkish badgers have a relatively high genetic diversity.

Key words

Eurasian badger, *Meles*, Cytochrome *b*, Turkey, Iran.

Introduction

The genus *Meles* (Eurasian badger) is one of the most widespread mustelids in the Palaearctic region. The Eurasian badgers inhabit densely vegetated areas and forests across Eurasia, from British Islands to Japanese Islands (Wilson & Reeder 1993, 2005). Based on morphological characters, Eurasian badgers (*Meles*) were previously considered a single species, *M. meles* (Heptner *et al.* 1967, Corbet 1978, Wilson & Reeder 1993, Nowak 1999, Kurose *et al.* 2001), but studies using mitochondrial and nuclear genes (Marmi *et al.* 2006 and Del Cerro *et al.* 2010, Tashima *et al.* 2011a,b) have confirmed that the genus *Meles* consists of four species, which were also proposed on the basis of a recent morphological study (Abramov & Puzachenko 2013): (1) the European badger (*Meles meles*); (2) the Caucasian-Pamir badger (*M. canescens*); (3) the Asian badger (*M. leucurus*) and (4) the Japanese badger (*M. anakuma*). Moreover, based on cranial morphometrics Abramov & Puzachenko (2013) suggested the existence of two Eurasian badger species in Turkey (*M. meles* in the European part and *M. canescens* in the Asian part), and the presence of *M. canescens* in Iran.

In general, the Eurasian badgers (*Meles meles, M. leucurus* and *M. anakuma*) are listed as Least Concern (LC) in the IUCN Red List (Abramov & Wozencraft 2008, Kaneko & Sasaki 2008, Kranz *et al.* 2008). However, in

recent years, the Turkish population of Eurasian badger has decreased as a consequence of unplanned urbanization, noise and light pollution, intensive agriculture, excessive use of fertilizers and pesticides, road-kill, and floods (Özen & Uluçay 2010, Pamukoğlu & Sezginer 2011, İnaç *et al.* 2012, Pamukoğlu & Albayrak 2014).

The Southwestern part of the Asian continent, including Turkey and Iran, is Eastern region of the Mediterranean area in Palaearctic (Kryštufek & Vohralik 2001, Özdikmen 2011). There are few studies regarding the ecology, distribution, morphology and anatomy of the Eurasian badgers distributed in this region (Pamukoğlu & Çakir 2001, Özen & Uluçay 2010, İnaç *et al*. 2012, Pamukoğlu & Albayrak 2014). Also, only few Turkish and Iranian carnivore species were investigated by using mitochondrial DNA (Fadakar *et al.* 2013, Hassan-Beigi *et al.* 2014, Farhadinia *et al.* 2015, Hirata *et al.* 2014, İbiş & Tez 2014, İbiş *et al.* 2014, Statham *et al.* 2014). The aim of this study is to investigate the systematic status of Iranian and Turkish badger populations, which are assigned to *M. meles* by the current mammal check-lists of Turkey and Iran (Kryštufek & Vohralik 2001, 2009 Karami *et al.* 2008), by using a molecular marker.

Material and Methods

Sampling and DNA isolation

Tissue samples (ear, muscle or tail) from road-killed badgers were collected from 11 localities (Table 1, Fig. 1). Total genomic DNA was isolated from ear, muscle or tail tissues fixed in 99% ethanol using the DNeasy Blood and Tissue Kit (QIAGEN), following the manufacturer's instructions.

PCR reaction and Sequencing

The complete cytochrome *b* gene was amplified by Hot Start PCR using primer pair, Cb-M1 (5'-CTCACATGG AATCTAACCATGAC-3) and Cb-MR1 (5'-TCTTCCTT GAGTCTTAGGGAG-3), designed by Kurose *et al.* (2000). PCR amplifications were performed in 50 µl reaction mixture (1X Taq buffer with $(NH_4)_2SO_4$, 200 µM dNTP mix, 1.5 u Hot Start Taq DNA polymerase (Thermo Scientific), 1.5 mM $MgCl_2$, 0.8 µM of each primers, 1 µl DNA extract). The Hot Start PCR program included a pre-denaturation procedure consisting of 15 min. at 95 °C by 1 cycle, a denaturation step of 1 min. at 94 °C, an annealing step of 1 min. at 50 °C, an extension step of 2 min. at 72 °C by 35 cycles and an ending step of 10 min. at 72 °C by 1 cycle.

To verify the quality of total DNA and PCR products, 1% and 1.5% agarose gels were run and stained with ethidium bromide, respectively.

The PCR products purified by High Pure PCR Product Purification Kit (Roche) were sequenced by using Cb-M1 (Forward) and Cb-MR1 (Reverse) with following the internal primers, Cb-L3 (5'-CTTACATGTAGGA CGAGGCCT-3'), Cb-L4 (5'TCCCATTCCATCCATAT TACAC-3'), Cb-LR3 (5'GATTGCGTATGCGAATAA GAA-3'), Cb-LR4 (5'-CGGTTGCACCTCAAAAAGA CA-3'), Cb-LR5 (5'-AGGGGATACCAGAGGGGTT-3) and Cb-LR6 (5'-GTAAGATTGCGTATGCGAATAAG-3'), for the complete cytochrome *b* gene reported by Kurose *et al.* (2001) with an ABI 3100 Genetic Analyzer (RefGen, METU, Technopark-Ankara, Turkey).

Sequence analysis

To determine the lineages of Turkish and Iranian haplotypes, Turkish and Iranian badger sequences were compared with those from the GenBank database (NCBI: The National Center for Biotechnology Information) that were registered by Ledje *et al*. (1996): X94922, by Kurose *et al*. (2001): AB049790-809, by Arnason *et al*. (2007): AM711900 (= NC_011125), by Yonezawa *et al*. (2007): AB291075 (= NC_009677), by Fernandes *et al.* (2008a): EF689063–66, by Del Cerro *et al*. (2010): HQ711941–51, by Sato *et al*. (2012): AB285330, and by Koh *et al*. (2014): KF944283–87 and KF891475–83. The hog badger, *Arctonyx collaris*, (AB049810: Kurose *et al*. 2001) and the honey badger, *Mellivora capensis* (EF987755: Koepfli *et al*. 2008) were used as out-group.

To align and edit mitochondrial cytochrome *b* sequences, we used Geneious v.6.1 (available from http://www.geneious.com), yielding a sequence of 1140 bp. The number of segregating sites (S), haplotype diversity (*Hd*), nucleotide diversity (π) and frequency of each haplotype were calculated using DnaSP v. 5.10.01 (Librado & Rozas 2009). Genetic distances were estimated under the Kimura 2-parameter (K2P) nucleotide substitution model (Kimura 1980) in MEGA5 v. 5.01 (Tamura *et al*. 2011).

Phylogenetic analyses were performed using the Maximum Likelihood (ML) and Neighbor-Joining (NJ) methods with MEGA5 (Tamura *et al*. 2011), and the Bayesian (BI: Bayesian Inference of phylogeny) method with Mr. Bayes v. 3.2.3 (Ronquist & Huelsenbeck 2003). Before Bayesian and Maximum Likelihood analyses, the HKY (Hasegawa-Kishino-Yano) + G (Gamma) substitution model was selected as the most appropriate model according to both Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) using jModelTest2 (Guindon & Gascuel 2003, Darriba *et al*. 2012). The ML and NJ trees were constructed on the basis of the HKY+G and K2P substitution models, respectively, by 10000 bootstrap replicates.

The Bayesian tree was also generated on the basis of the HKY+G substitution model. The Bayesian posterior probabilities for 0.3 million generations with trees sampled every 100 generations were calculated by using four Monte Carlo Markov chains. The first 25% of samples

Table 1. List of the Turkish and Iranian badger samples and sequences obtained from the GenBank database.

Turkish and Iranian samples No.	Haplotype/ Sequence code	Map number	Locality	Reference
1405, 1421	MEMETR1	1	Kırıkköy, Lüleburgaz, Kırklareli, Turkey	This study
1537	MECATR1	2	Çandır, Kalecik, Ankara, Turkey	This study
1349	MECATR2	3	Feke, Adana, Turkey	This study
1521	MECATR2	4	Tuzluca, Iğdır, Turkey	This study
1571	MECATR2	5	Göle, Ardahan, Turkey	This study
1420	MECATR3	6	Karakurt, Kars, Turkey	This study
1519	MECATR3	7	Koyunyurdu, Selim, Kars, Turkey	This study
1508	MECATR4	8	Mammals Collection, Department of Biology, Dicle University, Diyarbakır, Turkey (collected from the Southeast part of Turkey)	This study
1572	MECATR5	9	Bodrum, Muğla, Turkey	This study
1578	MECATR6	10	Çiftlikköy, Banaz, Uşak, Turkey	This study
1526	MECAIR1	11	Gilan Province, Iran	This study
	HQ711945–46	12	The Levant	Del Cerro *et al*., 2010
	HQ711947–48	13	Crete Island, Greece	Del Cerro *et a* ., 2010
	AB049808-9	14	Russia	Kurose *et al*., 2001
	HQ711941	15	Germany	Del Cerro *et al*., 2010
	HQ711942	16	England	Del Cerro *et al*., 2010
	EF689063-64	17	Portugal	Fernandes *et al*., 2008
	EF689065-66; HQ711943	18	Spain	Fernandes *et al*., 2008; Del Cerro *et al*., 2010
	AM711900; X94922	19	Sweden	Arnason *et al*., 2007; Ledje *et al*., 1996
	HQ711944	20	Norway	Del Cerro *et al*., 2010
	AB049790-806; AB291075; AB285330	21	Japan	Kurose *et al*., 2001 Yonezawa *et al*., 2007; Sato *et al*., 2012
	AB049807; HQ711949; HQ711951	22	Russia	Kurose *et al*., 2001 Del Cerro *et al*., 2010
	HQ711950	23	Mongolia	Del Cerro *et al*., 2010
	KF944283–87; KF891475–83	24	South Korea	Koh *et al*., 2014

were discarded as burn-in (average standard deviation of split frequencies <0,01). After discarding burn-in trees and evaluating convergence, the remaining samples were retained for generating consensus trees (50% majority rule), calculating 95% Bayesian credible intervals and posterior probabilities. Bayesian tree was pictured with tree figure drawing tool, FigTree v1.3.1 (Rambaut 2009). The median-joining (MJ) network was revealed using the Network 4.6.1.1 software (Bandelt *et al.* 1999, http://www.fluxus-engineering.com) in order to represent the intra-specific genealogy of the haplotype dataset.

Results

Mitochondrial DNA sequences

The complete mitochondrial cytochrome *b* gene sequences (1140 bp) were obtained from the 12 badger samples, including Turkish (11) and Iranian (1) samples (Table 1, Fig. 1). A total of eight haplotypes (MEMETR1, MECATR1-MECATR6, MECAIR1) were determined among the 12 Eurasian badgers (Table 1, Fig. 1). The haplotypes MEMETR1, which represented the European part of Turkey, and MECATR1- MECATR6, which represented the Asian part of Turkey, were observed in the Turkish samples. For the Iranian sample, MECAIR1 haplotype was found (Table 1, Fig. 1). All haplotypes (MEMETR1, MECATR1-MECATR6, MECAIR1) were new and so far have not been reported in any Eurasian badger population from different geographic regions. The newly determined cytochrome *b* sequences have been deposited in the GenBank database (KT988010–KT988017). Based on mitochondrial sequences, haplotype diversity (*Hd*) (0.9091) and nucleotide diversity (π) (0.01767) demonstrated relatively high levels of genetic diversity for the 11 Turkish badgers. In addition, the number of polymorphic (segregating) sites was S=60. The genetic divergence of the mitochondrial cytochrome *b* sequences ranged from 0.001 to 0.054, with an average of 0.017 (1.7%) among all the Turkish badgers, using K2P distance. Furthermore, the mean sequence divergence was 0.053 (5.3%) between haplotypes from the European and the Asian parts of Turkey, ranging from 0.052 to 0.054.

In comparing the Turkish and Iranian haplotypes with those from the GenBank database, the haplotypes MECATR1-MECATR6 and MECAIR1, which were

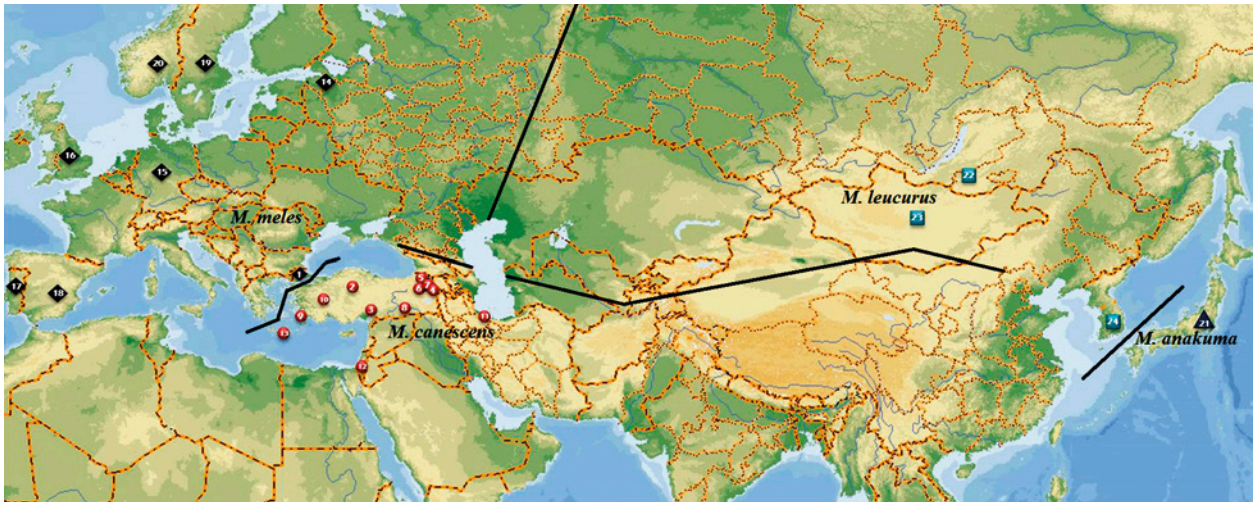

Fig. 1. Localities of the Turkish and Iranian badger samples and sequence distributions from the GenBank database. (For map numbers, see Table 1).

found in ten badger samples from the Asian part of Turkey and Iran, were determined to belong to *Meles canescens*. On the other hand, the haplotype MEMETR1, which was found in the two samples from the European part of Turkey, was observed to belong to *M. meles*.

Phylogenetic analyses of the Eurasian badger mitochondrial cytochrome *b* haplotypes

The phylogenetic trees constructed by Bayesian Inference, Maximum Likelihood and Neighbor-Joining methods resulted in the same topology according to the clustering of haplotypes. Therefore, phylogenetic relationships between the complete mitochondrial cytochrome *b* (1140 bp) haplotypes found in this study and the GenBank database were shown only in Bayesian tree, which resulted in four phylogroups (Fig. 2). From top to bottom in Figure 2, these phylogroups corresponded to the Transcaucasian badger *M. canescens* (from the Asian part of Turkey, Iran, the Levant and Crete Island of Greece), the European badger *M. meles* (from the European part of Turkey, Germany, England, Portugal, Spain, Norway, Sweden and the European part of Russia), the Japanese badger *M. anakuma* (from Japan), and the Asian badger *M. leucurus* (from the Asian part of Russia, Mongolia and South Korea), respectively. Network analysis was also applied for haplotypes used in the Bayesian analysis. The Median-Joining network revealed that the haplotype grouping picture of the genus *Meles* was in agreement with that of Bayesian analysis, with a clear genetic pattering within the genus *Meles* (Fig. 3). Moreover, these analyses grouped all haplotypes into four geographical phylogroups compatible with previous studies (Marmi *et al.* 2006, Del Cerro *et al.* 2010, Tashima *et al.* 2011a, b). According to the clustering status in Bayesian and network analyses, the haplotypes found in this study were positioned in the two allopatric phylogroups, which correspond to *M. canescens* containing MECATR1-MECATR6 and MECAIR1, as well as *M. meles* comprising MEMETR1 (Figs. 2, 3). Bayesian and network analyses indicated that the European part of Turkey differed genetically from Asian part of Turkey and Iran, based on the complete cytochrome *b* sequences.

Discussion

The Eurasian badger (*Meles*) is a widespread genus of the family Mustelidae (Wilson & Reeder 1993, 2005). There have been numerous studies based on different genetic markers; allozyme (Pertoldi *et al.* 2000), microsatellites (Pope *et al.* 2006, Van de Zande *et al.* 2007, Huck *et al.* 2008, O'Meara *et al.* 2012, Frantz *et al.* 2014), ZFX and ZFY genes (Statham *et al.* 2007, Tashima *et al.* 2011b), *SRY* gene (Tashima *et al.* 2011a), nuclear genes (Sato *et al.* 2003, Del Cerro *et al.* 2010), mitochondrial control region (Marmi *et al.* 2006, Del Cerro *et al.* 2010, Tashima *et al.* 2011a, O'Meara *et al.* 2012, Frantz *et al.* 2014) and cytochrome *b* gene (Kurose *et al.* 2001, Sato *et al.* 2003, Koh *et al.* 2014), throughout the range of Eurasian badger. However, to date, as genetic data have not been reported from Iran and Turkey, the phylogenetic relationships of Iranian and Turkish badgers are still unclear.

In order to determine the phylogenetic status of the Eurasian badger populations native to Iran and Turkey, we presented genetic data obtained from the mitochondrial cytochrome *b* gene.

The Iranian and Turkish badgers were relatively polymorphic, since all the haplotypes (MEMETR1, MECATR1-MECATR6, MECAIR1) within sampling area were new and thus far have not been reported from any Eurasian badger population in different geographic regions (Figs. 2–3).

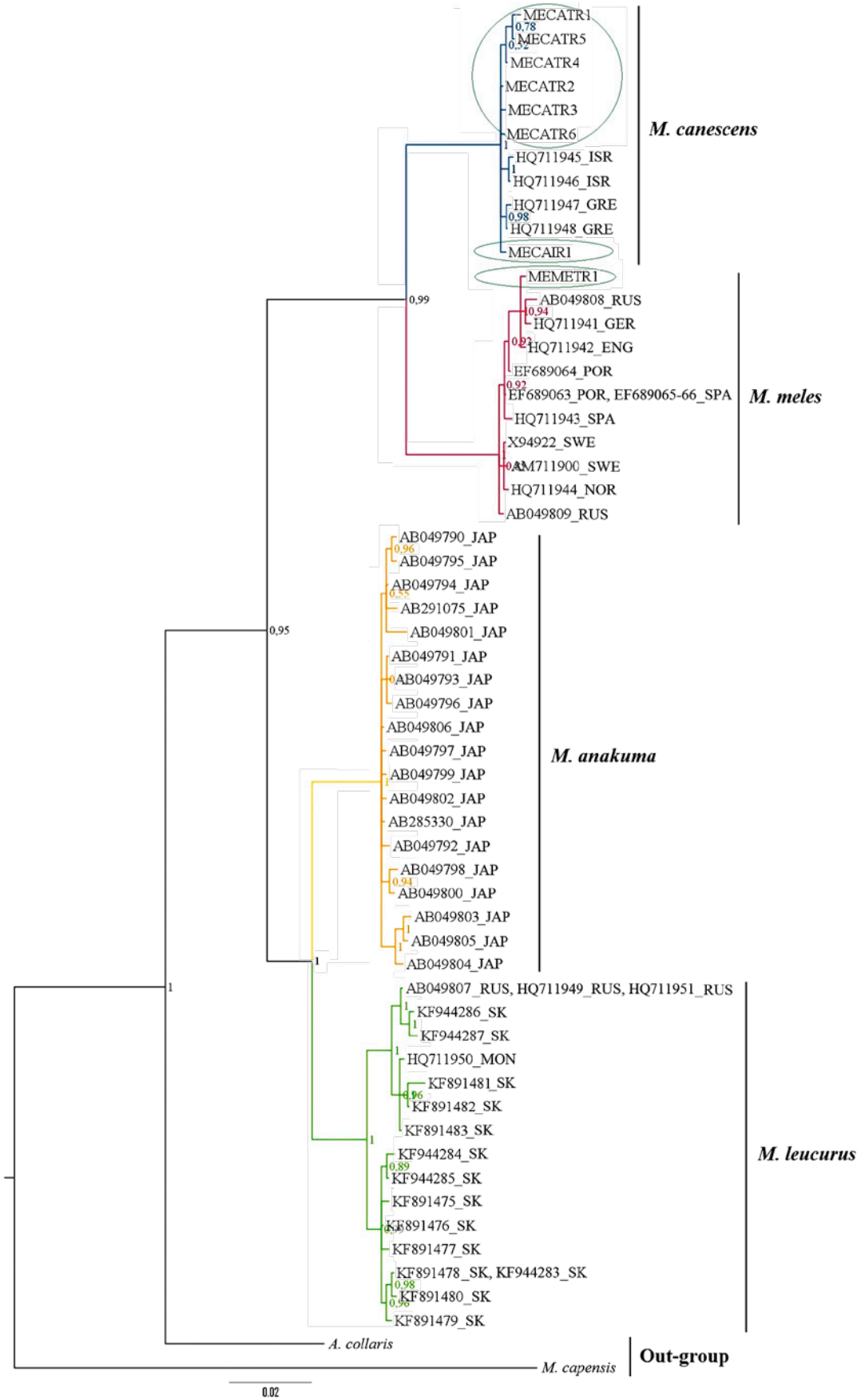

Fig. 2. Bayesian tree generated from haplotypes of the complete mitochondrial cytochrome *b* gene (1140 bp) of Eurasian badgers, rooted with *A. collaris* and *M. capensis*. Numbers above branches show the Bayesian posterior probabilities. Circles indicate the Turkish and Iranian badger haplotypes.

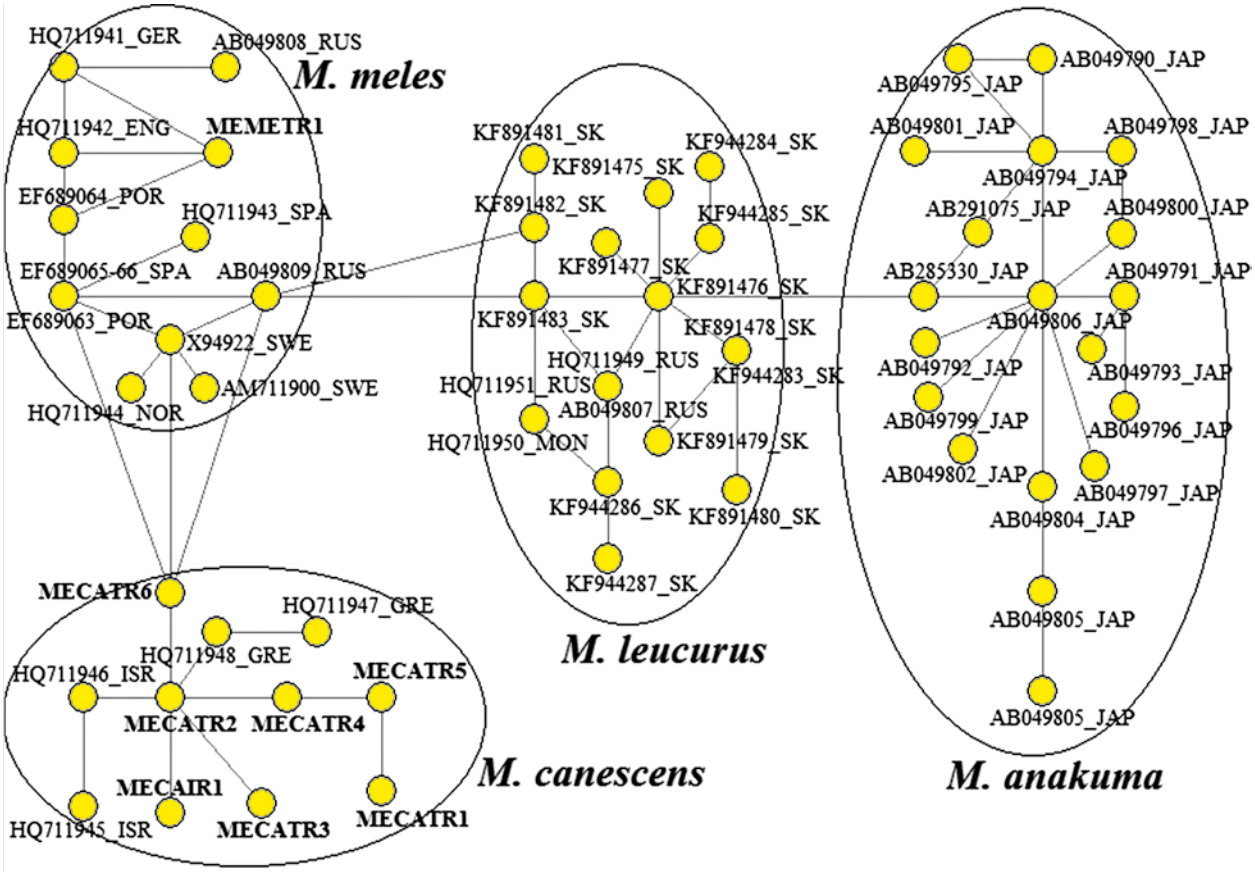

Fig. 3. Median-joining network generated from haplotypes of the complete mitochondrial cytochrome *b* gene (1140 bp) of Eurasian badgers. The bold haplotypes indicate the Turkish and Iranian badger.

This study confirmed significant genetic differences corresponding to *M. meles* from the European part of Turkey, and *M. canescens* from the Asian part of Turkey and Iran (Figs. 2, 3), as reported by the previous genetic (Marmi *et al.* 2006, Del Cerro *et al.* 2010, Tashima *et al.* 2011a, b) and morphometric results (Abramov & Puzachenko 2013). Additionally, our work provided an example of the barrier effect of the Bosphorus and Dardanelles straits (Fig. 1). Turkey is divided into two geographical areas by these straits: (1) The European part of Turkey and (2) The Asian part of Turkey. In addition to two Eurasian badger species; *M. meles* and *M. canescens*, the barrier effect of these straits in the west is also valid for some mammalian species, such as striped field mouse *Apodemus agrarius* (Yiğit *et al.* 2000, Kefelioğlu *et al.* 2003), European ground squirrel *Spermophilus citellus* (Gündüz *et al.* 2007), and European polecat *Mustela putorius* (Kurtonur *et al.* 1994, Fernandes *et al.* 2008b, İbiş 2013) occurring in the European part of Turkey, and probably Anatolian ground squirrel *Spermophilus xanthoprymnus* (Gündüz *et al.* 2007) occurring in the Asian part of Turkey. In a similar example, *A. agrarius* is distributed in the northeastern of the Caucasus Mountains (Corbet 1978, Wilson & Reeder 2005, Kaneko *et al.* 2008), although it is absent in the Asian part of Turkey (Yiğit *et al.* 2000, Kefelioğlu *et al.* 2003). In this context, the Caucasus Mountains puts a barrier effect for some mammalian species in the East and also limits the distribution of *M. meles* and *M. canescens* (Fig. 1) (Marmi *et al.* 2006, Del Cerro *et al.* 2010, Tashima *et al.* 2011a,b, Abramov & Puzachenko 2013, This Study), as such the Bosphorus and Dardanelles straits.

Finally, based on the complete mitochondrial cytochrome *b* sequences, the present study confirms that the Eurasian badgers consist of four species and that Turkey is inhabited by *M. meles* and *M. canescens* (Figs. 2–3), as has been previously found by Marmi *et al.* (2006), Del Cerro *et al.* (2010), Tashima *et al.* (2011a,b), and Abramov & Puzachenko (2013). To elucidate the genetic diversity and phylogenetic relationships of Turkish and Iranian badgers in detail, it would be a great importance to analyze additional data from badgers throughout Southwest Asia, and these data should consider a multilocus study.

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