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Inferring phylogenetic relationships in the common vole (*Microtus arvalis*) based on mitochondrial and nuclear sequence diversities

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Abstract: The common vole *Microtus arvalis* (Pallas, 1778) is the most widespread *Microtus* species. It has two forms – the European arvalis form (2n: 46, NF: 84) and the Asian obscurus form (2n: 46, NF: 72). The present study aimed to clarify the taxonomic status of *M. arvalis* populations distributed in Eastern Turkey, Europe and Asia by analysing two mitochondrial (*CYTB* and *COX1*) and one nuclear (*IRBP*) markers. Phylogenetic dendrograms (median-joining networks and Bayesian trees) constructed using the mitochondrial markers clearly separated the Anatolian population from the European and Asian populations. Contrarily, any explicit differentiation was not shown in *IRBP* analyses. Mean and net genetic distance values (*d*) were found to be notably low for three markers. Species delimitation test (Automatic Barcode Gap Discovery Method) supported these results. Our results indicate that the arvalis and obscurus forms are not sufficiently differentiated to be considered different species, while the Anatolian population has only recently split from the Asian population. Together, these findings demonstrate that the speciation process is ongoing.

Keywords: Common vole, *Microtus arvalis*, Anatolia, *CYTB*, *COX1*, *IRBP*

1. Introduction

Microtus is one of the most diversified mammalian groups. It consists of 14 subgenera and 65 extant species in the Holoarctic Region (Musser and Carleton, 2005; Golenishchev and Malikov, 2006). The common vole, *Microtus arvalis* (Pallas, 1778), has the largest distribution area, compared to other *Microtus* species, ranging from Western Europe to the east of Russia (Mitchell-Jones et al., 1999; Shenbrot and Krasnov, 2005), and occupies many habitats such as moist meadows, pastures, forest steppes, and moist forests (Zima, 1999). With regard to chromosomal differences, *M. arvalis* has two karyotype forms: the European arvalis form (2n: 46, NF: 84) and the Asian obscurus form (2n: 46, NF: 72) (Malygin, 1974; Malygin and Orlov, 1974; Kral and Liapunova, 1975). While the arvalis form is found in a region from Europe to Northeast Russia, the obscurus form is distributed in the east of Russia and the Middle East, and both forms come into contact with each other in the European part of Central Russia (Bulatova, 2007, 2010).

Up to now, various karyological and molecular studies about these two forms have been published (Mazurok et al., 2001; Haynes et al., 2003; Fink et al., 2004; Jaarola et al., 2004; Heckel et al., 2005; Bulatova et al., 2007, 2010; Lavrenchenko et al., 2009; Buzan et al., 2010; Barbosa et al., 2018). However, it has been a matter of debate as to whether or not the arvalis and obscurus forms are different species.

Microtus arvalis has been previously recorded in Eastern Anatolia in Turkey by Kefelioğlu (1995) and reviewed by Kryštufek and Vohralík (2005). Taxonomic studies on *M. arvalis* in Turkey are mainly karyological and morphological (Kefelioğlu, 1995; Markov et al., 2009, 2014; Yorulmaz et al., 2013). Molecular systematic studies of the Turkish common vole are almost absent, with the exception of that of Tougard et al. (2013) who studied Turkish *M. arvalis* samples using a single molecular marker (*CYTB*) and defined the western and eastern populations as *M. arvalis* and *M. obscurus*, respectively. Therefore, two mitochondrial (Cytochrome-*b*/*CYTB* and Cytochrome

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oxidase-I/*COX1*) and one nuclear (Interphotoreceptor retinoid binding protein/*IRBP*) markers were used here to compare Anatolian specimens with other Palearctic populations, in order to determine the phylogenetic status of these forms of common vole.

2. Material and methods

2.1 Sampling

Eleven samples from Eastern Anatolia, 15 samples from Serbia and Hungary, and 61 gene sequences from Genbank were used in analyses of the *CYTB* gene region (Figure 1). For the *COX1* gene, 13 samples from Eastern Anatolia, 16 samples from Serbia and Hungary and 8 gene sequences from Genbank were analysed.

In addition, 11, 14 and 4 *IRBP* sequences were analysed from Eastern Anatolia, Serbia and Hungary and Genbank, respectively.

Microtus arvalis specimens were captured by live animal traps and killed using diethyl ether, and their tissues and skulls were taken, with legal permission approved by Ankara University Local Ethics Committee for Animal Experiments (Document no: 2016-21-184). DNA isolation and the polymerase chain reaction of Eastern Anatolian, Serbian and Hungarian samples were performed. The skull and tissues of Turkish samples were stored in the Ankara University Mammalian Research Collection (AUMAC, Ankara, Turkey). AUMAC samples and sequences obtained from Genbank[1] are shown in Table S1.

2.2 DNA isolation and PCR

DNA samples were isolated from frozen kidney and liver tissues (stored at –80 °C) using a GeneMATRIX TISSUE & BACTERIAL DNA Purification Kit E3551-02 (BMLabosis, Ankara, Turkey). Two mitochondrial (*CYTB* and *COX1*) and one nuclear (*IRBP*) gene regions were amplified, and primers were used as follows: BatL5310/ R6036R primers (*COX1*, Robins et al., 2007), L14727-SP/ H15915-SP primers (*CYTB*, Jaarola and Searle, 2002) and MSB-PRBBF/MSB-PRBBR primers (*IRBP*, Sawyer et al., 2014).

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[1] National Center for Biotechnology Information (2021). GenBank [online]. Website https://www.ncbi.nlm.nih.gov›genbank [accessed 00 Month Year].

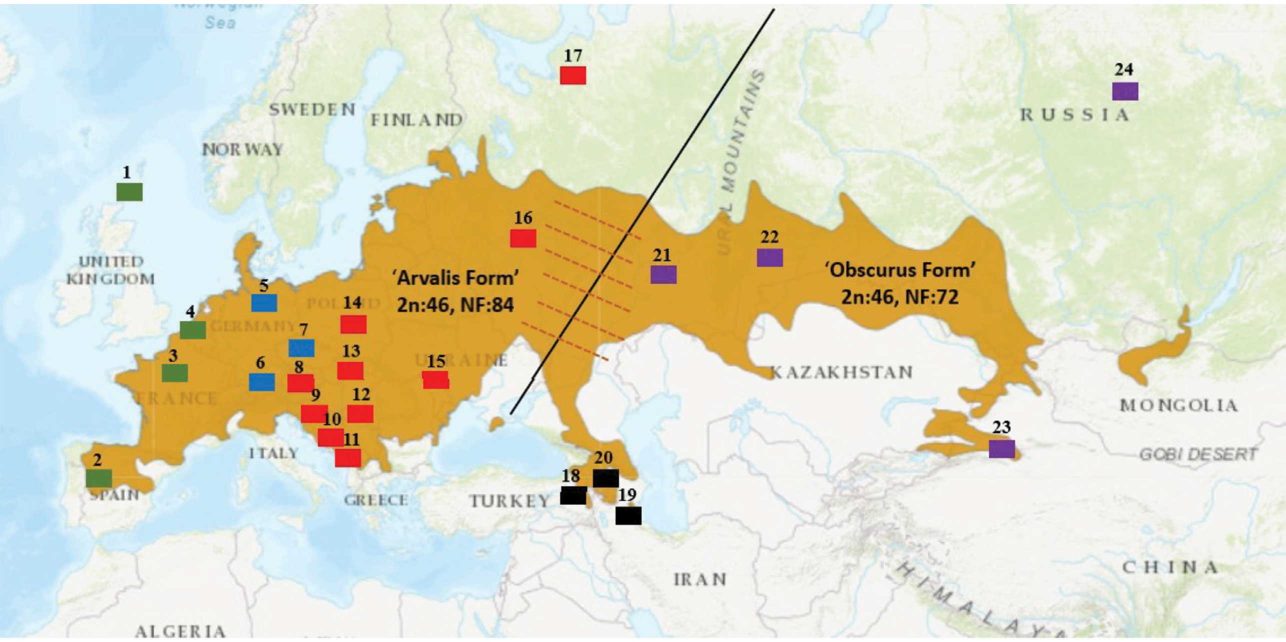

Figure 1. Figure 1. Location map of AUMAC samples and GenBank Sequences (modified from Yiğit et al. 2016[1]). Black line is the border of arvalis and obscurus forms; dotted lines show possible hybridization zone of the two forms. Western Europe (1: Orkney Island, 2: Spain, 3: France, 4: Belgium), Central Europe (5: Germany, 6: Switzerland, 7: Czech Republic), Eastern Europe (8: Austria, 9: Slovenia, 10: Bosnia, 11: Montenegro, 12: Serbia, 13: Hungary, 14: Poland, 15: Ukraine, 16: European Russia/Vladimir, 17: European Russia/ Arkhangelsk Oblast) groups are 'arvalis' form. Anatolia and its surroundings (18: Anatolia/Ardahan, Kars and Erzurum provinces, 19: Iran, 20: Armenia) and Asia (21: Russia/Orenburg Oblast, 22: Russia/ Chelyabinsk Oblast, 23: China/Xinjiang, 24: Siberia) belong to 'obscurus' form.

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[1] IUCN (2021). The IUCN Red List of Threatened Species [online]. Website https://www.iucnredlist.org/ [accessed 00 Month Year].

Reaction mixtures and conditions were obtained from Yiğit et al. (2017) for the *COX1* and *CYTB* markers, and Sawyer et al. (2014) for the *IRBP* marker. The PCR products were electrophoresed in 0.8% agarose gel for 1 h at 70 V in 1 × TAE, and the PCR bands were viewed with a SYNGENE Bio Imaging System (Synoptics Group, Frederick, MD, USA). Forward and reverse sequencing was performed by Medsantek (İstanbul, Turkey) and BMLabosis as a part of project 17L0430003 of the Scientific Research Projects Council of Ankara University.

2.3 Mitochondrial and nuclear gene analysis

Chromas Lite 2.1.1 (Technelysium Pty Ltd., South Brisbane, QLD, Australia) was used for visualising forward and reverse sequences. The alignment of sequences, the calculation of means and net genetic distance values (*d*) based on the *p*-distance parameter (Hamming, 1950) were performed using the MEGA X program (Kumar et al., 2018).

The determination of haplotypes, the calculation of genetic diversity values (haplotype diversity (*H*d), the nucleotide diversity (*pi*), the number of mutations, the number of parsimony informative and singleton sites) were performed using DNASP v6 software (Rozas et al., 2017).

Using all haplotypes belonging to the *COX1*, *CYTB* and *IRBP* regions, median-joining networks were drawn in Network 10.2.0.0 (Bandelt et al., 1999) to analyse relationships among populations. Default options were regarded and outgroups were not included.

A Bayesian tree was constructed using BEAST 1.75 (Drummond and Rambaut, 2007) and determining appropriate evolutionary models based on Akaike information criterion (AIC) and Bayesian information criterion (BIC) parameters were inferred by jModelTest 0.1 (Posada, 2008) (HKY+I+G parameter (Hasegawa et al., 1985) for *CYTB*, HKY + I parameter (Hasegawa et al., 1985) for *COX1* and HKY + G parameter (Hasegawa et al., 1985) for *IRBP*. Five independent runs, each of 10 million iterations, were carried out and the first 5000 samples from each were removed as burn-in. The performance of the Bayesian analyses was tested using Tracer 1.5 software[2], and effective sample size (ESS) values of 200 or higher were considered.

The *M. arvalis* populations' divergence times were determined for each gene region according to a mtDNA divergence rate (3.27×10^{-7} mutations/site/year; inferred by Martinkova et al., 2013) and a nuclear DNA divergence rate (0.8% per 1 my; Ochman and Wilson, 1987). The *Microtus longicaudus* – *Microtus pennsylvanicus* split (0.92 ± 0.02 mya, Conroy and Cook, 2000) was used as a calibration point. The acquired dendrograms were visualised using FigTree 1.4[3].

In addition, Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012) was applied as a species delimitation test. With the ABGD method[4], a barcode gap is determined when the divergence among organisms of the same species is smaller than the divergence among organisms from different species. The Jukes–Cantor parameter (Jukes and Cantor, 1969) and parameters were used as follows: a proxy for the minimum gap width (X): 1.0, pmin (prior minimum distance): 0.008 (*CYTB/COXI*) – 0.001 (*IRBP*), Pmax (prior maximal distance): 0.05 (*CYTB/COXI*) – 0.006 (*IRBP*), steps: 10, number of bins: 20. Outgroups were removed from data files in ABGD analyses.

3. Results

3.1. Mitochondrial DNA analysis

The genetic diversity obtained from *CYTB* (928 base pair) and *COX1* (550 base pair) sequences are shown in Table 1. Eighty-seven specimens formed 70 haplotypes for *CYTB* gene (20 haplotypes were found in the Turkish specimens, 30 in the European and 20 in the Asian); overall haplotype diversity (*H*d) was found to be 0.997 and nucleotide diversity (*pi*) was calculated as 0.030. For COX1 sequences, 14 haplotypes were present in 37 samples (3 haplotypes from Anatolia, 9 haplotypes from Europe and 2 haplotypes from Asia); haplotype diversity (*H*d) and nucleotide diversity (*pi*) were defined as 0.876 and 0.013, respectively.

Genetic diversity values were found to be higher in *CYTB* than in *COX1*. Mean and net genetic distance values (*d*) were calculated according to the *p*-distance parameter (Hamming, 1950) and were notably low for interspecific ranges of mtDNA (< 5%) as shown in Table 2. Genetic distance values between European and Asian or Anatolian populations were found to be higher than those between Asian and Anatolian populations, for both genes.

Median-joining networks and Bayesian trees constructed using *CYTB* and *COX1* sequences showed a similar topology. In the median-joining network for the *CYTB* gene (Figure 2), European samples were located in a different group. Nevertheless, one Ukraine sample was counted in the European group, but clustered with Asian samples. In addition, some samples from Iran, which neighbours Turkey, were located in the Asian group. As shown in the Bayesian tree for *CYTB* (Figure 3), samples belonging to Anatolian, Asian and European populations

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[2] Rambaut A (2007). Tracer 1.5 software [online]. Website http://beast.bio.ed.ac.uk/Tracer [accessed 00 Month Year].

[3] Rambaut A (2007). FigTree 1.4 [online]. Website http://tree.bio.ed.ac.uk/software/figtree [accessed 00 Month Year].

[4] Museum National d'Histoire Naturelle (MNHN???) (2021). ABGD method [online]. Website https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html [accessed 00 Month Year].

Table 1. Genetic diversity values of *M. arvalis CYTB*, *COX1* and *IRBP* regions. (Nr: number of samples; NH: number of haplotypes; *H*d: haplotype diversity; *pi*: nucleotide diversity; PS: total number of polymorphic sites; NM: number of mutations; PIS: number of parsimony-informative sites; NS: number of singleton sites).

		Nr	NH	*H*d	*pi*	PS	NM	PIS	NS
CYTB (928 bp)	Anatolia and proximate locations	31	20	0.966	0.010	55	56	15	40
	Europe	32	30	0.996	0.020	110	117	59	51
	Asia	24	20	0.982	0.005	33	35	12	21
	Total	87	70	0.997	0.030	151	162	98	53
COX1 (550 bp)	Anatolia	13	3	0.295	0.0005	2	0	0	2
	Europe	18	9	0.850	0.007	33	34	23	10
	Asia	6	2	0.333	0.006	1	0	1	1
	Total	37	14	0.876	0.013	37	26	11	38
IRBP (571 bp)	Anatolia	11	6	0.891	0.006	16	16	3	13
	Europe	18	11	0.954	0.006	14	14	11	3
	Total	29	20 (including 3 common haplotypes)	0.953	0.006	27	27	13	14

Nr: number of samples; NH: number of haplotypes; *H*d: haplotype diversity; *pi*: nucleotide diversity; PS: total number of polymorphic sites; NM: number of mutations; PIS: number of parsimony-informative sites; NS: number of singleton sites.

Table 2. Mean and net genetic distance values (*d*) and standard errors among *M. arvalis* populations of *CYTB*, *COX1* and *IRBP* regions.

		Mean genetic distance	Net genetic distance
CYTB	Anatolia-Europe	0.041 ± 0.005	0.025 ± 0.004
	Anatolia-Asia	0.027 ± 0.005	0.019 ± 0.004
	Europe-Asia	0.045 ± 0.009	0.032 ± 0.005
COX1	Anatolia-Europe	0.027 ± 0.005	0.020 ± 0.005
	Anatolia-Asia	0.011 ± 0.004	0.010 ± 0.004
	Europe-Asia	0.027 ± 0.005	0.021 ± 0.005
IRBP	Anatolia-Europe	0.006 ± 0.001	0

formed different clades, with around 99% posterior probability (pp) values. When *CYTB* dendrograms are examined in detail, it could be seen that *M. arvalis* clades are separated into subclades, and these subclades show geographical proximity.

In median-joining network and Bayesian trees for COX1, three *M. arvalis* clades were present, corresponding to haplotypes from Europe, Asia and Anatolia. These were well supported by Bayesian posterior probabilities (0.94–0.99). However, samples from Arkhangelsk Oblast in the northern part of European Russia clustered among those from the Asian obscurus, rather than the European arvalis form (Figures 4 and 5).

According to ABGD results, no barcode gaps showing interspecific distances were determined for either of the mtDNA genes (Figures S1 and S2). ABGD retrieved 2 lineages for *COXI* and *CYTB* genes (Europe and Asia/Anatolia), when only initial partitions were taken into account.

The evolutionary divergence times of the populations were calculated considering a mammal mtDNA divergence ratio (3.27×10^{-7} mutations/site/year; Martinkova et al., 2013) and using the *Microtus longicaudus* – *Microtus pennsylvanicus* split time of 0.92 ± 0.02 MYA (Conroy and Cook, 2000) as the calibration point. Accordingly, European populations have differentiated from Anatolian and Asian populations at 0.295 MYA (95% highest posterior density interval; HPD: 0.2148–0.3938) and the splitting of Anatolian and Asian populations has started at 0.187 MYA (95% highest posterior density interval; HPD: 0.1239–0.2628) for *CYTB*. Based on *COX1*, European populations split from Asian and Anatolian populations at 0.298 MYA (95% highest posterior density interval; HPD: 0.1795–0.4517), and Anatolian and Asian populations were separated at 0.141 MYA (95% highest posterior density interval; HPD: 0.072–0.2253).

3.2. Nuclear DNA analysis

Genetic diversity values as well as mean and net genetic distance values (*d*) acquired using the 571 base-pair *IRBP* gene region are given in Tables 1 and 2. A total of 29 samples were used in analyses and yielded 17 haplotypes which consisted of 6 Anatolian and 11 European haplotypes.

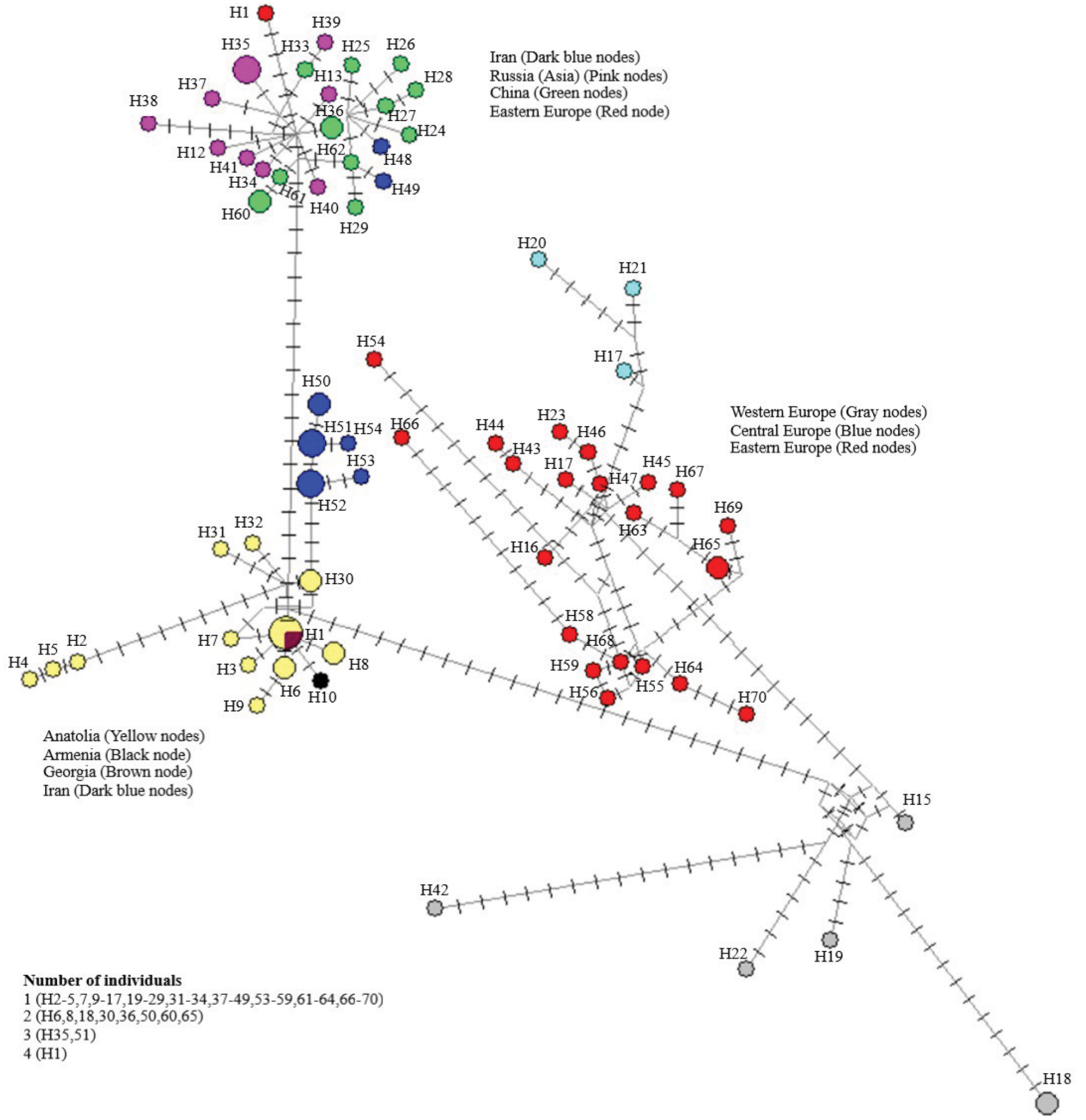

Figure 2. Median-joining network obtained from *CYTB* haplotypes of Anatolian, Asian and European populations of *M. arvalis*. Number of mutations are shown by black lines on the branches.

Additionally, three similar haplotypes were shared by both Anatolian and European specimens. While haplotype diversity was calculated as being high (0.953), nucleotide diversity was found to be low (0.006 ± 0.001). Other genetic diversity values (PS: total number of polymorphic sites; NM: number of mutations; PIS: number of parsimony-informative sites; NS: number of singleton sites) of the *IRBP* gene were higher than in *COX1* for the Anatolian populations, but lower for the European populations.

The mean genetic distance value between the Anatolian and European populations was extremely low (0.006 ± 0.001), and the net genetic distance value was 0 (zero).

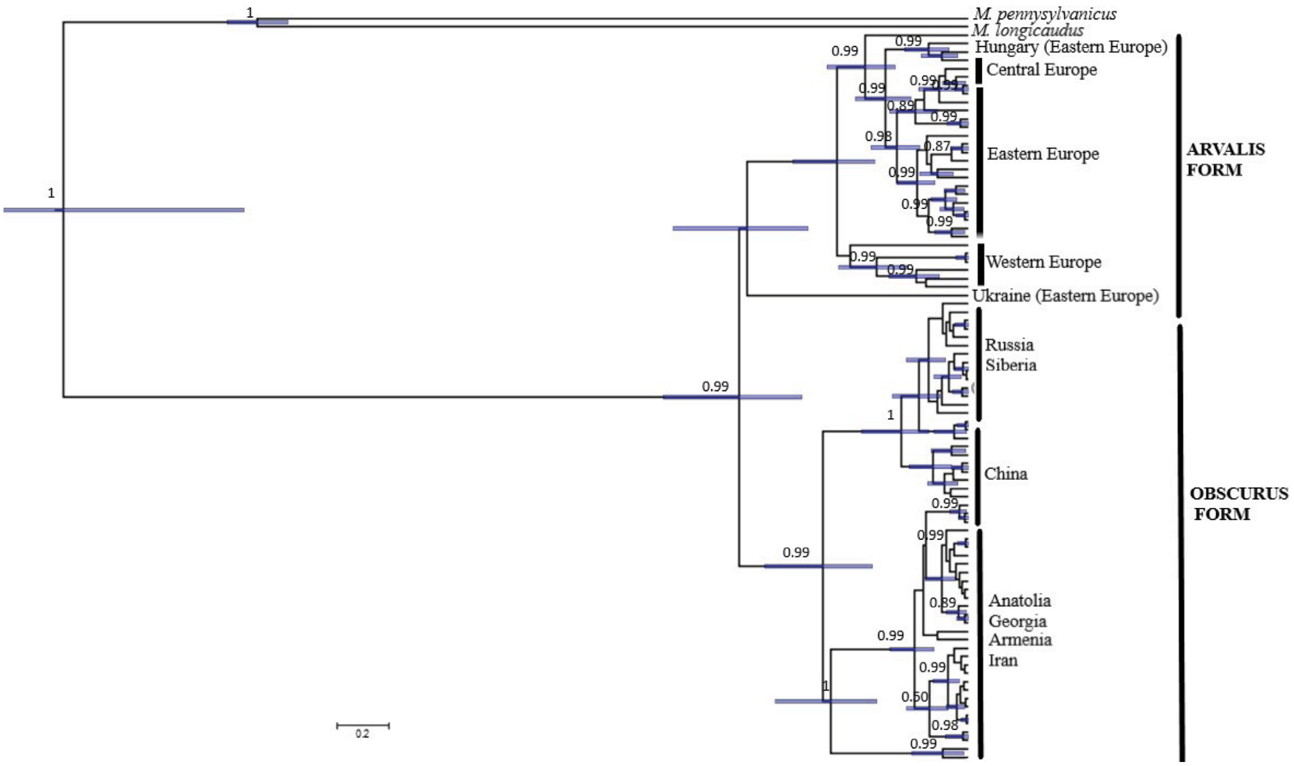

Figure 3. Bayesian tree obtained from *CYTB* sequences based on HKY+I+G parameter (Hasegawa et al., 1985). Numbers on branches show posterior probability (pb) values above 50%.

The median-joining network and Bayesian coalescence did not resolve relationships of *M. arvalis* from the nuclear IRBP sequences. In the median-joining network (Figure 6), Anatolian, European and common haplotypes clustered together. European and Anatolian samples were not separated, with 3.9%–99% pp values in the Bayesian tree (Figure 7).

In ABGD distributions, 2 lineages for initial partitions were defined (Figure S3). One lineage has one for each sample from Ardahan, whereas the other lineage has the remaining samples. The genetic distance histogram implies intraspecific relationships among *M. arvalis* populations.

The evolutionary divergence times of the Anatolian and European populations were calculated using the mammal nuclear DNA divergence ratio (0.8% per 1 my; Ochman and Wilson 1987) and was found to be 0.114 MYA (95% highest posterior density interval; HPD: 0.2817–0.8956).

4. Discussion

The distribution range of the common vole is confined to Eastern Anatolia in Turkey (Kefelioğlu 1995). This pattern of distribution might be due to competition between other *Microtus* species found to occupy the same niche in Anatolia or due to restricted ecological tolerance (Kryštufek and Vohralík, 2005). Kefelioğlu (1995) stated that *M. arvalis* and *M. levis* occur sympatrically in eastern Turkey. Also *M. levis* is found almost all over Turkey. Similarly, Major's pine vole (*M. majori*) has spread throughout the eastern Black Sea region (Yiğit et al., 2006). So far, there has been no study published that indicates that these two species are sympatric in eastern Anatolia. Orlov and Malygin (1969) indicated that the range boundaries between the arvalis and obscurus forms, were primarily reported to be allopatric in the European part of Russia and in Armenia. Kryštufek and Vohralík (2005) reported that the name *M. arvalis* is used in a broad sense to refer to several sibling species in the Palearctic region, and they considered the Turkish population to be *Microtus obscurus*. In terms of chromosomal arm differences, the west (arvalis form) and the east (obscurus form) populations have similar diploid chromosome numbers (2n: 46) with two chromosomal arm numbers (NF in arvalis: 86, NF in obscurus: 72).

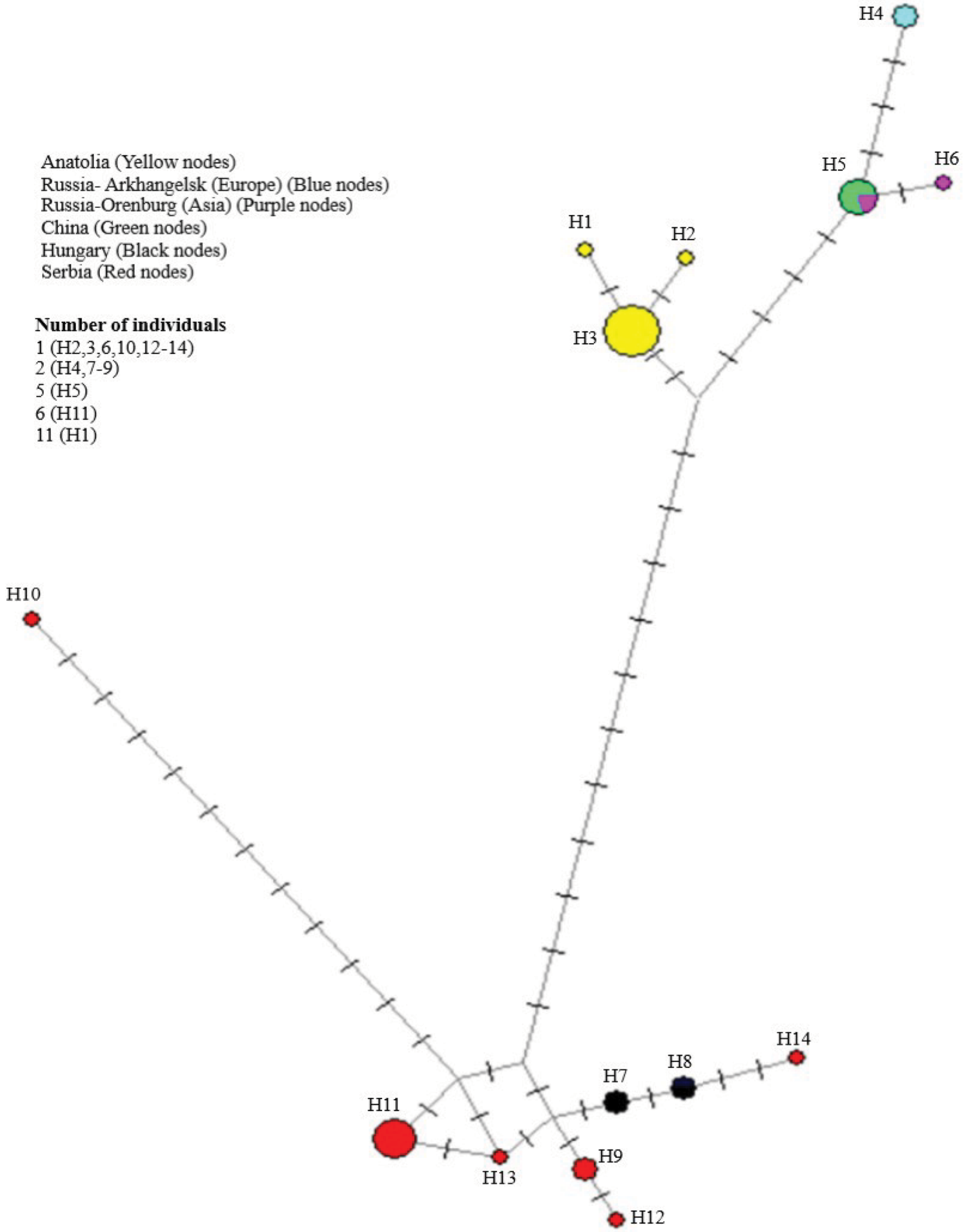

Figure 4. Median-joining network obtained from *COXI* haplotypes of Anatolian, Asian and European populations of *M. arvalis*. Number of mutations are shown by black lines on the branches.

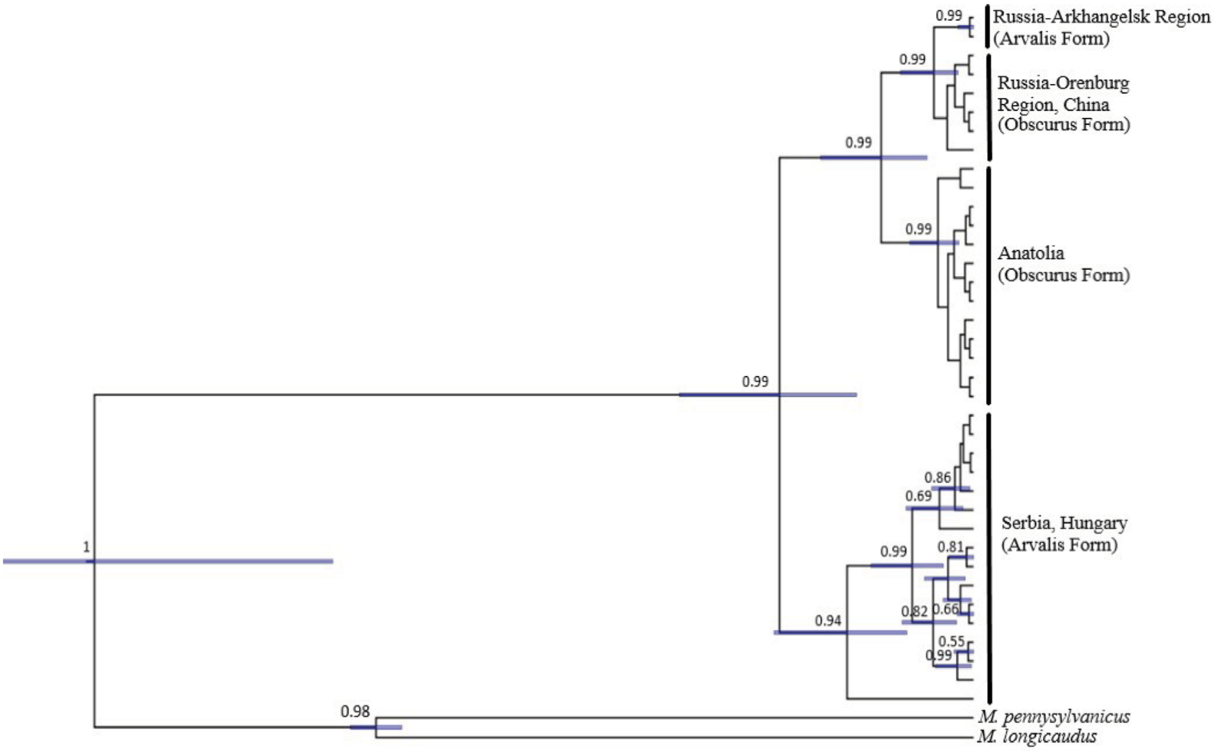

Figure 5. Bayesian tree obtained from *COXI* sequences based on HKY + I parameter (Hasegawa et al., 1985). Numbers on branches show posterior probability (pb) values above 50%.

Especially typical in terms of karyologic characteristics are four acrocentric pairs in arvalis and ten pairs in obscurus (Malygin and Orlov, 1974). The Turkish population is also reported to have ten pairs of acrocentrics as in the obscurus form (Kefelioğlu, 1995). Apart from this, Mazurok et al. (2001) also found differences between arvalis and obscurus forms based on GTG-banding and Ag-staining methods. In accordance with these findings, our results support the view that Anatolian populations are phylogenetically close to Asian obscurus populations.

In comparisons based on *CYTB*, the net genetic distances between two forms were given as 1%–1.19% by Haynes et al. (2003), 2%–4% by Jaarola et al. (2004), 2.37%–3.52% by Fink et al. (2004), 2.3%–3.6% by Buzan et al. (2010), 3.3%–5.4% by Tougard et al. (2013) and 2.7% by Barbosa et al. (2018). These reports are consistent with our findings that the mean genetic distance values were found to be 1.1%–4.5% and the net genetic distance values were 1.0%–3.2% (Table 2), and that the median-joining network and Bayesian tree of the *CYTB* marker separated the forms explicitly (Figures 2 and 3). Similar to Haynes et al. (2003), Fink et al. (2004) and Buzan et al. (2010), subclades of Western Europe (France, Spain, Belgium and Orkney Islands), Central Europe (Germany, Czech Republic and Switzerland) and Eastern Europe (Austria, Slovenia, Bosnia, Serbia, Hungary, Poland, Ukraine and European Russia) were retrieved; the Montenegro population from Eastern Europe was clustered in the Western Europe subclade, however. The similar tree topology also appeared in the dendrograms given by Jaarola et al. (2004). This finding, obtained from *CYTB* sequences, was supported by the *COX1* marker (Figures 4 and 5).

Tougard et al. (2013) stated that haplotype diversity values were notably high (0.929-1.000) in the South Caucasian region, and 0.0048- 0.0133 in European specimens for the *CYTB* marker. These are consistent with our results (Table 1). In our results, the median-joining networks and Bayesian trees from *CYTB* and *COX1* markers showed a similar topology (Figures 2–5), with Anatolian and Asian samples establishing a closer clade than in European populations. Interestingly, one Ukraine haplotype was also clustered with Asian haplotypes in median-joining network of *CYTB* region and Arkhangelsk Oblast (European Russia) specimens were clustered with Asian populations in median-joining network and Bayesian trees of *COXI* gene. These results provide some

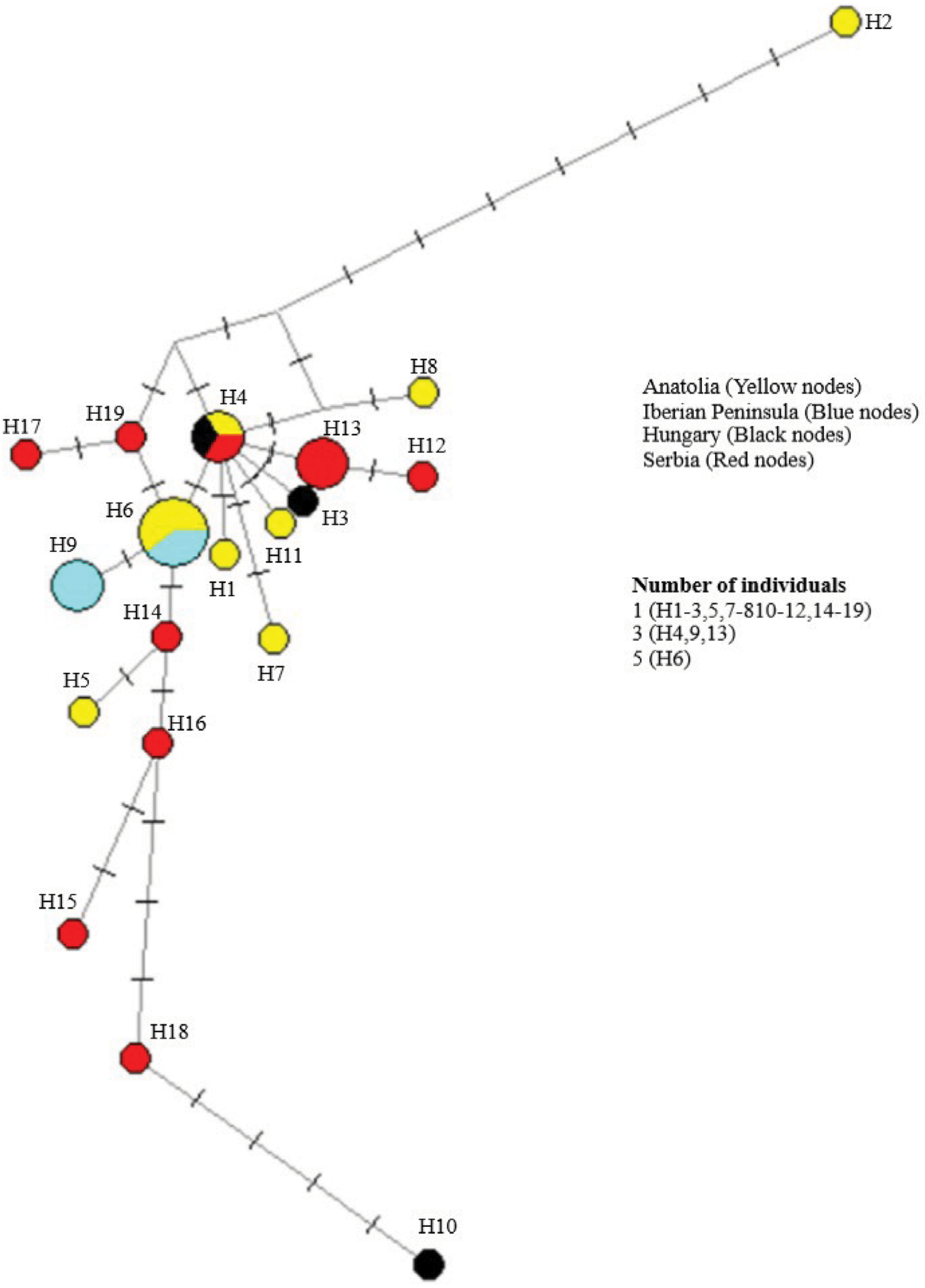

Figure 6. Median-joining network obtained from *IRBP* haplotypes of Anatolian, Asian and European populations of *M. arvalis*. Number of mutations are shown by black lines on the branches.

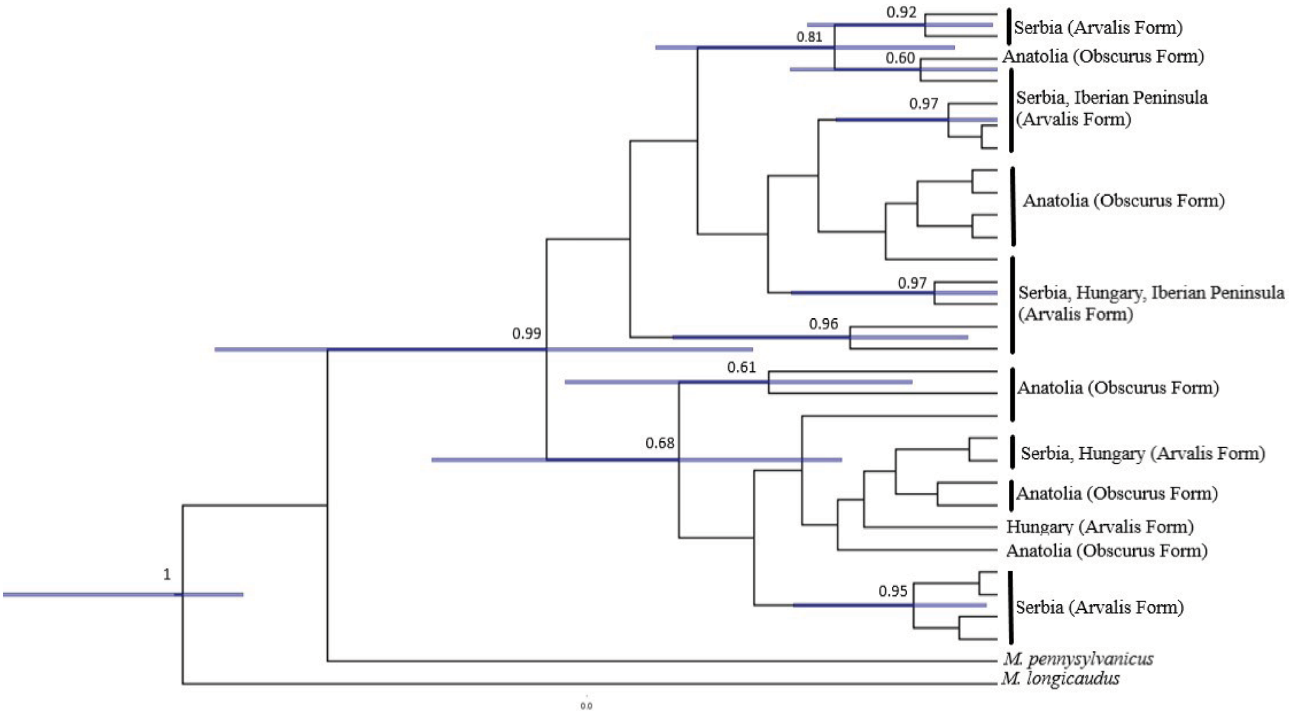

Figure 7. Bayesian tree obtained from *IRBP* sequences based on HKY + G parameter (Hasegawa et al., 1985). Numbers on branches show posterior probability (pb) values above 50%.

clues that the obscurus form may have spread further west than has been stated in the literature. Apart from this, in *IRBP* sequences, Anatolian and European samples were located together in the median-joining network and Bayesian tree (Figures 6 and 7). It is remarkable that the results obtained for the *IRBP* gene region are quite low and the Anatolian (Ardahan) samples form common haplotypes with European (Serbia, Hungary and Iberian Peninsula) samples (Figure 6). The *IRBP* gene region has recently been preferred in studies on the formation of rodent phylogeny and species identification and in the establishment of placental phylogeny at various taxonomic levels (Stanhope et al., 1992; 1996; Springer et al., 1997, 1999; Jansa and Voss, 2000; DeBry and Sagel, 2001; Huchon et al., 2002; Voss and Jansa, 2003; Michaux et al., 2005; Galewski et al., 2006; Chaval et al., 2010; Pages et al., 2012; Barbosa et al., 2013). On the other hand, reduced signal and incomplete lineage sorting in the *IRBP* gene may cause inconsistency with the mitochondrial DNA results (Salichos and Rokas, 2013).

Fink et al. (2004) calculated that the obscurus form split from other arvalis populations at 0.587–0.237 mya. Tougard et al. (2013) noted that divergence times were estimated as 0.478 mya for arvalis-obscurus, 0.109 mya for Sino-Russian, and 0.012 mya for South Caucasian populations. According to the divergence times based on our findings, they ranged between 0.298–0.141 mya for 3 arvalis populations in mtDNA, and 0.114 mya for Anatolian and European populations based on the *IRBP* gene. These results are consistent with the divergence time (3.5 my) given for *Microtus* species by Lemskaya et al. (2010). Such a divergence time was reported to be involved in an ongoing speciation process (Jaarola et al., 2004; Tryfonopoulos et al., 2008).

According to Bradley and Baker (2001), genetic distance values among either conspecific populations or valid species ranges from 2% to 11% in general. The average intraspecific genetic distance is 1.5 % (0.0%–4.7%) for rodents and, in particular, 2.0% (0.2%–4.4%) for *Microtus* species (Baker and Bradley, 2006). Jaarola et al. (2004) also suggested that there is a large-scale range in intraspecific variation in the genus *Microtus*, such that overlaps exist between inter- and intraspecific *CYTB* distance values. In support of this, the distance values based on *CYTB* for arvalis and obscurus in the present study (Table 2) exceed the intraspecific level of variation and correspond instead to the lower limit of the interspecific range in *Microtus*.

Bulatova (2007, 2010) reported that arvalis and obscurus forms come into contact with one another in the European part of Central Russia and produce hybrid individuals. Akhverdyan et al. (1999) and Meyer et al. (1999) observed that these two forms could breed with each other in captivity. Malygin and Panteleichuk (2003) defined that F1, F2 and following generations of individuals of arvalis and obscurus forms, showed fertility in spite of decreasing efficiency. Lavrenchenko et al. (2009) considered arvalis and obscurus forms to be semispecies in terms of mitochondrial (*CYTB*) and nuclear gene (*p53* gene) analyses and cytogenetic studies. On the other hand, Tougard et al. (2013) suggested that the distribution area of arvalis and obscurus forms are quite wide and their hybrid zone is narrow and unique, and their mobility is also limited; for these reasons, arvalis and obscurus should be defined as different species. As mentioned above, chromosomal forms can produce fertile individuals in nature and in the laboratory. In support of intraspecific fertility with chromosomal arm variations, the Turkish hamster (*Mesocricetus brandti*) provides a good example of this case, in that this species has a constant diploid number of chromosomes (2n: 42) with two NFs (NF: 82 in west to eastern Turkey and FN: 84 in eastern Turkey) (Yiğit et al., 2000), and these two chromosomal forms gave fertile offspring in the laboratory with NF: 83 (Çam et al., 2015). NF: 83 was also found in the contact zone of the distributional area.

By considering these findings, the Anatolian population of arvalis could be evaluated as being a border population for the species, and a low value of nucleotide diversity could normally be expected. Mean and net genetic distance values (*d*) ranged between 1.1 % and 4.5 % (Table 2). In particular, the genetic distance values between the Anatolian and Asian populations were nearly half the value of between the European and the Asian or Anatolian populations for both genes. These values are rather low to consider arvalis and obscurus forms as different species. Mitochondrial genetic distance values over 10% are generally considered as evidence for distinct species. Certainly, the results are consistent with the intraspecies distance values given by Nicholas et al. (2012). Our results also showed that genetic distance values were calculated as notably low, especially between Anatolia-Asia. As an alternative approach, the ABGD method is used to define species limits, and considering the histograms of distances obtained (Figures S1–S3), intraspecific relationships could be suggested. With respect to initial partitions, European and Asian/Anatolian lineages for *COXI* and *CYTB* are consistent with the separation of arvalis and obscurus forms in median-joining network and Bayesian tree. The *IRBP* gene region did not reveal a clear group distinction in ABGD analysis as in other analyses. It provides good evidence that as well as the Anatolian population being separated from other populations, other populations, there is an ongoing process of divergence between the arvalis and obscurus forms. It could also be concluded that the arvalis and obscurus populations are 2 separate evolutionarily significant units (ESUs) within *M. arvalis* and that there were 2 ESUs [Anatolia and Asia (Russia-China)] in the obscurus form. However, it is hard to consider that they are distinct species. Based on the genetic data from mtDNA, nucDNA, karyology and hybridization studies, *M. arvalis* is a valid species which includes both western and eastern populations. The characteristics that distinguish the two forms are currently only NF numbers. There are no clear studies to identify geographic races in such a large distribution area, extending from western Europe to China. Kryštufek and Vohralík (2005) considered this species as *M. obscurus*. In contrast to Kryštufek and Vohralík (2005), we used *M. arvalis* as a valid taxon based on the priority rule. In these circumstances, assigning Anatolian and other populations into a subspecies is only a superficial approach. The type location of *M. arvalis obscurus* is the Altai Mountains in Siberia, whereas there are many subspecies described in locations in the proximity of eastern Anatolia, especially in Caucasia (Kryštufek and Vohralík, 2005). However, it is unclear whether or not these are true *M. arvalis*. No doubt, in such a large distribution area, there should be distinctive characteristics in populations other than NF numbers, but these need to be looked for. Therefore, we submit that it is valid that the subspecific name (*M. arvalis obscurus*) be sustained, based on the NF number of the Anatolian population.

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Supplementary material:

Table S1. *M. arvalis* sequences stored in Genbank used in this study.

Accession Numbers	Localities	Gene region	References
MW454918-MW454930	Turkey	*COX1*	This study
MW454931-MW454934	Hungary	*COX1*	This study
MW454935-MW454946	Serbia	*COX1*	This study
MW456004-MW456014	Turkey	*CYTB*	This study
MW456015-MW456019	Hungary	*CYTB*	This study
MW456020-MW456028	Serbia	*CYTB*	This study
MW490055-MW490065	Turkey	*IRBP*	This study
MW490066-MW490068	Hungary	*IRBP*	This study
MW490069-MW490070	Serbia	*IRBP*	This study
JF499315.1-JF499317.1	Russia-Arkhangelsk_Region	*COX1*	Lissovsky et al. (unpublished)
KP190308.1-KP190312.1	China	*COX1*	Liu et al. (unpublished)
JX457686.1-JX457689.1	Iberian Peninsula	*IRBP*	Barbosa et al. (2013)
EU439459.1	Poland	*CYTB*	Borkowska et al. (2008)
AY708485.1	Switzerland	*CYTB*	Fink et al. (2004)
AY708491.1	Germany	*CYTB*	Fink et al. (2004)
AY708522.1	France	*CYTB*	Fink et al. (2004)
AY708497.1 AY708523.1	Spain	*CYTB*	Haynes et al. (2003)
GU187385.1	Bosnia	*CYTB*	Buzan et al. (2010)
GU187381.1	Slovenia	*CYTB*	Buzan et al. (2010)
GU187378.1	Montenegro	*CYTB*	Buzan et al. (2010)
AY708517.1	Czech Republic	*CYTB*	Fink et al. (2004)
AY708508.1	Belgium	*CYTB*	Fink et al. (2004)
AY708460.1	Austria	*CYTB*	Fink et al. (2004)
AY220785.1	Orkney Islands	*CYTB*	Haynes et al. (2003)
KP255617.1 KP255618.1 KP255620.1 AY220762.1	Ukraine	*CYTB*	Stojak et al. (2015)
AY220771.1	Russia Vladimir Region	*CYTB*	Haynes et al. (2003)
FR865416.1-FR865417.1, FR865420.1-FR865427.1	Russia: Aytuar Orenbourg oblast	*CYTB*	Tougard et al. (2013)
FR865428.1 FR865429.1	Russia: Bredy Chelyabinsk oblast	*CYTB*	Tougard et al. (2013)
AY220764.1 AY220765.1	Siberia	*CYTB*	Tougard et al. (2013)
AY220761.1	Armenia	*CYTB*	Haynes et al. (2003)
AY220760.1	Georgia	*CYTB*	Haynes et al. (2003)
MN183141.1-MN183144.1	China	*CYTB*	Chen et al. (2019)
KX581042.1, KX581044.1-KX581047.1	China	*CYTB*	Mahmoudi et al. (2017)
KX058268.1-KX058269.1, KX581042.1-KX581052.1	Iran	*CYTB*	Guo et al. (unpublished)
FR865413.1	Erzurum	*CYTB*	Tougard et al. (2013)

Table S1. *(continued)*

Accession Numbers	Localities	Gene region	References
FR865408.1-FR865412.1	Kars	*CYTB*	Tougard et al. (2013)
JF456725.1	*Microtus longicaudus*	*COX1*	Eger et al. (unpublished)
KX455523.1	*Microtus longicaudus*	*IRBP*	Abramson and Petrova (unpublished)
KY754039.1	*Microtus longicaudus*	*CYTB*	Steppan and Schenk (2017)
JF456745.1	*Microtus pennsylvanicus*	*COX1*	Eger et al. (unpublished)
KJ556734.1	*Microtus pennsylvanicus*	*IRBP*	Kohli et al. (2014)
KJ556623.1	*Microtus pennsylvanicus*	*CYTB*	Kohli et al. (2014)

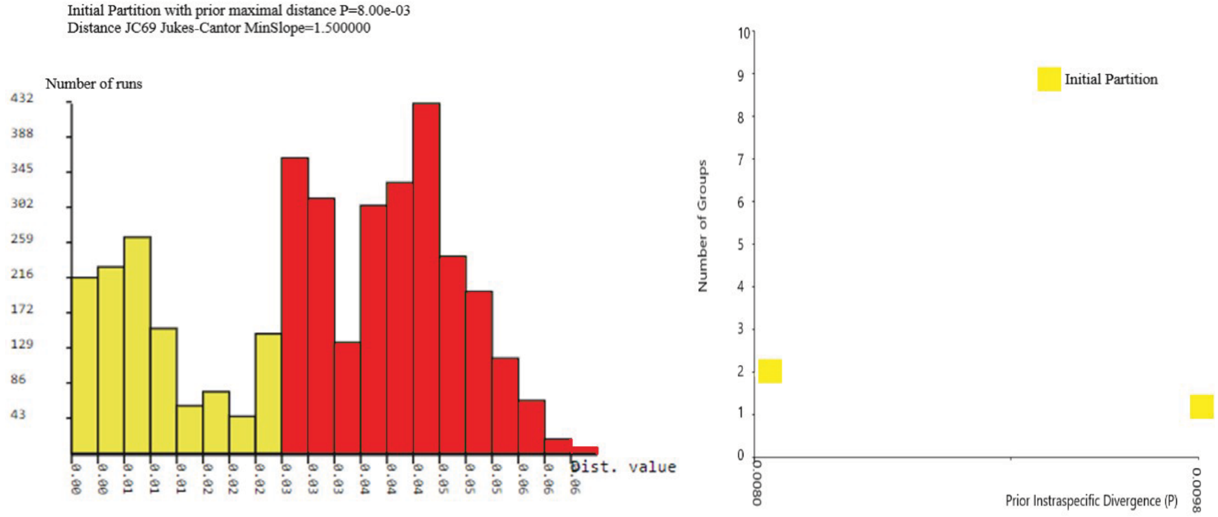

Figure S1. ABGD results of *CYTB* gene region. The histogram shows distribution of genetic distances among *M. arvalis* samples; yellow columns show mean intralineage distances and red columns show interlineage distances. The diagram implies the lineages according to initial partitions = Group 1 (n: 56): Anatolia, Georgia, Armenia, Iran, Siberia, China, Asian parts of Russia and one sample from Ukraine; Group 2 (n: 31): European parts of Russia, three samples from Ukraine, Western, Central and Eastern Europe.

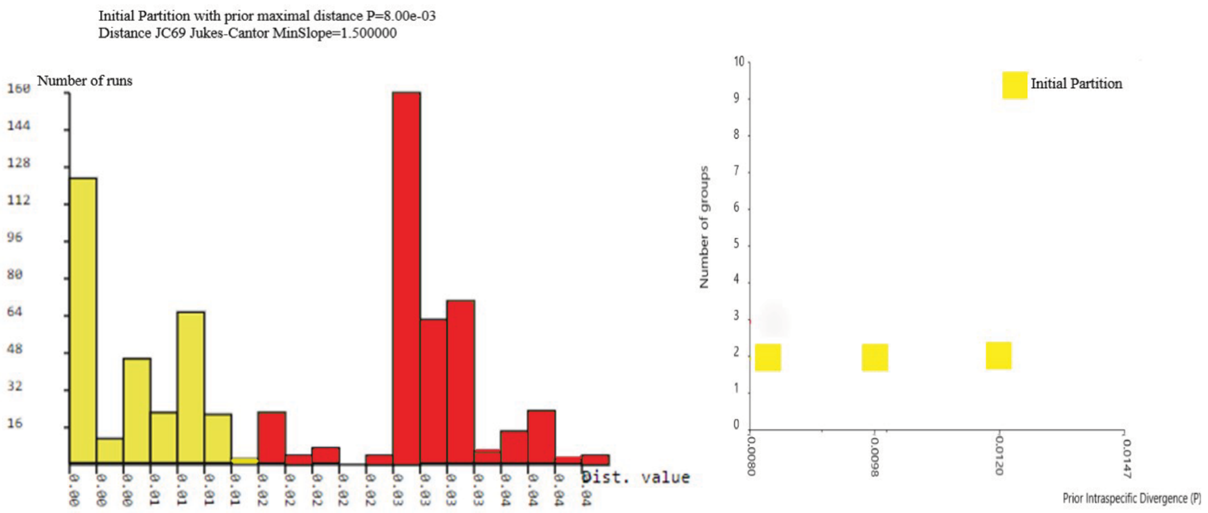

Figure S2. ABGD results of *COXI* gene. The histogram shows distribution of genetic distances among *M. arvalis* samples; yellow columns show mean intralineage distances and red columns show interlineage distances. The diagram implies the lineages according to initial partitions = Group 1 (n: 21): Anatolia, China, Asian and European parts of Russia; Group 2 (n: 16): Serbia, Hungary.

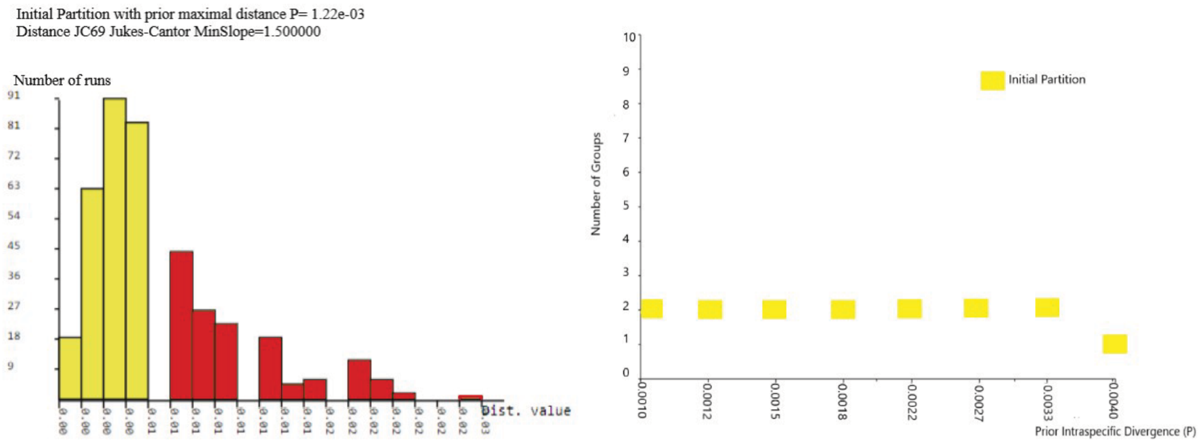

Figure S3. ABGD results of *IRBP* gene region. The histogram shows distribution of genetic distances among *M. arvalis* samples; yellow columns show mean intralineage distances and red columns show interlineage distances. The diagram implies the lineages according to initial partitions = Group 1 (n: 28): Anatolia, Serbia, Hungary, Iberian Peninsula; Group 2 (n: 1): one sample from Anatolia.