

Molecular assessment of the taxonomic status of pine vole species *Microtus subterraneus* and *Microtus majori* in southeastern Europe and Turkey

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Abstract

Molecular assessment of the taxonomic status of pine vole species *Microtus subterraneus* and *Microtus majori* in southeastern Europe and Turkey. Intra- and inter-specific phylogenetic relationships and the taxonomic status of pine vole populations have been controversial for years, and cryptic species are thought to exist, especially concerning the species' distribution area. To clarify the taxonomic status of Turkish populations we analysed mitochondrial (cytochrome-*b* and cytochrome oxidase-*I*) and nuclear (interphotoreceptor retinoid binding protein) gene markers, adding GenBank data from Europe and Caucasia. Considering the data obtained based on mean genetic distance, genetic diversity and fixation index values, Bayesian trees and Median-joining networks, we found that *M. subterraneus* and *M. majori* are valid species which have diverged since 1.28 Mya. Findings also suggested that although Anatolian, Thracian (Turkish Thrace and Greece) and European populations of *M. subterraneus* and Anatolian and Caucasian populations of *M. majori* have been in the process of divergency since 0.528–0.337 Mya correspond to the Pleistocene glacial periods, these intrapopulations do not appear to be different species. Besides, considering the high intraspecific variation in *M. subterraneus*, it remains likely that new species could be identified in future studies.

Key words: *Microtus subterraneus*, *Microtus majori*, Pine vole, Anatolia, mtDNA, nucDNA

Resumen

Evaluación molecular de la situación taxonómica del campañol de pino europeo, Microtus subterraneus, y el campañol de pino de Major, Microtus majori, distribuidos en Europa sudoriental y Turquía. Las relaciones filogenéticas intra e interespecíficas y la situación taxonómica de las poblaciones de campañoles han sido controvertidas durante años; asimismo, se cree que existen especies crípticas, especialmente en la zona de distribución de *M. subterraneus*. Para esclarecer la situación taxonómica de las poblaciones turcas de estas especies, se analizaron marcadores genéticos mitocondriales (citocromo *b* y subunidad 1 de la citocromo oxidasa) y nucleares (proteína de unión a retinoides del espacio interfotorreceptor) añadiendo datos de GenBank procedentes de Europa y el Cáucaso. Considerando los datos obtenidos a partir de la distancia genética media, la diversidad genética y los valores del índice de fijación, árboles bayesianos y redes basadas en el algoritmo de unión por medianas, se pudo demostrar que *M. subterraneus* y *M. majori* son especies válidas que divergieron hace 1,28 millones de años. También se sugirió que, aunque las poblaciones de *M. subterraneus* de Anatolia, Tracia (Tracia oriental y Grecia) y Europa y las poblaciones de *M. majori* de Anatolia y el Cáucaso han estado en proceso de divergencia desde hace entre 0,528 y 0,337 millones de años, esto es, los períodos glaciales del Pleistoceno, estas subpoblaciones no parecen ser especies diferentes. Además, considerando la variación intraespecífica de *M. subterraneus*, aún es posible que la nueva especie se pueda identificar en estudios futuros.

Palabras clave: *Microtus subterraneus*, *Microtus majori*, Campañol de pino europeo, Anatolia, ADNmt, ADNnuc

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Introduction

The subgenus *Terricola* Fatio, 1867 comprises approximately 14 pine vole species in Europe, Transcaucasia, and areas of Asia Minor in the western parts of the Palearctic Region (Musser and Carleton, 2005; Gornung et al., 2011). In Turkey, three *Terricola* species have been recorded as *Microtus subterraneus* (de Selys Longchamps, 1836), *Microtus majori* Thomas, 1906 and *Microtus daghestanicus* Schidlovsky, 1919 (Yiğit et al., 2006). Distribution of *M. subterraneus* (common pine vole) –type locality Belgium– includes Anatolia, the Balkan Peninsula, Estonia, southern and southwestern Europe, left bank Ukraine, and parts of Russia (Onega, right bank of Don and Middle and Upper Volga). *M. subterraneus* is found in northern Turkey, including Thrace and western Anatolia (Yiğit et al., 2006). Apart from this, *M. majori* (Meryemana pine vole) was first recorded in Trabzon Province (Turkey) and spread only in northern Anatolia and Caucasia (Ellermann and Morrison–Scott, 1965; Corbet, 1978; Kryštufek et al., 1994; Çolak et al., 1998; Yiğit et al., 2006). According to Tougard (2017) *M. subterraneus* and *M. majori* could have diverged in Anatolia, Caucasia and Iran and *M. subterraneus* spread throughout western Europe.

M. subterraneus was previously considered a polytypic species and has been divided into 25 subspecies, including *M. subterraneus majori* and *M. subterraneus daghestanicus* (Ellermann and Morrison–Scott, 1965). However, differences between *M. majori* versus and *M. daghestanicus* have been detected and they have been accepted as species in their own right (Baskevich et al., 2007). *M. subterraneus* and *M. majori* show differences with regard to external morphological, dental, karyological and electrophoretic features (Kryštufek et al., 1994; Yiğit et al., 2006), but their cranial and baculum morphologies are similar (Çolak et al., 1998). Besides, *M. subterraneus* populations in their distribution area indicate a high level of variation in terms of dental, cranial, karyology, ecology and allozyme data (Çolak et al., 1998; Zagorodnyuk, 1992; Macholan et al., 2001; Kryštufek and Vohralík, 2004). Karyological differences are especially noteworthy; Çolak et al. (1998) reported $2n = 52$, $Nfa = 56$, $NF = 60$ in Thracian *M. subterraneus* populations; $2n = 54$, $NFa = 56$, $NF = 60$ in Anatolian *M. subterraneus* population and $2n = 54$, $NFa = 56$, $NF = 60$ in *M. majori* ($2n$ = diploid number of chromosomes, Nfa = the number of autosomal arms, NF = the total number of chromosomal arms). Also, the specimens with $2n = 54$ in the south of eastern Europe were accepted as *Microtus dacius* Miller, 1912 as a different species (Zagorodnyuk, 1992).

In molecular studies in which the *CYTB* gene region was analyzed, Jaarola et al. (2004) determined a high level of intra-specific gene diversity in *M. subterraneus* with 6–7% genetic distance values among haplotypes and separation of *M. subterraneus* and *M. majori* in phylogenetic dendrograms. According to Baskevich et al. (2007), *M. subterraneus* Anatolian and European samples were not split in phylogenetic dendrograms and genetic distance values were remarkably low (3.7%). Thanou et al. (2012), calculated net genetic

distance values between Anatolian–Greek and Anatolian–European *M. subterraneus* samples as 3.3% and 2.8%, respectively, and these samples were separated from *M. majori* samples and clustered together. Tougard (2017) calculated the origin of the subgenus *Terricola* as 4.05 Mya (Early Pliocene) and *M. subterraneus* and *M. majori* were considered distinct species. Martínková and Moravec (2012) established phylogenetic dendrograms by analyzing many mitochondrial and nuclear gene regions and *M. subterraneus* and *M. majori* diverged in these dendrograms. A study based on the nuclear *BRCA1* gene Exon 11 regions and *XIST* gene performed by Bogdanov et al. (2020) showed that *M. subterraneus* and *M. majori* were separate. Similarly, Bogdanov et al. (2021) studied mitochondrial *CYTB* and nuclear *BRCA1* gene Exon 11 regions and *IRBP* genes and showed the splitting of *M. subterraneus* and *M. majori*.

In this study we focused mainly on *M. subterraneus* distributed in the Thrace and Anatolia (western Black Sea) regions of Turkey and *M. majori* found in the eastern Black Sea. From a phylogeographic perspective, Bosphorus and Dardanelle Straits are located in the Marmara Sea in Turkey and have acted as a potential barrier for the differentiation of terrestrial and underground mammals since the Late Pliocene (approximately 2 Mya) (Bacescu, 1985; Tortonese, 1985; Çağatay et al., 2000; Yaltrak et al., 2000). Further, evolution times of *Microtus* species date back to 0.5–3.5 Mya (Lemskaya et al., 2010) and an important part of speciation of these species occurred less than 1 million years ago (Bannikova et al., 2010; Tougard, 2017). The morphological and karyological differences between Thracian/European and Anatolian populations of *M. subterraneus* suggest they were isolated by the effect of these straits and evolved as different species. The fact that the western Anatolian *M. subterraneus* populations have similar karyological and morphological features to *M. majori* indicates the possibility that these two populations belong to the same species. As has been seen, there are differences between *M. subterraneus* and *M. majori* species as well as *M. subterraneus* Thracian/European and Anatolian populations. However, comprehensive molecular studies are needed to clarify the systematics of these species. Here we evaluated the taxonomic status of *M. subterraneus*/*M. majori*, *M. subterraneus* Thracian/European and Anatolian populations and *M. majori* Anatolian and Caucasian populations, analyzing mitochondrial cytochrome-*b* (*CYTB*) and mitochondrial cytochrome-*b* (*CYTB*) (*COXI*) markers and nuclear interphotoreceptor retinoid binding protein (*IRBP*).

Material and methods

Sampling of terricola samples, DNA isolation and amplification of genes

The specimens used in this study are stored in the Ankara University Mammalian Research Collection (AUMAC, <http://mammalia.ankara.edu.tr/>) and were collected with the permission of Ankara University

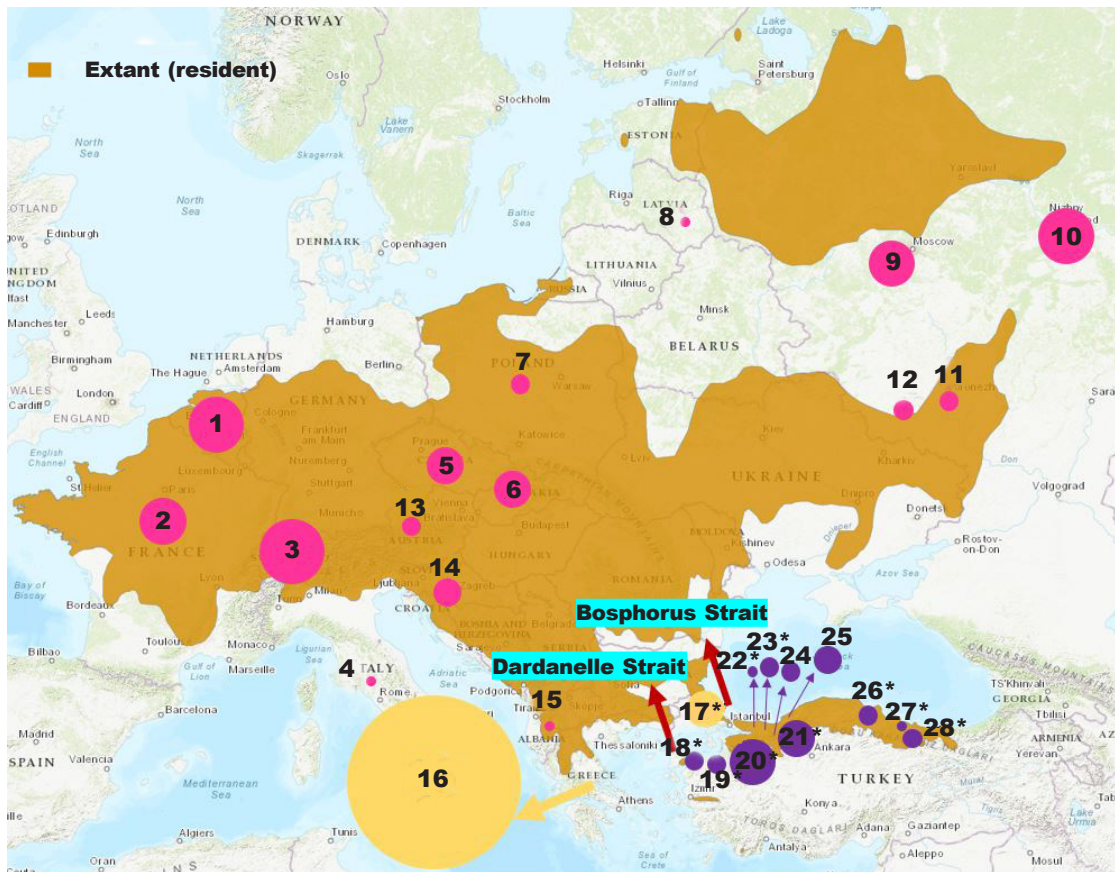


Fig. 1. Locations of *Microtus subterraneus* AUMAC samples (numbers with asterisk) and GenBank sequences based on mtDNA lineages (Europe: 1, Belgium; 2, France; 3, Switzerland; 4, Italy; 5, Czech Republic; 6, Slovakia; 7, Poland; 8, Latvia; 9, Russia–Kaluga Oblast; 10, Russia–Novgorod Oblast; 11, Russia–Voronezh Oblast; 12, Russia–Belgorod Oblast; 13, Austria. Balkan: 6, Slovakia; 14, Croatia; 15, Albania. Thrace: 16, Greece; 17, Kırklareli Province. Anatolia: 18, Kazdağı Region; 19, Balıkesir Province; 20, Bursa Province; 21, Karabük Province; 22, Kocaeli Province; 23, Sakarya Province; 24, Bolu Province; 25, Zonguldak Province; 26, Samsun Province; 27, Giresun Province; 28, Gümüşhane Province). The yellow areas show the distribution area of *M. subterraneus* according to IUCN data (Hutterer et al., 2021). The sizes of the circles are proportional to the number of specimens. Purple circles show Anatolian specimens, yellow circles show Thracian samples and pink circles show European circles.

Fig. 1. Ubicaciones de las muestras de *Microtus subterraneus* AUMAC (números con asterisco) y secuencias de GenBank basadas en linajes de ADNmt (Europa: 1, Bélgica; 2, Francia; 3, Suiza; 4, Italia; 5, República Checa; 6, Eslovaquia; 7, Polonia; 8, Letonia; 9, Rusia–Óblast de Kaluga; 10, Rusia–Óblast de Novgorod; 11, Rusia–Óblast de Voronezh; 12, Rusia–Óblast de Belgorod; 13, Austria. Balcanes: 6, Eslovaquia; 14, Croacia; 15, Albania. Tracia: 16, Grecia; 17, provincia de Kırklareli. Anatolia: 18, región de Kazdağı; 19, provincia de Balıkesir; 20, provincia de Bursa; 21, provincia de Karabük; 22, provincia de Kocaeli; 23, provincia de Sakarya; 24, provincia de Bolu; 25, provincia de Zonguldak; 26, provincia de Samsun; 27, provincia de Giresun; 28, provincia de Gümüşhane). Las zonas amarillas indican el área de distribución de *M. subterraneus* según datos de la UICN (Hutterer et al., 2021). El tamaño de los círculos es proporcional al número de muestras. Los círculos morados representan ejemplares de Anatolia; los amarillos representan muestras de Tracia y los rosas, muestras de Europa.

Local Ethics Committee for Animal Experiments (Document no: 2016–21–184). Twenty-four individuals captured with Sherman traps were killed using diethyl ether in field experiments, and skulls, furs and muscle, heart, liver and kidney tissue were collected. Four-

teen AUMAC samples (eight *M. subterraneus* and six *M. majori*) for the *CYTB* gene region, 24 AUMAC samples (17 *M. subterraneus* and seven *M. majori*) for the *COXI* gene region, and 23 AUMAC samples (16 *M. subterraneus* and seven *M. majori*) for the

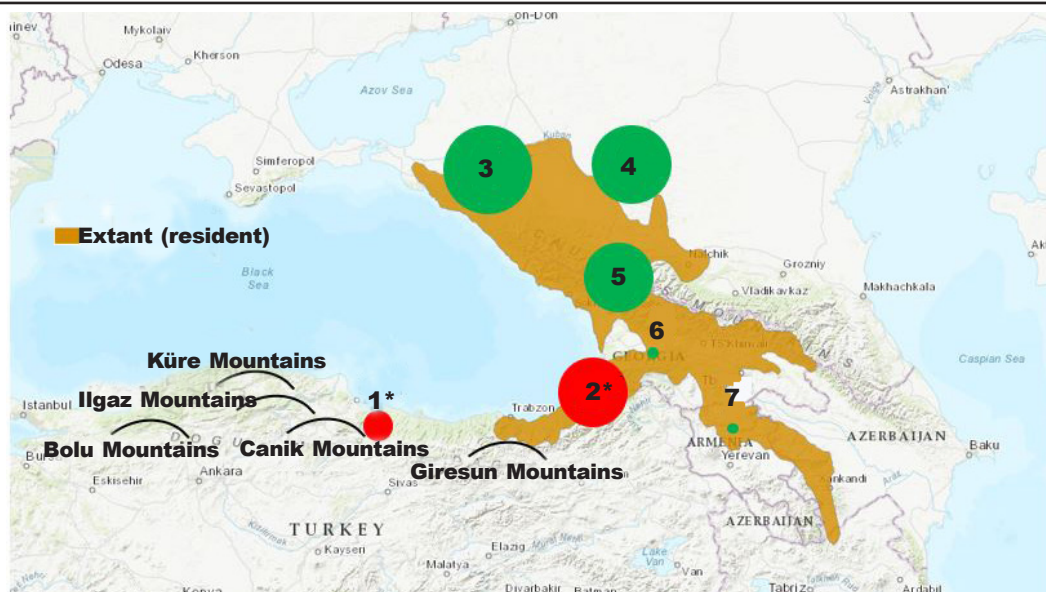


Fig. 2. Locations of *M. majori* AUMAC samples (numbers with asterisk) and GenBank sequences based on mtDNA lineages (Anatolia: 1, Ordu Province; 2, Artvin Province. Caucasia: 3, Russia–Krasnodar; 4, Russia–Stavropol; 5, Russia–Balkaria; 6, Georgia; 7, Armenia). Yellow areas show the distribution area of *M. majori* according to IUCN data (Kryštufek et al., 2016). The sizes of the circles are proportional to the number of specimens. Red circles show the Anatolian samples whereas green circles imply the Caucasian samples.

Fig. 2. Ubicaciones de las muestras de *M. majori* AUMAC (números con asterisco) y secuencias de GenBank basadas en linajes de ADNmt (Anatolia: 1, provincia de Ordu; 2, provincia de Artvin. Cáucaso: 3, Rusia–Krasnodar; 4, Rusia–Stavropol; 5, Rusia–Balkaria; 6, Georgia; 7, Armenia). Las zonas amarillas indican el área de distribución de *M. majori* según datos de la UICN (Kryštufek et al., 2016). El tamaño de los círculos es proporcional al número de muestras. Los círculos rojos representan las muestras de Anatolia y los verdes, las del Cáucaso.

IRBP gene region were amplified for analyses. Using a GeneMATRIX TISSUE and Bacterial DNA Purification Kit E3551–02 (BMLabosis, Ankara, Turkey), we isolated DNA samples from frozen kidney and liver tissue (stored at -80°C).

Amplification of *COXI*, *CYTB* and *IRBP* genes was carried out using the following primers, respectively: BatL5310/R6036R primers (*COXI*, 720 base–pair, Robins et al., 2007), L14727–SP/ H15915–SP primers (*CYTB*, 1000 base–pair, Jaarola and Searle, 2002) and MSB–PRBBF/MSB–PRBBR primers (*IRBP*, 900 base–pair, Sawyer et al., 2017). Steps for the PCR and electrophoresis stages followed Çetintürk et al. (2021). Forward and reverse sequencing of PCR products was performed by MEDSANTEK and BMLabosis as a part of project the Scientific Research Projects Council of Ankara University (project no: 17L0430003).

Furthermore, 121 *CYTB* gene sequences (91 *M. subterraneus* and 30 *M. majori*), one *M. subterraneus* *COXI* gene sequence and 13 *IRBP* gene sequences (seven *M. subterraneus* and six *M. majori*) were downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/genbank>, table 1s in supplementary material). Figures 1 and 2 show the locations of the specimens.

Sequence analyses

Forward and reverse sequences were visualized with Chromas Lite 2.1.1 (www.technelysium.com.au). In the MEGAX Program (Kumar et al., 2018), we performed sequence alignments and calculated mean genetic distance values (d) based on the p –distance Parameter (Hamming, 1950). In the DNASP v6 Software (Rozas et al., 2017), besides haplotypes, we determined and calculated genetic diversity values (haplotype diversity (H_d), nucleotide diversity (P_i), number of mutations, number of parsimony informative and singleton sites, and fixation index values (F_{ST}).

Using all *COXI*, *CYTB* and *IRBP* haplotypes, we constructed median–joining (MJ) networks in Pop Art version 1.7 (Bandelt et al., 1999) to determine phylogenetic relationships among populations and species mentioned in this study. Outgroups were not taken into consideration in the networks.

A Bayesian tree was generated using MrBayes 3.2.7a (Ronquist et al., 2012). The Markov Chain Monte Carlo (MCMC) approach was performed for two different runs with 50.000.000 generations sampling

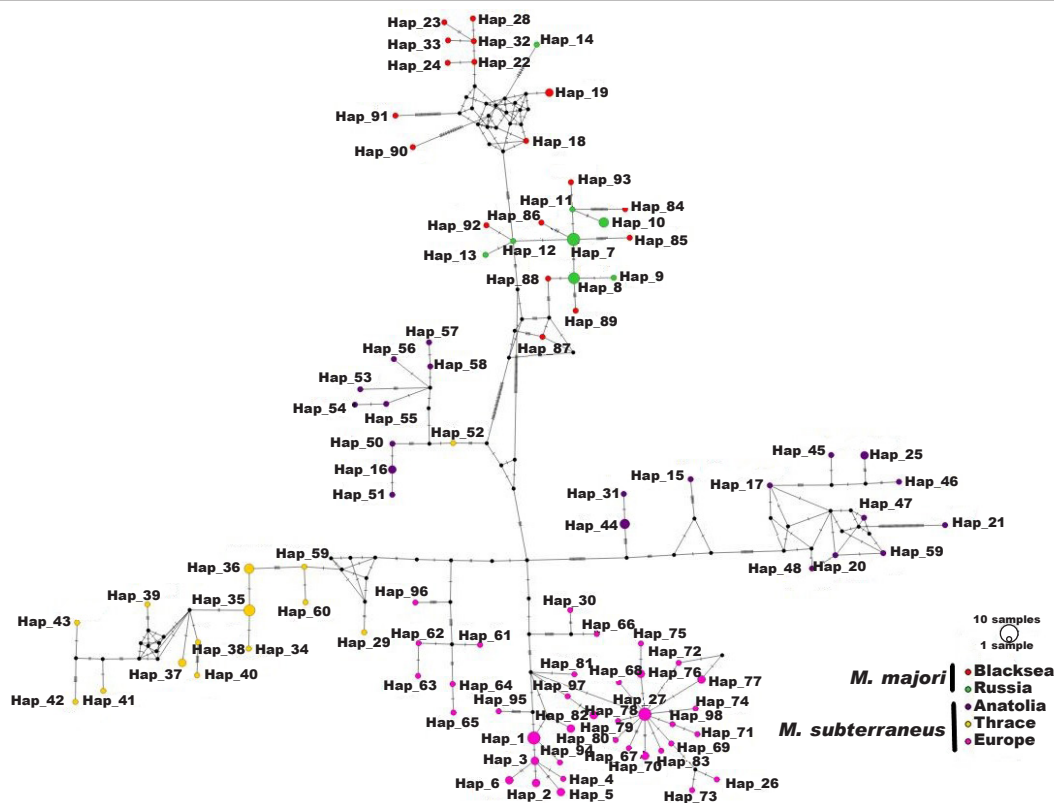


Fig. 3. Median-joining network derived from *CYTB* haplotypes. Numbers of mutations are indicated with black lines on branches.

Fig. 3. Red basada en el algoritmo de unión por medianas derivada de haplotipos *CYTB*. El número de mutaciones se mostró con líneas negras en las ramas.

each 100 generations with a 25% burn-in. Outgroups (*Microtus longicaudus* and *Microtus pennsylvanicus*) were included in phylogenetic tree analysis. HKY + G parameter (Hasegawa et al., 1985) was determined as an appropriate evolutionary model for *CYTB*, *COXI* and *IRBP* genes in Jmodeltest 0.1 (Posada, 2008) based on the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). Divergence times of the lineages based on coalescent method were determined in BEAST 1.75 (Drummond and Rambaut, 2007) with five independent runs, each of 10 million iterations and the first 5,000 samples from each run were eliminated as burn-in. The acquired dendrograms were viewed in FigTree 1.4 (<http://tree.bio.ed.ac.uk/software/figtree>). Only *CYTB* and *COXI* data were used and a *CYTB* divergence rate (3.27×10^{-7} mutations/site/year; inferred by Martinkova et al., 2013) and the *Microtus longicaudus*–*Microtus pennsylvanicus* split (0.92 ± 0.02 Mya; Conroy and Cook, 2000) were chosen as a calibration point. The accuracy of the BEAST analyses was examined with the aid of Tracer 1.5 Software (<http://beast.bio.ed.ac.uk/Tracer>). The results were accepted as long as effective sample size (ESS) values were 200 or higher.

Results

Mitochondrial DNA (mtDNA) *CYTB* results

We analysed a 576 base-paired part of the amplified *CYTB* gene region of 135 samples. The genetic diversity values given in table 2s in supplementary material show total haplotype diversity values (H_d) were 0.991 (*M. subterraneus*)–0.968 (*M. majori*) and varied between 0.946–0.983 for the populations and species being studied. Total nucleotide diversity values (P_i) were calculated as 0.034 (*M. subterraneus*)–0.020 (*M. majori*) and the highest in *M. subterraneus* (Anatolia) ($P_i = 0.042$), whereas the lowest were in *M. majori* (Anatolia) ($P_i = 0.007$). Other genetic diversity values (such as polymorphic sites and number of mutations) were highest in *M. subterraneus* (Anatolia) and *M. majori* (Caucasia–Russia and Georgia) and lowest in *M. majori* (Anatolia), consistent with P_i values.

Mean genetic distance values (d) ranged from 9.1% to 9.6% among *M. majori* and *M. subterraneus* populations (table 3s in supplementary material). The d values among *M. subterraneus* Anatolian, Thrace

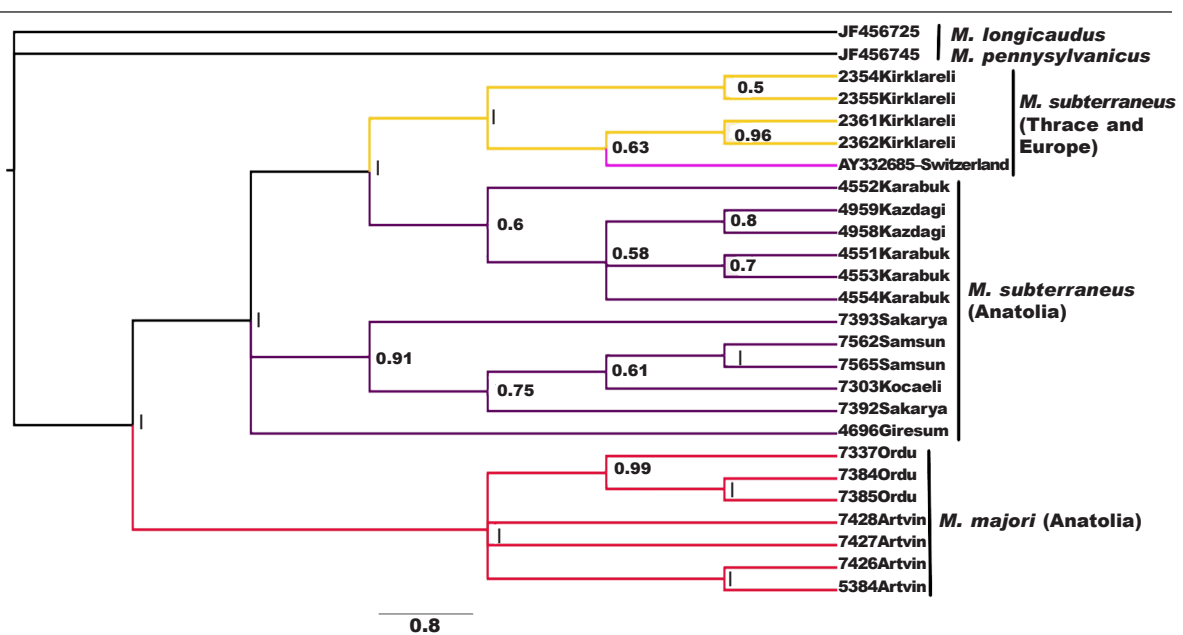


Fig. 4. Bayesian inference tree derived from COXI gene sequences. Numbers on nodes (bold) show posterior probability (pp) values and numbers on branches imply mean branch lengths.

Fig. 4. Árbol de inferencia bayesiano derivado de secuencias del gen COXI. Los números en los nodos (en negrita) representan los valores de probabilidad posterior (pp) y los números en las ramas representan la longitud media de las ramas.

and European populations varied between 3.6–5.3% and the genetic distance between *M. majori* Anatolian and Caucasian populations was 2.6%. Fixation index (F_{ST}) values were estimated as 0.387–0.594 (among *M. subterraneus* populations), 0.496 (between *M. majori* populations) and 0.666–0.816 (among *M. subterraneus* and *M. majori* populations) (table 3s in supplementary material).

As shown in the median-joining (MJ) network (fig. 3), *M. subterraneus* and *M. majori* populations diverged while *M. majori* Anatolian and Caucasian haplotypes remained together. Furthermore, *M. subterraneus* Anatolian, Thracian and European haplotypes clustered in a way that shows no clear distinction. One sample from the Turkish Thrace was located closer to the Anatolian haplotypes, and haplotypes of the Greek Thrace formed a distinct group. Anatolian haplotypes yielded Lineage A1 (Bursa, Kazdağı, Balıkesir provinces and Kırklareli province, Turkish Thrace) and Lineage A2 (Kocaeli, Sakarya, Bolu, Zonguldak, Samsun, Giresun, Gümüşhane provinces); European haplotypes established Lineage E1 (Albania, Croatia, Slovakia) ve Lineage E2 (Italy, Switzerland, Belgium, Austria, Czech Republic, France, Poland, Slovakia, Latvia, Germany, European Russia), and Slovakian haplotypes were common to E1 and E2 lineages.

Similar results were obtained with the MJ Network in the Bayesian Tree with regard to splitting of *M. subterraneus* and *M. majori* (pp = 1.00) as well as grouping *M. majori* Anatolian and Caucasian popula-

tions (pp = 0.58–1.00). Lineages A1, A2, E1, and E2 were defined (pp = 0.93–1.00) as for the MJ network.

Divergence time estimates yielded the following results: *M. subterraneus* and *M. majori*–0.990 Mya, *M. subterraneus* Anatolian and Thracian/European populations–0.528 Mya, *M. subterraneus* Thracian/European populations–0.337 Mya and *M. majori* Caucasian and Anatolian populations–0.365 Mya.

Mitochondrial DNA (mtDNA) COXI results

Our analysis included 529 base-paired regions of 24 samples. Because only one European (Switzerland) sequence could be used, Swiss and Turkish Thracian samples were grouped under the 'Europe' group. Total haplotype diversity (Hd) was calculated as 0.993 (*M. subterraneus*)–0.905 (*M. majori*) (table 4s in supplementary material) and this value was higher in *M. subterraneus* (Anatolia) (Hd = 0.985) than in *M. subterraneus* (Europe) (Hd = 0.900) and *M. majori* (Hd = 0.905). The total nucleotide diversity (Pi) was 0.038 (*M. subterraneus*)–0.044 (*M. majori*). The number of polymorphic sites, mutations, parsimony informative and singleton sites were relatively higher in *M. majori* and *M. subterraneus* (Anatolia) than in *M. subterraneus* (Europe).

Mean genetic distance values (d) were determined as 13.4% (*M. Majori*–*M. subterraneus*) and 5.6% (*M. subterraneus* Europe–Anatolia). Fixation index (F_{ST}) values were defined as 0.44 between *M. subterraneus*

Anatolian and European populations and 0.736–0.822 between *M. Majori*–*M. subterraneus* populations (table 5s in supplementary material).

The MJ network showed that *M. majori*, *M. subterraneus* (Anatolia) and *M. subterraneus* (Europe) populations diverged completely. The *M. subterraneus* Anatolian populations comprised two lineages (Kazdağı, Karabük (A1) and Kocaeli, Sakarya, Samsun, Giresun (A2)) and *M. subterraneus* European population (Turkish Thrace and Switzerland) joined the A1 lineage.

The studied populations were also split in Bayesian Tree (pp = 0.91–1.00). Kazdağı, Karabük (A1) and Kocaeli, Sakarya, Samsun, Giresun (A2) lineages appeared similar to the MJ Network (pp = 1.00) although the A1 lineage was closer to the European population than to the A2 lineage (pp = 1.00) (fig. 4).

M. majori diverged from *M. subterraneus* at 1.28 Mya, while the separation of the Anatolian and European populations of *M. subterraneus* caused 0.420 Mya.

Nuclear DNA (nucDNA) *IRBP* results

We analysed 440 base-pairs at the *IRBP* gene region from 36 samples. Total haplotype diversity (Hd) was 0.557 (*M. subterraneus*)–0.603 (*M. majori*). The highest Hd value was found in *M. subterraneus* (Anatolia) (Hd = 0.782). Hd values of the *M. subterraneus* Turkish Thracian and European populations were calculated as 0 (zero) (table 6s in supplementary material). Total nucleotide diversity (Pi) was determined as 0.001 (*M. subterraneus*)–0.002 (*M. majori*) and Pi values of *M. majori* versus *M. subterraneus* (Anatolia) were similar (0.002); Pi values of *M. subterraneus* Turkish Thracian and European) were 0. Compared to *CYTB* and *COXI* values, genetic diversity values such as the number of polymorphic sites, mutations, parsimony informative and singleton sites were notably lower than expected.

Mean genetic distance values (*d*) between *M. majori* and *M. subterraneus* Anatolian, Turkish Thracian and European populations were defined as 0.4–0.6%. The *d*-values between *M. subterraneus* populations were 0–0.1% and between *M. majori* populations were 0.1% (table 7s in supplementary material). Fixation index (F_{ST}) values were 0–0.266 between *M. subterraneus* populations, 0.023 between *M. majori* populations and 0.558–0.680 between *M. majori*–*M. subterraneus* populations (table 7s in supplementary material).

M. majori and *M. subterraneus* were diverged with several mutations in the MJ Network, Anatolian, Thracian and European samples of *M. subterraneus* shared a common haplotype that clustered with other Anatolian haplotypes. Anatolian and Caucasian *M. majori* specimens formed two common haplotypes and grouped with a Caucasian haplotype. In the Bayesian tree, two species were separated (pp = 0.99); however, subgroups of *M. subterraneus* (pp = 0.91–1.00) and *M. majori* (pp = 0.91) were not split.

Discussion

The aim of the present study was to clarify the taxonomic status of pine vole species (*M. subterraneus*

and *M. majori*) in Turkey taking into account additional data from other locations in Europe and Caucasia. Considering studies reported to date, genetic diversity values yielded consistent results. Haplotype and nucleotide diversity values of *M. subterraneus* (0.9605 and 0.022) and *M. majori* (0.9737 and 0.013) were calculated following Bogdanov et al. (2021) as close to the present study results. Studies analyzing the *CYTB* gene in the literature also showed that *M. subterraneus* and *M. majori* samples clearly differed whereas *M. subterraneus* Anatolian and European populations were not separated in phylogenetic dendrograms (Jaarola et al., 2004; Baskevich et al., 2007; Thanou et al., 2012; Tougard, 2017). Furthermore, *M. subterraneus* and *M. majori* were diverged in the dendrograms constructed based on many mitochondrial and nuclear genes (Martínková and Moravec, 2012). Nuclear *BRCA1* gene Exon 11 region and *XIST* gene analyses by Bogdanov et al. (2020) and *CYTB*, *BRCA1* gene exon 11 and *IRBP* analyses by Bogdanov et al. (2021) also showed that *M. subterraneus* and *M. majori* were split. In our study, *M. subterraneus* and *M. majori* were separated in the Bayesian trees and median-joining networks created for mtDNA markers. Furthermore, although the splitting of *M. subterraneus* intra-populations was not clear, different clades were formed in these populations in Bayesian trees and median-joining networks (fig. 3–4). Subjected pine vole species also diverged in the *IRBP* Bayesian tree, but *M. subterraneus* Anatolian, Thracian and European samples were clustered. Haplotypes were diverged with a small number of mutations in the median-joining network. In this regard, our results obtained in phylogenetic approaches are consistent with previous studies. According to the divergence times, while Macholan et al. (2001) calculated the splitting of two pine vole species as 0.350 Mya for allozyme data, Tougard (2017) detected the origin of the subgenus *Terricola* as 4.05 Mya (Early Pliocene) based on the *CYTB* gene. The present study reports the divergence times of *M. majori* and *M. subterraneus* (*CYTB*/1.003 Mya, *COXI*/1.28 Mya), and *M. subterraneus* intra-populations (*CYTB*/0.587–0.260 Mya, *COXI*/0.420 Mya).

Mean genetic distances between these species were 9.1–15.6% for mtDNA and 0.4–0.6% for nucDNA (table 3s and 5s in supplementary material), congruent with the values in the literature (9.68% (*CYTB*), 0.08% (*IRBP*) (Bogdanov et al., 2021), 16.9% (allozyme data, Macholan et al., 2001). Intraspecific variation is accepted as below 10% for *COXI* (Waugh, 2007). Aside from this, interspecific genetic distance values are 2–11% in general (Bradley and Baker 2001), 0.0–4.7% (mean 1.5%) for rodents, and 0.2–4.4% (2.0%) for genus *Microtus* (Baker and Bradley, 2006). Furthermore, fixation index (F_{ST}) values of 0.25 and above are accepted as a signal of significant differentiation between populations (Wright, 1978), as demonstrated by the F_{ST} data in our study, except for *M. majori* (Anatolian and Caucasian) *IRBP* results (F_{ST} = 0.023). Results found in this study and other recent studies considering molecular systematic, karyological, ecological, morphological and allozyme data

indicate that *M. subterraneus* and *M. majori* are valid species. It is also assumed that the *M. subterraneus* population extended to the eastern Black Sea (Giresun and Gümüşhane Provinces) and that *M. majori* spread to Ordu Province in the west. Under these circumstances, distributions of these species intersect in the eastern Black Sea region of Turkey and are found as allopatric populations in this area. Divergence times of these species coincide with Pleistocene glacial cycles. Pine voles could have differentiated in Anatolia, Caucasasia and Iran, and *M. subterraneus* spread to western Europe from this region in this age (Tougaard et al., 2017). Due to the collision of the Eurasian and Arabian plates, mountain rising occurred in the western parts of Eastern Europe, Anatolia, and Central Asia during the Late Miocene–Pliocene, and the *Terricola* populations may have been isolated (Ruban et al., 2007; Mosar et al., 2010). As the north of Anatolia is a mountainous region, the Canik Mountains, Küre Mountains, Ilgaz Mountains and Eastern Black Sea mountains in this region (fig. 2) are important barriers for species that prefer low altitudes, such as pine voles. Increasing global cooling in the following periods may have led to the formation of temperate coniferous and deciduous forests, and suitable vegetation for the colonisation of *Terricola* species towards Europe (Fauquette and Bertini, 2003; Pross and Klotz, 2002; Thompson and Fleming, 1996; Willis et al., 1999). These events demonstrate that Anatolia is an important centre of speciation and differentiation for rodent species. It could also be seen that the differentiation between *M. subterraneus* intra-populations was low in terms of the mean distance values, being 3.6–5.6% for mtDNA (table 3s and 5s in supplementary material), similar to genetic distance values as 3.7% (*CYTB*, Baskevich et al., 2007), 2.8–3.3% (*CYTB*, Thanou et al., 2012), 7.3% (allozyme data, Macholan et al., 2001). These results mainly correspond to interspecific distance values (Baker and Bradley, 2006). Values above 4.4% could result from high intraspecific diversity of *M. subterraneus*, but Bayesian trees and median-joining networks did not show any differentiation at the subspecies level. The splitting of *M. subterraneus* into different groups within the Anatolian and European populations is also an indication that the intraspecific diversity is high and that there is an ongoing separation process. Although the obtained results indicate that the European and Anatolian populations belong to the same species (*M. subterraneus*), considering the populations in the distribution area of *M. subterraneus* differ from each other in terms of karyology and morphology (Çolak et al., 1998; Selçuk and Kefelioğlu, 2018) and the high intraspecific diversity (Macholan et al., 2001; Jaarola et al., 2004; Bogdanov et al., 2021), cryptic species are probably present in *M. subterraneus*. Because *M. subterraneus* exists in coniferous and deciduous forests that are undergoing destruction because of fragmentation, isolation could be mentioned, especially for the southeast Balkans (Thanou et al., 2012). For Anatolian and Thracian *M. subterraneus* populations, the Bosphorus and Dardanelles Straits are influential barriers for the speciation of terrestrial and underground mammal species (Bacescu, 1985; Torto-

nese, 1985; Çağatay et al., 2000; Yaltırak et al., 2000). In our study, divergence times of *M. subterraneus* populations are 0.528–0.337 Mya and as they were isolated and differentiated from each other in the Balkan and Anatolian refugiums during the Pleistocene glacial periods (Bilton et al., 1998; Taberlet et al., 1998; Schmitt, 2007), they remain separate today due to the effect of the Bosphorus and Dardanelles Straits (fig. 1), and the process of speciation is ongoing. Furthermore, Brunet–Lecomte et al. (1992) reported *M. grafi* from Bulgaria and Brunet–Lecomte et al. (2001) suggested that *Microtus grafi* is a subspecies of *M. subterraneus*. This finding suggests that the Thracian population belongs to the *M. subterraneus grafi* subspecies. Finally, Baskevich et al. (2018) suggested that the cytogenetic differences in $2n = 52$ and $2n = 54$ karyotypes observed in eastern European *M. subterraneus* populations were due to Robertsonian translocation; however, this was insufficient for reproductive isolation among them. In conclusion, comprehensive molecular studies covering the distribution area are required to clarify the systematics of *M. subterraneus* and its populations and reveal cryptic species.

Additionally, nucDNA results were considerably lower than mtDNA results. Although the *IRBP* marker has frequently been used in studies of rodent phylogeny and species identification (Stanhope et al., 1996; Springer et al., 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; Pagès et al., 2010; Barbosa et al., 2013; Bogdanov et al., 2021), inconsistent results with mtDNA are highly possible because of the lack of signal and incomplete lineage classification in the *IRBP* gene (Salichos and Rokas, 2013). It could therefore be inferred that nucDNA is more useful for solving phylogenetic relationships in higher taxa than intra- and interspecific levels (Springer et al., 1997). The low genetic distance values at both intra- and interspecific levels in this study show that this genetic marker is not useful for distinguishing the taxa. However, this study has shown that a *COXI* marker accepted as the only barcoding gene (Li et al., 2014) could be effective in barcoding *M. subterraneus* and *M. majori* species.

Finally, divergence times of the studied groups based on the coalescent method differed for *CYTB* and *COXI* markers. Phylogenies of different genes including the divergence times of the same species may not be congruent (Edwards and Beerli, 2000; Hilton and Hey, 1997; Nichols, 2001; Takahata and Satta, 1997; Wang et al., 1997), fundamentally due to gene duplication, horizontal gene transfer, and deep coalescence (Edwards, 2009). The discrepancy between times of gene and population divergence arises because prior to species divergence, a degree of gene divergence had already accrued in the ancestral species (Arbogast et al., 2000) and coalescence of gene lineages from separate species can only occur more anciently than the splitting times of the species to which they belong (Degnan and Rosenberg, 2009). For this reason, *Terricola* species/populations could have diverged more recently. Furthermore, a *CYTB* divergence rate (3.27×10^{-7} mutations/site/year; infe-

red by Martinkova et al., 2013) was accepted for both *CYTB* and *COXI* divergence time data in this study. The discrepancy between the divergence times of different genes may therefore be expected.

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Supplementary material

Table 1s. Accession numbers and locations of the sequences obtained from GenBank

Tabla 1s. Número de accesoión y ubicación de las secuencias obtenidas de GenBank.

Species			
Gene	Accession number	Location	Reference
<i>M. subterraneus</i>			
CYTB	MZ198155.1–MZ198158.1	Russia: Novgorod Oblast	Bogdanov et al. (2021)
CYTB	MZ198159.1–MZ198163.1	Russia: Kaluga Oblast	Bogdanov et al. (2021)
CYTB	MZ198164.1, MZ198164.1	Russia: Voronezh Oblast	Bogdanov et al. (2021)
CYTB	MZ198168.1–MZ198169.1	Russia: Belgorod Oblast	Bogdanov et al. (2021)
CYTB	KR107033.1–KR107034.1	Russia: Novgorod Oblast	Baskevich et al. (2016)
CYTB	AJ717745.1	Italy	Tougaard et al. (2008)
CYTB	AY332714.1	Switzerland	Pfunder et al. (2004)
CYTB	FR869869.1–FR869874.1	Switzerland	Martínková et al. (unpublished)
CYTB	KY620210.1	Albania	Jablonsky (unpublished)
CYTB	AY513832.1	Greece	Jaarola et al. (2004)
CYTB	AY513833.1	Austria	Jaarola et al. (2004)
CYTB	FR869861.1	Austria	Martínková et al. (unpublished)
CYTB	FR869856.1–FR869858.1	Croatia	Martínková et al. (unpublished)
CYTB	FR869859.1–FR869860.1	Slovakia	Martínková et al. (unpublished)
CYTB	FR869885.1–FR869887.1	Slovakia	Martínková et al. (unpublished)
CYTB	LT222310.1	Slovakia	Tougaard (2017)
CYTB	FR869862.1, FR869864.1–FR869868.1	Belgium	Martínková et al. (unpublished)
CYTB	FR869875.1–FR869878.1	Czech Republic	Martínková et al. (unpublished)
CYTB	FR869879.1–FR869882.1	France	Martínková et al. (unpublished)
CYTB	LT222311.1	France	Tougaard (2017)
CYTB	FR869883.1–FR869884.1	Poland	Martínková et al. (unpublished)
CYTB	GU291956.1	Latvia	Baltrūnaitė (2010)
CYTB	JF318998.1	Germany	Schlegel et al. (2012)
CYTB	FJ641162.1–FJ641177.1	Greece	Thanou et al. (unpublished)
CYTB	FR869854.1–FR869855.1	Greece	Martínková et al. (unpublished)
CYTB	AY513836.1	Gümüşhane (Turkey)	Jaarola et al. (2014)
CYTB	FR869836.1–FR869838.1	Gümüşhane (Turkey)	Martínková et al. (unpublished)
CYTB	FR869839.1, FR869840.1, FR869842.1	Zonguldak (Turkey)	Martínková et al. (unpublished)
CYTB	FR869843.1–FR869844.1	Bolu (Turkey)	Martínková et al. (unpublished)
CYTB	FR869845.1–FR869846.1	Balıkesir (Turkey)	Martínková et al. (unpublished)
CYTB	FR869847.1	Kırklareli (Turkey)	Martínková et al. (unpublished)
CYTB	FR869848.1–FR869853.1	Bursa (Turkey)	Martínková et al. (unpublished)
COXI	AY332685.1	Switzerland	Pfunder et al. (2004)

Table 1s. (Cont.)

Species

Gene	Accession number	Location	Reference
<i>RBP</i>	MZ222031.1	Russia: Novgorod Oblast	Bogdanov et al. (2021)
<i>IRBP</i>	MZ222032.1–MZ222033.1	Russia: Kaluga Oblast	Bogdanov et al. (2021)
<i>IRBP</i>	MZ222034.1	Russia: Voronezh Oblast	Bogdanov et al. (2021)
<i>IRBP</i>	MZ222035.1	Russia: Belgorod Oblast	Bogdanov et al. (2021)
<i>IRBP</i>	MZ222036.1	Kırklareli (Turkey)	Bogdanov et al. (2021)
<i>IRBP</i>	MZ222037.1	Balıkesir (Turkey)	Bogdanov et al. (2021)
<i>M. majori</i>			
<i>CYTB</i>	MZ198192.1, MZ198194.1	Russia: the Kabardino–Balkar Republic	Bogdanov et al. (2021)
<i>CYTB</i>	MZ198195.1–MZ198199.1	Russia: Stavropol Krai	Bogdanov et al. (2021)
<i>CYTB</i>	MZ198200.1–MZ198205.1	Russia: Krasnodar Krai	Bogdanov et al. (2021)
<i>CYTB</i>	KM656468.1, KM656477.1, KM656478.1	Russia: Krasnodar Krai	Baskevich et al. (2016)
<i>CYTB</i>	KM656469.1–KM656473.1	Russia: the Kabardino–Balkar Republic	Baskevich et al. (2016)
<i>CYTB</i>	KM656474.1–KM656476.1	Russia: Stavropol Krai	Baskevich et al. (2016)
<i>CYTB</i>	KP013582.1–KP013583.1	Russia	Tkachenko et al. (2015)
<i>CYTB</i>	MZ198206.1	Georgia	Bogdanov et al. (2021)
<i>CYTB</i>	AY513814.1	Artvin (Turkey)	Jaarola et al. (2004)
<i>CYTB</i>	DQ841703.1–DQ841704.1	Artvin (Turkey)	Martínková et al. (2007)
<i>IRBP</i>	MZ222057.1	Russia: Stavropol Krai	Bogdanov et al. (2021)
<i>IRBP</i>	MZ222058.1–MZ222059.1	Russia: Krasnodar Krai	Bogdanov et al. (2021)
<i>IRBP</i>	MZ222062.1	Armenia	Bogdanov et al. (2021)
<i>IRBP</i>	MZ222061.1	Artvin (Turkey)	Bogdanov et al. (2021)
<i>IRBP</i>	AM919409.1	–	Galewski et al. (unpublished)
<i>M. pennsylvanicus</i>			
<i>CYTB</i>	KJ556623.1	Canada	Kohli et al. (2004)
<i>COXI</i>	JF456745.1	Canada	Eger et al. (unpublished)
<i>IRBP</i>	KJ556734.1	Canada	Kohli et al. (2014)
<i>M. longicaudus</i>			
<i>CYTB</i>	KY754039.1	USA	Steppan and Schenk (2017)
<i>COXI</i>	JF456725.1	Canada	Eger et al. (unpublished)
<i>IRBP</i>	KX455523.1	Canada	Abramson and Petrova (direct submission)

Table 2s. Genetic diversity values of *M. majori* and *M. subterraneus* based on *CYTB* data: Ns, number of samples, Nh, number of haplotypes; Hd, haplotype diversity; Nd, nucleotide diversity (P_i); Ps, polymorphic sites; Nps, number of parsimony informative sites; Nss, number of singleton sites; Nm, number of mutations

Tabla 2s. Valores de la diversidad genética de *M. majori* y *M. subterraneus* basados en datos del gen *CYTB*: Ns, número de muestras; Nh, número de haplotipos; Hd, diversidad de haplotipos; Nd, diversidad nucleotídica (P_i); Ps, sitios polimórficos; Nps, número de sitios informativos de parsimonia; Nss, número de sitios singleton; Nm, número de mutaciones.

	Ns	Nh	Hd	Nd	Ps	Nps	Nss	Nm
<i>M. subterraneus</i> (Europe)	54	37	0.980	0.015	73	45	28	74
<i>M. subterraneus</i> (Thrace)	20	14	0.947	0.015	45	15	30	48
<i>M. subterraneus</i> (Anatolia)	25	21	0.983	0.042	88	52	36	89
<i>M. subterraneus</i> (total)	99	72	0.991	0.034	152	101	51	174
<i>M. majori</i> (Anatolia)	9	8	0.972	0.007	12	6	6	14
<i>M. majori</i> (Caucasia)	27	18	0.946	0.018	82	30	52	94
<i>M. majori</i> (total)	36	26	0.968	0.020	87	33	54	101
Total	135	98	0.993	0.056	198	132	66	255

Table 3s. Mean genetic distance (with standard errors) and fixation index (F_{ST}) values based on *CYTB* data.

Tabla 3s. Valores de la distancia genética media (con el error estándar) y del índice de fijación (F_{ST}) basados en datos del gen *CYTB*.

	Mean genetic distance (d)	F_{ST}
<i>M. subterraneus</i> (Europe)– <i>M. subterraneus</i> (Anatolia)	0.046 ± 0.005	0.387
<i>M. subterraneus</i> (Thrace)– <i>M. subterraneus</i> (Anatolia)	0.053 ± 0.006	0.461
<i>M. subterraneus</i> (Europe)– <i>M. subterraneus</i> (Thrace)	0.036 ± 0.006	0.594
<i>M. majori</i> – <i>M. subterraneus</i> (Europe)	0.091 ± 0.009	0.673
<i>M. majori</i> – <i>M. subterraneus</i> (Thrace)	0.096 ± 0.011	0.816
<i>M. majori</i> – <i>M. subterraneus</i> (Anatolia)	0.095 ± 0.010	0.666
<i>M. majori</i> (Anatolia)– <i>M. majori</i> (Caucasia)	0.026 ± 0.004	0.496
<i>M. subterraneus</i> (total)– <i>M. majori</i> (total)	0.093 ± 0.010	0.706

Table 4s. Genetic diversity values of *M. majori* and *M. subterraneus* based on COXI data. (For the abbreviations see table 1s).

Tabla 4s. Valores de la diversidad genética de *M. majori* y *M. subterraneus* basados en datos del gen COXI. (Para las abreviaturas, véase tabla 1s).

	Ns	Nh	Hd	Nd	Ps	Nps	Nss	Nm
<i>M. subterraneus</i> (Europe)	5	4	0.900	0.040	40	24	16	42
<i>M. subterraneus</i> (Anatolia)	12	12	0.985	0.041	52	38	14	55
<i>M. subterraneus</i> (total)	17	16	0.993	0.038	55	42	13	60
<i>M. majori</i> (total–Anatolia)	7	5	0.905	0.044	51	45	6	51
Total	24	20	0.986	0.086	130	117	13	149

Table 5s. Mean genetic distance (with standard errors) and fixation index (F_{ST}) values based on COXI data.

Tabla 5s. Valores de la distancia genética media (con el error estándar) y del índice de fijación (F_{ST}) basados en datos del gen COXI.

	Mean genetic distance (d)	F_{ST}
<i>M. subterraneus</i> (Europe)– <i>M. subterraneus</i> (Anatolia)	0.056 ± 0.007	0.440
<i>M. majori</i> – <i>M. subterraneus</i> (Europe)	0.154 ± 0.002	0.822
<i>M. majori</i> – <i>M. subterraneus</i> (Anatolia)	0.156 ± 0.001	0.736
<i>M. subterraneus</i> (total)– <i>M. majori</i> (total)	0.134 ± 0.013	0.729

Table 6s. Genetic diversity values of *M. majori* and *M. subterraneus* based on IRBP data. (For the abbreviations see table 1s).

Tabla 6s. Valores de la diversidad genética de *M. majori* y *M. subterraneus* basados en datos del gen IRBP. (Para las abreviaturas, véase tabla 1s).

	Ns	Nh	Hd	Nd	Ps	Nps	Nss	Nm
<i>M. subterraneus</i> (Thrace)	5	1	0	0	0	0	0	0
<i>M. subterraneus</i> (Europe)	5	1	0	0	0	0	0	0
<i>M. subterraneus</i> (Anatolia)	13	5	0.782	0.002	4	2	2	4
<i>M. subterraneus</i> (total)	23	5	0.557	0.001	4	2	2	4
<i>M. majori</i> (Anatolia)	8	2	0.571	0.002	3	0	3	3
<i>M. majori</i> (Caucasia)	5	3	0.700	0.002	2	2	0	2
<i>M. majori</i> (total)	13	3	0.603	0.002	3	1	2	3
Toplam	36	8	0.773	0.003	8	5	3	8

Table 7s. Mean genetic distance (with standard errors) and fixation index (F_{ST}) values based on *IRBP* data.

Tabla 7s. Valores de la distancia genética media (con el error estándar) y del índice de fijación (F_{ST}) basados en datos del gen IRBP.

	Mean genetic distance (d)	F_{ST}
<i>M. subterraneus</i> (Europe)– <i>M. subterraneus</i> (Anatolia)	0.001 ± 0.001	0.266
<i>M. subterraneus</i> (Europe)– <i>M. subterraneus</i> (Thrace)	0	0
<i>M. subterraneus</i> (Thrace)– <i>M. subterraneus</i> (Anatolia)	0.001 ± 0.001	0.266
<i>M. majori</i> – <i>M. subterraneus</i> (Europe)	0.004 ± 0.001	0.680
<i>M. majori</i> – <i>M. subterraneus</i> (Anatolia)	0.006 ± 0.002	0.558
<i>M. majori</i> – <i>M. subterraneus</i> (Thrace)	0.004 ± 0.002	0.680
<i>M. majori</i> (Anatolia)– <i>M. majori</i> (Caucasia)	0.001 ± 0.001	0.023
<i>M. subterraneus</i> (total)– <i>M. majori</i> (total)	0.005 ± 0.028	0.578