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ORIGINAL ARTICLE

High molecular variability in three pine vole species of the subgenus *Terricola* (*Microtus*, Arvicolinae) and plausible source of polymorphism

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Abstract

We studied molecular variability to clarify intraspecific differentiation and phylogenetic relationships in three pine vole species (genus *Microtus*, subgenus *Terricola*): *Microtus subterraneus*, *Microtus daghestanicus*, and *Microtus majori*. Multilocus analysis was performed using the entire mitochondrial *cytb* gene and fragments of nuclear *BRCA1*, *IRBP*, and *XIST* genes. Results confirmed separation of the species, especially *M. majori* compared with *M. daghestanicus* and *M. subterraneus*. These species showed different molecular polymorphism in the genetic markers. We identified two close forms of *M. majori*, differing in *cytb* gene and the nuclear gene *XIST*; one form inhabits the northern slopes of the Greater Caucasus, another the Transcaucasia. Separation of *M. daghestanicus* populations from North Ossetia and the others was clear. *Microtus subterraneus* populations from southern Europe and Asia Minor were characterized by maximal genetic heterogeneity; the specimen from Samsun (northern Asia Minor) appeared to be most distant from the others. Despite polymorphism in the chromosome number in *M. subterraneus* populations from the East European Plain, they possess a depleted gene pool. Results indicated that *M. subterraneus* colonized the East European Plain in the Holocene, and chromosome variability originated in this part of the species' range as a result of chromosomal fission and quick fixation of the arrangement in northern populations. We argue that differences in the genetic differentiation patterns of *Terricola* species are mainly due to their ecological peculiarities.

KEYWORDS
genetic differentiation, intraspecific molecular variability, multilocus analysis, pine voles

Abstrait

Nous avons étudié la variabilité moléculaire pour clarifier la différenciation intraspécifique et les relations phylogénétiques chez trois espèces de campagnols des pins (genre *Microtus*, sous-genre *Terricola*): *Microtus subterraneus*, *Microtus daghestanicus* et *Microtus majori*. L'analyse multilocus a été réalisée en utilisant l'intégralité du gène *cytb* mitochondrial et des fragments des gènes nucléaires *BRCA1*, *IRBP* et *XIST*. Les résultats

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ont confirmé la séparation des espèces, en particulier *M. majori* par rapport à *M. daghestanicus* et *M. subterraneus*. Ces espèces ont montré un polymorphisme moléculaire différent dans les marqueurs génétiques. Nous avons identifié deux formes proches de *M. majori*, différant par le gène *cytb* et le gène nucléaire *XIST*; une forme habite les pentes nord du Grand Caucase, une autre la Transcaucasie. La séparation des populations de *M. daghestanicus* d'Ossétie du Nord et des autres était claire. Les populations de *Microtus subterraneus* du sud de l'Europe et de l'Asie Mineure étaient caractérisées par une hétérogénéité génétique maximale; le spécimen de Samsun (nord de l'Asie Mineure) semblait le plus éloigné des autres. Malgré le polymorphisme du nombre de chromosomes dans les populations de *M. subterraneus* de la plaine d'Europe de l'Est, elles possèdent un pool génétique appauvri. Les résultats ont indiqué que *M. subterraneus* a colonisé la plaine d'Europe de l'Est à l'Holocène et que la variabilité chromosomique est née dans cette partie de l'aire de répartition de l'espèce en raison de la fission chromosomique et de la fixation rapide de l'arrangement dans les populations du nord. Nous soutenons que les différences dans les modèles de différenciation génétique des espèces *Terricola* sont principalement dues à leurs particularités écologiques.

1 | INTRODUCTION

Studying the genetic variability of individual species as well as different species groups is of paramount importance for the elucidation of many theoretical problems in modern biology. Among these problems, the first that should be mentioned is the definition of the term "species" and its criteria, speciation mechanisms, and factors, which affect the process of speciation. The question as to what degree changes in different genetic and morphological features are gradual and synchronous is also intriguing. Small mouse-like rodents are optimal model objects of studies focused on these questions because their high reproduction rate and fast alternation of generations promote fast changes in population gene pools. Species whose ranges cover territories, which are various in climatic and geographical aspects, are of particular interest.

Pine voles are small rodents of the subfamily Arvicolinae Gray, 1821. Until the 1970s, pine voles were united in the single Holarctic genus or subgenus *Pitymys* McMurtrie, 1831 (e.g., Ellerman & Morrison-Scott, 1951; Gromov & Polyakov, 1977; Miller, 1912; Ognev, 1950). Later, the independent evolution of pine voles from the Old and New Worlds was proved by paleontological and molecular genetic data. Presently, Palearctic species are considered to be representatives of the distinct subgenus *Terricola* Fatio, 1867 of the genus *Microtus* Schrank, 1798 (Chaline, 1987; Chaline et al., 1988; Potapov et al., 1999; Jaarola et al., 2004; Musser & Carleton, 2005; Tougard, 2017). According to Musser and Carleton (2005), the subgenus *Terricola* is represented by 14 species. However, their set, the number, and taxonomic position are still disputed.

The distribution of subgenus *Terricola* covers the mountains and flat landscapes of most of Europe, Transcaucasia (up to northeastern Turkey and possibly northwestern Iran), and Asia Minor. Pine voles lead a semi-underground life and inhabit meadows and forests, mainly broad-leaf forests, although they sometimes penetrate into mixed and coniferous woods (Gromov & Polyakov, 1977; Kryštufek & Vohralík, 2005; Ognev, 1950; Shvarts, 1985; Tougard, 2017).

The application of karyotyping and molecular genetic methods effected a breakthrough in the taxonomy of this vole group and has resolved many disputed points. For example, some researchers (e.g., Ellerman & Morrison-Scott, 1951) included the forms majori, daghestanicus, and subterraneus into a polytypic species *Pitymys subterraneus* sensu lato. The forms majori and daghestanicus are sympatric; they were found in the Greater Caucasus, Transcaucasia, and the Pontic Mountains. The form subterraneus has a mosaic distribution from western France, the Alps, and the Balkan Peninsula to Upper–Middle Volga in the meridional direction, and from Asia Minor to Lake Onega and Baltic Sea in the latitudinal direction (Shenbrot & Krasnov, 2005; Tougard, 2017) (Figure 1). Other scientists (Corbet, 1978; Gromov & Polyakov, 1977; Ognev, 1950) recognized subterraneus and majori forms as distinct species, and daghestanicus form as a subspecies of majori. Later, different karyotypes were described in all of these forms: 2n = 54, NF = 60 in majori; 2n = 38–54, NF = 58 in daghestanicus (Akhverdyan et al., 1992; Baskevich et al., 1984; Baskevich, Potapov, Khlyap, et al., 2016; Ivanov & Tembotov, 1972; Khatoukhov et al., 1978; Lyapunova et al., 1988); 2n = 52–54, NF = 60 in subterraneus (Baskevich et al., 2007, 2018; Bulatova et al., 2007; Macholán et al., 2001; Meylan, 1972; Mitsainas et al., 2010; Sablina et al., 1989; Zagorodnyuk, 1988; Zima & Kral, 1984). These karyotypic peculiarities served as the basis for recognition of all of the forms as distinct species: *Microtus majori* Thomas, 1906 (Major's pine vole), *Microtus daghestanicus* Shidlovsky, 1919 (the Caucasus or Daghestan pine vole), and *Microtus subterraneus* de Selys-Longchamps, 1836 (the common or European pine vole) (Ivanov & Tembotov, 1972; Khatoukhov et al., 1978; Niethammer & Krapp, 1982; Baskevich et al., 1984). Further studies demonstrated that *M. majori*, *M. daghestanicus*, and *M. subterraneus* differ from each

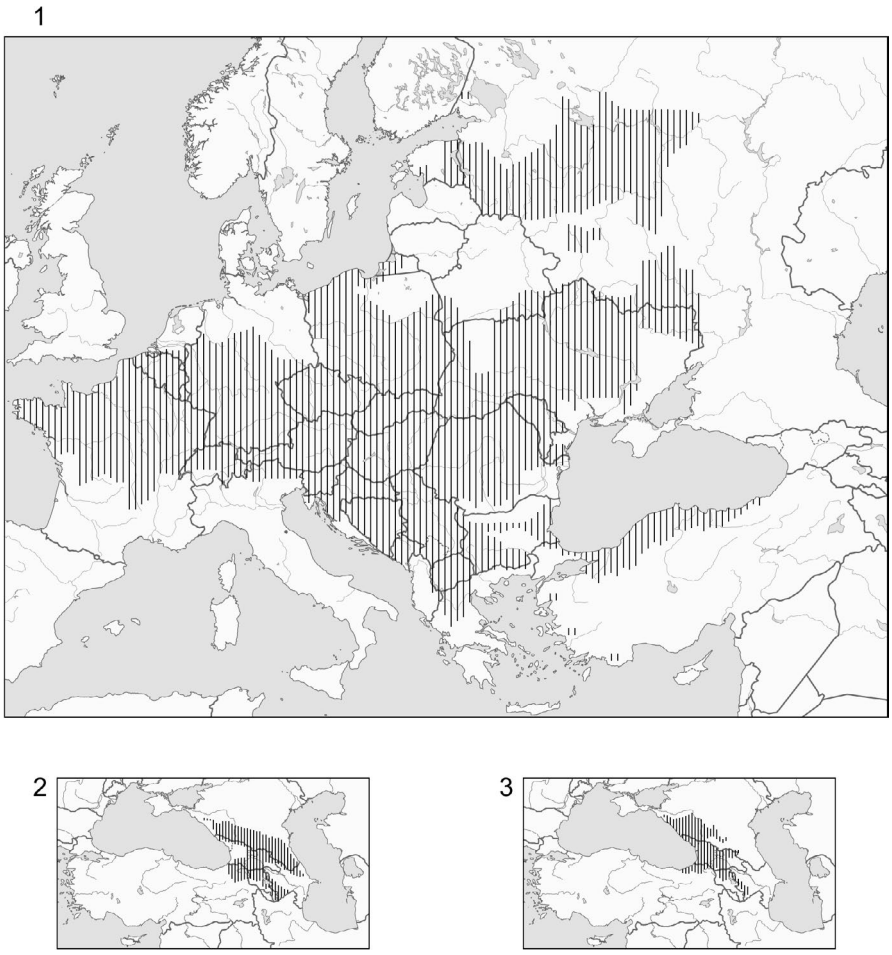

FIGURE 1 Approximate geographic distribution of (1) *Microtus subterraneus*, (2) *Microtus daghestanicus*, and (3) *Microtus majori* according to own and published data (Gromov & Erbajeva, 1995; Tougard, 2017)

other in their molecular genetic characteristics (Baskevich et al., 2018; Baskevich, Potapov, Khlyap, et al., 2016; Baskevich, Potapov, & Mironova, 2016; Bogdanov, Khlyap, et al., 2020; Jaarola et al., 2004; Macholán et al., 2001; Martínková & Moravec, 2012; Mezhzherin et al., 1995; Tougard, 2017) as well as in their ecological preferences (Baskevich, 1997; Shvarts, 1985; Tembotov & Khatoukhov, 1979; Zagorodnyuk, 1992). Recent taxonomic checklists (Musser & Carleton, 2005; Pavlinov & Lissovsky, 2012) supported the specific status of *M. majori*, *M. daghestanicus*, and *M. subterraneus*.

The taxonomic status of some chromosome forms, which were discovered in the widely distributed and genetically polymorphic species *M. subterraneus* and *M. daghestanicus*, are still in debate. In the latter species, different Robertsonian chromosome translocations led to the emergence of 11 karyomorphs: 2n = 54, 53, 52, 46, 45, 44, 43, 42″A″, 42″B″, 40, 38; NF =58 in all cases (Akhverdyan et al., 1992). Two of these versions (2n = 38, 42″A″), from the Karabakh and Zangezur mountain ranges of Transcaucasia, were considered as a separate species *Microtus nasarovi* Shidlovsky, 1938 (Khatoukhov et al., 1978; Zagorodnyuk, 1988). Three chromosome forms are revealed in *M. subterraneus*: two with 2n = 54, differing in the heterochromatin band size of the X chromosome and its morphology, and one with 2n = 52, which supposedly emerged due to a Robertsonian fusion (Baskevich et al., 2007, 2018; Bulatova et al., 2007; Macholán et al., 2001; Meylan, 1972; Mitsainas et al., 2010; Sablina et al., 1989). According to Zagorodnyuk (1991), the 52 chromosome form inhabiting southern Europe may be assigned to a separate species, *Microtus dacius* Miller, 1911, while the former name, *M. subterraneus* s. str., should be maintained for both 54 chromosome forms, one of them being distributed in northeast Europe and the other in Asia Minor.

Assessments of the relationships between representatives of the subgenus *Terricola* are equally ambiguous. Kratochvil and Kral (1974) proposed combining *M. majori* and *M. daghestanicus* into the Pontian-Caucasian species group and this point of view was later supported by craniological analysis (Mironova et al., 2013). On the other hand, some authors noted the closeness of *M. subterraneus* and *M. majori*, on the basis of morphological and zoogeographical studies, and included these species into a "subterraneus" group together with *Microtus multiplex* Fatio, 1905, *Microtus tatricus* Kratochvil, 1952 and *Microtus bavaricus* Konig, 1962 (Chaline et al., 1988). Allozyme data obtained by Macholán et al. (2001) also demonstrated the similarity of *M. subterraneus* and *M. majori*. Zagorodnyuk (1988), analyzing routinely stained chromosomes, proposed to distinguish six supraspecific groups. One of them (the "subterraneus" group) is represented by *M. subterraneus*, *M. daghestanicus*, and *M. nasarovi*, while *M. majori* forms another group (the "majori"). Jaarola et al. (2004), studying mitochondrial *cytochrome b* gene (*cytb*) variability, came to a similar conclusion. Based on the multilocus analysis, Martínková and Moravec (2012) demonstrated that the subgenus *Terricola* is subdivided into two supraspecific groups: an eastern group, which

includes *M. daghestanicus*, *M. subterraneus*, and the more distant *M. majori*, and a western group, combining all the other species. Despite differences between the subgenus structure schemes suggested in these three studies (Jaarola et al., 2004; Martínková & Moravec, 2012; Zagorodnyuk, 1988), they unanimously noted the separation of *M. majori* from *M. daghestanicus* and *M. subterraneus* that was confirmed by molecular (Baskevich et al., 2018; Baskevich, Potapov, Khlyap, et al., 2016; Bogdanov, Khlyap, et al., 2020; Tougard, 2017), karyotypic (Baskevich, 1997) and allozyme studies (Mezhzherin et al., 1995). Nevertheless, it should be stressed that the majority of these studies of molecular genetic variability within subgenus *Terricola* considered only one mitochondrial gene (*cytb*), and the analyses were conducted on only a few specimens of each species. Thus, assessments of intraspecific polymorphism and interspecific differences might be very rough. All three considered *Terricola* species occupy areas, which are very diverse in climatic and geographic terms. Mountain regions (the Alps, the Carpathians, the Greater Caucasus) and the Turkish Straits are significant biogeographic barriers. So, genetic variabilities of *M. subterraneus*, *M. daghestanicus* and *M. majori* expect to be more complex than they are presently known and need the analysis of the representative material.

The current study was undertaken to assess *M. subterraneus*, *M. daghestanicus*, and *M. majori* with regard to their genetic variability, precise differentiation level between the species, and their relationships using multiple molecular markers: the entire mitochondrial *cytb* gene and fragments of the nuclear *BRCA1* (breast and ovarian cancer type 1 susceptibility protein) gene, exon 11, the *IRBP* (interphotoreceptor retinoid-binding protein) gene, exon 1, and the *XIST* (X-inactive specific transcript) gene. Recently, the sequencing of protein-coding nuclear *BRCA1* and *IRBP* genes has been actively pursued for similar studies of various mammalian groups (e.g., Adkins et al., 2001; Bannikova et al., 2013; Bogdanov, Maltsev, et al., 2020; Lebedev et al., 2018, 2020; Martínková & Moravec, 2012). The product of the *XIST* gene, localized on the X chromosome in rodents, is a non-coding RNA, which, in interaction with some proteins, takes part in regulating the inactivation of one X chromosome in females (Nesterova et al., 2001). An *XIST* gene fragment has been used successfully to discriminate specimens of sibling species of mole voles (Bakloushinskaya et al., 2019; Lebedev et al., 2020), which are rodent groups related to pine voles. Preliminary analysis of limited samples of *M. subterraneus*, *M. daghestanicus*, and *M. majori* (five specimens of each pine vole species) demonstrated that fragments of *BRCA1* and *XIST* genes are promising nuclear markers for studying this rodent group. For example, significant variability of the *BRCA1* gene was detected in *M. daghestanicus* sample, even among specimens from the same locality, and differentiation between Major's pine voles from the Greater Caucasus and Transcaucasia was traced in *XIST* gene fragment (Bogdanov, Khlyap, et al., 2020). It can be expected that enlargement of sample size and a set of nuclear markers as well as an analysis of their longer fragments will allow to more accurately determine the patterns of genetic polymorphism.

2 | MATERIALS AND METHODS

2.1 | Experimental material

Data regarding the material, used in the study, are presented in Table 1. Geographical locations of capture points of the subgenus *Terricola* voles are shown in Figure 2. One Altai vole *Microtus obscurus* Eversmann, 1841 and one East European vole *Microtus rossiaemeridionalis* Ognev, 1924 from our collection were chosen as an outgroup. We followed international, national, and institutional guidelines for animal care.

We analyzed *cytb* gene sequences in the total sample. In addition to our own material, we used previously published data (Jaarola et al., 2004; Jaarola & Searle, 2002; Martínková et al., 2007) and data available in the GenBank database (Appendix 1) for the entire *cytb* gene sequences of *M. subterraneus* (AY513832–AY513835), *M. daghestanicus* (AY513790–AY513792), and *M. majori* (AY513814, DQ841703, DQ841704), as well as two specimens of the field vole *Microtus agrestis* Linnaeus, 1761 (AY167180, AY167187) and one root vole *Microtus (Alexandromys) oeconomus* Pallas, 1776 (AY220018), to enlarge the outgroup. Thus, *cytb* gene sequences were analyzed in 69 voles: 64 pine voles of three species (54 sequences were obtained by us and 10 sequences were published earlier) and five voles of the outgroup (two of which were studied by us, and three, by other scientists).

The sequencing of nuclear genes was selectively performed on those representatives of the species that demonstrated the largest differences in the *cytb* analysis: eight *M. subterraneus*, 18 *M. daghestanicus*, six *M. majori*, one *M. obscurus*, and one *M. rossiaemeridionalis* (34 animals in total; all these specimens were taken from our own collections).

Sequences of all the genes that we have analyzed have been deposited in GenBank. Accession numbers are as follows: MZ198155–MZ198210 for *cytb* gene, MZ221997–MZ222030 for *BRCA1* gene, MZ222031–MZ222064 for *IRBP* gene, MZ222065–MZ222098 for *XIST* gene first fragment, and MZ222099–MZ222132 for *XIST* gene second fragment. Accession numbers, related to each specimen, are listed in Appendix 1.

2.2 | Molecular and statistical analyses

DNA was extracted from ground liver and kidney samples, stored in alcohol, after treatment with proteinase K, phenol-chloroform deproteinization, and final precipitation in isopropanol (Sambrook et al., 1989). Primers for amplification and sequencing of the entire *cytb* gene, two overlapping fragments of the *BRCA1* gene, exon 11, two non-overlapping fragments of the *XIST* gene, and one fragment of the *IRBP* gene, exon 1, are listed in Table 2. Polymerase chain reaction (PCR) was carried out in a mixture containing 25–35 ng of DNA, 2 μl of 10 × Taq buffer, 1.6 μl of 2.5 mM dNTP (Sileks, Russia), 4 pM of each primer, one unit of Taq polymerase (Syntol), and deionized water to a final volume of 20 μl. Amplification was conducted in

a TERTSIK thermal cycler (DNA-Technology). PCR, briefly, included preheating at 94°C (3 min) and then 35 cycles as follows: 30 s at 94°C; 1 min at 55–63°C (see the exact annealing temperatures in Table 2); 1 min at 72°C; finally, extension of the PCR products was performed at 72°C (6 min). The lengths of the PCR products are indicated in Table 2. Automatic sequencing was carried out using the ABI PRISM® BigDye™ Terminator v. 3.1 Kit (Applied Biosystems) in the AB 3500 genetic analyzer (Applied Biosystems) at the Core Centrum of Koltzov Institute of Developmental Biology, Russian Academy of Sciences. After alignment, the length of the *cytb* gene nucleotide sequence, determined in all voles, was equal to 1143 bp (Alignment S1); the *BRCA1* gene comprised 1698 bp (Alignment S2); the *IRBP* gene was 807 bp (Alignment S3); and the two non-overlapping parts of the *XIST* gene comprised 409–413 and 583–585 bp, 994–998 bp in total (Alignment S4). Differences in the length of the latter gene fragments are related to deletions. The sequences of all protein-coding genes are presented by entire codons. Marginal codon boundaries (i.e., the first nucleotide of the initial codon and the third nucleotide of the last codon) in fragments of nuclear genes *BRCA1* and *IRBP* were determined from their annotated sequences in genomes of the house mice *Mus musculus* (Bogdanov, Maltsev, et al., 2020; Sharan et al., 1995; Stanhope et al., 1992). Sites, in which two overlapped peaks were reproducibly registered in chromatograms, were coded and treated as heterozygous.

The uncorrected mean and pairwise genetic *p*-distances (*D*) between vole species, intraspecific groups, and single specimens were calculated using Mega X software (Kumar et al., 2018). The values of the genetic variability parameters (the number of mitotypes and polymorphic nucleotide sites, the mean number of substitutions per nucleotide site, haplotype and nucleotide diversities) in the summarized samples of each species of the subgenus *Terricola* were determined using Arlequin software, version 3.5.2.2 (Excoffier & Lischer, 2010). Dendrograms were built based on the Maximum Likelihood (ML) method using IQTree software, version 2.0-rc2 (Nguyen et al., 2015; Minh et al., 2019); the ModelFinder option (Kalyaanamoorthy et al., 2017) was applied to achieve optimal model evaluation of nucleotide substitutions for each gene. Before statistical analysis, non-extended deletions, revealed in the *XIST* gene sequences of several specimens, were filled in by random nucleotides, which were absent in the total vole sample; the replacement did not affect the nucleotide substitution model, as was proved by repeated testing. Furthermore, we conducted an analysis of the concatenated nuclear gene sequences, partitioning the dataset by gene and applying the gene-specific substitution models selected using ModelFinder (Chernomor et al., 2016); each partition had its own set of branch lengths. Standard nonparametric bootstrapping was conducted throughout 1000 pseudo replications for all reconstructions.

Bayesian inference for a sequence of each gene separately and combined sequence of all nuclear genes was additionally evaluated in MrBayes software, version 3.2.7 (Ronquist et al., 2012; Ronquist et al., 2020); analyses were run for 1 million generations with Markov chains sampled every 1000 generations, 25% of trees were discarded ("burn-in"), and node support was assessed using posterior probability values. The analysis included two independent runs. The Tracer 1.7.1 software (Rambaut et al., 2018) was used to check for convergence and determine the necessary burn-in fraction, which was 10% of the chain length. In all calculations, the effective sample size exceeded 200 for all estimated parameters.

Final phylogenetic tree images were rendered using FigTree software, version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

3 | RESULTS

3.1 | *Cytb* gene polymorphism: Interspecific variability

The topologies of the phylogenetic reconstructions built using IQTree (Figure 3) and MrBayes (Figure S1) software were similar, differing just in clustering of the *M. daghestanicus* intraspecific groups and outgroup species. Both the ML-tree and Bayesian tree clearly demonstrated the distribution of *cytb* gene mitotypes of the subgenus *Terricola* representatives into three general clades, with high bootstrap (99–100) and Bayesian (one in all cases) support. The clades correspond to the recognized species *M. majori*, *M. daghestanicus*, and *M. subterraneus* (Musser & Carleton, 2005; Pavlinov & Lissovsky, 2012). Among these species, *M. majori* maximally differs from *M. daghestanicus* ($D = 0.0965$) and *M. subterraneus* ($D = 0.0968$); the lowest value of average genetic distance (0.0783) was determined in comparison with the two latter species (Table S1). Thus, our data confirmed the closer affinity of *M. daghestanicus* and *M. subterraneus* as well as the isolation of *M. majori* that coincides with the results of the same gene analysis performed by other authors on limited material (Baskevich, Potapov, Khlyap, et al., 2016; Jaarola et al., 2004). Genetic distances between the three studied species of the subgenus *Terricola* significantly exceeded the minimal values (2%–5%) that were determined for mammalian species comparison and proposed as the "mark" for discrimination in the genetic species concept (Baker & Bradley, 2006; Bradley & Baker, 2001).

3.2 | *Cytb* gene polymorphism: Intraspecific variability

In each of the studied *Terricola* species (Figure 3, Figure S1), the *cytb* gene demonstrates high variability, but its characteristics are different in *M. majori*, *M. daghestanicus*, and *M. subterraneus*. Between two and six major clades or separate branches may be traced in these species. The average pairwise intraspecific genetic distance (Table S2) is minimal in *M. majori* ($D = 0.0139$), but it is 1.5 times higher in *M. daghestanicus* ($D = 0.0232$) and *M. subterraneus* ($D = 0.0226$). In the same order (from *M. majori* to other two species), the intraspecific differentiation character becomes more complex. Calculation of the genetic variability parameters in each species of subgenus *Terricola* (Table S3) produces similar results.

TABLE 1 Material used in the study

Species Geographic origin	ID	Voucher	Sex	Karyotype
Microtus subterraneus				
1. Russia, Novgorod oblast, Valdaysky district, vicinities of Krenye Lake (57.98°N; 33.38°E)	11-56	SIEE, 11-56	f	54, 60; our data
The same locality	11-57	SIEE, 11-57	f	54, 60; our data
The same locality	11-59	SIEE, 11-59	m	54, 60; our data
The same locality	13-70	SIEE, 13-70	f	54, 60; our data
2. Russia, Kaluga oblast, Ulyanovsky district, vicinities of the Nagaya village (53.57°N; 35.74°E)	13-146	SIEE, 13-146	f	54, 60; our data
The same locality	13-179	SIEE, 13-179	f	54, 60; our data
The same locality	13-180	SIEE, 13-180	m	54, 60; our data
The same locality	MS-1	SIEE, MS-1	m	
The same locality	MS-2	SIEE, MS-2	f	
3. Russia, Voronezh oblast, Verkhnekhavsky district, the right bank of Usmanka River (51.94°N; 39.68°E)	03-67	SIEE, 03-67	f	52, 60; our data
The same locality	03-78	SIEE, 03-78	m	52, 60; our data
The same locality	03-153	SIEE, 03-153	m	52, 60; our data
The same locality	03-192	SIEE, 03-192	m	52, 60; our data
4. Russia, Belgorod oblast, Gubkinsky district, about 10 km southeast of Gubkin town (51.18°N; 37.65°E)	694	SIEE, 694		
The same locality	696	SIEE, 696		
5. Austria, Glocknerhaus (47.07°N; 12.77°E)	2[a]			
6. Greece, Seli (40.55°N; 22.03°E)	1[a]			
7. Turkey, Kirklareli (41.73°N; 27.23°E)	TRS-3	OMU, 1425		52, 60; our data
8. Turkey, Balikesir (39.64°N; 27.88°E)	TRS-2	OMU, 1424		54, 60; our data
9. Turkey, Bursa (40.18°N; 29.07°E)	TRS-4	OMU, 1426		
10. Turkey, Samsun (41.27°N; 36.33°E)	TRS-1	OMU, 1423		54, 60; our data
11. Turkey, Çiğlikara (36.52°N; 29.82°E)	3[a]			
The same locality	4[a]			
Microtus daghestanicus				
12. Turkey, Bağdaşan (41.05°N; 42.38°E)	2[a]			
13. Turkey, Handere (40.27°N; 42.45°E)	3[a]			
14. Turkey, Kars, Sarikamiş (40.60°N; 43.08°E; 2200 m above see level)	TRD-1	OMU, 1422	f	
15. Georgia, Bediani (41.54°N; 44.26°E)	1[a]			
16. Russia, the Republic of North Ossetia–Alania, Alagirsky district, vicinities of the Nizhny Tsey village (42.80°N; 43.95°E)	10-88	SIEE, 10-88	m	54, 58; our data
The same locality	10-90	SIEE, 10-90	m	54, 58; our data
17. Russia, the Republic of North Ossetia–Alania, Alagirsky district, vicinities of the Verkhny Tsey village (42.80°N; 43.94°E)	R1707-69	SSC, R1707-69	m	
The same locality	R1707-70	SSC, R1707-70	f	
The same locality	R1707-71	SSC, R1707-71	f	
18. Russia, the Karachay-Cherkess Republic, about 8 km northeast of Dombay village, the valley of Gonachkhir River, point 1 (43.30°N; 41.76°E; 1750 m above see level)	R1608-72	SSC, R1608-72	m	
The same locality	R1608-73	SSC, R1608-73	f	

(Continues)

TABLE 1 (Continued)

Species Geographic origin	ID	Voucher	Sex	Karyotype
19. Russia, the Karachay-Cherkess Republic, about 8 km northeast of Dombay village, the valley of Gonachkhir River, point 2 (43.28°N; 41.80°E; 1900 m above see level)	R1608-88	SSC, R1608-88	m	
20. Russia, the Kabardino-Balkar Republic, Elbrussky District, vicinities of Adyl-Su River, point 1 (43.21°N; 42.68°E)	10-2	SIEE, 10-2	m	54, 58; our data
The same locality	10-12	SIEE, 10-12	m	54, 58; our data
The same locality	10-51	SIEE, 10-51	f	54, 58; our data
The same locality	10-62	SIEE, 10-62	f	54, 58; our data
21. Russia, the Kabardino-Balkar Republic, Elbrussky District, vicinities of Adyl-Su River, point 2, "Poushka" (43.22°N; 42.69°E)	10-50	SIEE, 10-50	m	54, 58; our data
The same locality	10-59	SIEE, 10-59	f	54, 58; our data
The same locality	10-70	SIEE, 10-70	f	54, 58; our data
22. Russia, the Kabardino-Balkar Republic, Elbrussky District, the outskirts of Terskol village (43.25°N; 42.54°E)	26949	KIDB, 26949	f	54, 58; our data
23. Russia, the Kabardino-Balkar Republic, Zolsky District, Ekiptsoko (43.68°N; 43.08°E)	11-22	SIEE, 11-22	m	
Microtus majori				
The same locality	11-20	SIEE, 11-20	f	54, 60; our data
The same locality	11-26	SIEE, 11-26	f	54, 60; our data
The same locality	11-31	SIEE, 11-31	m	54, 60; our data
24. Russia, Stavropol Krai, Shpakovsky District, the Strizhament Mountain (44.82°N; 42.03°E)	13-15	SIEE, 13-15	f	54, 60; our data
The same locality	13-19	SIEE, 13-19	m	54, 60; our data
The same locality	13-27	SIEE, 13-27	m	54, 60; our data
The same locality	13-53	SIEE, 13-53	f	54, 60; our data
The same locality	13-56	SIEE, 13-56	m	54, 60; our data
25. Russia, Krasnodar Krai, the city of Sochi, Adlersky City District, vicinities of Beshenka River (43.69°N; 40.2°E)	453	SIEE, 453		
The same locality	455	SIEE, 455		
26. Russia, Krasnodar Krai, the city of Sochi, Adlersky City District, Khmelevskiye Lakes (43.71°N; 40.2°E)	252	SIEE, 252	f	
The same locality	253	SIEE, 253	f	
27. Russia, Krasnodar Krai, the city of Sochi, Adlersky City District, the Aibga Ridge (43.65°N; 40.25°E)	381	SIEE, 381	f	
28. Russia, Krasnodar Krai, the city of Sochi, Adlersky City District, the Psekhako Ridge (43.54°N; 39.95°E)	312	SIEE, 312	f	
29. Abkhazia, Sukhumi (43.00°N; 40.98°E)	16-7	SIEE, 16-7	f	54, 60; our data
30. Turkey, Hopa (41.41°N; 41.44°E)	MM388[b]			
31. Turkey, Damar (41.25°N; 41.60°E)	–[a]			
The same locality	TU601[b]			
32. Turkey, Artvin (41.17°N; 41.82°E)	TRM-1	OMU, 1420		
33. Armenia, Lori Province, vicinities of Lermontovo village (40.76°N; 44.61°E)	24710	KIDB, 24710	f	
Microtus obscurus				
The same locality	24709	KIDB, 24709	f	

(Continues)

TABLE 1 (Continued)

Species Geographic origin	ID	Voucher	Sex	Karyotype
Microtus rossiaemeridionalis				
34. Russia, Novosibirsk Oblast, vicinities of Novosibirsk city (54.82°N; 83.10°E)	24221	KIDB, 24221	m	54, 56; our data
Microtus agrestis				
35. Russia, Sverdlovsk Oblast, Serovsky District, Serov town, vicinities of Kakva River	–[c]			
36. Spain, Pyrenees	4[a]			
Microtus oeconomus				
37. Russia, Krasnoyarsk	2[a]			

Note: ID = collection number of specimen. Collection location and voucher numbers of specimens are presented in the column "Voucher." SIEE = Severtsov Institute of Ecology and Evolution, Moscow, Russia; OMU = Ondokuz Mayis University, Kurupelit, Samsun, Turkey; SSC = The Southern Scientific Center of the Russian Academy of Sciences, Rostov-on-Don, Russia; KIDB = Collection of tissues of wild animals for fundamental, applied, and environmental researches of Koltzov Institute of Developmental Biology, Moscow, Russia. F = female, m = male. 2n and NF values, separated by commas, are presented in the column "Karyotype."

[a]Published earlier (Jaarola et al., 2004).

[b]Published earlier (Martínková et al., 2007).

[c]Published earlier (Jaarola, Searle, 2002).

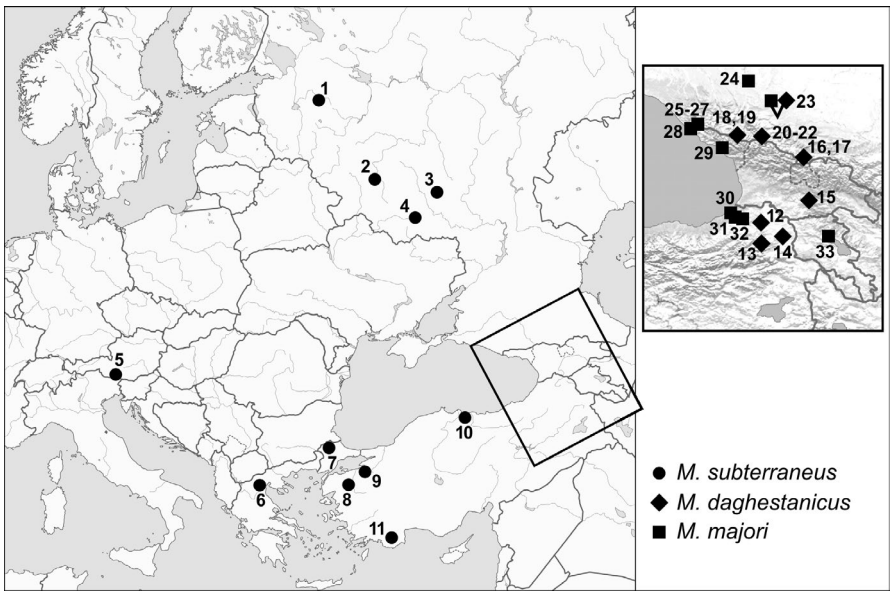

FIGURE 2 Sampling sites of the subgenus *Terricola* pine voles. Site numbers, with detailed geographical information and data on specimens studied, are given in Table 1. Neighboring capture points are designated by a single symbol

In the total *M. majori* sample, represented by 20 specimens, we detected 16 mitotypes, which were divided into two distinct and compact clades in the dendrograms; nevertheless, the clades were strongly supported by Bayesian inference only. The average genetic distance between them amounted to 0.0245. The first clade (I) was represented by specimens from populations of the northern slopes of the Greater Caucasus Mountain Range (Kabardino-Balkar Republic, Stavropol Krai, and Krasnodar Krai, Russia), the second clade (II), by Major's pine voles from Transcaucasia (Abkhazia, Armenia) and northeastern Turkey.

The *M. subterraneus* total sample of 23 voles yielded 16 mitotypes, distributed between six clades or individual branches. Common pine voles originated from the East European Plain (Novgorod, Kaluga, Voronezh, and Belgorod Oblasts in Russia) and belonged to two chromosome forms (see Table 1) compose the first clade (I). Despite karyotypic heterogeneity and maximal sample size, the first clade is the least variable. The second branch (II), represented by a specimen from Austria, is adjacent (sister) to the previous clade. The common pine vole sample from Asia Minor was characterized by maximal genetic differentiation. Specimens from the western provinces of Turkey (in both Europe and Asia) compose the third clade (III) and adjacent Branch IV. The specimen from Samsun, in the northern part of Asia Minor, forms basal branch VI, which is maximally distant from the other populations of the species ($D = 0.0549–0.0636$). Common pine vole from Greece forms Branch V, which occupies an intermediate position between Branches IV and VI. Thus, genetic variability

TABLE 2 Primers used for PCR conduction and sequencing *cytb*, *BRCA1*, *IRBP*, and *XIST* genes

Gene / Fragment	Primers	Temperature	Source
cytb			
1235 bp	L14727-SP (GACAGGAAAAATCATCGTTG) H15915-SP (TTCATTACTGGTTTACAAGAC)	55°C	Jaarola and Searle (2002)
BRCA1, exon 11			
First fragment, 1189 bp	BRCA1-1F-EII (GATGTAACAAATACTGAGCAGCATCA) BRCA1-M1R-EII (GACTTGGATTCCTACCGACTG)	60°C	Present study
Second fragment, 656 bp	BRCA1-M1F (ACGTCCACAGTTCAAAAGCACCTA) BRCA1-M2R-EII (GCTACTTTCGTGTCTCGGTGGAT)	63°C	Present study
IRBP, exon 1			
942 bp	F11 (CAGCCATTGAGCAGGCTATGAA) R22_cric (AGACCACGGCTGAGTAGTCCAT)	63°C	Lebedev et al. (2018)
XIST			
First fragment, ~660 bp or	Xist1-L11841 (GGGGTCTCTGGGAACATTTT) Xist1-R12504 (TGCAATAACTCACAAAACCAAC) or	63°C	Bakloushinskaya et al. (2019)
First fragment, ~400 bp	Xist1-L11841 Xist1-Rint2dag (TTAGAAGAAGAAAAAGAAGAGAAG)	63°C	Present study
Second fragment, ~1170 bp or	Xist1-L11841 Xist-R13010 (TAGAATAAAGGTGGGGTTGTCG) or	63°C	Present study
Second fragment, ~540 bp	Xist2-Fint (GTGGATGGATATATGTTGGTTTTG) Xist-R13010	63°C	Present study

Note: The lengths of PCR products are presented in the column "Fragment." Sequences of forward (upper line) and reverse (bottom line) primers (5′–3′) are presented for each studied gene fragment in the column "Primers." Annealing temperatures are listed in the column "Temperature."

among common pine voles in Asia Minor significantly exceeds differences between the species populations from the western part of the peninsula and Europe.

We determined 19 mitotypes in the total sample of 21 specimens of *M. daghestanicus*, which were distributed between four clades or individual branches. Caucasus pine voles from the Kabardino-Balkar and Karachay-Cherkess Republics of Russia compose the first clade (I). In the ML dendrogram, the second clade (II), which is sister to Clade I, includes specimens from Turkey. The third clade (III) is represented by Caucasus pine voles from the North Ossetia-Alania Republic of Russia and adjacent Branch IV, by a specimen from Georgia. It is noteworthy that maximal genetic distances were determined between Clades II and IV (D = 0.0379), composed of specimens from quite near sites in Turkey and Georgia, as well between Clades I and III (D = 0.0341), represented by Caucasus pine voles from neighboring republics in the central part of the Greater Caucasus. The Bayesian tree differs from the ML reconstruction only in that the basal clade for *M. daghestanicus* includes specimens from Asia Minor (Figure S1).

3.3 | Analysis of nuclear genes polymorphism

Fragments of nuclear genes *BRCA1*, *IRBP*, and *XIST* appeared to be differently variable both in the subgenus *Terricola* and in each of its species. Therefore, the ML and Bayesian phylogenetic reconstructions built using the nuclear genes only partially coincide, both with each other and with trees obtained from the *cytb* gene analysis. The number of fixed substitutions and the genetic distance values determined in comparison of the pine vole species for the *BRCA1*, *IRBP*, and *XIST* genes vary significantly too (Table S4).

The *IRBP* gene fragment appears to be least suitable for discrimination of studied species in subgenus *Terricola* due to weak genotype fixation in all of them. Specific substitutions were revealed only in two nucleotide sites: one nucleotide site in *M. majori* and another in *M. subterraneus*. The minimum value of the average interspecific genetic distance (D = 0.0048) was (unexpectedly) determined in the comparison of *M. majori* and *M. daghestanicus*, and the latter species rather than the former occupied the closest position to the outgroup (Figure 4, Figure S2). It should be noted that some intraspecific groups appeared to be even more differentiated in the studied fragment of the *IRBP* gene than the species. For example, three fixed substitutions (two transitions and one transversion) divide common pine voles from the East European Plain and Asia Minor (D = 0.0050), as well as East European *M. subterraneus* specimens and other vole species. Two transitions are specific only to Caucasus pine voles from North Ossetia; the substitutions were revealed in four specimens in the homozygotic state and, in one vole, in the heterozygotic state. As a result, both the ML and Bayesian trees demonstrated full separation of the aforementioned *M. subterraneus* and *M. daghestanicus* populations, excluding the heterozygous vole.

In total, the heterozygous vole ratio for the *IRBP* gene was high: 22 voles of 34 specimens of all species (i.e., about 65% of the entire sample). In six specimens (common pine voles TRS-1 and TRS-3 from

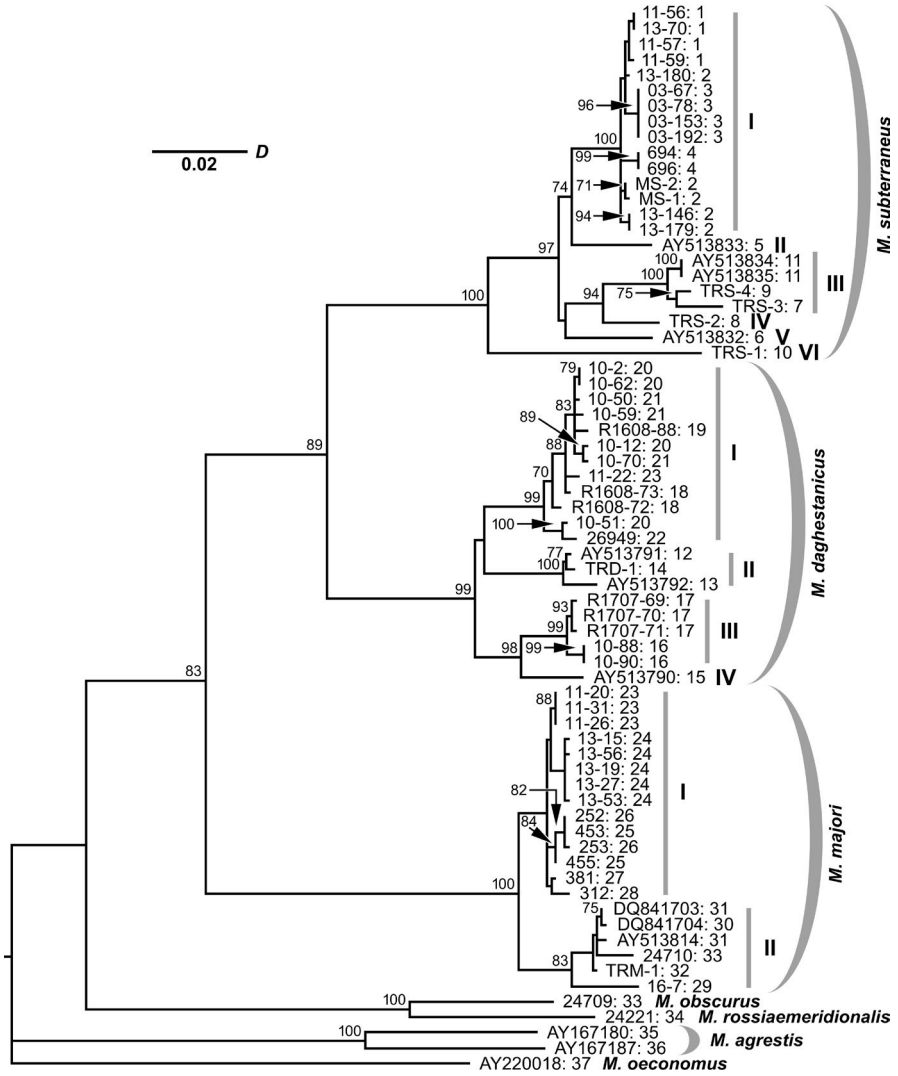

FIGURE 3 The ML dendrogram constructed from a comparison of the entire mitochondrial *cytb* gene sequences (1143 bp) of the subgenus *Terricola* pine voles, as well as other vole species of the genus *Microtus*. The collection numbers of the animals and, following the colon, the collection site numbers (see Table 1) are indicated to the right of the branches. Bootstrap index values exceeding 70% are indicated above the branching nodes of the dendrogram. *D*, genetic distance scale

Turkey, Caucasus pine vole R1608-88 from the Karachay-Cherkess Republic, and all three studied Major's pine voles from the Greater Caucasus, 13-15, 253, and 312) 5–9 nucleotide sites were simultaneously heterozygous. The divergence of Major's pine voles into North Caucasian and Trans-Caucasian groups, which is better traced in the ML dendrogram, related to variability in nucleotide sites, which were heterozygous in at least one specimen, i.e., no fixed substitutions were revealed. The presence of animals, which were simultaneously heterozygous in multiple nucleotide sites, may be due to the invasion of migrants with significantly different genotypes to a native population. Therefore, our data indicate highly differentiated common pine voles in Asia Minor and the Caucasus pine voles and Major's pine voles in the Caucasus, as well as active gene exchange between conspecific populations. No specimen was found to be heterozygous for the *IRBP* gene among the studied common pine voles from the East European Plain.

The *BRCA1* gene fragment allows clear distinction between the subgenus *Terricola* species because each of them is characterized by a number of fixed substitutions. The phylogenetic relationships of the species are similar to those that were established from the *cytb* gene. The average genetic distances between the species vary from 0.0049 to 0.0058. *M. majori* maximally differs from two other species of the subgenus and occupies the basal position in the ML and Bayesian trees before the outgroup (Figure 5, Figure S3). Differentiation in *M. majori* on the *BRCA1* gene is not apparent. A genetically uniform group of common pine voles from the East European Plain differs from a polymorphic sample from Asia Minor by one transition ($D = 0.0028$). Among common pine voles, a specimen from Samsun is maximally distant from other samples ($D = 0.0035–0.0047$). In *M. daghestanicus*, two clades with high statistic support stand out. The first clade includes three specimens (R1707-69, R1707-70, 10-88) from two neighboring sites in North Ossetia; genotypes of this clade are characterized by two transitions, which are absent from all the rest of the total vole sample. The second clade is represented by three specimens (10-2, 10-50, 10-51) whose genotypes contain three unique transitions; these specimens originate from two neighboring sites in Kabardino-Balkar Republic, Adyl-Su River valley. It is noteworthy that the difference between the clades in terms of average genetic distance ($D = 0.0049$) and the number of fixed substitutions (five) reaches up to the interspecific level. The rest of the Caucasus pine voles from Adyl-Su River valley, together with conspecific specimens from other localities, have

similar genotypes and compose a background group and one clade slightly separated from it. The results correspond to those obtained earlier (Bogdanov, Khlyap, et al., 2020). Therefore, not only interspecific differences but also significant intraspecific differentiation (in *M. subterraneus* and *M. daghestanicus*), and even an intrapopulation polymorphism (in *M. daghestanicus*) were revealed on the *BRCA1* gene, despite the fact that we analyzed a protein-coding fragment of the gene. An equally complex, "multilayered" variability of this gene was earlier determined in the house mouse *M. musculus* (Bogdanov, Maltsev, et al., 2020).

Of 34 voles of all species, 20 (about 60% of the total sample) were heterozygous for the *BRCA1* gene. In eight specimens (one common pine vole TRS-2 from Turkey and Caucasus pine voles 10-12, 10-59, 10-70, 26949, R1608-72, R1608-73, and TRD-1 from sites in the Greater Caucasus and Turkey), five to nine nucleotide sites were simultaneously heterozygous. One Caucasus pine vole (10-2) from Adyl-Su River valley was heterozygous for a three-nucleotide deletion. No individual was heterozygous for the *BRCA1* gene among the studied common pine voles from the East European Plain.

Analysis of the *XIST* gene sequences, including two separate fragments, exhibited maximal differentiation of *M. majori* from the other *Terricola* species (9–10 fixed substitutions, $D = 0.0148–0.0149$), as well as the closeness of *M. subterraneus* and *M. daghestanicus*, which can be distinguished by only one fixed transition ($D = 0.0076$). Within each species, the *XIST* gene appeared to be highly polymorphic, and in some cases its population variability was similar with minimal interspecific differences or even exceeded them. In contrast to the *BRCA1* and *IRBP* genes, a clear division between North Caucasian and Trans-Caucasian Major's pine voles (through two fixed transitions and one transversion, $D = 0.0033$) with high statistic support was revealed using the *XIST* gene (Figure 6, Figure S4), which confirms the results of the previous study (Bogdanov, Khlyap, et al., 2020). Genotypes of common pine voles from the East European Plain and western Turkey were quite close and grouped in one compact clade in dendrograms. However, a specimen from Samsun was so distinctive that a branch corresponding to it formed a star-like structure together with clades represented by *M. subterraneus* and *M. daghestanicus* samples. In the latter species, up to six clades or individual branches could be distinguished. Genotypes of Caucasus pine voles from two neighboring sites in North Ossetia, which formed one clade and an adjacent branch, are maximally distant from other *M. daghestanicus* samples ($D = 0.0043–0.0091$). The rest of the clades included Caucasus pine voles from different localities, i.e., any coincidence with the geographical origin of specimens cannot be traced.

Although the *XIST* gene is not protein-coding, heterozygosity on it was detected least often, likely due to localization of the gene on the X chromosome. Consequently, only females are able to possess heterozygous genotypes. Indeed, of 34 voles, only seven females (about 21% of the total sample) were heterozygous for the *XIST* gene: common pine vole 11–57, four Caucasus pine voles, 10-51, 10-62, R1608-73, R1707-71, and two Major's pine voles, 253, and 16-7 (Table 1). The number of nucleotide sites that were heterozygous

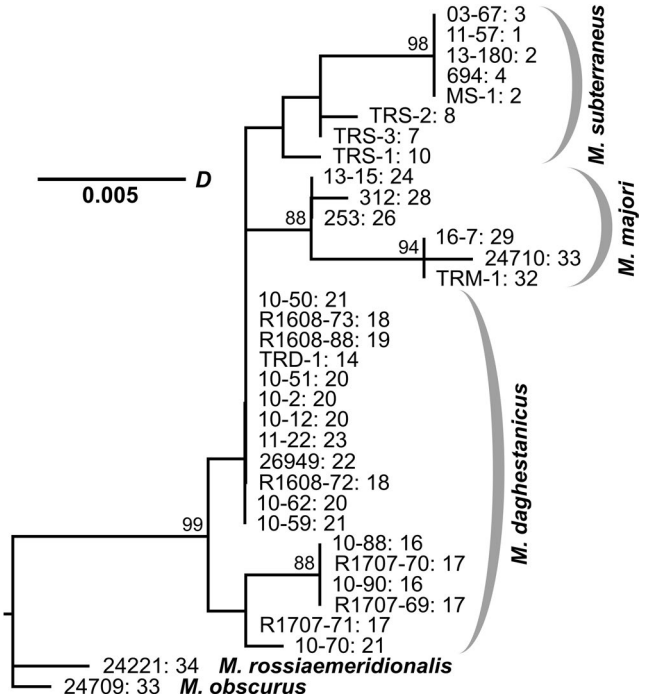

FIGURE 4 The ML dendrogram constructed from a comparison of the nuclear *IRBP* gene fragments (807 bp) of the subgenus *Terricola* pine voles, as well as *M. obscurus* and *M. rossiaemeridionalis* representatives. The collection numbers of the animals and, following the colon, the collection site numbers (see Table 1) are indicated to the right of the branches. Bootstrap index values exceeding 70% are indicated above the branching nodes of the dendrogram. *D*, genetic distance scale

for nucleotide substitutions did not exceed three per specimen. One Caucasus pine vole (R1707-71) from Verkhny Tsey village in North Ossetia was heterozygous for a four-nucleotide deletion and a specimen TRD-1 from Kars (Turkey), for a three-nucleotide deletion.

The compilation of nuclear DNA analysis data allowed us to conclude the following. Of three studied *Terricola* species, *M. majori* is more distant from the other two, which is more easily visible from the *BRCA1* and *XIST* genes. Differentiation of *M. majori* North Caucasian and Trans-Caucasian population groups, which are genetically quite close, is weakly traced. The high variability of all the nuclear genes in both *M. subterraneus* and *M. daghestanicus*, comparable with interspecific differences, blurs the boundary between these species. It is possible that future study of additional material will allow us to discover even more divergent genotypes. Populations of *M. subterraneus* in Asia Minor are characterized by maximal genetic polymorphism; among them, the population from Samsun is the most distant from the others. The gene pool of populations from the East European Plain is depleted, which is indicated by genotype similarity and rare detection of heterozygous specimens. In *M. daghestanicus*, a population from North Ossetia is distinctive, beyond doubt. Variability, which is observed among other populations of the species, does not clearly coincide with the geographical origin of the samples; together with an abundance of specimens, heterozygous on multiple nucleotide sites, this could indicate

an active exchange of individuals and genes between *M. daghestanicus* populations in Kabardino-Balkar and Karachaevo-Cherkess Republics. Intensive contacts may be proposed for North Caucasian *M. majori* populations and *M. subterraneus* populations in Asia Minor. All of the above was demonstrated by the ML and Bayesian dendrograms, which were reconstructed on united sequences of the three nuclear genes (Figure 7, Figure S5) as well as on all studied mitochondrial and nuclear markers (Figures S6 and S7). It is noteworthy that the trees are similar to those obtained from mitochondrial DNA analysis but reflect population differences in *M. daghestanicus* less distinctly due to the slower evolution of nuclear genes compared to mitochondrial genes.

4 | DISCUSSION

Using single genetic traits (i.e., genes, especially those that are insufficiently variable, a limited set of allozymes or karyotypic features that are applied separately from molecular markers) may significantly misrepresent estimates of phylogenetic relationships. Thus, according to our data, the *XIST* gene is not adequate to produce a reliable distinction between *M. daghestanicus* and *M. subterraneus*, due to the outlying position of the common pine vole from Samsun, and the *IRBP* gene, is inadequate in distinguishing among all three pine vole species. So, only a complex study using a number of genetic markers can be successful in researching the animal group. In general, analysis of one mitochondrial gene and three nuclear genes, as was performed in our study, confirms the specific status of *M. daghestanicus*, *M. subterraneus*, and *M. majori* as well the isolated position of the latter species from the other two, as was revealed in the majority of previous publications (Baskevich, 1997; Baskevich et al., 2018; Baskevich, Potapov, Khlyap, et al., 2016; Jaarola et al., 2004; Martínková & Moravec, 2012; Mezhzherin et al., 1995; Tougard, 2017). The result is in accordance with paleontological data and the ecological preferences of extant species of the subgenus *Terricola* and their ancestral fossil forms. Inhabiting broad-leaf forests, as found in the Mediterranean region, in middle and low altitude mountain ranges, and occasionally filtering into alpine meadows (i.e., archaic ecological specialization) are characteristic for both *M. majori* (Kryštufek & Vohralík, 2005; Tembotov & Khatoukhov, 1979) and representatives of the ancient genus *Allophaiomys* Kormos, 1932, which gave rise to pine voles (Chaline, 1987; Tougard, 2017). *M. daghestanicus* occupies the alpine meadow zone and *M. subterraneus* occurs in various (broad-leaf, mixed, and coniferous) forests of low mountain belts and flats as well forest-steppes, i.e., ecological niches that significantly differ from those of *M. majori* and the ancestral species. There is a high probability that ecological peculiarity was one of major factors that formed the unique pattern of

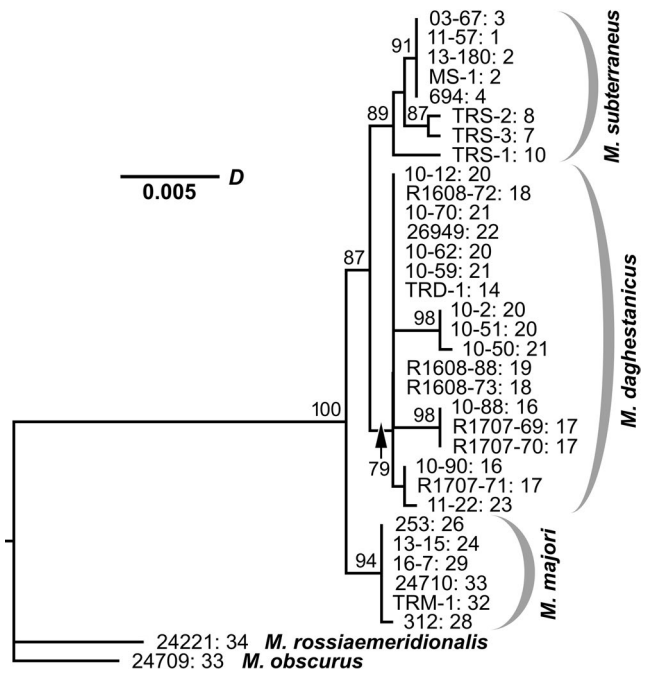

FIGURE 5 The ML dendrogram constructed from a comparison of the nuclear *BRCA1* gene fragments (1698 bp) of the subgenus *Terricola* pine voles, as well as *M. obscurus* and *M. rossiaemeridionalis* representatives. The collection numbers of the animals and, following the colon, the collection site numbers (see Table 1) are indicated to the right of the branches. Bootstrap index values exceeding 70% are indicated above the branching nodes of the dendrogram. *D*, genetic distance scale

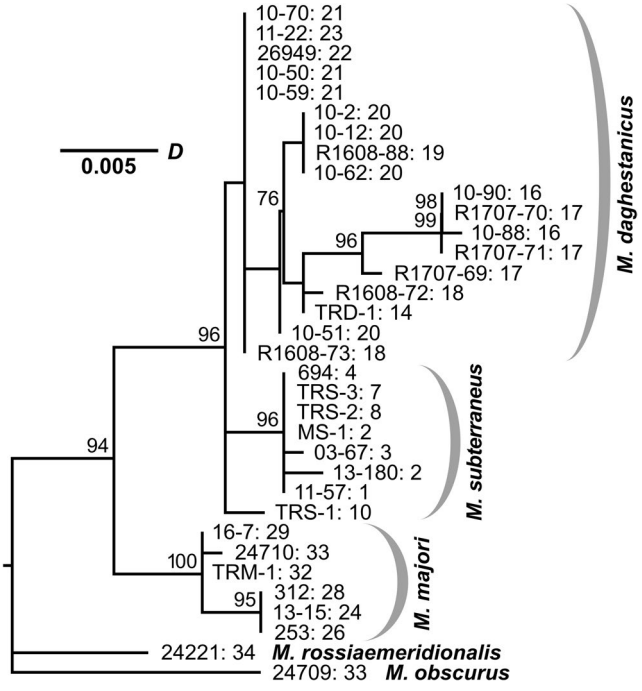

FIGURE 6 The ML dendrogram constructed from a comparison of two joined non-overlapping fragments (998 bp in total) of the nuclear *XIST* gene of the subgenus *Terricola* pine voles, as well as *M. obscurus* and *M. rossiaemeridionalis* representatives. The collection numbers of the animals and, following the colon, the collection site numbers (see Table 1) are indicated to the right of the branches. Bootstrap index values exceeding 70% are indicated above the branching nodes of the dendrogram. *D*, genetic distance scale

intraspecific genetic differentiation in each of the *Terricola* species, as proposed earlier for *M. daghestanicus* and *M. majori* (Bogdanov, Khlyap, et al., 2020).

Analysis of mitochondrial DNA and nuclear *XIST* gene has established that there are two closely related intraspecific forms, distributed on different sides of the Greater Caucasus Ridge, in the forest species *M. majori*. The Greater Caucasus, with abundant very high peaks, seems to be a formidable geographic barrier for *M. majori*, which has prevented migration and genetic exchange between the North Caucasian and Transcaucasian population groups of the species for a long time. It should be noted that the molecular genetic variability of *M. majori* does not coincide with its subspecific taxonomy. Considering fur color and body size, Gromov and Erbajeva (1995) distinguished the following subspecies of Major's pine vole: *Microtus majori majori* Thomas, 1906, which is distributed in the eastern part of the Pontian Mountains and in the western part of the Greater Caucasus, including the Black Sea coast; *Microtus majori ciscaucasicus* Ognev, 1924, inhabiting Transcaucasia and the northern foothills of western and central parts of the Greater Caucasus; and *Microtus majori suramensis* Heptner, 1948 from Transcaucasia, the southern slopes of the Greater Caucasus and central Greater Caucasus. Therefore, the distribution of each of the subspecies overlaps with both of the intraspecific forms that we have identified. *M. majori* subspecific taxonomy was in fact built based on morphs,

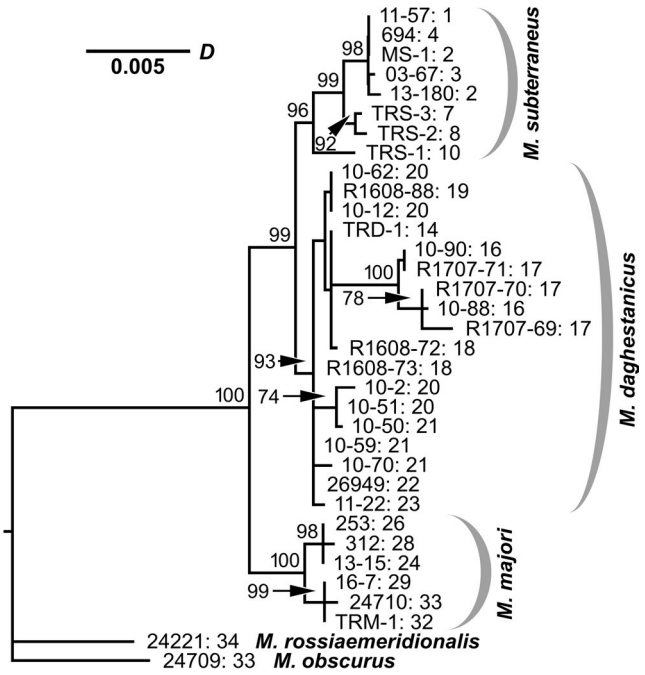

FIGURE 7 The ML dendrogram constructed from a comparison of joined fragments of all studied nuclear genes (3503 bp in total) of the subgenus *Terricola* pine voles, as well as *M. obscurus* and *M. rossiaemeridionalis* representatives. The collection numbers of the animals and, following the colon, the collection site numbers (see Table 1) are indicated to the right of the branches. Bootstrap index values exceeding 70% are indicated above the branching nodes of the dendrogram. *D*, genetic distance scale

which are often formed as results of adaptations to concrete environmental conditions in local areas. *M. majori* subspecies taxonomy needs to be revised using genetic data, which make it possible to determine population differences more accurately and to avoid species misidentification because of morphological similarity and the sympatry of *M. majori* and *M. daghestanicus*.

Although Caucasus pine voles from the territory where Robertsonian chromosome variability was revealed (southern regions of Armenia and Azerbaijan as well as Dagestan) were not involved in our work, the intraspecific molecular genetic variability of the total sample of *M. daghestanicus* appeared to be high and quite complicated. At least four intraspecific groups may be recognized in the species from the mitochondrial DNA and just two based on all the nuclear genes. Because we had no material from Georgia and were therefore not able to study nuclear genes for Georgian pine vole populations, we can still identify at least two significantly differing intraspecific groups in *M. daghestanicus*. One group includes populations from North Ossetia, and the other, genetically highly polymorphic group, the remaining populations, both from the Greater Caucasus and from northeastern Turkey. The presence of specimens with sharply differing genotypes or heterozygous on many nucleotide sites in populations may be due to active contacts between them inside a population group or, probably, both groups. In our opinion, such high population variability in *M. daghestanicus* is due to its ecological preferences, but in this case, mountain valleys with thick forests formed an isolation barrier, unlike the case of *M. majori*. Inhabiting high mountain ranges (alpine meadows) creates all the necessary prerequisites for the long-term isolation of Caucasus pine vole populations and, hence, the intensification of divergence processes, especially when forests were fully developed and extended across higher slopes. The most ancient fossils of representatives of the subgenus *Terricola* from Transcaucasia (Armenia) have been traced to the early Pleistocene (Agadzhanyan & Yatsenko, 1984), and recent species formation, according to different estimations, began about 0.8 Mya (Tougard, 2017) or even 0.235 Mya (Macholán et al., 2001). It can be argued that the most intensive phases of population divergence in *M. daghestanicus* fell in the interglacial Riss-Würm epoch of the Upper Pleistocene, about 0.13–0.115 Mya, and the Atlantic period of the Holocene, approximately 8000–4500 ya (Glushankova, 1998; Hewitt, 1996; Hewitt, 1999; Khotinsky, 1989; Velichko, 1993; Velichko, 1973). Aridization and cooling in the Upper Pleistocene, as well after the Atlantic period of the Holocene, may, conversely, have promoted a subalpine mountain belt expansion on account of the degradation of the forest belt and its shifting down slopes to the valleys. Under such conditions, contact between *M. daghestanicus* populations, which would have accumulated a number of mutations in their gene pools during their time of isolation, might be renewed. Ancestral polymorphism could also be the cause of genetic heterogeneity of the Caucasus pine vole samples. However, mosaic habitats, which are characteristic for *M. daghestanicus*, don't favor maintaining ancestral variability.

The subspecies taxonomy of the Caucasus pine vole has not been elaborated, and the very complicated pattern of its genetic population variability does not facilitate the task.

Compared to *M. daghestanicus* and *M. majori*, *M. subterraneus* is ecologically more flexible, allowing common pine voles to colonize a wide territory and various landscapes, both mountainous, and flat. However, the *M. subterraneus* range was fragmented. "Mosaicism" of the species range, together with significant population decline in unfavorable climatic epochs, seemed to be essential features in the history of *M. subterraneus*. Molecular genetic data, obtained by us and other authors (Jaarola et al., 2004; Macholán et al., 2001), indicate that the species survived the Pleistocene glacial epochs in some refugia in southern Europe (Balkan Peninsula) and Asia Minor because just these populations maximally differ both each from other and common pine voles from Eastern Europe, based on the *cytb* gene. Moreover, taking into account the very high variability of mitochondrial and nuclear genes in common pine voles from Asia Minor, the existence of several refugia in this territory, and even the origin of the species there, may be proposed, which agrees with the latest biogeographic information (Tougard, 2017). Nevertheless, study of additional material of *M. subterraneus*, both from Asia Minor and southern Europe, is necessary to check this hypothesis.

A depleted gene pool and very small population differences among common pine voles from the East European Plain indicate that the species colonized the area swiftly in the recent past, likely in the Atlantic period of Holocene, when broad-leaf forests were maximally developed (Glushankova, 1998; Hewitt, 1999; Khotinsky, 1989; Velichko, 1993; Velichko, 1973). Balkan Peninsula is the most likely postglacial colonization root for East European populations of *M. subterraneus*, like many other animal species (Taberlet et al., 1998). Low genetic polymorphism of the common pine vole populations from northeastern Europe was apparently due to the founder and/or bottleneck effects.

A comparison of molecular genetic and karyotypic polymorphism patterns exhibits weak coincidence only. As mentioned above, in karyotypes of common pine voles from central, southern, and southeastern Europe 52 chromosomes were revealed (Macholán et al., 2001; Mitsainas et al., 2010; Niethammer & Krapp, 1982; Sablina et al., 1989; Zima & Kral, 1984), specimens from the northern part of East Europe presented 54 chromosomes (Baskevich et al., 2007; Bulatova et al., 2007; Macholán et al., 2001; Sablina et al., 1989), and specimens from Asia Minor also presented 54 chromosomes, but with a different X chromosome morphology (Macholán et al., 2001). Dendrograms built using the results of *cytb* gene analysis demonstrated no clear division between the 52 chromosome form and the two 54 chromosome forms, either in the territory of the East European Plain where DNA variability in common pine voles is very low in total or in the Mediterranean region. Therefore, Zagorodnyuk's view (Zagorodnyuk, 1991) that common pine vole forms with 2n = 52 and 2n = 54 may be elevated to the species *M. dacius* and *M. subterraneus* s. str, respectively, is confirmed neither by our data nor the results of breeding experiments. According to the latter, the forms produced fertile and viable offspring and reproduction intensity was not decreased in following generations, including back-crossing (Meylan, 1972). The origin of two European chromosome forms, which in fact do not differ in molecular genetic markers, further corresponds to the principles of a theory of "sudden" evolution, with chromosome rearrangement as its initial stage (Vorontsov & Lyapunova, 1989). The question of which of the forms was ancestral and what chromosome rearrangement was responsible for the karyotype change is quite difficult. According to the conventional notion, karyotypic evolution, accompanied by chromosome number changes, more often happens by Robertsonian fusions of acrocentrics to metacentrics (Baker & Bickham, 1986; Bakloushinskaya, 2016; King, 1993); in this case, the 54 chromosome form from the northern part of Eastern Europe would be admitted as ancestral to the form with 2n = 52. Nevertheless, DNA analysis results obtained in this study indicate that *M. subterraneus* populations from Greece and Austria (2n = 52) are more ancient than the form with 2n = 54 from the northern part of Eastern Europe, which was recently colonized by the species. The origin of the East European form with 2n = 54 from Asia Minor common pine voles seems to be doubtful, as the populations, separated by the Caucasus and Transcaucasia, had no direct contact. Hence, the European 54 chromosome form more likely derived from the 52 chromosome form through fission of one metacentric pair into two acrocentric pairs. Compared to Robertsonian fusions, chromosome fissions are less often recorded, although they have been found in humans (Perry et al., 2004) as well as in several animal species and groups, including voles (Capanna & Civitelli, 1970; Fredga et al., 1980; Yosida, 1983; Perry et al., 2004; Baskevich, Khlyap, et al., 2016; Travenzoli et al., 2019; Singchat et al., 2020).

The converse interrelation of karyotype and gene evolution rates is observed in *M. subterraneus* populations in Asia Minor, which significantly differ in mitochondrial and nuclear DNA but belong to the same chromosome form.

Recognition of the Alpine *Microtus subterraneus incertoides* Wettstein, 1927 subspecies (terra typica: Austria, Tyrol, Gschnitztal that is very close to the locality 5 in our study) (Ellermann & Morrison-Scott, 1951) does not contradict the results of our study or others yet, but the association of genetically very heterogeneous Asia Minor populations with the same subspecies *Microtus subterraneus fingeri* Neuhäuser, 1936 (Kryštufek & Vohralík, 2005) seems to be doubtful. Moreover, it may be argued that a quite peculiar population from Samsun belongs to a new cryptic species. However, *M. subterraneus* subspecific taxonomy and taxonomical conclusions in relation to genetic forms of the species are premature at present. Resolution of these problems needs a complex study of the species throughout its range and discovery of the total spectrum of its genetic variability.

Thus, analysis of several genes of mitochondrial and nuclear DNA confirmed the genetic distinctiveness of *M. daghestanicus*, *M. subterraneus*, and *M. majori* as well as the most separation of the latter. Each of the species is characterized by a unique pattern and measure of polymorphism due to differences in their ecological preferences. Maximal intraspecific differentiation, comparable with interspecific differentiation, was determined within *M. daghestanicus* and *M. subterraneus*. The genetic variability of *M. daghestanicus* does not exhibit a clear relation with the geographic population distribution, based

on nuclear genes, with the exception of samples from North Ossetia. So significant genetic polymorphism in the species gives reason to believe that we have not completely described it. Analysis of additional material from little studied parts of the species ranges (primarily, the area of *M. daghestanicus* Robertsonian chromosome fan, as well as populations of *M. subterraneus* in southern Europe and Asia Minor) will promote the discovery of many hidden genetic forms and significantly supplement knowledge of pine vole differentiation.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Figure S1. Bayesian inference dendrogram constructed from a comparison of the entire mitochondrial *cytb* gene sequences (1143 bp) of the subgenus *Terricola* pine voles, as well as other vole species of the genus *Microtus*.

Figure S2. Bayesian inference dendrogram constructed from a comparison of the nuclear *IRBP* gene fragments (807 bp) of the subgenus *Terricola* pine voles, as well as *Microtus obscurus* and *Microtus rossiaemeridionalis* representatives.

Figure S3. Bayesian inference dendrogram constructed from a comparison of the nuclear *BRCA1* gene fragments (1698 bp) of the subgenus *Terricola* pine voles, as well as *Microtus obscurus* and *Microtus rossiaemeridionalis* representatives.

Figure S4. Bayesian inference dendrogram constructed from a comparison of two joined non-overlapping fragments (998 bp in total) of the nuclear *XIST* gene of the subgenus *Terricola* pine voles, as well as *Microtus obscurus* and *Microtus rossiaemeridionalis* representatives.

Figure S5. Bayesian inference dendrogram constructed from a comparison of joined fragments of all studied nuclear genes (3503 bp in total) of the subgenus *Terricola* pine voles, as well as *Microtus obscurus* and *Microtus rossiaemeridionalis* representatives.

Figure S6. The ML dendrogram constructed from a comparison of joined fragments of all studied mitochondrial and nuclear genes (4646 bp in total) of the subgenus *Terricola* pine voles, as well as *Microtus obscurus* and *Microtus rossiaemeridionalis* representatives.

Figure S7. Bayesian inference dendrogram constructed from a comparison of joined fragments of all studied mitochondrial and nuclear genes (4646 bp in total) of the subgenus *Terricola* pine voles, as well as *Microtus obscurus* and *Microtus rossiaemeridionalis* representatives.

Table S1. Average genetic distances (*p*-distances) calculated by

comparing sequences of the *cytb* gene (1143 bp) of different vole species.

Table S2. Average values of pairwise genetic distances, calculated by comparing sequences of the *cytb* gene (1143 bp) within a sample, an intraspecific group, or an entire species of pine voles.

Table S3. Genetic variability parameters calculated for united samples of the subgenus *Terricola* species, based on total sequences of the *cytb* gene.

Table S4. Average *p*-distance values and the number of fixed substitutions (in brackets) calculated by comparing fragments of three nuclear genes of the vole species.

Alignment S1. Alignment of all analyzed nucleotide sequences of the *cytb* gene.

Alignment S2. Alignment of all analyzed nucleotide sequences of the *BRCA1* gene.

Alignment S3. Alignment of all analyzed nucleotide sequences of the *IRBP* gene.

Alignment S4. Alignment of all analyzed nucleotide sequences of the *XIST* gene.

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APPENDIX 1 GenBank accession numbers for sequences of all genes used in the study (including published earlier sequences from the GenBank database)

Species ID	*cytb*	*BRCA1*	*IRBP*	*XIST* (f)	*XIST* (s)
Microtus subterraneus					
11-56	MZ198155				
11-57	MZ198156	MZ221997	MZ222031	MZ222065	MZ222099
11-59	MZ198157				
13-70	MZ198158				
13-146	MZ198159				
13-179	MZ198160				
13-180	MZ198161	MZ221998	MZ222032	MZ222066	MZ222100
MS-1	MZ198162	MZ221999	MZ222033	MZ222067	MZ222101
MS-2	MZ198163				
03-67	MZ198164	MZ222000	MZ222034	MZ222068	MZ222102
03-78	MZ198165				
03-153	MZ198166				
03-192	MZ198167				
694	MZ198168	MZ222001	MZ222035	MZ222069	MZ222103
696	MZ198169				
2	AY513833[a]				
1	AY513832[a]				
TRS-3	MZ198170	MZ222002	MZ222036	MZ222070	MZ222104
TRS-2	MZ198171	MZ222003	MZ222037	MZ222071	MZ222105
TRS-4	MZ198172				
TRS-1	MZ198173	MZ222004	MZ222038	MZ222072	MZ222106
3	AY513834[a]				
4	AY513835[a]				
Microtus daghestanicus					
2	AY513791[a]				
3	AY513792[a]				
TRD-1	MZ198174	MZ222005	MZ222039	MZ222073	MZ222107
1	AY513790[a]				
10-88	MZ198175	MZ222006	MZ222040	MZ222074	MZ222108
10-90	MZ198176	MZ222007	MZ222041	MZ222075	MZ222109
R1707-69	MZ198177	MZ222008	MZ222042	MZ222076	MZ222110
R1707-70	MZ198178	MZ222009	MZ222043	MZ222077	MZ222111
R1707-71	MZ198179	MZ222010	MZ222044	MZ222078	MZ222112
R1608-72	MZ198180	MZ222011	MZ222045	MZ222079	MZ222113
R1608-73	MZ198181	MZ222012	MZ222046	MZ222080	MZ222114
R1608-88	MZ198182	MZ222013	MZ222047	MZ222081	MZ222115
10-2	MZ198183	MZ222014	MZ222048	MZ222082	MZ222116
10-12	MZ198184	MZ222015	MZ222049	MZ222083	MZ222117
10-51	MZ198185	MZ222016	MZ222050	MZ222084	MZ222118
10-62	MZ198186	MZ222017	MZ222051	MZ222085	MZ222119
10-50	MZ198187	MZ222018	MZ222052	MZ222086	MZ222120
10-59	MZ198188	MZ222019	MZ222053	MZ222087	MZ222121
10-70	MZ198189	MZ222020	MZ222054	MZ222088	MZ222122

(Continues)

APPENDIX 1 (Continued)

Species ID	*cytb*	*BRCA1*	*IRBP*	*XIST* (f)	*XIST* (s)
26949	MZ198190	MZ222021	MZ222055	MZ222089	MZ222123
11-22	MZ198191	MZ222022	MZ222056	MZ222090	MZ222124
Microtus majori					
11-20	MZ198192				
11-26	MZ198193				
11-31	MZ198194				
13-15	MZ198195	MZ222023	MZ222057	MZ222091	MZ222125
13-19	MZ198196				
13-27	MZ198197				
13-53	MZ198198				
13-56	MZ198199				
453	MZ198200				
455	MZ198201				
252	MZ198202				
253	MZ198203	MZ222024	MZ222058	MZ222092	MZ222126
381	MZ198204				
312	MZ198205	MZ222025	MZ222059	MZ222093	MZ222127
16-7	MZ198206	MZ222026	MZ222060	MZ222094	MZ222128
MM388	DQ841704[b]				
–	AY513814[a]				
TU601	DQ841703[b]				
TRM-1	MZ198207	MZ222027	MZ222061	MZ222095	MZ222129
24710	MZ198208	MZ222028	MZ222062	MZ222096	MZ222130
Microtus obscurus					
24709	MZ198209	MZ222029	MZ222063	MZ222097	MZ222131
Microtus rossiaemeridionalis					
24221	MZ198210	MZ222030	MZ222064	MZ222098	MZ222132
Microtus agrestis					
–	AY167180[c]				
4	AY167187[a]				
Microtus oeconomus					
2	AY220018[a]				

ID = collection number of specimen. *XIST* (f) = first fragment of the *XIST* gene, *XIST* (s) = second fragment of the *XIST* gene. [a]Published earlier (Jaarola et al., 2004), [b]Published earlier (Martínková et al., 2007), [c]Published earlier (Jaarola & Searle, 2002).