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ANIMAL GENETICS

# Genetic Diversity of *Chionomys* Genus (Mammalia, Arvicolinae) and Comparative Phylogeography of Snow Voles

**A. A. Bannikova[a], A. M. Sighazeva[b], V. G. Malikov[c], F. N. Golenishchev[c], and R. I. Dzuev[b]**

[a] *Lomonosov Moscow State University, Department of Vertebrate Zoology, Moscow, 119991 Russia*
*e-mail: hylomys@mail.ru*

[b] *Kabardino-Balkarian State University, Department of General Biology, Ecology, and Nature Management, Nalchik, Kabardino-Balkaria, 360004 Russia*

[c] *Zoological Institute, Russian Academy of Sciences, St. Petersburg, 199034 Russia*

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**Abstract**—In the present study, the genetic polymorphism of the *Chionomys* genus was examined based on the sequencing of the mitochondrial *cytb* gene and two nuclear exons, including *GHR* exon 10 and *BRCA1* exon 11. The distinct subdivision of the genus of snow voles into five lineages, including *Ch. nivalis, Ch. gud, Ch. roberti,* and *Ch.* aff. *nivalis* from Turkey, as well as *Ch.* aff. *gud* from Turkey, was demonstrated. The branching order in the trees constructed based on the data for different genes was ambiguous, which was probably the consequence of recent and rapid radiation of the major lineages from a common ancestor. However, the data of the mitochondrial and nuclear gene analyses definitely indicated that the genetic and taxonomic diversity of the *Chionomys* genus was higher than it was expected before. The genetic divergence of some populations was so deep that they probably deserved the statuses of independent species. Despite that the range of the European snow vole *Ch. nivalis* is larger and more fragmented than the Gudaur vole *Ch. gud*, the latter species with its relatively small range, which is limited to the Caucasian and Pontic Mountains, was characterized by a similarly expressed phylogenetic structure. At the same time, Robert's vole *Ch. roberti* was less structured genetically than the first two species. The data obtained supported the Near Eastern, rather than the European origin of the *Chionomys* genus.

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## INTRODUCTION

The snow voles, genus *Chionomys* Miller, 1908, are represented to varying degrees by petrophilous forms mosaically distributed in the mountains of Europe, the Caucasus, Asia Minor, the Middle East, and the Near East. Phylogenetic relationships of snow voles have always remained disputable. According to the literature data, for a long time, snow voles were treated as the subgenus of *Microtus* [1–4]. At the same time, other authors recognized the snow voles as an independent genus in the Microtini Miller tribe in 1886 [5]. Various more recent data have confirmed that *Chionomys* represented a lineage that is considerably distant from other voles of the Microtini tribe [6–15]. However, no definite conclusion could be drawn based on the relationships between gray voles and snow voles on the mtDNA data because of the polytomy of *Microtus*, mostly determined by unstable position of *M. gregalis* Pallas, 1779 in the mitochondrial trees [16–18]. The data on nuclear genes supported the monophyly of the Microtini tribe (*Chionomys*, *Microtus, Blanfordimys* Argyropulo, 1933, *Lasiopodomys* Lataste, 1887), its early branching off within the genus *Arvicola*, and sister relationships of *Chionomys* and *Microtus* sensu lato [19]. Thus, the special closeness of certain Microtini species to snow voles is no longer a question. Due to the accumulation of morphological and molecular data on inter- and intraspecific variation of *Chionomys*, special attention is now focused on interspecific phylogenetic relationships in the genus, as well as to the phylogeographic structures of the species.

In *Chionomys* genus, three species are usually recognized, including *Ch. nivalis* Martins, 1842 (European snow vole); *Ch. gud* Satunin, 1909 (Gudaur snow vole); and *Ch. roberti* Thomas, 1906 (Robert's snow vole) [20]. According to established morphological concepts, these species are organized in two groups. The *nivalis* group includes *Ch. nivalis*, and the *roberti* group consists of *Ch. roberti* and *Ch. gud* [5, 11, 20–24]. *Ch. nivalis* Martins, 1842 inhabits subalpine and alpine mountain belts of Europe, Near East, Northwestern Caucasus, Transcaucasia, Asia Minor, Kopet Dag, and some mountain ranges of the Iranian Plateau. *Ch. gud* Satunin, 1909 is found in the Northern and Central Caucasus with relict fragments of the range in Pyatigor'e (Stavropol krai), Transcaucasia, and Asia Minor. *Ch. roberti* Thomas, 1906 is usually found in the forest and subalpine zones of the Caucasus and Asia Minor. In addition to the species described, a new species of snow vole from Zagros Mountains (Iran), *Ch. layi* Zykov, 2004 was described.

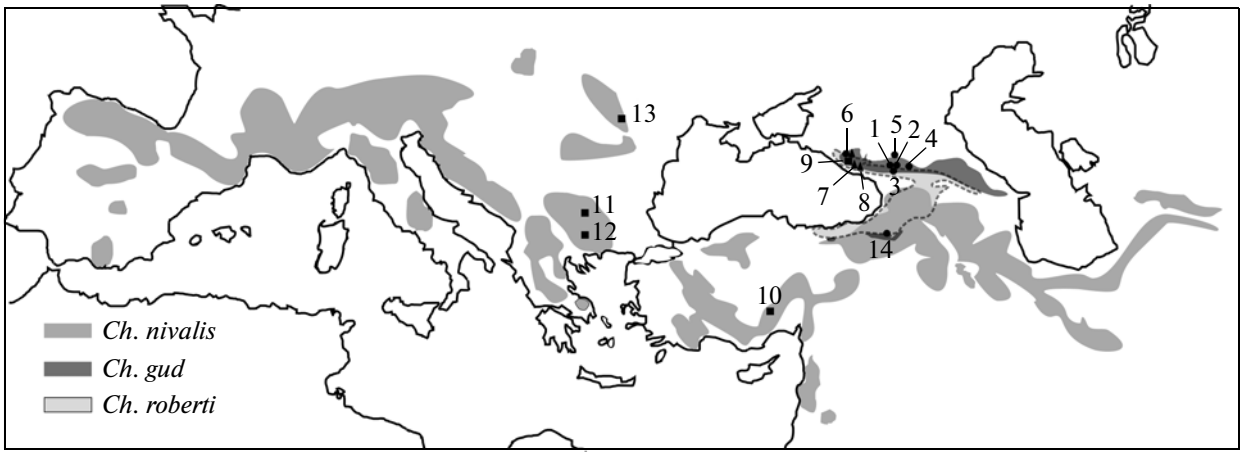


**Fig. 1.** Distribution of *Ch. nivalis, Ch. gud*, and *Ch. roberti* (according to [26] with modifications) and sampling localities (1–13). Squares are *Ch. nivalis*; circles are *Ch. gud*; triangles are *Ch. roberti.* Sampling locality of GenBank *Ch. gud* specimen from Turkey is also shown (14). Localities are numbered as in Table 1.

Based on morphological characters, Lay's snow vole, or the Zagros snow vole, belongs to the *nivalis* group [25]. Thus, according to the modern data, the center of genus diversity is located in the Caucasus–Asia Minor region, where all currently known forms are found [26]. Although the number of species recognized in the genus is small, for almost all of these species, from 2–3 to 25–26 subspecies were described [11]. The diversity of the group as a whole is extremely difficult to interpret systematically. The mosaic distribution of snow voles and complex and differently directed patterns of their morphological variation, along with the absence of representative samples from some peripheral populations, makes it difficult to establish the taxonomic status of the forms from the periphery of the range of genus distribution. It seems likely that some of these populations are rather ancient isolates that deserve certain interest with respect to the problem of intraspecific differentiation and speciation.

It should be noted that a great deal of data concerning intraspecific genetic variation and phylogeography of *Ch. nivalis* has been accumulated [27–29]. At the same time, the genetic diversity of *Ch. gud* and *Ch. roberti* remains much more poorly investigated. For all three species, the investigation of even single samples from the poorly studied territories of the Near East can considerably change the ideas on the genus volume.

The aim of this study was to analyze phylogenetic relationships in the *Chionomys* genus and the patterns of intraspecific variation based on the mitochondrial cytochrome *b* gene (*cytb*) and exons of two nuclear genes, *BRCA1* and *GHR*. In the comparative phylogenetic section of the study close attention was paid to the extended geographic sample of poorly examined species of *Ch. gud* and *Ch. roberti*.

## MATERIALS AND METHODS

### *Description of the Material, DNA Extraction, Amplification, and Sequencing*

The original sample consisted of 46 specimens of *Chionomys* snow voles from 13 localities (Fig. 1). In addition, we obtained sequences of the *BRCA1* exon 11 for *Arvicola amphibius, Clethrionomys glareolus, Clethrionomys glareolus, Eothenomys melanogaster, Microtus oeconomus,* and the *cytb* sequences for *Microtus arvalis* and *Microtus gregalis*, which were used as outgroup in phylogenetic trees. Most of voucher samples are stored at the Zoological Museum of the Lomonosov Moscow State University, Zoological Institute of the Russian Academy of Sciences, St. Petersburg, and in the collection of the Kabardino-Balkarian State University. A list of samples, geographical localities, museum-catalogue reference numbers, and symbols of the samples undergoing genetic analysis and their GenBank accession numbers are presented in Table 1.

In addition to the original material, 42 *cytb* sequences (AY513845 to AY513849; GQ150786 to GQ150788; GQ150791 to GQ150802; HQ901791, HQ901792, HQ901794 to HQ901807) and one *GHR* sequence (AM392378) of snow voles were taken from GenBank. The *cytb, BRCA1*, and *GHR* sequences of the other nine representatives of Arvicolinae used as the outgroup were also taken from GenBank (see Appendix).

Genomic DNA was extracted from ethanol-fixed muscle and liver samples using the standard method of phenol–chloroform deproteinization after the treatment of tissue homogenate with proteinase K [30].

The complete sequence of the mitochondrial *cytb* gene (1140 bp) was amplified with a combination of L14729/H15906arvic primers [31]. In the case of degraded DNA samples and in order to control the

**Table 1.** Description of original material used in the study

| Species | Geographic locality (number is given in brackets according to Fig. 1) | Sample code (according to Figs. 2–4) | Museum, catalogue reference number or specimen identification number | GenBank accession number | | |
|---|---|---|---|---|---|---|
| | | | | *cytb* | *BRCA1* | *GHR* |
| *Chionomys gud* | Kabardino-Balkaria, vicinity of the Mt. Elbrus, Narzan Valley 43°15′39″ N, 42°35′31″ E (1) | Ch gud 1-09 | KBSU S-3164 | JN244677 | JN244726 | JN244708 |
| | | Ch gud 2-09 | KBSU S-3165 | JN244678 | JN244721 | JN244709 |
| | | Ch gud 3-09 | KBSU S-3163 | JN244679 | – | – |
| | | Ch gud 4-09 | KBSU S-3166 | JN244680 | – | – |
| | | Ch gud 5-09 | KBSU S-3159 | JN244681 | – | – |
| | | Ch gud 6-09 | KBSU S-3160 | JN244682 | – | – |
| | Kabardino-Balkaria, vicinity of the Mt. Elbrus, Tegenekli 43°09′41″ N, 42°35′57″ E (2) | Ch gud 2-06 | ZMMU S-179565 | GQ352458 | – | – |
| | | Ch gud 3-06 | ZMMU S-179566 | JN244683 | – | – |
| | | Ch gud 4-06KB | ZMMU S-179567 | JN244684 | – | – |
| | | Ch gud 1/8-06 | KBSU S-2426 | JN244687 | – | – |
| | | Ch gud 1/27-06 | KBSU S-2427 | JN244686 | – | – |
| | | Ch gud 18-10 | KBSU S-3300 | JN244695 | JN244724 | JN244713 |
| | | Ch gud 19-10 | KBSU S-3299 | JN244696 | – | – |
| | Kabardino-Balkaria, vicinity of the Mt. Elbrus, Shkhelda 43°14′57″ N, 42°37′43″ E (3) | Ch gud 32-99 | ZMMU S-179565 | GQ352457 | – | – |
| | | Ch gud 42-99 | ZMMU S-180660 | JN244685 | – | – |
| | Kabardino-Balkaria, Bezengi 43°11′57″ N, 43°14′59″ E (4) | Ch gud 14-10 | KBSU S-453 | JN244692 | | |
| | | Ch gud 15-10 | KBSU S-2243 | JN244693 | JN244723 | JN244712 |
| | | Ch gud 16-10 | KBSU S-465 | JN244694 | | |
| | Stavropol krai, Zheleznovodsk, Razvalka Mountain 44°05′57″ N, 43°01′04″ E (5) | Ch gud M-07 | ZIN 100239 | GQ352460 | JN244727 | JN244714 |
| | | Ch gud 5-10 | KBSU S-3308 | JN244689 | JN244722 | JN244711 |
| | | Ch gud 6-10 | KBSU S-3269 | JN244690 | – | – |
| | | Ch gud 7-10 | KBSU S-3351 | JN244691 | – | – |
| | Adygea, Laganaki 44°00′06″ N, 40°14′59″ E (6) | Ch gud 4-06Ad | ZIN 100358 | GQ352463 | – | – |
| | | Ch gud 1-06 | ZIN 100359 | GQ352461 | – | – |
| | | Ch gud A | ZIN 100237 | JN244688 | – | – |
| *Chionomys roberti* | Adygea, Laganaki 44°00′06″ N, 40°14′59″ E (6) | Ch rob M-07 | ZIN 100234 | GQ352459 | – | – |
| | Adygea, Guzeripl 43°50′00″ N, 40°12′06″ E (7) | Ch rob 3-06 | ZIN 100236 | GQ352462 | – | – |
| | | Ch rob 1-10 | KBSU S-3303 | JX440343 | – | – |
| | | Ch rob 2-10 | KBSU S-3304 | JN244697 | JN244728 | JN244716 |
| | | Ch rob 3-10 | KBSU S-3211 | JN244698 | – | – |
| | | Ch rob 4-10 | KBSU S-3893 | JN244699 | – | – |
| | Abkhazia, Lake Ritsa 43°33′17″ N, 40°06′45″ E (8) | Ch rob 8-10 | KBSU S-3307 | JN244700 | JN244729 | JN244717 |
| | | Ch rob 9-10 | KBSU S-3305 | JN244701 | – | – |
| | | Ch rob 10-10 | KBSU S-3306 | JN244702 | – | – |
| | | Ch rob 11-10 | KBSU S-3206 | JN244703 | – | – |
| | | Ch rob 12-10 | KBSU S-3207 | JN244704 | – | – |
| | | Ch rob 13-10 | KBSU S-3392 | JN244705 | – | – |

**Table 1.** (Contd.)

| Species | Geographic locality (number is given in brackets according to Fig. 1) | Sample code (according to Figs. 2–4) | Museum, catalogue reference number or specimen identification number | GenBank accession number | | |
|---|---|---|---|---|---|---|
| | | | | *cytb* | *BRCA1* | *GHR* |
| *Chionomys nivalis* | Adygea, Caucasian Reserve, Abago Mountain 43°54′ N, 40′08′ E (9) | Ch niv 11-1 | ZIN 100617 | JX440341 | JX440344 | JX440339 |
| | | Ch niv 11-2 | ZIN 10618 | JX440342 | – | JX440340 |
| | | Ch niv 11-3 | ZIN 10619 | JX440342 | JX440344 | JX440340 |
| | Turkey, Central Taurus 37°43′17″ N, 35°10′53″ E (10) | ZIN98639 | ZIN 98639 | JN244707 | JN244731 | JN244718 |
| | Bulgaria, Vitosha Mountain 42°37′10″ N, 23°22′59″ E (11) | ZIN73190 | ZIN 73190 | GQ352464 | – | JN244719 |
| | | ZIN73189 | ZIN 73189 | JN244734 | – | – |
| | Bulgaria, Pirin 41°49′43″ N, 23°32′23″ E (12) | ZIN78540 | ZIN 78540 | JN244706 | – | – |
| | Eastern Carpathians, Breskul Mountain, 45°46′53″ N, 26°45′31″ E (13) | ZIN73183 | ZIN 73183 | GQ352465 | – | – |
| *Arvicola amphibius* | Tver oblast, Kolchevatiki | | ZMMU S-182598 | – | JX440345 | – |
| *Clethrionomys glareolus* | Chuvashia | | 20/11_15 | – | JX440346 | – |
| *Eothenomys melanogaster* | North Vietnam, Sa Pa 22°21′ N, 103°46′ E | | ZIN 96316 | – | JX440348 | – |
| *Microtus oeconomus* | Mongolia 48°17′15″ N, 99°47′50″ E | | ZMMU S-189125 | – | JX440347 | – |
| *Microtus arvalis* | Azerbaijan, Talysh, Lerik | | Ma_Leric2004 | GQ352469 | – | – |
| *Microtus gregalis* | Buryatia | | ZMMU S-182598 | GQ352466 | – | – |

Note: ZMMU is Zoological Museum of the Lomonosov Moscow State University; ZIN is Zoological Institute of the Russian Academy of Sciences, St. Petersburg; KBSU is Kabardino-Balkarian State University.

possible amplification of pseudogenes, two PCR reactions were performed that enable the generation of overlapping fragments. For these purposes, original primers Chi_L426_gud, Chi_H604_gud, Chi_L455_rob, Chi_H604_rob, Chi_L444_niv, Chi_H604_niv were designed. Primer sequences are demonstrated in Table 2. The amplification of *cytb* included 35 cycles and was carried out under the following conditions: denaturation at 94°C for 30 s, annealing at 60 to 62°C for 1 min, and extension at 72°C for 1 min. Initial denaturation was carried out for 3 min at 94°C and final extension was carried out for 6 min at 72°C.

**Table 2.** Primers designed for amplification and sequencing of *cytb* and *BRC1* exon 11 in *Chionomys* species

| Primer | Sequence (5'-3') |
|---|---|
| | *cytb* |
| Chi_H604_gud | gtc-cag-ttg-ggt-tgt-tag-atc-ctg-ttt-c |
| Chi_L426_gud | ggc-aac-agt-aat-tac-aaa-tct-tct-atc-agc |
| Chi_L455_rob | cca-tcc-cct-aca-tcg-gca-caa-c |
| Chi_H604_rob | gtc-cag-ttg-gat-tat-tgg-atc-ctg-ttt-c |
| Chi_H604_niv | gtc-cgg-ttg-ggt-tat-tgg-atc-ctg-ttt-c |
| Chi_L444_niv | cct-ctt-atc-agc-cat-ccc-ata-cat-cg |
| | *BRCA1* |
| F180_arv | cgg-aac-aga-tgg-gct-gaa-agt-aaa-g |
| R1240_arv | ggc-atc-tgc-tgc-agg-ttc-tgt-gt |

The tenth exon of the *GHR* gene was amplified with Chr_arv_F/Chr_arvic_R primers [19]. The amplification of the *BRCA1* exon 11 was carried out using a combination of original primers F180_arv/R1240_arv (Table 2). The amplification conditions for both nuclear genes were the same as for *cytb*, except for the annealing temperature (65°C).

The experiments were performed using the Bio-Rad and Eppendorf devices. Amplification products were tested in 1% agarose gel and precipitated with a mixture of ammonium acetate and 70% ethanol. Automated sequencing was carried out using an ABI 3100-Avant sequencer and ABI PRISM BigDye Ter-

minator kit v. 3.1 at the laboratory of the Genome Center for Collective Use.

### *Sequences, Phylogenetic, and Phylogeographic Analysis*

Sequences were aligned using the SeqManII module of the DNASTAR Lasergene9 software package and the ClustalW module of the BioEdit 7.0 software package with manual adjustment. The sizes of the final aligned sequences made up 910, 974, and 1140 bp for *GHR*, *BRCA1*, and *cytb*, respectively. In addition to complete sequences, an analysis of *cytb* also included fragments of about 1000 bp (mostly from GenBank) and three short fragments (426, 821, and 573 bp). The largest *Chionomys* sample used in the *cytb* analysis consisted of 88 specimens (46 original sequences and 42 GenBank sequences) from 33 localities of the genus distribution range.

The hypothesis on the nucleotide composition homogeneity was tested in the MEGA4 software program [32].

Phylogenetic analysis using maximum likelihood (ML) was performed in the Treefinder, v.October 2008 software program [33], while maximum parsimony (MP) and neighbor-joining (NJ) analyses were carried out as implemented in the PAUP* v.40b10 software program [34]. The clade robustness was assessed using the bootstrap analysis based on 1000 pseudoreplicates.

The ML reconstructions were preceded by determining the best-fit model of sequence evolution for each codon position using the Modeltest version 3.7 software program [35] based on the Bayesian information criterion (BIC). For each partition, which corresponds to three codon positions, a separate model of evolution was used. The models used for the *cytb*, the first and third codon positions were represented by TN+G and, for the second codon position, the models were represented by HKY+G. The best-fit model for the first and second codon positions of *BRCA1* and *GHR* was HKY, while for the third position of both nuclear genes, it was the HKY+G model. The data for nuclear genes were examined individually for each of the genes and for the concatenated sequence of two genes.

The MP analysis was conducted using equal weights for all substitution variants and the main options as follows: heuristic search, star=stepwise, addseq=random, nreps=20, swap=tbr, multrees=yes. NJ trees were reconstructed based on uncorrected *p* distance.

In addition, the relationships between the *cytb* haplotypes were examined using the method of median-joining network of haplotypes as implemented in the NETWORK v.4.5.0.0 software program [36]. For samples that consisted of more than five individuals, based on the *cytb* sequencing, the indices of haplotype ($H$) and nucleotide ($\pi$) diversity were calculated, and Tajima's $D$ and Fu's $F_s$ tests of neutrality were performed as implemented in the ARLEQUIN 3.11 software program [37].

## RESULTS

### *Sequence and Nucleotide Composition of cytb*

The original sequences used in the study represent the mitochondrial *cytb* gene; they are not pseudogene sequences, since they were generated as a result of reamplification with different primer systems and contained no abnormalities typical of nuclear copies of mtDNA [38, 39].

An analysis of the *cytb* nucleotide composition in *Chionomys* showed that *Ch. gud* differed from *Ch. roberti* and *Ch. nivalis* in the nucleotide frequencies at the third codon position. This conclusion was based on the data of pairwise haplotype comparison tests between *Ch. gud, Ch. roberti*, and *Ch. nivalis*, which denied the null hypothesis ($p < 0.05$) based on the consistency of nucleotide composition within the group. Compared to *Ch. nivalis, Ch. roberti*, and other species of the Arvicolinae subfamily, the nucleotide composition of the third codon position in *Ch. gud* was shifted toward a deficiency of cytosine (C was 30, 36, and 37% in *Ch. gud*, *Ch. nivalis*, and *Ch. roberti*, respectively), along with the excess thymidine (T was 24, 18.6, and 18.4% in *Ch. gud, Ch. nivalis*, and *Ch. roberti*, respectively). At the same time, the contents of guanidine and adenine were more balanced. In addition, the *Ch. gud* EU700087 and *Ch. nivalis* ZIN98639 samples from Turkey differed overall from the other *Chionomys* and Arvicolinae samples in their higher guanine contents (5.3 and 4.6%, respectively).

### *Phylogenetic Analysis of cytb*

The topologies of the trees reconstructed using three different phylogenetic algorithms (NJ, MP, ML) were basically similar. Alternative branching patterns concerning the divergence order of the main three species of the genus and of some intraspecific groupings had no statistically significant support. A maximal parsimonious (ML) tree constructed from the *cytb* sequences in the species of *Chionomys* is demonstrated in Fig. 2.

In all trees, haplotypes of the *Chionomys* genus formed three major groups. The groups had high bootstrap support and were separated from each other by considerable genetic distances. These groups corresponded to the three currently recognized species *Ch. gud, Ch. roberti*, and *Ch. nivalis.* Moreover, special attention should be paid to the two additional, well differentiated branches, represented by single samples. One of the branches is represented by a specimen from Ardahan in northwestern Turkey, which is deposed in GenBank (EU700087) under the name *Ch. gud.* Although this specimen is a sister to the *Ch. gud* sample from the Caucasus (bv: ML/MP/NJ = 98/97/87%), it is considerably distant from this sam-

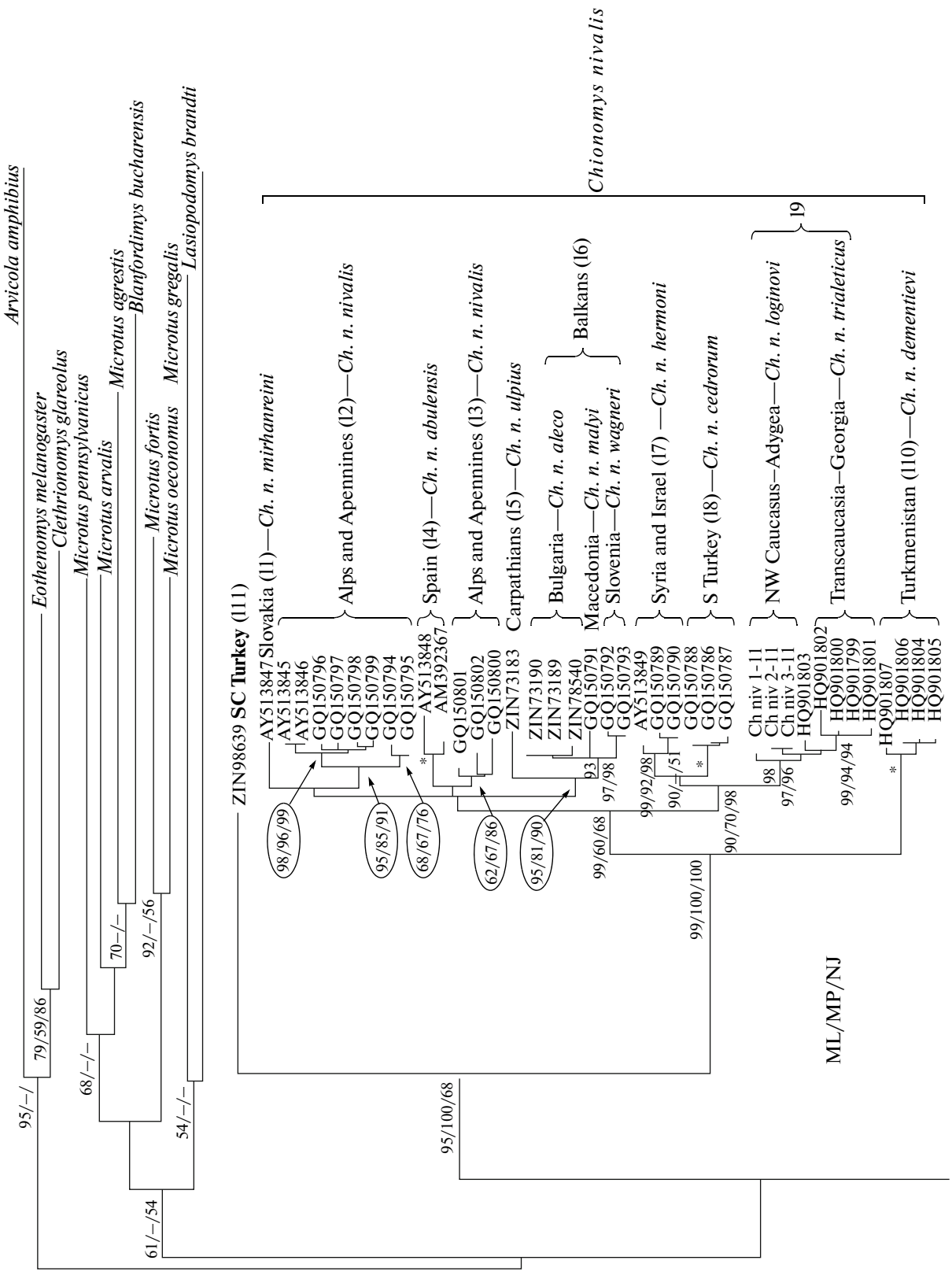


**Fig. 2.** ML phylogenetic tree of the *Chionomys* voles generated from the *cytb* data. Bootstrap values (≥ 50%, 1000 replications) in maximum likelihood (ML), parsimony (MP), and distance (NJ) analyses are shown at the nodes in the order of ML/MP/NJ or *ML/(MP/NJ)*; 100% bootstrap values in all analyses are designated by asterisks. *Arvicola amphibious*, *Eothenomys melanogaster*, *Clethrionomys glareolus*, and eight *Microtus* species were used as the outgroup.

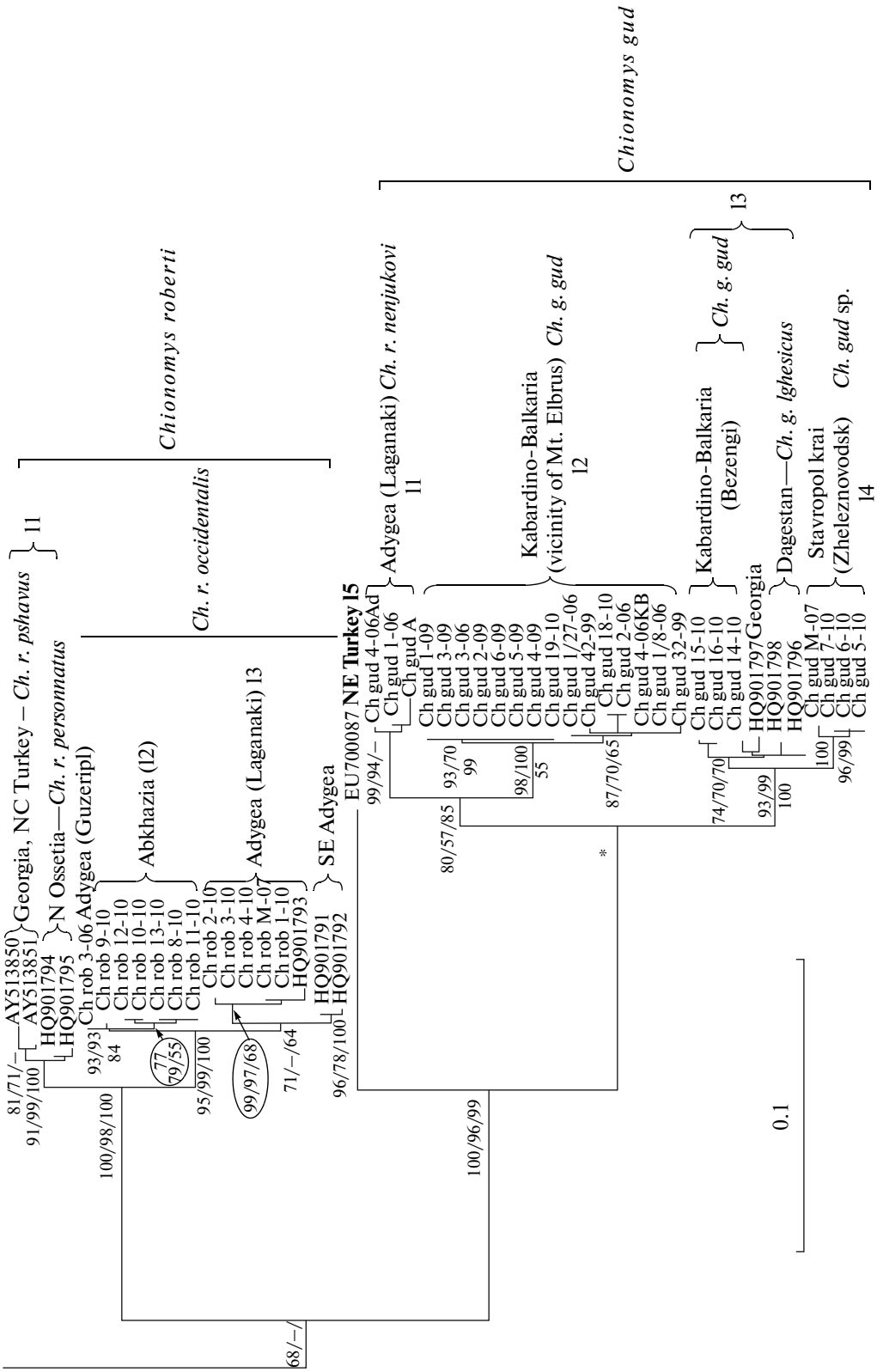


**Fig. 2.** Contd.

ple ($p$ distance = 10.80 ± 0.84). Another branch is formed by the specimen from the Central Taurus Mountain Range in southern Turkey. This specimen was morphologically diagnosed as *Ch. nivalis* (ZIN98639). The tree position of this specimen is clearly basal relative to all other samples of *Ch. nivalis*

**Table 3.** Genetic distance (% ± S.E.) between geographic populations of *Ch. gud* from the Caucasus and the mean values of intrapopulation genetic distances (below the diagonal, uncorrected *p* distance; above the diagonal, net distance; the mean values of intrapopulation genetic distances are shown in bold type above the diagonal)

| Populations | Near Mt. Elbrus ($n = 15$) | N. Caucasus (Bezengi) ($n = 4$) | Stavropol krai ($n = 4$) | Adygea (Laganaki) ($n = 3$) | Dagestan and Georgia ($n = 3$) |
|---|---|---|---|---|---|
| Near Mt. Elbrus | **0.39 ± 0.11** | 3.93 ± 0.52 | 4.12 ± 0.54 | 3.59 ± 0.60 | 3.80 ± 0.60 |
| N. Caucasus (Bezengi) | 4.50 ± 0.57 | **0.76 ± 0.20** | 1.54 ± 0.35 | 3.88 ± 0.60 | 3.80 ± 0.60 |
| Stavropol krai | 4.45 ± 0.57 | 2.05 ± 0.38 | **0.27 ± 0.10** | 4.27 ± 0.62 | 1.7 ± 0.42 |
| Adygea (Laganaki) | 4.20 ± 0.61 | 4.63 ± 0.64 | 4.78 ± 0.63 | **0.76 ± 0.30** | 4.50 ± 0.68 |
| Dagestan and Georgia | 4.20 ± 0.61 | 4.20 ± 0.61 | 2.0 ± 0.42 | 4.80 ± 0.68 | **0.42 ± 0.20** |

(ML/MP/NJ = 95/100/68%). The genetic distance that separates this specimen from the other specimens of *Ch. nivalis* averages to be 9.70 ± 0.79%, which two times higher than the maximum genetic distance within the species (4.70 ± 0.63%). The genetic distance between the individual from Central Taurus and the most distant sample from Kopet Dag is even higher, 10.4 ± 0.94%, which is consistent with the *p* distance between *Ch. roberti* and *Ch. nivalis* from Western Europe and Near East (10.45 ± 0.80%). It should be noted in this respect that the distances between the three known species of *Chionomys* constitute 10–12%, while the mean intraspecific mitochondrial distances (without considering the deviating Turkish samples ZIN98639 and EU700087) constitute 3.60 ± 0.50% (*Ch. gud*) and 1.78 ± 0.34% (*Ch. roberti*). Due to the isolated tree position and considerable genetic distances, which were much higher than the mean level of intraspecific differentiation of snow voles the specimens from Central Taurus and Ardahan (ZIN98639 and EU700087) were examined further outside of any species of *Chionomys*.

Attempts to unambiguously determine the order of clade divergence that corresponds to *Ch. gud, Ch. roberti*, and *Ch. nivalis* were unsuccessful. The *Ch. gud* and *Ch. roberti* grouped together only in maximum likelihood analysis (Fig. 2), albeit with low bootstrap support (bv = 68%). In distance analysis, the NJ tendency to group together was demonstrated only by *Ch. nivalis* and *Ch. roberti* (although with almost no support, bv = 45%). The parsimonious analysis did not support any of the topologies.

### *GHR Exon 10 and BRCA1 Exon 11*

The topologies of both nuclear trees reconstructed using three different phylogenetic algorithms (NJ, MP, ML) were basically the same. The summarized tree inferred from concatenated sequence of two nuclear genes and using the method of maximum likelihood (ML) is demonstrated in Fig. 3. The support values obtained in parsimonious (MP) and distance (NJ) analyses are demonstrated at the branches. In the tree, the three clades that correspond to *Ch. gud, Ch. roberti*, and *Ch. nivalis* were identified. *Ch. gud* forms sister group with *Ch. roberti* with moderate bootstrap support. The clades that correspond to *Ch. gud* and *Ch. roberti* were homogenous, while the specimens of *Ch. nivalis* from the Caucasus, Western Europe, the Near East, and Central Taurus in Turkey (ZIN98639) were substantially distant from each other.

### *Intraspecific Variations in mtDNA*

*Ch. gud.* In the *Ch. gud* sample examined ($n = 29$), a total of 17 *cytb* haplotypes were identified. These haplotypes formed five haplogroups with high bootstrap support. Leaving out the specimen from Ardahan, Turkey, three well-defined groups were identified. These groups clearly corresponded to different geographical localities of the Northern Caucasus (Fig. 2), i.e., Adygea (l1, locality 6 in Fig. 1), near Mt. Elbrus (l2, localities 1 to 3), and Zheleznovodsk (l3, loc. 5). Haplotypes from the Bezengi mountain area in the Central Caucasus (loc. 3) were mixed with haplotypes from Georgia and Dagestan, which form another moderately supported grouping l4. In the ML tree, this group was sister to the Zheleznovodsk group with high bootstrap support (93/99/100%). The largest sample from near Mt. Elbrus was heterogeneous and consisted of two subgroups of haplotypes, which, nevertheless, did not correlate with their geographic affiliation. Genetic distances between geographic populations of *Ch. gud* from the Caucasus, as well as interpopulation genetic distances are demonstrated in Table 3. The highest genetic distances were observed between the Adygea sample and other samples. Adygea and Bezengi samples were found to be the most heterogenous, while the Zheleznovodsk sample was the most homogenous. The sample from near Mt. Elbrus was characterized by a normal haplotype and nucleotide diversity ($H = 0.75$; $\pi = 0.0045 \pm 0.0033$). Based on Tajima's $D$ and Fu's $F_s$ tests, for this group, the null hypothesis on population stability could not be rejected. Median-joining haplotype network (Fig. 4a) supported the results of phylogenetic analysis using different algorithms with tree constructions and indicated the common origin of the Dagestani–Georgian and Bezengi haplotypes, and to the closeness of this

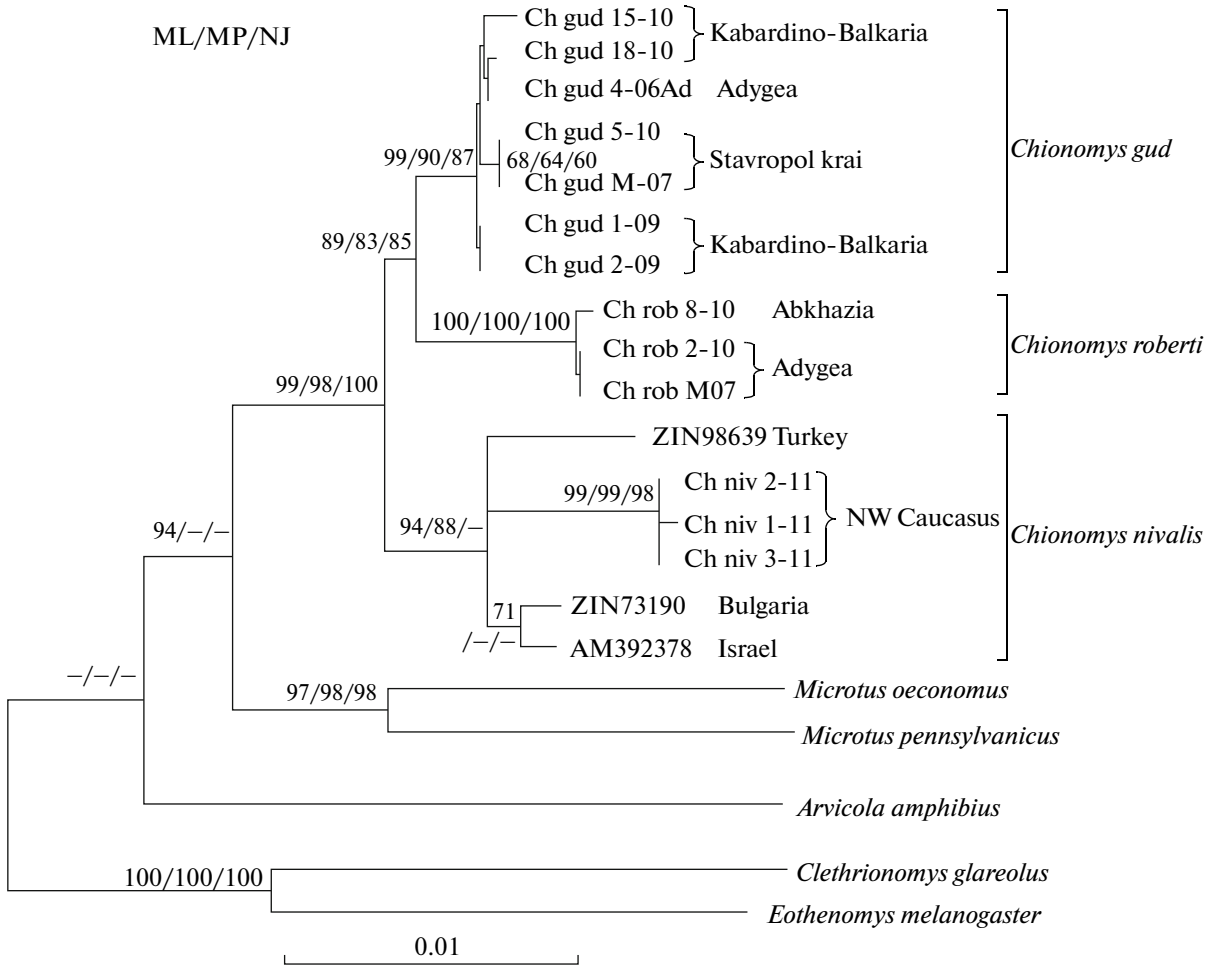


**Fig. 3.** ML phylogenetic tree of the *Chionomys* voles generated from concatenated sequence of 910 bp from the *GHR* exon 10 and 974 bp from the *BRCA1* exon 11. Bootstrap values (≥50%, 1000 replications) in maximum likelihood (ML), parsimony (MP), and distance (NJ) analyses are shown at the nodes in the corresponding order. *Arvicola amphibious*, *Eothenomys melanogaster*, *Clethrionomys glareolus*, *Microtus pennsylvanicus*, and *M. oeconomus* were used as outgroup.

haplogroup to that from Zheleznovodsk. On the contrary, the Adygei haplogroup, as well as that from vicinity of the Mt. Elbrus were isolated from the haplogroups mentioned above and from each other.

*Ch. roberti*. Among the 12 original and 6 GenBank *cytb* sequences of *Ch. roberti*, a total of 14 haplotypes were identified. The haplotypes were mainly grouped into the following geographical regions: North Ossetia, Georgia, and northern central Turkey (l1); Abkhazia (l2); Adygea–Laganaki (l3); and Adygea–Caucasian Reserve (Fig. 2). The first two haplogroups were monophyletic; their relationships were resolved using all three algorithms of phylogenetic analysis, and were highly supported in bootstrap analysis. The sample from Adygea was peculiar because it contained a clearly defined haplogroup from the Laganaki area (southwest of Adygea), while haplotypes from other Adygea localities did not form a monophyletic group, which demonstrates incomplete lineage sorting (Figs. 2 and 4b). Genetic distances between geographic samples of *Ch. roberti* are demonstrated in Table 4. The haplotype and nucleotide diversity of the total sample from Adygea ($n = 9$) constituted $H = 1.0 \pm 0.050$, $\pi = 0.0050 \pm 0.0030$, which was higher than the values of these indices obtained for the representative sample from the Abkhazian population ($H = 0.93 \pm 0.12$, $\pi = 0.0016 \pm 0.0012$).

*Ch. nivalis*. Unlike the two other species, phylogeography of *Ch. nivalis* is studied reasonably well [28, 29]. At present, taking into account the original and GenBank data (without deviating from the total group sample from Turkey (ZIN98639)), at least ten mitochondrial lineages can be distinguished (Fig. 2). These lineages are associated with different mountain systems, including the Tatra Mountains (l1), the Alps and Apennines (two clades, l2 and l3), the Pyrenees (l4), the Carpathians (l5), the Balkans (l6), mountain ranges of the Near East (l7 and l8), the Northern Caucasus and Transcaucasia (l9), and Kopet Dag (l10). The extension of the total sample (compared to the

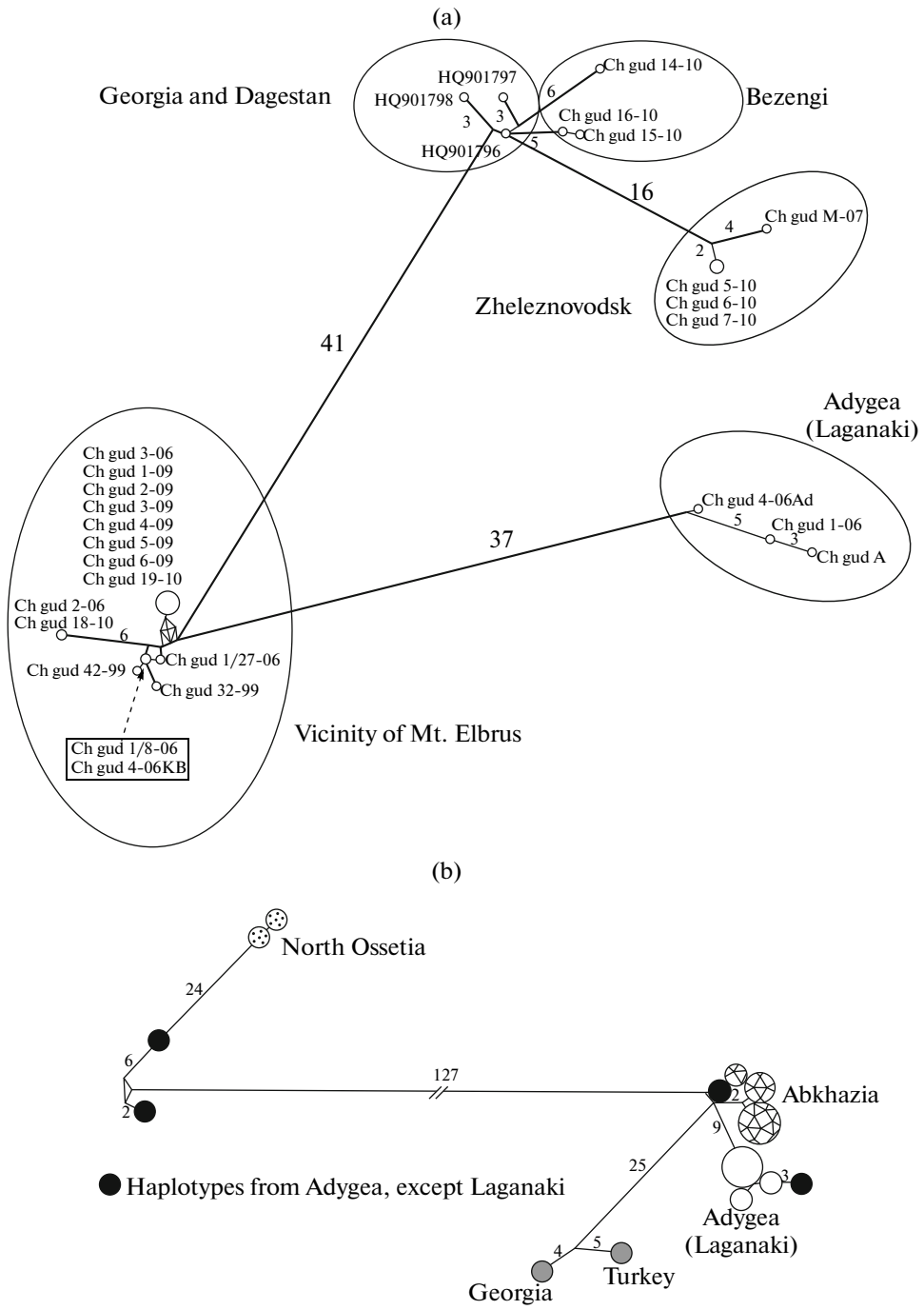


**Fig. 4.** Median-joining network of haplotypes. (a) *Ch. gud*; (b) *Ch. roberti*. Samples are coded as in Table 1. Circle sizes are proportional to number of specimens with given haplotype. Absolute numbers of nucleotide substitutions are shown on the lines that connect the haplotypes.

material used in [28]) showed that the Near Eastern haplotypes were grouped into one clade; however, with weak statistical support. At the same time, within this sample, the both specimens from Southern Turkey and specimens from Syria and Israel formed distinct monophyletic lineages (17 and 18). Conversely, the Caucasian branch (19) was strictly monophyletic, but less structured because neither Georgian, nor Adygei haplotypes formed monophyletic groups. In our study, the genetic data on the European snow vole were supplemented with haplotypes from Bulgaria (*Ch. n. aleco*), Eastern Carpathians (*Ch. n. ulpius*), and Northwestern Caucasus (*Ch. n. loginovi*). The genetic distance (net distance) between specimens that belong to different mountain systems (Pyrenees, Alps, Apennines, Carpathians, Caucasus, Pontic Mountains, Kopet Dag)

**Table 4.** Genetic distance (% ± S.E.) between geographic populations of *Ch. roberti* and the mean values of intrapopulation genetic distances (the designations are the same as in Table 3)

| Populations | Adygea ($n = 9$) | North Ossetia, Georgia, and northern central Turkey ($n = 4$) | Abkhazia ($n = 6$) |
|---|---|---|---|
| Adygea | **0.62 ± 0.14** | 2.47 ± 0.44 | 0.58 ± 0.15 |
| North Ossetia, Georgia, and northern central Turkey | 3.18 ± 0.48 | **0.80 ± 0.21** | 2.28 ± 0.43 |
| Abkhazia | 0.98 ± 0.21 | 2.77 ± 0.46 | **0.16 ± 0.08** |

varies from 1.22 ± 0.33% (Spain/Western Alps and Apennines) to 4.7 ± 0.63% (Kopet Dag/Caucasus).

Thus, in the three recognized species of *Chionomys*, three to ten haplogroups were identified. In most cases, these haplogroups corresponded to certain geographical localities and determined the presence of distinct phylogeographic structure in all species of the *Chionomys* genus.

## DISCUSSION

### *Interspecific Variation and Systematics*

Phylogenetic analysis of the mitochondrial *cytb* gene and two nuclear exons identified five distinct phyletic lineages in the examined snow vole sample. However, the divergence order of these lineages cannot be established. mtDNA and the combination of the *BRCA1* and *GHR* support grouping of *Ch. gud* + *Ch. roberti* and the earlier radiation of *Ch. nivalis* from a common ancestor. It should be noted that the described relationships are only a tendency because none of the given genes have received strong support for any topology. These findings suggest the rapid and almost simultaneous radiation of the three species. It seems likely that the revealed shift in the nucleotide composition of *Ch. gud* (towards a higher content of T and lower content of C compared to other species) also explains the polytomy of the species as a result of the effect of the *Ch. nivalis* and *Ch. roberti* branch attraction.

In addition to clades that correspond to the three known species of *Ch. gud*, *Ch. roberti*, and *Ch. nivalis*, the examined sample contained two more phylogenetic lineages. One of these lineages was represented by "*Ch. nivalis*" from the Central Taurus Mountain Range, Turkey (ZIN98639), which was considerably different from Western European snow voles. The second lineage was only present in the mitochondrial tree because it was represented by the GenBank sequence EU700087 obtained from the Ardahan specimen as described under the name *Ch. gud* [17]. Due to specific positions in molecular phylogenetic trees and considerable genetic differences, which are far above the mean intraspecific level, it has been suggested that the described specimens can belong to the independent species of *Chionomys* sp. 1 (ZIN98639) and *Chionomys* sp. 2 (EU700087). The discovery of genetically distant forms of snow voles on the territory of Turkey suggests that the genetic and taxonomic diversity of the genus is much higher than was expected before. It was previously considered that the center of origin and diversity of *Ch. gud* and *Ch. roberti* was the Caucasus and adjacent mountain areas [5, 11, 40], while the origin of *Ch. nivalis* was associated with Western European mountain systems, including the Alps, Carpathians, and Pyrenees [11, 28]. The appearance in the phylogenetic tree of snow voles of eastern Turkish branches that represent the forms basal to *Ch. gud*, as well as to *Ch. nivalis*, supports the recent suggestion that the origin of the whole *Chionomys* genus may be Near Eastern [29].

### *Intraspecific Variation, Phylogeography, and Subspecies*

*Ch. nivalis.* Based on the morphological characteristics, in the European snow vole, a total of 25 subspecies distributed across all mountain systems of Europe and Asia Minor have been described [11, 41]. For geographical forms from the territory of Iran, the subspecies assignment was not established, while for many other subspecies, the molecular genetic data are now available. Most of the mitochondrial lineages of the European snow vole are associated with certain geographical regions and mostly correspond to the previously identified subspecies (Fig. 2). For instance, the subspecies *Ch. n. mirhanreini* distributed in the Tatra Mountains is represented by a separate mitochondrial lineage from Slovakia; haplotypes of the two Italian lineages from Alps and Apennines belong to the nominative subspecies *Ch. n. nivalis*; the Balkan group of haplotypes belong to the subspecies of *Ch. n. aleco* Paspalev, Martino, Peshev, 1952, *Ch. n. malyi* Bolkay, 1925, and *Ch. n. wagneri* V. et. Martino, 1940 (from Bulgaria, Macedonia, and Slovenia, respectively); the Spanish haplogroup belongs to the subspecies *Ch. n. abulensis* Morales Agacino, 1936; and the haplotype from Transcarpathia represents the subspecies *Ch. n. ulpius* Miller, 1908. One of the lineages from the Near East includes the snow voles from Israel and Syria, which belong to the subspecies *Ch. n. hermonis.* Another lineage (Ciglikara, Turkey) corresponds to *Ch. n. cedrorum* Spitzenberger, 1973. A highly isolated lineage from Kopet Dag belongs to *Ch. n. dementievi* Miller, 1908. In our study, *Ch. nivalis* from the Caucasus was represented by a sample from Abago Mountain, Adygea, which corresponded to *Ch. n. longinovi*, Ognev, 1950.

The two haplotypes identified in three individuals from that sample were close to the Transcaucasian haplotypes that characterize another subspecies, *Ch. n. trialeticus* Shidlovsky, 1919. It is considered that the population from Central Taurus in Turkey corresponds to. This form is characterized by a tendency to complicate the third upper molar ($M^3$) and by elongated tail. The morphological specificity of this form is so high that it was initially described as *Ch. gud spitzenbergerae* Spitzenberger, 1971 [11]. *Ch. n. cedroum*, described from the Taurus Mountain Range, was characterized by the species-specific simplified $M^3$ (typica form) and a relatively short tail. However, due to the lack of external measurements of our specimen from the Taurus mountain range, its subspecies assignment remained unclear. In the case when the ZIN98639 specimen belongs to the form *spitzenbergerae*, its molecular genetic isolation from all other *Ch. nivalis* is even higher than its morphological specificity. The data on rapidly evolving mtDNA, as well as those on conservative exons of nuclear genes pointed to deep diversification of *Ch. nivalis*. At the level of nDNA, the specimens of Balkan, Caucasian, and Israeli mitochondrial lineages available were found to diverge from each other less than from the specimen from Central Taurus (0.3–0.8% versus 1.1–1.2%). At the same time, the divergence level of the latter specimen actually corresponds to the distance between *Ch. nivalis*/*Ch. roberti*/*Ch. gud*/*Ch. nivalis* (1.4/1.2/0.8/1.0%).

*Ch. gud*. The correspondence between subspecies and mitochondrial lineages in *Ch. gud* is more complex than in *Ch. nivalis*. A comparison of the subspecies and mitochondrial haplotypes distribution showed that nominative subspecies (*Ch. g. gud*) found in the central part of the Greater Caucasus Mountain Range includes two haplogroups, i.e., one near Mt. Elbrus and one at Bezengi. Moreover, these haplogroups are rather distant from each other, since the haplogroup of Bezengi groups, together with the specimens from Dagestan (*Ch. g. lghesicus*) and with high statistical support, forms a sister group with the haplotypes of the relict population from Razvalka Mountain, near Zheleznovodsk (the subspecies was not described for this part of the range). The Adygea haplogroup from the Laganaki area belongs to the subspecies from the western part of the Greater Caucasus Mountain Range, *Ch. g. nenjukovi* Formozov, 1931.

The populations from Bezengi and Zheleznovodsk are the most distant from the other examined samples. Moreover, the sample from Bezengi was the most heterogeneous, while the sample from Zheleznovodsk was the most homogeneous. The population of Gudaur snow vole from the Razvalka Mountain is the isolated peripheral grouping with a small number of individuals. It seems likely that this population often experiences the periods of decline in number, which explain the decrease in genetic diversity. On the contrary, the population from near Mt. Elbrus is located in the geographical center and ecological optimum of the species range; it communicates with the other populations, where the indices of haplotype and nucleotide diversity and the tests of neutrality indicate the long-lasting and stable existence of this grouping in the corresponding area.

*Ch. roberti*. It is currently considered that the majority of the Caucasus and Transcaucasia is inhabited by nominative subspecies of Robert's snow vole, *Ch. roberti roberti*. Furthermore, the morphological characteristics that served as the basis for isolating subspecies, such as *Ch. r. pshavus* Schidlovski, 1919 (Iori River, Northern Georgia), *Ch. r. personnatus* Ognev, 1924 (North Ossetia), and *Ch. r. occidentalis* Turov, 1928 (Mzymta River, Krasnodar krai), are nothing more than individual variations [41]; compared to morphological variations, their intraspecific genetic structure is somewhat more distinct. The subspecies *Ch. r. occidentalis*, which is represented in our study by the samples from Laganaki, Guzeripl, and other Adygei localities, is extremely genetically polymorphic and includes more than one mitochondrial lineage. According to our data, the subspecies *Ch. r. pshavus* and *Ch. r. personnatus* are the most diverged intraspecific forms of Robert's snow vole. Haplotypes from northern central Turkey, Georgia, and North Ossetia, which probably belong to these taxa, are noticeably distant from the haplotypes of North Caucasian populations, which indicates the possible recent origin of the latter populations. Interestingly, similar to the case with *Ch. gud*, the mitochondrial haplotypes of *Ch. roberti* from the Laganaki area in Adygea differ substantially from Central-Caucasian and Transcaucasian haplotypes.

It should be noted that the exact match of mitochondrial lineages to the subspecies identified based on morphological characters is not so common in animal phylogeography. For instance, five mitochondrial haplogroups of tundra shrew *Sorex tundrensis* ambiguously overlap with the subspecies taxonomic structure [42]. Four *cytb* lineages of *Microtus oeconomus* [43] include two to seven subspecies, the total number of which constitutes more than 15 [41]. The genetic variation of *Microtus fortis* is so low that it is impossible to differentiate morphologically recognized subspecies *M. f. michnoi* from Buryatia and *M. f. uliginosus* from Korea [18]. Compared to these examples, to a great extent, the structure of intraspecific morphological variations in species of *Chionomys* coincides with their phylogeographic structure, which indicates the contingency of morphological and molecular evolution, and makes it possible to consider the identified mitochondrial haplogroups to be phylogroups.

In general, comparison of the level of genetic differentiation in the *Chionomys* genus with that in other closely related Arvicolinae voles of the genus *Microtus* showed that some interpopulation distances (e.g., about 4.7% for *Ch. gud* and *Ch. nivalis*) were close to those determined in gray voles from the group of cryp-

tic species *Microtus longicaudus* [44], *M. savii* [45], *Microtus subterraneus*, and *M. agrestis* [16].

### *Phylogeography and Ecology*

A comparison of the intraspecific genetic variation in *Ch. nivalis, Ch. gud*, and *Ch. roberti* showed that, although the range of the European snow vole was larger and more fragmented, the Gudaur vole, which had a relatively small range limited to the Caucasian and Pontic Mountains, was characterized by similarly distinct phylogenetic structure. Among the three species, phylogenetic structure of *Ch. roberti* was most indistinct.

*Ch. gud* and especially *Ch. nivalis*, are adapted to the conditions of extreme aridity and increased insolation of open biotopes. It seems likely that, for this reason, the European snow vole could populate the arid and snowless Kopet Dag mountains and/or initially form in these conditions. Compared to strictly stenobiontic character of *Ch. nivalis* and *Ch. gud*, as petrophilous forms, Robert's snow vole is more flexible. *Ch. roberti* is characterized by the genus-maximum range of vertical distribution, which ranges from deciduous forest belt to upper subalpine limit [5]. *Ch. roberti*, which is obviously a petrophilous species, occupies more humid stations and can live in the conditions of high shadiness of the Caucasian coniferous–deciduous forests and tall grasses. To find refuge, these voles can use the trunks of fallen trees and dig simple pits in soft ground. Among the three species, Robert's snow voles are the most closely associated with mountain rivers and streams and they settle along the beds. It is known that, as a subaquatic form, Robert's vole is a good swimmer [46]. It is suggested that, due to the specific pattern of the *Ch. roberti* biotopic distribution, its distribution range is less fragmented, the gene flow between individual populations is heavier, and the phylogenetic structure is weaker than other snow vole species.

In general, in all snow vole species, the main role in the establishment of contemporary phylogenetic structure and population differentiation is played by the insular effect of the isolated mountain regions to which they are assigned, as well as by the stenobiontic character of the species. It seems likely that the widespread distribution of *Ch. nivalis* in the mountains of Western Europe and the Near East, but their sporadic presence in the Caucasus can be explained in terms of the inclination of this species to the conditions of increased aridity and insolation, which are missing in the Caucasus. On the contrary, the distribution of the *Ch. roberti* exclusively in the Caucasus and in the mountains of Turkey adjacent to the coastline of the Black Sea seems to be associated with the inability to pass over the waterless territories. The spread of *Ch. gud* to Western Europe was probably hampered by the success of *Ch. nivalis*.

Thus, extensive genetic diversification of snow voles is clearly seen at all taxonomic levels. As follows from the data obtained, due to the broad intraspecific genotype variations, the *Chionomys* genus includes more cryptic forms (possibly, of species rank) than is accepted in the group systematics.

At the same time, mitochondrial distance and/or reciprocal monophyly of mtDNA lineages can hardly be an absolute criterion of species recognition due to the limited information obtained from a single gene locus. The data on a few conservative nuclear genes can also be uninformative or contradictory. This determines the need to accumulate data on many nuclear loci and requires caution in the taxonomic interpretation of molecular genetic data with regard to the history of recently diverged forms [47].

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## APPENDIX

The GenBank sequences used in phylogenetic analysis as outgroup were as follows: *Arvicola amphibious* AF119269 (*cytb*), AM392380 (*GHR*); *Eothenomys melanogaster* AY426682 (*cytb*), AM392399 (*GHR*); *Clethrionomys glareolus* AM392368 (*cytb*), AM392384 (*GHR*); *Microtus pennsylvanicus* AF119279 (*cytb*), AM392376 (*GHR*), AY295009 (*BRCA1*); *M. agrestis* AF119271 (*cytb*); *M. oeconomus* FJ986325 (*cytb*), AM392388 (*GHR*); *M. fortis* AF163894 (*cytb*); *Lasiopodomys brandti* GQ352472 (*cytb*); *Blanfordimys bucharensis* AM392369 (*cytb*).

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