Organisms Diversity & Evolution (2022) 22:821–841
https://doi.org/10.1007/s13127-022-00559-7

ORIGINAL ARTICLE

Complete mitochondrial genomes of *Chionomys roberti* and *Chionomys nivalis* (Mammalia: Rodentia) from Turkey: Insight into their phylogenetic position within Arvicolinae

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Received: 5 November 2021 / Accepted: 1 April 2022 / Published online: 2 May 2022

Abstract

The complete mitochondrial DNA (mitogenome) sequences of *Chionomys nivalis* and *C. roberti* were first presented as reference mitogenomes by the current study using Long-Range PCR and Next-Generation Sequencing. The structure and organization of the circular mitogenomes were similar for each species. Each mitogenome included 22 tRNA genes, two rRNA genes, 13 protein-coding genes (PCGs), a control region (D-loop), and an origin of the light-strand region (OL), with the mitogenome lengths of 16.293 for *C. nivalis* and 16.300 for *C. roberti*. Phylogenetic analyses based on whole mitogenomes sequences allowed us to see better the relative position of the *Chionomys* within Arvicolinae. According to this, *Chionomys* was in a close phylogenetic relationship with *Microtus* rather than *Arvicola*. Contrary to the results of past studies, the relative positions of the species within *Chionomys* varied in the cytochrome *b* sequence-based phylogenetic analyses. Additionally, the presence of 10 genetic lineages determined by previous works within *C. nivalis* was approved. Among them, the Central Taurus lineage genetically was the lineage most distant from others. On the other hand, Turkish *C. roberti* specimens were clustered with the Transcaucasian specimens (Datvisi- Georgia and Alania-North Ossetia-Russia) splitting from other GenBank specimens of Russia and Georgia. Divergence-time analyses demonstrated that the first appearance of Arvicolinae dates back to the Early Pliocene (4.96 mya, 95% HPD: 4.09–5.82, BPP: 1), compatible with the past findings. Also, it was detected that the split of *Arvicola* / *Chionomys* + *Microtus* took place at the beginning of the Quaternary period (2.35 mya, 95% HPD: 1.97–2.75, BPP: 0.98). This corresponded to the Early Pleistocene divergence (approximately 2.4 mya) of these three genera suggested by the fossil record. In addition to this, molecular dating analyses demonstrated that the divergence between *C. nivalis* and *C. roberti* occurred in the Middle Pleistocene (1.06 mya, 95% HPD: 0.87–1.25, BPP: 1). Further studies using other molecular markers are needed to make definite judgments on the taxonomy and evolution of *Chionomys*.

Keywords Arvicolinae · *Chionomys* · Mitogenome · Phylogeny

Introduction

The current taxonomic status of the genus *Chionomys*, which comprises snow voles living in mountainous areas, has started to become clearer, leaving a somewhat complex past behind. The snow voles were identified by Miller (1908) as a genus under the name *Chionomys*. This taxon was for a long time considered to be a subgenus by authors such as Miller (1912), Corbet (1978), and Ellerman and Morisson-Scott (1966). However, *Chionomys* was considered a genus by many other authors (Aharoni, 1932; Gromov & Polyakov, 1992; Musser & Carleton, 1993), and therefore, the taxonomy of *Chionomys* has been controversial. Previous studies based on different markers, such as isozymes (Graf, 1982; Graf & Scholl, 1975), paleontological data (Chaline & Graf, 1988), dental traits (Nadachowski, 1991), morphological features (Gromov & Polyakov, 1992), and molecular data (Jaarola et al., 2004), have supported that *Chionomys* is a separate genus. Contrary to this, the more recent studies based on both mitochondrial and nuclear gene sequences and morphological data could not fully confirm the conclusion that *Chionomys* and *Microtus* are separate genera, in spite of the basal position of *Chionomys* in *Microtus* phylogeny, and thus the controversy

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continued. In one of two studies with similar content, it has been emphasized that these genera are sister taxa (Galewski et al., 2006), while in another study it has been suggested that *Chionomys* should be classified as separate genera to avoid more taxonomic complexity (Robovský et al., 2008). It has also been recommended that *Chionomys* be evaluated as a subgenus of *Microtus* in order not to cause more complex taxonomic problems (Yannic et al., 2012). Although the controversial situation regarding its taxonomy remains, ultimately there are many studies in which *Chionomys* is largely considered to be a separate genus (Bannikova et al., 2013; Chaline & Graf, 1988; Jaarola et al., 2004; Kryštufek & Vohralík, 2005; Mahmoudi et al., 2017; Nadachowski, 1991; Wilson & Reeder, 2005).

The genus *Chionomys* is represented by the *Chionomys gud*, *Chionomys nivalis*, and *Chionomys roberti* species. The members of the genus are mainly distributed in mountainous areas in Europe, Asia Minor, and parts of Western Asia. According to the previous phylogeographic scenarios, the snow vole species of the genus *Chionomys* were divided into two lineages, one of European and the another of Near Eastern or Caucasian descent. The European snow vole, *C. nivalis,* represents the European lineage within the genus, while the Robert's vole, *C. roberti*, and the Gudaur vole, *C. gud*, correspond to the eastern lineage. General phylogeographic patterns for *C. nivalis* have claimed this species has European origin and expanded towards the east from Europe in course of time (Bužan & Kryštufek, 2008; Castiglia et al., 2009; Kryštufek, 1999; Nadachowski, 1991). Evidence revealed by paleontological data, stating that this species had lived in Europe and also Asia Minor in the same time period, did not reinforce these suggested phylogeographic patterns (Kowalski, 2001). Contrary to the previously postulated phylogeographic scenarios, other phylogeographic models proposed by Yannic et al. (2012) and Bannikova et al. (2013) have introduced evidence demonstrating a Caucasian and Middle Eastern origin of the three species within the genus, and also of a subsequent westward expansion of *C. nivalis* from the east.

C. nivalis inhabits patchy habitats because of its habitat requirements (mountainous and rocky areas) and has the widest distribution range when compared to those of the other two species within the genus. Its geographic distribution extends from southwest Europe to the Caucasus, Turkey, Israel, Lebanon, Syria, and Iran (Kryštufek & Vohralík, 2005). This species has approximately 20 subspecies and at least ten allopatric lineages determined by morphological and molecular evaluations (Bannikova et al., 2013; Nadachowski, 1991; Yannic et al., 2012). It is mostly postulated that the emerging and evolution of *C. roberti* and *C. gud* occurred in the Near East or Caucasus. Both species are endemic to the Caucasus and the Eastern Black Sea Mountains (Kryštufek & Vohralík, 2005; Yannic et al., 2012). *C. gud* lives in the Russian, Georgian and, Azerbaijanian regions of the Caucasus, and in the Eastern Black Sea Mountains located in the northeast of Turkey. In Turkey, it was reported that this species was obtained from the same localities as *C. nivalis*, but that it lives in moister habitats (Kryštufek & Vohralík, 2005; Wilson & Reeder, 2005). *C. roberti* is found in the western Caucasus including Russia, Georgia, and Azerbaijan, and in the Eastern Black Sea Mountains in northeast Turkey. When compared to *C. roberti* and *C. gud*, which have limited distribution ranges within the genus, *C. nivalis* lives in a broader range in discontinuous habitats across the Palearctic region. Therefore, this species has been the focus of the previous studies modelling the comparative phylogeography and phylogeny of *Chionomys* (Bannikova et al., 2013; Castiglia et al., 2009; Yannic et al., 2012). In this context, intense efforts have been made to explain the phylogeny, phylogeography, and molecular characterization of this species. On the other hand, the number of studies on the intraspecific diversity within *C. roberti* and *C. gud* is few, and they are mostly based on morphology (size differences, differentiation in the fur color, cranial differences, and the enamel pattern of molar teeth) and karyology (Kryštufek & Vohralík, 2005 and references therein; Sözen et al., 2009; Arslan & Zima, 2014). There are very few studies using mitochondrial DNA (mtDNA) sequences of *C. roberti* and *C. gud* (Bannikova et al., 2013; Yannic et al., 2012). Therefore, molecular data belonging to *C. roberti* and *C. gud* is scarce. Other studies presenting molecular data have included a limited number of specimens, and in terms of content, they are studies mostly aimed at phylogenetic inference at the genus level, molecular genotyping, or presenting new molecular data of these species (Balakirev et al., 2018; Bužan & Kryštufek, 2008; Jaarola et al., 2004). Hence, the phylogenetic relationships among the three snow voles and the inferences concerning their phylogeography have remained controversial and uncertain.

Nowadays, phylogenetic studies revealing the history of particular organismal evolution are mostly based on determining the variations in specific genetic systems (DNA in the nucleus or in organelles) borne by that organism. One of those genetic systems is the mitochondrial DNA (mtDNA or mitogenome) of the organism. (Avise, 1986). This genetic system has been commonly and easily characterized at the molecular level for a long time. The mammalian mitogenome is about 16.5 kb in length and has been used as an important microevolutionary phylogenetic marker due to some of its properties, including maternal inheritance, rapid evolution level, widespread intraspecific variation, and absence of intermolecular genetic recombination (Avise, 2000). Previous studies intended to determine the evolutionary history, genetic diversity, and phylogenetic relationships of mammalian species or population genetics have mostly employed sequences of a single or a couple of genes or non-coding

region of the mitogenome (Avise, 1987; Irwin et al., 1991; Pool et al., 2010). Although these studies have presented acceptable perspectives for the inference of evolutionary backgrounds of organisms, molecular studies in the last decade have started to include whole-genome sequencing of the organisms due to the development of technologies (Next Generation Sequencing, NGS) and their affordability (Pool et al., 2010). Moreover, molecular systematics principally aims to build a species tree rather than a gene tree in order to avoid the errors arising from incomplete lineage sorting. The only method to reduce errors in the constructed species tree is to increase the number of genes used. (Tateno et al., 1982). Therefore, it is clear that whole genome sequencing can offer more options for coming up with solutions to complex problems in population genetics and phylogenetic studies, rather than partial sequencing of the coding or non-coding regions in the mitogenome (Li et al., 2016; Yuan et al., 2016). In this context, and given the aforementioned situation, the whole mitogenome sequencing has started to be extensively used in phylogenetic studies including those of mammalian species from different families (Ding et al., 2019; İbiş, 2020; İbiş et al., 2020; Krause et al., 2008; Mitra et al., 2019; Wada et al., 2010). However, the whole mitogenome sequences of the snow vole species within the genus *Chionomys* are still unavailable. In addition, the available partial mitogenome sequence data of the *Chionomys* species may not be sufficient on its own to reveal the more precise phylogeographic and phylogenetic relationships among the species of this taxon. Of course, the determination of mitogenome sequences of *Chionomys* species will also be useful for revealing phylogenetic relationships with those genera closely related to this genus.

The current study aimed to: i) obtain complete mitogenome sequences of *C. nivalis* and *C. roberti* by means of Long-Range PCR and Next Generation Sequencing; ii) characterize the mitogenome organization of two species as a reference genome; and iii) contribute to inferring phylogenetic relationships and phylogeography within the genus *Chionomys*.

Material and methods

Sampling, DNA extraction, sequencing, and mitogenome assembly

This study used already existing tissues from samples of *C. nivalis* and *C. roberti*. *C. nivalis* samples from Kars (N40°47′00′′—E43°00’,00′′, one sample), Ardahan (N41°29′00′′—E42°41′00′′, one sample) and Niğde provinces (N37°26′00′′—E34°37′00′′, one sample), and *C. roberti* samples from Rize (N40°51′48.36″—E40°56′06.59″ and N41°00′04.68″—E41°03′25.37″, two samples) and Trabzon (N41°41′50.62″—E39°39′13.71″, one sample) provinces in the northeast of Turkey had been collected in previously performed fieldwork. The IUCN Red List Category and Criteria of both species is Least Concern (LC). Muscle tissues belonging to the specimens were used in the molecular studies. The DNeasy Blood and Tissue Kit (QIAGEN) was used for the extraction of the genomic DNA (gDNA), following the manufacturer's protocol. The Qubit® 3.0 Fluorometer (Thermo Fisher Scientific) and Qubit™ dsDNA BR Kit (Thermo Fisher Scientific) were used for the determination of the quantity of the gDNA. To check the quality of the gDNAs, 10 µL of gDNA was electrophoresed in 1% agarose gel and visualized under UV light. The whole sequences of the mitogenomes were gained from the gDNAs by means of Long-Range PCR with NEB LongAmp® Taq 2×Master Mix (M0287S, New England Biolabs). Two overlapping fragments (~11 kb and ~7.1 kb in length) were acquired. The Long-Range PCR mixture contained 1×NEB LongAmp® Taq 2×Master Mix (12.5 µL), 0.6 µM of each primer (1.5 µL of each 10 µM primer), ~30 ng of gDNA (1 µL) and 8.5 µL ddH_2O. The PCR conditions included a pre-denaturation at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C (CrocAL1-2024 LCrocBH1-13002H) and 55 °C (ScVu-11712L-LuLu-2503H) for 45 s, and extension at 65 °C for 11 min (CrocAL1-2024L-CrocBH1-13002H) and 7.5 min (ScVu-11712L-LuLu-2503H), respectively (İbiş et al., 2020). The final extension step was carried out at 65 °C for 10 min. Negative control (no gDNA) was included in the PCR run to determine possible contamination. 5 µL of the PCR product was run on 1% agarose gel, and 4 µL of the PCR product was used to determine the DNA concentration with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The standardized PCR products were diluted to 0.2 ng/ µL with ddH_2O; 5 µL of PCR product (1 ng) was reserved for the sequencing library.

Data preparation and phylogenetic analyses

PCR products were used to construct the sequencing libraries by using the Nextera XT DNA Library Prep Kit (FC-131–1096, Illumina, San Diego, USA) and Nextera XT DNA Library Preparation Index Kit v2 Set A (FC- 131–2001, Illumina, San Diego, USA), following the manufacturer's protocols. Bead-based normalization was utilized for the normalization of the quantity of each library. Qubit dsDNA HS Kit was used for determining the loading volume and concentration of the library. Sequencing of the library was carried out by using MiSeq Reagent Kit v2 (500 cycles) (Illumina, San Diego, USA) and Illumina MiSeq platform (Illumina, San Diego, USA) at Genome and Stem Cell Center, GENKOK, Erciyes University.

Geneious Prime® V2021.0.3 software (Kearse et al., 2012) was used for the analysis of the obtained raw sequences (average 200–230 bp in length, ~ 600.000–800.000 reads for each sample). Trimming and quality filtering operations comprised short reads (< 50 bp), low-quality bases (Q-score < 25), and adapters in raw sequence, which were executed using BBDuk Trimming Tool in the Geneious Prime software. Thereafter, Geneious Mapper Algorithm was used to assemble the resultant reads to the mitogenome of *Microtus arvalis* (NC_038176) (Sensitivity: Highest sensitivity/Medium, Fine Tuning: Iterate up to 25 times, S1). Contig sequences were acquired, and annotations of the genes were carried out. Furthermore, the border of the genes was controlled via MITOS2 software (Bernt et al., 2013) and manually curated. For the determinations of the repeated sequence motifs in the control region (D-loop), the Tandem Repeats Finder Web Server was employed (Benson, 1999). The AT Skew and GC Skew analyses were estimated according to the formulas [A-T] / [A + T] and [G-C] / [G + C], respectively.

For phylogenetic analysis, two different data sets were used; the first set included the sequences of whole protein-coding sequences (PCGs) minus control region (D-loop) of the mitogenomes, the second set consisted of the cytochrome *b* sequences of the samples. The mitogenome data set was used to reveal the phylogenetic position of the genus *Chionomys* within Arvicolinae, while the *CYTB* dataset was used to see better the relative position of Turkish *Chionomys* species in the intrageneric phylogeny of *Chionomys*. Additional mitogenome (S2) and cytochrome *b* sequences (S3) obtained from NCBI were also used in the analyses. The whole mitogenome and cytochrome *b* sequences of *Cricetulus griseus* and *Mesocricetus auratus* downloaded from the NCBI were also included in the analyses as outgroup sequences (S2 and S3). Alignments of both data sets were performed by the MAFFT, a multiple sequence alignment algorithm (Katoh et al., 2002). The ambiguous regions within the mitogenome dataset were masked with Gblocks v0.91b (Castresana, 2000) using default parameters. Both the mitogenome and *CYTB* datasets were used for the two model-based phylogenetic analyses; Maximum Likelihood (ML) implemented in MEGA X (Kumar et al., 2018), and Bayesian Inference (BI) executed in BEAST v1.8.0 (Drummond et al., 2012), were used to evaluate evolutionary relations and divergence-time dating. According to the Bayesian Information Criteria (BIC) using jModeltest 2.1.10 (Darriba et al., 2012), the GTR + G + I for the mitogenome data set and the HKY + G + I for the *CYTB* dataset were determined to be the best nucleotide substitution models. A nonparametric bootstrap (1000 replicates) and the Bayesian Posterior Probability (BPP) tests were performed to appraise the reliability of constructing tree topologies. For dating divergence time, the Yule tree prior was selected and a strict molecular clock was used. One independent MCMC (Markov Chain Monte Carlo) for 10,000,000 generations with a sampling frequency of every 1000 generations was executed in BEAST v1.8.0 (Drummond et al., 2012). Tracer v1.6 (part of the BEAST package) was used to check whether the ESS > 200 (the lower bound of effective sample size) or not in the independent run. The acquired tree file containing 10,000 trees was summarized by TreAnnotator v1.8.0 (part of the BEAST package) by removing the initial 10% (1000 trees) of the sampled trees as burn-in and a 50% majority-rule consensus tree was constructed to calculate the BPPs and divergence times of the tree nodes. As a calibration point, the divergence time of *Arvicola*/*Microtus*, alleged to have occurred 2.4 million years ago (mya) with the standard deviation of 0.2, was taken into account (Chaline & Graf, 1988; Yannic et al., 2012). The tree constructed from the BI analysis was visualized and modified by FigTree v1.4.0 (part of the BEAST package). The overall DNA polymorphism in the mitogenomes was estimated by MEGA X (Kumar et al., 2018). The genetic distance estimations between the lineages of *C. nivalis* were calculated using the Kimura-2 parameter (K2P) model with 1000 bootstrap replicates implemented in MEGA X (Kumar et al., 2018).

Results

1. Organizations of *C. nivalis* and *C. roberti* mitogenomes

Whole mitogenomes of *C. nivalis* and *C. roberti* were provided by the current study, with ~ 6.500 ×—~ 10.000 × mean coverages. It was detected that the mitogenomes of both species from each locality bore unique haplotypes (GenBank accession numbers: OK323269, OK323270, OK323271, OK323272, OK323273, and OK323274). The length of the complete mitogenomes for two samples belonging to *C. nivalis* was 16.293 bp, whereas the complete mtDNA mitogenomes for three samples of *C. roberti* were 16.300 bp in length. The pattern of the whole mitogenomes for both species was the same as previously determined mitogenomes of other mammalian species, and they were composed of 22 tRNA genes, 2 rRNA genes, 13 protein-coding genes (PCGs), a control region (D-loop), and an origin of the light-strand region (O_L). It was detected that the *ND6* and 8 *tRNA* genes ($tRNA^{Gln}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Ser2}$, $tRNA^{Glu}$, and $tRNA^{Pro}$) localized on the light chain (L) of the mtDNAs of two species. However, the O_L origin, D-loop, 12 protein-coding genes (PCGs), 14 tRNA, and 2 rRNA genes were coded on heavy chain (H) (Fig. 1).

The mitogenomes of both species possessed 8 overlapping regions, with a total of 63 bp in length (between 1 and 43), and 15 intergenic spacers, a total of 30 bp in

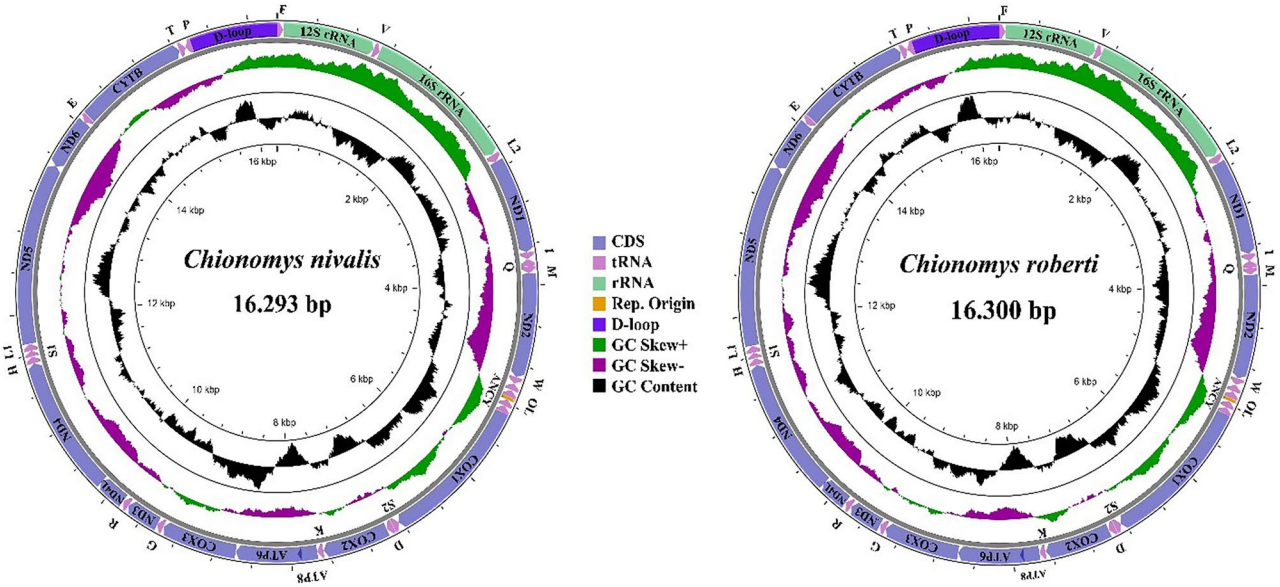


Fig. 1 Circular mitogenome map of *C. nivalis* (1778_Kars) and *C. roberti* (1775_Trabzon). The map shows the 13 protein-coding, two rRNAs, 22 tRNAs genes and Dloop- O_L regions in the mtDNA genome of the *Chionomys* species

length (between 1 and 5). It was seen that the open reading frame of the *ND1* gene started with the GTG triplet, whereas *ND2* began with ATC, *ND3* with ATT/ATA, and *ND5* with ATA/ATT triplets in both mtDNAs. On the other hand, it was detected that most of the PCGs (9 genes, 69.2%) started with the ATG triplets. TAA was identified as the most common stop codons found in a total of 9 of the 10 PCGs, which were terminated with the full termination triplets. However, the *ND5* gene ended with the TAG triplet. Additionally, it was determined that the *ND1*, *COXIII*, and ND4 genes ended with the T- incomplete termination codon (Table 1).

2. Nucleotide contents

Most parts of the mitogenome of the *C. nivalis* were constituted by protein-coding genes (PCGs), which were a total of 11.391 bp in length and with a percentage of 69.90% of the whole mitogenome. Also, the total length and the proportion of PCGs in the structure of the mitogenome of *C. roberti* were nearly the same as that of *C. nivalis* except for small proportional differences, which can be ignored (11.391 bp and 69.90%). The mitogenomes of the two species had the typical mammalian mtDNA features in terms of the nucleotide composition and content of the entire genomes. They were richer in A + T content than G + C, and the frequency of the guanine was low (A > T > C > G). The lowest A + T content was in the PCGs and the highest was in the *tRNA* genes for the two species. The skew analysis, showing the replication origin with the leading strand tending as G-biased and T-biased, demonstrated that A/T skew was positive and G/C skew was negative for both species (Table 2).

3. tRNA genes, rRNAs, non-coding regions, and origin of replication for the light strand (OL)

The mitogenomes of the two species included 22 tRNA genes (1.497 bp length in *C. nivalis*, and 1498 bp length in *C. roberti*) whose length ranged from 59 bp ($tRNA^{Ser1}$) to 75 bp ($tRNA^{Leu2}$). Also, $tRNA^{Thr}$ (67 bp in *C. nivalis* and 69 bp in *C. roberti*) had different sequence lengths in the two species (Fig. 2). The length of the *12S* and *16S rRNAs* was different in the mtDNAs of the two species. While these genes were respectively 949 bp and 1563 bp in length in the mtDNA of *C. nivalis*, they were 948 and 1565 in length in that of *C. roberti*. Both of the rRNA genes in the mtDNAs belonging to *C. nivalis* and *C. roberti* held a rich A + T content in accordance with the rich A + T content previously observed in the mtDNAs of other mammalian species. The non-coding component of the mtDNA, known as the control region or D-loop, was between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes. The length of this region was 896 bp in the mtDNA of *C. nivalis,* while that of *C. roberti* was 901 bp in length. In terms of A + T content, this region was the second richest region after *tRNA* genes in the mitogenomes of both species (61.8% in *C. nivalis* and 60.9% in *C. roberti*). For both species, the O_L region, which is a small non-coding region corresponding to the replication for the light strand, was a length of 30 bp and between $tRNA^{Asn}$ and $tRNA^{Cys}$ (Tables 1 and 2).

Table 1 Mitogenome organization of *C. nivalis* (1778_Kars) and *C. roberti* (1775_Trabzon). Characters in bold are differences between the mitogenomes (S: strand, I.S.: intergenic spaces, ni: *C. nivalis*, ro: *C. roberti*)

Gene	S	Position	Size (bp)	Codon			I.S
				Start	**Stop**	**Anticodon**	
		ni/ro	**ni/ro**	**ni/ro**	**ni/ro**	**ni/ro**	**ni/ro**
tRNA^{Phe}	H	1–66	66	-	-	GAA	2
12 s (s)rRNA	H	**69–1.017 / 69–1.016**	**949 / 948**	-	-	-	0
tRNA^{Val}	H	**1.018–1.084 / 1.017–1.084**	**67 / 68**	-	-	TAC	0
16S(l)rRNA	H	**1.085–2.647 / 1.085–2.649**	**1.563 / 1.565**	-	-	-	1
tRNA^{Leu2}	H	**2.649–2.723 / 2.651–2.725**	75	-	-	TAA	0
ND1	H	**2.724–3.678 / 2.726–3.680**	955	GTG	T–	-	0
tRNA^{Ile}	H	**3.679–3.747 / 3.681–3.749**	69	-	-	GAT	-3
tRNA^{Gln}	L	**3.745–3.816 / 3.747–3.818**	72	-	-	TTG	2
tRNA^{Met}	H	**3.819–3.887 / 3.821–3.889**	69	-	-	CAT	0
ND2	H	**3.888–4.922 / 3.890–4.924**	1.035	ATC	TAA	-	1
tRNA^{Trp}	H	**4.924–4.990 / 4.926–4.992**	67	-	-	TCA	1
tRNA^{Ala}	L	**4.992–5.060 / 4.994–5.062**	69	-	-	TGC	2
tRNA^{Asn}	L	**5.063–5.132 / 5.065–5.134**	70	-	-	GTT	2
O_L	H	**5.135–5.164 / 5.137–5.166**	30	-	-		-1
tRNA^{Cys}	L	**5.164–5.231 / 5.166–5.233**	68	-	-	GCA	0
tRNA^{Tyr}	L	**5.232–5.298 / 5.234–5.300**	67	-	-	GTA	1
COXI	H	**5.300–6.844 / 5.302–6.846**	1.545	ATG	TAA	-	-3
tRNA^{Ser2}	L	**6.842–6.910 / 6.844–6.912**	69	-	-	TGA	3
tRNA^{Asp}	H	**6.914–6.981 / 6.916–6.983**	68	-	-	GTC	1
COXII	H	**6.983–7.666 / 6.985–7.668**	684	ATG	TAA	-	3
tRNA^{Lys}	H	**7.670–7.733 / 7.672–7.735**	64	-	-	TTT	0
ATP8	H	**7.734–7.937 / 7.736–7.939**	204	ATG	TAA	-	-43
ATP6	H	**7.895–8.575 / 7.897–8.577**	681	ATG	TAA	-	-1
COXIII	H	**8.575–9.358 / 8.577–9.360**	784	ATG	T–	-	0
tRNA^{Gly}	H	**9.359–9.426 / 9.361–9.428**	68	-	-	TCC	0
ND3	H	**9.427–9.774 / 9.429–9.776**	348	**ATT/ATA**	TAA	-	1
tRNA^{Arg}	H	**9.776–9.842 / 9.778–9.844**	67	-	-	TCG	3
ND4L	H	**9.846–10.142 / 9.848–10.144**	297	ATG	TAA	-	-7
ND4	H	**10.136–11.513 / 10.138–11.515**	1.378	ATG	T–	-	0
tRNA^{His}	H	**11.514–11.582 / 11.516–11.582**	**69 / 67**	-	-	GTG	0
tRNA^{Ser1}	H	11.583–11.641	59	-	-	GCT	-1
tRNA^{Leu1}	H	11.641–11.710	70	-	-	TAG	0
ND5	H	11.711–13.522	1.812	**ATA / ATT**	TAG	-	-4
ND6	L	13.519–14.043	525	ATG	**TAA / TAG**	-	0
tRNA^{Glu}	L	14.044–14.112	69	-	-	TTC	5
CYTB	H	14.118–15.260	1.143	ATG	TAA	-	2
tRNA^{Thr}	H	**15.263–15.329 / 15.263–15.331**	**67 / 69**	-	-	TGT	0
tRNA^{Pro}	L	**15.330–15.397 / 15.332–15.399**	68	-	-	TGG	0
D-loop	H	**15.398–16.293 / 15.400–16.300**	**896 / 901**	-	-	-	0

4. Protein-coding genes and codon usage

Relative synonymous codon usage (RSCU) rates were shown in Fig. 3. The total codon frequency for *C. nivalis* (1778 Kars) and *C. roberti* (1775 Trabzon) was 3796. For *C. nivalis*, the three amino acids with the highest frequency in the total codon were Leu (582), Isoleucine (369), and Threonine (304), while the three amino acids with the lowest frequency were Cysteine (31), Arginine (64), and Aspartate (72). The more detailed analysis emphasized that CUA, AUC, and AUU were the most frequently used codons and that the three codons less

Table 2 Nucleotide composition and Skew analysis of the mtDNAs belonging to the *C. nivalis* (1778_Kars) and *C. roberti* (1775_Trabzon)

	Species	Size (bp)	% A	% T	% G	% C	% A + T	% G + C	AT-Skew	GC-Skew
Whole mtDNA	*C. nivalis*	16.293	33.1	27.3	13.4	26.3	60.4	39.6	0.09	–0.32
	C. roberti	16.300	33.0	27.2	13.5	26.3	60.2	39.8	0.10	–0.32
PCGs	*C. nivalis*	11.391	32.2	27.7	11.9	28.2	59.9	40.1	0.07	–0.40
	C. roberti	11.391	32.1	27.5	12.2	28.3	59.6	40.4	0.08	–0.40
tRNA genes	*C. nivalis*	1.497	35.2	28.5	15.6	20.7	63.7	36.3	0.10	–0.14
	C. roberti	1.498	35.2	29.0	15.4	20.4	64.2	35.8	0.0	–0.14
rRNA genes	*C. nivalis*	2.512	36.9	23.7	18.0	21.3	60.6	39.4	0.22	–0.08
	C. roberti	2.513	36.9	23.5	18.2	21.4	60.4	39.6	0.22	–0.08
D-loop	*C. nivalis*	896	30.9	30.9	14.0	24.2	61.8	38.2	0.001	–0.27
	C. roberti	901	30.7	30.2	13.8	25.3	60.9	39.1	0.009	–0.29

likely to be represented were CGG, AAG, and GCG. The most frequent initiation codon in the PCGs of the *C. nivalis* mitogenome was ATG (76.92%). For *C. roberti*, the three amino acids with the highest frequency in the total codon were Leu (578), Isoleucine (372), and Threonine (304), while the three amino acids with the lowest frequency were Cysteine (31), Arginine (64), and Aspartate (72). The more specific analysis highlighted that CUA, AUC, and AUA were the most frequently used codons and that the three codons less likely to be represented were CGG, ACG, and CCG. The most frequent initiation codon in the PCGs of the *C. roberti* mitogenome was ATG (69.23%) (Fig. 3).

5. Phylogenetic analyses and divergence-time dating

A total of 15,030 bp PCG sequences of complete mitogenomes were analyzed for BI, ML, and divergence-time dating analyses. The data set comprised 7295 variable sites, of which 6295 were parsimony informative and 1000 were singletons. Two different topologies were reconstructed by ML and BI because of the variability of positions of *Arvicola and Ellobius* (Figs. 4 and 5). The monophyletic position of a group consisting of four genera, *Microtus*, *Lasiopodomys*, *Neodon*, and *Proedromys*, was strongly supported by BPP:1 in BI and the bootstrap values: 100% in ML phylogenetic reconstructions. *Microtus* was paraphyletic compared to *Lasiopodomys*, *Neodon*, and *Proedromys* in this group in both trees. *Chionomys* was clearly monophyletic with the support of BPP:1 in the BI and the bootstrap values; 100% in the ML trees. This taxon was obviously in a close phylogenetic relationship with the *Microtus,* and it was more distant from *Arvicola* in the BI and ML trees. The position of *Arvicola* in both phylogenetic trees was variable. While *Arvicola* was in the most basal position in the ML tree (with low bootstrap value), it was included as an outgroup of the *Microtus-Lasiopodomys-Neodon-Proedromys* group plus *Chionomys* in the BI tree (BPP: 0.99). On the other hand, the four genera, *Myodes*, *Prometheomys*, *Dicrostonyx*, and *Ondatra* clustered as monophyletic groups supported with high BPP:1 and relatively moderate or lower bootstrap values. However, *Neodon*, *Ellobius*, and *Eothenomys* were paraphyletic in both trees. Divergence-time estimations of Arvicolinae based on Bayesian Inference are presented in Table 3. The divergence-time dating analysis revealed an initial divergence between *Arvicola* and *Chionomys*/*Microtus* about 2.35 mya with 95% HPD: 1.97–2.75. The next inner basal radiation of *Chionomys* realized about 1.96 mya with 95% HPD: 1.61–2.28. Diversification of *C. roberti* and *C. nivalis* was dated about 1.06 mya with 95% HPD: 0.87–1.25 (Fig. 5).

The *CYTB* data set belonging to the 132 taxa was subjected to ML and BI analyses. The data set comprised 442 variable sites, of which 351 were parsimony informative and 91 were singletons. Two different topologies were obtained by the BI and ML analyses (Figs. 6 and 7). According to the topology reconstructed by the BI analysis, *C. gud* formed the basal position and, *C. roberti* and *C. nivalis* were closely related to each other. Each clade had robust BPP support: 1 or 0.97. On the other hand, *C. roberti* was found in the basal position (100% bootstrap support), whereas *C. nivalis* and *C. gud* were more closely related species with the relatively lower bootstrap support (48%) in the ML tree. The obtained topologies demonstrated that each species were polytomic because of subtrees consisting of geographically distant populations. Within the *C. nivalis*, at least 10 different lineages, five of them of European origin, were detected throughout its distribution range by both analyses (Figs. 6 and 7). The Ardahan and Kars haplotypes of *C. nivalis*, obtained from northeast Turkey in the scope of the current study, were clustered with the haplotypes of the Caucasia lineage. Three *C. roberti* haplotypes obtained from this study involved one of two

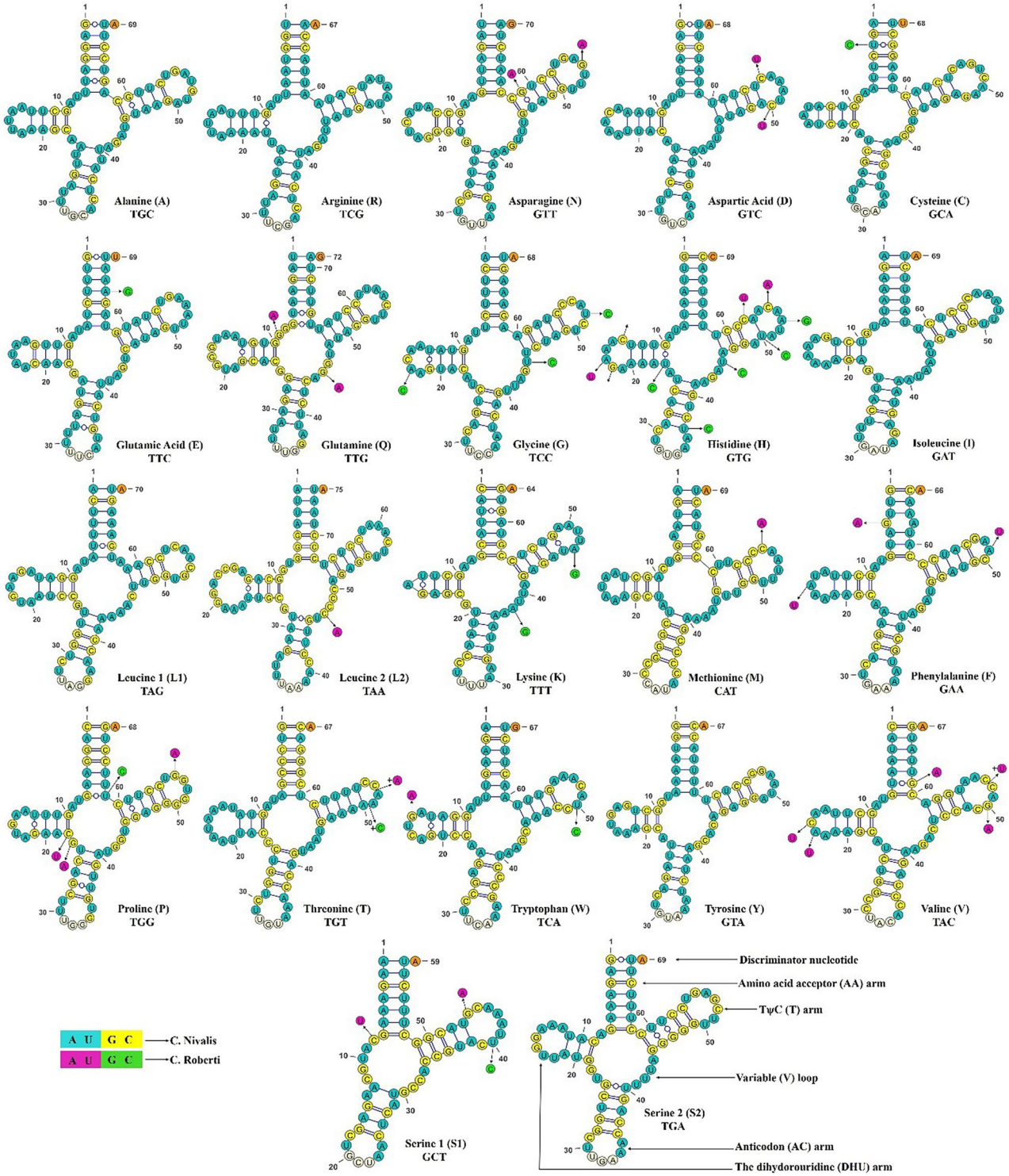


Fig. 2 Putative secondary structure of tRNAs for *Chionomys* haplotypes

main *C. roberti* clads including Georgian and Russian haplotypes.

Intraspecific relationships within *C. nivalis* revealed ten different lineages which are genetically distant to a certain degree. The most basal lineage, named the Central Taurus lineage in this study, included two haplotypes from the Central Taurus Mountains in the south of Turkey. One of them was a previously known GenBank haplotype,

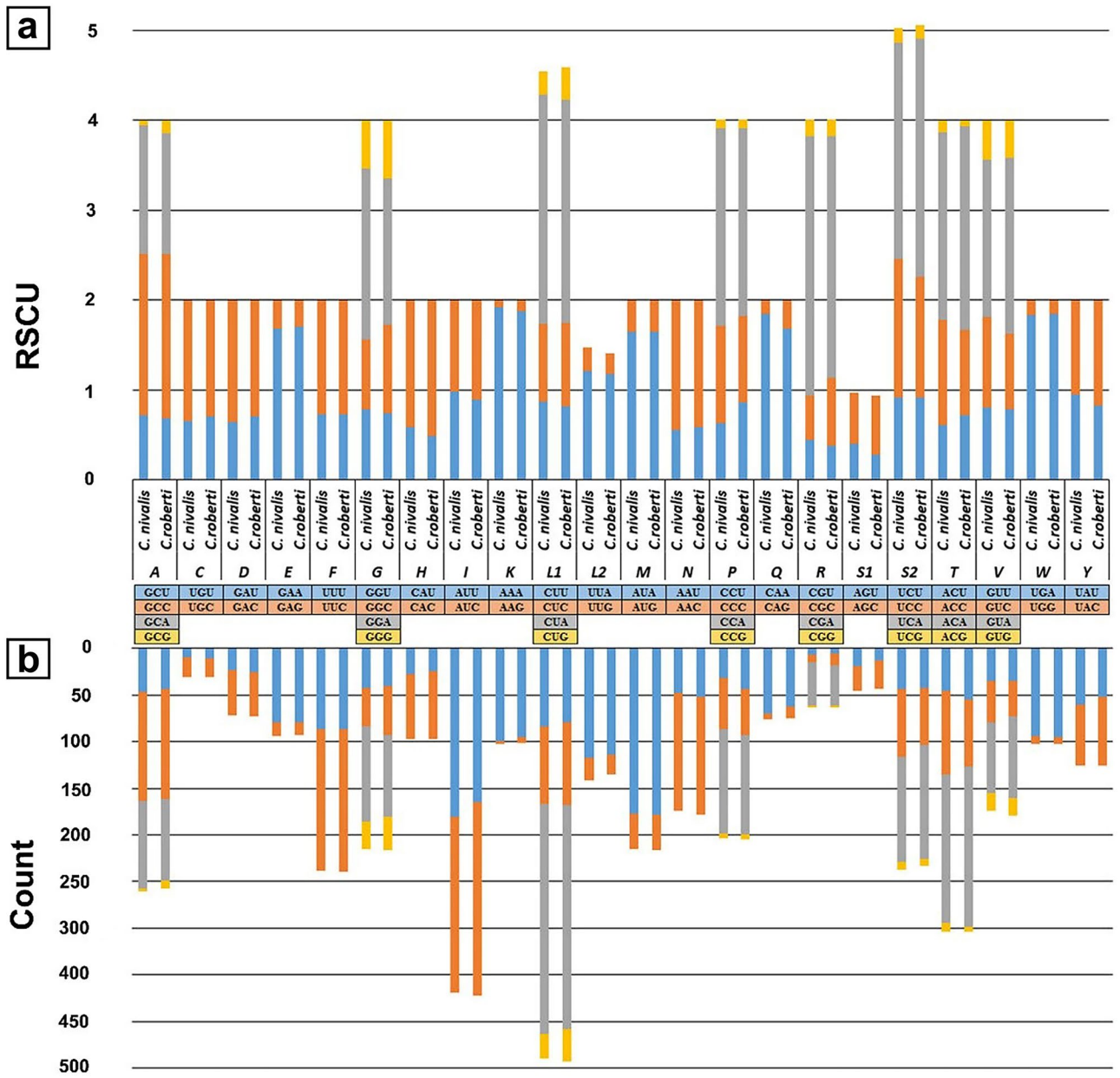
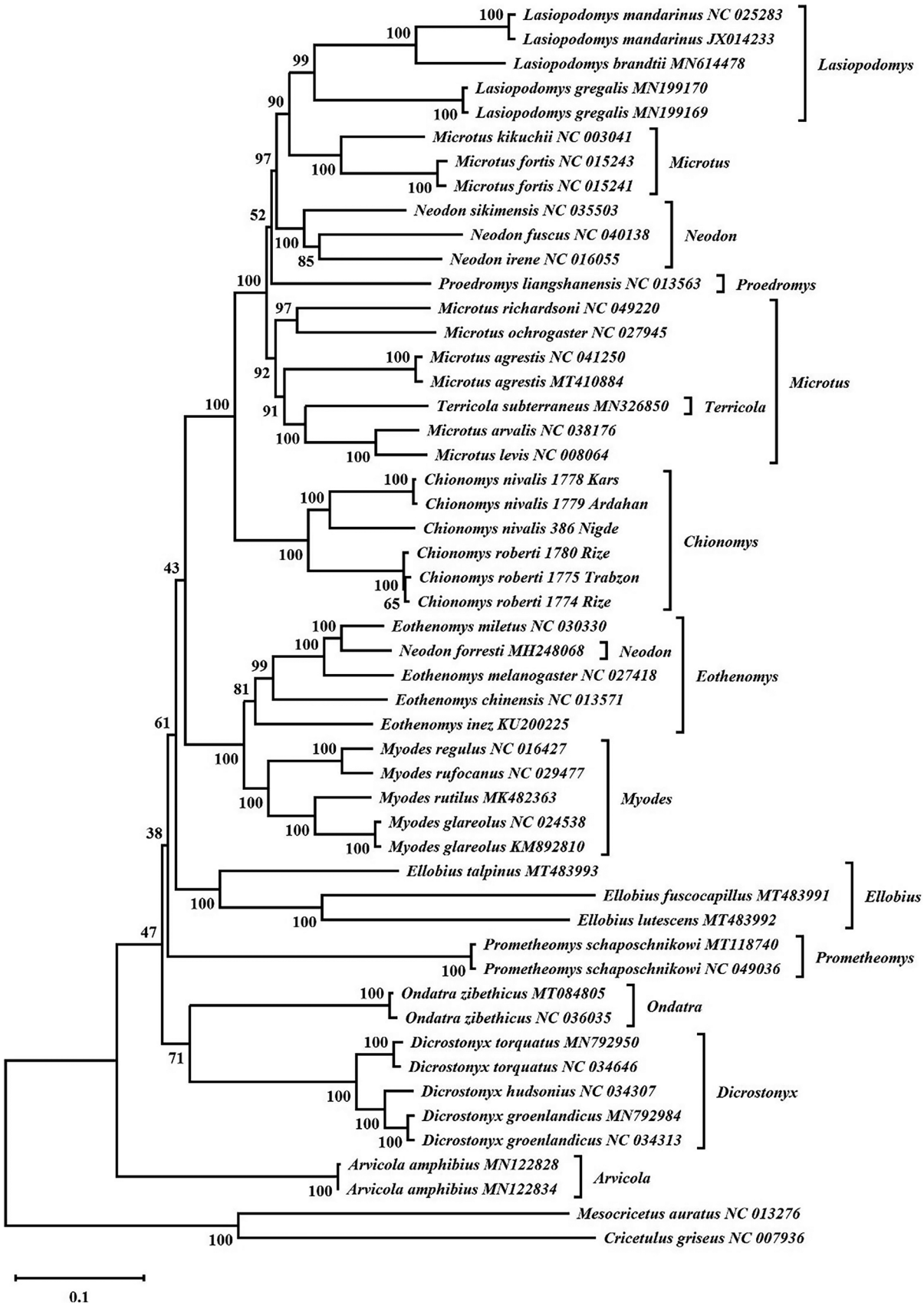


Fig. 3 Relative synonymous codon usage (RSCU) and codon usage of *C. nivalis* (1778_Kars) and *C. roberti* (1775_Trabzon) mitogenomes. Graphics above (**a**) give RSCU values; graphics below (**b**) give codon usage quantity. Amino acids were demonstrated according to the IUPACIUB one-letter abbreviation. Codon families were given on the X-axis. The stop codons were not indicated

while, another one was a newly detected haplotype from Niğde. This lineage was genetically highly different from all other lineages, such that there was a genetic distance of approximately 10% (K2P = min. 9.90% and max. 11.48%) between this lineage and other lineages (Table 4). Apart from this, Turkmenistan + northeastern Iran, the Middle East (haplotypes from Turkey, Syria, and Israel), and western Iran lineages were also involved in the basal position of the remaining lineages, and they corresponded to the relevant geographic subpopulations or lineages of the *C. nivalis* (Turkmenistan + northeastern Iran: *C. n. dementievi*; the Middle East/Turkey: *C. n. cedrorum*; Syria and Israel: *C. n. hermoni*; western Iran: *C. n. layi*). The northeastern Black Sea haplotypes of Turkey employed in the current study came together with the GenBank haplotypes of Georgia and haplotypes also from Russia in the Caucasus and created a separate lineage (the Caucasian lineage, Georgia and Turkey: *C. n. trialeticus*; Russia: *C. n. loginovi*). The K2P genetic distance values ranged from 2.7% – 5.22% between this


Lasiopodomys mandarinus NC 025283
Lasiopodomys mandarinus JX014233
Lasiopodomys brandtii MN614478
Lasiopodomys gregalis MN199170
Lasiopodomys gregalis MN199169
Lasiopodomys
Microtus kikuchii NC 003041
Microtus fortis NC 015243
Microtus fortis NC 015241
Microtus
Neodon sikimensis NC 035503
Neodon fuscus NC 040138
Neodon irene NC 016055
Neodon
Proedromys liangshanensis NC 013563
Proedromys
Microtus richardsoni NC 049220
Microtus ochrogaster NC 027945
Microtus agrestis NC 041250
Microtus agrestis MT410884
Terricola subterraneus MN326850
Terricola
Microtus arvalis NC 038176
Microtus levis NC 008064
Microtus
Chionomys nivalis 1778 Kars
Chionomys nivalis 1779 Ardahan
Chionomys nivalis 386 Nigde
Chionomys roberti 1780 Rize
Chionomys roberti 1775 Trabzon
Chionomys roberti 1774 Rize
Chionomys
Eothenomys miletus NC 030330
Neodon forresti MH248068
Neodon
Eothenomys melanogaster NC 027418
Eothenomys chinensis NC 013571
Eothenomys inez KU200225
Eothenomys
Myodes regulus NC 016427
Myodes rufocanus NC 029477
Myodes rutilus MK482363
Myodes glareolus NC 024538
Myodes glareolus KM892810
Myodes
Ellobius talpinus MT483993
Ellobius fuscocapillus MT483991
Ellobius lutescens MT483992
Ellobius
Prometheomys schaposchnikowi MT118740
Prometheomys schaposchnikowi NC 049036
Prometheomys
Ondatra zibethicus MT084805
Ondatra zibethicus NC 036035
Ondatra
Dicrostonyx torquatus MN792950
Dicrostonyx torquatus NC 034646
Dicrostonyx hudsonius NC 034307
Dicrostonyx groenlandicus MN792984
Dicrostonyx groenlandicus NC 034313
Dicrostonyx
Arvicola amphibius MN122828
Arvicola amphibius MN122834
Arvicola
Mesocricetus auratus NC 013276
Cricetulus griseus NC 007936
0.1

◂ **Fig. 4** The reconstructed ML phylogenetic tree, based on the GTR nucleotide substitution model and including the PCG sequences of *C. nivalis* and *C. roberti* along with the representatives of other genera in the Cricetidae family available in GenBank. The bootstrap values are indicated at each node

lineage and other lineages, except for the Central Taurus lineage. When the Turkish haplotypes from Caucasia and Central Taurus lineages were compared within themselves; it was determined that there was a high degree of genetic distance at the level of K2P = 11.1% between the Niğde (from the Central Taurus Mountains) and Ardahan/Kars haplotypes (from northeastern Turkey). The relative position of five European lineages consisting of different haplotypes across Europe in the phylogenetic trees was compatible with the results of previous studies and five main lineages were retrieved. (According to the literature the subspecific designations of the geographic populations within the lineages mentioned are as follows: 1. Western Europe, Bulgaria: *C. n. aleco*; Greece: *C. nivalis*; Slovenia: *C. n. wagneri*; Macedonia: *C. n. malyi*; Ukraine: *C. n. ulpius*; Albania: *C. nivalis. 2*. Alps: Italy and Switzerland: *C. n. nivalis*. 3. Italy: *C. n. nivalis*. 4. Slovakia: *C. n. mirhanreini*. 5. Iberia: *C. n. abulensis*).

Discussion

1. **Structure and organization of the mitogenomes**

This was the first study to present the complete mitogenomes of two snow vole species (*C. nivalis* and *C. roberti*) from Turkey. It also introduced phylogenetic, phylogeographic, and taxonomic implications about these species. The sequence length of the obtained mitogenomes differed more or less from previously reported mitogenomes for Arvicolinae species. However, their structure and organization were relatively similar to the previously reported mitogenomes. The *C. nivalis* and *C. roberti* mitogenomes were 16.293 bp and 16.300 bp in length respectively. However, most of the mitogenomes reported for the Arvicolinae species were close to each other in length. Of the Arvicolinae species, the complete mitogenomes of *Dicrostonyx groenlandicus*, *Myodes glareolus* and *Lasiopodomys mandarinus* were 16.341 bp, 16.353 bp and 16.375 bp in length respectively (Filipi et al., 2015; Cong et al., 2016; Fedorov & Goropashnaya, 2016). In the current study, the mitogenomes of the two snow vole species were similar structurally and organizationally to those of other mammals including rodents, carnivores, and artiodactyls (Ding et al., 2016; Yue et al., 2015; Zhong et al., 2010; Zhou et al., 2019). The mitogenomes of the two species included 13 protein-coding (PCGs), 22 transfer RNA (tRNA), and two ribosomal RNA (rRNA) genes, along with a light chain (L) replication origin (O_L), and also a non-coding control region (D- loop). The light chain (L chain) contained the *ND6* gene, O_L origin, and eight tRNA genes ($tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Glu}$, $tRNA^{Gln}$, $tRNA^{Pro}$, $tRNA^{Ser(UCN)}$, and $tRNA^{Tyr}$). However, the 12 PCGs, 14 tRNA, and two rRNA genes were on the heavy chain (H chain). Similar to the mitogenomes of some extinct and extant vertebrates (Krause et al., 2008; Rohland et al., 2007; Wada et al., 2010), the mitogenomes of the two *Chionomys* species were also comprised of the overlapping regions and intergenic spaces between protein-encoding genes and tRNA genes. Regarding the nucleotide composition in the mitogenomes of both species, it was observed that Guanine was the lowest in ratio, and this was compatible with many vertebrate species (Bendová et al., 2016; Fu et al., 2016; Gissi et al., 1998; Jiang et al., 2012, 2018; Yue et al., 2015; Zhong et al., 2010). The AT and GC skew indicate mutation or evolutionary pressure associated with the mechanism of DNA replication (Charneski et al., 2011; Martin, 1995). The determined AT and GC skews for the *C. nivalis* and *C. roberti* mitogenomes were consistent with those of previously studied Arvicolinae species (İbiş et al., 2020; Shi et al., 2021). The start and stop codons with the highest frequency were ATG and TAA, which were compatible with the studies including other mammalian mitogenomes (İbiş et al., 2020; Lamelas et al., 2020; Shi et al., 2021). The incomplete stop codons (T-) detected in three PCGs (*ND1*, *COXIII*, and *ND4*) in the current study were the common incomplete stop codons observed in other mammalian species (Bendova et al., 2016; Cong et al., 2016). Poly-adenylation of the 3'-end of the mRNA occurring after transcription may lead a complete functional TAA stop codon to occur (Ojala et al., 1981). Moreover, one reason for the presence of incomplete stop codons may be a tendency to reduce mitogenome size due to selection pressure (Rand, 1993). The RSCU values, known as the number of times a codon appears in a gene under the assumption of equal codon usage (60), were at the highest level for nine codons: GCC (A), GGA (G), CUA (L1), CCU (P), CGA (R), UCA (S2), ACA (T) for *C. roberti*, ACU (T) for *C. nivalis*, and GUA (V) for both *C. roberti* and *C. nivalis*. The origin of replication (O_L) is one of the non-coding regions of mitochondrial DNA (Fernández-Silva et al., 2003), and this region was 30 bp in length and located in the region consisting of five tRNA genes (W, A, N, C, and Y) in the mitogenomes

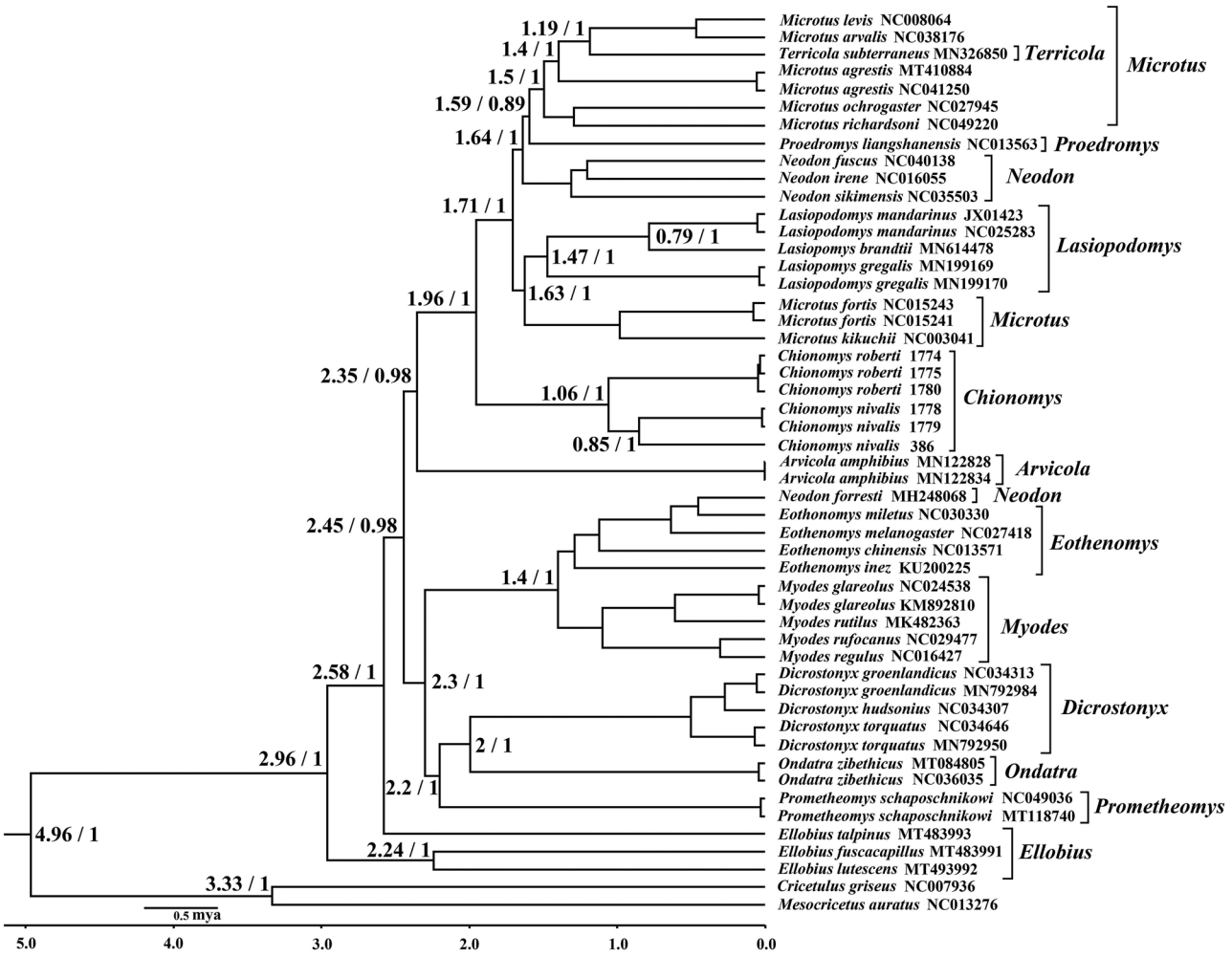


Fig. 5 The reconstructed BI phylogenetic tree, based on the GTR nucleotide substitution model and including the PCG sequences of *C. nivalis* and *C. roberti* along with the representatives of other genera in the Cricetidae family available in GenBank. The divergence times, Mya (left) and the Bayesian posterior probability values (right) are indicated at each node

of the two *Chionomys* species. The structure mentioned was in the same order as in the other Arvicolinae species (İbiş et al., 2020; Lamelas et al., 2020; Shi et al., 2021) and it was also observed in most mammal species (Hao et al., 2011; Kim et al., 2015; Folkertsma et al., 2018; Aleix-Mata et al., 2020). Apart from O_L, the major non-coding region including the main regulatory elements for mtDNA transcription and replication is the control region, often known as the D-loop (Fernández-Silva et al., 2003). The length of the control region (D-loop) is variable and is 657 bp in length for *Microtus ochrogaster* and 1089 bp in length for *Myodes rufocanus* from the Arvicolinae species (Cao et al., 2016; Lu et al., 2017). In the current study, it was determined that the control regions of the *C. roberti* and *C. nivalis* mitogenomes were 901 bp and 896 bp in length, respectively.

2. Phylogenetic relationships of *Chionomys*

The systematics of *Chionomys* is controversial, and this taxon has sometimes been considered as a subgenus and sometimes as a genus, according to different data such as paleontological and morphological features, and isozymes (Chaline & Graf, 1988; Graf, 1982; Graf & Scholl, 1975; Gromov & Polyakov, 1992; Miller, 1908; Nadachowski, 1991). In recent years, mitochondrial and nuclear DNA-based studies have focused on the relationships of *Chionomys* with other taxa within the subfamily Arvicolinae and have attempted to clarify the systematics of *Chionomys* (Jaarola et al., 2004; Galewski et al., 2006; Robovský et al., 2008; Bužan et al., 2008; Abramson et al., 2009; Castiglia et al., 2009; Yannic et al., 2012; Bannikova et al., 2013; Mahmoudi et al.,

Table 3 Divergence-time estimations of Arvicolinae in millions of years before present. The main nods described are illustrated in Fig. 5

Nodes	Estimated Node Age (mya)	Estimated %95 HPD (mya)	BPP
Cricetinae / Arvicolinae	4.96	4.09–5.82	1
Ellobius / Others	2.58	2.13–3.01	1
Prometheomys + *Ondatra* + … / *Arvicola* + *Chionomys* + …	2.45	2.04–2.87	0.98
Prometheomys + *Ondatra* + …/ *Eothenomys* + *Myodes*	2.3	1.91–2.69	1
Prometheomys / *Ondatra* + *Dicrostonyx*	2.2	1.83–2.58	1
Ondatra / *Dicrostonyx*	2	1.64–2.34	1
Eothenomys / *Myodes*	1.4	1.15–1.63	1
Arvicola / *Chionomys* + …	2.35	1.97–2.75	0.98
Chionomys / *Microtus* + …	1.96	1.61–2.28	1
C. nivalis / *C. roberti*	1.06	0.87–1.25	1
C. nivalis (Caucasus) / *C. nivalis* (Central Taurus)	0.85	0.7–1.01	1
Microtus + *Lasiopodomys* / *Neodon* + …	1.71	1.4–1.99	1
L. gregalis / *L. brandtii* + *L. mandarinus*	1.47	1.21–1.72	1
L. brandtii / *L. mandarinus*	0.79	0.65–0.93	1
Neodon / *Proedromys* + …	1.64	1.34–1.9	1
Proedromys / *Microtus*	1.59	1.32–1.86	0.89

2017). Some of these studies have mostly been concentrated on the phylogeography of *C. nivalis* due to its broad geographic range. (Bannikova et al., 2013; Castiglia et al., 2009; Mahmoudi et al., 2017; Yannic et al., 2012). Other studies have, however, looked from a wider perspective and have tried to determine the intergeneric phylogenetic relationships within Arvicolinae by dealing with the relationships at the genus and subgenus levels (Galewski et al., 2006; Robovský et al., 2008; Bužan et al., 2008; Abramson et al., 2009).

The present study was performed to determine the position of *Chionomys* within the subfamily Arvicolinae using the sequences of the 13 PCGs from the mitogenomes and the intrageneric phylogeny of *Chionomys* using the mitochondrial *CYTB* data of *C. nivalis* and *C. roberti*. The ML and BI trees reconstructed based on the PCGs sequences of the genera within Arvicolinae displayed similar topologies, except for the positions of *Arvicola* and *Ellobius*. In this context, the phylogenetic relationships were more strongly resolved in the BI tree with high posterior probability values in the main nodes (Fig. 5) when compared to the ML tree with relatively low bootstrap values in the main nodes (Fig. 4). Within the subfamily Arvicolinae, six genera (*Dicrostonyx*, *Ondatra*, *Prometheomys*, *Myodes*, *Proedromys*, and *Lasiopodomys*) were monophyletic, whereas *Microtus*, *Neodon*, *Eothenomys*, and *Ellobius* were paraphyletic. This was consistent with the previous findings (Conroy & Cook, 1999; Bužan & Kryštufek, 2008; Galewski et al., 2006; Abramson et al., 2009; İbiş et al., 2020; Shi et al., 2021; Kirkland & Farré, 2021). In many studies on the Arvicolinae phylogeny, there has been either no data or not enough data on the *Chionomys* members (Conroy & Cook, 1999; Galewski et al., 2006; Bužan et al., 2008; Abramson et al., 2009; Shi et al., 2021; Kirkland & Farré, 2021). Therefore, when considering the *Chionomys* species in the current study, the ML and BI tree topologies allowed us to better see the possible position of *Chionomys* within Arvicolinae and showed that *Chionomys* was monophyletic. *Chionomys* was in close relation to the *Microtus* group consisting of *M. richardsoni*, *M. ochrogaster*, *M. agrestris*, *M. subterraneus*, *M. arvalis* and *M. levis*. In the BI tree (Fig. 5), the position of *Arvicola* was in the close basal position of the *Chionomys* and *Microtus* phylogroups, and the close relation of *Arvicola* to these two genera was compatible with the results of the previous studies, which reconstructed the Arvicolinae phylogeny based on mitochondrial genes, nuclear genes, and morphology or its combinations (Galewski et al., 2006; Robovský et al., 2008; Abramson et al., 2009). On the other hand, in the ML tree (Fig. 4), the position of *Arvicola* was in the most basal position according to the phylogenetic positions of the *Chionomys* and *Microtus* phylogroups, and this was consistent with the previous phylogeny of Arvicolinae reconstructed by using the cytochrome *b* gene sequences (Bužan et al., 2008; Galewski et al., 2006).

Based on the mitochondrial *CYTB* data, two different phylogenies of *Chionomys* were reconstructed by means of the ML and BI analyses (Figs. 6 and 7). This difference arose from different species found in the basal positions of both BI and ML trees. According to this, *C. roberti* was in the most basal position of the ML tree (Fig. 6), *C. gud* was in the most basal position of the

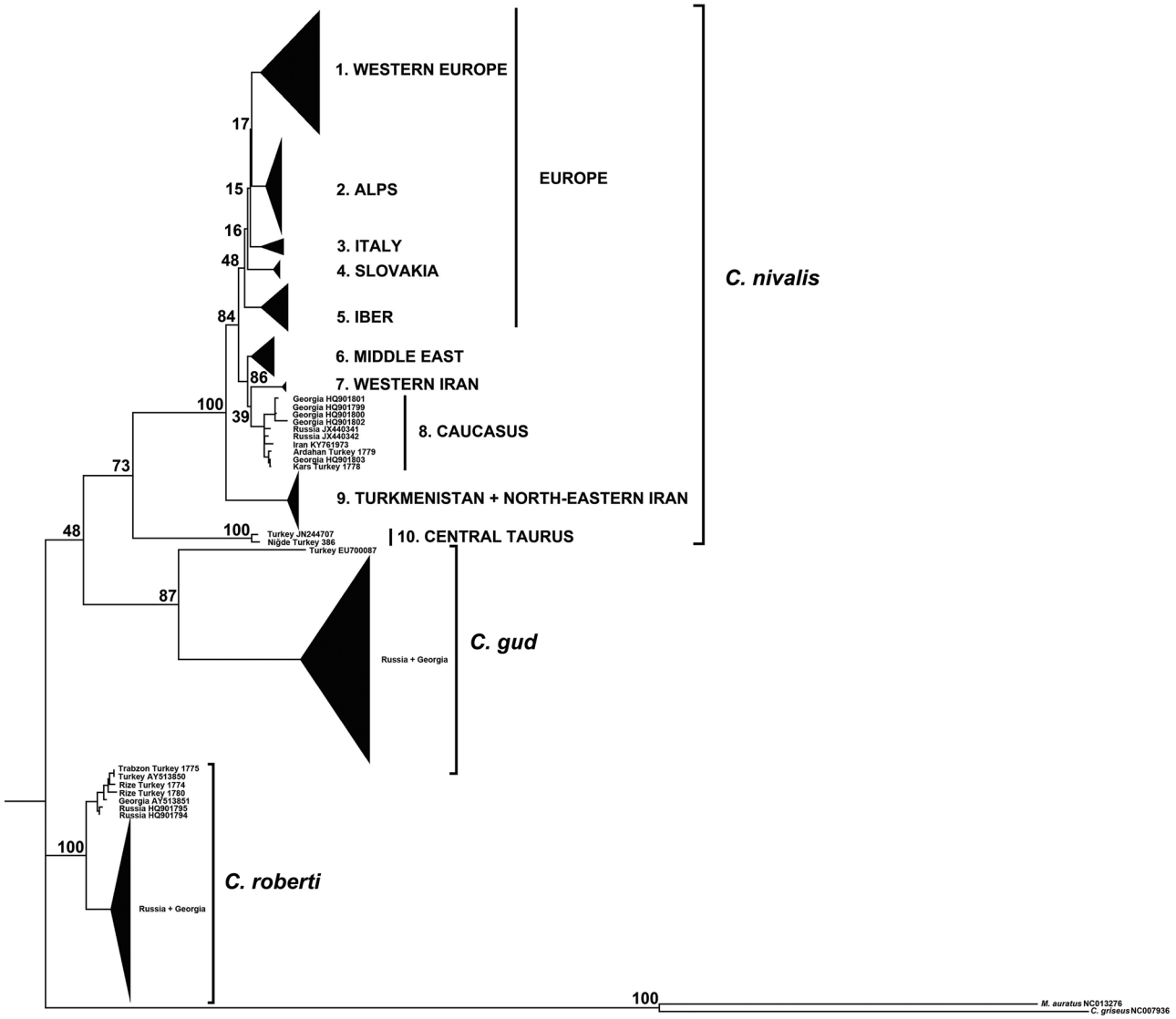


Fig. 6 ML tree reconstructed from *CYTB* data set of the genus *Chionomys*. Bootstrap values are shown at nodes. The symbols and numbers indicate the compressed lineages within the snow vole species including cytochrome *b* sequences obtained from GenBank and this study. The relative position of the snow vole haplotypes from Turkey determined by the current study is shown without compressing in the topology for a better understanding

BI tree (Fig. 7). What causes this phylogeny to produce such a topology may be the use of all cytochrome *b* sequences, including the shortest sequences nearly 340 bp in length, found in GenBank in the analyses. Despite this, the case was generally compatible with the previous results (Bannikova et al., 2013; Bužan & Kryštufek, 2008; Nadachowski, 1991; Yannic et al., 2012).

The intraspecific phylogeny of *C. nivalis* determined in the current study was compatible with the phylogenies revealed in the previous studies. The presence of the 10 *CYTB* lineages was determined within this species and this confirmed the results of Bannikova et al. (2013). Of the 10 *CYTB* lineages, the Central Taurus lineage was in the most basal position of all the remaining *C. nivalis* lineages, as reported by Bannikova et al. (2013). This lineage had remarkable genetic differentiation from other lineages, which was almost at the level of 10%. Bannikova et al. (2013) noted that genetically such a different lineage could be considered a new taxon. The results of the current study also showed strong signals supporting this suggestion. According to some authors (Bradley & Baker, 2001; Baker & Bradley, 2006), genetic differentiation at this level is noteworthy because it is over the interspecific genetic differentiation limit and indicates two separate biological species. In this

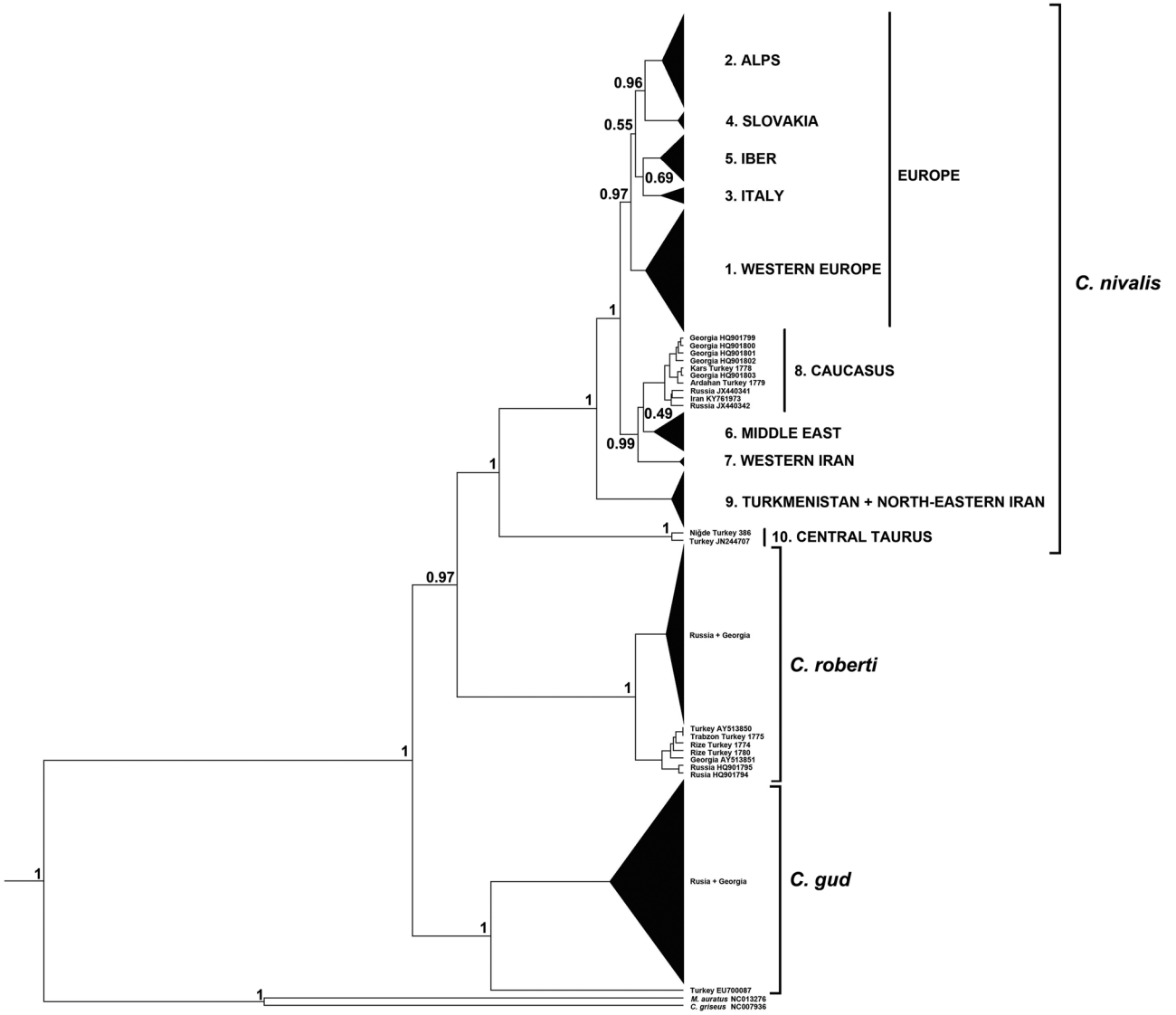


Fig. 7 BI tree reconstructed from *CYTB* data set of the genus *Chionomys*. Posterior probability values are shown at nodes. The symbols and numbers indicate the compressed lineages within the snow vole species including cytochrome *b* sequences obtained from GenBank and this study. The relative position of the snow vole haplotypes from Turkey determined by the current study is shown without compressing in the topology for a better understanding

context, data from other markers should also be used to clarify this situation. The *C. nivalis* specimens obtained from Ardahan and Kars, geographically located within the Caucasian ecological region, were grouped with the specimens from Georgia, Russia, and northern Iran in the Caucasian lineage (Figs. 6 and 7). Moreover, the positions of the five lineages in Europe and the other lineages in the phylogenetic trees were consistent with the results in previous studies (Bannikova et al., 2013; Castiglia et al., 2009; Mahmoudi et al., 2017; Yannic et al., 2012). The intraspecific relationships in *C. gud* and *C. roberti* were compatible with phylogenies obtained in previous studies (Bannikova et al., 2013). *C. gud* was mainly divided into two lineages. The GenBank sequence of this species from Turkey was distinct from the GenBank sequences from Russia and Georgia and created the first *C. gud* lineage. The second *C. gud* lineage included samples from Russia and Georgia and was divided into several subclades (the data was compressed in the tree to avoid complexity), just as reported by Bannikova et al. (2013). Unfortunately, the lack of any *C. gud* specimens to use in the scope of this study meant that we did not have the opportunity to make any assessment for this species. This study is, regrettably, lacking in this respect. Similar to *C. gud*, the presence of two main clades was detected within the *C. roberti* as well.

Table 4 Estimates of the genetic distance over sequence pairs between lineages of *C. nivalis* according to the Kimura 2-parameter model with 1000 bootstrap replicates

	Lineages	1.	2.	3.	4.	5.	6	7.	8.	9.	10.
1.	**Central Taurus**		0.0134	0.0103	0.0097	0.0100	0.0100	0.0102	0.0113	0.0101	0.0109
2.	**Western Europe**	0.1112		0.0076	0.0065	0.0070	0.0065	0.0064	0.0093	0.0083	0.0100
3.	**Caucasus**	0.1082	0.0419		0.0039	0.0057	0.0056	0.0053	0.0077	0.0047	0.0074
4.	**Middle East**	0.0997	0.0386	0.0246		0.0052	0.0051	0.0050	0.0063	0.0046	0.0073
5.	**Iber**	0.1089	0.0412	0.0405	0.0370		0.0047	0.0043	0.0065	0.0059	0.0071
6.	**Alps**	0.1006	0.0329	0.0374	0.0346	0.0314		0.0041	0.0059	0.0057	0.0070
7.	**Italy**	0.1056	0.0330	0.0368	0.0339	0.0280	0.0245		0.0057	0.0056	0.0075
8.	**Slovakia**	0.0990	0.0443	0.0428	0.0343	0.0366	0.0288	0.0296		0.0067	0.0093
9.	**Western Iran**	0.1079	0.0461	0.0287	0.0295	0.0428	0.0393	0.0379	0.0360		0.0073
10.	**Turkmenistan + Iran**	0.1148	0.0619	0.0573	0.0573	0.0557	0.0530	0.0600	0.0604	0.0569	

The Turkish *C. roberti* specimens obtained in the scope of this study in the first clade were clustered with the specimens of Datvisi (Georgia) and Alania-North Ossetia (Russia) splitting from other GenBank specimens from Russia and Georgia. While this clade included the Transcaucasian specimens, the second clade consisted of the northern specimens. It was consequently observed that there was no discrepancy from the results of past studies in the intraspecific phylogenies of both species (Bannikova et al., 2013).

3. Evolutionary divergence events

The beginning of the evolutionary divergence events within Arvicolinae was dated differently in mitochondrial (Conroy & Cook, 1999) and paleontological (Chaline & Graff, 1988) studies; 5–9 mya and 3–5 mya, respectively. In the current study, the beginning of the evolutionary divergence within the subfamily Arvicolinae was dated to 4.96 mya by means of molecular divergence analysis. This divergence dating was consistent with the results of the paleontological and relatively molecular studies. On the other hand, the estimates based on nuclear genes suggested that the genetic divergence within Arvicolinae began much earlier than mitochondrial and paleontological dating, approximately 18.5 mya (Abramson et al., 2009). In this context, representatives of the subfamily Arvicolinae might have emerged in the Pliocene (3–5 mya) (Chaline & Graf, 1988; Conroy & Cook, 1999; this study). Also, based on the mean uncorrected pairwise divergences of nuclear and mitochondrial genes, Galewski et al., (2006) stated that the divergence events among some Arvicolinae genera occurred 2–10 mya.

Molecular divergence analyses revealed that the split of the main clades (generic diversification) leading to radiation within Arvicolinae started about 2.96 mya and lasted till 1.4 mya. According to evolutionary divergence dates, the six genera, *Ellobius*, *Prometheomys*, *Ondatra*, *Dicrostonyx*, *Eothenomys*, and *Myodes*, were ranked in this order from the most basal position to the inner basal positions of the remaining taxa in the BI tree (Fig. 5). Although paleobiogeography of the genus *Ellobius* is controversial, the fossil evidence for representatives of this genus was recorded in a time interval extending from the Pliocene to Pleistocene (Rey-Rodríguez et al., 2021). In accordance with this information, the molecular dating analyses pointed out that this genus started diversification at about 2.96 mya. The first fossil evidence of *Prometheomys* was dated to the Middle Pleistocene by the paleontological study (Gromov & Polyakov, 1992). In the current study, the split of this taxon occurred approximately 2.2 mya, and this confirmed the finding of Gromov and Polyakov, (1992). It has been known that the successive muskrat fossils dated at a time that started from the Plio-Pleistocene transition to 0.6 mya were evaluated as a single *Ondatra* species (*Ondatra zibethicus*) (Martin, 1996). As a result of the current study, the *Ondatra* divergence pointed to 2 mya, which also was compatible with these results (Martin, 1996). Additionally, the first fossil findings belonging to the genus *Dicrostonyx* are known to date from about 1.8 or 1.9 mya (Chaline & Graf, 1988). In the current study, the divergence-time estimations of this taxon based on PCGs showed that the radiation of *Dicrostonyx* started about 2 mya. Molecular data (Liu et al., 2019) suggested that speciation processes in *Eothenomys* occurred between 2.52 and 0.52 mya, this

was a time period that also coincided with our findings (1.4 mya). According to the fossil evidence, the emergence and diversification process of the *Myodes* species occurred between 2 and 1 mya (Chaline & Graf, 1988). The current study found that the genus *Myodes* started to be diversified about 1.4 mya and it was also consistent with this finding of Chaline and Graf (1988). The molecular dating analyses that were conducted according to the calibration point used by Chaline and Graff (1988) and Yannic et al. (2012) pointed out that the split of *Arvicola*/*Chionomys* + *Microtus* took place at the beginning of the Quaternary period (2.35 Mya, 95% HPD: 1.97–2.75, BPP: 0.98). This aforementioned evolutionary divergence time coincided with the Early Pleistocene divergence (approximately 2.4 mya) that was suggested for these three genera based on the fossil record (Repenning, 1983; Chaline & Graf, 1988). Additionally, the division detected by the current study between *Chionomys* and *Microtus* that happened 1.96 mya is consistent with the divergence between these taxa that occurred in the Early Pleistocene (2.35 mya), as suggested by Yannic et al. (2012). Contrary to the findings of Yannic et al. (2012), who suggested that the basal radiation of *Chionomys* occurred in the Early Pleistocene (1.77 mya), the result of the current study suggests that *C. nivalis* and *C. roberti* diverged in the Middle Pleistocene (1.06 mya). This is compatible with the finding of Nadachowski and Baryshnikov (1991). In addition to this, the separation within *C. nivalis* (into two main lineages, the Central Taurus and Caucasus lineages) corresponding to the Middle Pleistocene is compatible with the Middle Pleistocene record of *C. nivalis* reported from Emirkaya, Central Anatolia, Turkey (Montuire et al., 1994), whereas it is partly incompatible with the estimated radiation of Yannic et al. (2012), who pointed to the Late Pleistocene. It has been estimated that the split of *Microtus* from the fossil genus *Allophaiomys* occurred 2.4 or 2 mya (Chaline & Graf, 1988). The result of our study is compatible with this estimation and demonstrated that the main radiation leading to *Arvicola*/*Chionomys* + *Microtus* happened 2.35 mya. Moreover, the *Chionomys*/*Microtus* split happened 1.96 mya according to our findings. In addition to this, the split of the genus *Lasiopodomys* was dated to 1.71 mya and the primary diversification between *L. gregalis* / *L. brandtii* and *L. mandarinus* also started at 1.47 mya. Moreover, it was found that the separation of *L. brandtii* from *L. mandarinus* occurred at about 0.79 mya (Shi et al., 2021). Our results indicating the time of molecular divergence processes among and within this genus supported these three divergence-time estimation well.

Conclusions

The current study first presented the reference mitogenome sequences of the two representatives of the *Chionomys*. In this way, it was possible that clearer inferences could be made regarding the phylogeny, phylogeography, and taxonomy of *Chionomys*, which has hitherto been incomplete or controversial. Accordingly, given the position of *Chionomys* in phylogenetic trees with the 100% bootstrap and BPP = 1 supports, its divergence from *Microtus* occurred approximately 1.96 mya in the Early Pleistocene, and the view that *Chionomys* is an independent genus within Arvicolinae was well supported. When looking at the phylogenetic relationships among *Chionomys* representatives, contrary to usual and known expectations, the cytochrome *b* sequence-based analyses indicated that the more complex phylogenetic relationships among *Chionomys* species may be. Because, it was the case an unstable closeness of the *C. roberti* and *C. gud* to *C. nivalis*, rather than their closeness to each other in ML or BI tree. It is thought that what causes this phylogeny to produce such a topology may be the use of all cytochrome *b* sequences, including the shortest sequences nearly 340 bp in length, found in GenBank in the analyses. The Central Taurus lineage of *C. nivalis* bearing a high degree of genetic differences was a different lineage. Increasing both the number of specimens and the use of different additional markers will allow more precise assessments of the taxonomy, phylogeny, and phylogeography of this lineage. The basal positions of the Central Taurus and Turkmenistan + northeastern Iran lineages in the *C. nivalis* phylogeny support the view that the Near East might be the origin of this species rather than Europe. As expected, *C. roberti* specimens from Turkey clustered with the specimens from Transcaucasia. It is certain that the inclusion of *C. gud* samples in subsequent studies will be useful for clarifying the taxonomic problems related to this genus.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s13127-022-00559-7.

Data availability Mitogenome sequence data is available at GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore). The following information was supplied regarding data availability: GenBank accession numbers: OK323269, OK323270, OK323271, OK323272, OK323273, and OK323274. Also, detailed information about the sequence data downloaded from GenBank used in phylogenetic analyses is provided in the S1 and S2.

Declarations

Ethics approval We declare that the studies have been approved by the Animal Experiments Local Ethics Committee of Erciyes University. Relevant permit number: 21/218. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Conflicts/competing of interest The authors declare no conflict/competing of interest.

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