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Mitochondrial Genetic Diversity and Historical Population Dynamics of the Bank Vole *Clethrionomys glareolus* in Northern Anatolia: Insights from *Cytb* and *COI* Gene Sequences

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Research Article

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Abstract

This study presents a genetic analysis of *Clethrionomys glareolus* populations across northern Anatolia, utilizing mitochondrial *Cytb* and *COI* gene sequences. Phylogenetic analysis identified two distinct Turkish clades, Clade 1 and Clade 2. Neutrality tests and mismatch distribution analyses indicate a recent population expansion following a genetic bottleneck associated with Pleistocene climatic changes. Interestingly, bank voles from Uludağ in Bursa Province of northwestern Anatolia exhibited a closer phylogenetic relationship with northeastern populations than with other northwestern populations, suggesting complex historical population dynamics, possibly involving a population replacement in northern Anatolia and relic survival in Uludağ. The geographic boundaries between the clades did not align closely with main rivers, suggesting that these geographic features did not act as strong long-term barriers to gene flow among bank vole populations. Our mtDNA study lays the foundation for future research to evaluate these scenarios using phylogeographic patterns of genome-wide diversity for a comprehensive understanding of the complex evolutionary history of bank voles in Türkiye.

Keywords: Bank vole, mitochondrial DNA, Turkish Pleistocene refugia, inland water barriers, Türkiye

Kuzey Anadolu'da Bank Vole (*Clethrionomys glareolus*) Populasyonlarının Mitokondriyal Genetik Çeşitliliği ve Tarihsel Populasyon Dinamikleri: *Cytb* ve *COI* Gen Dizilerinden Elde Edilen Bilgiler

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Öz

Bu çalışma, Kuzey Anadolu'daki *Clethrionomys glareolus* populasyonlarının mitokondriyal *Cytb* ve *COI* gen dizileri kullanılarak yapılan genetik analizlerini sunmaktadır. Filogenetik analizler, iki ayrı Türkiye kladını, Klad 1 ve Klad 2'yi ayırt etmiştir. Nötralite testleri ve uyumsuzluk analizleri, Pleistosen dönemi iklim değişiklikleri ile ilişkili genetik bir darboğazın ardından yakın zamanda bir populasyon genişlemesi olduğunu ortaya koymuştur. Dikkat çekici bir şekilde, Bursa'nın Uludağ bölgesinden alınan kızıl sırtlı orman faresi örnekleri, diğer kuzeybatı populasyonları yerine kuzeydoğu populasyonları ile daha yakın filogenetik ilişkiler sergilemiştir. Bu durum, kuzey Anadolu'da potansiyel bir populasyon değişimi ve Uludağ'da bir kalıntı populasyonun

hayatta kalmasını içeren karmaşık tarihsel populasyon dinamiklerine işaret etmektedir. Kladlar arasındaki coğrafi sınırlar, ana nehirlerle doğru orantılı olmayıp, bu coğrafi özelliklerin kızıl sırtlı orman faresi populasyonları arasındaki gen akışına ciddi engeller oluşturmadığını göstermektedir. Bu mitokondriyal DNA çalışması, Türkiye'deki kızıl sırtlı orman farelerinin evrimsel tarihini derinlemesine anlamak amacıyla gelecekte yapılacak araştırmalar için sağlam bir temel oluşturarak, genetik çeşitlilik gösteren filocoğrafik desenlerin kullanılmasını önermektedir.

Anahtar Kelimeler: Kızıl sırtlı orman faresi, mitokondriyal DNA, Türkiye Pleistosen sığınakları, iç su bariyerleri, Türkiye

Introduction

During the Pleistocene glaciations, many European species found refugia in the Mediterranean region of Europe. However, some species also survived in temperate areas in northern Anatolia [1, 2]. The bank vole, *Clethrionomys glareolus* Schreber, 1780 (sometimes also called *Myodes glareolus*; Kryštufek et al. [3]), is a species extensively studied to comprehend the response of the European fauna to Pleistocene climate changes [4-12]. This species is found in temperate and boreal forests across Europe and western Asia, including northern Anatolia [13, 11]. Recent phylogenetic and phylogeographic studies by Deffontaine et al. [4], Filipi et al. [7], Çolak et al. [8], Lu et al. [9], Chiocchio et al. [11] and Kotlik et al. [12] have highlighted the significant role of climatic fluctuations and environmental changes during the Pleistocene period (starting around 1.81 million years ago) in influencing the genetic differentiation of *Clethrionomys glareolus*. Bank voles are distributed along the boreal forests of Anatolia. According to Osborn [14], the bank vole exhibits a fragmented distribution in Anatolia, with noticeable morphological differences observed among specimens in the western and eastern parts of northern Anatolia. Several major geographic features, including the Sakarya River, Kızılırmak River, Melet River, İkizdere stream, and the Izmit Gulf–Lake Sapanca–Sakarya Valley waterway [15], may have contributed to the fragmentation of the geographic range of terrestrial species in Anatolia, potentially leading to phylogeographical breaks. However, the impact of these barriers on the differentiation of *C. glareolus* remains unknown. Çolak et al. [16] identified two distinct groups in the northern Anatolia region based on allozyme data, with the differentiation largely associated with altitudinal differences. Similarly, Beteş et al. [17] revealed the existence of two lineages as western and eastern, in the northern Anatolia through RAPD-PCR analysis. Çolak et al. [8] identified two lineages in Anatolia as northeastern and northwestern, using phylogenetic analysis of the mitochondrial DNA (mtDNA) *Cytb* and D-*loop* regions, which were separated by the Kızılırmak valley. Interestingly, the study showed that specimens from Uludağ region (N=2) in the northwestern Anatolia clustered together with the northeastern Anatolia lineage. Uludağ, a prominent mountain located in the Marmara Region of the northwestern Anatolia, reaches an elevation of 2543 meters and presents a distinct ecological setting that may contribute to genetic differentiation within the species. In a parallel vein, İbiş et al. [18], through their study on *Crocidura leucodon*, suggested the existence of a glacial refuge in the Uludağ-Bursa region for this species. It is noteworthy that *C. glareolus* and *Crocidura leucodon* shares similar

characteristics as having a small size and a limited mobility, traits that allow these species to potentially respond rapidly to environmental and climatic factors. Building upon the insights presented in the study by İbiş et al. [18], we aim to investigate whether Uludağ and its surroundings served as a Pleistocene refugium for *C. glareolus.* For the purpose to achieve this, we expanded the sample size in this area incorporating additional gene regions into our analysis, complementing the findings of Çolak et al. [8]. The primary objective of our study is to investigate the genetic differentiation and past population dynamics of bank vole populations in Anatolia. Specifically, we aim to reassess the distinctions of the Uludağ population as a unique genetic entity, utilizing sequences of the *COI* gene region selected for its lower variability relative to the *D-loop* region used in previous studies [8]. By expanding our sample size from Uludağ and its surrounding areas, our aim is to achieve a more comprehensive understanding of the genetic diversity and population structure across the region spanning from Uludağ to northeastern Anatolia, and to explore potential drivers such as geographical barriers and climatic changes during the Pleistocene.

Materials and Method

Sample Collection

We examined a total of 66 cytochrome b (*Cytb*) gene sequences for bank voles collected from 24 different locations across northern Anatolia, covering the species' distribution in Türkiye (Table 1, Figure 1).

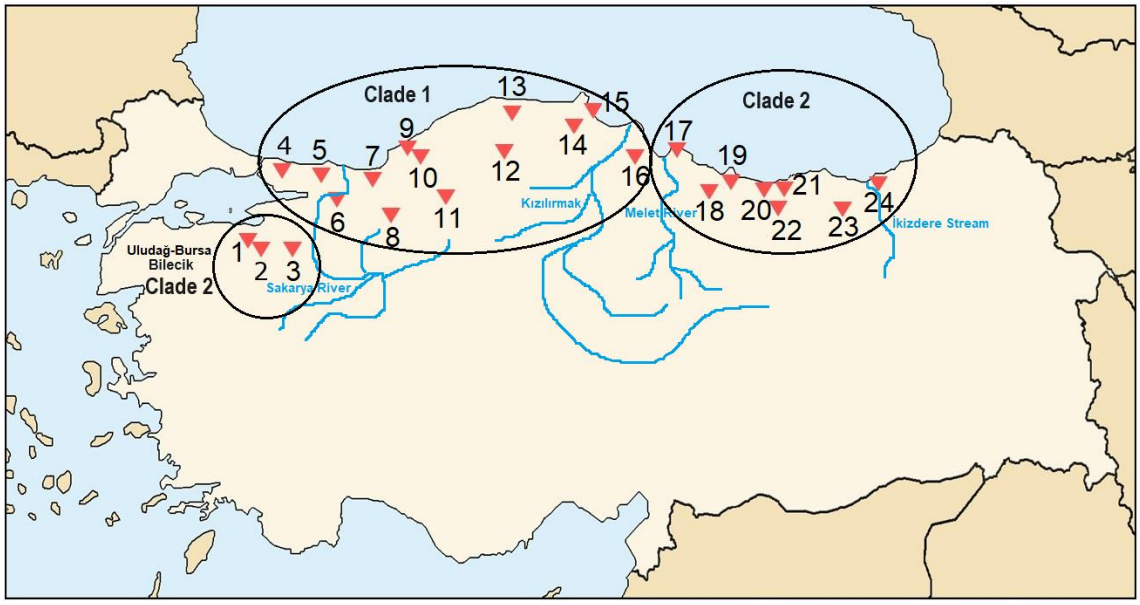

Figure 1. *Sampling localities of the bank voles from Anatolia (see the map references in Table 1)*

Among these, 34 samples had been previously analyzed for the *Cytb* gene by Çolak et al. [8]. In this study, we extended the *Cytb* analysis by including additional 32 specimens. Furthermore, we conducted the first analysis of the cytochrome c oxidase subunit I (*COI*) gene region using 63 samples in Türkiye. Ethical permits were obtained from the Animal Experiments Local Ethics Committee of Ankara

University (no: 2019-11-108) for the collection of all specimens.In addition to the Turkish samples, specimens from GenBank were included in phylogenetic tree construction and genetic distance calculations (Appendix E). *Clethrionomys centralis* (KY968281 for *Cytb* and KY968255 for *COI*) [17] was used as the outgroup.

Table 1. *Map references, locality name, number of specimens from each locality and GenBank Accession numbers of the bank vole haplotypes from Anatolia*

Map references	Locality	Total number of specimens	GenBank Accession Number	
			Cytb	***COI***
1	Bursa	9	KM508997, KM508998 OM674439	OM674417
2	Uludağ-Bursa	3	KM508997, KM508998, KM508997	OM674417, OM674418
3	Muratdere-Bilecik	3	OM674439	-
4	Şile-İstanbul	4	KM508989, KM508990	OM674420, OM674421
5	Kandıra-Kocaeli	3	KM508989, KM508991, KM508992	OM674417, OM674420
6	Kartepe-Kocaeli	3	KM509000, KM509001, KM509002	OM674420, OM674427
7	Akçakoca-Düzce	3	KM508993, KM508990, KM508994, OM674428	OM674420
8	Abant-Bolu	4	KM508995, KM508995, KM508996	OM674419, OM674420 OM674423
9	Zonguldak	4	KM508999, KM508989	OM674419, OM674420
10	Çaycuma-Zonguldak	1	OM674433	OM674419
11	Kızılcahamam-Ankara	1	KM509003	OM674420
12	Ilgaz-Çankırı	4	OM674435, OM674436 OM674437, OM674438	OM674420
13	Küre-Kastamonu	3	KM509005, KM508996	OM674419, OM674422
14	Bürnük-Sinop	2	KM509006	OM674419, OM674420
15	Göktepe-Sinop	4	KM509007, KM509008 OM674431	OM674419, OM674420 OM674423
16	Çakallı-Samsun	1	KM509009	OM674417
17	Ünye-Ordu	2	KM509011	OM674417
18	Gürgentepe-Ordu	2	KM509010, OM674429	OM674424
19	Ulubey-Ordu	2	KM509010, OM674432	OM674417
20	Bulancak-Giresun	1	KM509012	OM674425
21	Bicik-Giresun	1	KM509012	OM674417
22	Barça village-Giresun	2	KM509013	OM674417, OM674424
23	Sümela-Trabzon	3	KM509014, OM674430	OM674417
24	İkizdere-Rize	1	OM674434	OM674426

Laboratory Methods

Genomic DNA was extracted from muscle and kidney tissues of the samples using Doyle [20]'s Cetyltrimethyl Ammonium Bromide (CTAB) isolation protocol. The *Cytb* gene was amplified using the universal primers L14724 and H15915 [21]. Additionally, a 700 bp fragment of the *COI* gene was

amplified using the primers LCO1490 and HCO2198 [22]. Sanger sequencing was performed, and both forward and reverse sequences were obtained for each amplicon. The sequences have been deposited in GenBank under accession numbers OM674417-OM674439 (Table 1). The polymerase chain reaction (PCR) mixture and amplification of the *Cytb* gene region were conducted following the procedures described in Çolak et al. [8]. For the *COI* gene region, the PCR protocol consisted of an initial denaturation step at 96 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min.

Phylogenetic Inferences, Genetic Distances and Network Analyses

The sequences were aligned using the ClustalW alignment tool implemented in BioEdit (ver. 7.2.5) [23]. Unique haplotypes were identified using DnaSP (ver.6.12.03) [24]. We reconstructed the phylogenetic relationships among haplotypes separately for each gene region, *Cytb* and *COI*, using both maximum likelihood (ML) and Bayesian inference (BI) approaches. For ML analysis, we employed IQ-TREE (ver. 1.6) [25]. The best-fitting model of sequence evolution for each gene region was determined by applying the Bayesian information criterion (BIC) in MEGAX. The HKY+G models were chosen for the *COI* and *Cytb* regions, respectively. This model is used for the ML and BI reconstructions. The Nearest-Neighbor Interchange (NNI) algorithm was employed as the tree-building method for ML analysis. Bootstrap resampling (BP) was used to generate the ML tree with 1000 replicates, providing a measure of the tree's robustness. For the Bayesian analysis, we utilized MrBayes (version 3.2.7a) [26]. Separate Markov chain Monte Carlo (MCMC) Bayesian analyses were conducted for *COI* and *Cytb* gene regions. In each analysis, we performed 2 million iterations for the *COI* gene region and 5 million iterations for the *Cytb* gene region to ensure robust results. Empirically determined, the initial 25% of each run was discarded as a burn-in. Bayesian posterior probabilities (BPP) were calculated based on the 50% majority rule consensus of trees sampled every 1000 generations. We used a significance level of $P < 0.01$ for Bayesian posterior probabilities to determine the statistical support for clades. The median-joining networks (MJN) were constructed to illustrate the relationships between haplotypes for each gene region using Network (ver. 10.2) [27]. The genetic distance between the groups appearing in the phylogenetic trees was estimated with the Kimura-2 parameter (K2P) using the MEGA X software. To explore the connection between geographical distances and genetic differentiation among *C. glareolus* populations, we conducted a Mantel test using the IBD: Isolation By Distance v1.52 software [28]. For this purpose, pairwise differences (pi) were computed among three groups representing Eastern haplotypes, Western haplotypes, and Uludağ-Bilecik haplotypes. Distance data between populations were determined by measuring the geographical distances between the Sümela (representing Eastern haplotypes), Şile (representing Western haplotypes), Abant, and Uludağ localities using the distances in kilometres obtained through Google Earth. Uludağ locality was chosen due to the clustering of

haplotypes with Eastern haplotypes in the phylogenetic tree. This allowed us to assess whether geographical distance has an effect on genetic distance among populations.

Demographic History and Neutrality

Nucleotide diversity (π), haplotype diversity (h), and mismatch distribution analyses were assessed using DnaSP (ver. 6.12.03) [24]. The Arlequin software (ver. 3.5.2.2) [29] was utilized for neutrality analyses, including Tajima's D [30] and Fu's Fs [31] tests, which provide insights into population expansion and bottlenecks.

Mutation Rates

The parameter τ (Tau) estimated by DnaSP was utilized to determine the time since the last population expansion. The time (T) elapsed since the last population expansion for each group, based on the studied gene regions, was calculated using the formula "$T = \tau/2u$" (as described by Honda et al. [32]). Three different mutation rates, 0.11, 0.047, and 0.028 substitutions/site/My [32-34], were used to estimate the expansion times of *C. glareolus* groups in Anatolia during the Pleistocene period. According to Ho et al. [35], using mutation rates for molecular dating is particularly suitable when investigating intraspecific variations and time frames within the last 2 million years.

Results

A total of 716 base pairs (bp) of the *COI* gene were analyzed for 63 specimens, along with 1,110 bp of the *Cytb* gene from 32 new specimens. Additionally, 34 *C. glareolus* samples (KM508990-KM509014) from the study of Çolak et al. [8] were included. In total, the *COI* sequences revealed 11 haplotypes, while the *Cytb* sequences exhibited 35 haplotypes.

Phylogenetic Analysis

Phylogenetic trees constructed from the *COI* and *Cytb* gene regions revealed consistent clustering patterns among bank vole populations in Türkiye, placing them in two distinct clades: Clade 1 and Clade 2. Additional clades were identified for populations from other regions, aligning with previous studies (Figures 2 and 3, Appendix C and D). Clade 1 comprises the majority of specimens from the northwestern Anatolia region, while Clade 2 includes specimens from both the southern Marmara region in northwestern Anatolia (including Uludağ, Bursa and Bilecik; haplotypes 01, 02, and 35) as well as from the northeastern Anatolia region. In the *Cytb* tree (Figure 2), both Clade 1 and 2 are moderately to well supported, with moderate bootstrap support for them being sister clades and thus monophyly of Turkish bank voles. In contrast, in the *COI* tree, the monophyly of Turkish bank voles is well supported (85%), but Clade 2 is not recovered as monophyletic, with some haplotypes placed at the base of the entire Turkish clade (Figure 3). Additionally, the phylogenetic trees constructed using the *Cytb* data (no

Balkan haplotypes were present in *COI* data) support clustering of the Balkan and Anatolian populations in one clade (BS=93% and BI=0.88%), consistent with the previous studies.

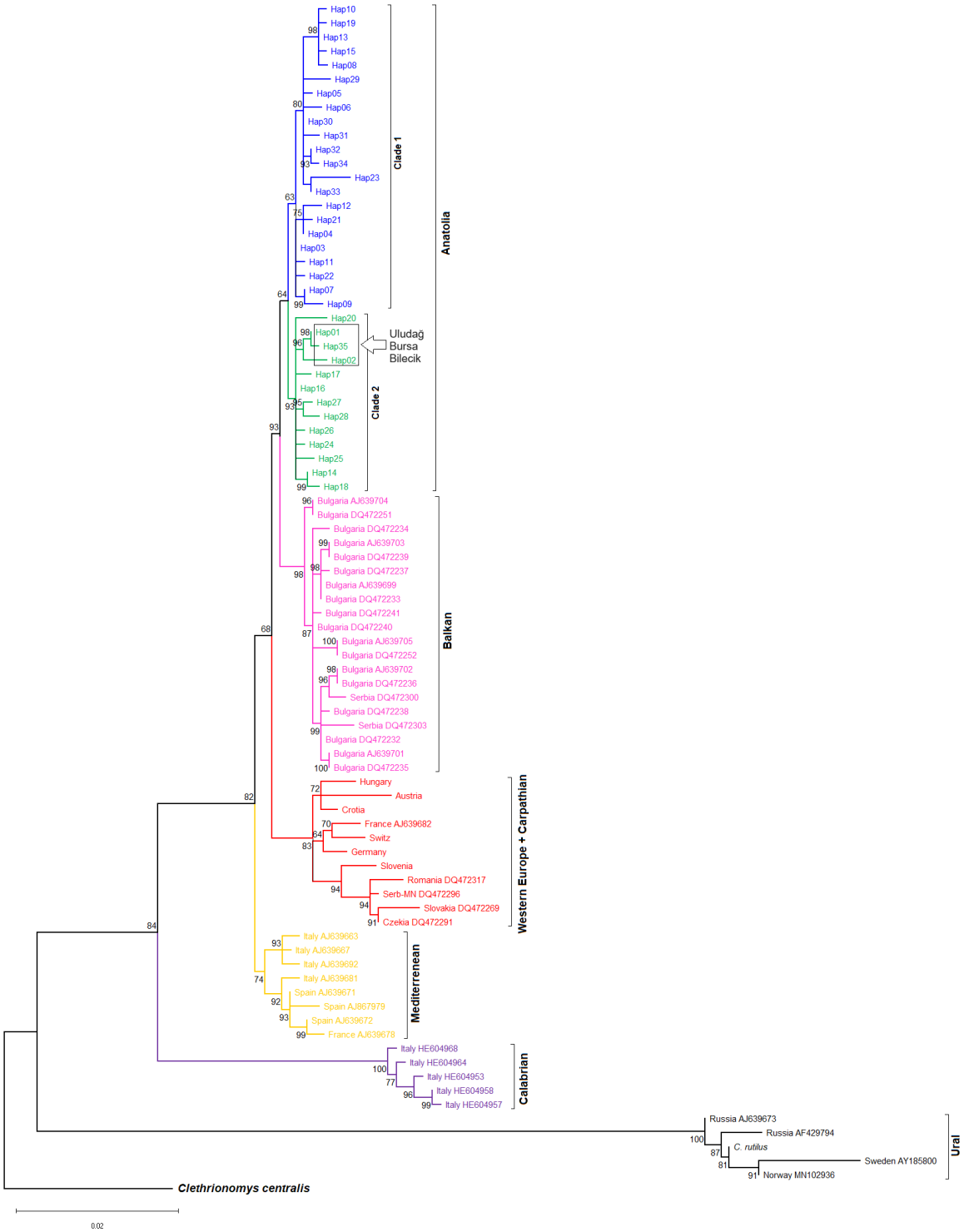

Figure 2. *Maximum likelihood tree obtained for Cytb gene. Clethrionomys centralis is used as an outgroup. Bootstrap supports are showed on each node for each main group*

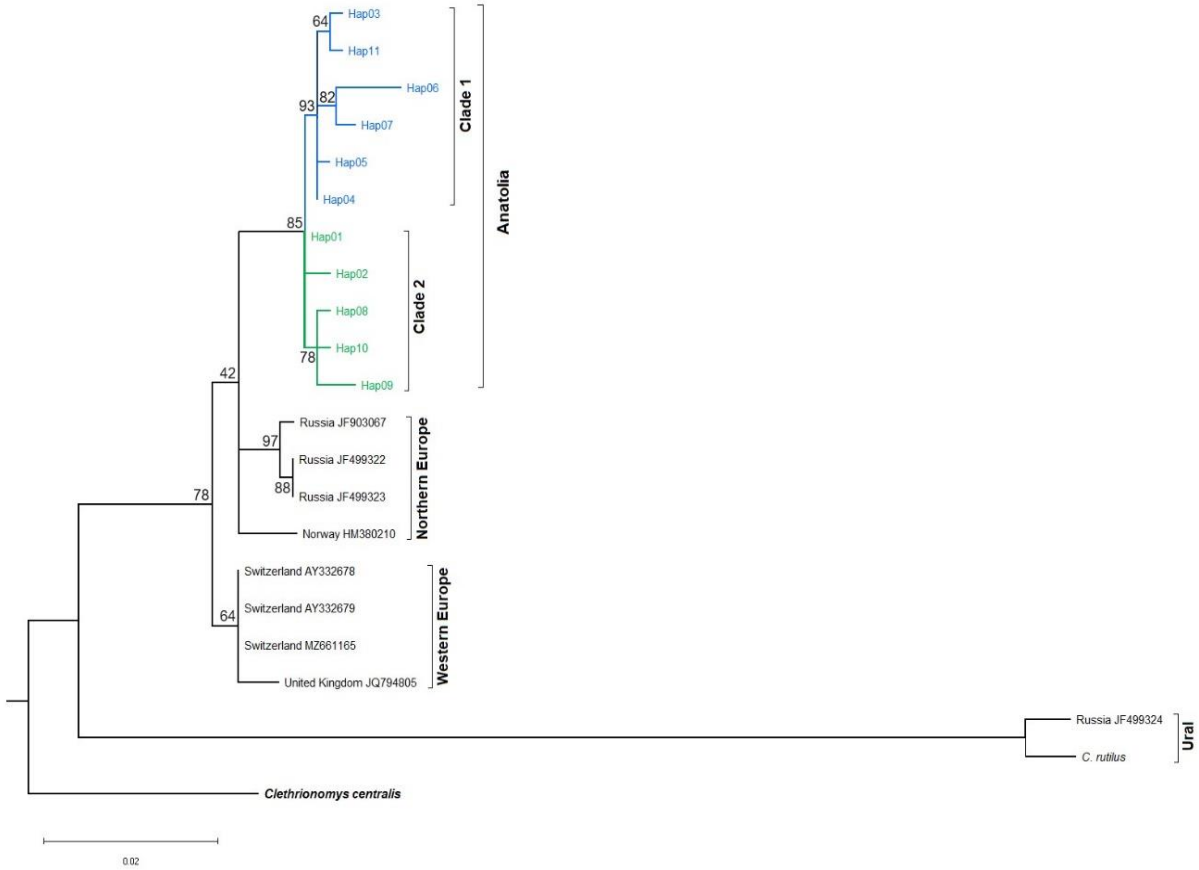

Figure 3. *Maximum likelihood tree obtained for COI gene. Clethrionomys centralis is used as an outgroup. Bootstrap supports are showed on each node for each main group*

In the MJN constructed for *Cytb* and *COI* haplotypes, Turkish haplotypes formed two distinct groups, consistent with the phylogenetic trees, and were separated by two and single mutational steps in the *Cytb* and *COI* MJNs, respectively (Appendix G and F). The *Cytb* groups each exhibited a star-like topology (Appendix G). The group of *Cytb* haplotypes from the Balkans was separated from Clade 1 by three mutational steps (no Balkan haplotypes were present in *COI* MJN), while the European group was differentiated from Clade 2 by six and three mutational steps in the *Cytb* and *COI* networks, respectively.

Genetic Diversity

Haplotype diversity was relatively high for both of the northwestern and northeastern Anatolia samples, while nucleotide diversity was low in both gene regions. In contrast, the Uludağ population showed notably lower haplotype diversity and nucleotide diversity when compared to other populations (Table 2).

Table 2. *Genetic diversity within bank vole populations (N: number of specimens, H: Number of haplotypes, Hd: Haplotype diversity, π: Nucleotide diversity, SD: Standard deviations, NW: northwestern, NE: northeastern)*

Lineage	*Cytb*				*COI*			
	N	H	Hd (±SD)	π (±SD)	N	H	Hd (±SD)	π (±SD)
NW Anatolia	36	22	0.96 (±0.01)	0.003 (±0.0003)	36	22	0.94 (±0.02)	0.003 (±0.0003)
NE Anatolia	15	10	0.92 (±0.05)	0.002 (±0.0005)	15	12	0.96 (±0.04)	0.003 (±0.0005)
Uludag-Bursa	15	3	0.73 (±0.06)	0.002 (±0.0004)	12	2	0.72 (±0.11)	0.002 (±0.0003)

Genetic Distance

The genetic distance within the Turkish clades of *C. glareolus*, as determined by the Kimura 2-parameter (K2P) model, was very low (K2P% = 0.63 for *Cytb* and K2P% = 0.72 for *COI*). However, the K2P value was relatively high between the Turkish and European populations (K2P% = 2.21 for *Cytb* and K2P% = 1.79 for *COI*). Specifically, based on the *Cytb* sequences, 0.98% differentiation was observed between the Balkan and Anatolian populations. (Table 3).

Table 3. *Degree of sequence divergence (in per cent) with Kimura 2-parameter distance for the Cytb and COI between clades (The standard errors (in parenthesis) are based on 10.000 bootstrap replicates of the Kimura 2-parameter)*

Clades	*Cytb*	*COI*
Clade 1/Clade 2	0.63 (0.16)	0.72 (0.21)
Anatolia/Europe	2.21 (0.26)	1.79 (0.46)
Anatolia/Balkan	0.98 (0.23)	-

Mantel Test

The Mantel Test analysis allowed us to assess the correlation between pairwise geographical distances and genetic distances based on the *Cytb* and *COI* gene regions. While the calculated correlation coefficient (r) indicated a negative relationship between geographical and genetic distances, the relationship was not statistically significant ($r = -0.2618$, $P <= 0.7140$).

Mismatch Distribution and Neutrality Tests

Mismatch distribution analysis and neutrality tests were conducted for both *COI* and *Cytb* sequences, considering the presence of two clusters in the phylogenetic tree (Clade 1 and Clade 2). In the case of *COI*, Fu's test statistic FS exhibited significantly negative values different from zero in both Clade 1 ($FS = -16.9$, $P < 0.01$) and Clade 2 ($FS = -10.2$, $P < 0.01$), indicating an excess of recent mutations or rare alleles. However, Tajima's D values for Clade 1 ($D = -0.71$, $P = 0.26$) and Clade 2 ($D = -0.99$, $P = 0.17$)

were not statistically significant. For *Cytb*, Tajima's D and Fu's test statistic Fs displayed significantly negative values different from zero in Clade 1 (D = -1.81, $P < 0.05$, FS = -15.86, $P < 0.01$). Although Tajima's *D* and Fu's test statistic Fs values were negative for Clade 2, the values were not statistically significant (D = -1.54, $P = 0.07$, FS = -4.57, $P = 0.99$). The mismatch distributions of both Clade 1 and Clade 2 were unimodal, providing support for rapid expansion (Figure. 4).

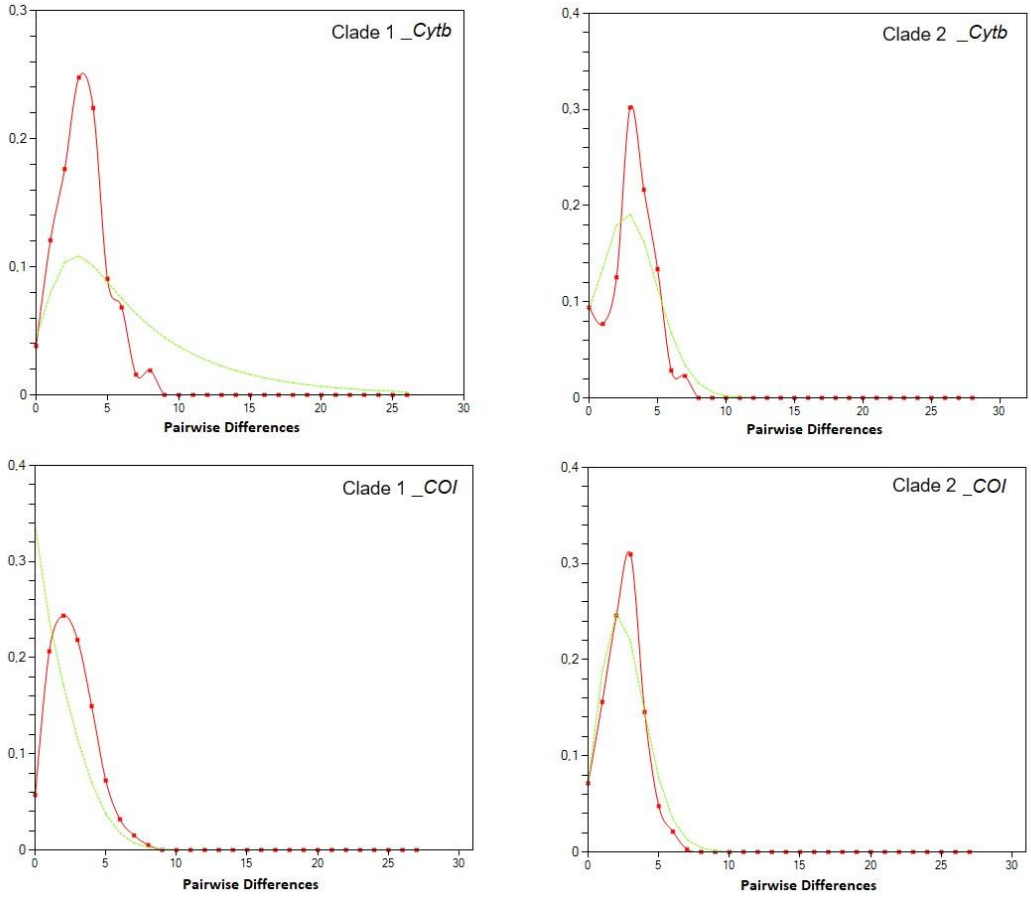

Figure 4. *Observed (red line) and expected (green line) mismatch distributions of the bank vole group for Cytb and COI showing the demographic history in panels.*

Expansion Times

The τ values obtained to estimate the last expansion time of the groups were between 6.126 (Clade 1) and 6.350 (Clade 2) for the *COI* gene region. Based on three different mutation rates (0.11, 0.047, and 0.028 substitutions/site/My), the elapsed time since the last expansion was approximately 40.000, 90.000, and 150.000 years ago, respectively (Table 4). For the *Cytb* gene region, the τ values ranged from 3.237 (Clade 1) to 3.213 (Clade 2), and the estimated expansion times were 13.000, 30.000, and 50.000 years ago (Table 4).

Table 4. *Expansion time estimates inferred from Cytb and COI genes for bank voles from Anatolia with three potential evolutionary rates (* Expansion times of populations were calculated with three different evolutionary rates stated in literature. These evolutionary rates are the rate of mutation in 1 million years, substitutions/site/My. (My, Million years.)*

		Tau (τ)	Expansion times (My)*		
			0.028	0.047	0.11
COI					
	Clade 1	6.126	0.152	0.091	0.038
	Clade 2	6.35	0.158	0.094	0.04
Cytb					
	Clade 1	3.237	0.052	0.03	0.013
	Clade 2	3.213	0.051	0.03	0.013

Discussion

Phylogeny

The bank vole, *Clethrionomys glareolus* has been used as a model organism in many genetic studies to investigate the effects of climatic fluctuations during the Pleistocene period on mammalian species (reviewed by Kotlík et al. [36]). The most comprehensive phylogenetic study to date on the bank vole population in Türkiye was conducted by Çolak et al. [8], using the *Cytb* and *D-loop* gene regions, identifying two haplotype groups in northern Anatolia. They also asserted that despite the distant geographical separation, the Uludag population in southern Marmara showed closer genetic affinity with samples from the northeastern Anatolia rather than the adjacent northwestern Anatolian populations. However, the scarcity of samples from important regions, and the use of the *D-loop* region with its high mutation rate and highly variable nature, may have constrained the study by Çolak et al. [8]. Therefore, this study aimed to reassess the phylogenetic analyses of Anatolian bank vole populations by expanding sampling efforts in the Uludağ region and nearby areas, and comparing results obtained from different additional mtDNA gene. Specifically, the *COI* gene was chosen to explore the long-term connectivity between bank vole populations in Anatolia due to its low mutation rates, contributing to a better understanding of population dynamics and evolutionary processes. The phylogenetic and MJN analyses of the *COI* and *Cytb* datasets consistently support the presence of two main groups within the bank vole population in Anatolia, which aligns with previous studies, including those utilizing RAPD-PCR [17] and *Cytb* [8]. These groups, referred to as Clade 1 and Clade 2, are located in the northwestern Anatolia region (Clade 1) and the southern Marmara region (Uludağ, Bursa and Bilecik) and the northeastern Anatolia region (Clade 2), respectively (Figure 2, 3). The observed lower haplotype and nucleotide diversity in the Uludağ-Bursa-Bilecik population may suggest that this population underwent a stronger bottleneck than other populations (or experienced a stronger selective sweep), potentially during cold periods of the Pleistocene [37]. The clustering of Uludağ-Bursa-Bilecik haplotypes within Clade 2, predominantly found in northeastern Anatolia, is intriguing due to its discontinuous distribution

compared to Clade 1, which is widespread throughout central Türkiye (Figure 1). The minimal haplotype differences within Clade 2 and its star-like structure suggest a recent origin from a single ancestor. It is plausible that Clade 2 initially expanded from a glacial refugium, covering much of northern Anatolia, with subsequent replacement of central populations by those from Clade 1. Whether this replacement involved only mtDNA or entire populations cannot be determined from the present data. Similar population replacements, initially identified through mtDNA phylogeography, have been documented in other regions of the bank vole range, such as Britain [38, 39] and Fennoscandia [40]. This evidence suggests that population dynamics in bank voles have been climate-driven, reflecting adaptations of populations from different refugia to varying environmental conditions [41]. An alternative explanation that the Uludağ-Bursa-Bilecik populations share a more recent ancestor with those of Clade 1 and the discordance between phylogeny and geography is due to incomplete lineage sorting, seems less likely. This is indicated by the small differences among Clade 2 haplotypes, suggesting a recent common ancestor between Uludağ-Bursa-Bilecik and northeastern Anatolia. Our results do not support the role of altitude in the observed geographic structure suggested by isoenzyme studies [16] as the high-altitude populations (altitude 1020-1650 m above sea level) in Abant (Hap09-Hap10), Kartepe (Hap 29-Hap30), and Ilgaz (Hap 31-Hap34) were clustered with populations at lower altitudes. These results highlight the complexity of population structure and genetic diversity in bank voles in Anatolia and possible differences between mtDNA and nuclear genetic structure that require further investigation to uncover the underlying factors shaping these patterns. In phylogenetic analyses by Ledevin et al. [42] the specimens from Uludağ (the only bank vole locality from Türkiye included in that study) were clustered with the Balkan lineage. In our study, the samples from Uludağ were grouped together with specimens from the northeastern Anatolia region and the remaining samples from northern Anatolia. Our findings are consistent with Ledevin et al. [42] in that the Anatolian clades form a sister clade to the Balkan clade, highlighting regional genetic affinities within the bank vole populations across these areas.

Genetic Divergence

The genetic distance within the Turkish clades of *C. glareolus*, as determined by the Kimura 2-parameter (K2P) model, was remarkably low (K2P% = 0.63 for *Cytb* and K2P% = 0.72 for *COI*). These values indicate a relatively low genetic divergence among the studied populations. Similar patterns of low genetic divergence have been observed in other rodent species distributed in Anatolia, such as squirrels and hamsters [43-46]. According to Baker and Bradley [47], populations with genetic divergence values below 2% are unlikely to represent different species. While the genetic divergence values suggest the specimens within the Turkish groups of bank vole are closely related, the lack of shared haplotypes between the two clades indicates that ongoing gene flow between these populations is unlikely. However, comparatively higher K2P values were observed between the Turkish and European

populations (K2P% = 2.21 for *Cytb* and K2P% = 1.79 for *COI*). Specifically, based on the *Cytb* sequences, a distance of 0.98% was observed between the Balkan and Anatolian populations.

Past Population Demography

The nucleotide and haplotype diversities observed in the Spanish and Italian phylogroups of the bank voles were low, attributed to population fragmentation during glaciations [4]. In contrast, the two groups in Anatolia exhibited high haplotype diversity and low nucleotide diversity in both gene regions, which could indicate a rapid expansion following the Last Glacial Maximum (LGM) [37]. The MJN constructed from *Cytb* haplotypes revealed a star-like topology for each of the two Turkish clades, which may have been a result of such an expansion. Consistent with such a scenario, Tajima's *D* was found to be significantly negative only for *Cytb* sequences, supporting the evidence of recent demographic expansion in Clade 1. Although Tajima's *D* was negative in Clade 2 for *Cytb* and in all groups for *COI*, the values were not statistically significant. On the other hand, Fu's F_S was negative in all groups for both *Cytb* and *COI*, but it was statistically insignificant only in Clade 2 for the *Cytb* gene. The differences between Tajima's *D* and Fu's F_S values can be attributed to the calculation of these two neutrality tests using different parameters. Tajima's *D* estimates the nucleotide differences in the sequences, while Fu's F_S considers the haplotype diversity in the population [48]. In this study, nucleotide diversity in the bank vole groups was found to be lower than haplotype diversity, leading to differences in the neutrality calculations. Moreover, the number of segregating sites (S: segregating sites) differed between the gene regions. The highest number of segregating sites was observed in the *Cytb* sequences of Clade 1 (S=28), which explains why Tajima's *D* value was significantly negative only in this group. The significantly negative Fu's Fs values suggest an excess of rare haplotypes, likely reflecting a recent population expansion following a bottleneck, while the less significant Tajima's D values indicate that the nucleotide diversity has not fully recovered from past events. This discrepancy underscores the complex demographic history of *C. glareolus*, with the combination of these tests providing a more nuanced understanding of population growth after the Last Glacial Maximum.

Timing of Population Expansion

Based on the population expansion analyses conducted in our study, the bank vole populations in Anatolia exhibit a complex demographic history shaped by the climatic fluctuations during the Pleistocene. The presence of glacial refugia and subsequent population expansions during interglacial periods has been documented in various species [1, 6, 49, 50]. We estimated the expansion time of bank vole groups in Anatolia by analyzing mismatch distribution and using various mutation rates reported in literature. The results consistently indicated rapid population growth towards the end of the Pleistocene, with estimates ranging from approximately 13.000 to 50.000 years ago (MIS1 and MIS3) based on *Cytb* sequences, and from approximately 40.000 to 150.000 years ago (MIS3 and MIS6) based

on *COI* sequences. These patterns of expansion likely indicate the dynamic response of bank vole populations in Anatolia to changing climatic conditions over time. The discrepancies between the two gene regions may be attributed to differences in mutation rates. Further studies should consider specific mutation rates for the *COI* gene in expansion time analyses, as previous studies have focused primarily on *Cytb* gene analyses [32-34].

Geographic Barriers

Geographic barriers in northern Anatolia, including the major river systems Sakarya River, Kızılırmak River, Melet stream, İkizdere stream, and Izmit Gulf–Lake Sapanca–Sakarya Valley, have been implicated in shaping the phylogeography of various animal species during the Plio-Pleistocene period [50-55]. However, our phylogenetic analysis and the relatively low support values suggest that these barriers did not play significant roles in separating bank vole populations in Anatolia. The Melet River, specifically examined in relation to dormouse populations [53], did not appear to have acted as a strong barrier affecting the phylogenetic patterns of bank voles. Similarly, the closure of the Izmit Gulf-Lake Sapanca-Sakarya Valley waterway approximately 11.7 thousand years ago [15] did not seem to act as a barrier for the bank vole population in the Sakarya Valley (Localities 4-8 in Figure. 1), as indicated by the clustering of specimens from surrounding locations (Hap03, Hap04 and Hap12 for *Cytb*, Hap03-Hap07 for *COI*). This suggests a potential rapid westward expansion of Clade 1 specimens following the closure of the waterway. In the case of the Kızılırmak River, although considered a potential barrier for gene flow in the region [50, 55], haplotypes from the localities at the west of the river such as Bürnük and Göktepe (Hap13, Hap15, and Hap19 for *Cytb*; Hap04 for *COI*) and those from Çakallı on the east (Hap26 for *Cytb*; Hap01 for Çakallı) were found in different clades in both *Cytb* and *COI* phylogenetic trees. This discrepancy underscores the need for more comprehensive sampling and the use of microsatellite markers to detail the polymorphism between haplotypes on either side of the Kızılırmak River. On the other hand, the absence of bank vole specimens in the eastern part of the İkizdere stream suggests the need for further investigations in this region to determine the relationships of bank voles in this area.

Conclusion

Our examination of mitochondrial DNA variation in bank voles from northern Türkiye has yielded several noteworthy findings. Phylogenetic and network analyses of *Cytb* and *COI* sequences support the existence of two genetically distinct clades in northern Anatolia, which are closely related to the Balkan populations from Europe. These Turkish clades likely occupied major refugia during the Pleistocene glaciations, one in the western part and the other in the eastern part of northern Anatolia. Evidence of recent population expansion and the broadly estimated times of this expansion highlight the impact of climatic fluctuations towards the end of the Pleistocene on bank vole demography in Türkiye. The close relationship between the Uludağ-Bursa-Bilecik haplotypes and those from northeastern Anatolia

indicates a recent common ancestor for the populations from these regions. This suggests that the survival of a relic population in the vicinity of Uludağ, probably after a mitochondrial DNA or population replacement in northern Anatolia. Main Turkish rivers except for the Kızılırmak River, do not seem to have acted as strong long-term barriers inhibiting gene flow among bank vole populations. The clustering of haplotypes near the Kızılırmak River in different clades suggests it may influence gene flow, pointing to the need for further detailed analysis with increased sampling. These insights not only deepen our understanding of the evolutionary history of bank voles in this region but also underscore the complex interplay between geographic and climatic factors in shaping genetic diversity and structure. Moving forward, integrating nuclear DNA markers or genome-wide diversity studies would allow for a more comprehensive understanding of both historical and contemporary population dynamics. Such studies could also help to uncover the roles of selective pressures on these populations, particularly in light of current climatic changes.

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Conflict of Interests No conflict of interest or common interest has been declared by the authors.

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