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Complete mitogenomes of Turkish tree squirrels, *Sciurus anomalus* and *S. vulgaris*, (Sciuridae: Rodentia: Mammalia) and their phylogenetic status within the tribe Sciurini

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ABSTRACT

The genus *Sciurus*, a member of the family Sciuridae, is widely distributed in the Holarctic region. To better understand mitogenomic characteristics and to reveal internal phylogenetic relationships of the genus, 20 complete mitogenomes of Turkish tree squirrels were successfully sequenced for the first time, including 19 for *S. anomalus* (from 16,505 bp to 16,510 bp) and one for *S. vulgaris* (16,511 bp). The mitogenomes of two species were AT-biased. All tRNAs for two species displayed a typical clover-leaf structure, except for $tRNA^{Ser(AGY)}$. The tRNA Serine1 (S1)-GCT structure lacked the dihydrouridine (DHU) loop and stem. Based on mitogenomic dataset for phylogeny of Sciurinae, phylogenetic analyses (Bayesian Inference and Maximum Likelihood) did not support monophyly of *Sciurus* and proposed that *S. anomalus*, the most basal taxa in the Sciurini tribe, had at least five mitogenome lineages, which were also supported by network analysis. The dissimilarities among the five lineages of *S. anomalus* ranged from 0.0042 (0.42%) to 0.0062 (0.62%) using K2P sequence pairwise distances. In addition to this mitogenomic analysis result, phylogenetic analyses using the *CYTB* + D-loop dataset proposed the existence of at least nine lineages for *S. anomalus*, which was different than those of the previous studies. The current study proposed that the use of mitogenomic data for reconstructing the phylogeny of Turkey's *Sciurus* holds an important value for revealing evolutionary relationships.

1. Introduction

Sciurus is one of 50 genera within the family Sciuridae, covering tree squirrels. The genus *Sciurus*, clustered into the tribe Sciurini, is widely distributed in Eurasia, North and South America, and also Australia introduced by humans, having seven subgenera and at least 28 species. The species diversity of tree squirrels is at its maximum level in the Nearctic and Neotropical zoogeographical regions, where 25 species are distributed. In particular, the Neotropical region is home to 20 species. However, *S. anomalus*, *S. lis* and *S. vulgaris* are natively distributed in the Palearctic region, whereas *S. carolinensis* was introduced into Britain, Scotland, Ireland and Italy by humans (Thorington and Hoffman, 2005; IUCN, 2020). Additionally, Wauters et al. (2017) reported that *S. meridionalis* is distributed in South Italy.

In the last two decades, to better understand molecular evolution and phylogeny of the genus *Sciurus*, mitogenomes of 14 species (*S. aberti*, *S. anomalus*, *S. arizonensis*, *S. carolinensis*, *S. colliaei*, *S. granatensis*, *S. griseus*, *S. ignitus*, *S. lis*, *S. nayaritensis*, *S. pucheranii*, *S. variegatoides*,

Abbreviations: CYTB, Cytochrome *b*; PCR, Polymerase Chain Reaction; O_L, origin of light-strand region; NCBI, National Center for Biotechnology Information; *Rag1*, Recombination activating gene 1; *C-myc*, Myelocytomatosis; *COI*, Cytochrome Oxidase Subunit I.

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S. vulgaris, and *S. yucatanensis*) have been sequenced and annotated in gene order (Reyes et al., 2000; Madsen et al., 2015; Jin et al., 2016; Kim et al., 2017; Abreu-Jr et al., 2020; Anonymous, 2020; Boukhdoud et al., 2021; Margaryan, 2019). The low species diversity of the Eurasian *Sciurus* in the Palearctic region has attracted phylogenetically less attention, and no recent attempt has been made to assess the phylogenetic relationships of the Eurasian species (*S. anomalus* and *S. vulgaris*) based on mitogenomic sequences. Of the Eurasian species, although there are studies including the *S. vulgaris* mitogenomes (Reyes et al., 2000; Madsen et al., 2015; Kim et al., 2017; Abreu-Jr et al., 2020; Anonymous, 2020; Margaryan, 2019), a first study including the *S. anomalus* mitogenome that is a partial mitogenome was performed by Boukhdoud et al. (2021) from Lebanon in the species range.

Mitochondrial genome or mitogenome is commonly used as a molecular tool in studies such as genetic diversity, phylogeography, phylogeny, and population structure of animals (Anderson et al., 1981; Moritz, 1994; Wallace, 1995; Ankel-Simons and Cummins, 1996; Sorenson et al., 1999). Since the last decade, numerous studies have especially focused on sequencing and using complete mitogenomes rather than a single gene or partial sequences of mitochondrial DNA in explaining phylogenetic relationships among or within mammalian and other vertebrate species (Thalmann et al., 2013; Filipi et al., 2015; Ding et al., 2016a,b; Frank et al., 2016; Sarvani et al., 2018; *etc.*). When compared with short or highly conserved mitochondrial sequences at locus level that provide less phylogenetic information, complete mitogenomes are more useful and informative (Fenn et al., 2008; Jacobsen et al., 2012; Barbosa et al., 2018).

Of the *Sciurus* species, two species (*Sciurus anomalus* and *S. vulgaris*) are distributed in Turkey and inhabit in woodlands, broad-leaved forests, coniferous forests, and mixed forests (Kryštufek and Vohralik, 2005; Shar et al., 2016; Yigit et al., 2016). *S. anomalus* is distributed throughout the Anatolian part of Turkey (also introduced to the Belgrad Forest near Istanbul in the Turkish Thracian), whereas *S. vulgaris* is found in the Turkish Thracian and was introduced to the eastern-most Black Sea Mountains in Northeast Turkey (Kryštufek and Vohralik, 2005, 2009). Although numerous phylogeny studies included the tree squirrels under the name *Sciurus* throughout the genus' range (Oshida and Masuda, 2000; Oshida et al., 2009; Pečnerová and Martínková, 2012; Villalobos and Gutierrez-Espeleta, 2014; Asadi Aghbolaghi et al., 2019, 2020a,2020b; *etc.*), there are currently three studies investigating the phylogenetic relationships of the Turkish *Sciurus* populations (Oshida et al., 2009; Asadi Aghbolaghi et al., 2019; Demirtaş, 2022). While Oshida et al. (2009) used only *CYTB* sequences from a small number of samples for the Turkish *S. anomalus*, Asadi Aghbolaghi et al. (2019) used both mitochondrial (*COI*, *CYTB* and D-loop) and nuclear (*Rag1* and *C-myc*) DNA sequences obtained from more samples from a large part of the Anatolian geography for the Turkish *S. anomalus*. The Turkish samples of *S. anomalus* were more closely related to the American species (The New World *Sciurus*: *S. aestuans*, *S. carolinensis*, *S. niger* and *S. stramineus*) in the study of Oshida et al. (2009), whereas the Turkish *S. anomalus* samples were clustered into one lineage with three groups in the study of Asadi Aghbolaghi et al. (2019). In the last study based on the network and phylogenetic analyses by used four mitochondrial regions (*CYTB*, *tRNA-Thr*, *tRNA-Pro*, and partial D-loop), Demirtaş (2022) reported that the Turkish *S. anomalus* consisted of eastern and western mitochondrial lineages, which were grouped with the Greek and Lebanese haplotypes, respectively. In this context, molecular genetic studies for Turkey's *Sciurus* are relatively limited (Oshida et al., 2009; Asadi Aghbolaghi et al., 2019; Demirtaş, 2022) as well as mitogenomic data for *S. anomalus* is scarce (Boukhdoud et al., 2021).

In the current study, 20 complete mitogenomes of Turkish *Sciurus* were successfully sequenced by means of the high-throughput next-generation sequencing technology (NGS). We aimed: (i) to characterize mitogenomes of Turkish *Sciurus*; *S. anomalus* and *S. vulgaris*, (ii) to review molecular phylogenetics presenting further molecular insights into the tribe Sciurini phylogeny that could be used as a framework for further evolutionary studies by comparing to partial and complete mitogenomes of the 14 *Sciurus* species, and also (iii) to compare intra-specific relationships of *S. anomalus* with the results of previous studies using *CYTB* + D-loop sequences.

2. Materials and methods

2.1. Sampling and genomic DNA extraction

Tissue samples such as tail and muscle were obtained from 21 dead individuals of Turkey's *Sciurus* (Fig. 1 and Table 1), and they were used to extract genomic DNA (gDNA) by using the DNeasy Blood & Tissue Kit (QIAGEN, Germany), following the manufacturer' protocol. The extracted gDNAs were standardized to ~ 25 ng/μL with sterile water and then stored at −20 °C until mitogenome amplification. The Qubit™ dsDNA BR Assay Kit, the Qubit® 2.0 Fluorometer Quantitation Platform (Invitrogen, Thermo Fisher Scientific), and a 1% agarose gel electrophoresis stained with ethidium bromide were used to assess concentration and quality of gDNA. The tissue samples of the Turkish *Sciurus* are stored at Department of Biology, Faculty of Sciences, Erciyes University in Kayseri, Turkey.

2.2. Long-Range PCR amplification

A Long-Range PCR method with NEB LongAmp® Taq 2x Master Mix (M0287S, NEB), following the protocols of the manufacturer, was used to amplify complete mitogenomes of the Turkish tree squirrels. PCR products for two overlapping fragments of ~ 11 kb and ~ 7.5 kb in length were generated by using two primer pairs; (i) CrocAL1-2024L: 5′-GACCGTGCAAAGGTAGCATAATC-3′ – CrocBH1-13002H: 5′-TAATTAAAAGGGCTCAGGCGTTG-3′, and (ii) ScVu-11712L: 5′- AGAAGTAATCCATTGGTCTTAGGA-3′ - LuLu-2503H: 5′-CTCAGATCACGTAGGACTTTAATC-3′. The reaction mixture Long-Range PCR amplifications was in a 25 μL volume, including 12.5 μL of LongAmp Taq 2 × Master Mix, 2 μL for each of 10 μM forward and reverse primers, 1 μL (~25 ng) of gDNA, and 7.5 μL of ddH_2O. Thermal cycling conditions included a pre-denaturation step of 1 cycle at 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 30 sec, 30 cycles of annealing at 58/55 °C for 30 sec, 30 cycles of extension at 65 °C for 11/7.5 min, and a final extension step of 1 cycle at 65 °C for 10 min. No gDNA (negative control) was included during the PCR procedures for detecting possible contamination. An agarose gel of 1% was to run 5 μL of the PCR. We quantified PCR products using 4 μL of the amplicons and the Qubit dsDNA BR Assay Kit (Cat. No: Q32850, Invitrogen, Thermo Fisher Scientific, USA). We diluted the PCR products to a concentration of 0.2 ng/μL with sterile dH_2O, and then used 1 ng of amplicon (5 μL in volume) to prepare the sequencing library products. An AMPure XP kit (Beckman Coulter) was used to purify the PCR products.

2.3. Preparation of Libraries, sequencing and data analysis

Next-generation sequencing libraries were constructed by using Nextera XT DNA Library Prep Kit (Illumina, San Diego, USA) and Nextera XT DNA Library Preparation Index Kit v2 Set A (Cat. No: FC-131–2001, Illumina, San Diego, USA), following the manufacturer's protocols. Quantity of each library was normalized with bead-based normalization. The library loading volume and concentration were determined by using Quibt dsDNA HS Kit. Sequencing of the library was carried out by using MiSeq Reagent Kit v2 (500 cycles) (Illumina) and MiSeq platform (Genome and Stem Cell Centre, GenKok, Erciyes University). Analysis of the obtained raw sequences extracted in the FASTQ format was verified for quality by means of Geneious Prime® 2021.0.1 software (Kearse et al., 2012). Trimming and quality filtering operations contained short readings (<30 bp), low quality bases (Q-score < 20) and adapters in raw sequences, which were performed using BBDuk Trimming Tool in the Geneious Prime software. In the next step, clear and

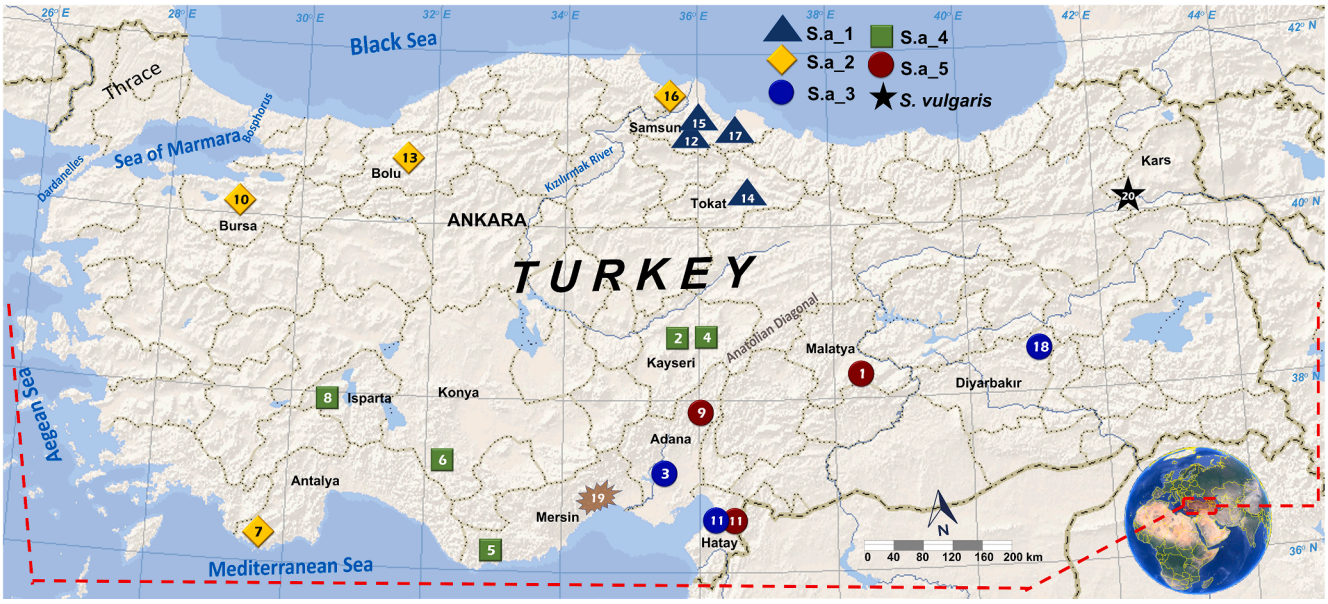

Fig. 1. Map displaying collection localities of Turkish *Sciurus* samples. S.a_1 – S.a_5 refer to lineages in Fig. 3, Fig. 4 and supplement data 5. The locality 19 belongs to the sample that failed mitogenome sequencing as shown in Table 1.

Table 1
A list of Turkish *Sciurus* samples amplified complete mitogenomes using Long-Range PCR (Nr.: number).

Map Nr. (Fig. 1)	Taxon	Gender (♀/♂)	Collection Nr.	Haplotype code	Accession Nr.	Mitogenome in length	Locality
1	*S. anomalus*	♂	78	S.a_Tr.1	ON620273	16,506 bp	Malatya, Turkey
2	*S. anomalus*	♀	177	S.a_Tr.2	ON620274	16,507 bp	Hacılar, Kayseri, Turkey
3	*S. anomalus*	♀	308	S.a_Tr.3	ON620275	16,509 bp	Adana, Turkey
4	*S. anomalus*	♀	345	S.a_Tr.2	ON620274	16,507 bp	Hisarcık, Kayseri, Turkey
5	*S. anomalus*	♂	486	S.a_Tr.4	ON620276	16,510 bp	Anamur, Mersin, Turkey
6	*S. anomalus*	♀	522	S.a_Tr.5	ON620277	16,509 bp	Yazdamı, Bozkır, Konya, Turkey
7	*S. anomalus*	♂	1251	S.a_Tr.6	ON620278	16,508 bp	Yeşilköy, Kaş, Antalya, Turkey
8	*S. anomalus*	♂	1498	S.a_Tr.7	ON620279	16,509 bp	Isparta, Turkey
9	*S. anomalus*	♂	1625	S.a_Tr.8	ON620280	16,508 bp	Konakkuran Köyü, Feke, Adana, Turkey
10	*S. anomalus*	?	1734	S.a_Tr.9	ON620281	16,507 bp	Uludağ, Bursa, Turkey
11	*S. anomalus*	♂	1754	S.a_Tr.10	ON620282	16,506 bp	Yayladağ, Hatay, Turkey
11	*S. anomalus*	♀	1755	S.a_Tr.11	ON620283	16,505 bp	Yayladağ, Hatay, Turkey
12	*S. anomalus*	♂	1756	S.a_Tr.12	ON620284	16,510 bp	Celallı Köyü, Kavak, Samsun, Turkey
13	*S. anomalus*	?	1770	S.a_Tr.13	ON620285	16,508 bp	Abant, Bolu, Turkey
14	*S. anomalus*	?	1771	S.a_Tr.14	ON620286	16,510 bp	Tokat, Turkey
15	*S. anomalus*	?	1772	S.a_Tr.15	ON620287	16,508 bp	Nebian, Samsun, Turkey
16	*S. anomalus*	?	1785	S.a_Tr.16	ON620288	16,506 bp	Emenli, Bafra, Samsun, Turkey
17	*S. anomalus*	?	1823	S.a_Tr.17	ON620289	16,507 bp	Çarşamba, Samsun, Turkey
18	*S. anomalus*	?	1885	S.a_Tr.18	ON620290	16,509 bp	Kulp, Diyarbakır, Turkey
19	*S. anomalus*	?	1769	———	———	Failure	Erdemli, Mersin, Turkey
20	*S. vulgaris*	?	1418	S.v_Tr.1	ON620291	16,511 bp	Sarıkamış, Kars, Turkey

quality reads were assembled to the mitogenome of *Sciurus vulgaris* (NC_002369: Reyes et al., 2000) using Geneious Mapper Algorithm (with fallowing parameters; Sensitivity: Highest sensitivity/Medium, Fine Tuning: Iterate up to 25 times), and contig sequences were obtained, and gene annotations were carried out. Moreover, gene borders were checked by using MITOS2 (Bernt et al., 2013) and manually curated. Tandem Repeats Finder Web Server (Benson, 1999) was used to determine the repeated sequence motifs in the control region (D-loop). AT and GC Skew analyses were performed with formulas [A − T]/[A + T] and [G − C]/[G + C], respectively. All the mitogeneomic sequences were aligned with MAFFT multiple sequence alignment algorithm (Katoh et al., 2002).

2.4. Evolutionary analyses

Phylogenetic analyses were performed using two data sets consisting of complete mitogenomes and mitochondrial *CYTB* + D-loop sequences, which were presented in Table 1 and supplement data 1. Firstly, we analyzed a dataset consisting of complete mitogenomes, which included 65 sequences from the subfamily Sciurinae species obtained from the GenBank database plus 20 sequences from the Turkish tree squirrels. Secondly, we extracted *CYTB* + D-loop sequences from the mitogenomes of *Sciurus anomalus* and included them in a dataset. MAFFT algorithm (Katoh et al., 2002) in Geneious Prime, using default parameters (Algorithm: Auto, Scoring matrix: 200 PAM/K = 2, Gap open penalty: 1.53, Offset value: 0.123), was used to align for mitogenome and *CYTB* + D-loop datasets. Haplotypes for two datasets were determined by means of DnaSP Ver. 5.10.01 (Librado and Rozas, 2009) and then used to carry out phylogenetic analyses by using two methods (BI: Bayesian Inference and ML: Maximum Likelihood). The best-fitting evolutionary model of nucleotide substitution for the phylogenetic analyses was GTR + G + I for two datasets, which was chosen with the jModeltest v.2.1.10 program (Darriba et al., 2012) based on the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). MrBayes v.3.2.6

(Ronquist et al., 2012) was used to perform Bayesian analyses, where posterior probabilities (pp) were calculated by using four MCMC (Markov Chain Monte Carlo) chains iterated for 3 million and 4 million generations for the mitogenome and the *CYTB* + D-loop datasets, respectively, with trees sampled every 100 generations. After evaluating convergence with average standard deviation of split frequencies < 0.01 and discarding the first 10% as burn-in phase, a 50% majority rule consensus tree was calculated 95% Bayesian credibility intervals and posterior probabilities. FigTree v1.3.1 (Rambaut, 2009) was used to draw tree diagrams obtained from the Bayesian analyses. The ML analyses, with 10,000 bootstrap replicates for the mitogenomes and the *CYTB* + D-loop dataset, were performed by using MEGAX (Kumar et al., 2018). The complete mitogenomes of two species from the family Dipodidae (*Scarturus williamsi*: NC_049035 and *Dipus sagitta*: NC_027499) and the mitochondrial *CYTB* + D-loop sequences of three species from the family Sciuridae (*Sciurus ignitus*: NC_050032; *S. carolinensis*: NC_050012; *Simosciurus stramineus*: NC_050024) were used as outgroups. By using mitogenomes, haplotype network for *S. anomalus* was drawn by using median-joining method by means of PopART v.1.7 (*http://popart.otago.ac.nz*; Bandelt et al., 1999; Leigh and Bryant, 2015).

Genetic distances among mitogenome haplotypes of Turkey's *Sciurus* and mitogenome lineages of *S. anomalus* were calculated by using the Kimura 2-Parameter (K2P) model (Kimura, 1980), with a bootstrap method (10,000 replicates) in MEGAX (Kumar et al., 2018).

3. Results

3.1. Complete mitogenome sequences of Turkish tree squirrels

Of 21 Turkish tree squirrels, 20 complete mitogenomes were successfully sequenced by means of NGS platform, with minimum 1,924 × and maximum 19,485 × coverage, but one sample was not sequenced due to the highly degradation of sample. The mitogenomes of the Turkish tree squirrels included 19 different haplotypes, in which 18 haplotypes (S.a_ Tr.1 – S.a_ Tr.18; accession number: ON620273 – ON620290) and one haplotype (S.v_Tr.1; accession number: ON620291) belonged to *Sciurus anomalus* and *S. vulgaris*, respectively. In addition, two mitogenomes from Kayseri in Central Anatolia that belonged to *S. anomalus* shared the same mitogenome sequence (S.a_Tr.2, ON620274). The mitogenomes of *S. anomalus* and *S. vulgaris* ranged from 16,505 bp to 16,511 bp in length and had probably small length variation due to short deletion(s) / insertion(s) in the control region (D-loop), and/or in the rRNA and/or tRNA genes (Table 1 and Table 2). Based on K2P sequence pairwise distances of mitogenome haplotypes, the dissimilarity between *S. anomalus* and *S. vulgaris* was at least 16.45%, ranging from 0.1645 (16.45%) to 0.1659 (16.59%) with an average of 0.1652 (16.52%). Pairwise distances within the Turkish *S. anomalus* ranged from 0.0002 (0.02%) to 0.0072 (0.72%).

3.2. Characterization of mitogenomes

For the mitogenome features of Turkey's *Sciurus*, we presented the general characteristics of only one mitogenome for each of the two species: *S. anomalus* (S.a_Tr.2, ON620274) and *S. vulgaris* (S.v_Tr.1, ON620291). The complete mitogenomes of Turkey's *Sciurus* included 37 coding regions; 13 protein-coding genes (PCGs), 22 tRNAs and two rRNAs, a non-coding region; control region (D-loop) and an origin of light-strand region (O_L) (Fig. 2). The 12 PCGs, 14 tRNAs, 2 rRNAs, O_L origin and D-loop were located on heavy chain (*H*), whereas the one PCG and 8 tRNAs were located on light chain (*L*) (Fig. 2 and Table 2). The *S. anomalus* mitogenome had seven overlapping regions with a total length of 73 bp, ranging from 1 to 43 bp, and 12 intergenic spaces with a total length of 42 bp, ranging from 1 to 8 bp. In the *S. anomalus* mitogenome, the longest intergenic region was between *ATP8* and *ATP6* genes, and it was 43 bp in length (Table 2). In the *S. vulgaris* mitogenome, there were seven overlapping regions with a total length of 73 bp, ranging from 1 to 43 bp, and 12 intergenic spaces with a total length of 39 bp, ranging from 1 to 8 bp. And also, the longest intergenic region was between *ATP8* and *ATP6* genes, and it was 43 bp in length (Table 2). Additionally, no tandem repeat elements were detected in both of the Turkish *Sciurus* mitogenomes. The *S. anomalus* and *S. vulgaris* mitogenomes comprised intergenic spaces and overlapping regions among the PCGs and the tRNA genes (Table 2).

3.3. Nucleotide compositions of mitogenomes

Nucleotide composition features of *S. anomalus* and *S. vulgaris* mitogenomes were presented in Table 3, in which rate of Adenine (A) was the highest in nucleotide composition, but rate of Guanine (G) was the lowest (for the mitogenome haplotypes of both species; A > T > C > G). According to Skew analysis, G/C Skews were negative for the complete mitogenomes, the PCGs, the tRNAs, the rRNAs and the D-loops. On the other hand, A/T Skews were positive for the complete mitogenomes, the tRNAs and the rRNAs, whereas A/T Skews were negative for the PCGs and the D-loops (Table 3).

3.4. Codon usage and protein-coding genes of mitogenomes

The values of Relative Synonymous Codon Usage (RSCU) for the Turkish *Sciurus* mitogenomes were displayed in Supplementary Fig. S1. Regarding the number of codon usage, the three amino acids [Leucine (624 and 615), Isoleucine (347 and 341), and Serine (305 and 302)] for the both species had the highest frequency, while Cysteine (27 and 26) had the lowest frequency (supplement data 2). In 10 of the 13 PCGs, start / initiation codon was ATG, and that of the two PCGs was ATA (*ND3* and *ND5*). In *ND2* from the PCGs, start codon for *S. anomalus* and *S. vulgaris* was ATC and ATT, respectively. Moreover, in the mitogenomes of the two species, T– was an incomplete stop codon for *COX3*, *ND2* and *ND4*, TA- was an incomplete stop codon for *ND1* and *ND3* (Table 2).

3.5. Transfer RNA and ribosomal RNA genes, control region and origin of replication for the light strand of mitogenomes

The mitogenomes of *S. anomalus* and *S. vulgaris* included 22 tRNA genes that have different in total length, 1.507 bp and 1511 bp, respectively. The six tRNAs ($tRNA^{Ala}$, $tRNA^{Lys}$, $tRNA^{Gly}$, $tRNA^{Arg}$, $tRNA^{Thr}$, and $tRNA^{Pro}$) of the two species also were different in length (Table 2). When compared secondary structure predictions for the tRNAs of the *S. anomalus* and *S. vulgaris* mitogenomes, there was no significant difference. All of the tRNAs for two species displayed a typical clover-leaf structure, except for $tRNA^{Ser(AGY)}$, and the tRNA Serine1 (S1)-GCT structure lacked the dihydrouridine (DHU) loop and stem (supplement data 3 and supplement data 4). As observed in the six tRNAs, the *12S rRNA* and *16S rRNA* of the *S. anomalus* and *S. vulgaris mitogenomes* also were different in length 970 bp and 968 bp, and 1577 bp and 1579 bp, respectively (Table 2).

Control region (D-loop), the longest non-coding region, was located between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes in the mitogenomes of *S. anomalus* and *S. vulgaris*, and 1054 bp and 1058 bp in length, respectively (Table 2). O_L region, the origin of light strand replication, was within the WANCY tRNA region in the Turkish *Sciurus* mitogenomes, with 31 bp in length for both species (Table 2). Moreover, the stem-loop structure of O_L of *S. anomalus* and *S. vulgaris* started with 5′-TCTCC −3′.

3.6. Phylogeny of tree squirrels based on mitogeneomes

87 mitogenome sequences (85 Sciurinae and 2 outgroup mitogenomes) (with a 15,347 bp alignment) from the current study and the GenBank database were used in phylogenetic analyses. As a result of the BLAST analysis, the mitogenome under the name *Spermophilus dauricus*

Table 2
Annotations for Turkish *Sciurus anomalus* (S.a_Tr.2) and *S. vulgaris* (S.v_Tr.1).

Gene	Haplotype	Direction	Position	Lengh (bp)	Intergenic Spacer	Start Codons	Stop Codons	Anticodon
tRNA^{Phe}	S.a_Tr.2	H	1–66	66	0			GAA
	S.v_Tr.1							
12S rRNA	S.a_Tr.2	H	67–1036	**970**	0			
	S.v_Tr.1		67–1034	**968**				
tRNA^{Val}	S.a_Tr.2	H	1036–1104	69	−1			TAC
	S.v_Tr.1		1034–1102					
16S rRNA	S.a_Tr.2	H	1105–2681	**1577**	0			
	S.v_Tr.1		1103–2681	**1579**				
tRNA$^{Leu(UUR)}$	S.a_Tr.2	H	2682–2756	75	0			TAA
	S.v_Tr.1							
ND1	S.a_Tr.2	H	2761–3716	956	+4	ATG	TA-	
	S.v_Tr.1							
tRNA^{Ile}	S.a_Tr.2	H	3717–3785	69	0			GAT
	S.v_Tr.1							
tRNA^{Gln}	S.a_Tr.2	L	3783–3854	72	−3			TTG
	S.v_Tr.1							
tRNA^{Met}	S.a_Tr.2	H	3857–3925	69	+2			CAT
	S.v_Tr.1							
ND2	S.a_Tr.2	H	3926–4967	1042	0	**ATC**	T–	
	S.v_Tr.1					**ATT**		
tRNA^{Trp}	S.a_Tr.2	H	4968–5034	67	0			TCA
	S.v_Tr.1							
tRNA^{Ala}	S.a_Tr.2	L	5038–5106	**69**	+3			TGC
	S.v_Tr.1		5038–5107	**70**				
tRNA^{Asn}	S.a_Tr.2	L	5109–5181	73	**+2**			GTT
	S.v_Tr.1		5111–5183		**+3**			
O_L	S.a_Tr.2	H	5184–5214	31	+2			
	S.v_Tr.1		5186–5216					
tRNA^{Cys}	S.a_Tr.2	L	5214–5280	67	−1			GCA
	S.v_Tr.1		5216–5282					
tRNA^{Tyr}	S.a_Tr.2	L	5281–5346	66	0			GTA
	S.v_Tr.1		5283–5348					
COX1	S.a_Tr.2	H	5355–6896	1542	+8	ATG	TAA	
	S.v_Tr.1		5357–6898					
tRNA$^{Ser(UCN)}$	S.a_Tr.2	L	6898–6966	69	**+1**			TGA
	S.v_Tr.1		6902–6970		**+3**			
tRNA^{Asp}	S.a_Tr.2	H	6970–7038	69	+3			GTC
	S.v_Tr.1		6974–7042					
COX2	S.a_Tr.2	H	7039–7728	**690**	0	ATG	TAA	
	S.v_Tr.1		7043–7726	**684**				
tRNA^{Lys}	S.a_Tr.2	H	7732–7800	**69**	+3			TTT
	S.v_Tr.1		7730–7797	**68**				
ATP8	S.a_Tr.2	H	7802–8005	204	+1	ATG	TAA	
	S.v_Tr.1		7799–8002					
ATP6	S.a_Tr.2	H	7963–8643	681	−43	ATG	TAA	
	S.v_Tr.1		7960–8640					
COX3	S.a_Tr.2	H	8643–9426	784	−1	ATG	T–	
	S.v_Tr.1		8640–9423					
tRNA^{Gly}	S.a_Tr.2	H	9427–9496	**70**	0			TCC
	S.v_Tr.1		9424–9492	**69**				
ND3	S.a_Tr.2	H	9497–9843	347	0	ATA	TA-	
	S.v_Tr.1		9493–9839					
tRNA^{Arg}	S.a_Tr.2	H	9844–9910	**67**	0			TCG
	S.v_Tr.1		9840–9907	**68**				
ND4L	S.a_Tr.2	H	9911–10207	297	0	ATG	TAA	
	S.v_Tr.1		9908–10204					
ND4	S.a_Tr.2	H	10201–11578	1378	−7	ATG	T–	
	S.v_Tr.1		10198–11575					
tRNA^{His}	S.a_Tr.2	H	11579–11647	69	0			GTG
	S.v_Tr.1		11576–11644					
tRNA$^{Ser(AGY)}$	S.a_Tr.2	H	11648–11706	59	0			GCT
	S.v_Tr.1		11645–11703					
tRNA$^{Leu(CUN)}$	S.a_Tr.2	H	11707–11776	70	0			TAG
	S.v_Tr.1		11704–11773					
ND5	S.a_Tr.2	H	11777–13594	1818	0	ATA	TAA	
	S.v_Tr.1		11774–13591					
ND6	S.a_Tr.2	L	13578–14102	525	−17	ATG	AGA	
	S.v_Tr.1		13575–14099					
tRNA^{Glu}	S.a_Tr.2	L	14103–14171	69	0			TTC
	S.v_Tr.1		14100–14168					
CYTB	S.a_Tr.2	H	14176–15315	1140	**+4**	ATG	AGA	
	S.v_Tr.1		14172–15311		**+3**			
tRNA^{Thr}	S.a_Tr.2	H	15316–15382	**67**	0			TGT
	S.v_Tr.1		15312–15380	**69**				

(*continued on next page*)

Table 2 (*continued*)

Gene	Haplotype	Direction	Position	Lengh (bp)	Intergenic Spacer	Start Codons	Stop Codons	Anticodon
tRNA^{Pro}	S.a_Tr.2	L	15387–15453	**67**	+4			TGG
	S.v_Tr.1		15385–15453	**69**				
D-loop	S.a_Tr.2	H	15454–16507	**1054**	0			
	S.v_Tr.1		15454–16511	**1058**				

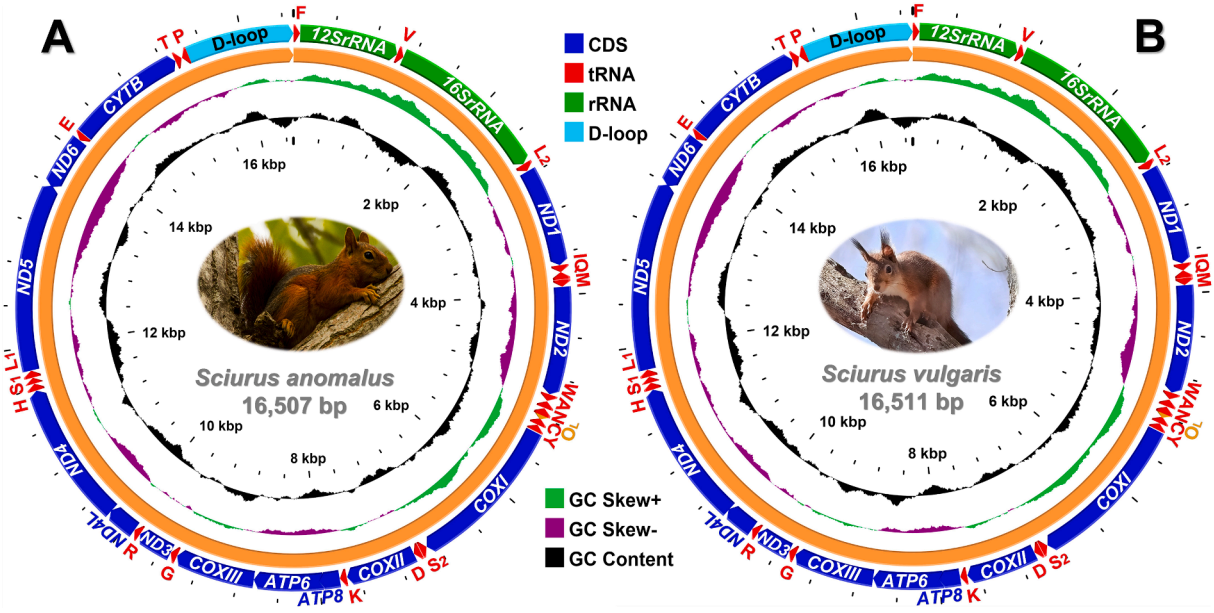

Fig. 2. The circular maps of Turkish *Sciurus anomalus* and *S. vulgaris* mitogenomes.

Table 3
Nucleotide composition features of Turkish *Sciurus anomalus* and *S. vulgaris* mitogenomes.

	Sequence	Size (bp)	A (%)	C (%)	G (%)	T (%)	A+T (%)	G+C (%)	AT Skew	GC Skew
Whole genome	345	16507	31.5	25.2	13.1	30.2	61.7	38.3	0.022	**−0.320**
	1418	16511	32.1	24.5	12.5	30.9	63.0	37.0	0.020	**−0.323**
PCGs	345	11404	30.4	26.6	11.8	31.2	61.6	38.4	**−0.013**	**−0.385**
	1418	11398	31.0	26.0	11.4	31.6	62.6	37.4	**−0.012**	**−0.391**
rRNA genes	345	2547	35.3	21.9	17.5	25.3	60.6	39.4	0.164	**−0.113**
	1418	2547	35.8	20.8	16.8	26.6	62.4	37.6	0.147	**−0.106**
tRNA genes	345	1507	35.5	20.6	14.9	29.0	64.5	35.5	0.101	**−0.159**
	1418	1511	35.8	19.7	14.5	30.0	65.8	34.2	0.087	**−0.151**
D-loop	345	1054	29.5	25.3	12.3	32.9	62.4	37.6	**−0.055**	**−0.348**
	1418	1058	30.1	24.5	11.8	33.6	63.7	36.3	**−0.056**	**−0.349**

(NC_027283) indicated a query cover of 100% and a percent identity of 97.88 with the *Sciurus vulgaris* mitogenome (AJ238588), and it might possibly belong to *S. vulgaris*. Based on the GTR + G + I substitution model, and Bayesian and ML phylogenetic analyses, mitogenomic haplotype trees were relatively resolved and displayed substantially identical topologies for the subfamily Sciurinae that included the tribes Sciurini and Pteromyini. Each of the two tribes was a monophyletic clade, with relatively high posterior probability and bootstrap scores for main nodes. As expected, the two outgroup taxa (*S. williamsi* and *D. sagitta*) that separated first before the Sciurinae taxa rooted the Bayesian and ML trees.

The tribe Pteromyini that was located at the basal was the first clade that was split within the subfamily Sciurinae, whereas the tribe Sciurini formed the second clade that was clustered in the phylogenetic trees (Fig. 3 and supplement data 5). By viewing the phylogenetic trees, the haplotypes under the name *Sciurus* within the tribe Sciurini did not form a monophyletic group, and the *Sciurus* tree squrriels were paraphyletic relative to the sciurines such as *Hadrosciurus*, *Microsciurus*, *Guerlinguetus*, *Simosciurus* and *Syntheosciurus* within the tribe Sciurini with respect to mitogenome. Of the tree squirrel species distributed in Turkey, *S. anomalus* was the first diverged species within the tribe Sciurini and formed the most basal phylogroup, and other species, *S. vulgaris*, was sister species with *S. lis*.

All of the complete mitogenomes of *S. anomalus*, except for the partial mitogenome of the Lebanese *S. anomalus* (MW027641), belonged to the Turkish *S. anomalus*. The *S. anomalus* mitogenomes could be separated into five main lineages in both Bayesian and ML trees: S.a_1 – S.a_5, in which the lineage naming was done according to the Bayesian tree (Fig. 3 and supplement data 5). Based on K2P sequence pairwise distances, the dissimilarities among the five mitogenome lineages of *S. anomalus* ranged from 0.0042 (0.42%) to 0.0062 (0.62%): 0.0062 between S.a_1 and S.a_2, 0.0055 between S.a_1 and S.a_3, 0.0052 between S.a_1 and S.a_4, 0.0054 between S.a_1 and S.a_5, 0.0060 between S.a_2 and S.a_3, 0.0057 between S.a_2 and S.a_4, 0.0059 between S.a_2 and S.a_5, 0.0050 between S.a_3 and S.a_4, 0.0052 between S.a_3 and S.a_5, 0.0060 between S.a_2 and S.a_3, and 0.0042 between S.a_4 and S.

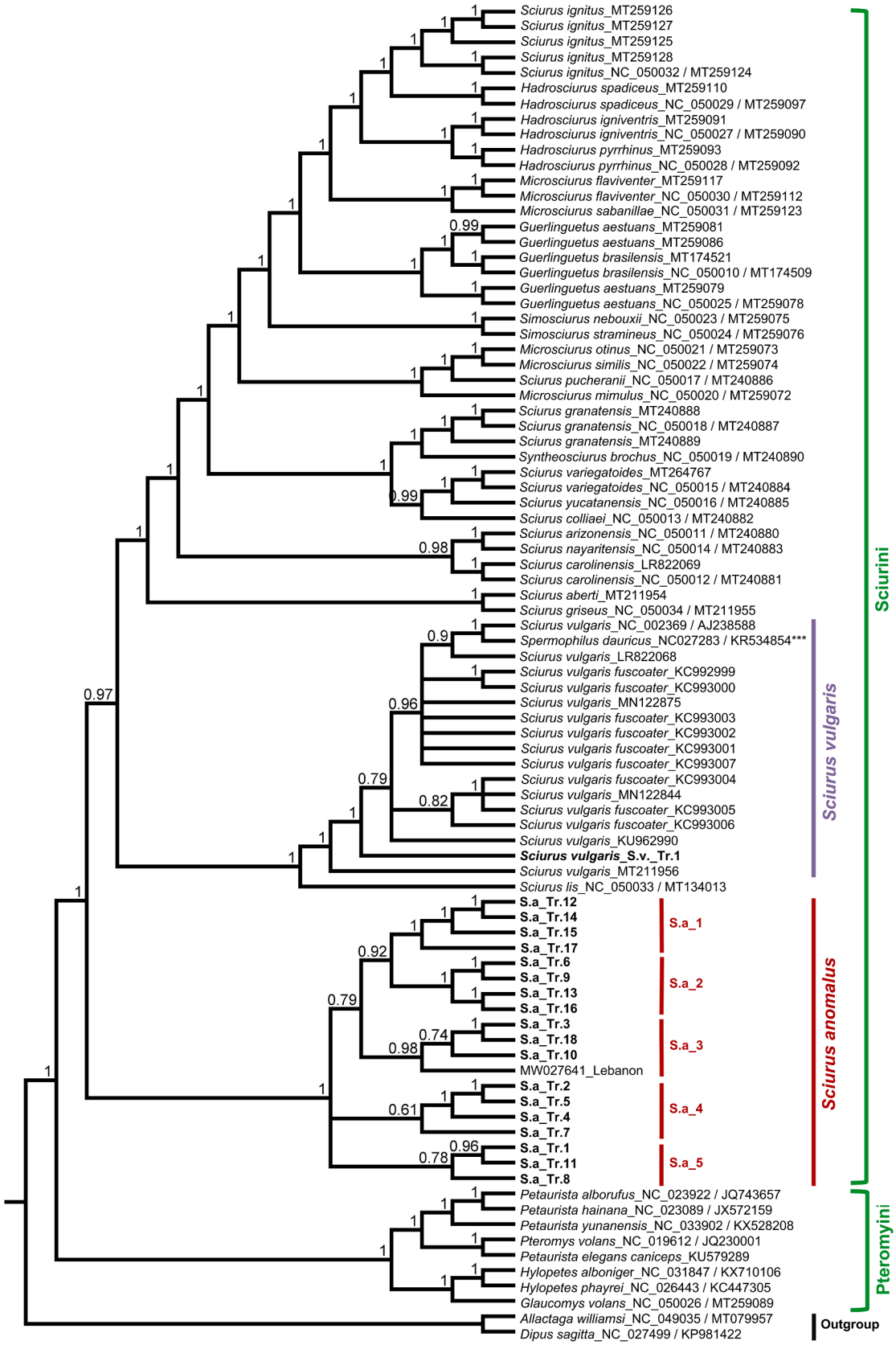

Fig. 3. Phylogenetic tree reconstructed by using Bayesian method of Turkish tree squirrels (*Sciurus*) based on mitogenomes and GTR + G + I model. [*Spermophilus dauricus*_NC_027283 / KR534854*** indicated a query cover of 100% and a percent identity of 97.88 with the *Sciurus vulgaris* mitogenome (AJ238588)].

a_5. According to the distributional map of mitogenomic lineages (Fig. 1), the lineage S.a_1 consisted of haplotypes from the central-northern Anatolia, the lineage S.a_2 contained haplotypes from the western and north-western Anatolia, the lineages S.a_3 and S.a_5 included haplotypes from the southern and southern-eastern Anatolia, the lineage S.a_4 comprised haplotypes from the central and southern Anatolia. The existence of the *S. anomalus* mitogenomic lineages might be caused from barriers such as the Kızılırmak River and the Anatolian Diagonal. For *S. anomalus*, such phylogeographic breaks could be the result of long-term barriers to gene flow. In this context, the Kızılırmak River might be a barrier between the S.a_2 + S.a_4 lineages (distributed in the western and the southern of the River) and the S.a_1 lineage (distributed in the eastern and the northern of the River), whereas the Anatolian Diagonal might be a barrier between the S.a_4 lineage (distributed in the western of the Diagonal) and the S.a_3 and S.a_5 lineages (distributed in the eastern and the southern of the Diagonal).

Based on the 19 Anatolian and the one Lebanese mitogenomes,the same lineages of the *S. anomalus* haplotypes as on the trees were also found in the haplotype network, in which there were at least three hypothetical haplotypes and more than one mutational steps that separated the five main lineages (Fig. 4). The haplotype network was entirely compatible with the topology of the Bayesian tree (Fig. 3), but not quite in agreement with that of the ML tree (supplement data 5) due to clustering of the Isparta haplotype (S.a_Tr.7). In the Bayesian tree (Fig. 3) and the network (Fig. 4), the Isparta haplotype (S.a_Tr.7) was clustered with haplotypes from Kayseri (S.a_Tr.2), Mersin (S.a_Tr.4), and Konya (S.a_Tr.5) within S.a_4 lineage, but the Isparta haplotype (S.a_Tr.7) was clustered with haplotypes from Malatya (S.a_Tr.1), Adana (S.a_Tr.8) and Hatay (S.a_Tr.11) within S.a_5 lineage in the ML tree (supplement data 5). In the lineage S.a_3, the haplotypes from the south-southeastern Anatolian part (S.a_Tr.3: Adana, S.a_Tr.10: Hatay, and S.a_Tr.18: Diyarbakır) of Turkey were grouped together with the Lebanese haplotype (MW027641) to match the geographic pattern. Moreover, two haplotypes found in the same locality (S.a_Tr.10 and S.a_Tr.11) from Yayladağ, Hatay, Turkey (locality number 11 in Fig. 1 and Table 1) were clustered into two distinct lineages, S.a_3 and S.a_5, respectively, which partially overlapped geographically.

The phylogenetic analyses (Bayesian and ML) revealed unresolved haplotype trees for *S. vulgaris*, in which there were a different pattern, and therefore, it was relatively difficult to distinguish potential lineages for *S. vulgaris* (Fig. 3 and supplement data 5). The Turkish *S. vulgaris* that sampled from the north-eastern Anatolian region was clustered alone on

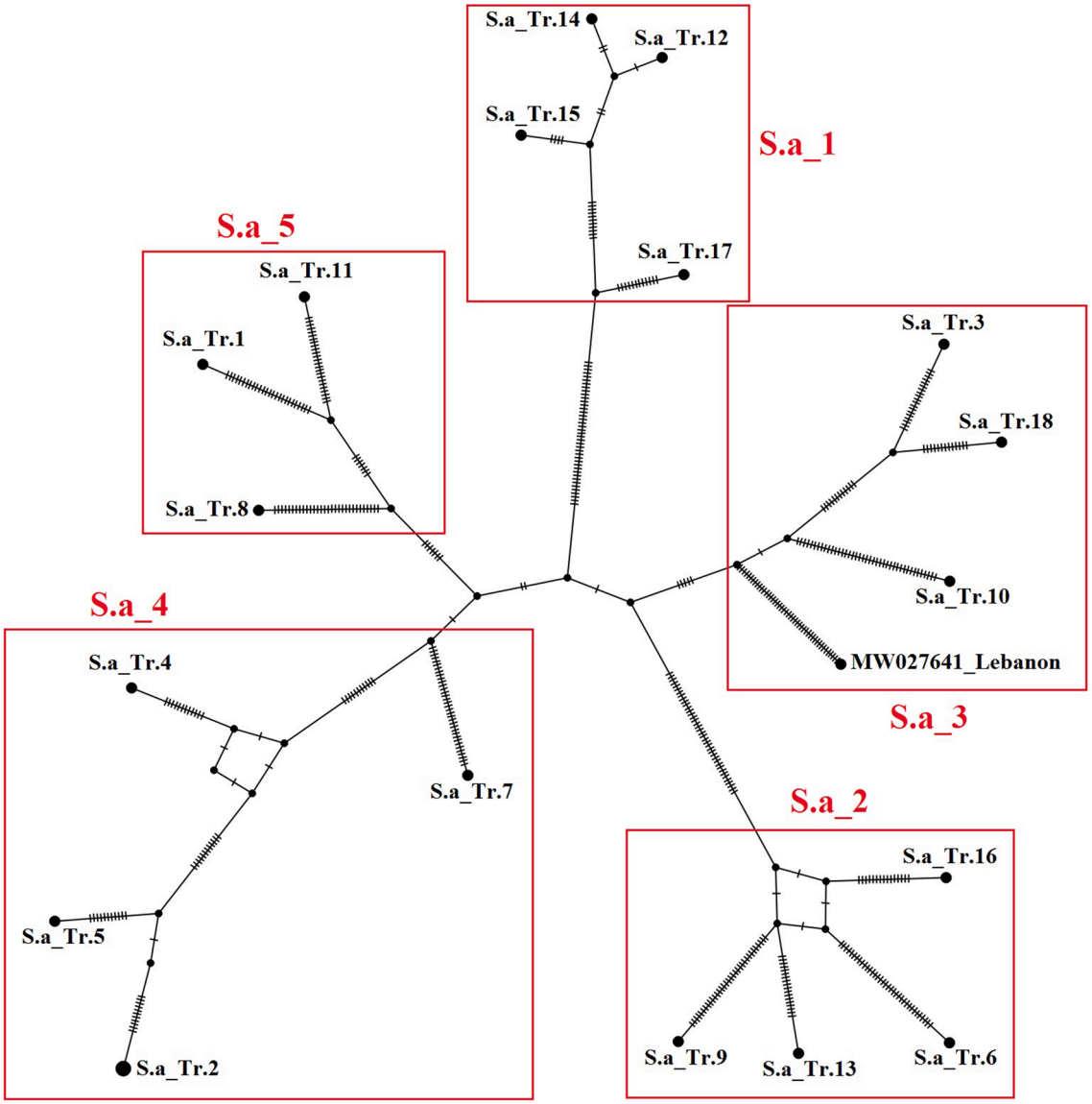

Fig. 4. Haplotype network *of S. anomalus* based on mitogenomes.

a different branch in the Bayesian tree (Fig. 3), whereas The Turkish *S. vulgaris* was grouped with the European red squirrel, *S. vulgaris* (*S. vulgaris fuscoater* KC993004 from Denmark, *S. vulgaris* MN122844 from unknown locality, *S. vulgaris fuscoater* KC993005 from Denmark, *S. vulgaris fuscoater* KC993006 from Denmark, *S. vulgaris* LR822068 from unknown locality, *S. vulgaris* MN122875 from unknown locality, *S. vulgaris fuscoater* KC993002 from Denmark, *S. vulgaris fuscoater* KC993007 from Denmark, *S. vulgaris fuscoater* KC993003 from Denmark, *S. vulgaris fuscoater* KC993001 from Denmark, *S. vulgaris fuscoater* KC992999 from Denmark, and *S. vulgaris fuscoater* KC993000 from Denmark) in the ML tree (supplement data 5).

3.7. Phylogeny of Sciurus anomalus based on mitochondrial CYTB and D-loop sequences

The combined sequences of mitochondrial *CYTB* and D-loop regions for *S. anomalus* from the current study and the GenBank database were aligned and trimmed by producing a 1,198 bp alignment with 39 sequences (36 for *S. anomalus* and 3 for outgroup). Based on the *CYTB* + D-loop dataset and the GTR + G + I substitution model, the Bayesian and Maximum Likelihood analyses produced trees with relatively high posterior probability and low bootstrap scores for main nodes, respectively. Both the phylogenetic trees displayed relatively similar topologies for the *S. anomalus* phylogeny, with at least nine lineages (Fig. 5 and supplement data 6). The Turkish haplotypes of *S. anomalus* were fall into eight lineages. In the phylogenetic trees, the haplotypes from the western (Antalya and Bursa) and north-west Anatolian parts of Turkey and Lesvos-Greece were positioned in basal lineages, whereas the haplotypes from the south (Adana) and south-east (Diyarbakır) Anatolian parts of Turkey were closely clustered with the haplotypes from the northern Zagros region of Iran (Fig. 5 and supplement data 6).

4. Discussion

The current study focused to shed light into the mitogenomic characterization and the phylogenetics of Turkish tree squirrels, by performing a comparative analysis of mitogenomes of Turkish *Sciurus* with other sciurines.

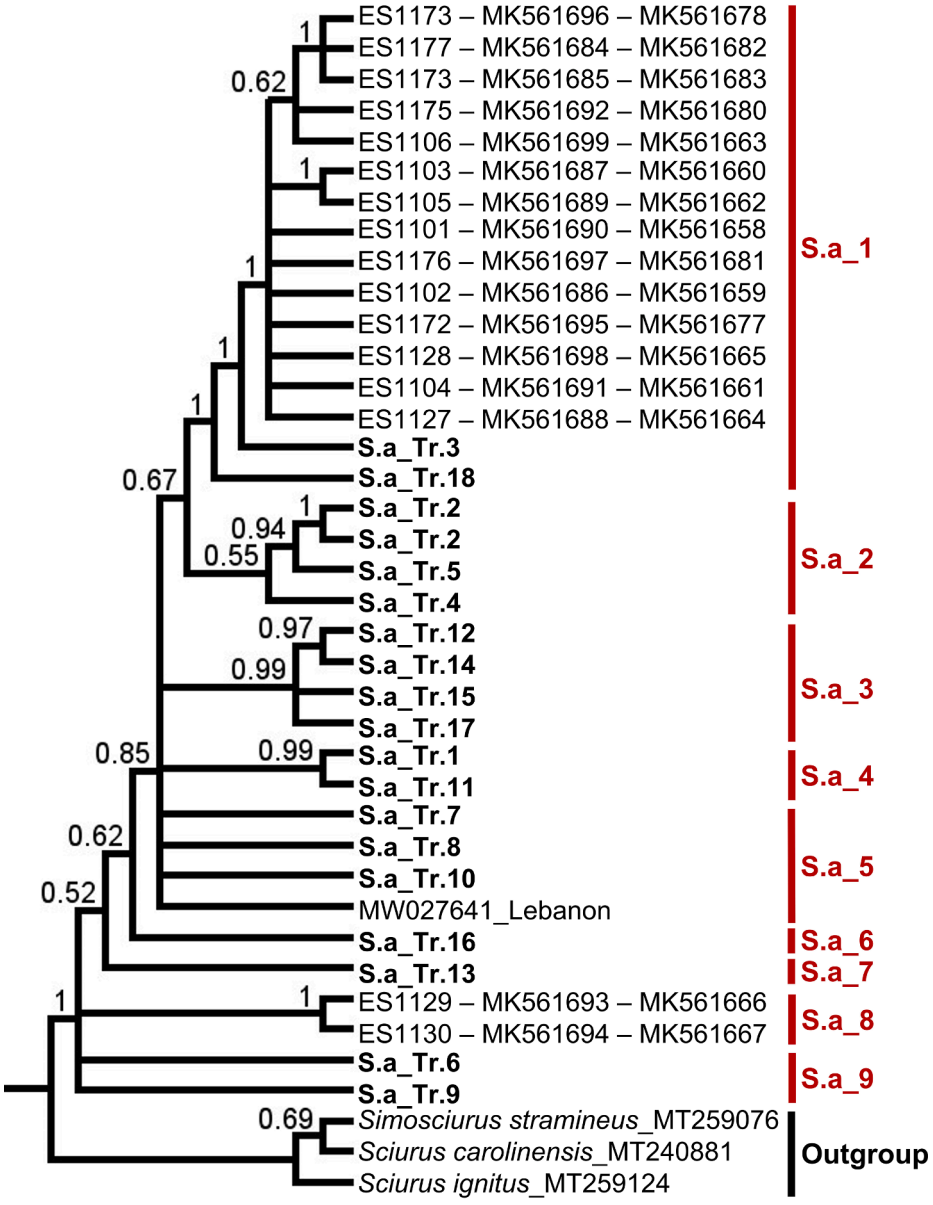

Fig. 5. Phylogenetic tree reconstructed by using Bayesian method of *Sciurus anomalus* based on mitochondrial *CYTB* + D-loop sequences and GTR + G + I model.

4.1. Comparing Turkey's Sciurus mitogenomes with other rodent mitogenomes

Although a great number of *S. vulgaris* mitogenomes are available, there is only one partial mitogenome of the Lebanese *S. anomalus* registered by Boukhdoud et al. (2021) (supplement data 1). In this context, the complete mitogenome of *S. anomalus* is scarce and the complete mitogenomes of the Turkish *Sciurus* (*S. anomalus* and *S. vulgaris*) are not currently available. In this study, we found that the mitogenomes of the Turkish *S. anomalus* and *S. vulgaris* had different sequence lengths (Table 1), which were probably due to control region / D-loop and tRNAs as reported for the *Sciurus* species (Reyes et al., 2000; Madsen et al., 2015; Kim et al., 2017; Anonymous, 2020; Abreu-Jr et al., 2020; Boukhdoud et al., 2021; Margaryan, 2019). The Turkish *S. anomalus* mitogenomes were lengthly different than that of the Lebanese *S. anomalus* (Boukhdoud et al., 2021). The Turkish *S. vulgaris* mitogenome (S.v_Tr.1, ON620291: 16,511 bp) was the same in length to those of the Korean (KU962990) (Kim et al., 2017) and the Danish (MN122875) (Margaryan, 2019) *S. vulgaris*, but different than those of *S. vulgaris* from Europe (Reyes et al., 2000; Madsen et al., 2015; Margaryan, 2019), and Eastern Russia (Abreu et al., 2020).

Since the Turkish *Sciurus* mitogenomes included 37 coding regions (13 PCGs, 22 tRNAs and 2 rRNA genes), a non-coding region (control region / D-loop) and an origin of light-strand region (O_L), they were compatible with partial and complete mitogenomes of the 14 *Sciurus* species (*Sciurus aberti, S. anomalus, S. arizonensis, S. carolinensis, S. colliaei, S. granatensis, S. griseus, S. ignitus, S. lis, S. nayaritensis, S. pucheranii, S. variegatoides, S. vulgaris,* and *S. yucatanensis*) (Reyes et al., 2000; Madsen et al., 2015; Kim et al., 2017; Anonymous, 2020; Abreu-Jr et al., 2020; Boukhdoud et al., 2021; Margaryan, 2019). For the intergenic spaces and overlapping regions among the PCGs and the tRNAs, the mitogenomes of the Turkish *S. anomalus* and *S. vulgaris* were similar to those of many mammalian species (Rohland et al., 2007; Krause et al., 2008; Wada et al., 2010; Chen et al., 2016a,2016b; Ding et al., 2016b; *etc.*). Regarding the initiation codon generally in the 13 PCGs, an observed frequency of for ATG (76.9%) in the current study was higher than that of the other rodent species (72.22%) (Yue et al., 2015), whereas it was similar to those of the Williams's jerboa *Scarturus williamsi* (İbiş, 2020) and the long-clawed mole vole *Prometheomys schaposchnikowi* (İbiş et al., 2020).

There are common incomplete stop codons observed in mitogenome of many other rodent species (Partridge et al., 2007; Jiang et al., 2012; Yue et al., 2012, 2015; Bendová et al., 2016; Ding et al., 2016a, 2016b, 2016c; İbiş, 2020; İbiş et al., 2020), and it has been suggested that the incomplete stop codon could be formed a complete termination codon owing to the post-transcriptional polyadenylation (Ojala et al., 1981; Cha et al., 2007). Moreover, Rand (1993) and Selosse et al. (2001) stated that the incomplete stop codons could have emerged because of selective pressures, and so, mitogenome size was reduced due to the loss of unnecessary genes. Similarly, the incomplete stop codons were also determined in the mitogenomes of the Turkish *Sciurus*.

In terms of base composition, if compared, the mitogenomes of the Turkish *S. anomalus* and *S. vulgaris* were AT-biased and compatible with the complete mitogenome of the Korean *S. vulgaris* (Kim et al., 2017). This result also was in agreement with rodent species such as the Chinese hamster *Cricetulus griseus* (Partridge et al., 2007), the false zokor *Myospalax aspalax* (Yuan et al., 2016), and *P. schaposchnikowi* (İbiş et al., 2020).

Some of the tRNAs have a missing cloverleaf structure that may play a functional role in the structural compensation mechanism among the Aminoacid Acceptor (AA), the Anticodon (AC), the Dihydorouridine (DHU), and the Tψ C (T) arms and the variable or extra arm that is different in size and not present for all the tRNAs (Steinberg and Cedergren, 1994). All of the tRNAs for the Turkish *S. anomalus* and *S. vulgaris* displayed a typical clover-leaf structure, except for $tRNA^{Ser\ (AGY)}$. The tRNA Serine1 (S1)-GCT lacked the dihydrouridine (DHU) loop and stem. Moreover, a similar case was also displayed in the secondary structure predictions for the tRNAs of rodent species such as *Microtus fortis calamorum* (Jiang et al., 2012), the gray dwarf hamster *Cricetulus migratorius* (Ding et al., 2016a), *S. williamsi* (İbiş, 2020), and *P. schaposchnikowi* (İbiş et al., 2020).

In the majority of mammalian species, the D-loop region is a regulatory region of mitochondrial DNA for expression and replication, and it has different lengths. In terms of base composition, this region is in the form of A + T > G + C (Sbisà et al., 1997). The D-loop region in the Turkish *Sciurus* mitogenomes (1054 bp and 1058 bp) was longer than that of the *P. schaposchnikowi* mitogenome (871 bp). Also, the A + T contents of the D-loop region in the Turkish *Sciurus* mitogenomes (62.4% and 63.7%) were relatively compatible with those of the two *P. schaposchnikowi* mitogenomes (62.57% and 63.03%) (İbiş et al., 2020).

O_L region has an important task for the correct initiation of DNA replication (Wanrooij and Falkenberg, 2010), and the stem-loop structure of O_L can differ among species (Ding et al., 2016c). The stem-loop structure of O_L region in the Turkish *Sciurus* mitogenomes that started with 5′-TCTCC-3′ was different than those of *S. williamsi* (İbiş, 2020) (5′-GCCGG-3), *C. migratorius* (Ding et al., 2016a), and *P. schaposchnikowi* (İbiş et al., 2020) (5′-CTTCT-3′). Moreover, the O_L region in the Turkish *Sciurus* mitogenomes was shorter than that of *P. schaposchnikowi* (34 bp) (İbiş et al., 2020).

4.2. Phylogenetic implications of Turkey's Sciurus based on mitogenomes

Phylogenetic studies including the Turkish *Sciurus* are limited to *S. anomalus*, using sequences from mitochondrial (*CYTB* and D-loop) (Oshida et al., 2009) (*CYTB*, *tRNA-Thr*, *tRNA-Pro*, and partial D-loop) (Demirtaş, 2022) and nuclear (*Rag1* and *C-myc*) (Asadi Aghbolaghi et al., 2019) regions. Based on the Turkish *Sciurus* (*S. anomalus* and *S. vulgaris*) mitogenomes, a phylogenetic study is currently not yet available. In the current study based on the Bayesian and ML methods using the mitogenomes and followed the generic classification suggested by Abreu-Jr et al. (2020), the phylogenetic analyses revealed substantially similar tree topologies for the subfamily Sciurinae including the two tribes Sciurini and Pteromyini. In the phylogenetic trees, each of the two tribes was a monophyletic clade (Fig. 3 and supplement data 5). *S. anomalus* was the first diverged species within the genus *Sciurus* and formed the most basal phylogroup within the tribe Sciurini. *S. lis and S. vulgaris* were sister species and more closely related to the northern American Sciurini species rather than *S. anomalus* (Fig. 3 and supplement data 5). This was compatible with the study of Abreu-Jr et al. (2020). Moreover, our results proposed; (i) that the two Eurasian Sciurini species (*S. lis* and *S. vulgaris*) and the northern American Sciurini species shared a common ancestor, and (ii) that *S. anomalus* shared a common ancestor with the two Eurasian (*S. lis* and *S. vulgaris*) and the northern American Sciurini species. On the other hand, based on the mitochondrial *CYTB* sequences, Oshida et al. (2009) reported that the *S. anomalus* samples from the Central Anatolian region of Turkey was in a closer relationship with the American species (the New World *Sciurus* species: *S. aestuans*, *S. stramineus*, *S. carolinensis* and *S. niger*) rather than the Eurasian species (the Old World *Sciurus* species: *S. lis* and *S. vulgaris*). To reconstruct the phylogenetic relationships within the tribe Sciurini, Pečnerová and Martínková (2012) used partial sequences of the mitochondrial *12S rRNA, CYTB* and D-loop regions, and they reported that the Eurasian Sciurini species are not monophyletic as presented in the study of Oshida et al. (2009), and that *S. anomalus* is in a closer relationship with the American species (*Microsciurus flaviventer*, *S. aberti*, *S. aestuans*, *S. stramineus*, *S. carolinensis* and *S. niger*). Moroever, based on mitochondrial (*COI, CYTB,* and D-loop) and nuclear (*C-myc* and *Rag1*) sequences for *S. anomalus* and the other *Sciurus* species, Asadi Aghbolaghi et al. (2020a) also reported that *S. lis* and *S. vulgaris* formed the most basal phylogroups, and they also emphasized that *S. anomalus* is in a closer relationship with the New World species rather than the Eurasian

species. When compared to the results of Oshida et al. (2009), Pečnerová and Martínková (2012), and Asadi Aghbolaghi et al. (2020a), the results of the current study were different. This might be due to the use of different markers.

As for the phylogenetic relationships of Turkey's *Sciurus,* the Turkish tree squirrels were clustered into two phylogroups, *S. vulgaris* and of *S. anomalus*. Based on the mitogenome haplotypes from the Anatolian region of Turkey and Lebanon, the phylogenetic and the network analyses revealed the existence of five main lineages for *S. anomalus* (Fig. 3 and supplement data 5). When compared to the result of the current study, Asadi Aghbolaghi et al. (2019), who used mitochondrial and nuclear sequences, reported one lineage with three groups for the Anatolian part of Turkey and another lineage with two groups for the Levant region and Zagros forests. Recently, Demirtaş (2022), who used four mitochondrial regions, suggested that the Anatolian *S. anomalus* haplotypes were clustered into western and eastern mitochondrial lineages, which also included the Greek and the Lebanese haplotypes. However, Demirtaş (2022) reported that none of the Anatolian *S. anomalus* haplotypes was grouped with the Iranian or Syrian haplotypes. In terms of lineage number of the Turkish *S. anomalus*, the result with five main lineages for *S. anomalus* in the current study partially displayed a contradiction with that of Asadi Aghbolaghi et al. (2019), but different than the result of Demirtaş (2022) with western and eastern mitochondrial lineages. This might possibly be due to the difference in the sampling locations from the Anatolian part of Turkey and the used markers. Moreover, Asadi Aghbolaghi et al. (2019) suggested that the ancestral population of *S. anomalus* might have evolved from the western Anatolian part of Turkey and the Lesvos Island of Greece (possibly also from other Greek Islands in the eastern Aegean Sea). In the current study, the ML analysis supported the suggestion of Asadi Aghbolaghi et al. (2019) related to the ancestral population of *S. anomalus* and proposed a wider geographical area including the northern-western (Bolu and Samsun) and the western (Antalya and Bursa) Anatolian parts of Turkey (supplement data 5). However, the suggestion of Asadi Aghbolaghi et al. (2019) was not confirmed by the result of the Bayesian analysis (Fig. 3). Our results proposed that use of the complete mitogenomes in phylogenetic and network analyses might be particularly useful for testing and improving phylogenetic hypotheses and evolutionary scenarios fictionalized based on partial sequences of mitochondrial genome.

4.3. Phylogenetic analysis of S. anomalus using mitochondrial CYTB and D-loop sequences

Considering the sampling from known distributional area of *S. anomalus*, phylogenetic studies are relatively limited (Oshida et al., 2009, Asadi Aghbolaghi et al., 2019, 2020a; Demirtaş, 2022). Nevertheless, the number of samples in the studies of Oshida et al. (2009) and Asadi Aghbolaghi et al. (2020a) was in small size. Based on mitochondrial *CYTB* + D-loop sequences (Table 1 and supplement data 1), phylogenetic analyses revealed that *S. anomalus* was assigned to at least nine lineages (Fig. 4 and supplement data 6). In the previous studies based on the mitochondrial regions, Asadi Aghbolaghi et al. (2019) suggested one lineage consisting of three haplogroups in the Anatolian part of Turkey and another lineage including two haplogroups in the Levant region and the Zagros forests in Iran for *S. anomalus*, whereas Demirtaş (2022) empahised the existence of the eastern and western lineages for *S. anomalus*. In terms of the number of mitochondrial lineages, the result of the current study was not consistent with the results of Asadi Aghbolaghi et al. (2019) and Demirtaş (2022).

Additionally, Asadi Aghbolaghi et al. (2019) also suggested that the ancestral population attributed to *S. anomalus* evolved in a geographical region covering the western Anatolian part of Turkey and the Greek Island, Lesvos. By hypothesizing that *S. anomalus* may have survived in refugia with convenient climatic conditions in low forest areas in the Anatolian geography due to reduced forest cover during cold and semi-arid glacial periods, the phylogeographic study of Asadi Aghbolaghi et al. (2019) was confirmed by Asadi Aghbolaghi et al. (2020a), who emphasized that this species originated in Eurasia. Asadi Aghbolaghi et al. (2019) also stated that no haplotype for the mitochondrial *CYTB* and D-loop regions was shared among the five different groups belonging to the two lineages, and they also expressed that the three phylogenetic groups in one lineage of *S. anomalus* seem parapatric in the northern and the western Anatolian parts of Turkey. On the other hand, Demirtaş (2022) stated that two the main mitochondrial lineages of *S. anomalus* were resulted from climatic fluctuations during the Chibanian geological stage, and he suggested that the western lineage originated in the Aegean Islands and the western Anatolia region, and that the eastern lineage originated in the eastern Anatolian region or the Caucasus region despite insufficient sampling. There were differences between the current study and that of Aghbolaghi et al. (2019) in terms of the sampling locations of *S. anomalus* from Anatolia and the mitochondrial *CYTB* + D-loop sequences in length. In the current study, the haplotypes from the western (Antalya and Bursa) and northern-west (Bolu and Samsun) Anatolian parts of Turkey and the Lesvos Island of Greece were positioned in basale of both the Bayesian and the ML trees (Fig. 4 and supplement data 6). This result was in full agreement with the suggestion of Asadi Aghbolaghi et al. (2019) related to the originated region for the ancestral population of *S. anomalus*. However, there was an unagreement between the results of Demirtaş (2022) and the current study due to differences in lineage topologies in terms of origin of lineages and the ancestral population for *S. anomalus*.

4.4. Insights for the intraspecific relationships in the Turkish S. anomalus

The Anatolian part of Turkey hosts many mammalian species having relatively high intraspecific genetic diversity (Gündüz et al., 2005, 2007; Gür, 2013; İbiş et. al., 2018; İbiş et al., 2014; Kankılıç et al., 2017; Stamatis et al., 2009; Arslan et al., 2020; *etc.*) due to topographic structure and climatic differences and being a contact area of three hotspots (Caucasus, Iran-Anatolian and Mediterranean) in respect to biodiversity (Mittermeier et al., 2005; Bilgin, 2011; Şekercioğlu et al., 2011). The phylogenetic analyses based on both the complete mitogenome and the mitochondrial *CYTB* and D-loop data revealed the existence of lineage more than one within *S. anomalus*. This might be an indication of the intraspecific maternal variation for the Turkish *S. anomalus* due to differentiating among its populations. Bilgin (2011) described three major patterns by analyzing geographical distribution of intraspecific genetic variation in the 29 Anatolian species. One of the patterns is "pattern II", and according to that, phylogeographic breaks that occur between or among main clades/lineages within Anatolia have probably been differentiating the Anatolian animal populations. The results of the current study also complied with "pattern II". As seen in the phylogenetic trees and the haplotype network, the *S. anomalus* lineages were separated due to possible phylogeographic breaks, which could be the result of long-term barriers to gene flow. By viewing the distributional map (Fig. 1), the existence of the *S. anomalus* mitogenomic lineages might be caused from barriers such as the Kızılırmak River and the Anatolian Diagonal.

4.5. Conclusion

To summarize the results of the current study, the mitogenomes of the *S. anomalus* and *S. vulgaris* from Turkey were newly sequenced and annotated. The mitogenome structures of the Turkish *S. anomalus* and *S. vulgaris* were the same as those of the tribe Sciurini. The phylogenetic trees displayed that *Sciurus* was not a monophyletic genus, and that *S. anomalus* was in the most basale position. The newly sequenced mitogenomes of *S. anomalus* and *S. vulgaris* could be used as very important basic information for evolutionary relationship analysis, genetic monitoring, and spatial distributional range in the future. This mitogenomic data would also support further genetic investigations,

which would contribute to improving *in-situ* and *ex-situ* management strategies towards better conservation of the *Sciurus* species. To draw a more comprehensive picture in this context, further phylogeographical and population studies should include both mitochondrial and nuclear genome molecular markers of the *Sciurus* squirrels.

CRediT authorship contribution statement

Osman İbiş: Conceptualization, Project administration, Resources, Supervision, Methodology, Data curation, Investigation, Validation, Writing – original draft, Writing – review & editing. **Ahmet Yesari Selçuk:** Resources, Methodology, Data curation, Investigation, Validation, Writing – review & editing. **Saffet Teber:** Methodology, Data curation, Validation. **Mehmet Baran:** Methodology, Data curation, Validation. **Alaettin Kaya:** Resources, Investigation, Writing – review & editing. **Servet Özcan:** Resources, Investigation, Writing – review & editing. **Haluk Kefelioğlu:** Resources, Investigation, Writing – review & editing. **Coşkun Tez:** Conceptualization, Project administration, Resources, Supervision, Methodology, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data accessibility

The mitogenomic data presented in this study can be found in the GenBank database (NCBI) under accession numbers: ON620273 – ON620291.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.146773.

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