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Revealing the Genetic Differentiation of *Rattus norvegicus* (Berkenhout 1769) Populations by Analyzing Two Mitochondrial Markers

Rattus norvegicus (Berkenhout 1769) Populasyonlarının Genetik Farklılaşmasının İki Mitokondriyal Belirtecin Analiz Edilmesi İle Ortaya Çıkarılması

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ABSTRACT

Rattus norvegicus (Brown rat) has a significant importance for public health and economy due to its close association with human populations. However, there have been very few molecular systematic studies on global populations of *R. norvegicus*. In this study, sequences obtained from Türkiye, Europe, Asia, Africa, and America regions were analyzed using mitochondrial Cytochrome-b and Cytochrome oxidase-I gene regions and genetic differentiation levels between these populations were revealed. Accordingly, samples belonging to the studied populations did not split in Bayesian Inference trees and Median-joining networks; these samples also formed common haplotypes, and the mean genetic distance and fixation index values were generally low. The results of the study showed that gene flow between these populations may continue due to human transportation activity.

Key Words
Rattus norvegicus, Brown rat, cytochrome-b, Cytochrome oxidase-I.

ÖZ

Rattus norvegicus (Kahverenkli sıçan) insan popülasyonlarıyla yakın ilişkisi nedeniyle halk sağlığı ve ekonomi açısından büyük öneme sahiptir. Ancak, *R. norvegicus*'un küresel popülasyonları üzerine çok az sayıda moleküler sistematik çalışma yapılmıştır. Bu çalışmada, Türkiye, Avrupa, Asya, Afrika ve Amerika bölgelerinden elde edilen diziler, mitokondriyal Sitokrom-b ve Sitokrom oksidaz-I gen bölgeleri kullanılarak analiz edilmiş ve bu popülasyonlar arasındaki genetik farklılaşma düzeyleri ortaya çıkarılmıştır. Buna göre, çalışılan popülasyonlara ait örnekler Bayesian Çıkarım Ağaçları ve Median-joining ilişki ağlarında ayrılmamıştır; bu örnekler aynı zamanda ortak haplotipler oluşturmuştur ve ortalama genetik uzaklık ve fiksasyon indeksi değerleri genel olarak düşüktür. Çalışmanın sonuçları, bu populasyonlar arasındaki gen akışının, insanların taşımacılık aktivitesi nedeniyle devam ediyor olabileceğini göstermiştir.

Anahtar Kelimeler
Rattus norvegicus, Kahverenkli sıçan, Sitokrom-b, Sitokrom oksidaz-I.

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INTRODUCTION

Rattus norvegicus (Berkenhout 1769) (Brown rat) is one of the most common synanthropic rodent species along with *Rattus rattus* (Linnaeus 1758) and *Mus musculus* (Linnaeus 1758) and it is found in almost every part of the World except for Antarctica [1-3]. Although *R. norvegicus* was originally native to south-east Siberia, north-east China, and parts of Japan, it has spread throughout the World as human populations have carried rats as a result of events such as transportation and colonization [1-3,4-6]. This species has the ability to easily adapt to the areas it enters and invade as an alien species [7]. Besides, since *R. norvegicus* lives close to human populations in urban areas, it also has negative effects such as transmitting diseases (as a vector), consumption and contamination of foods, and damaging properties [8-17]. *R. norvegicus* is frequently seen throughout Türkiye, especially in cities and rarely in rural areas [18]. Despite the cosmopolitan distribution of *R. norvegicus* and its significant impacts on public health and the economy, molecular studies are notably few and mostly involve local populations [19-23]. Molecular studies are important in terms of studying invasive species to identify source populations of the original colonization and contemporary dispersal into the invaded range, so routes of dispersal and future spread of these species can be predicted. In this way, pest management plans can be effective in the future [24-26]. With this study, it was aimed to reveal the genetic diversity and differentiation levels of the Asian, European, American, and African populations of *R. norvegicus* with the help of analyses of the mitochondrial gene data (Cytochrome-b (CYTB) and Cytochrome oxidase-I (COI) markers). Furthermore, for the first time, samples of the Turkish population were used in order to reveal the genetic relationship between the Turkish population and other populations, as well as to contribute to the literature with data from a new geographical region.

MATERIALS and METHODS

In this study, sequences obtained from Asian, European, American, and African regions were used. The samples from Türkiye (n: 28) are part of the AUMAC (Ankara University Mammalian Research Collection - https://mammalia.ankara.edu.tr/) and were obtained with approval from the Ankara University Local Ethics Committee for Animal Experiments (Document no: 2018-14-81). Other sequences were acquired from GenBank (https://www.ncbi.nlm.nih.gov/) (Table 1, Figure 1). DNA isolation of Turkish samples was performed using the GeneMATRIX TISSUE and Bacterial DNA Purification Kit E3551–02 (BMLabosis, Ankara, Türkiye). CYTB and COI gene regions were amplified with the primers L14727-SP (5′-GACAGGAAAAATCATCGTTG-3′)/ H15915-SP (5′-TTCATTACTGGTTTACAAGAC-3′) [27] and BatL5310 (5′-CCTACTCRGCCATTTTACCTATG-3′)/R6036R (5′-ACTTCTGGGTGTCCAAAGAATCA-3′) [28]. PCR mix, PCR conditions, and electrophoresis stages were modified from Yiğit et al. [29]. Forward and reverse sequencing was performed by BMLabosis (Ankara, Türkiye).

After the sequences were viewed and controlled in Chromas Lite 2.1.1 Software (www.technelysium.com.au), all sequences were aligned in MEGAX Software [30]. Mean genetic distance (*d*) values between populations were calculated based on *p*-distance parameter [31] in MEGAX Software [30]. Haplotype diversity (Hd), nucleotide diversity (Pi) and fixation index (Fst) values as well as the number of polymorphic sites and mutations were determined using the DNASP 6.0 Software [32]. Median-joining networks were created using haplotypes in the software Network 10.2.0.0 [33]. With the help of jModeltest [34-35], based on AIC (Akaike Information Criterion) and BIC (Bayesian Information Criterion), as the most suitable model to build Bayesian Inference (BI) trees Kimura-2 Parameter [36] and Hasegawa, Kishino, and Yano (HKY) Parameter [37] were defined for CYTB and COI genes, respectively. Bayesian Inference (BI) trees were constructed in MrBAYES 3.1.2 Program [38] with 500.000 generations and visualized in FigTree 1.4 Program (http://tree.bio.ed.ac.uk/software/figtree).

RESULTS and DISCUSSION

The 285 base pair CYTB gene region was analyzed with 48 sequences, and the 580 base pair COI gene region was analyzed with 54 sequences. Considering the CYTB results, the haplotype and nucleotide diversity values were found to be highest in the Asian population (Hd: 0.873, Pi: 0.009) and lowest in the European population (Hd: 0.286, Pi: 0.001). Similarly, the number of polymorphic sites and mutations was determined as highest in the Asian population and lowest in the European population (Table 2). Mean genetic distance (*d*) values varied between 0.06-0.9%, and Fst values were 0-0.111 (Table 3). The studied populations shared common haplotypes, and in the Median-joining Network, all haplotypes were closely located with a small number of

Table 1. Sequences obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Gene Region	Location	Accession Number	Reference
CYTB	Türkiye	PQ009837.1- PQ009838.1 (Samsun Province)	This study
		PQ009839.1- PQ009840.1 (Zonguldak Province)	
		PQ009841.1, PQ009842.1 (Ankara Province)	
		PQ009842.1 (Tekirdağ Province)	
	Switzerland	OQ885473.1	Ruedi et al. [39]
	Italy	OM502400.1	Sciandra et al. [40]
	Germany	JX887164.1	Song et al. [41]
	France	JX887162.1, JX887163.1	Song et al. [41]
	Canary Islands	HE820126.1, HE820127.1	López et al. [42]
	South Africa	MH794439.1, MH794442.1, MH794455.1, MH794459.1- MH794461.1	Moseley et al. [43]
	Madagascar	LC147010.1- LC147012.1	Sakuma et al. [44]
	India	AB973106.1, AB973107.1	Chingangbam et al. [45]
	Vietnam	FJ842277.1, FJ842278.1	Bastos et al. [46]
	Indonesia	FJ842279.1	Bastos et al. [46]
	Thailand	HM217429.1, HM217470.1, HM217473.1	Pagès et al. [47]
	Cambodia	HM217481.1	Pagès et al. [47]
	Sri Lanka	KY697996.1	Hemamali and Boyagoda [48]
	Sri Lanka	KY986748.1	Hemamali and Meegaskumbura (unpublished)
	Malaysia	MH818043.1- MH818047.1	Mohamad Ikbal, Omar, and Bhassu (unpublished)
	South Korea	OK746255.1	Park et al. [49]
	China	OP149415.1, OP149451.1, OP149475.1, OP149486.1, OP149490.1,-OP149492.1	Jing et al. [50]
	USA	JQ814283.1	Conroy et al. [51]
	Mus musculus (Outgroup)	HM222709.1	Naidu et al. [52]

mutations (Figure 2a). In the BI tree (Figure 3), samples from Thailand, Cambodia, and Vietnam were separated as the most distinct clade (pp: 0.58 and 0.62) and samples from other populations were grouped without any geographical discrimination (pp: 0.64-1.00). Besides, Turkish samples were clustered with samples from Europe, Asia, Africa and America (pp: 0.74-1.00).

COI gene region genetic diversity values (when the European and African populations were not taken into account due to the low sample size) showed that Hd and Pi values, the number of polymorphic sites and mutations were highest in the Asian population (Hd: 0.924, Pi: 0.006) and lowest in American population (Hd: 0.689, Pi: 0.002) (Table 2). *d* and Fst values were calculated as 0.2-0.6% and 0.024-0.666, respectively (Table 3). In the Median-joining Network, similar to CYTB gene results, all haplotypes, including common haplotypes formed by samples from different geographic regions were placed with a small number of mutations (Figure 2b). In the BI tree (Figure 4), the Japanese population (n: 1) split from other samples as a basal clade (pp: 0.96), and the other clades formed did not show geographical proximity, and samples from four populations gathered together (pp: 0.96-1.00). In addition, sequences belonging to Türkiye clustered with Asian, European, and American samples (pp: 0.96-0.98).

Table 1. (Continued)

Gene Region	Location	Accession Number	Reference
COI	Türkiye	PP967868.1- PP967871.1, PP967876.1- PP967882.1, PP967892.1, PP967893.1 (Ankara Province)	This study
		PP967872.1- PP967875.1 (Zonguldak Province)	
		PP967883.1 (Tekirdağ Province)	
		PP967884.1- PP967886.1 (Iğdır Province)	
		PP967887.1- PP967890.1 (Edirne Province)	
		PP967891.1 (Istanbul Province)	
		PP967894.1- PP967895.1 (Samsun Province)	
	Switzerland	MZ661173.1	Wyler (unpublished)
	Austria	KY754542.1	Schäffer et al. [53]
	South Africa	MZ353142.1, MZ353143.1	Shivambu et al. [54]
	Russia	JF499337.1-JF499339.1	Lissovsky et al. (unpublished)
	Thailand	HM217501.1, HM217504.1	Pagès et al. [47]
	China	KU182943.1, KU182944.1	Yin et al. (unpublished)
	China	KT335596.1	Sun et al. (unpublished)
	China	JQ043460.1, JQ043462.1	Ma and Lu (direct submisson)
	Japan	AB451019.1	Nakamura and Ohnuma (unpublished)
	USA	HM102311.1, HM102312.1	Cooper et al. [55]
	USA	EF568655.1, EF568688.1, EF568689.1, EF568706.1	Lorenz et al. (unpublished)
	Canada	JF457097.1, JF457098.1	Eger et al. (unpublished)
	French Polynesia	EF186576.1, EF186577.1	Robins et al. [28]
	Mus musculus (Outgroup)	KC617843.1	Jones et al. [56]

R. norvegicus has a worldwide distribution and because it affects the human populations in terms of public health and economy, evaluation of *R. norvegicus* populations has importance in driving the spread of zoonotic diseases, and helping pest control management [57]. Studies performed on wild brown rats mostly focused on ecology [58-65], and anticoagulant rodenticide resistance [66-73]. Molecular systematics and population genetic studies generally included local populations [19-23]. Broadly, studies evaluating *R. norvegicus* populations are very scarce [42, 57, 74].

In the first study to reveal genetic variation in this species, 22 allozyme loci were studied, and, contrary to mitochondrial data results, genetic variation was found to be high, gene flow was low, and the mean Fst value was 0.34 among sub-populations distributed in Türkiye [19]. Lack et al. [20]. suggested high frequencies of long-distance dispersion according to mitochondrial DNA analysis of U.S. populations. They also found that haplotype diversity values were 0.100-0.905 and nucleotide diversity values were 0.00018-0.00560 in U.S. populations. Song et al. [41] CYTB and D-loop regions and proved that the Norway rat emerged from Asia (near China) 1.3 Mya according to the CYTB analysis and found common haplotypes belonging to different geographical regions for both CYTB and D-loop regions. They calculated haplotype diversity values as 0.11-0.96 (CYTB) and 0.45-0.87 (D-loop); nucleotide diversity values were found to be $0.50\text{-}6.41 \times 10^{-3}$, (CYTB) and $0.88\text{-}9.03 \times 10^{-3}$ (D-loop). They also measured the Fst value as 0.27 (p,0.01) between Europe and Asia, 0.38 (p,0.01) between Europe and Africa, and 0.15 (p,0.01) between Asia and Africa. Puckett et al. [57] offered that *R. norvegicus* populations originating from China and Mongolia spread throughout the world through 5 possible expansion routes based on mitochondrial and nuclear SNPs. They also defined two clades in nuclear data as Asian

Table 2. Genetic diversity values derived from *R. norvegicus* sequences (NS: Number of samples, NH: Number of haplotypes, Hd: Haplotype diversity, Pi: Nucleotide diversity, PS: Number of polymorphic sites, MT: Number of mutations).

	CYTB GENE REGION					COI GENE REGION				
	NS(NH)	Hd	Pi	PS	MT	NS(NH)	Hd	Pi	PS	MT
Türkiye	7(5)	0.857	0.008	5	5	28(5)	0.728	0.005	8	8
Europe	7(2)	0.286	0.001	1	1	2(2)	1.000	0.003	2	2
Asia	24(12)	0.873	0.009	12	13	12(8)	0.924	0.006	14	14
Africa	9(4)	0.694	0.004	4	4	2(1)	0.000	0.000	0	0
America	1(1)	-	-	-	-	10(3)	0.689	0.002	3	3
Total	48(16)	0.761	0.007	16	17	54(13)	0.874	0.005	17	17

Table 3. Mean genetic distance (*d*) and fixation index (Fst) values of *R. norvegicus* populations.

	CYTB GENE REGION		COI GENE REGION	
	d values ± Standard errors	Fst values	d values ± Standard errors	Fst values
Türkiye-Europe	0.005±0.002	0.111	0.005±0.002	0.098
Türkiye-Asia	0.009±0.003	0.047	0.007±0.002	0.109
Türkiye-Africa	0.007±0.002	0.100	0.006±0.002	0.563
Türkiye-America	0.004±0.002	-	0.005±0.002	0.245
Europe-Asia	0.005±0.002	0.077	0.005±0.001	0.013
Europe-Africa	0.003±0.001	0	0.005±0.002	0.666
Europe-America	0.0006±0.001	-	0.003±0.001	0.106
Asia-Africa	0.007±0.003	0.048	0.005±0.002	0.384
Asia-America	0.005±0.001	-	0.005±0.002	0.024
Africa-America	0.002±0.002	-	0.002±0.001	0.393

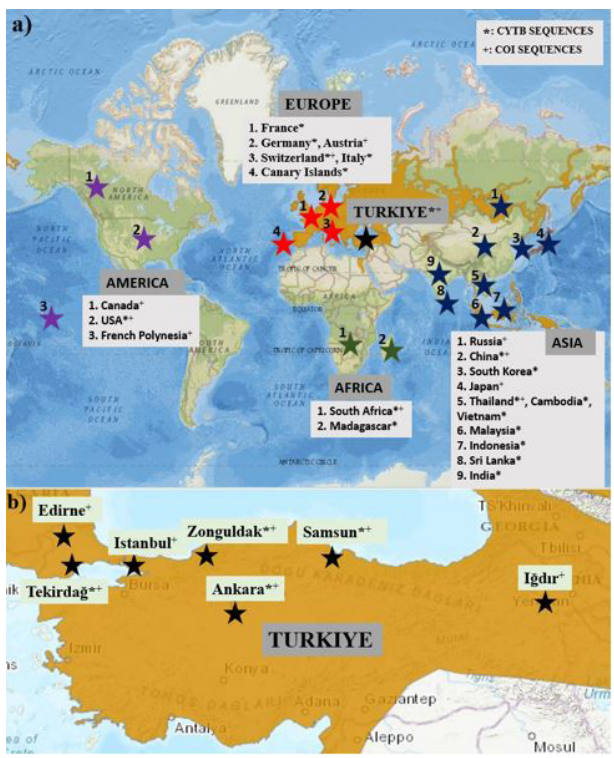

Figure 1. IUCN distribution map (https://www.iucnredlist.org/species/19353/165118026) of R. norvegicus and sampling locations of this study (a) and province information of Turkish specimens (b).

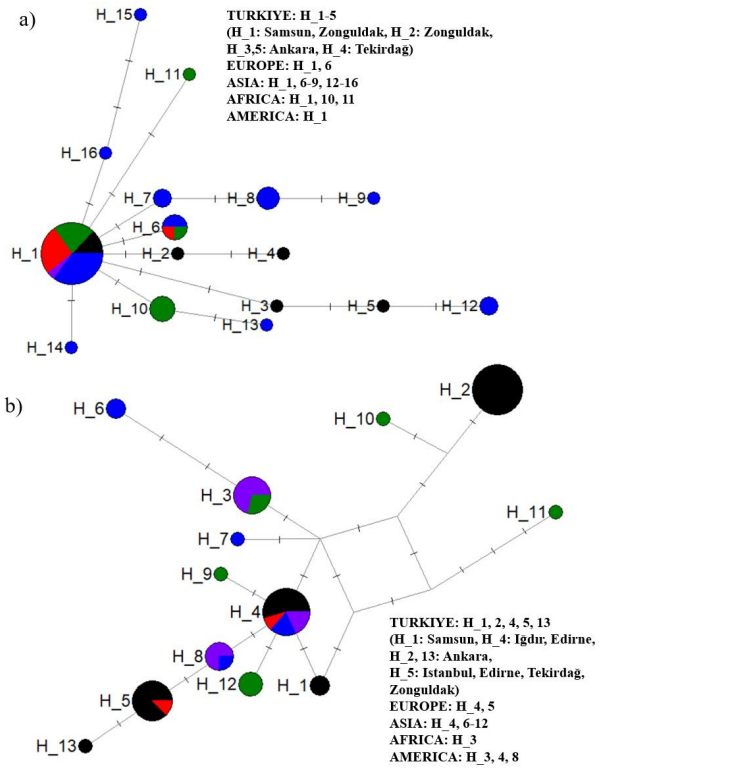

Figure 2. Median-joining networks of CYTB (a) and COI (b) gene regions derived from haplotypes. Mutations are shown with lines on branches and the sizes of circles are directly proportional to the number of samples

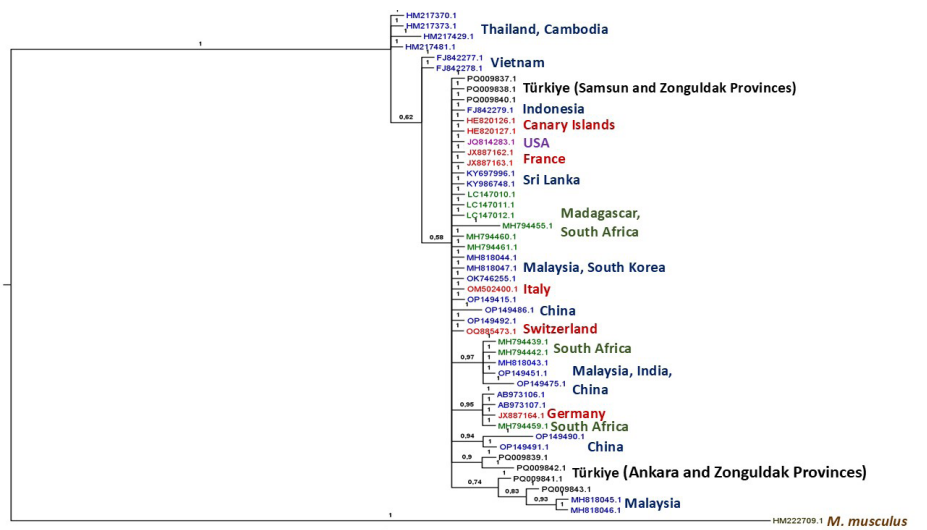

Figure 3. Bayesian Inference tree of CYTB gene region considering the Kimura-2 [36] parameter. Numbers on branches are posterior probability (pp) values.

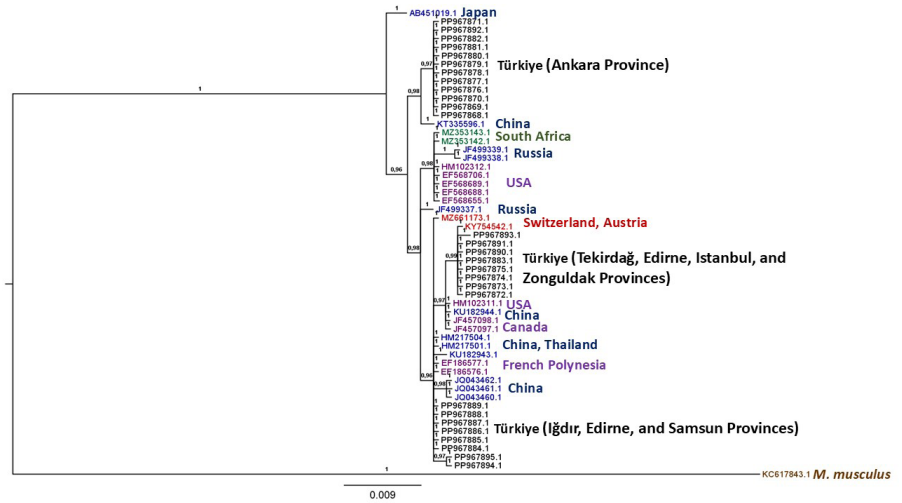

Figure 4. Bayesian Inference tree of COI gene region considering the HKY [37] parameter. Numbers on branches are posterior probability (pp) values.

and non-Asia (Europe, Africa, the Americas, and New Zealand), and observed sub-clades within these clades. In the mitochondrial dataset, 10 clades containing common haplotypes were observed. Hadjisterkotis et al. [22] determined that some of the Cyprus samples were clustered with French Polynesia samples according to the D-loop analysis. Chen et al. [23] calculated nucleotide diversity values as 0.0011-0.0015 and Fst values as 0.034-0.103 among Chinese populations. Liu et al. [74] did not define any clear distinction between Asian and European samples in their study where they analyzed the CYTB and D-loop gene regions in the Hubei population of China.

In this study, Asian, European, African, and American populations were not separated in phylogenetic approaches (Figure 2-4), and genetic distance values were low (0.06-0.9%, Table 3). Genetic diversity values were higher in European and Asian populations than in African and American populations (Table 2). The higher nucleotide diversity values and number of polymorphic sites and mutations, as well as the fact that Thailand and Japanese samples were located as distinct clades in the Bayesian Inference trees (Figure 3-4), may indicate a relative intra-population differentiation. Fst values used to identify intra-species genetic differentiation were found to be 0-0.111 for the CYTB gene region and 0.024-0.666 for the COI gene region (Table 2). Fst values lower than 0.25 are accepted as a sign of a low level of differentiation, whereas higher Fst values point a significant differentiation [75]. Türkiye-Africa, Europe-Africa, Asia-Africa, and Africa-America Fst values of COI gene region higher than 0.25 may imply a possible differentiation between these populations, despite low levels of genetic distance. However, in any case, the studied populations cannot be separated from each other.

It has been proposed that *R. norvegicus* have been spread to longer distances such as from Asia into Europe [76] and European individuals were then transported to America, Oceania, and Africa [46,76]. *R. norvegicus* started to be transferred across the globe in the 15th century [13] and it migrated to Europe in the 18th century [74]. New settlers moved the brown rats to North America in the 18th century by shipping [78].

The spread of the brown rat to many parts of the World has been continued by human activities so far [79-80], it is also possible to consider the gene flow caused by this. Therefore, it can be expected that the differentiation levels of *R. norvegicus* populations are low. Consequently, results of CYTB and COI gene region analyses showed that there is no significant genetic differences among *R. norvegicus* populations due to the effect of gene flow. Even isolated island population samples such as the Canary Islands (Europe), Madagascar (Africa), and French Polynesia (America) used in the study did not cause any important separation in terms of genetic diversity and distance, or phylogenetic approaches. On the other hand, Turkish specimens evaluated as a local population had the highest values after the Asian population in terms of genetic diversity (Table 2) and were located in different clades in Bayesian Inference trees (Figure 3-4). Although these clades do not show any geographical discrimination, this situation also points to a possible differentiation within the Turkish population.

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