Systematics and Phylogenetics
Research Article

ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., 70 (1), 2018: 19-30

Geographical Distribution Pattern of Mitochondrial DNA Cytochrome *b* Diversity in Populations of *Arvicola amphibius* (Linnaeus, 1758) (Mammalia: Rodentia) in Turkey as Determined by PCR-RFLP

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Abstract: *Arvicola amphibius* (Linnaeus, 1758) is a semiaquatic rodent living in and around wetland habitats covered by rich vegetation. It has a broad distribution in the Palearctic and three subspecies live in Turkey. In order to identify the geographical distribution of mitochondrial DNA cytochrome *b* diversity in *A. amphibius* populations in Turkey, the restriction fragment length polymorphism method was applied. The digestion patterns of four restriction enzymes (*Alu* I, *Rsa* I, *Sau3A* I and *EcoR* V) in 100 samples of *A. amphibius* from 20 localities in Turkey brought out four mtDNA lineages [Thrace (THR), central Anatolia (CA), southern Anatolia (SA) and eastern Anatolia (EA)], each having high genetic diversity. In contrast to the three lineages in THR, CA and SA, respectively, corresponding to the known subspecies *A. a. cernjavskii*, *A. a. persicus* and *A. a. hintoni*, separate populations constituting the fourth *A. amphibius* lineage in EA were determined. The results proved that the variable topography and geomorphological conglomeration process of Anatolia lead to an allopatric differentiation and had a large impact on the appearance and shaping of intraspecific genetic variations in water voles. Moreover, the existing high genetic variability in Turkish water vole populations propounds the importance of Anatolia as a potential refuge in the Pleistocene.

Key words: *Arvicola amphibius*, PCR-RFLP, mitochondrial DNA, genetic diversity, Turkey

Introduction

Turkey is surrounded on three sides by seas and has served as a natural bridge for the movement of species between Europe and the Middle East. The territory of Turkey also provides a link to the Arabian Peninsula and the Caucasus, thus allowing exchange of faunal elements in both west-east and north-south directions (Çıplak 2003, Bĭlgı 2011). On the other hand, the territory of the country contains several geographic barriers allowing vicariance events and facilitating allopatric differentiation. Such barriers are the Western Anatolia Mts, Black Sea Mts, Taurus Mts, Eastern Anatolia Mts, considered to form the "Anatolian Diagonal" as well as the "Turkish Straits System" (Davıs 1971, Bĭlgĭn 2011, Korkmaet al. 2014). Due to the limitation of species movements arising from the non-uniform topography, variable climate and active tectonics, which has caused mixing faunal elements of diverse geographic origins and the rich intraspecific genetic diversity, the territory of Turkey (both Thrace and Anatolia) has been recognised as a biodiversity hotspot (Bĭlgĭn 2011, Şekercĭoğet al. 2011). Additionally, apart from the three well-known

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European glacial refuges (Iberian, Italian and Balkan peninsulas), the importance of Anatolia in the southeastern part of this continent as a potential refuge during glacial periods has been frequently emphasised (Kosswig 1955, Hewitt 2000, Rokas et al. 2003, Çıplak 2004, Michaux et al. 2004, Kaya et al. 2015). When the above-mentioned circumstances are considered, it should be expected that there is more intraspecific variability in Anatolia. However, the number of studies on intraspecific variability in Anatolia, especially in mammal species, are limited. Therefore, more genetic data reflecting the genetic diversity of species are needed to understand the importance of Anatolia as a glacial refuge and to reveal its impacts on species differentiation.

The Eurasian water vole *Arvicola amphibius* (Linnaeus, 1758) is a semiaquatic small mammal. This rodent is widely distributed in the Palaearctic, including Turkey, both Thrace and Anatolia, living next to wetlands covered by rich reed mace and bulrush vegetation (Kryštufek & Vohralík 2005, Wilson & Reeder 2005). The nomenclature of this taxon seems complex due to the alternate usage of two synonymous names, *A. terrestris* and *A. amphibius*, as valid in recent studies (Üstünbaş et al. 2011, Tez et al. 2011, Arslan et al. 2011, Kryštufek et al. 2015). In this paper, the name *A. amphibius* is used as suggested by previous authors (Corbet 1978, Wilson & Reeder 2005).

A generalization about the scope of the studies performed on European populations of *A. amphibius* divides them into two parts: 1) phylogenetic and phylogeographic studies based on variations of the nuclear and mitochondrial DNA, and 2) mostly conservation biology studies dealing with the changes in the genetic structure of this species using polymorphic microsatellite loci (Taberlet et al. 1998, Stewart et al. 1998, Telfer et al. 2003, Berthier et al. 2005, Piertney et al. 2005, Aars et al. 2006, Centeno-Cuadros et al. 2009, Kryštufek et al. 2015). Phylogenetic and phylogeographic studies showing the connections and movement of *A. amphibius* lineages indicate that current lineages of this species in Italy and Central Europe may have emerged from pre-Pleistocene population divergence, while the more northern populations in the UK must have been derived from two different sources, the Iberian Peninsula and more eastern refuges (Taberlet et al. 1998, Piertney et al. 2005, Castiglia et al. 2016). Previous studies on this species from Turkey have mainly been determined its taxonomy using morphometry, morphology, karyology and distribution records. Three subspecies of this rodent in Turkey have been determined based on morphometric and morphological differentiation of its populations: *A. amphibius cernjavskii* Petrov, 1949 in THR, *A. amphibius persicus* de Phillipi, 1865 in most parts of Anatolia and *A. amphibius hintoni* Aharoni, 1932 in Hatay Province in the southern part of Anatolia (Mursaloğlu 1975). Morphometric and morphological studies are not comprehensive and have been mostly performed on only local populations. Therefore, they are insufficient for the evaluation of populations at a subspecific level. There is no karyological revision at the subspecies level of this species, a variable morphology of the autosomal chromosomes in karyological structure has often been found in populations from different geographical regions within the distribution range of known subspecies in Turkey (Özkurt et al. 1999, Gözcelïoğl et al. 2006, Tez et al. 2011, Arslan et al. 2011, Arslan & Zima 2014). A local biochemical study and a recent study based on randomly amplified polymorphic DNA (RAPD-PCR) also proved the presence of *A. a. cernjavskii* in THR and *A. a. persicus* in Anatolia, but they did not explore in detail the phylogeny and genetic features of the species because of the small scale of the geographical sampling and the marker used (İyïgün & Çolak 2004, Üstünba et al. 2011). Although previous studies using morphometry, morphology, karyology, biochemical and molecular data on *A. amphibius* in Turkey are the initial steps in revealing genetic variations of the species, they are not adequate to determine the geographical distributions of genetic variations and solve problems with the movements of this species in Turkey. For this purpose, the primarily aim of the present study was to explore the distribution of genetic diversities and differentiations in the cytochrome *b* region of mitochondrial genome in *Arvicola amphibius* populations in Turkey by employing the PCR-restriction fragment length polymorphism (PCR-RFLP) method.

Materials and Methods

Sample collection

A total of 100 samples of *Arvicola amphibius* were collected from 20 localities in Turkey for the PCR-RFLP analysis performed within the scope of the study (Table 1, Fig. 1). Tissue samples were obtained from the captured animals during the field work performed in accordance with the legal permission (no: B.18.0.DMP.0.02.510.02-59992) given by the Ministry of Environment and Forests, General Management of Nature Conservation and National Parks of the Republic of Turkey and a decision (no.

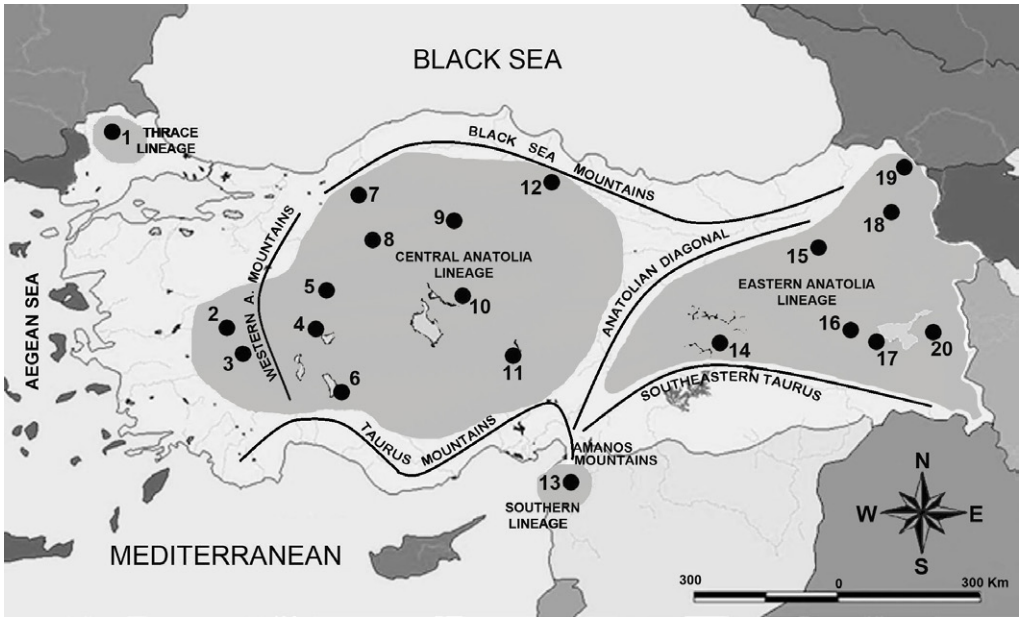

Fig. 1. Map of the sampling sites of *Arvicola amphibius* populations. Numbers in the map represent the localities given in Table 1. Black lines indicate some mountain chains in Turkey. Shaded areas match the approximate distribution of *A. amphibius* lineages determined by PCR-RFLP in the current study.

Table 1. Sampling locations of *Arvicola amphibius* specimens used in the study. All locations are in order consistent with the map number in Figure 1. (N = sample size, THR = Thrace, CA = central Anatolia, SA = southern Anatolia, EA = eastern Anatolia, m = meter).

No.	Localities	Lineages	N	Coordinates		Altitude (m a.s.l.)
				X	Y	
1	İnece Creek, Kırklareli	THR	10	41°40′	27°4′	101
2	Yenişehir, Uşak	CA	4	38°39′	29°0′	926
3	Çivril, Denizli	CA	8	38°16′	29°54′	886
4	Eber Lake, Afyon	CA	5	38°37′	31°12′	968
5	Gülçayır, Eskişehir	CA	7	39°14′	31°23′	843
6	Beyşehir Lake, Konya	CA	6	37°40′	31°43′	1148
7	Yeniçağa, Bolu	CA	4	40°49′	32°5′	1001
8	Ayaş, Ankara	CA	5	40°1′	32°13′	981
9	Kızılırmak, Çankırı	CA	2	40°20′	33°58′	552
10	Kılıçözü Creek, Kırşehir	CA	8	39°11′	34°8′	1008
11	Ovaçiftliği-Kuşçu, Kayseri	CA	6	38°14′	35°9′	1080
12	Ladik, Samsun	CA	3	40°54′	35°52′	916
13	Reyhanlı, Hatay	SA	6	36°18′	36°32′	87
14	Sivrice, Elazığ	EA	2	38°27′	39°16′	1252
15	20 km. east of Erzurum	EA	5	39°56′	41°21′	1936
16	Altınova crossroad, Muş,	EA	2	38°37′	41°55′	1279
17	Tatvan, Bitlis	EA	4	38°28′	42°28′	1654
18	Selim, Kars	EA	5	38°37′	41°55′	1858
19	Çamlıçatak, Ardahan	EA	4	41°7′	42°50′	1800
20	Erçek Lake, Van	EA	4	38°36′	43°34′	1808

2011-127-492) given by the Animal Experiments Local Ethics Committee of Ankara University. All skulls, skins and variable tissues were deposited in the Ankara University Mammalian Research Collection (AUMAC, www.mammalia.ankara.edu.tr).

Laboratory protocols

Total genomic DNA was isolated from the kidney, liver or muscle tissues compatible with the CTAB isolation method (Doyle & Doyle 1990). Approximately 1140 bp of cytochrome *b* gene region

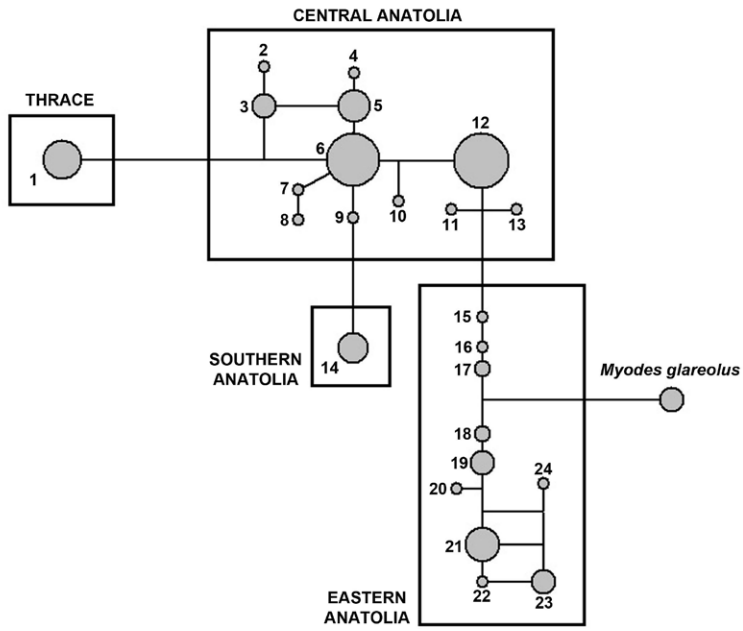

Fig. 2. A median-joining network constructed using 24 mitochondrial DNA cytochrome *b* RFLP haplotypes identified in the *Arvicola amphibius* populations. An explanation of each haplotype is indicated in Table 2. The size of the circles is proportional to the frequencies of the haplotypes.

of mitochondrial DNA of *A. amphibius* was amplified with primers L14724[a] and H15915R (IRWIN et al. 1991). Amplification reactions were carried out in a 37.5 µL total volume, containing 21.9 µL sterile water, 3.75 µL buffer (750 mM Tris-HCl pH 8.8 at 25°C) 200mM $(NH_4)_2SO_4$, 0.1% Tween 20 (Fermentas), 6 µL dNTP mix (A, C, G, T 200 µM), 3 µL $MgCl_2$ (25 mM), 0.45 µL forward and reverse primers (20 pmol, Fermentas), 0.45 µL Taq polymerase (500 U, Fermentas) and 1.5 µL DNA (at least 500 ng/µL for each sample). After an initial denaturation of 5 min at 94 °C, 30 cycles of PCR were performed in a Thermo Scientific Hybaid P × 2 thermal cycler. The reaction conditions were as follows: denaturation for 1 min at 93 °C, annealing for 1 min at 50 °C, extension for 5 min at 72 and a final extension for 1 min at 72 °C.

Following amplification, the PCR products were digested using four different endonucleases (*Alu* I, *Rsa* I, *Sau3A* I and *EcoR* V, FastDigest Fermentas). For each sample, a reaction mix containing 18 µL nuclease-free water, 2 µL buffer (10 × FastDigest Buffer/FastDigest Green Buffer) and 1 µL restriction enzyme was prepared, then 10 µL PCR products were added to the mix. After incubation at 37 °C, which was the optimum temperature for all of the endonucleases used in the study, for 15–30 min, all of the digested DNA fragments were loaded on 2% agarose gel and run by applying 100 V for 60–80 min in an electric field. After electrophoresis, all of the gels were stained with ethidium bromide (EtBr) for approximately 30 min and then visualised by a KODAK Gel Logic 100 System.

Statistical analyses

For statistical analyses, a data set including 100 samples of *A. amphibius*, along with four samples of *Myodes glareolus* used as the outgroup, was prepared according to the band profiles of each sample on agarose gel, and a matrix was constructed by scoring bands as present (1) or absent (0). To show phylogenetic relationships among the populations, a neighbour-joining (NJ) (Saitou & Nei 1987) phylogenetic tree based on Nei-Li's genetic distance (*D*) and the BIONJ method (Gascuel 1997) with 1000 bootstrap replicates (Felsenstein 1985) was constructed with PAUP version 4.0b10 (Swofford 2002). For determining RFLP haplotypes and their evolutionary relationship, a median-joining network analysis (Bandelt et al. 1999) was realised using Network 4.6.1.2. Nei's *D* (Nei 1978) was calculated with POPGENE version 1.32 (Yeh et al. 1997) for measuring the genetic divergence of the populations. To assess the parameters of the genetic variation and differentiation, the average number of observed alleles per locus (Na), the average number of effective alleles (Ne), Shannon's information index (I), observed heterozygosity (Ho) and expected heterozygosity (He) values and their standard errors were computed. Moreover, *F* statistics (Wright 1951)

Table 2. Mitochondrial DNA cytochrome *b* haplotype list of *Arvicola amphibius* in Turkey based on PCR-RFLP. Numbers in parenthesis following locality names indicate the sample size in shared haplotypes.

Haplotypes	Frequency	Localities
1	10	Kırklareli
2	1	Samsun
3	4	Kayseri
4	1	Konya
5	7	Konya (1), Kırşehir (5), Ankara (1)
6	19	Samsun (1), Kayseri (2), Afyon (3), Denizli (4), Eskişehir (2), Konya (2), Kırşehir (3), Çankırı (2)
7	1	Konya
8	1	Konya
9	1	Afyon
10	1	Samsun
11	1	Uşak
12	21	Afyon (1), Denizli (4), Eskişehir (5), Bolu (4), Uşak (4), Ankara (3)
13	1	Ankara
14	6	Hatay
15	1	Van
16	1	Van
17	2	Van
18	2	Elazığ
19	4	Bitlis (3), Muş (1)
20	1	Erzurum
21	8	Ardahan (4), Kars (4)
22	1	Kars
23	4	Erzurum
24	1	Muş

were calculated for measuring the genetic differentiation between the populations at three different levels: 1) F_{IS}, to demonstrate the degree of inbreeding within individuals relative to the rest of their subpopulation; 2) F_{ST}, to estimate the degree of inbreeding within a subpopulation relative to the total population and 3) F_{IT}, to determine an overall inbreeding coefficient for an individual by measuring its heterozygosity relative to the total population. Additionally, gene flow (N_m) statistics were calculated among the populations. All of the genetic variation and differentiation statistics were derived as implemented in GenAlEx 6.5 (Peakall & Smouse 2012).

Results

Distribution of genetic diversity

An approximate 1140-bp length of cytochrome *b* gene in mitochondrial DNA of *A*. *amphibius*, digested by four endonucleases (*Alu* I, *Rsa* I, *Sau3A* I, and *EcoR* V), yielded 24 haplotypes, comprising four main lineages, including samples from THR, CA, SA and EA in Network analysis. No shared haplotype was found between the lineages. The most common and shared haplotypes were within the CA lineages (haplotypes 12, 6 and 5, respectively). The EA lineages had two shared haplotypes (Table 2, Fig. 2).

The NJ phylogenetic tree based on the Nei-Li *D* and BIONJ method (GASCUEL 1997) brought out four main lineages, as was determined in the phylogenetic network. The first lineage comprised only samples from EA with a high bootstrap value (100%). The second lineage, with a moderate bootstrap value (60%), consisted of CA populations. The third lineage included Hatay samples in SA and had a bootstrap value of 93%. As for the fourth lineage, it involved samples in THR with a bootstrap value as high as 97% (Fig. 3).

The first three highest values of Nei's *D* (NEI 1978) were found between SA and the other lineages, while the lowest value was computed between the EA and CA lineages (Table 3).

Allele diversity and genetic variability

Digestion of the cytochrome *b* gene of 100 samples of *Arvicola amphibius* by endonucleases *Alu* I, *Rsa* I, *Sau3A* I and *EcoR* V, revealed 32 loci in total. All of the loci were polymorphic. The results of the allele diversity and genetic variability are shown in Table 4. The average number of different alleles per

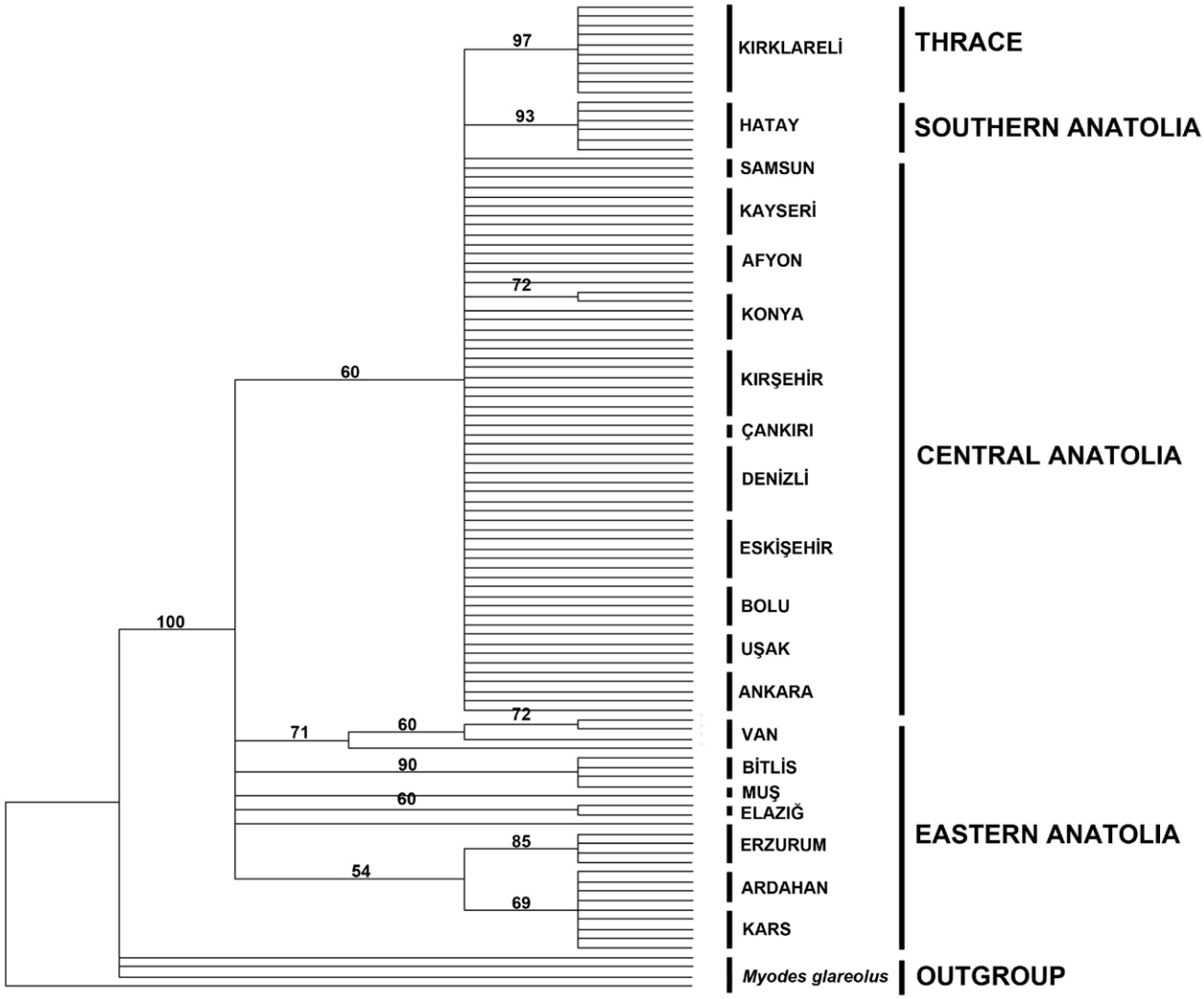

Fig. 3. NJ tree displays inferred phylogenetic relations based on PCR-RFLP digestion patterns in 20 populations of *Arvicola amphibius* in Turkey. Bootstrap resampling support from 1000 replications is demonstrated above the branches.

locus (Na) was high and ranged between 10.38 and 31.13 in all of the lineages. Similarly, the average effective number of alleles per locus (Ne) was also considerable and the values for all of the lineages were between 9.49 and 25.08. The He was slightly lower than the Ho in all of the lineages and the average Ho and He were 0.97 and 0.91, respectively. The highest and lowest values of the average number of private alleles were 1.656 in CA and 0.063 in THR, while it was 0.188 in EA. SA had no unique alleles. Shannon's Information index (I) varied from 2.29 in SA to 3.32 in CA and demonstrated that all of the lineages had a high level of genetic diversity. The average value of Wright's *F* statistics calculated at three different levels, F_{IS}, F_{IT} and F_{ST}, supplied a perspective about the distribution of genetic differentiation in all of the examined lineages. The mean value of the inbreeding coefficient (F_{IS} = –0.061) was negative and reflected less inbreeding than expected or sufficiency of heterozygosity in all of the lineages. Moreover, F_{IT} had a negative mean value (F_{IT} = –0.013) and showed increasing heterozygosity in the lineages as well. The mean value of F_{ST} was 0.045, which indicated the presence of little genetic differentiation in all of the lineages (4.5%). Gene flow values (N_m) between all of the lineages changed from 7.15 to 32.89 (Table 5).

Discussion

Taxonomic implications at the subspecific level

Few detailed molecular markers using on the phylogeny, genetic structure and geographic distribution of the genetic diversity of the *A. amphibius* populations in Turkey exist. Three subspecies of this species has been proposed based only on variations in the dental, cranial and fur structures (Mursaloğlu 1975). Although it is difficult to identify subspe-

Table 3. Nei's *D* (below diagonal) and genetic identity (*I*) (above diagonal) (Nei, 1978) values between the *Arvicola amphibius* lineages.

Lineages	1	2	3	4	5
1. THR		0.325	0.502	0.563	0.289
2. SA	1.123		0.433	0.483	0.243
3. EA	0.688	0.836		0.688	0.394
4. CA	0.574	0.728	0.374		0.408
5. Outgroup	1.242	1.415	0.932	0.897	

Table 4. Genetic variability in *Arvicola amphibius* lineages in Turkey. Na = average number of different alleles per locus, Ne = average number of effective alleles per locus, I = Shannon's information index, Ho = observed heterozygosity, He = expected heterozygosity, uHe = unbiased expected heterozygosity, F = fixation index.

Lineages		N	Na	Ne	I	Ho	He	uHe	F
THR	Mean	10	15.09	12.76	2.63	0.96	0.92	0.97	–0.05
	SE		0.20	0.25	0.02	0.01	0.00	0.00	0.01
SA	Mean	6	10.38	9.49	2.29	0.97	0.89	0.97	–0.09
	SE		0.18	0.24	0.02	0.01	0.00	0.00	0.01
EA	Mean	25	25.50	19.62	3.10	0.96	0.95	0.97	–0.02
	SE		0.32	0.32	0.01	0.01	0.00	0.00	0.01
CA	Mean	59	31.13	25.08	3.32	0.97	0.96	0.97	–0.01
	SE		0.18	0.29	0.01	0.00	0.00	0.00	0.00
Total	Mean	100	17.88	14.77	2.66	0.97	0.91	0.97	–0.06
	SE		0.72	0.54	0.04	0.00	0.00	0.00	0.01

Table 5. Pair-wise F_{ST} (below diagonal) and N_m values (above diagonal) between all of the *Arvicola amphibius* lineages.

Lineages	1	2	3	4	5
1. THR		7.15	14.04	16.82	5.40
2. SA	0.034		9.73	10.99	4.50
3. EA	0.017	0.025		32.89	6.91
4. CA	0.015	0.022	0.008		7.25
5. Outgroup	0.044	0.053	0.035	0.033	

cies while considering only these features, the first available karyological differences arising from variable autosomal chromosome morphology in the standard karyotype (2n = 36) of *A. amphibius* has facilitated the separation of *A. a. cernjavskii* in Thrace from *A. a. persicus* in Anatolia (Özkurt et al. 1999, Gözcelíoğl et al. 2006). Moreover, a display of new chromosomal variations by subsequent traditional and banded karyotype studies, including samples from some locations being close to the previously known localities in both in Thrace and central Anatolia, has supported and even heralded such a subspecific differentiation in *A. amphibius* populations (Tez et al. 2011, Arslan et al. 2011). In a recent comprehensive study, RAPD-PCR analysis of the populations from Thrace and some parts of Anatolia has presented two major *A. amphibius* groups representing the two mentioned subspecies in Thrace and Anatolia with relatively low genetic distance (*D*), but surprisingly high genetic differentia-

tion (G_{ST}) between all of the populations (Üstünbaş et al. 2011). This was the first DNA-based study on *A. amphibius* populations in Turkey and it could not be expected to reflect a general subspecific evaluation due to the relatively limited geographic range of the sampling. Due to the large geographic sampling and PCR-RFLP analysis of this study, we could assess the subspecies of *A. amphibius* in Turkey. According to our results, the determined 24 haplotypes in the populations of *A. amphibius* in Turkey formed four main genetically differentiated and highly variable lineages. Those lineages had no shared haplotypes among them and their geographical distributions were non-overlapping. Thus, we have confirmed that three of the known subspecies, *A. a. cernjavskii* from Thrace, *A. a. persicus* from most parts of Anatolia and *A. a. hintoni* in and around Hatay Province in the southern part of Anatolia, live in Turkey. However, it seems that the determination of the fourth lineage formed by the separate populations in from eastern Anatolia may be the main issue for taxonomical reassessment of *A. amphibius* in Turkey. It may be considered to be early for describing a new taxon at this stage, because this requires additional studies, such as the usage of more and powerful genetic markers, and detailed statistical analysis.

Genetic variability of *A. amphibius* in Turkey

The main parameters of genetic variability in the water vole lineages showed that all of the lineages had significant genetic diversity (Table 4). The CA and EA lineages were the most diversified lineages. They included more specimens from different localities, whereas both THR and SA had a small specimen size from only one locality. A limited specimen size from each locality in those lineages may be the cause of the lower level of genetic diversity when compared to the other lineages. Moreover, the average number of private allele values for the CA and EA lineages was higher than that for THR and SA lineage had no private alleles. In addition, another indicator of a high level of genetic diversity in all of the lineages is that Ho is always higher than He. The negative values of inbreeding coefficients F_{IS} = –0.06 and F_{IT} = –0.013, corresponding to the increasing heterozygosity in all of the lineages, is another indicator of genetic richness in all of the *A. amphibius* lineages, which is consistent with the results of a previous local biochemical study performed by İyiğün & Çola (2004). According to the results of the RAPD-PCR study by Üstünbaş et al. (2011), genetic diversity is at a low level in the populations from Thrace and Anatolia, as opposed to our results. The average pair-wise F_{ST} statistics showed little genetic differentiation among all of the lineages of *A. amphibius*, contrary to the results of Üstünbaş et al. (2011). This low F_{ST} level is also supported by the N_m values derived from the F_{ST} value. We have recorded low F_{ST} and high N_m levels which are moderate in terms of subspecific differentiation in the studied populations *of A. amphibius*.

In a general frame, the high overall intraspecific genetic diversity in populations of *A. amphibius* living in fragmented and generally disconnected wetland habitats in Turkey is a good signal for the continuity of the species in future. The high level of karyotype diversity, arising from a differentiation in the morphology of autosomal chromosomes in the populations from distinct geographical regions in Turkey, is another signal supporting the high genetic diversity in *A. amphibius* (Özkurt et al. 1999, Gözcelïoğl et al. 2006, Tez et al. 2011, Arslan et al. 2011; Arslan & Zima 2014). Therefore, it can also be concluded that the variable topography and active tectonism of Anatolia, which led to allopatric differentiation for many taxa, has had a large impact on the appearance and shaping of intraspecific genetic variations for water voles.

Biogeographical perspective for diversification of *A. amphibius* lineages

The most striking result is the position of the EA lineage, which seems to be a separate lineage in the NJ phylogenetic tree and Network analysis. Samples bearing haplotypes of this lineage were collected from the east of the mountain chains known as the Anatolian Diagonal in eastern Anatolia. This significant geographic barrier reaches from north-eastern Anatolia to south-western Anatolia and comprises consecutive mountains lying in a north-south and east-west direction. In south-eastern Anatolia, it is divided into two arms, one is linked to the Taurus Mts. and the other is connected to the Amanos Mts. In terms of biogeography, the Anatolian Diagonal is a significant geographical formation playing a role in the spread and differentiation of many plant and animal species by allowing both dispersal and vicariance events (Davis 1971, Kapli et al. 2013, Ahmadzadeh et al. 2013). Our results indicated that the formation of the Anatolian Diagonal may have had a considerable impact giving rise to a vicariance event, and thus to allopatric differentiation of the EA lineages from the others.

Diversification of the THR lineage from the other lineages in Anatolia could be explained by climatic changes during the Middle Pleistocene, which have led to a discrete linkage between the straits, water flow events and geomorphological formation

of the Turkish Strait system (Korkmaz et al. 2014). It comprises the Bosporus and the Dardanelle Straits and the Marmara Sea, and is known to be an important geographical barrier that prevents a continental linkage between Thrace and Anatolia. Phylogeographic studies including some rodent species from Thrace and Anatolia disclosed conflicting results comprising genetic homogeneity or high genetic differences between the populations on both sides of this system (Michaux et al. 2003, 2004, Dubey et al. 2007, Gündüz et al. 2007, Hurner et al. 2010, Helvaci et al. 2012). The genetic homogeneity of the populations in those studies has been interpreted as a result of a recent population expansion, taking place shortly before water began flowing. On the other hand, high genetic differences might have occurred as a result of the alternating submersion and rise of the Bosporus Strait during the glacial cycles of the Pleistocene and the geomorphological formation of the straits. In principle, it should be expected that a recent diversification caused by water flowing rather than the effect of climatic changes in the Pleistocene and geomorphological processes shaping the current geology of the straits may have occurred, if the degree of genetic differentiation between the populations is low, or vice versa (Demirso 2002). When this statement is considered, Nei's *D*, genetic similarity (*I*) and F_{ST} values may give us a clue about which event had affected to a greater extent the diversification of the THR lineage from the other two lineages in Anatolia. The second lowest *D* value, due to the highest *I* value, was between the geographically close THR and CA lineages. Supporting this, the F_{ST} value was similar for these two lineages as compared with the others. Even if it seems difficult to make a definite inference about the approximate diversification time, due to the power of the marker used in the current study, low *D* and F_{ST} and high genetic similarity values between the mentioned lineages indicate that a recent diversification affected by water flowing may be more possible, provided that the effect of other events is not ignored.

The SA lineage, including samples from Reyhanlı (Hatay), may have diversified as the result of two concurrent tectonics events occurring approximately 3 Mya in the Late Pliocene (Popov et al. 2006). The first is the rise of the Amanos Mts., which isolate the populations in the south from those in South-western and Central Anatolia. The second is the formation of the south-eastern parts of the Taurus Mts., which prevents the linkage of populations in the south to eastern Anatolia. The highest *D* and F_{ST} values between this lineage and others may indicate such long-term isolation. The active tectonic movements triggering the formation of the Amanos and South-eastern Taurus Mts. in the region have also been suggested as possible mechanisms for the splitting of the two rodent species: *Mesocricetus auratus* in south-eastern Anatolia and *Mesocricetus brandtii* in the rest of Anatolia (Neumann et al. 2006).

The CA lineages contain samples from 11 different localities inside of a wide geographical area surrounded by the Black Sea Mts. in the north, the Western Anatolia Mts. in the west, the Central and Western Taurus Mts. in the south and the Anatolian Diagonal in the east. This geographical area is mostly involved by Kırşehir, the Menderes-Taurus blocks and Sakarya continent, which are three of the five main geological units in central Anatolia. Previous studies on the diversification of *Pseudophoxinus* and *Aphanius* species complexes have revealed that the geomorphological formation process of central Anatolia was significant factor for the divergence of the populations living outside of those geological units and occupying these areas (Hrbek et al. 2002, 2004). The presence of the three most common mtDNA haplotypes in the CA lineage of *A. amphibius* showed that interaction of the individuals within the distribution limits of this lineage was possible. However, strong geographical barriers encircled the area and suggested formation process shaping the area may have prevented interactions with other lineages to a certain extent and, apparently, they are the most possible reason for the isolation of the lineage.

Based on comparison of the values of *D* and *I* between all of the lineages, it was seen that the SA lineage, which was thought to have been isolated by the impact of two main geographical barriers, is the most distant lineage. In addition, the F_{ST} and N_m values are also confirming this conclusion. The constituent EA lineage formed one separate branch, while the CA lineage was clustered together with the THR and SA lineages in the NJ phylogenetic tree, which were the closest lineages according to the values of *D* and *I*. This may have resulted from the wide geographical distribution of the CA lineage, which may have interacted with the EA lineage via possible corridors in the impassable mountains of the Anatolian Diagonal. Additionally, it is also possible that central Anatolia was on one of the passageways providing a connection between populations in Europe and both the southern and eastern Anatolian refuges in Anatolia during the Tertiary (Kosswig 1955). These possible connections can also be supported by the lowest F_{ST} value and the highest N_m value between the two lineages. Nevertheless, additional sampling from near localities in both the west and east sides of the Anatolian Diagonal is needed to take this thought

to the forefront for possible interaction. Apart from this, EA in the east of the Anatolian Diagonal and the THR lineage remaining on the European side of the Turkish Strait system were significantly distant in conformity with the *D* or similarity, F_{ST} and N_m values between them.

Consequently, the geographic distribution of genetic diversity and genetic differentiation in the populations of *A. amphibius* living in Turkey has revealed that this semiaquatic small mammal species has four main genetically diversified lineages and high intraspecific genetic variability. The obtained results show that the Anatolian Diagonal, the Amanos Mts., the South-eastern Taurus Mts. and the Turkish Strait system are the most considerable geographical barriers leading to the allopatric diversification of this species. The detection of THR, CA and SA lineages, including samples from the distribution areas of the three previously determined subspecies of *A. amphibius* (Mursaloğlu 1975), confirmed the presence of all subspecies in Turkey, which is generally consistent with the results of a former study based on RAPD data (Üstünbaş et al. 2011). However, *A. amphibius* also has separate populations in eastern Anatolia, showing a significant differentiation in their mtDNA. Therefore, the existence of the fourth lineage, including populations in eastern Anatolia apart from the three known subspecies, was the most striking result of this research. In this case, it is useful to remember the thought suggested by Kryštufek et al. (2015) that "species limits in the genus *Arvicola* is still far from final". Therefore, more genetic markers should be employed and more detailed evaluations are required on the taxonomy of *A. amphibius* in Turkey. Despite the fact that it is not possible to compare the results with regard to the genetic diversity of *A. amphibius* in Turkey presented by this study to that of its counterparts in Europe due to the used marker, it should be considered that the existing high genetic variability in Turkish water vole populations can propound the importance of Anatolia as a potential refuge in the Pleistocene, as has been demonstrated in broad phylogeographic studies (Taberlet et al. 1998, Hewitt 1999, 2000). It is also clear that additional genetic markers, such as both organelle and nuclear DNA sequences and microsatellites enabling data comparison, should be employed for exploring this question further through more defined evaluations.

Acknowledgments: We would like to thank Dr. Tolga KANKILIÇ and Dr. Kubilay TOYRAN for their help during the field work, Dr. Başar KARACA for his help conducting the experimental protocols in the laboratory, Dr. Münir UÇAK for preparing the map and Dr. Sarp KAYA for constructing the phylogenetic tree. This research was partially supported by the Coordinatorship of the Scientific Research Projects of Ankara University (No. 12B4240010) and Coordinatorship of Scientific Research Projects of Artvin Çoruh University (No. 2015. F10.02.05).

References

Aars J., Dallas J. F., Piertney S. B., Marshall F., Gow J. L., Telfer S. & Lambin X. 2006. Widespread gene flow and high genetic variability in populations of water voles *Arvicola terrestris* in patchy habitats. Molecular Ecology 15: 1455-1466.

Ahmadzadeh F., Flecks M., Rödder D., Böhme W., Ilgaz Ç., Harris D. J., Engler J. O., Üzüm N. & Carretero M. A. 2013. Multiple dispersal out of Anatolia: biogeography and evolution of oriental green lizards. Biological Journal of the Linnean Society 110: 398-408.

Arslan A., Yorulmaz T., Toyran K., Gözütok S. & Zima J. 2011. C-heterochromatin variation and NOR distribution in the karyotype of water vole *Arvicola terrestris* (Mammalia, Rodentia). Caryologia 64 (2): 215-222.

Arslan A. & Zima J. 2014. Karyotypes of the mammals of Turkey and neighbouring regions: a review. Folia Zoologica 63 (1): 1-62.

Berthier K., Galan M., Foltête J. C., Charbonnel N. & Cosson J. F. 2005. Genetic structure of the cyclic fossorial water vole (*Arvicola terrestris*): Landscape and demographic influences. Molecular Ecology 14: 2861-2871.

Bandelt H., Froster P. & Rohl A. 1999. Median joining network for inferring intraspecific phylogenies. Molecular Biology and Evolution 16 (1): 37-48.

Bilgin R. 2011. Back to the Suture: The distribution of intraspecific genetic diversity in and around Anatolia. International Journal of Molecular Sciences 12: 4080- 4103.

Castiglia R., Aloise G., Amori G., Annesi F., Bertolino S., Capizzi D., Mori E. & Colangelo P. 2016. The Italian peninsula hosts a divergent mtDNA lineage of the water vole, *Arvicola amphibius s.l.*, including fossorial and aquatic ecotypes. Hystrix, the Italian Journal of Mammalogy (Online) **27** (2).

Centeno-Cuadros A., Delibes M. & Godoy J. A. 2009. Dating the divergence between southern and European water voles using molecular coalescent-based methods. Journal of Zoology 279: 404-409.

Corbet G. B. 1978. The mammals of the Palaearctic region: a taxonomic review. London: Cornell University Press. 314 p.

Çıplak B. 2003. Distribution of Tettigoniinae (Orthoptera, Tettigoniidae) bush-crickets in Turkey: the importance of the Anatolian Taurus Mountains in biodiversity and implications for conservation. Biodiversity and Conservation 12: 47-64.

Çıplak B. 2004. Systematics, phylogeny and biogeography of *Anterastes* (Orthoptera, Tettigoniidae, Tettigoniinae): evolution within a refugium. Zoologica Scripta 33: 19-44.

Davis P. H. 1971. Distribution patterns in Anatolia with particular reference to endemism. In: Davis P.H., Harper P. C. & Hedge I. C. (ed.) Plant life of southwest Asia. Aberdeen:

Aberdeen University Press. 335 p.

Demirsoy A. 2002. Genel Zoocoğrafya ve Türkiye Zoocoğrafyası. Ankara: Meteksan. 1007 p.

Doyle J. J. & Doyle J. L. 1990. Isolation of plant DNA from fresh tissiue. Focus 12: 13- 15.

Dubey S., Cosson J., Vohralík V., Kryštufek B., Diker E. & Vogel P. 2007. Molecular evidence of Pleistocene bidirectional faunal exchange between Europe and the Near East: The case of the bicoloured shrew (*Crocidura leucodon*, Soricidae). Journal of Evolutionary Biology 20: 1799-1808.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39 (4): 783-791.

Gascuel O. 1997. BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. Molecular Biology and Evolution 14: 685-695.

Gözcelioğlu B., Çolak E. & Çolak R. 2006. Karyotype of *Arvicola terrestris* (Mammalia: Rodentia) in Turkish Thrace. Pakistan Journal of Biological Sciences 9 (12): 2387-2388.

Gündüz İ., Jaarola M., Tez C., Yeniyurt C., Polly P. D. & Searle J. B. 2007. Multigenic and morphometric differentiation of ground squirrels (*Spermophilus*, Scuiridae, Rodentia) in Turkey, with a description of a new species. Molecular Phylogenetics and Evolution 43: 916-935.

Helvaci Z., Renaud S., Ledevin R., Adriaens D., Michaux J., Çolak R., Kankiliç T., Kandemir İ., Yiğit N. & Çolak E. 2012. Morphometric and genetic structure of the edible dormouse (*Glis glis*): a consequence of forest fragmentation in Turkey. Biological Journal of the Linnean Society 107: 611-623.

Hewitt G. M. 1999. Post-glacial re-colonization of European biota. Biological Journal of the Linnean Society 68: 87-112.

Hewitt G. M. 2000. The genetic legacy of the Quaternary ice ages. Nature 405: 907-913.

Hrbek T., Küçük F., Frickey T., Stölting K. N., Wildekamp R. H. & Meyer A. 2002. Molecular phylogeny and historical biogeography of the *Aphanius* (Pisces, Cyprinodontiformes) species complex of central Anatolia, Turkey. Molecular Phylogenetics and Evolution 25: 125-137.

Hrbek T., Stölting K. N., Bardakçi F., Küçük F., Wildekamp R. H. & Meyer A. 2004. Plate tectonics and biogeographical patterns of the *Pseudophoxinus* (Pisces: Cypriniformes) species complex of central Anatolia, Turkey. Molecular Phylogenetics and Evolution 32: 297-308.

Hürner H., Kryštufek B., Sarà M., Ribas A., Ruch T., Sommer R., Ivashkina V. & Michaux J. R. 2010. Mitochondrial phylogeography of the edible dormouse (*Glis glis*) in the western Palearctic region. Journal of Mammalogy 91 (1): 233 – 242.

Irwin D. M., Kocher T. D. & Wilson A. C. 1991. Evolution of the cytochrome *b* gene of mammals. Journal of Molecular Evolution 32: 128-144.

Iyigün C. & Çolak R. 2004. An electrophoretic study on esterase and blood serum proteins of the water vole *Arvicola terrestris* (L., 1758) (Mammalia: Rodentia), in Kırşehir Province. Turkish Journal of Biology 28: 47-53.

Kaya S., Boztepe Z. & Çiplak B. 2015. Phylogeography of the *Poecilimon luschani* species group (Orthoptera, Tettigoniidae): a radiation strictly correlated with climatic transitions in the Pleistocene. Zoological Journal of the Linnean Society 173: 1-21.

Kapli P., Botoni D., Ilgaz Ç., Kumlutaş Y., Avci A., Rastegar-Pouyani N., Fathinia B., Lymberakis P., Ahmadzadeh F. & Poulakakis N. 2013. Molecular phylogeny and historical biogeography of the Anatolian lizard *Apathya* (Squamata, Lacertidae). Molecular Phylogenetics and Evolution 66: 992-1001.

Korkmaz E. M., Lunt D. H., Çiplak B., Değerli N. & Başibüyük H. H. 2014. The contribution of Anatolia to European phylogeography: the centre of origin for the meadow grasshopper *Chorthippus parallelus*. Journal of Biogeography 41: 1793-1805.

Kosswig C. 1955. Zoogeography of the Near East. Systematic Zoology 4 (2): 49-73+96.

Kryštufek B. & Vohralík V. 2005. Mammals of Turkey and Cyprus. Rodentia I: Sciuridae, Dipodidae, Gliridae, Arvicolinae. Koper: Založba Annales. 292 p.

Kryštufek B., Koren T., Engelberger S., Horváth G. F., Purger J. J., Arslan A., Chişamera G. & Murariu D. 2015. Fossorial morphotype does not make a species in water voles. Mammalia 79 (3): 293-303.

Michaux J. R., Magnanou E., Paradis E., Nieberding C. & Libois R. M. 2003. Mitochondrial phylogeography of the woodmouse (*Apodemus sylvaticus*) in the Western Palearctic region. Molecular Ecology 12: 685-697.

Michaux J. R., Libois R., Paradis E. & Filippucci M. G. 2004. Phylogeographic history of the yellow-necked fieldmouse (*Apodemus flavicollis*) in Europe and in the Near and Middle East. Molecular Phylogenetics and Evolution 32: 788-798.

Mursaloğlu B. 1975. Türkiye su sıçanlarının *Arvicola* coğrafik varyasyonları. TÜBİTAK V. Bilim Kongresi Tebliğleri, pp. 353-368.

Nei M. 1978. Molecular Population Genetics and Evolution. Amsterdam: North Holland Publishing Company. 288 p.

Neumann K., Michaux J., Lebedev V., Yiğit N., Çolak E., Ivanova N., Poltoraus A., Surov A., Markov G., Maak S., Neumann S. & Gattermann R. 2006. Molecular phylogeny of the Cricetinae subfamily based on the mitochondrial cytochrome *b* and 12S rRNA genes and the nuclear vWF gene. Molecular Phylogenetics and Evolution 39: 135-148.

Özkurt Ş., Çolak E., Yiğit N., Sözen M. & Verimli R. 1999. Contributions to the karyology and morphology of *Arvicola terrestris* (Lin. 1758) (Mammalia: Rodentia) in central Anatolia. Turkish Journal of Zoology 23: 253-257.

Peakall R. & Smouse P. E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28: 2537-2539.

Piertney S. B., Stewart W. A., Lambin X., Telfer S., Aars J. & Dallas J. F. 2005. Phylogeographic structure and postglacial evolutionary history of water voles (*Arvicola terrestris*) in the United Kingdom. Molecular Ecology 14: 1435-1444.

Popov S. V., Shcherba I. G., Ilyina L. B., Nevesskaya L. A., Paramonova N. P., Khondkarian S. O. & Magyar I. 2006. Late Miocene to Pliocene palaeogeography of the Paratethys and its relation to the Mediterranean. Palaeogeography, Palaeoclimatology, Palaoecology 238: 91-106.

Rokas A., Atkinson R., Webster L., Csoka G. & Stone G. N. 2003. Out of Anatolia: longitudinal gradients in genetic diversity support an eastern origin for a circum-Mediterranean oak gallwasp *Andricus quercustozae*. Molecular Ecology 12: 2153-2174.

Saitou N. & Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425.

Stewart W. A., Piertney S. B. & Dallas J. F. 1998. Isolation and characterization of highly polymorphic microsatellites in the water vole, *Arvicola terrestris*. Molecular Ecology 7: 1258-1259.

Swofford D. L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Şekercioğlu Ç. H., Anderson S., Akçay E., Bilgin R., Can O. E., Semiz G., Tavşanoğlu Ç., Yokeş M. B., Soyumert A., Ipekdal K., Sağlam İ. K., Yücel M. & Dalfes H. N. 2011. Turkey's globally important biodiversity in crisis. Biological Conservation 144: 2752-2769.

Taberlet P., Fumagalli L., Wust-Saucy A. G. & Cossons J. F. 1998. Comparative phylogeography and post-glacial colonization routes in Europe. Molecular Ecology **7**: 453-461.

Telfer S., Dallas J. F., Aars J., Piertney S. B., Stewart W. A. & Lambin X. 2003. Demographic and genetic structure of fossorial water voles *Arvicola terrestris* on Scottish islands. Journal of Zoology 259: 23-29.

Tez C., Ibiş O., Tez R., Kiliç M. & Telcioğlu M. 2011. Distributional, morphological and karyotypic contributions for the Eurasian water vole, *Arvicola amphibius* (Linnaeus, 1758) (Rodentia: Mammalia), from Turkey. Archives of Biological Sciences 63: 407-412.

Üstünbaş S., Çolak R., Karacan G. O. & Çolak E. 2011. RAPD-PCR analysis of water vole *Arvicola amphibius* (Linnaeus, 1758) (Mammalia: Rodentia) distributed in Turkey. Journal of Animal and Veterinary Advances 10 (13): 1673-1677.

Wilson D. E. & Reeder D. M. 2005. Mammal Species of the World: A Taxonomic and Geographic Reference. Third Edition. Baltimore, MD: The Johns Hopkins University Press. 2142 p.

Wright S. 1951. The genetical structure of populations. Annals of Eugenics 15: 323-354.

Yeh F. C., Yang R. C., Boyle T. B. J., Ye Z. H. & Mao J. X. 1997. POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada.

Received: 10.01.2017
Accepted: 03.05.2017