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COI GENE ANALYSES OF THE DAGHESTAN PINE VOLE (*Microtus daghestanicus* Shidlovsky, 1919) POPULATION FROM NORTHEASTERN TURKEY

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ABSTRACT. Daghestan pine vole (*Microtus daghestanicus* Shidlovsky, 1919) is spread in Caucasia, Turkey and Northwestern Iran and distribution of this species is limited to Northeastern Anatolia in Turkey. Few molecular studies on *M. daghestanicus* have been performed so far, and it was analysed in this study with the mitochondrial cytochrome oxidase subunit I (*COI*) region and compared with other *Terricola* species (*Microtus subterraneus* and *Microtus majori*) and other *Microtus* species found in its distribution area (*Microtus arvalis* and *Microtus mystacinus*). For this purpose, mean genetic distance values and fixation index values were calculated. Also, Bayesian Inference tree and Median-joining network were constructed. The acquired results showed that *M. daghestanicus* was clearly separated in the Pleistocene Period and was closer to *M subterraneus* than *M. majori* in the subgenus *Terricola*.

1. INTRODUCTION

Microtus Schrank, 1798 is one of the largest rodent genus and due to the unsolved taxonomic problems, high number of studies have been performed on this genus [1,2,3,4,5,6,7]. *Microtus daghestanicus* Shidlovsky, 1919 whose type location is Daghestan is a species of pine vole (subgenus *Terricola*) recorded from Southwestern parts of European Russia, Georgia, Armenia, Northeastern Turkey, Azerbaijan and Northwestern Iran [8,9]. Although *M. daghestanicus* and *M. majori* Thomas, 1906 were considered as subspecies of *M. subterraneus* de Selys Longchams, 1836 before [10,11], differences of these species were determined by karyological studies and they were accepted as valid species [12]. Conducted molecular studies showed that *M. daghestanicus* split from *M. subterraneus* and *M. majori* as

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closer to *M. subterraneus* in mitochondrial cytochrome-*b* (*CYTB*) and nuclear *IRBP, BRCA1* and *XIST* analyses [3,13,14].

Mitochondrial cytochrome oxidase subunit I (*COI*) is a barcod gene frequently used in eukaryotes. Since *COI* gene has a fast rate of evolution [15], few insertions and deletions, provides enough variation between species [16], this gene has been often preferred for species determination [17] and phylogeny construction of recently diverged species [18], including studies on rodents [6,7,17,19,20,21]. It was also suggested that species identification of rodents belong to the Murinae and Arvicolinae subfamilies is difficult due to rapid radiation, high morphological similarity, and high intraspecies and interspecies diversity [22]. Therefore, effective and reliable methods are required for the identification of these species, and the *COI* gene region could be remarkably effective to solve this problem.

In this paper, *M. daghestanicus* specimens obtained from Northeastern Turkey (Rize Province) were compared with other *Terricola* species (*M. subterraneus* and *M. majori*) as well as *Microtus arvalis* Pallas, 1778 and *Microtus mystacinus* de Filippi, 1865 species found in Daghestan pine vole's distribution area using *COI* marker. It was attempted to obtain evidence that will strengthen the validity of *M. daghestanicus* in debate and to contribute to the literature containing a limited number of molecular studies.

2. MATERIALS AND METHODS

Two *M. daghestanicus* samples were collected from Ovit Mountain region of Rize Province (Latitude: 41.02633572, Longitude: 40.51527612) in northeastern part of Turkey during the field studies in August 2017 with the permission of Ankara University Local Ethics Committee for Animal Experiments (Document no: 2016-21-184) and Republic of Turkey Ministry of Agriculture and Forestry (Document no: 72784983-488.04-117392). Besides, *M. subterraneus* (3 samples, Samsun and Giresun provinces), *M. majori* (3 samples, Ordu and Artvin provinces), *M. mystacinus* (3 samples, Erzurum and Muş provinces) and *M. arvalis* (2 samples, Ardahan Province and Hungary) sequences belong to Ankara University Mammalian Research Collection (AUMAC, http://www.mammalia.ankara.edu.tr) from previous studies. As an outgroup, one *Myodes rufocanus* sequence (Accession number: HM380211.1) acquired from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) was used.

DNA samples were isolated from liver tissue using the GeneAll® ExgeneTM Tissue SVmini kit (Atlas Biotechnology, Ankara, Turkey) and 720 base pair *COI* gene region was amplified with BatL5310 and R6036R primers [23]. For this purpose, reaction mix and PCR conditions were modified from Çetintürk et al. [7]. PCR products were electrophoresed on 0.8% agarose gel for 1 hr at 70 V in 1× TAE [Tris-Acetate-EDTA (ethylenediaminetetraacetic acid)] and PCR bands were viewed in the SYNGENE Bio Imaging system (Ankara, Turkey). Forward and reverse sequencing was carried out by BM LABOSIS (Ankara, Turkey).

Sequences were displayed and controlled in Chromas Lite 2.1.1 (www.technelysium.com.au), and the 507 base-pair region was formed for analysis by aligning using the software MEGAX [24]. Mean genetic distance values (*d*) between species according to the *p*-distance Parameter [25] were calculated in MEGAX [24], and fixation index values (F_{ST}) were defined in the DnaSP 6 Programme [26]. Bayesian Inference tree was generated in MrBayes 3.2.7a [27] and displayed using FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree). HKY+I Parameter [28] was chosen as an appropriate evolutionary model based on the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) with the help of jModelTest 2.1.7 [29,30]. The Markov Chain Monte Carlo (MCMC) approach was performed for two different runs with 10.000.000 generations with 100 samples each, with a 25% burn-in. Further, Median-joining network was constructed using haplotypes in the software POPART version 1.7 [31]. Using BEAST 1.75 Programme [32], evolutionary divergence times of *Microtus* species were also found considering the mammalian mtDNA divergence rate (2% per 1 million year; [33]) the divergence times of the Asian-Anatolian and European populations of *M. arvalis* based on *COI* gene (0.298 MYA [7]) were used as a calibration point. BEAST analyses were controlled in terms of effective sample size (ESS) values in Tracer 1.5 Software (http://beast.bio.ed.ac.uk/Tracer), and effective sample size (ESS) values of 200 or higher were accepted.

3. RESULTS

According to the results which Table 1 showed that mean genetic distance values (*d*) between *M. daghestanicus* and other *Microtus* species varied between 7.6-16.0%. The *d* value between *M. daghestanicus* and *M. arvalis* (16.0%) was the highest and the *d* value between *M. daghestanicus* and *M. subterraneus* (7.6%) was the lowest. In total, the mean distance values between all *Microtus* species ranged from 5.9 to 16.3. Fixation index values

(F_{ST}) were calculated as between 0.552 (*M. daghestanicus* and *M. subterraneus*) and 0.911 (*M. majori* and *M. mystacinus*).

Bayesian Inference tree and Median-joining network gave similar results in phylogenetic approaches. In Bayesian Inference tree (Figure 1), all species were split with high posterior probability values (pp=0.96-1.00), and *M. daghestanicus* was located, separately as closer to *M. subterraneus* (pp=0.96) than *M. majori*. Similarly, *M. daghestanicus* and *M. subterraneus* haplotypes were diverged as closer to each other than other species in Median-joining network (Figure 2), and *M. majori* was also separated from *M. arvalis* and *M. mystacinus* with more mutations than from *M. daghestanicus* and *M. subterraneus*.

Evolutionary divergence times of the studied species were defined as follows: *M. daghestanicus* and *M. subterraneus*: 0.766 MYA; *M. majori* and *M. daghestanicus/M. subterraneus*: 1.583 MYA; *M. majori/M. daghestanicus/M. subterraneus* and *M. arvalis/M. mystacinus*: 1.883 MYA; *M. arvalis* and *M. mystacinus*: 0.618 MYA.

TABLE 1. Mean genetic distance values (*d*) with standard errors and Fixation index values (F_{ST}).

SPECIES	***d*** **VALUES WITH STANDARD ERRORS**	**FIXATION INDEX VALUES (F_{ST})**
M. daghestanicus-M. subterraneus	0.076±0.011	0.552
M. daghestanicus-M. majori	0.141±0.017	0.784
M. daghestanicus-M. mystacinus	0.149±0.018	0.857
M. daghestanicus-M. arvalis	0.160±0.018	0.770
M. subterraneus-M. majori	0.134±0.016	0.799
M. subterraneus-M. mystacinus	0.150±0.018	0.882
M. subterraneus-M. arvalis	0.163±0.019	0.797
M. majori-M. mystacinus	0.140±0.017	0.911
M. majori-M. arvalis	0.145±0.017	0.811
M. mystacinus-M. arvalis	0.059±0.010	0.715

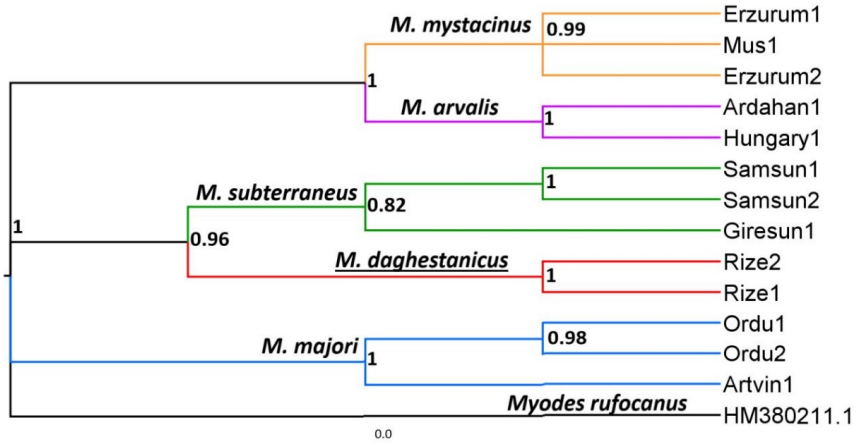

FIGURE 1. Bayesian Inference tree acquired using *COI* gene sequences. Numbers on branches indicate posterior probability (pp) values.

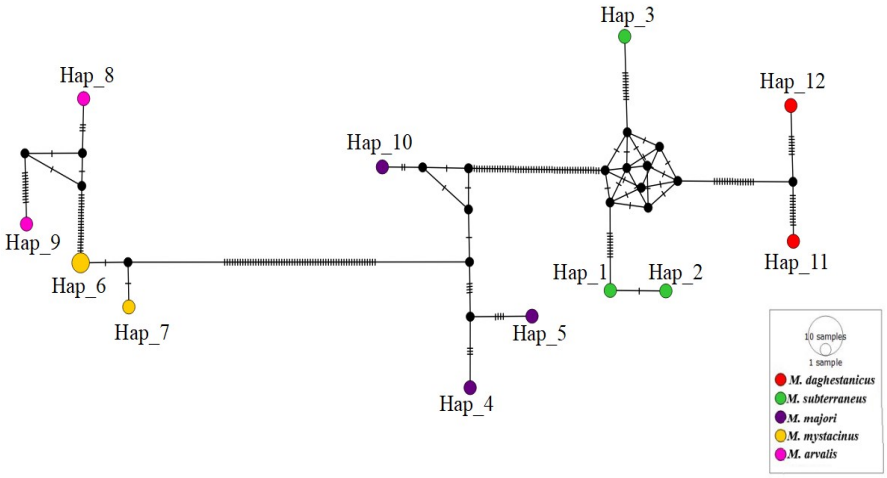

FIGURE 2. Median-joining network constructed with *COI* haplotypes. Numbers of mutations were given with black lines on branches.

4. DISCUSSION

Microtus daghestanicus Shidlovsky, 1919 has been recorded from a limited area in Caucasia, Northeastern Turkey and Northwestern Iran and is controversial regarding its taxonomic status [8,9]. Some authors [10,11]

offered that similar to *M. majori,* Daghestan pine vole is a subspecies of *M. subterraneus*. However, karyological [12] and molecular studies consisting *CYTB*, *IRBP, BRCA1* and *XIST* analyses [3,13,14] suggested that *M. daghestanicus* is a separate species as closely-related to *M. subterraneus*. According to the mitochondrial *CYTB* results in these studies, Jaarola et al. [3] found that phylogenetic trees supported the split of three pine vole species with *M. majori*'s separation from *M. daghestanicus* and *M. subterraneus.* Baskevich et al. [13] obtained results showed the divergence of *M. daghestanicus* and *M. majori* in phylogenetic dendrograms as well as 9.15% genetic distance. Likewise, Bogdanov et al. [14] analysed *CYTB*, *IRBP, BRCA1* and *XIST* gene markers and determined the similar separation in dendrograms. They also calculated the genetic distance values (7.8%, 9.65% and 9.68% between *M. daghestanicus* and *M. subterraneus, M. daghestanicus-M. majori* and *M. subterraneus-M. majori*, respectively) for *CYTB* gene. Results in this study yielded similar results with these studies regarding that *M. daghestanicus* was diverged from *M. subterraneus* and *M. majori,* and *M. majori* is the first separated taxon. Mean genetic distance values were found as 7.6% (*M. daghestanicus-M. subterraneus*), 14.1% (*M. daghestanicus-M. majori*) and 13.4% (*M. subterraneus-M. majori*). With respect to accepted intraspecies variation as <10% for *COI* [16] as well as interspecific genetic distance values (2%-11% in general [34], 0.0%–4.7% (mean 1.5%) for rodents and 0.2%–4.4% (2.0%) for genus *Microtus* [35]), obtained data implies interspecific genetic distance data. In addition, fixation index values (F_{ST}) of 0.25 and above are regarded to indicate high level of differentiation [36]. Klaus et al. [37] calculated the F_{ST} value between *Microtus richardsoni* U.S.A. populations as 0.624 and accepted this value as high. Heckel et al. [38] found the high mean F_{ST} value between *Microtus arvalis* European populations as 0.70. Sheremetyeva et al. [39] also determined the high F_{ST} value (0.624) between *Microtus maximowiczii* populations in Middle Amur River Region. Çetintürk et al. [40] analysed *Microtus mystacinus* interpopulations from Asia and Europe and F_{ST} values were found from moderate (0.195) to high (0.741). These findings are similar to high F_{ST} values in Table 1. Moreover, evolutionary divergence times coinciding with Pleistocene Period pointed that *M. majori* first diverged from *M. daghestanicus/M. subterraneus* group 1.583 MYA and *M. daghestanicus* split from *M. subterraneus* 0.766 MYA.

In conclusion, this performed study accepted the species status of *Microtus daghestanicus* Shidlovsky, 1919 and offered that mitochondrial *COI* gene is

effective to identify *Microtus* species that consist of recently diverged species.

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Declaration of Competing Interests. The author declares no conflict of interest.

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