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ANIMAL GENETICS

High Diversity of mtDNA Haplotypes Confirms Syntopic Occurrence of Two Field Mouse Species *Apodemus uralensis* and *A. witherbyi* (Muridae: *Apodemus*) in Armenia[1]

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Abstract—Wood mice of the genus *Apodemus* belong to the most frequent and epidemiologically important rodents of Europe and adjacent regions. Previous studies showed that in the Middle East region species of this genus exhibit extraordinary morphological similarity precluding their proper determination without application of molecular characters. In order to determine the species of the studied populations and to obtain an insight into their phylogeographic history, we analyzed their genetic variation. We sequenced 1139 bp fragment of the mitochondrial DNA control region and flanking tRNA genes in samples collected from six localities. Phylogenetic analyses revealed presence of distinct clades corresponding to species *A. uralensis* and *A. witherbyi*. In most localities we confirmed presence of both species which suggests their large sympatric and syntopic occurrence. We recognized an extensive genetic variability, 38 specimens of *A. uralensis* belong to 32 distinct haplotypes, while 19 specimens of *A. witherbyi* to 14 haplotypes. We confirmed presence of several distinct haplotypes that may originate from multiple wood mouse colonization waves from distinct geographic regions.

Keywords: Apodemus, Transcaucasus, phylogeography, mitochondrial DNA, D-loop

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INTRODUCTION

The Republic of Armenia is a biogeographically distinctive territory with significantly diverse habitats despite of its small area. It lies between Anatolian and Iranian plateaus and nearly half of its territory is consisted by the Lesser Caucasus. Within a relatively limited region, we can find arid subtropical semi-deserts at the very south, alpine landscapes at altitudes of 4000 meters above the sea level, or humid mixed forests at the north. The local biodiversity has been substantially influenced by geological processes, ice ages and related climatic changes in the past, therefore various species from Europe, Middle East and Central Asia meet in this region, as well as many others are endemic to local mountains. Numerous mountain ridges and valleys may have played role in dispersal and speciation of fauna by functioning as barriers, migration routes or refugia [1–8]. However, relatively little research has been done on this topic. Due to complexity of the local biodiversity, not only genetic affinities and variation of the Armenian populations of particular species, but also species identity of some populations, has remained obscure up to these days and often much of accessible information on the local fauna is decades old and very obsolete.

This applies also to the complex of murid species which we aimed on in our study. Wood mice of the genus *Apodemus* Kaup, 1929 sensu lato belong to the most widespread rodents of Eurasia [9, 10]. Their ability to inhabit wide spectrum of habitats from semi-deserts and steppes to woodlands allows these animals to be present in relatively high densities across the landscapes. This predetermines these mice to become valuable component of mammalian communities and food webs and consequently also agricultural and forestry pests and transmitters of diseases (zoonoses). In the Western Palearctics, the wood-mouse species of the genus *Apodemus* (except for *A. agrarius, A. epimelas* and *A. mystacinus*) form a monophyletic group of species which are closely related and morphologically similar to each other [9, 11–17]. Over the past few decades there has been significant number of research on the evaluation of genetic and morphological criteria enabling identification of traditionally recognized European species, i.e., *A. sylvaticus* (Linnaeus, 1758), *A. flavicollis* (Melchior, 1834), and *A. microps* (Krato-

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chvíl and Rosický, 1952) which was currently synonymized with *A. uralensis* (Pallas, 1811) [18–30]. Within the main European clade of the genus *Apodemus* there is a low level of interspecific morphological variation, which can be a result from a bush-like pattern of radiation [31] leading to nearly simultaneous emergence of many species. However, because of this pattern it is also difficult to infer mutual relations of the species, although they are well defined monophylla themselves [12, 30, 32]. Using molecular data along with morphological assessment is especially important in the case of research on wood mice in the Middle East, where there is no accurate taxonomy within the genus *Apodemus*. The latter is a consequence of the difficulty in identifying the species because of the high morphological and ecological similarities of representatives of various species. Studies concerning *Apodemus* phylogeny published over 10–15 years until today used such molecular methods like allozyme analysis (which was one of the first biomolecular methods used by many authors for species determination) [11, 33], restriction fragment length polymorphism (RFLP) [32], random amplified polymorphic DNA (RAPD) [34], nuclear and mitochondrial DNA sequencing [12, 15, 16, 31, 32, 35–39]. Different studies often gave contradictory results about the relationships among *Apodemus* species, but all agree on the distinctness of examined species. Phylogeographic analyses were performed in *A. sylvaticus* and *A. flavicollis* [12, 40–42].

Information on the taxonomy and distribution of *Apodemus* species in the territory of Middle East and Transcaucasus is still incomplete, because published data are based mainly on morphology patterns and molecular methods were used at a limited number of localities across the country [12, 25, 43].

In the neighbourhood of Armenia, five valid species of the genus *Apodemus* have been reported until now [cf. 11, 14, 16, 38, 44–47]: *A. uralensis* (Pallas, 1811), *A. witherbyi* (Thomas, 1902), *A. flavicollis* [42], *A. hyrcanicus* [44] and *A. mystacinus* [43, 48]. Based on a large scale phylogenetic comparison of mitochondrial cyt b gene sequences of *A. uralensis* across the Europe and Central Asia, the eastern populations have been recently recognized as a separate species *A. tokmak* (Severtsov, 1873). The range of this new species is, however, restricted to the Central Asia and separated from the Transcaucasus region by the Caspian Sea.

In this paper, we present new genetic data for wood mice of the genus *Apodemus* from Armenia. We sequenced a fragment of mitochondrial DNA including highly variable control region, performed phylogenetic analysis and visualized haplotype networks. The aim of this study was to (1) verify putative species identity of the collected mice using molecular characters; (2) analyze sequence variation on population level and (3) discuss phylogeographic and demographic interpretations of the results.

MATERIALS AND METHODS

A total of 58 mouse specimens belonging to *Apodemus* species were examined, including pygmy wood mice *A. uralensis* and steppe field mice *A. witherbyi* [49] (Table 1). The geographic origin of samples is presented in Fig. 1. Aghavnadzor (40°35′03″ N 44°41′29″ E; number of specimens $n = 30$), Hankavan (40°37′09″ N, 44°34′29″ E; $n = 11$) Kotayk province, central Armenia, 1900 m a.s.l. *Biotope*: the localities Hankavan and Aghavnadzor include a valley of the river Marmarik and the several altitudinal zonation such as foothill, mountain, subalpine and alpine zones of the Pambak and Tsaghkunyats ridges. River valley is surrounded by mountain meadows and mixed forests at higher elevations, mainly on the northern slopes. Aygut (40°41′03″ N, 45°10′23″ E; $n = 1$), Gegharkunik province, NE Armenia, 1400 m a.s.l. *Biotope*: Lesser Caucasus, river valley surrounded by semi-arid mountain steppe with dispersed mixed forests at higher altitudes. Dilijan (40°44′27″ N, 44°51′47″ E; $n = 6$), Tavush province, NE Armenia, 1300 m a.s.l. *Biotope*: Lesser Caucasus, humid mixed forests. Animals were captured in the Dilijan town and the territory along the Agstev River. Khosrov State Reserve (39°56′42.1″ N, 44°51′35.5″ E; $n = 9$), Ararat province, central-south Armenia, 1300 m a.s.l. *Biotope*: slopes descending to the Ararat valley, arid foothills, slopes covered with dispersed woodlands. It is a noted area in the Caucasus region for unique European and Asian flora and fauna. Yerevan (40°11′ N, 44°31′ E; $n = 1$), Capital district, south-central Armenia, 1000 m a.s.l. *Biotope*: Ararat valley, predominantly arid with dry steppe vegetation. One individual was caught in the south-western suburbs of the capital Yerevan. In addition, one specimen of *Mus macedonicus* from Yerevan was also included.

Total genomic DNA was isolated from finger or tail tip preserved in absolute ethanol at –20°C. The DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol.

The DNA amplification was performed with polymerase chain reaction (PCR) using primers suggested by Bellinvia (2004) for the mitochondrial DNA control region (D-loop) and flanking tRNA genes (about 1000 bp). Selected part of mtDNA was amplified in two overlapping segments. PCR reactions were carried out in 25 μL volume including 1 μL of each 10 μM primer, 12.5 μL of PPP Master Mix (TopBio), 9.5 μL of PCR H_2O and 1 μL of template DNA following manufacturer's protocol. The PCR amplification protocol consisted of 31 cycles of denaturation at 95°C for 30 s, annealing at 50°C (using primers 1 + 2bis) or 55°C (using primers 3 + 4) for 1 min, and extension at 72°C for 1 min; a further 15 min elongation step at 72°C followed the last cycle. For some samples the temperature of annealing had to be decreased to 46°C in case of using combination of primers 1 + 2bis to

Table 1. List of localities of genetically determined specimens of *Apodemus uralensis* and *A. witherbyi*

	A. uralensis		*A. witherbyi*	
	number of individuals (N)	number of haplotypes (N_h)	number of individuals (N)	number of haplotypes (N_h)
Hankavan	11	11	–	–
Dilidjan	3	3	3	3
Aygut	1	1	1	1
Aghavnadzor	23	18	5	5
Khosrov Reserve	–	–	9	6
Yerevan, SW	–	–	1	1

obtain usable PCR products. All the PCR products were purified using the ethanol precipitation.

The entire D-loop and flanking region sequences were aligned and manually checked using Chromas Pro 1.7.5 (Technelysium Pty Ltd), BioEdit [50] and Clustal X 2.0.11 [51]. An online toolbox FaBox 1.41 (http://users-birc.au.dk/biopv/php/fabox/index.php) was also used for work with the sequences. We prepared three alignments, the first one in length of 1139 bp for phylogenetic analyses where we included sequences of all 59 samples—and also sequences of *Apodemus* species from GenBank (numbers AY588254, AY588250, AY588251, AY623063, AY623064, AY623065, AY623066, AY623067, AY588263, AY588260, AY588252, AY588255, AY588256, AY588257, AY588259, AY588264, AY588253, AY588262, AY588258, AY588261). We added two sequences of *Mus macedonicus macedonicus* as outgroup to this alignment (collected specimen and a GenBank sequence EU106248.1).

The second and third alignment was prepared for construction of haplotype networks. The second alignment included only 19 of our samples marked as *A. witherbyi*, its total length was 1041 bp. The third alignment included 38 of our samples marked as *A. uralensis* and its total length was 1024 bp.

Neighbour-joining (NJ) and maximum parsimony (MP) analyses were performed under PAUP* version 4.0b10 [52], and Bayesian analysis (BA) was con-

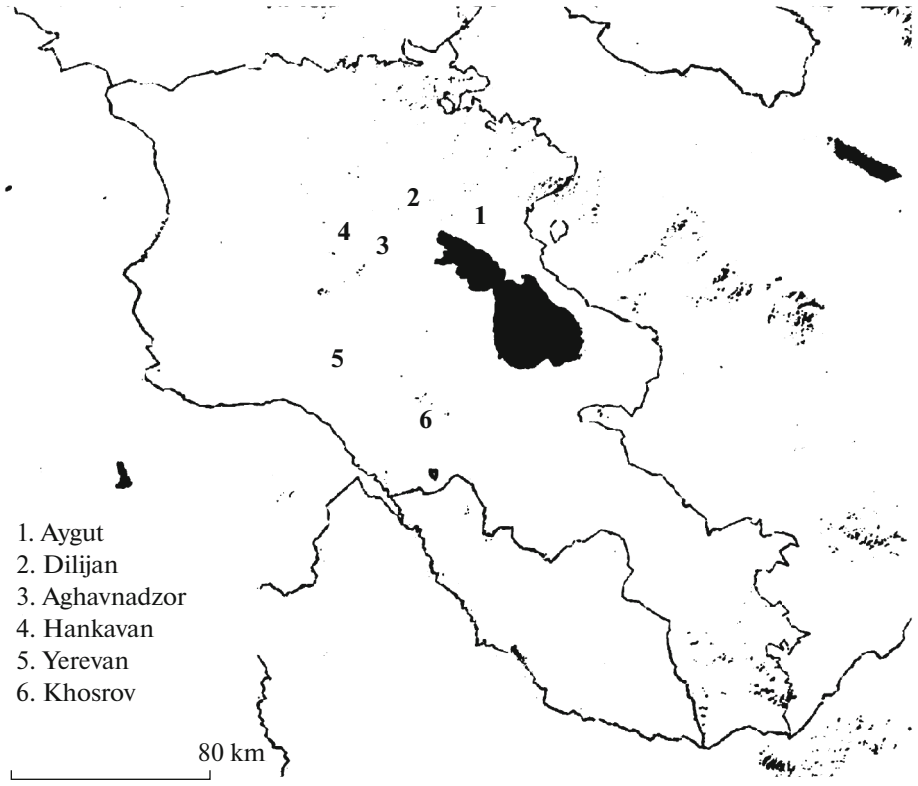

Fig. 1. Map of collection localities. 1—Aygut, 2—Dilijan, 3—Aghavnadzor, 4—Hankavan, 5—Yerevan, 6—Khosrov Reserve.

ducted with MrBayes 3.1 [53, 54]. Tree search with NJ algorithm was done with Kimura two-parameter distance and support within the final topology was assessed through 10000 bootstrap pseudoreplicates. For MP, we conducted heuristic search analyses using tree-bisection and reconnection (TBR) branch swapping and 1000 random replicates of taxa additions. The branch support was evaluated using 10000 bootstrap pseudoreplicates [55]. All characters were equally weighted and unordered. Bayesian analysis was conducted with a random starting tree and run for 15×10^6 generations, with trees sampled every 100 generations. The burn-in command was used to discard the first 15000 trees (1500000 generations). As the best-fit model, TVM+I+G by hLRT was selected in Modeltest 3.7 [56]. In addition, we applied the median-joining method available in NETWORK, version 4.6.1.2 [57] to construct haplotype networks for each examined *Apodemus* species.

Polymorphism for each of populations *A. witherbyi* and *A. uralensis* was detected by the statistic software DnaSP v5 5.10.01 [58] which quantified the following: haplotype diversity (*h*), segregating sites (S), nucleotide diversity (π) and performed neutrality tests [59]: Tajima's *D*, Fu and Li's *F**, Fu and Li's *D**, and Fu's *Fs* tests [59–61]. According to Russell et al. (2005) [62], high values of *h* and π indicate a constant large size of population. However, a low value of π and high value of *h* signify recent expansion. To detect signals of population expansion, we also used simple expansion coefficients [60], defined as S/Π, where Π is the average number of pairwise nucleotide differences (also quantified in DnaSP v5 5.10.01).

RESULTS

In this study we sequenced a fragment of the mitochondrial DNA containing the entire D-loop region and flanking tRNA genes: part of the $tRNA^{Thr}$ and the entire $tRNA^{Pro}$ at 5', as well as part of the $tRNA^{Phe}$ at 3'. For 59 specimens this part of DNA varied from 1019 to 1076 bp.

The phylogenetic analyses were based on the alignment of 1139 bp including our samples and 22 sequences from GenBank (including 12 of the genus *Apodemus*). We performed Bayesisan, maximum parsimony and neighbour joining analyses; these methods mutually agreed in main topology of the resulting trees (see Fig. 2 for Bayesian tree and node supports). Monophyly of two distinct branches including 38 and 19 *Apodemus* samples from the territory of Armenia was strongly supported (Bayesian posterior probabilities >0.98, MP and NJ bootstraps >99 and 91, respectively). Inclusion of the GenBank sequences of known species identity into the phylogenetic analyses allowed us to unequivocally assign 57 of 58 sequenced *Apodemus* specimens (except the sample no. 1421, see below) into *A. uralensis* (38 specimens) or *A. witherbyi* (19 specimens). A sequence of the additional sample belonging to the genus *Mus* (no. 1386; locality Yerevan) fits *M. m. macedonicus*; this conclusion was further confirmed by a separate phylogenetic analysis of this sequence in the context of *Mus macedonicus* samples previously studied by Macholán et al. (2007) [63], in which no. 1386 fell within the clade of *M. m. macedonicus* samples closely to the sequences from Iran (data not shown).

The detailed phylogenetic placement of the sequence of the specimen no. 1421 has remained unstable, although it obviously belongs to the *Apodemus* clade. The sequence has no clear affinities to those of any previously sequenced *Apodemus* species. Uncorrected p-distances of this sequence to those of other *Apodemus* species range from 4.04 to 21.00% (for *A. uralensis* and *A. agrarius*, respectively). The supports of the detailed branching patterns within *A. uralensis* and *A. witherbyi* clades were rather low, although the Armenian samples tend to cluster together and/or with samples from geographically close areas of the Middle East (Fig. 2).

Next we separately analyzed the sequences from Armenian populations of *A. uralensis* and *A. witherbyi*. For *A. uralensis* we constructed a nucleotide alignment consisting of 1025 bp, of which 106 were variable and 64 were parsimony-informative. The uncorrected *p*-distance among haplotypes within this dataset ranged from 0.10 to 4.91%. Similarly, we obtained an alignment for *A. witherbyi* consisting of 1041 bp, of which 50 were variable and 20 were parsimony-informative. Within this dataset the uncorrected *p*-distance among haplotypes ranged from 0.20 to 2.37%.

These alignments were further used for construction of haplotype networks (Fig. 3 and Fig. 4) and computation of indices characterizing haplotype diversity. In both clades, the results revealed presence of some very distinct haplotypes, as well as the possibility of recent expansion. The latter suggestion was supported by high haplotype diversities, low nucleotide diversities and high values of simple expansion coefficients, also the neutrality tests resulted in negative, although non-significant values (Table 2).

External characters of speciemens possessing haplotypes belonging to *A. uralensis* and *A. witherbyi* clades overlapped considerably. The mean body length was 90.7 (range 82–96) and 94.6 mm (90–101); the ear length 15.0 (14.0–16.1) and 14.9 mm (14.2–16.1); and hind-foot lengths 20.61 (19.9–21.9) and 21.17 mm (20.3–22.5), for *A. uralensis* and *A. witherbyi*, respectively. The only examined measurement with significant between-species difference was the tail length (*t*-test: $t = -6.00$, $P < 0.0001$, *A. uralensis*: mean = 85.7 mm, range 77–95, *A. witherbyi*: mean = 99.9 mm, range 93–110). The individuals identified as *A. witherbyi*, were characterized by a bit paler and more yellowish dorsal surface than in *A. uralensis*. They tend to possess a bit larger and smoother yellow pectoral

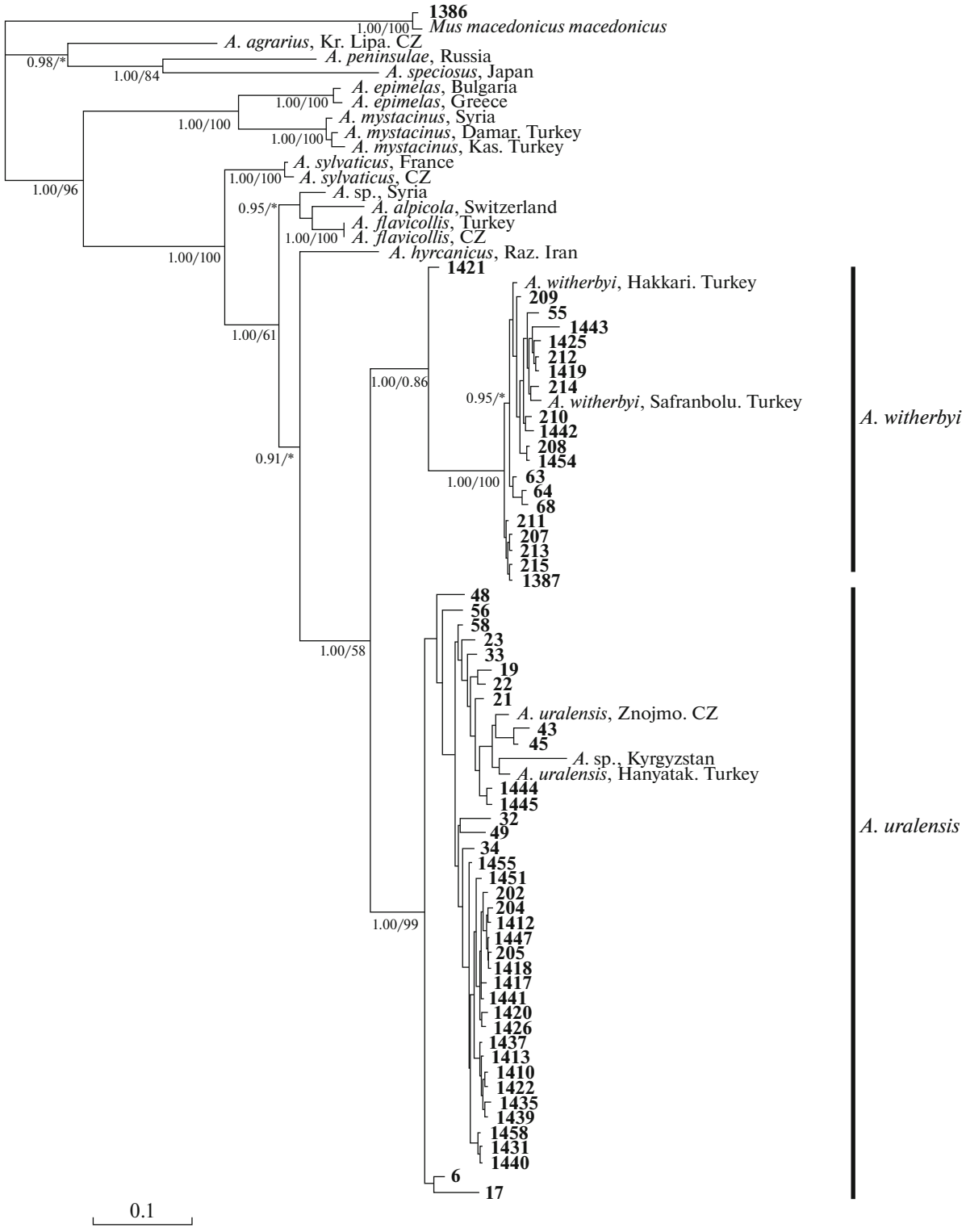

Fig. 2. Phylogenetic tree constructed using Bayesian analysis with Maximum parsimony posterior probability (posterior probability >0.90/bootstrap values >50 shown only, * posterior probability <50).

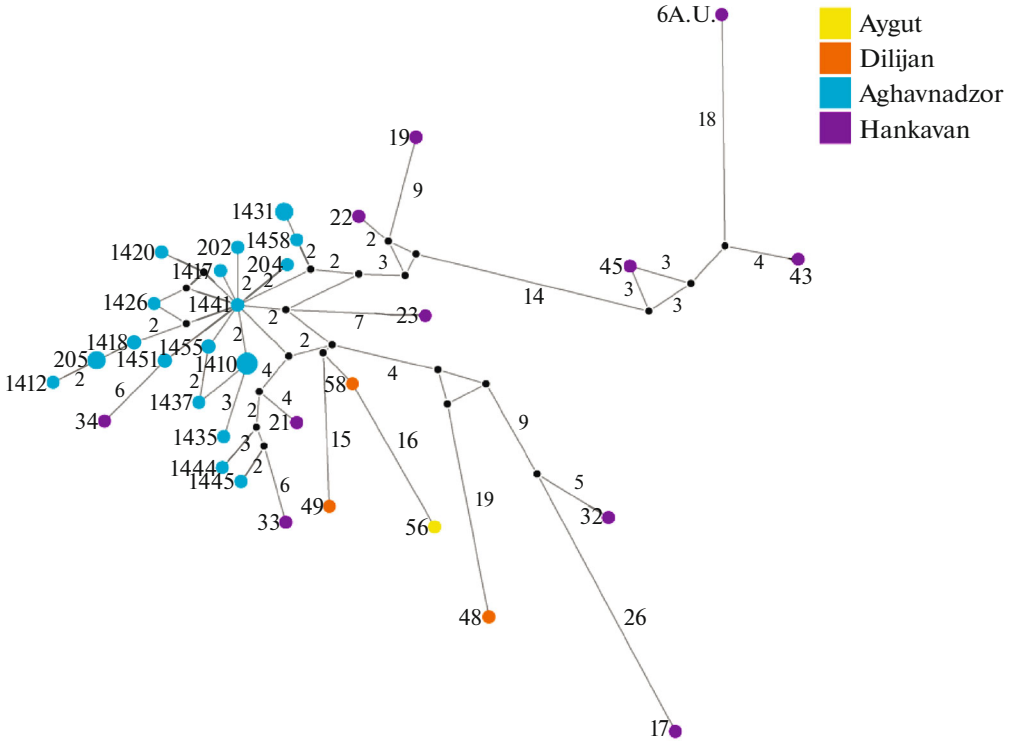

Fig. 3. Median-joining network obtained for the D-loop sequences of *A. uralensis*. Circle sizes are proportional to the number of the same haplotypes observed in the data set. Values at branches represent numbers of mutational steps (displayed for $n > 1$).

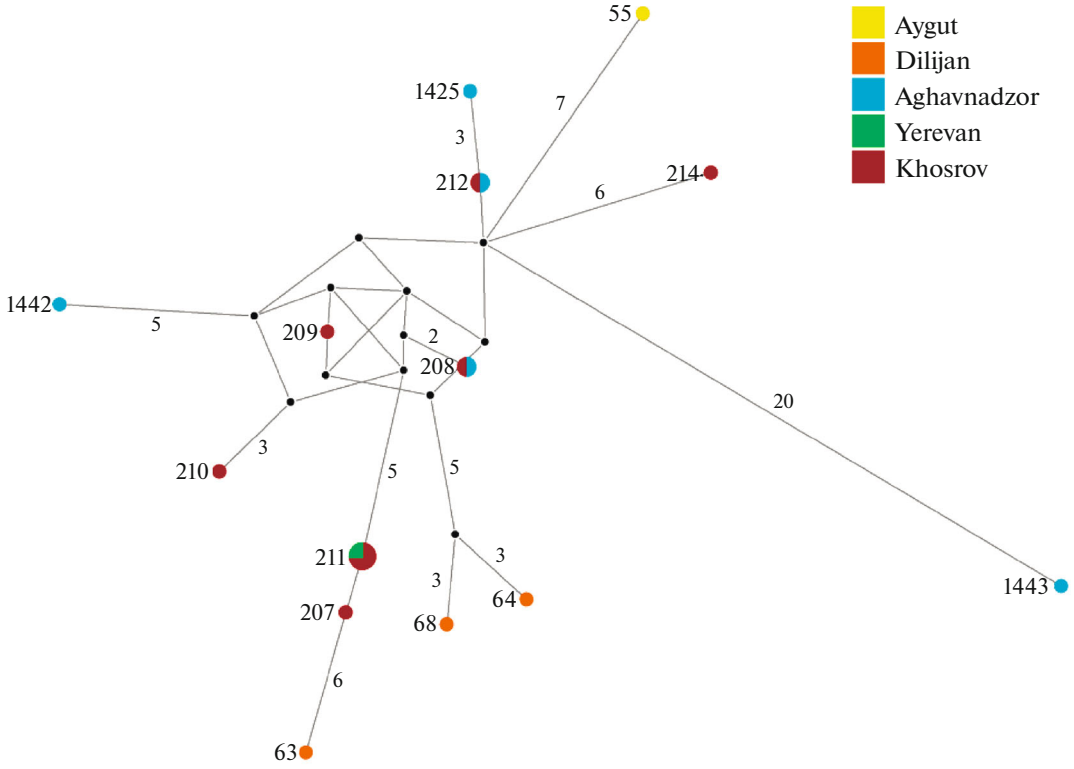

Fig. 4. Median-joining network obtained for the D-loop sequences of *A. witherbyi*. Circle sizes are proportional to the number of the same haplotypes observed in the data set. Values at branches represent numbers of mutational steps (displayed for $n > 1$).

Table 2. Sequence polymorphism and demographic characteristics for *A. uralensis* and *A. witherbyi* based on mitochondrial D-loop

	N_s	S	N_h	H	π	Fu and Li's *F**	Fu and Li's *D**	Fu's *Fs*	Tajima's *D*	Exp
A. witherbyi	19	45	14	0.95	0.01	−1.7612	−1.6579	−2.352	−1.1711	4.70354
A. uralensis	38	96	29	0.98	0.015	−1.6442	−1.2774	−8.009	−1.5749	6.69876

Samples are defined as species in phylogenetic tree. Sequences: number of individuals sequenced (N_s), number of segregating sites (S), number of haplotypes (N_h), haplotype diversity (h), nucleotide diversity (π), Fu and Li's *F**, Fu and Li's *D**, Fu's *Fs*, Tajima's *D* and expansion coefficient (exp).

spots. Nevertheless, these differences in colouration were not distinct enough to reliably predict species identity as revealed by sequence data.

DISCUSSION

Five *Apodemus* species are currently recognized to inhabit the regions neighbouring Armenia. (1) *A. uralensis* (Pallas, 1811) a widespread species ranging from the Central Europe [19, 64] usually referred to as *A. microps* to the Central Asia [65]. The geographic variation of this taxon was examined by Orlov (1996). (2) *A. witherbyi* (Thomas, 1902). This species was described from Israel [66, 67] as *A. hermonensis* [66], but it was later found to be widespread throughout the non-desert areas of the Middle East (including Rhodos Island [68]). Later on Filippucci et al. (1996) [69] suggested that *A. hermonensis* probably can be conspecific with the species reported as *A. falzfeini* (Mezhzherin & Zagorodnyuk, 1989) or *A. fulvipectus* (Ognev, 1924) from Turkmenistan, the Transcaucasus, the Caucasus, and neighboring steppes up to Crimea [70]. The presence of *A. hermonensis* in Armenia was reported by Suzuki et al. (2008) [26]. Currently, *A. hermonensis* is treated as a junior synonym of *A. witherbyi* [43, 45, 48, 71]. (3) *A. flavicollis*. The phylogeographic analyses of the entire distribution range of this species suggest that Middle East populations represent a distinct clade [42]. Wood mice populations from the Transcaucasus referred to as *A. ponticus* (Sviridenko, 1936) may probably represent a sister lineage to *A. flavicollis* and therefore are often treated as conspecific [43, 72]. (4) *A. hyrcanicus* (Vorontsov et al., 1992), a recently described species, probably restricted to the area of Hyrcanian forests on the Southern shore of the Caspian Sea [14, 43, 44]. Its occurrence in Armenia is thus improbable. (5) *A. mystacinus* (Danford and Alston, 1877) inhabiting rocky habitats in urban or woodland areas throughout Anatolia, Levant, Georgia and Crete, sometimes attributed together with its sister species *A. epimelas* to the subgenus *Karstomys* [43, 48, 49].

The phylogenetic analyses performed by Bayesian and maximum parsimony methods mutually agreed and both revealed presence of two distinct *Apodemus* clades corresponding to species *A. uralensis* and *A. witherbyi*. Thus, the species determination using the sequenced fragment of mitochondrial DNA including the control region is unequivocal. The sequence obtained from the specimen 1421, however, does not fit to any of the previously sequenced species. This may be caused by a possible presence of nuclear pseudogenes [73], though we cannot rule out it may represent a new mitochondrial lineage. On the other hand, topology of both species within the phylogeny of *Apodemus* species remains unclear, when compared with the data in literature. In our results, *A. uralensis* and *A. witherbyi* grouped together into a clade, while *A. hyrcanicus, A. flavicollis* and *A. sylvaticus* formed ancestral groups. According to other authors, whose studies were based on cytochrome b, 12S rRNA or nuclear genes, *A. uralensis* usually groups together with *A. sylvaticus*, *A. flavicollis* and *A. hyrcanicus*, though their exact positions may vary, leaving *A. witherbyi* (or *A. hermonensis*, respectively) as a sister species to the whole group [12, 38, 43].

In both examined species, the patterns revealed by haplotype network analyses may suggest recent demographic expansion of the populations. The presence of some very distinct haplotypes in both examined species requires further examination. Geography and climatic history of Armenia (glacial history of the Caucasus [4]) allows us to speculate that this pattern may be a result of successive colonization waves.

In most localities we confirmed the presence of both *A. uralensis* and *A. witherbyi* which suggests their considerable sympatric and syntopic occurrence. This further supports previous results from Eastern Turkey [11, 13] where these two species, and sometimes also *A. flavicollis* were frequently found on the same localities. We found no obvious microhabitat separation in sympatric populations. Nevertheless, the predominance of *A. witherbyi* in Khosrov Reserve and *A. uralensis* in Hankavan, conforms to a general tendency of the former species to prefer steppe while the latter species rather forest and/or mountainous habitats.

The genetic distinctness of sympatric *Apodemus* species sometimes sharply contrasts with their morphological uniformity, the phenomenon that was previously demonstrated, e.g., in the Balkans (cf. [74]). Also in Armenia and adjacent regions, high degree of genetic differentiation of sympatric *Apodemus* species [11, this paper] contrasts with their apparent mutual

similarity in appearance (colouration, body dimension) and craniodental morphometry (cf. [13, 14]). The external dimensions and colouration of our genetically determined specimens of *A. uralensis* and *A. witherbyi* fit the variation reported by Kryštufek and Vohralík (2009) [71]. The only little overlapping trait was the tail length, however, its reliability for field determination requires further verification in larger sample of genetically determined specimens.

There may be three possible explanations for this kind of variation: (1) gene introgression among sympatric species in the Middle East [75–77] and/or (2) convergent morphological evolution of multiple clades in the Middle East [78, 79] and/or (3) divergent morphological evolution in European taxa combined with persistence of ancestral phenotypes in the Middle East [14]. These let us to discuss some possible ecological causes of small interspecific variation in the Middle East when compared to that in Europe. Habitats suitable for the survival of *Apodemus* species can be more differentiated in European landscapes (like field and forest) than in the Middle East.

Our results based on maternally inherited mitochondrial genes are unable to detect genetic introgression. Although, Macholán et al. (2001) [11] failed to detect interbreeding among sympatric *Apodemus* species in the Middle East, nuclear genes have to be examined to prove their findings. In conclusion, our study confirmed that the two syntopic species belong to the species *A. uralensis* and *A. witherbyi*, each characterized by a distinct cluster of haplotypes.

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