

# Phylogenetic Relationships of *Apodemus* Kaup, 1829 (Rodentia: Muridae) Species in the Eastern Mediterranean Inferred from Mitochondrial DNA, with Emphasis on Iranian Species

Jamshid Darvish<sup>1,2</sup> · Zeinolabedin Mohammadi<sup>1</sup> · Fatemeh Ghorbani<sup>1</sup> · Ahmad Mahmoudi<sup>1</sup> · Sylvain Dubey<sup>3</sup>

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**Abstract** Wood mice of the genus *Apodemus* are widely distributed in Eurasia, with the Eastern Mediterranean being considered as a hotspot. Indeed, numerous species have been documented in Iran, including *A. witherbyi*, *A. hyrcanicus*, *A. uralensis*, *A. avicennicus*, *A. hermonensis*, and *A. arianus*. In this study, 129 specimens were collected from different Iranian localities and two specimens from Afghanistan. The animals were identified taxonomically and their phylogenetic relationships were investigated using cytochrome b mitochondrial DNA sequences. Five species of the genus *Apodemus* were identified in Iran, including *A. hyrcanicus*, *A. witherbyi*, *A. cf. ponticus*, *A. uralensis*, and *A. mystacinus*, beside, *A. pallipes* from Afghanistan. This

study found no evidence of *A. flavicollis* or *A. sylvaticus* in Iran, despite their occurrence in Turkey, shedding doubt on the status of *A. flavicollis* in Iran, Asia Minor, and the Levant. Phylogenetic analyses imply that *A. witherbyi* has priority over *A. avicennicus*, *A. hermonensis*, and *A. iconicus*. Estimation of the divergence time for these taxa suggests a separation at around 7.2 Ma for the subgenera *Karstomys* (including *A. mystacinus* and *A. epimelas*) and *Sylvaemus* (including *A. flavicollis*, *A. sylvaticus*, *A. uralensis*, *A. pallipes*, *A. hyrcanicus*, *A. witherbyi*, and *A. cf. ponticus*). Within the subgenus *Karstomys*, the divergence times for *A. mystacinus* and *A. epimelas* were between 3.0 and 6.1 Ma, and divergence times within the subgenus *Sylvaemus* were between 5.2 and 6.9 Ma for *A. witherbyi* and other species in this subgenus. It is postulated that vicariance events including the uplifting of the Zagros Mountains and Anatolian Plateau in the middle Miocene and climate oscillations during the Messinian Salinity Crisis besides formation of the Hyrcanian tertiary forests during the Neogene probably played substantial roles in the radiation and distribution of the genus *Apodemus* in the Eastern Mediterranean.

Jamshid Darvish and Zeinolabedin Mohammadi contributed equally to this work.

✉ Zeinolabedin Mohammadi  
Mohammadi.zeinal@gmail.com

Jamshid Darvish  
Darvish\_2001@yahoo.com

Fatemeh Ghorbani  
biosys2050@gmail.com

Ahmad Mahmoudi  
yakamuz.alpay@gmail.com

Sylvain Dubey  
sylvain.dubey[@]unil.ch

<sup>1</sup> Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, P.O. Box: 9177948974, Mashhad, Iran

<sup>2</sup> Applied Animal Institute (AAI), Rodentology Research Department (RRD), Ferdowsi University of Mashhad, P.O. Box: 9157948974, Mashhad, Iran

<sup>3</sup> Department of Ecology and Evolution, University of Lausanne, 1015 Lausanne, Switzerland

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## Introduction

Paleontological data have identified the Eastern Mediterranean as a crossroads for many taxa from the early Miocene (Koufos et al. 2005). This area shows high endemism in the Palearctic Region (Kryštufek et al. 2009). Its current geographical aspect was formed between the middle Miocene and the present (Macey et al. 1998; Koufos et al. 2005), and

the distributions of many taxa in this area are tightly related to geological events that occurred during the Neogene (Macey et al. 1998). From the middle Miocene about 15–5 Ma (million years ago), a counter-clockwise movement of the Arabian platform and its collision with Eurasia led to the uplifting of the Zagros and Alborz Mountains, considered as parts of the Alpine-Himalayan mountain chain (Dercourt et al. 1993; Sengor and Natalin 1996; Mouthereau 2011). The Alborz range was subsequently compressed and folded (Guest et al. 2007) and a deep depression was formed between the south Caspian Sea and the Alborz Mountains during the Neogene, while the south Caspian microcontinent moved northwards and reached its maximum subsidence during the late Miocene and Quaternary (Golonka 2004). These tectonic and orogenic activities create some barriers and corridors in the Iranian Plateau for gene flow (Macey et al. 1998; Ahmadzadeh et al. 2013; Rajaei Sh. et al. 2013). In the Near East, the central Anatolian Plateau was also formed as a result of volcanic activity during the Neogene (8 Ma) (Yilmaz et al. 1998; Aydar et al. 2013). In addition, in the late Miocene, the Mediterranean Sea underwent desiccation during the Messinian Salinity Crisis (5.96–5.33 Ma), which also impacted on the Near East (Clauzon et al. 1996; Krijgsman et al. 1999). The onset of the Praetiglian cold stage in Europe (2.5–2.2 Ma) was then preceded by the formation of ice sheets and glaciers at the early Pliocene/late Pliocene boundary (about 3 Ma) (Ruddiman and Raymo 1988), resulting in the retreat of forests and the prevalence of cold, dry habitats in the Mediterranean region. These climatic oscillations following geographical events formed the biological aspects of the Circa-Mediterranean regions.

Wood mice of the genus *Apodemus* are widely distributed in the Eastern Mediterranean and are adapted to different types of habitats. In total, 20 species of the genus *Apodemus* attributed to three subgenera (*Apodemus*, *Karstomys*, *Sylvaemus*) are known in Eurasia (Michaux et al. 2002; Bellinva 2004; Musser and Carleton 2005; Suzuki et al. 2008), six of which are supposed to exist in Iran, including *A. witherbyi*, *A. hyrcanicus*, *A. uralensis*, *A. flavicollis*, *A. ponticus*, and *A. mystacinus* (Musser and Carleton 2005; Kryštufek and Hutterer 2006; Darvish et al. 2014; Mohammadi et al. 2014). Five species were attributed to the subgenus *Sylvaemus*, which ranges through Europe, Near East, and Middle East, and one (*A. mystacinus*) was included in the subgenus *Karstomys* (Musser and Carleton 2005; Kryštufek and Hutterer 2006).

In Iran, habitats used by *Apodemus* species range from the Hyrcanian forests of the northern Alborz Mountains and the steppes of the southern slopes of the Alborz to farmlands and gardens in mountainous ranges, as well as grasslands beside shrub lands and scrub in the Zagros range. This genus was considered to be absent from the Persian Gulf Basin, Lut Desert, and the Kavir Plain in the central part of Iran, as well

as from the Sistan depression in the southeast of Iran. However, Blanford (1875) described a new taxon from Kuhrud, Isfahan, as *Mus erythronotus*, which was subsequently recognized as a junior name for *A. flavicollis* (Kryštufek 2002), since when this genus has undergone numerous revisions (Darvish et al. 2014).

Goodwin (1940) recognized two different morphs of the genus *Apodemus* in northeastern Iran, from where specimens captured from deciduous forest were identified as *A. arianus* and were later treated as *A. hyrcanicus* (Musser and Carleton 2005). Conversely, specimens collected from higher elevations were considered to be *A. sylvaticus chorassanicus*, but were later attributed to *A. witherbyi* (Musser and Carleton 2005). Further, Lay (1967) misidentified and reported *A. sylvaticus* from the northern parts of Iran. A biochemical study of the genus *Apodemus* demonstrated a different allozymic pattern in specimens from central north Iran compared with *A. sylvaticus* and *A. flavicollis* (Darviche et al. 1979).

Macholán et al. (2001) reported *A. hyrcanicus* and *A. flavicollis* from the north and west parts of Iran, respectively. The dental and cranial characteristics of *A. witherbyi* (under the name of *A. hermonensis*) and *A. hyrcanicus* of the northern parts of Iran were investigated by Javidkar et al. (2005). Geometric morphometric comparisons of the specimens from Fakhrrabad-Yazd with *A. witherbyi* and *A. hyrcanicus* led to the description of *A. avicennicus* as a new species from the Central Iranian Plateau (Darvish et al. 2006; Siah sarvie and Darvish 2008), and its status was confirmed by random-amplified polymorphic DNA–restriction fragment length polymorphism (RAPD-RFLP) analysis (Naseri et al. 2006). Darvish et al. (2010) conducted the first multivariate analysis of morphometric characters, combined with RFLP, for three species of the genus *Apodemus* in Iran. Moreover, *A. witherbyi* from the north of Iran was investigated using molecular and geometric morphometric analyses (Pour Feizi et al. 2009; Jangjoo et al. 2011). Finally, Mohammadi et al. (2012) investigated the karyotype of this genus in Iran. However, controversies remain because of overlaps in identification keys, the lack of discriminant characteristics between species, and misidentifications. *Apodemus hyrcanicus* and *A. ponticus* have rarely been included in phylogenetic analyses, and their phylogenetic status relative to other species of the genus *Apodemus* remains vague.

In this study, we performed phylogenetic analyses using the cytochrome b gene (*cyt b*) to: (i) clarify the species composition and the distributions of the various species of the genus *Apodemus* in Iran; (ii) address the enigmatic taxonomic situation of *A. ponticus*; and (iii) reconstruct the evolutionary history of *Apodemus* spp. in the Eastern Mediterranean.

## Materials and Methods

### Sampling

We collected 129 specimens from different Iranian localities and two additional specimens were collected in Afghanistan from 2010 to 2013 (Table 1 and Fig. 1). The materials were identified using available identification keys (Corbet 1978; Kryštufek and Vohralik 2009). Taxonomic names followed Musser and Carleton (2005), except for the subgenus ranks, which are based on Michaux et al. (2002). Phylogenetic relationship of five species of the subgenus *Sylvaemus* including *A. hyrcanicus*, *A. cf. ponticus*, *A. uralensis*, *A. witherbyi* from Iran, as well as *A. pallipes* from Afghanistan and *A. mystacinus* attributed to subgenus *Karstomys* were investigated. All specimens were deposited in the Zoology Museum of Ferdowsi University of Mashhad (ZMUFM), Mashhad, Iran.

In addition to the *cyt b* sequences resulting from this study, 59 additional sequences of the genus *Apodemus*, retrieved from GenBank, were included in our phylogenetic analyses. It comprised the species *A. flavicollis*, *A. uralensis*, *A. witherbyi*, *A. pallipes*, *A. ponticus*, *A. sylvaticus*, *A. mystacinus*, and *A. epimelas*. Finally, sequences of *Rattus rattus* and *Tokudaia osimensis* were used as outgroups.

### DNA Extraction, PCR Amplification and Sequencing

Tissue samples were available from 131 specimens belonging to the genus *Apodemus* and one *Mus musculus* (Table 1, Fig. 1), and comprised muscles preserved in 90 % ethanol. Total genomic DNA for polymerase chain reaction (PCR) was extracted using a standard salt extraction method (Bruford et al. 1992). Double-stranded DNA amplifications of the whole cytochrome *b* gene (*cyt b*) were performed with the primer pair *cyt b* L7 (5'-ACT AAT GAC ATG AAA AATC AT CGT T-3')/ *cyt b* H6 (5'-TCT TCA TTT TTG GTT TAC AAGAC-3'; Montgelard et al. 2002) following the protocol of Chevret et al. (2005). The sequences were deposited in Genbank database under the accession numbers that are given in Table 1.

### Phylogenetic Analyses

The nucleotide sequences were aligned using ClustalW (Thompson et al. 1994) as implemented in the software Bioedit, ver. 7.0.9 (Hall 1999). Genetic distance was calculated with MEGA v4.0 (Tamura et al. 2007) and the Kimura 2-parameter (K2P) model (Kimura 1980). We used PAUP\* 4.0b 10 software (Swofford 2000) to perform maximum parsimony (MP) and maximum likelihood (ML) analyses. Bayesian analyses (BA) were conducted using MrBayes ver. 3.2.2 (Huelsenbeck et al. 2011). The model best fitting our data

for the ML and BA analyses was determined using the Akaike information criterion, as implemented in Modeltest (Posada and Crandall 1998). Modeltest proposed a general time-reversible substitution model (GTR+I+G), with a proportion of invariable sites  $I=0.5478$ , variable sites  $G=1.8152$ , empirical base frequencies (A: 0.3223; C: 0.2946; G: 0.1098; T: 0.2733), and substitution rates (rate [A–C] 1.9917, rate [A–G] 7.5046, rate [A–T] 3.1752, rate [C–G] 0.6178, rate [C–T] 24.5240, rate [G–T] 1.0000) estimated from the data set. These parameters were used for the ML analyses.

ML analyses were carried out under heuristic tree search with ten random addition sequence replicates, and tree bisection reconnection (TBR) branch swapping. Parsimony analyses were performed using heuristic searches with tree-bisection-reconnection (TBR) branch swapping and random addition sequence with 1000 replicates. To assess support for internal nodes, we ran nonparametric bootstrapping (500 and 5000 replicates) (Felsenstein 1985) under ML and MP, respectively, with a single random addition sequence replicate per bootstrap replicate.

Bayesian inference (BI) reconstructions were performed with MrBayes 3.2.2 (Huelsenbeck et al. 2011) using a GTR model. Four simultaneous Markov chain Monte Carlo (MCMC) chains with incremental heating temperature of 0.2 were run 6,000,000 generations and sampled every 100 generations. The burn-in size was determined by checking the convergence of  $-\log$  likelihood ( $-\ln L$ ) values, and the first 5000 generations were discarded as burn-in. The Bayesian posterior probability (BPP) was evaluated from the remaining trees. Haplotypes were estimated using the program DnaSP v5 (Librado and Rozas 2009).

### Divergence Time

We performed dating analyses using Beast 2.1.3 (Bouckaert et al. 2014) with an uncorrelated lognormal relaxed clock and a yule branching process as a tree prior. This tree prior is the most suitable for trees describing the relationships between individuals from different species. The coefficient of variation frequency histogram did not abut against zero, meaning that there was among-branch rate heterogeneity within our data (Drummond et al. 2007). Consequently, as suggested by Drummond et al. (2007), we used a relaxed molecular clock. The analysis was performed with two independent chains and 60 million generations; chains were sampled every 1000 generations with a burn-in of three million generations.

We used one calibration point derived from fossil records, i.e., the split between the genus *Rattus* and *Mus*, which occurred 12 Ma (see e.g., Michaux et al. 2003), and two secondary calibration points from Michaux et al. (2005b), i.e., the

**Table 1** Tissue samples and GenBank accession numbers and locality information for the genus *Apodemus* specimens used in this study

Species	Collecting station		N	No. of the map	Accession number	Reference	
	Country	Locality					
<i>A. witherbyi</i>	Iran	Tabriz	10	1,2,3,4	KR003087; KR003090; KR003091	This study	
		Kordestan	26	5,6,7,8	KR003093; KR003094; KR003096; KR003102		
		Kermanshah	4	9,10	KR003088; KR003095		
		Zanjan	6	12	KR003092		
		Qazvin	1	13	KR003092		
		Shiraz	5	25	KR003100		
		Kerman	1	26	KR003100		
		Yazd	14	22,23,24	KR003089; KR003101		
		Khorasan Razavi	4	20, 21	KR003098		
		Khorasan Shomali	10	16,17,18,19	KR003097; KR003099		
	Turkey	Hakkari	1	32	AJ311156	Michaux et al. 2002	
		Dogubayazit	1	33	AJ311157	Kryštufek and Mozetic Francky 2005	
		Bozcaada	1	46	DQ000290		
	Armenia		1	58	HS1102	Suzuki et al. 2008	
	Levant	Mt. Hermon		2	29	AB303227	
					AB303228		
Greece	Rhodes		2	43	DQ000288	Kryštufek and Mozetic Francky 2005	
					DQ000289		
<i>A. uralensis</i>	Iran	Gorgan	17	14,15	KR003080; KR003081; KR003082; KR003083	This study	
	Turkey	Yalnizcam gecidi	1	35	AJ311155	Michaux et al. 2002	
	Kazakhstan		1		AB096837	Suzuki et al. 2003	
	Russia	Saratov	1	56	DQ844683	Balakirev et al. 2007	
<i>A. hyrcanicus</i>	Iran	Gorgan	9	14,15	KR003084; KR003085; KR003086	This study	
<i>A. ponticus</i>	Iran	Kordestan	7	7,8	KR003103; KR003104; KR003105	Michaux et al. 2004	
		Khoramabad	2	11	AJ605687		
Russia	Krasnodar		3	55	FN433629	Chelomina and Atopkin 2010	
					FN433630		
					FN433632		
		Adygeya, the settlement of Khamyshki	2	54	DQ844689	Balakirev et al. 2007	
					DQ844691		
	Levant	Mt. Carmel		2	28	AJ605689	Michaux et al. 2004
						AJ605690	
	Turkey	Safranbolou	1	38	AJ605671		
		Oludeniz Fethiye	2	40	AJ605676		
		Oludeniz Fethiye			AJ605677		
Rize		1	36	AJ605678			
Eludag, Bursa		2	47	AJ605679			
				AJ605680			
Efirli Ordu		1	37	AJ605681			
Egridir		2	41	AJ605669			
			AJ605670				
	Datca	2	42	AJ605668			

**Table 1** (continued)

Species	Collecting station		N	No. of the map	Accession number	Reference
	Country	Locality				
					AJ605667	
		Caycuma,Zonguldak	1	39	AJ605682	
		Belsehir	2	44	AJ605684	
					AJ605685	
<i>A. mystacinus</i>	Iran	Kordestan	15	7,8	KR003107; KR003108	This study
	Turkey	Antalya	2	45	AJ748225	Michaux et al. 2005a
					AJ748226	
	Syria	Bloudan	1	30	AJ748236	
		Slanfah	2	31	AJ748237	
					AJ311146	
	Georgia		1	57	AJ748240	
	Levant		1		MAS2	Suzuki et al. 2008
<i>A. pallipes</i>	Afghanistan	Bamian	2	27	KR003106	This study
	Pakistan		1	59	AF160603	Hooper et al. 2007
<i>A. flavicollis</i>	Russia	Volgograd	1	53	AJ605652	Michaux et al. 2004
	Turkey	Damar	1	34	AJ605666	
		Thrace	4	52	AJ605673	
					AJ605674	
					AJ605672	
					AJ605675	
	Greece	Peloponnese	5	49	AJ605626	
					AJ605624	
					AJ605625	
					AJ605628	
					AJ605627	
		Epirus	1	50	AJ605632	
<i>A. sylvaticus</i>	Turkey	Caycuma Zonjuldak	1	39	AJ511939	Michaux et al. 2002
<i>A. epimelas</i>	Bulgaria	Ploski, Blagoevograd	1	51	AJ748227	Michaux et al. 2005a
	Greece	Peloponissos, Taygetos	1	48	AJ748228	
		Peloponissos, Delphes	2	49	AJ748229	
					AJ748230	
<i>R. rattus</i>	Indonesia		1		AB033702	Suzuki et al. 2000
<i>M. musculus</i>	Iran	Kordestan	1	8	KR003109	This study
<i>T. osimensis</i>			1		AB033703	Suzuki et al. 2000

split between *A. sylvaticus* and *A. flavicollis* (4 Ma) and the split between *A. mystacinus* and *A. flavicollis*-*A. sylvaticus* (7 Ma).

## Results

### Haplotype Selection

A total of 61 unique haplotypes were identified from the 190 *Apodemus cyt b* sequences from Genbank and this

study. Within the Iranian samples, a total of 28 haplotypes were detected among the five studied species and 131 specimens (129 belong to this study and two obtained from Michaux et al. 2004), i.e., four in *A. uralensis*, three in *A. hyrcanicus*, 16 in *A. witherbyi*, two in *A. mystacinus*, and three in *A. cf. ponticus*. Additionally, a haplotype belonging to *A. pallipes* from Afghanistan was identified. The analyzed alignment (1076 bp) showed 403 variable sites; 327 of them were parsimony-informative. Gaps were missing and no ambiguity remained in our final dataset.



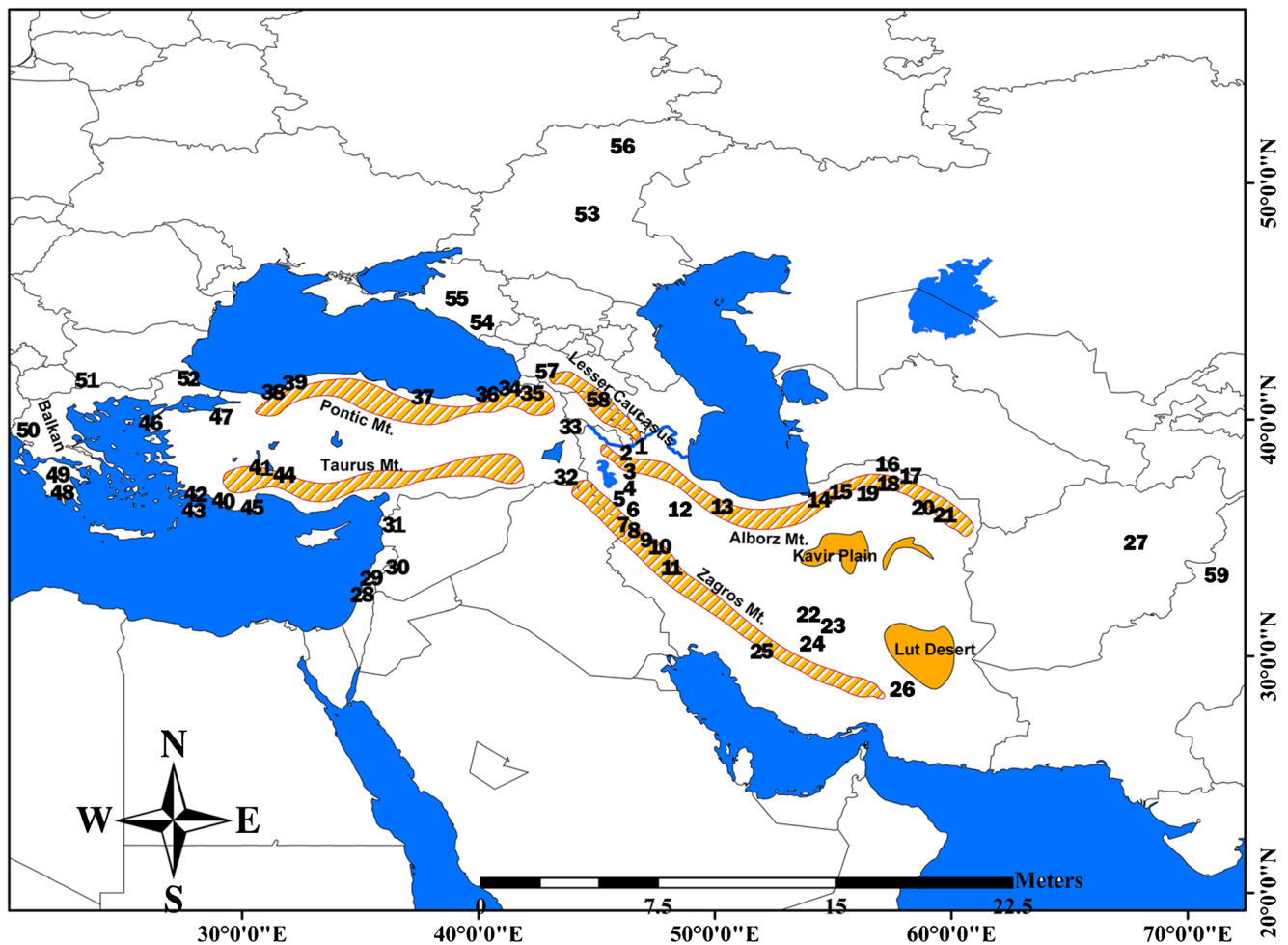


Fig. 1 Map showing the sampling localities of the specimens used for this study

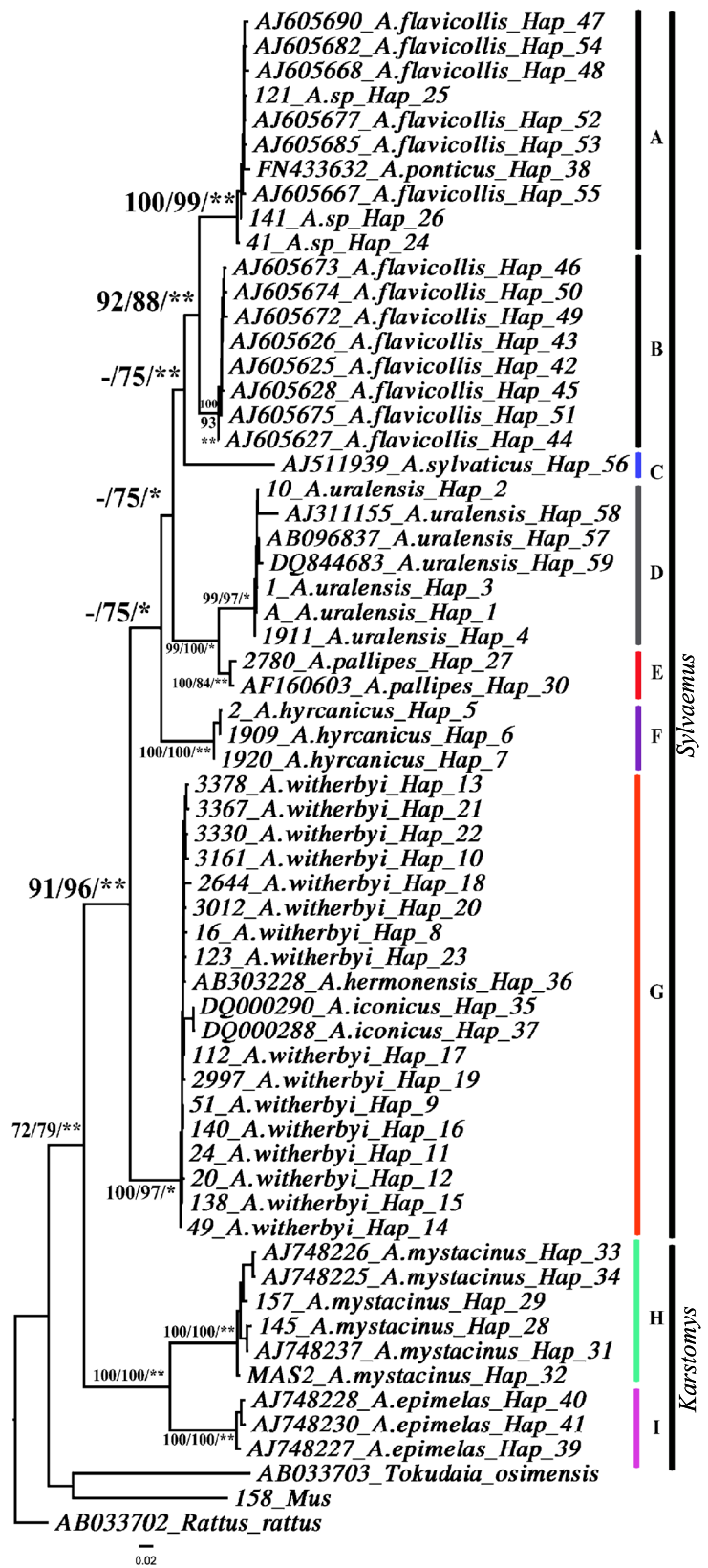
### Phylogenetic Relationships

The ML, MP, and BA analyses showed very similar relationships and our phylogeny was well resolved (Fig. 2). Indeed, the branching orders of the deeper clades were similar and only small differences were observed at the intra-specific level. Maximum likelihood analysis revealed nine lineages (A to I) that corresponded to the species *A. flavicollis*, *A. cf. ponticus*, *A. sylvaticus*, *A. uralensis*, *A. pallipes*, *A. hyrcanicus*, *A. witherbyi*, *A. mystacinus*, and *A. epimelas* (Fig. 2). Kimura 2-parameter distances among the nine clusters ranged from 6.4 % (*A. pallipes* versus *A. uralensis*) to 19.2 % (*A. cf. ponticus* versus *A. epimelas*). Distances within each of the nine lineages range from 0.8 % (within *A. hyrcanicus*) to 2.2 % (within *A. mystacinus*; Table 2). Support for higher-level relationships between *Karstomys* and *Sylvaemus* subgenera were of 72, 79, and 1.0 for MP, ML, and BA analyses, respectively. The supports for the *Karstomys* group, including *A. epimelas* and *A. mystacinus*, were of 100, 100, and 1.0 and for all *Sylvaemus*

species of 91, 96, and 1.0 for MP, ML, and BA, respectively (supports will be given in the same order below). Finally, *A. witherbyi* had a basal position within the members of the *Sylvaemus* group (for more details see Fig. 2).

Lineage (A) included haplotypes previously identified as *A. flavicollis* from Turkey and the Levant besides haplotypes from the western part of Iran (which were identified as *A. cf. ponticus*), as well as a haplotype of *A. ponticus* from Russia (support of 100, 99, and 1.0). Lineage (B) consisted of haplotypes of *A. flavicollis* from Russia, Turkey, and Greece (support of 100, 93, and 1.0). Lineage (C) included the only *A. sylvaticus* haplotype from Turkey, (D) haplotypes of *A. uralensis* (99, 97, and 1.0), (E) haplotypes of *A. pallipes* (100, 84, 1.0), and (F) haplotypes of *A. hyrcanicus* (100, 100, and 1.0), this last being basal to *A. cf. ponticus*, *A. flavicollis*, *A. sylvaticus*, *A. uralensis*, and *A. pallipes*. Lineage (G) included haplotypes of all the specimens of *A. witherbyi* from different regions of Iran, besides published sequence of *A. witherbyi*, *A. iconicus*, and *A. hermonensis* from Genbank. Lineage (H) (100,

**Fig. 2** Maximum likelihood phylogenetic reconstruction of *Apodemus* (based on haplotypes of *cytb* gene sequences). Numbers represent MP and ML bootstrap values (MP/ML) (500/5000 replicates). The posterior probability values from the Bayesian analysis are indicated at the >99 % (\*\*) and >95 % (\*) significance levels



99, and 1.0) included haplotypes of *A. mystacinus* from the northwest of Iran, Syria, the Levant, and Turkey, and finally

lineage (I) (100, 100, and 1.0) consisted of haplotypes of *A. epimelas*.

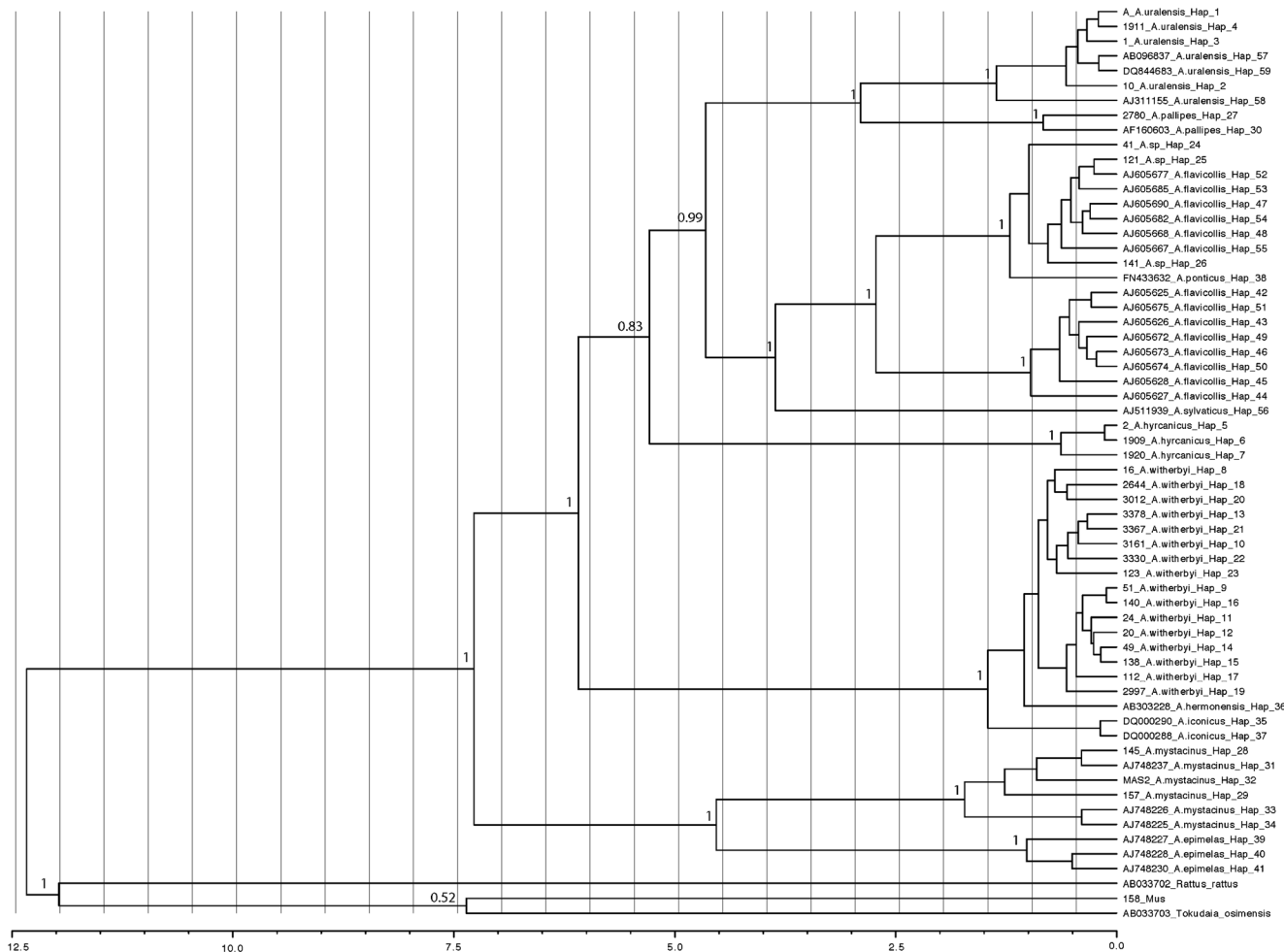
**Table 2** Intra- (bold numbers) and inter-species K2P genetic distances for the analysed *cyt b* gene fragment

	1	2	3	4	5	6	7	8	9
1. <i>A. uralensis</i>	<b>1.3</b>								
2. <i>A. hyrcanicus</i>	13.1	<b>0.8</b>							
3. <i>A. witherbyi</i>	13.9	12.9	<b>1.1</b>						
4. <i>A. cf. ponticus</i>	12.2	11.5	14.2	<b>1</b>					
5. <i>A. pallipes</i>	6.4	11.6	13.9	11.9	<b>1.4</b>				
6. <i>A. mystacinus</i>	17.4	18.6	17.9	17.3	17.5	<b>2.2</b>			
7. <i>A. epimelas</i>	18.2	18.2	16.9	19.2	18.9	13.4	<b>1.5</b>		
8. <i>A. flavicollis</i>	11.0	11.6	13.9	6.8	10.3	17.3	18.4	<b>0.8</b>	
9. <i>A. sylvaticus</i>	14.2	14.5	14.7	13.3	13.1	17.4	18.5	11.4	–

**Divergence Time**

Molecular dating based on *cyt b* revealed that the split between the subgenus *Karstomys* and *Sylvaemus* occurred approximately 7.2 Ma (95 % CI: 6.7–7.8; Fig. 3). Within the

subgenus *Karstomys*, the split between *A. mystacinus* (clade H) and *A. epimelas* (clade I) occurred 4.5 Ma (95 % CI: 3.0–6.1). Within the subgenus *Sylvaemus*, the split between *A. witherbyi* and the remaining species including (*A. hyrcanicus*, *A. cf. ponticus*, *A. uralensis*, *A. flavicollis*, *A. pallipes*, and *A. sylvaticus*) occurred 6 Ma (95 % CI: 5.2–6.9). The split between *A. hyrcanicus* (clade F) and the other clades (A, B, C, D, and E) occurred 5.2 Ma (95 % CI: 4.4–6.1), and the split between *A. pallipes* (clade E) and the other species (*A. hyrcanicus*, *A. cf. ponticus*, *A. uralensis*, *A. flavicollis*, and *A. sylvaticus*) occurred 4.6 Ma (95 % CI: 3.9–5.6). The split between *A. pallipes* (clade E) and *A. uralensis* (clade D) occurred 2.9 Ma (95 % CI: 1.9–4.0), whereas the splits between *A. sylvaticus* and the other two species (*A. cf. ponticus* and *A. flavicollis*) occurred between 3.8 Ma (95 % CI: 3.3–4.7). The split between the *A. cf. ponticus* and *A. flavicollis* occurred 2.6 Ma (95 % CI: 1.9–3.6) (Fig. 3). These findings were entirely congruent with the order of the analysed species in our phylogenetic tree, considering that the older clades took place as basal positions and the newest ones were arranged as terminal clades (Fig. 2).



**Fig. 3** Dating of the major splits in Ma and Bayesian support for the main branches from BEAST analyses



## Discussion

### Taxonomy

Despite its important diversity, data on the different species of the genus *Apodemus* from Iran are scarce. The results of this study thus provide important new information on these species. To date, the west of the Iranian Plateau was supposed to be inhabited by *A. witherbyi*, reported as *A. sylvaticus arianus* by Ellerman and Morrison-Scott (1951), with *A. uralensis* in the northwest and northeast of Iran (Kryštufek and Hutterer 2006; Darvish et al. 2010). Additionally, *A. flavicollis* was the only species of the genus *Apodemus* identified from the Zagros Mountains in western Iran (Etemad 1978; Kryštufek 2002).

The current study detected a high diversity of wood mice in Iran. Different lineages of the genus *Apodemus* belonging to five species were identified, four of which (*A. hyrcanicus*, *A. witherbyi*, *A. cf. ponticus*, *A. uralensis*) belonged to *Sylvaemus* and one (*A. mystacinus*) was included in the subgenus *Karstomys*. This study provided no evidence to support the presence of *A. flavicollis* or *A. sylvaticus* in Iran, despite their occurrence in Turkey.

The results of this study also shed light on the enigmatic situation of the taxon *A. avicennicus*, which was previously described as a new species based on morphological, morphometric, and RFLP analyses. Haplotypes of *A. witherbyi*, *A. avicennicus*, *A. hermonensis*, and *A. iconicus* constitute a monophyletic clade characterized by a mean genetic distance of only 1.1 %. This suggests that all three of these taxa, i.e., *A. avicennicus*, *A. hermonensis*, and *A. iconicus*, should be considered as synonyms of *A. witherbyi*. The priority of *A. witherbyi* over *A. hermonensis* and *A. iconicus* was previously affirmed by several authors (Filippucci et al. 1989; Kryštufek 2002; Kryštufek and Mozetic Francky 2005; Hofer et al. 2007).

Haplotypes of lineage (A) include two specimens from Khoramabad, western Iran, as well as haplotypes from the Levant and Turkey (except Thrace), which were previously recognized as *A. flavicollis* (Michaux et al. 2004) and haplotypes from Kordestan province, western Iran. Interestingly, this lineage is genetically distant (6.8 %) from lineage (B) including haplotypes of *A. flavicollis*. Hence, the status of *A. flavicollis* in Iran, Asia Minor, and Levant is questionable. Differences between eastern and western populations of *A. flavicollis* have previously been reported, but were attributed to the small sample size (Macholán et al. 2001). Michaux et al. (2004) demonstrated high genetic divergence between the western and eastern clades of *A. flavicollis* in relation to their origins from different Pleistocene refugia.

The Kordestan haplotypes in clade (A) were compared with European *A. flavicollis* mentioned in the literature (Reutter et al. 1999; Barčiová and Macholán 2009;

Kryštufek and Vohralik 2009) and were identified as *A. cf. ponticus* based on differences in coloration (smaller yellow spot on their chest), body and skull size (smaller mean), and pattern of the occlusal surface of the molars. This study thus provided good evidence for verifying *A. ponticus* as a distinct species, but closely related to *A. flavicollis*. In accordance with Suzuki et al. (2008), a sister relationship was confirmed between *A. ponticus* and *A. flavicollis*.

Furthermore, *A. uralensis* and *A. pallipes* from Afghanistan were also identified as sister taxa. Despite the low genetic distance between the lineages (E) representing *A. pallipes* and (D) *A. uralensis*, there were significant differences in their morphometric characteristics. Additionally, in agreement with Bellinvia (2004) and Michaux et al. (2002), the subgenus *Karstomys* was identified as a sister taxon to the *Sylvaemus* group.

### Geographic Distribution

*Apodemus witherbyi* was identified as the most widespread species of the genus *Apodemus* in Iran, occupying a range of habitats, except for deciduous forests of the south Caspian Sea, Persian Gulf shores, the central deserts, and southeast of Iran. This species inhabits juniper and coniferous forests on the northern slopes of the Alborz Mountains in the north and northeast Iran (Ghorbani et al. 2010), and sparse wild oak and pistachio forests in the Zagros Mountains, where it is sympatric with *A. cf. ponticus* (Mohammadi et al. 2014). *Apodemus witherbyi* also inhabits drier habitats on the southern slopes of the Alborz Mountains and in the highlands of Yazd near Shirkouh, central Iran, which is surrounded by the central deserts of Iran.

However, other species have more restricted ranges. For instance, *A. hyrcanicus* and *A. uralensis* are dense-forest dwellers and distributed sympatrically around the southern margin of the Caspian Sea (Ghorbani et al. 2010) in tertiary relict broad-leaf forests, so called Euxino-hyrcanian forests, on the northern slopes of the Alborz (Akhani et al. 2010). In addition, *A. cf. ponticus* is considered to be the least common species in Iran, with a restricted distributional range. This species is sympatric and syntopic with *A. witherbyi* in the thickets of the northwest Zagros Mountains (Mohammadi et al. 2014).

*Apodemus mystacinus* has been found in the high-altitude oak forests of the Zagros Mountains, where it was recently recorded in oak forests in Marivan (Darvish et al. 2014). However, Firouz (2008) also noted its presence in northwestern Iran, in the Alborz range. *Apodemus mystacinus* is sympatric with *A. cf. ponticus* on the western slopes of the Zagros, but these two species were not captured at the same elevation; *A. mystacinus* lives at higher elevations, i.e., above 1400 m above sea level. The western slopes of the Zagros Mountains

in Iran are thought to be the easternmost limit of *A. mystacinus*.

The south Caspian Sea depression formed during the Neogene (Golonka 2004), after which the region was gradually covered with late Tertiary deciduous forests and played a role as a refugium for many taxa during the Pleistocene. We can hypothesize that the south Caspian refugium had a profound impact on the speciation and divergence of *A. hyrcanicus*. Additionally, some refugial areas have been defined in the Zagros range (Djamali 2008; Ahmadzadeh et al. 2013; Rajaei Sh. et al. 2013) and could have had similarly essential roles in the genetic divergence of the population of *A. mystacinus* from Iran.

### Phylogeography of the Genus *Apodemus* in the Eastern Mediterranean

The orogenic activities in the Eastern Mediterranean and the squeezing of the Arabian and Turkish plates into the Iranian plate, which led to the uplifting of the High Zagros Mountains and the Anatolian Plateau from the middle and late Miocene (Dercourt et al. 1993; Sengor and Natalin 1996; Yılmaz et al. 1998), hampered biotic exchange between the central Iranian Plateau and the Near East. Further, the establishment of a marine barrier between Anatolia and the Balkan region, in addition to the uplifting of the Carpathians in the Tortonian age (11.62–7.2 Ma) (Rogl 1999), caused climate change and increased seasonality in the Eastern Mediterranean (Brachert et al. 2006). This phase was characterized by a gradual change from a subtropical to a warm temperate climate, and alteration of the vegetation from mixed mesophytic mountain forest to open habitats and savannah in Anatolia and the Middle East (Akgün et al. 2007; Pound et al. 2011). These tectonic events and the consequent climatic configuration of the late Miocene probably strongly reduced gene flow between different lineages of the genus *Apodemus*, resulting in diversification of the subgenus *Karstomys* in the Eastern Mediterranean (about 7.3 Ma). Interestingly, the split between the rodent clades *Spalax/Nannospalax* (Hadid et al. 2012) and different salamander lineages *Mertensiella luschani* (Weisrock et al. 2001), as well as the eastern/western Mediterranean clades of the plant genus *Haplophyllum*, occurred during the same period (Manafzadeh et al. 2013).

The estimated divergence times imply speciation of *A. witherbyi* at the Miocene/Pliocene transition (about 6 Ma). The late Miocene was characterized by extended savannah, called the Greco-Iranian Province, from the Balkan Peninsula to Iran and Afghanistan (De Bonis et al. 1993). During the late Miocene, seasonal rainfall led to the establishment of grasslands with small trees and replacement of the woodland by more open habitats (Koufos and Konidaris 2011). In turn, extension of the steppe area was responsible for a change in mammal diets from C3 plants to C4, leading to

a replacement of forest dwellers by open-adapted mammals in western Asia (Barry et al. 1985). This transition occurred in Pakistan between about 7.8 and 6 Ma (Cerling et al. 1997). Moreover, Koufos et al. (2005) suggested that extinction and immigration of woodland-dependent mammals at the Miocene/Pliocene boundary provided some unoccupied ecological niches for taxa such as *Apodemus* and *Parapodemus* in the Eastern Mediterranean. Additionally, Wessel (1955) proposed that southwestern Asia was a cradle for the evolution of different clades of rodents, as a result of their adaptation to new habitats. Based on our results, dispersal and the occupation of new habitats resulted in the radiation of the genus *Apodemus* to a new lineage (*A. witherbyi*) in the Eastern Mediterranean (6 Ma).

The split between *A. mystacinus* and *A. epimelas* (4.5 Ma) occurred after the Messinian Salinity Crisis (5.96–5.33 Ma), characterized by drought and cold climate (Hsu et al. 1977). During the Messinian Salinity Crisis (5.96–5.33 Ma) in the late Miocene, uplifting of Spain and Morocco closed the seaway between the Atlantic Ocean and the Mediterranean (Clauzon et al. 1996; Krijgsman et al. 1999). It is postulated that desiccation of Circa-Mediterranean regions trapped *A. epimelas* in islands (Mljet, Korcula) and restricted this taxon to Europe, i.e., the Balkan region, Macedonia, and Thrace, where the climate was still relatively humid about 5 Ma (Fortelius et al. 2006). In contrast, *A. mystacinus* occupied more southeastern regions (Anatolia, the Levant, some Aegean islands, and the western Zagros), which were drier under the effect of a north–south polarization of humidity (Fortelius et al. 2006). The results corroborate the diversification of two different clades of the subgenus *Karstomys* under the impact of late Pliocene climatic fluctuation, associated with the Messinian crisis (Michaux et al. 2005a).

The split between *A. sylvaticus* and *A. flavicollis* lineages (3.8 Ma) is thought to be related to the Zanclean phase (5.33–3.6 Ma), after the refilling of the Mediterranean Sea. Circa-Mediterranean regions were affected by a warm, humid climate during the early Pliocene and provided forest habitats in at least some parts of the Italo-Balkan/Near East regions (Michaux et al. 2004; Koufos et al. 2005; Kovar-Eder et al. 2006). This type of habitat supported the distribution of the ancestral *A. flavicollis* in the Italo-Balkan Peninsulas and the Near East, while the population of *A. sylvaticus* was restricted to the Iberian Peninsula (Michaux et al. 2003, 2004). This scenario is supported by the fact that the present seaway between the Black and Mediterranean seas (the Bosphorus Strait), which creates a barrier to terrestrial exchange, was not opened until the end of the Pliocene (Cheylan 1995; Dubey et al. 2007). This view was confirmed by the preference of *A. flavicollis* for forested habitats (Michaux et al. 2004) and of *A. sylvaticus* for bushy habitats (Kryštufek and Vohralik 2009). The current distributions of *A. sylvaticus* in the northern part of the Danube River (Bugarski-Stanojevic

et al. 2008) and *A. flavicollis* in the southern parts further support this hypothesis (Kryštufek and Vohralik 2009).

Divergence between the clades including *A. flavicollis* and *A. cf. ponticus* dates to 2.6 Ma, which is congruent with the starting of the Praetiglian cold stage in Europe, and forest withdrawal (Ruddiman and Raymo 1988). These events caused the formation of steppe habitats (Fauquette et al. 1998, 1999), resulting in the contraction and bounding of the ancestral populations of *A. flavicollis* and *A. cf. ponticus* to different refuge areas: one (*A. flavicollis*) in the south Balkans and another (*A. cf. ponticus*) in the Eastern Mediterranean, which was likely still covered with forests at that time. As noted above, our results are in concordance with those of Michaux et al. (2004), who recognized two different clades of *A. flavicollis* (Oriental and European ones) originating from different refugia during the Tertiary/Quaternary transition. (The oriental clade was recognized as *A. cf. ponticus* in our study).

The Hyrcanian forests were considered to act as refugia for many Arcto-Tertiary relict species (Zohary 1973; Leestmans 2005). From the middle late Miocene, the region had a humid, closed landscape (Mirzaie Ataabadi 2007), providing a suitable climate for expansion of the Pontic forests in the early Pliocene (5–2 Ma; Fortelius et al. 2006). These kinds of broad-leaf forest habitats remained stable during the Pleistocene (Weiss and Ferrand 2007), supporting the existence of forest-dwelling mammals. In this respect, the divergence of *A. hyrcanicus* (5.2 Ma) is postulated to have occurred after the formation of the Hyrcanian forests over the Caucasus and southern Caspian Sea at the Miocene/Pliocene boundary.

## Conclusions

The uplifting of the Zagros Mountains and Anatolian Plateau in the middle Miocene, the Messinian Salinity Crisis, and the formation of the Hyrcanian tertiary forests during the Neogene probably all played substantial roles in the radiation and subsequent distribution patterns of members of the genus *Apodemus* in the Eastern Mediterranean region. Alternative phases of climate fluctuations led to the formation and expansion of savannah, characterized by open-adapted mammals, and forest habitats in the Eastern Mediterranean, further driving the diversification of the genus *Apodemus*.

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