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Phylogeography of *Troglophilus* (Orthoptera: Troglophilinae) based on Anatolian members of the genus: radiation of an old lineage following the Messinian

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Active tectonic history of the Eastern Mediterranean, especially around Aegean area, through the Neogene led to interesting radiation patterns of animal lineages, allowing intriguing biogeographical hypotheses to be tested. Descendants of the ancestral stock in the Miocene Aegean Plate presently occur in the Anatolia, Aegean islands and the Balkan Penninsula. *Troglophilus* (Orthoptera, Rhaphidophoridae, Troglophilinae) is such a genus represented in these areas with approximately 15 species. The present study addresses the phylogeography of the genus, with a special emphasis on its Anatolian members, aiming to test the biogeographical patterns suggested for this area using mitochondrial [cytochome oxidase I (COI)] and nuclear (ITS1-5.8S–ITS2) markers. Data matrices obtained from sequences of COI and ITS1-5.8S–ITS2 were used for phylogenetic analyses using *Dolichopoda lycia* and *Dolichopoda sbordonii* as outgroups. All sets of the analyses suggested monophyly of the Anatolian haplotypes, although they are not congruent in revealing their relationships. Anatolian haplotypes constituted three main phylogroups in trees calculated from a matrix of short COI sequences: the ECMA (corresponding to the Eastern part of coastal Mediterranean Anatolia); the CWMA (from the Central and Western part of Mediterranean Anatolia); and NA (from Northern Anatolia). Trees obtained using longer sequences resulted in only two phylogroups, namely ECMA and CWMA + NA. The trees based on the ITS1-5.8S–ITS2 data matrix supported monophyly of Anatolian phylogroups. BEAST analysis of the COI estimated the time to most recent common ancestor for *Dolichopoda* and *Troglophilus* as 10.8 Mya, to that for the Anatolian + Balkan *Troglophilus* as 7.2 Mya, and to that for the Anatolian *Troglophilus* as 6.3 Mya. BEAST analysis of ITS1–ITS2 intron regions is largely congruent with that of COI. From these results, several conclusions were drawn. First, the divergence of *Dolichopoda* and *Troglophilus* possibly started with the opening of the Mid-Aegean Trench in the Tortonian. Second, *Troglophilus* possibly originated from an ancestral stock in the old Aegean Plate. It later diverged as Anatolian and Balkan lineages and, possibly, the Cretan population may be regarded the third lineage. Divergence within the Anatolian lineage is estimated to have occurred through the Pliocene and Pleistocene, although before the last four glacial periods in the late Pleistocene. Additionally, the northern Anatolian *Troglophilus* appears to originate from the dispersal of an ancestral stock from a mountainous lineage through the Taurus Way. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, **108**, 335–348.

ADDITIONAL KEYWORDS: Aegean area – Anatolia – cave crickets – evolution – Late Miocene – Taurus Way.

INTRODUCTION

Recently, the Eastern Mediterranean, mainly the lands surrounding the Aegean Sea, became a model area of historical biogeographical studies either for both terrestrial and aquatic animal lineages (Schilthuizen *et al*., 2004; Gentile & Argano, 2005; Kasapidis *et al*., 2005; Wahlberg, Brower & Nylin, 2005; Parmakelis *et al*., 2006; Douris *et al*., 2007; Allegrucci *et al*., 2009; Akın *et al*., 2010; Çıplak, Heller & Willemse, 2010; Velonà *et al*., 2010; Wielstra

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et al., 2010; Allegrucci, Trucchi & Sbordoni, 2011; Hernandez-Roldan *et al*., 2011; Simaiakis *et al*., 2012; others). This is especially the result of a dynamic palaeogeographical history of the region (Steininger & Rögl, 1984; Şengör & Natal'in, 1996; Robertson, 1998; Rögl, 1999; Bozkurt, 2001; Görür & Tüysüz, 2001; Meulenkamp & Sissingh, 2003; Jolivet *et al*., 2006). Successive changes in land/sea configuration through the Neogene led to repeated dispersal and vicariant separation of local populations; all these events produced interesting radiation patterns offering intriguing biogeographical hypotheses to be tested.

Most of the lands belonging to present Eastern Mediterranean area, namely Balkans and Western Anatolia, are derivatives of the old Aegean (Aegeid, Aegeis) plate, which had formed by merging of different archipelagos in the Tethys Ocean around the end of Eocene (Steininger & Rögl, 1984; Şengör & Natal'in, 1996; Robertson, 1998; Meulenkamp & Sissingh, 2003). Although Anatolia is the largest landmass that has originated from this plate, only the western part of the present Anatolia (or Asian Turkey) is derived from this plate, whereas its eastern half was formed from Persian, Caucasian, and Arabian tectonic plates (Steininger & Rögl, 1984; Bozkurt, 2001; Görür & Tüysüz, 2001). This multiple geological origin is also reflected in biodiversity patterns, where the Anatolian Diagonal appears to represent the border of western and eastern lineages (Davis, 1971; Ekim & Güner, 1986; Çıplak, Demirsoy & Bozcuk, 1993; Çıplak, 2003), although there are recent exchanges between both sides. The lineages of western origin primarily range in western Anatolia, Aegean Islands, and the Balkans. From a biogeographical point of view, the geographical changes around Anatolia or Aegean Sea that took place during the Miocene are more important for understanding the speciation pattern of lineages of western origin (Gantenbein & Keightley, 2004; Parmakelis *et al*., 2006; Douris *et al*., 2007; Allegrucci *et al*., 2009; Çıplak *et al*., 2010; Papadopoulou, Anastasiou & Vogler, 2010; Velonà *et al*., 2010; Allegrucci, Trucchi & Sbordoni, 2011). The main geological/geographical events that enormously influenced vicariant separation/dispersal in western Anatolia or in the Aegean area are: (1) the regression of the Aegean area in the late Tortonian (11–9 Mya); (2) the occurrence of numerous land bridges between Anatolia and Greece during the Messinian (7–5.5 Mya); and (3) the reflooding of the Mediterranean/Aegean during Pliocene (approximately 5 Mya) (Steininger & Rögl, 1984; Dermitzakis, 1990a, b; Meulenkamp & Sissingh, 2003; Anastasakis *et al*., 2006). Family Rhaphidophoridae is one of the oldest lineages of Orthoptera (Allegrucci *et al*., 2010) represented by two genera in the Northern Mediterranean. Of these, *Dolichopoda* includes approximately 50 species, whereas *Troglophilus* has approximately 15 (Allegrucci *et al*., 2009, 2011; Karaman *et al*., 2011; Eades *et al*., 2012). Their present ranges extend beyond areas derived from the old Aegean plate, although it is assumed that they originated from ancestral stock occurring in this old plate and later dispersed to their present range (Allegrucci *et al*., 2009; Karaman *et al*., 2011). *Troglophilus* is not represented in all lands derived from the old Aegean plate (Fig. 1). There are three species in the Aegean islands (Willemse, 1984; Kollaros, Paragamian & Legakis, 1991; Ketmaier *et al*., 2000; Rampini & di Russo, 2003a). Six species are recorded from the Balkans; in addition, the Italian species *Troglophilus andreinii* Capra also belongs to Balkan group (Karaman *et al*., 2011). Interestingly, this genus is not widespread in mainland Greece, except for a single locality in the central part and some in the north (Willemse, 1984; Willemse & Willemse, 2008; Karaman *et al*., 2011). The remaining five species are known only from Anatolia. The main range of *Troglophilus* species is southern Anatolia, its species always being endemic to the type locality or its neighbourhood: *Troglophilus adamovici* and *Troglophilus bicakcii* from the central Mediterranean Anatolia, and *Troglophilus escalerai* and *Troglophilus gajaci* from the eastern Mediterranean Anatolia. The only contradicting range pattern is that of *Troglophilus tatyanae* di Russo & Rampini, reported from the Black Sea, or the Euxinic part of Anatolia (Bolivar, 1899; Karabağ, 1958; Us, 1974; Cobolli *et al*., 1999; Çıplak *et al*., 2002; Rampini & di Russo, 2003b; di Russo, Rampini & Landeck, 2007). From this range pattern, Karaman *et al*. (2011) suggested that south Anatolia and the Balkans are two centres of origin for *Troglophilus*. However, it is possible that Crete is another centre of origin because the two species in Crete and Cyclades are similar to each other and differ from the remaining congenerics (Rampini & di Russo, 2003a).

By studying Anatolian *Troglophilus*, we aimed to address specific biogeographical issues. Karaman *et al*. (2011) considered South Anatolia and the Western Balkans as two radiation centres of the genus, and Black Sea Basin as the possible third. However, these assumptions need to be tested either for the whole genus or for its sublineages in Anatolia. Accordingly, it is necessary to know whether Anatolian species constitute one or more distinct lineages. Besides, if there is more than one sublineage either in Anatolia or within the whole genus, we need to estimate the time of their last common ancestors and their divergence, especially considering the Anatolian

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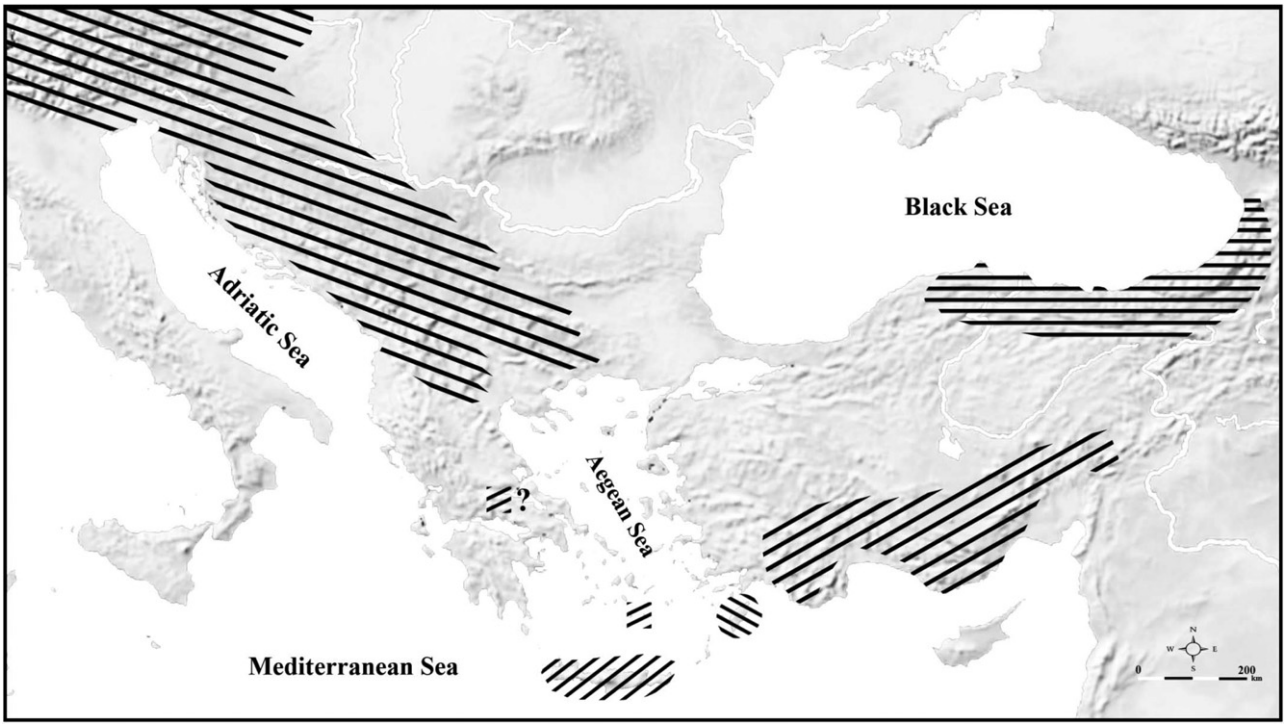

Figure 1. Eastern Meditetrranean distribution of *Troglophilus*.

sublineage(s). Answering these questions will allow us to correlate the radiation history of the genus *Troglophilus* or its Anatolian species with historic geological/climatic events in the Eastern Mediterranean. In the Mediterranean Basin, family Rhaphidophoridae is represented by genera *Troglophilus* and *Dolichopoda*, belonging to monotypic subfamilies Troglophilinae and Dolichopodinae, respectively. Allegrucci *et al*. (2009, 2011) estimated, using data mainly from *Dolichopoda*, that their divergence started approximately 12 Mya. Thus, we also aimed to test this dating using data from *Troglophilus* and to discuss the possible place of origin of this genus, whose range is almost limited to the areas derived from the old Aegean plate. To address these issues, we sequenced mitochondrial cytochrome oxidase I (COI) and nuclear ITS1-5.8S–ITS2 gene regions from representative populations belonging to both known and unknown taxa from different parts of Anatolia and representatives from the Balkans, aiming to conduct phylogenetic analyses and molecular clock estimations.

MATERIAL AND METHODS

SAMPLING

Different caves were visited to sample *Troglophilus*. Throughout Anatolia, we found members of genus *Troglophilus* in ten localities. After collection, specimens were preserved in 96% ethanol. Among these ten populations, five represent four known Anatolian species: *T. adamovici* Us, 1974, *T. gajaci* Us, 1974, *T. bicakcii* Rampini & di Russo, 2003b, and *T. tatyanae* di Russo & Rampini, 2007. Although the type locality of *T. escalerai* Bolivar, 1899 was visited, no specimens were found. The remaining five populations belong to two new species, designated as *Troglophilus* sp. nov. 1 and *Troglophilus* sp. nov. 2. Considerable intraspecies divergence was observed in each of these taxa, although further data are necessary for any taxonomic considerations. For phylogenetic and time estimation analyses, we also used samples or published sequences representing the Balkan lineage. Two sequences of COI of *Troglophilus cavicola* were obtained from GenBank (AY793624, AY507653; Allegrucci *et al*., 2009). In addition, sequences of the internal transcribed spacers (ITS1-5.8S rDNA–ITS2) of three specimens of *Troglophilus* from the Balkans (Kosovo, Macedonia, and Bulgaria) were used. In addition to *Troglophilus*, one sequence from *Dolichopoda sbordonii* (di Russo & Rampini, 2006) was included in the analyses as an outgroup. We attempted to obtain COI and ITS1-5.8S rDNA–ITS2 sequences from one to six individuals per population depending on the availability because haplotype diversity per cave is not high in these subterranean animals (Allegrucci *et al*., 2011; Karaman *et al*., 2011).

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DNA EXTRACTION, POLYMERASE CHAIN REACTION (PCR), AND SEQUENCING

Total DNA was extracted from ethanol-preserved tissue using the salt extraction method *sensu* Aljanabi & Martinez (1997). Two different markers, the mitochondrial COI and the nuclear ITS1-5.8S rDNA–ITS2, were sequenced. A 1153-bp region of the COI was amplified using the universal primers forward C1-J-1718, C1-J-1751 and reverse TL2-N-3014 as described previously (Simon *et al.*, 1994). Of the ITS1-5.8S rDNA–ITS2, a region of 833–923 bp was amplified using eukaryote-specific universal primers forward ITS5 and reverse ITS4 (White *et al.*, 1990). Amplification was performed in a 50-μL volume containing 0.3 μL of each primer (100 μM), 1 μL of dNTP mix (10 mM), 2 μL of 50 mM $MgCl_2$, 5 μL of 1 × Platinum PCR buffer (containing 200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1.25 U of Platinium *Taq* DNA polymerase, and 0.5–1 μL of template DNA for each reaction. Temperature cycling was carried out in an Eppendorf Mastercycler Personal. Cycling conditions for both markers were 2 min of denaturing at 95 °C followed by 45 s at 95 °C, 40 s at 49 °C, and 1.20 min at 72 °C (×35). The PCR products were purified and the amplification primers were used to generate sequences in both directions. Double-stranded amplification and purification was performed through the intake from Macrogen.

PHYLOGENETIC ANALYSIS

Sequences were checked, edited, and visually optimized using SEQUENCHER, version 4.1.4 (Gene-Codes Corp.) and alignment was accomplished using MEGA, version 5 (Tamura *et al.*, 2011) with default parameter settings. Each COI gene sequence was checked using DNASP, version 5 (Librado & Rozas, 2009) to distinguish numt or real mitochondrial genes, and to determine unique haplotypes and their frequency. Translation of the COI nucleotide sequences to amino acid sequences using DNASP did not reveal any stop codons. The obtained sequences were checked for homology using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

The sequences of both markers were deposited in GenBank (Table 1). Three different data matrices, two

Table 1. Localities of specimens (Fig. 4) and the haplotypes found and used in phylogenetic analyses of *Troglophilus* (NS, number of individual sharing respective haplotype)

Locality	Altitude (m)	COI			ITS1-5.8S-ITS2		
		Haplotype	NS	GenBank No	Haplotype	NS	GenBank No
Kastamonu, Pınarbaşı	1365	*Troglophilus tatyanae*	1	JX952192	*Troglophilus tatyanae*	1	JX952179
Denizli, Acıpayam	1110	***Troglophilus* sp. nov. 1-1**	2	JX952184	***Troglophilus* sp. nov. 1-1**	2	JX952173
Muğla, Fethiye	540	***Troglophilus* sp. nov. 1-2**	1	JX952188	***Troglophilus* sp. nov. 1-1**	1	JX952173
Konya, Hadim	1639	***Troglophilus* sp. nov. 1-3**	1	JX952185	***Troglophilus* sp. nov. 1-3**	1	JX952175
Mersin, Anamur	110	***Troglophilus* sp. nov. 2-1**	1	JX952187	***Troglophilus* sp. nov. 2-4**	4	JX952172
		***Troglophilus* sp. nov. 2-2**	1	JX952195			
		***Troglophilus* sp. nov. 2-3**	1	JX952196			
Antalya, Alanya	255	***Troglophilus* sp. nov. 2-4**	1	JX952182	***Troglophilus* sp. nov. 2-4**	4	JX952172
		***Troglophilus* sp. nov. 2-5**	1	JX952183			
Mersin, Silifke	141	*Troglophilus gajaci*	2	JX952191	*Troglophilus gajaci*	4	JX952178
Konya, Derebucak	1340	*Troglophilus bicakcii*-1	3	JX952189	*Troglophilus bicakcii*-1	3	JX952176
					Troglophilus bicakcii-2	3	JX952177
Konya, Seydişehir, Ferzene	1470	*Troglophilus adamovici*-1	1	JX952186	*Troglophilus adamovici*-1	4	JX952174
		Troglophilus adamovici-2	1	JX952190			
Konya, Seydişehir, Madenli	1549	*Troglophilus adamovici*-1	1	JX952186	*Troglophilus adamovici*-1	4	JX952174
		Troglophilus adamovici-3	1	JX952193			
		Troglophilus adamovici-4	1	JX952194			
Macedonia	368				*Troglophilus brevicauda*-1	2	JX952170
Kosovo	620				*Troglophilus cavicola*	1	JX952169
Bulgaria	585				*Troglophilus brevicauda*-2	4	JX952171
Out gropus							
Antalya Kemer		*Dolichopoda lycia*	1	JX952181			
Burdur		*Dolichopoda sbordonii*	1	JX952180	*Dolichopoda sbordonii*	1	JX952168

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from COI and one from ITS1-5.8S–ITS2, were prepared. Of the two matrices of COI, the first (COI-1) was produced from sequences trimmed according to 902-bp long sequences obtained from GenBank. The COI-1 matrix contains 25 sequences; 22 from Anatolian *Troglophilus*, two from Balkan *Troglophilus* (obtained from GenBank), and one from *Dolichopoda sbordonii* as outgroup. Because the sequences from GenBank are considerably shorter than those produced in our laboratory, we produced a second matrix of COI sequences (COI-2) to benefit from phylogenetic information within the remaining sites of DNA. In the second matrix, sequences from GenBank were excluded, although a sequence from *Dolichopoda lycia* (Galvagni, 2006) was added as second outgroup. The data matrix of ITS1-5.8S–ITS2 contains 47 sequences: 39 from Anatolian *Troglophilus*, seven from Balkan *Troglophilus*, and one from *Dolichopoda sbordonii* as an outgroup. Phylogenetic analyses were repeated for each matrix separately, as described below.

Phylogenetic analyses were conducted by maximum parsimony (MP) and maximum likelihood (ML) as implemented in PAUP, version 4.10b (Swofford, 2000), and Bayesian inference (BI) as implemented in MrBayes, version 3.1.2 (Ronquist & Huelsenbeck, 2003; Ronquist, Huelsenbeck & van der Mark, 2005). Parsimony analysis was performed using a heuristic search procedure with tree bisection–reconnection (TBR) branch swapping, and 100 random-addition replicates. Branch supports were estimated using 10 000 nonparametric bootstrap replicates (Felsenstein, 1985). The parameters and the best-fit models for each of the matrices were estimated using MODELTEST, version 3.7 (Posada & Crandall, 1998). The selected model was implemented in the ML and BI analyses performed with PAUP and MrBayes, respectively. ML analysis was performed using a heuristic search procedure with TBR branch swapping and as-is addition replicates. Branch supports were estimated using 1000 nonparametric bootstrap. For the Bayesian analyses of each matrix, four simultaneous searches and Markov chains were used for 3 000 000 generations, sampling every 100th generation. TRACER, version 1.5 (Rambout & Drummond, 2003) was used to examine the parameters and to determine the number of trees needed to reach stationary (burn-in). Bayesian posterior branch probabilities were obtained by taking the majority rule consensus of the sampled trees, excluding the first 3000 trees as burn-in.

Estimation of divergence time

Molecular clock analyses were conducted using mitochondrial sequences in the COI-1 and intron regions in ITS1-5.8S rDNA–ITS2 matrices. Substitution rate estimations for COI range from 0.0168 to 0.023 per site/million years (Papadopoulou *et al*., 2010; Allegrucci *et al*., 2011). Even so, there are extreme rate estimations such as 0.03–0.04 per site/million years for Tettigoniidae (Shapiro, Strazanac & Roderick, 2006). We concluded that it is more appropriate to calibrate the molecular clock analysis of COI-1 according to the general substitution rate for the insect mitochondrial genome suggested by Brower (1994; 0.023 substitutions/site/year), as widely used in several other studies (Percy, Page & Cronk, 2004; Kiyoshi & Sota, 2006; Papadopoulou *et al*., 2010). There are similar controversies for ITS1–ITS2. The substitution rate per site/million years varies from 0.00172 to 0.0258 in eukaryotes (Kasuga, White & Taylor, 2002; Percy *et al*., 2004; Bargues *et al*., 2006). However, Percy *et al*. (2004) suggested an insect specific rate ranging from 0.0025 to 0.07 for ITS regions. Thus, molecular clock analysis of nuclear ITS1–ITS2 sequences was repeated by calibrating for the lower and the upper limits, and the results of the analysis congruent with those of the COI chronogram were presented and discussed. Before running BEAST using nuclear ITS1-5.8S–ITS2 sequences, the exon of 5.8S rDNA region and the gaps were removed from the sequences because the substitution rates are different for exon and intron regions (Papadopoulou *et al*., 2010).

BEAST, version 1.6.1 (Drummond & Rambout, 2007) was used to estimate the divergence time of lineages under a Bayesian statistical framework. A ML scores was applied to each of mitochondrial COI-1 and nuclear ITS1–ITS2 sequences using MEGA, version 5 (Tamura & Nei, 1993; Tamura *et al*., 2011) to determine whether they behave clock-like or not. The time of lineage divergences was estimated using a strict molecular clock or the uncorrelated lognormal relaxed clock model. For each matrix, BEAST was run using a Yule speciation process and two independent MCMC chains for 300 000 000 and 80 000 000 generations for COI-1 and ITS1–ITS2, respectively, sampling every 1000th generation. The convergence to stationary and the effective sample size of model parameters were checked using TRACER. The maximum clade-credibility trees were built with TREEANNOTATOR (Drummond & Rambout, 2007), discarding the initial 10% of samples as burn-in. FIGTREE 1.3.1 (Rambout, 2008) was used to visualize the results, including confidence intervals.

RESULTS

After alignment and trimming, the final length of sequences in the COI-1 matrix was 902 bp. Of the 902 bp, 632 sites were constant, 270 were variable,

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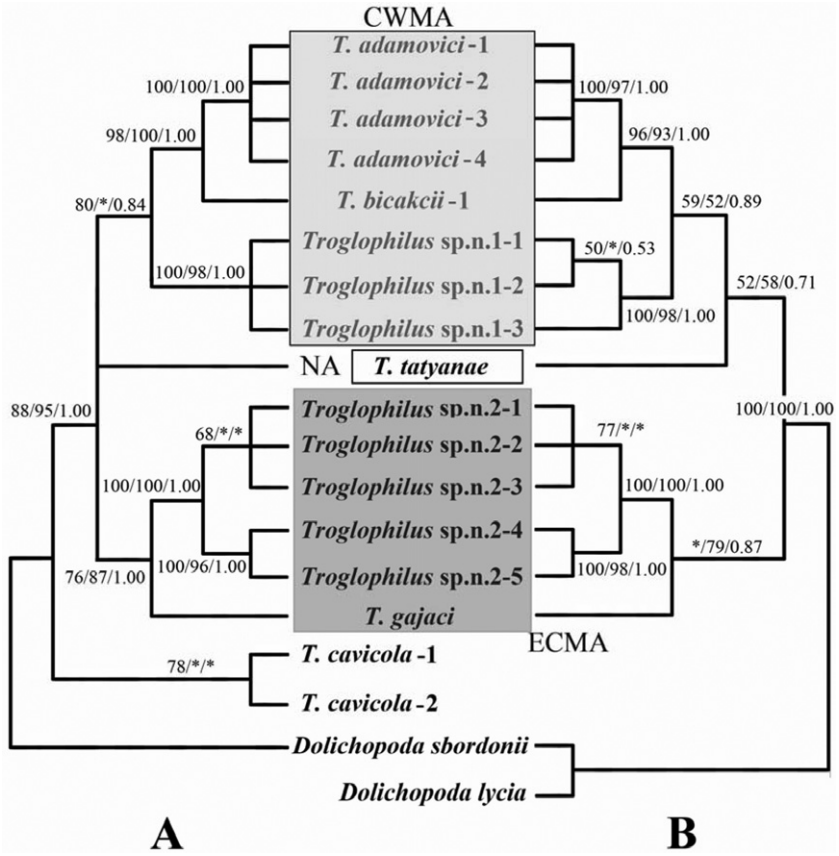

Figure 2. Results of maximum parsinomy (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses combined for strict consensus MP trees based on cytochrome oxidase I (COI) sequences (A, produced from 18 haplotypes of 902 bp, COI-1; B, produced from 17 haplotypes of 1122 bp, COI-2). Posterior probabilities (BI) and bootstrap supports (MP and ML) to branches are shown (MP, ML, and BI, respectively). Branches indicated with an asterisk (*) are not supported in the respective analyses. ECMA, phylogroup distributed in eastern coastal part of the Mediterranean Anatolia; CWMA, phylogroup distributed in the central and western part of Mediterranean Anatolia; NA, phylogroup distributed in North Anatolia.

and 198 were parsimony informative. Eighteen different haplotypes of COI sequences of ingroup + outgroup specimens were identified from 25 sequences. Of the 18 haplotypes of 902 bp sequences used in phylogenetic analyses, 15 are from Anatolian *Troglophilus*, two from Balkan *Troglophilus*, and one from *Dolichopoda sbordonii* as outgroup. For data including the outgroup, the GTR + Γ + I model with gamma correction (Γ) of 0.9840, the frequency of invariable sites (I) set at 0.5812, and a *ti/tv* ratio of 3.8571 were selected by both the hierarchical likelihood-ratio test (hLRT) and Akaike information criteria (AIC). MP analysis of haplotypes in the COI-1 matrix resulted in 30 equally parsimonious trees (tree length = 581, consistency index = 0.611, retention index = 0.729). All phylogenetic analyses (MP, ML, and BI) of 17 ingroup and one outgroup haplotype, combined in the strict consensus MP tree showing bootstrap (MP and ML) and posterior probability (BI) values, generated trees with rather similar topologies, except for the relationships of two haplotypes of the Balkan species (Fig. 2A). The Balkan haplotypes constitute a phylogroup basal to (MP), or branching off successively in the base (ML and BI), of the Anatolian ingroup haplotypes. All analyses support monophyly of the Anatolian haplotypes with high bootstrap (MP and ML) or posterior probability (BI) values. The Anatolian clade includes three major phylogroups: the NA, the ECMA, and the CWMA, consisting of one, six, and eight haplotypes, respectively. The ECMA phylogroup corresponds to the localities from the eastern coastal part of Mediterranean Anatolia, the CWMA phylogroup to that from the central + western part of Mediterranean Anatolia and the NA to the North Anatolian population (Fig. 2A). The NA phylogroup represents *T. tatyanae*. The ECMA phylogroup consists of *T. gajaci* and five haplotypes of an undescribed species. The CWMA phylogroup includes two infra

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phylogroups: the first includes *T. bicakcii* and *T. adamovici*, with the second a group of haplotypes representing an undescribed species.

After alignment and trimming, the final length of the sequences in the COI-2 matrix was 1122 bp. Of the 1122 bp, 782 sites were constant, 340 were variable, and 285 were parsimony informative. Fifteen different COI ingroup haplotypes out of total 24 sequences were identified. The GTR + I + Γ model with invariable sites (I) set at 0.5691, gamma correction (Γ) of 1.0656, and the frequency of the *ti/tv* ratio of 5.2595 were selected by both of hLRT and AIC. MP analysis of 1122-bp long haplotypes resulted in two equally parsimonious MP trees (tree length = 631, consistency index = 0.686, retention index = 0.766). The MP, ML, and BI analyses of these 15 haplotypes produced trees with almost identical topologies (Fig. 2B). The difference occurs for intraspecific relationships of haplotypes of *T. adamovici*: being polytomic (MP) or successively branched (ML and BI). On the other hand, the MP, ML, and BI trees calculated from COI-2 matrix are largely similar to those from COI-1. The only difference between COI-1 and -2 concerns the relationship of *T. tatyanae* with other phylogroups. The trees calculated from COI-1 suggest the haplotype of this species forms a separate phylogroup, whereas those of from COI-2 placed it in the base of CWMA, thus showing NA + CWMA as a single phylogroup.

The length of aligned and trimmed sequences of ITS1-5.8S–ITS2 was 870 bp. The highest substitution rate is found in the transcribed spacers ITS1 and ITS2, whereas the coding region of 5.8S (163 bp) is more conservative. The ITS1-5.8S–ITS2 region contained multiple length variants within each of the individuals and species sampled, which indicated the presence of internal repeats. ITS1 was highly diverged between species and was usually accompanied by significant intra-individual length variation formed by several long internal repeats. In the ITS1-5.8S–ITS2 region of nuclear rDNA, intra-individual sequence variation has been found in some individuals of *T. adamovici*, *T. bicakcii*, and *Troglophilus* sp. nov. 2.

Of the 870 aligned positions from the ITS1-5.8S–ITS2 region, 640 sites were constant, 109 were variable, 81 of which were phylogenetically informative, and 121 were indels. In phylogenetic analyses, all sites containing gaps were removed. Twelve haplotypes were identified from 47 sequences when indel sites were excluded, although this number rose to 14 including indels. The transversion model TVM + Γ with gamma correction (Γ) of 0.2534 and a *ti/tv* ratio of 0.7808, were selected by AIC running by MODELTEST for the matrix. MP analysis of 749-bp long haplotypes resulted in two equally parsimonious MP trees (tree length = 304, consistency index = 0.895, retention index = 0.921). The MP, ML, and BI analyses of these 12 haplotypes produced trees with similar topologies (Fig. 3). The MP and ML trees suggest a polytomy consisting of four phylogroups: (1) *T. bicakcii* + *T. adamovici*; (2) *T. gajaci* + *Troglophylus* sp. nov. 2; (3) *Troglophylus* sp. nov. 1; and (4) *T. tatyanae*. By contrast to MP and ML, BI tree suggests that (3) and (4) form a single phylogroup. Differences between COI and ITS1-5.8S–ITS2 trees also occur for the NA and CWMA phylogroups. The COI trees suggest NA and CWMA or NA + CWMA, whereas ITS1-5.8S–ITS2 splits NA + CWMA into two or three separate phylogroups (compare Figs 2, 3, 4).

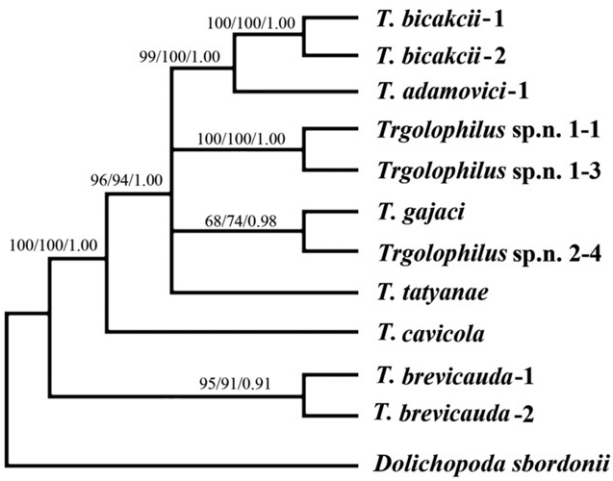

Figure 3. Results of maximum parsinomy (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses combined for strict consensus MP tree based on 12 haplotypes of ITS1-5.8S–ITS2 sequences. Posterior probabilities (BI) and bootstrap supports (MP and ML) to branches are shown (MP, ML, and BI, respectively).

Estimation of divergence times

The null hypothesis of an equal evolutionary rate throughout the tree calculated from COI-1 was not rejected at a 5% significance level (–lnL = 3782.816, $P > 0.05$) in ML analysis; therefore, the BEAST analysis for COI-1 dataset was carried out under a strict molecular clock rate variation model. Analysis was constrained with the tree topology given in Figure 2B, only for the monophyletic groups supported by COI-2. BEAST analysis under the GTR + I + Γ model (Fig. 5) estimated the time to most recent common ancestor (TMRCA) for *Dolichopoda* and *Troglophilus* as 10.860 Mya [95% highest posterior density (HPD) interval = 7.451–14.696] corresponding to opening of Mid-Aegean Trench in the Tortonian. BEAST also

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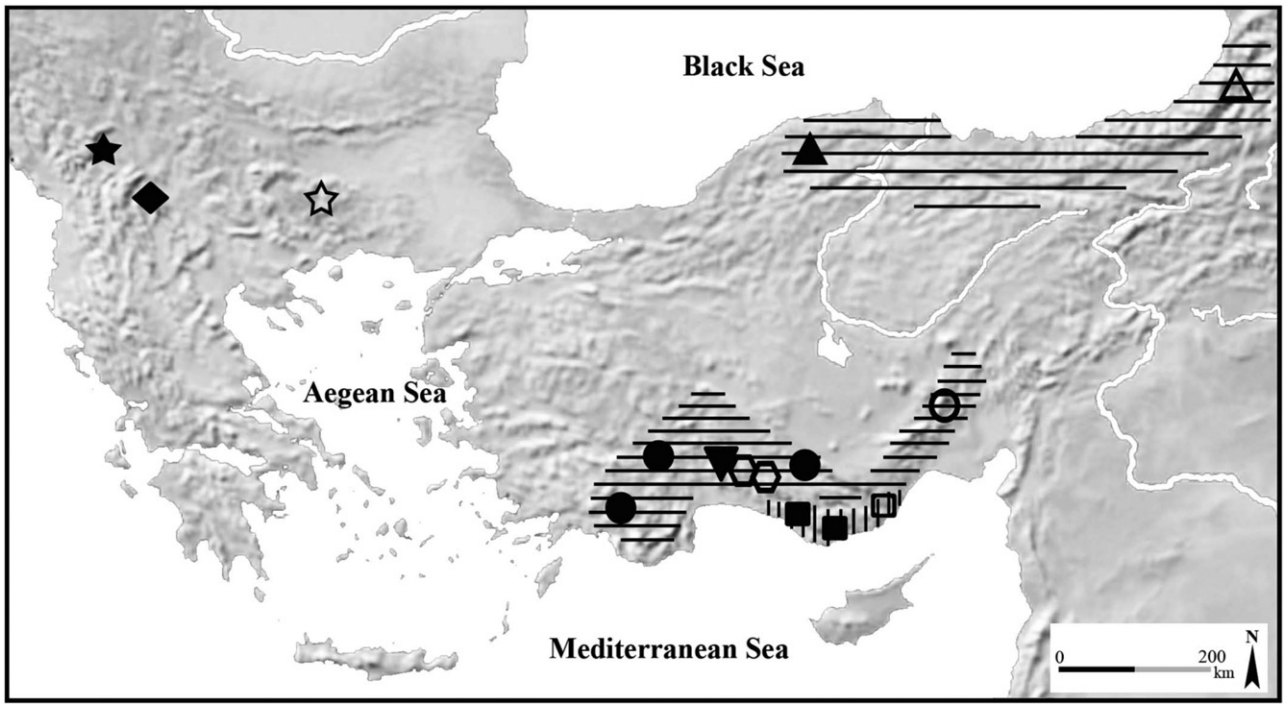

Figure 4. Distribution of main phylogroups of Anatolian *Troglophilus* and sampling sites given in Table 1. Solid and open triangle, *Troglophilus tatyanae* (open triangle represents a nonsampled record by di Russo *et al*., 2007); solid circle, ***Troglophilus* sp. nov. 1**; solid rectangle, ***Troglophilus* sp. nov. 2**; open rectangle, *Troglophilus gajaci*; solid reverse triangle, *Troglophilus bicakcii*; open hexagon, *Troglophilus adamovici*; open circle, *Troglophilus escalerai* (not sampled); solid star, Kosovo; solid diamond, Macedonia; open star, Bulgaria.

suggested the TMRCA for the Anatolian + Balkan *Troglophilus* as 7.223 Mya (95% HPD interval = 5.305–9.283) corresponding to Messinian Drought. The TMRCA for haplotypes of Anatolian *Troglophilus* is 6.357 Mya (95% HPD interval = 4.764–8.168). The TMRCA of haplotypes in ECMA phylogroup is 4.111 Mya (95% HPD interval = 2.804–5.501). Haplotypes in the CWMA + NA phylogroup shared a common ancestor 5.817 Mya (95% HPD interval = 4.381–7.650) and to that of CWMA alone 4.942 Mya (95% HPD interval = 3.611–6.417). The TMRCA for three haplotypes of *Troglophilus* sp. nov. 1 is 1.235 Mya (95% HPD interval = 0.832–1.668). The diversification within the Anatolian *Troglophilus* corresponds to the beginning of the Pliocene (Fig. 5).

The null hypothesis of an equal evolutionary rate throughout the tree calculated from ITS1–ITS2 was rejected at a 5% significance level (–lnL = –1612.770, $P < 0.05$) in ML analysis, indicating that this region has not evolved clock-like (ucld.stdev = 0.9995, 95% HPD interval = 0.6120–1.4558, mean rate = 0.0048, 95% HPD interval = 0.0024–0.0072, coefficient of variation = 0.932, 95% HPD interval = 0.5438–1.358). Therefore, the BEAST analysis was carried out under an uncorrelated lognormal relaxed clock rate variation model. BEAST analysis was constrained with the tree topology (Fig. 3). Of the two different analyses, the ITS1–ITS2 chronogram calibrated by the 0.007 substitution rate is largely similar to that obtained from COI-1 (Fig. 5) for the main nodes. BEAST analysis of ITS1–ITS2 under the TVM + Γ model estimated the TMRCA for *Dolichopoda* and *Troglophilus* as 10.337 Mya (95% HPD interval = 4.683–18.104), to TMRCA for the Anatolian + Balkan *Troglophilus* as 6.019 Mya (95% HPD interval = 2.786–10.248), and to TMRCA for haplotypes of Anatolian *Troglophilus* as 4.279 Mya (95% HPD interval = 2.013–7.391). Interestingly, the ITS1–ITS2 chronogram suggested TMRCA for the Balkan haplotypes as 8.600 (95 % HPD interval = 4.165–14.568), representing an estimation not present in the COI chronogram (Fig. 5) because we were unsuccessful in obtaining COI from these specimens.

DISCUSSION

Different phylogenetic analyses applied to two different markers suggested similar relationships for the Anatolian *Troglophilus* plus representative haplotypes from the Balkan species. An important result is the occurrence of all haplotypes from the Anatolian representatives as a single phylogroup supported by

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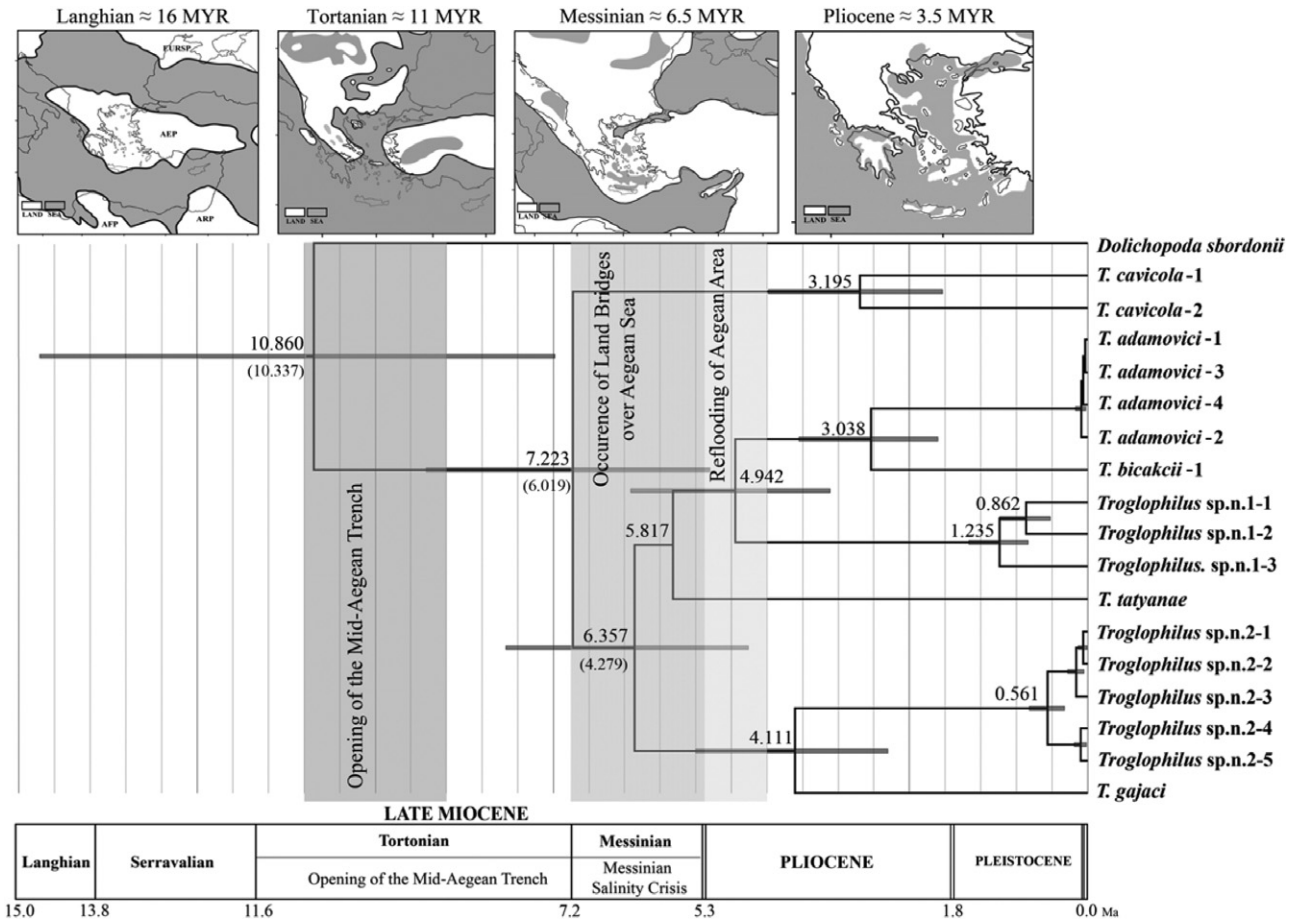

Figure 5. BEAST chronogram based on cytochrome oxidase I (COI) for *Troglophilus*. Divergence times inferred according to Bayesian inference with a strict molecular clock in BEAST. Bars at the nodes represent the 95% highest posterior density (HPD) credibility interval. The times to most recent common ancestor (TMRCAs) suggested by a BEAST chronogram for COI are shown on the upper part of the nodes. The values at the lower part of the nodes show TMRCAs of the ITS1–TS2 region (their HPDs are provided in the text). The main paleogeographical events in the Aegean area are shown on the upper portion of chronogram (*sensu* Steininger & Rögl, 1984; Dermitzakis, 1990a; Meulenkamp & Sissingh, 2003).

high bootstrap numbers and posterior probability values (Figs 2, 3, 4). Instability in different trees occurs for some basal branches of the Anatolian lineages. The trees calculated from ITS1-5.8S–ITS2 suggested three or four phylogroups within the Anatolian lineage. The trees obtained using the data matrix of COI-1 sequences suggested three intra-phylogroups. Two of these include the ECMA and CWMA, both having a range restricted to the Mediterranean Anatolia. The third group includes NA, represented by a single species, *T. tatyanae*, from the North Anatolia or the Black Sea Anatolia (Fig. 2). By contrast, trees produced from the matrix of COI-2 containing longer sequences suggested CWMA + NA as a single phylogroup. Incongruencies also occur for CWMA or CMWA + NA. Because COI-2 sequences are much longer, they possibly provide further resolution for the phylogenetic relationships.

The ECMA phylogroup includes *T. gajaci* and a new species, both restricted to the coastal line of the Mediterranean Anatolia. The new species was found in two different localities and, as suggested by BEAST analysis, these two populations shared the most common ancestor 561 kya. The second Mediterranean phylogroup is CWMA, which consist of two subgroups. One of these includes two known species, *T. adamovici* and *T. bicakcii*, which occur in the central part of Mediterranean Anatolia, namely the south end of Konya Province in the Mediterranean Taurus Mountains. The other includes three haplotypes, each from a different cave population, which are located far from each other: the haplotype *Troglophylus* sp. nov. 1-1 from Denizli Province, the *Troglophylus* sp. nov. 1-2 from Mugla Province, and the *Troglophylus* sp. nov. 1-3 from the south-eastern end of Konya Province. They constitute a phylogroup that sharing a common

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ancestor approximately 1.235 Mya. Each of these populations may represent distinct species. Karaman *et al*. (2011) stated that morphology is not productive in delimiting species, and our data are in agreement with this statement. However, further consideration is required before delimiting these taxa.

Unfortunately, we did not find any specimens of *T. escalerai* I. Bolivar, although the type locality was visited. Currently, this species is only known from type material. There are also some unconfirmed records of this species from other localities (Kunt, Yağmur & Elverici, 2008; Kunt *et al*., 2010) *sensu* Bolivar (1899). Additionally, Cobolli *et al*. (1999) reported two genetically different lineages from Ferzene Cave (Konya), both of which identified as *T. adamovici*; one of these may be *T. escalerai*. However, these records need confirmation because they are from or around the range of *T. adamovici*. On the other hand, *T. escalerai* was suggested to be similar to *T. adamovici* (Cobolli *et al*., 1999; Rampini & di Russo, 2003b) and, thus, it possibly belongs to the *T. adamovici* + *T. bicakcii* phylogroup. Considering the distribution of all members in the phylogroup and that of *Troglophylus* sp. nov. 1 in particular, the range of CWMA correlates well with the Taurus Mountains of the Mediterranean Anatolia. Most of the present records, except those from Mugla, are from a high altitude, indicating that it is a lineage that has dispersed and radiated along this mountain range (Table 1).

Karaman *et al*. (2011; 1036) suggested that the Balkans and Anatolia were two diversity centres for *Troglophylus*, and Northern Anatolia was possible a third one. The monophily of the Anatolian members is in agreement with the first suggestion by Karaman *et al*. (2011). Yet, the Northern Anatolian phylogroup belongs to the Anatolian lineage phylogenetically, and thus does not form a separate diversity centre. Moreover, trees produced from COI-2 suggested NA + CWMA as a single phylogroup. All these facts indicate that the northern Anatolian *Troglophilus* species results from a dispersal of the ancestral stock from southern Anatolia, and the period for this dispersal could be early Pliocene, as suggested by the BEAST results. Faunal exchange between north-east and south Anatolia, and even between Caucasus and the Balkans through the Anatolian Diagonal plus Southern Taurus (the geographical corridor called 'Taurus Way'; Çıplak, 2008), was observed in several other lineages of Orthoptera (Çıplak, 2000, 2008; Çıplak *et al*., 2005), as well as other animals such as frogs (Veith *et al*., 2003), ground squirrels (Gündüz *et al*., 2007), and gall wasps (Mutun, 2010).

Although the present data are in agreement with a hypothesis of two diversity or radiation centres, the published phylogenetic analyses do not include any representative from three species endemic to the Aegean islands: *Troglophilus spinulosus* from Crete, *Troglophilus marinae* from Santorini (the southernmost island of the Cyclades), and *Troglophilus lagoi* from Rhodes. The first two species were suggested to be closely related (Rampini & di Russo, 2003a) and considerably differentiated from the third one (Ketmaier *et al*., 2000). The suggested relationships appear plausible because Crete (and possibly Cyclades) was isolated geographically after the reflooding of the Aegean area by the start of the Pliocene, whereas Rhodes had a terrestrial connection with Anatolia until the Pleistocene (Dermitzakis, 1990a, b; Duermeijer *et al*., 1998; Gantenbein & Keightley, 2004; Kasapidis *et al*., 2005). From this historical geographical pattern, the *T. lagoi* may be considered to belong to the Anatolian lineage. Similar phylogeographical patterns between Anatolia and Rhodes were also observed in some other lineages (Gantenbein & Keightley, 2004; Kasapidis *et al*., 2005; Parmakelis *et al*., 2006; Çıplak *et al*., 2010). Thus, *T. spinulosus* and *T. marinae* may represent an independent lineage, and therefore Crete could be the third radiation centre in addition to the Balkans and Anatolia. Placement of the Cretan species in a different subgenus by Werner (1927) and similar phylogenetic patterns between Crete and Anatolia observed in some other animal lineages provide some support for this assumption. However, its confirmation requires further phylogenetic studies, including all members of the genus. Another point related to the biogeography of Cretan *Troglophylus* that requires careful consideration is the range of the genus in southern and central mainland Greece. There is a single record of this genus from central Greece concerning *T. cavicola* (Willemse, 1984; Willemse & Willemse, 2008; Karaman *et al*., 2011), whereas the main range of this species is the western part of the Balkan Peninsula. Molecular clock estimations using two different markers are largely congruent for the main branching events. However, a significant difference appears for the early radiation of *Troglophilus*. The COI-1 chronogram suggested the TMRCA as approximately 7 Mya for the genus, whereas the ITS1–ITS2 chronogram estimates 9 Mya. Because mitochondrial markers are generally considered to be more reliable (Avise, 2000, 2009) and the mitochondrial sequences used in BEAST analysis are longer than that of the nuclear marker, the time estimated by COI sequences appears to be more plausible. However, estimates of such deep divergence in *Troglophilus* require more taxa/samples. The molecular clock estimation by BEAST suggested strong correlations between speciation events in the genus and the main paleogeographical events in the Eastern Mediterranean. According to both BEAST chronograms, *Troglophilus* and *Dolichopoda* share a last common ancestor approximately

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11 Mya (Fig. 5). The estimation for TMRCA by Allegrucci *et al*. (2009) was 12 Mya using data mainly from *Dolichopoda*. Although there is a small difference between time estimates, both still would correspond to the major event: the opening of Mid-Aegean Trench in the Tortonian, approximately 9–11 Mya. From these data: (1) the old Aegean tectonic plate is the possible place of origin of these two lineages and (2) the divergence of these genera, and thus the divergence of the subfamilies Troglophilinae and Dolichopodinae, was triggered by the occurrence of the Aegean Sea in Tortonian. The main species diversity of *Dolichopoda* is at the western shore of the Aegean Sea, although a radiation from a Caucasian + Anatolian ancestral stock was suggested (Allegrucci *et al*., 2011). *Troglophilus* does not exhibit special dominancy in respect to species richness. However, a considerable number of species occur in Anatolia and the present data indicate the presence of undescribed species. Additionally, the Cretan + Cycladian species appear to represent a different lineage. Thus, the assumption that *Troglophilus* radiated from an ancestral stock present in the old Aegean Plate is plausible. There are two further possibilities. First, radiation started from the western part of the old Aegean Plate, when it split during the Tortonian, or from the south of the Aegean Plate, including Crete and south Anatolia (Fig. 5). The second fits with the pattern observed in some other lineages (Kasapidis *et al*., 2005; Çıplak *et al*., 2010; Velonà *et al*., 2010; Simaiakis *et al*., 2012). Yet, the present data provide some support for the first hypothesis because haplotypes from Balkan species constitute the most basal branches and there is a deep genetic divergence between them, especially as suggested by the BEAST chronogram of ITS1–ITS2.

The subsequent divergence event also appears to be related by geographical events in and around the Aegean Sea (Fig. 5). The TMRCA for the Anatolian and Balkan lineage was suggested as 7.22 and 6.37 Mya by BEAST chronograms of COI-1 and ITS, respectively (Fig. 5). This time estimate correlates well with the occurrence of land bridges between the two sister areas derived from the old Aegean Plate, namely Anatolia and Aegean Islands + the Balkans, during the Messinian Drought (Steininger & Rögl, 1984; Dermitzakis, 1990a, b; Roveri & Manzi, 2006). This is not only suggested by the BEAST chronogram, but also is consistent with the present phylogenetic results and the paleogeographical events that occurred in this area (Gantenbein & Keightley, 2004; Parmakelis *et al*., 2006; Douris *et al*., 2007; Akın *et al*., 2010; Çıplak *et al*., 2010). It is plausible, therefore, to assume the following scenario. The Balkan and Anatolian lineages, possibly plus the Cretan one, represented a single ancestral stock prior to the Messinian on one side of the Aegean Sea, on its western part. Later, when land bridges were re-established over the Aegean area during the Messinian, this ancestor dispersed to the south and east. When the Aegean area refilled with water by the beginning of Pliocene, the populations on both sides separated vicariantly (Fig. 5). However, currently, the main European diversity of the genus is in the Balkans, not in the central or southern mainland Greece. This indicates two possibilities; first, a dispersal only to the Anatolian side of the Aegean Sea, and then from there to Crete, and, second, a reduction of diversity in the central or southern mainland Greece. Data distinguishing one of these hypotheses requires the phylogenetic and molecular clock analysis of the Cretan + Cycladic lineage. The available data support the assumption that the post-Messinian western ancestral stock radiated to give birth of the Balkan lineage, and the eastern stock to the present Anatolian lineage. Divergence of three main lineages triggered by the post-Messinian reflooding of the Aegean area well fits with the above assumption. Dermitzakis (1990a, b) and Anastasakis *et al*. (2006) suggested that the Aegean Sea formed by progressive submergence of the Aegean area from the west to east and from the south to north after the end of the Messinian episode, a time consistent with the divergence period of our Anatolian and Balkan lineages. In such a reflooding pattern, Crete will be the first island to be isolated; thus, it is possible that the stock that remained there, isolated from other populations, evolved to the present Aegean or Cretan + Cycladic lineage (Fig. 5).

There are differences between chronograms from COI-1 and ITS for the TMRCA of the Anatolian lineage; the first estimated 6.357 Mya, whereas the second suggested 4.513 Mya, although their time ranges are overlapping (Fig. 5). In both cases, these data indicate that this lineage has diverged after the Late Messinian and Pliocene. Interesting in this respect is the presence of only two divergence events during the late Pleistocene or the last four glacial periods within the Anatolian lineage. One is the divergence of two haplotypes of *Troglophilus* sp. nov. 2 and the other is that of two haplotypes of *T. adamovici*. The *Troglophilus* sp. nov. 2 haplotypes are from the same locality, whereas those of *T. adamovici* are from two caves approximately 20 km apart. Divergence events other than these two are older than 800 kya (Fig. 5). These results indicate no effects of climatic shifts of the last four glacial periods on the speciation of *Troglophilus*, which is in agreement with the suggestions of Allegrucci *et al*. (2011) that climatic shifts of Pleistocene had no effect on the speciation of Rhaphidophoridae. Although this may be the case, there may be a correlation between the radiation of

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the genus and the humid climate of the Pliocene following the Messinian Drought. Thus, the possible scenario for this episode of speciation could be a reduction of *Troglophilus* diversity in the Messinian and its re-expansion under a more humid climate during the Pliocene. The inclusion of a Cretan and Cycladic specimens in phylogenetic and molecular clock analyses will allow further testing of these hypotheses.

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