

Phylogeography of Pholidopterini: Revising molecular clock calibration by Mid-Aegean Trench

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Abstract

The present study examines the phylogeography of Pholidopterini (Orthoptera, Tettigoniidae), a lineage distributed in the East Mediterranean and estimated substitution rates for the three mitochondrial and two nuclear gene segments. The last common ancestor of Pholidopterini was dated to 18 myr ago, in Early Miocene. Phylogeography of the lineage was marked with three waves of radiations, first during the Middle Miocene Climatic Optimum, the second during the Serravallian, and third during the ending of Messinian. The substitution rate estimations were 0.0187/0.018/0.0141/0.0010207 s/s/myr for COI/ND2/12S-tRNA^{Val}-6S/ITS1-5.8S rRNA-ITS2. The following main conclusions were drawn; (i) Radiation of Pholidopterini directed by the climatic shifts, (ii) signs of vicariant speciation were poor, contrary to the active tectonic history, (iii) the ultimate generic ancestors were dated to the Langhian and Serravallian, and (vi) the Tortonian transgression of Mid-Aegean Trench appears to be a reliable geographic calibration point for lineage splitting between Crete and Anatolia.

Keywords

Aegean phylogeography; Orthoptera; Pholidopterini; molecular clock; substitution rate estimation

Introduction

The tempestuous geological history of the East Mediterranean makes it a model area for biogeographic studies. The site evolved from a group of archipelagos that existed in the Late Mesozoic, between Laurasia and Gondwana, within the Tethys

Sea (Meulenkamp & Sissingh 2003; Popov et al. 2004). These archipelagos merged through the Eocene and the Oligocene and formed the lands starting from Zagros/Caucasus and extending to the Alps through Anatolia and Balkans by the beginning of the Miocene. However, tectonic instability of the region has continued throughout the Miocene up to the late Quaternary until becoming to the present form (Dermitzakis 1990; Rögl 1999; Meulenkamp & Sissingh 2003; Popov et al. 2004; Harzhauser & Piller 2007). Along with the turbulent tectonic dynamic process, the climate of the area has fluctuated, especially during the Miocene (Fauquetta et al. 2007; Ivanov et al. 2010; Suc et al. 2018), as an important evolutionary driver in the establishment of regional biodiversity. These large/small-scale tectonic/climatic events have resulted in a reticulate biogeography in the East Mediterranean.

By the 2000s, several studies on the phylogeography of the lineages distributed in the East Mediterranean, particularly in the Aegean Basin, were published (see the review by Poulakakis et al. 2015 for those before 2015; further news are Chobanov et al. 2017; Dufresnes et al. 2018; Jaros 2018; Kaya et al. 2013a; Kaya & Çıplak 2016, 2017; Kornilios et al. 2016, 2019; Papadopoulou & Knowles 2015). Among the majority of these publications, the species level lineages that radiated in the post-Tortonian period were considered, and vicariance was reported as the dominant pattern for radiation since the Tortonian transgression created several islands within the Aegean Sea (Poulakakis et al. 2015). However, again, these studies rarely attributed the divergence events to ecological changes, as the significant climatic fluctuations predated the Tortonian (Suc et al. 2018). Compared to the Aegean area, publications on the lineages distributed in other parts of the East Mediterranean, such as the Black Sea Basin, Caucasus, Zagros, and the Arabian Peninsula, are relatively rare (Wielstra et al. 2010; Ahmadzadeh et al. 2013; Guy-Haim 2018; Stahls et al. 2016; Kaya & Çıplak 2017; Salvi 2018; Solovyeva et al. 2018). Therefore, studying the phylogeography of the supra-species or supra-generic lineages is necessary to understand the radiation events derived by the far past, especially predating the Tortonian paleogeographic events in the Tethys Belt.

Although there are many publications on the biogeography of the East Mediterranean, there is still much to be done as the historical characteristic of the area may mislead biogeographers. For example, there is always a potential for mis-correlating nodes of a chronogram and tectonic events as there were several plates that frequently connected/disconnected through the Neogene (Meulenkamp & Sissingh 2003; Popov et al. 2004; Krijgsman et al. 2019). Second, ecological speciation in a continuous land could not be distinguished properly from vicariance/dispersal speciation. Third, poorly known geological fragments (e.g., marshes and lowlands can be confused; see Meulenkamp & Sissingh 2003; Popov et al. 2004). These handicaps are more probable when the far past is concerned. One such example comes from the usage of the Aegean Trench regression to calibrate molecular clock analyses. The Aegean Trench had regressed twice, first in the Tortonian (11–9 myr) and second in the Messinian-Pliocene transition (app. 5.5 myr) (Dermitzakis 1990; Anastasakis et al. 2006; Loget et al. 2006; Poulakakis et al. 2005). Some studies applied the first (Parmakelis et al. 2006a; Parmakelis et al. 2006b; Papadopoulou et al. 2010; Kornilios et al. 2016), some the second (Bitkau & Comes

2005; Allegrucci et al. 2011; Kyriazi et al. 2013) while some others both (Bittkau & Comes 2005; Kasapidis et al. 2005; Cellinese et al. 2009; Simaiakis et al. 2012) in calibration of their analyses. When it comes to insects, Papadopoulou et al. (2010) calibrated their rate estimation analyses by 10.5 ± 1.5 myr for a coleopteran lineage while Allegrucci et al. (2011) by 5–5.3 myr for an orthopteran lineage. However, their rates for cytochrome *c* oxidase subunit I (COI) were roughly similar to 0.0177 and 0.0168 substitution/site/million years (s/s/myr), respectively. The case was similar for 16S rDNA by 0.0054 and 0.0066 s/s/myr in respective studies. The rates reported by Papadopoulou et al. (2010) were considered almost a norm and have been widely used in dating phylogenies by entomologists when fossil or geographic calibration tools were unavailable. However, the obvious conflict between both studies challenges rate estimations or the phylogeographic hypotheses proposed in these studies.

The present study aims to address two complementary objectives. The first objective is the phylogeography of Pholidopterini. The tribe Pholidopterini (Orthoptera, Tettigoniinae), revised recently, is a lineage including approximately 130 species classified under nine genera distributed in the Western Palaearctic (Çıplak et al. 2021). Of these, *Exopholidoptera* Ünal and *Spinopholidoptera* Çıplak are monotypic, and their species are endemic to restricted ranges. The only species of *Exopholidoptera* is known from a single locality in central Anatolia and that of *Spinopholidoptera* in Eastern Pontides. The remaining seven genera are polytypic. The range of *Apholidoptera* Mařan and *Uvarovistia* Mařan, the first with two (Yahyaoglu et al. 2022) and the second with five species (Uluar et al. 2021), covers large parts of Zagros Mts. or associated highlands. *Aparapholidoptera* Çıplak includes four species ranging in Pontides and associated highlands with non-euxinic vegetation. Remaining four genera have higher species numbers and larger ranges. *Psorodonotus* Brunner von Wattenwyl includes 17 species, and most are endemic to the Lesser Caucasus and a few scattered to west half of Anatolia and Balkans. *Pholidoptera* Wesmaël includes 18 species, and its members are mainly confined to the Balkans, though two species also are distributed to the whole Black Sea Basin and large parts of Europe up to Scandinavia and Pyrenees. *Parapholidoptera* Mařan is mainly Anatolian, but three species are distributed in the Caucasus, two in the Levant, and Anatolian species also extend to the Balkans (Çıplak 2000). *Eupholidoptera* Mařan is the largest genus with 57 species. Its entire range starts from France in the west and reaches Sinai in the east/south along the Northern Mediterranean Basin (Çıplak et al. 2009, 2010). Regarding the phylogeography of Pholidopterini (see Fig. 1 for distribution and species richness of each genus), we aim to address the following: (i) to construct a chronogram of the tribe and to use it as a template for biogeographic assessments, (ii) to determine the ancestral places of the tribe and the general, and to estimate the subsequent establishment of peripheral ranges by considering the past tectonic events in the range area, and (iii) to accomplish the dominant pattern of speciation for the tribe and the genera, and the reasons underpinning by correlating the nodal ages with historical geological/climatological events.

Using the tribe as a model group, the study's second objective is to estimate the substitution rate for the genes studied. The calibration method is essential in dating phylogenies, and the biogeographic calibration was suggested to be one of the effective

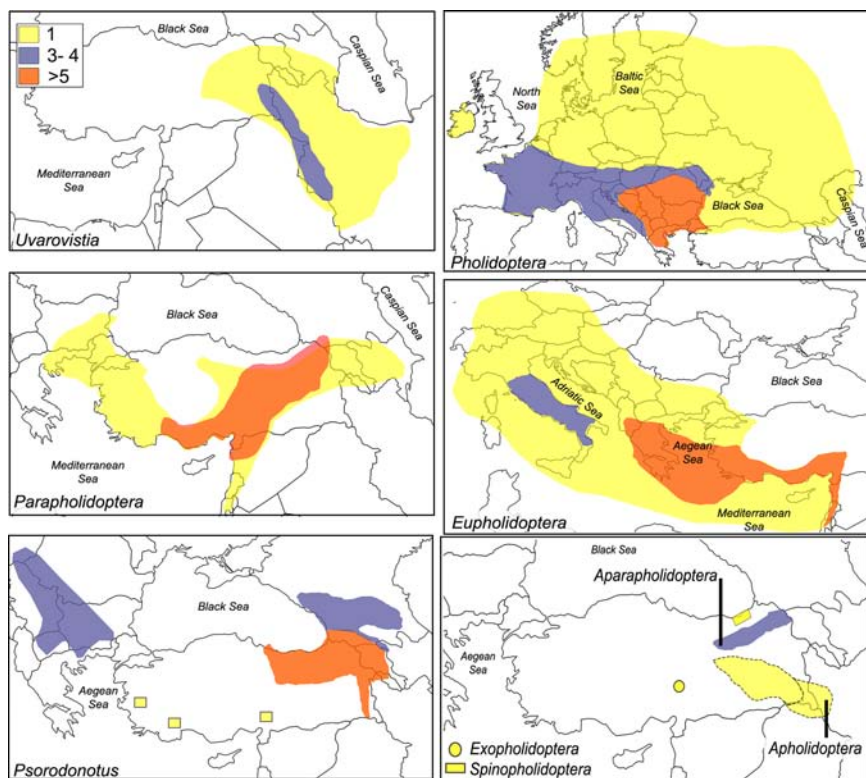


Fig. 1. Distribution of the genera in Pholidopterini according to their species richness (legend indicates to species number).

methods (Hipsley & Müller 2014; Ho et al. 2015). Calibrations by precise vicariant events allow a reliable estimation of substitution rates employed in other studies when geographic and/or fossil calibrations are unavailable (Kodandaramaiah 2011). The regression of Mid-Aegean Trench was suggested as a reliable geographic event to calibrate molecular clock analyses (Papadopoulou et al. 2010) as the paleogeography of the area is well known (Dermitzakis 1990; Anastasakis et al. 2006; Loget et al. 2006; Poulakakis et al. 2015). Distribution of the genus *Eupholidoptera* from Pholidopterini provides the opportunity to use this geographic event to estimate substitution rates for the genes studied. The genus *Eupholidoptera* occurs along the Northern Mediterranean Basin, and the primitive representatives of the genus are restricted to Crete with sister lineages in Anatolia, representing a vicariant division (Çıplak et al. 2009, 2010; Çıplak et al. 2021).

On the other hand, substitution rate estimations for the genes we studied are unknown or require re-examination. Several studies have reported the substitution rate of cytochrome *c* oxidase subunit I (COI) in insects (Brower 1994; Shapiro et al. 2006; Papadopoulou et al. 2010; Allegrucci et al. 2011; Kaya & Çıplak 2016), but these rates are highly variable and require verification. There has been no rate reported for the 12S rDNA-tDNAval-16S rDNA gene segments, though there were estimations for 12S and 16S parts separately (Papadopoulou et al. 2010; Allegrucci et al. 2011).

The substitution rate is unknown for the nicotinamide adenine dinucleotide (ND2) neither for Orthoptera nor other insects. The substitution rate of internal transcribed spacers 1 and 2 of ribosomal cistron (ITS1 - 5.8S rDNA - ITS2) has been reported for Diptera (Percy et al. 2004; Bargues et al. 2006), but it is not known for other insects. The present study aims to verify/estimate the substitution rates for these genes. As Crete disconnected twice from the mainland (in Tortonian and in Pliocene), we intend to determine the suitability of each as a calibration event.

Materials and methods

Taxa, outgroup, and data content

Previously published sequences (Çıplak et al. 2021; Kaya et al. 2015; Kaya & Çıplak 2016, 2017) were used to establish data sets for time and rate estimation analyses (Supplementary Table S1). These DNA sequences were generated from 47 (+2 new) species representing eight genera classified under Pholidopterini: *Apholidoptera*, *Aparapholidoptera*, *Pholidoptera*, *Psorodonotus*, *Spinopholidoptera*, *Eupholidoptera*, *Parapholidoptera* and *Uvarovistia* (Çıplak et al. 2021; Uluar et al. 2021; Yahyaoglu et al. 2022). Four outgroups were used for the analyses of phylogenetic dating and estimation of the substitution rates (Table 1): *Bolua turkiyae* Ünal as sister group to the Pholidopterini plus *Pachytrachis gracilis* Brunner von Wattenwyl, *Anabrus simplex* Haldeman and *Tettigonia chinensis* Willemse (Supplementary Table S1 for the GenBank accession numbers and taxa studied). Sequences of the three mitochondrial gene fragments (COI, ND2 and VAL consisting of a segment of 12S rDNA-rDNAval-16S rDNA) and a nuclear ribosomal cistron comprising ITS1-5.8S rDNA-ITS2 (ITS hereafter) were used in dating the phylogeny of Pholidopterini and estimating the substitution rates per genes. Multiple sequence alignment of each marker was done by

Table 1. Description of the sequences used in establishment of the concatenated matrix (+i, with indels; NS, number of sequences; NH, number of unique haplotypes; NBp, number of base pairs; NCS, number of conserved sites; NVS, number of variable sites; NI, number of indel sites; NCH, number of haplotypes established by concatenation; NN, number of gene sequence filled with 'N').

	Single gene matrices						
	NS	NH	NBp	NCS	NVS	NI	NN
COI	223	150	985	552	433	-	3
ND2	150	103	956	293	663	-	27
VAL+i	153	126	826	327	476	123	7
ITS+i	329	103	889	435	436	192	15
COI+ND2+VAL+ITS	NCH / NBp: 144 / 3656						
Data blocks and model parameters	GTR+I+G for eight data blocks: 1st, 2nd, 3rd codon positions for each of COI and ND2 plus VAL and ITS						
Outgroups	<i>Bolua turkiyae</i> , <i>Pachytrachis gracilis</i> , <i>Anabrus simplex</i> , <i>Tettigonia chinensis</i>						

MAFFT v.7.245 (Katoh & Standley 2013) online version (<http://align.bmr.kyushuu.ac.jp/mafft/online/server/>) applying the setups FFT-NS-i strategy (slow, iterative refinement method) and the rest of the settings left as default [scoring matrix 200PAM (k = 2), gap opening penalty = 1.53]. The unique haplotypes were detected by DNASP v.5 (Librado & Rozas 2009). The nucleotide composition, the number of variable/invariable, indels and parsimony-informative sites for each matrix were calculated by MEGA v.7.0 (Kumar et al. 2016). As trees produced from single gene matrices were inconsistent in topology (not presented here) we established a single matrix by concatenation and analysed under the coalescent approach. The concatenated matrix including all four gene fragments (see Supplementary Table S2 for sequence concatenation chart) was created using SEQUENCEMATRIX v.1.8 (Katoh et al. 2011). Missing data was allowed (filled with “N”s) as the percentage of the absent gene was expected to have a little influence (Zheng & Wiens 2015). The best-fit scheme and the evolutionary model for eight data blocks (1st, 2nd and 3rd codon position of COI and ND2; VAL and ITS) were estimated using PARTITIONFINDER v.1.1.1 (Lanfear et al. 2012).

Analyses for time and substitution rate estimations

Although mitochondrial genes inherit in connection, substitution rate estimation per gene is important for the following two reasons. First, COI has been a hallmark marker in molecular clock estimations and testing previously reported rates for insects, and particularly for Orthoptera (Brower 1994; Shapiro et al. 2006; Alleggrucci et al. 2011; Papadopoulou et al. 2010; Kaya & Çıplak 2016) has special importance. Second, these matrices produced some conflicting node topologies in the phylogenetic tree of Pholidopterini (Çıplak et al. 2021), indicating that each gene has experienced a different evolutionary past (Ho et al. 2015; Bromham et al. 2018). For example, the VAL matrices include indels while ND2 and COI do not and the amount of variation in VAL matrices constituted nearly half of the variation of ND2 matrix, and this is likely to cause a significant rate of variation across branches, leading to complications in analyses (Soares & Schrago 2015; Mendes & Hahn 2016). Two calibration points, fossils and the terrestrial disconnection of the Crete, were used for time calibration analyses. For the fossil-based calibration, the fossil record of *Decticus* sp. was used, dated to 23.1–33.9 million years (Mugleston et al. 2018). Thus, a basal internal node was constrained by selecting *Tettigonia chinensis* and *Anabrus simplex* as outgroups and a lognormal distribution (Ho 2007) was applied to this split with an offset of 23.1 and μ : 1.204 and σ : 0.6. Transgression of the Aegean Trench resulted in the disconnection of Crete from Anatolia, and this geological event proposed for the splitting of Anatolian–Cretan lineages of *Eupholidoptera* (Çıplak et al. 2010). However, this disconnection happened at least twice in Tortonian around 12–9 myr ago and second in the Messinian-Pliocene transition around 5.5 myr ago (Dermitzakis 1990; Anastasakis et al. 2006; Poulakakis et al. 2015). The XML file produced from the concatenated matrix was analyzed using BEAST v.2.6.1 (Bouckaert et al. 2019) under two different calibration settings: (i) Fossil + Tortonian, setting the time of the ancestral node of *Eupholidoptera* to 12–9 myr by applying a normal distribution with a mean of 10.5 and stdev: standard deviation of 0.7655 (Ho 2007) and (ii) Fossil +

Pliocene, setting the time of the ancestral node of *Eupholidoptera* to 5.5 ± 0.5 myr with a normal distribution. Prior to running the BEAST, the null hypothesis of an equal evolutionary rate throughout the tree was assessed by applying a maximum likelihood clock test (MLC) (Tamura & Nei 1993) using MEGA v.7.0. As the dataset was not clock-like (likelihood ratio test: $-\ln+c$ 265036.271, $-\ln-c$ 48724.228, d.f.= 138, $P = 0.0$), divergence time was estimated using the uncorrelated relaxed lognormal clock. BEAST analyses were performed using the partitioned dataset by genes and unlinking nucleotide substitution models and clock models for each gene, but linking the trees. Both BEAST analyses were run with the birth-death speciation model as all extant species were not represented in the matrices. Each of the BEAST analyses was carried out with two independent MCMC chains for 400 000 000 sampling every 40000th generation. BEAST analyses were monitored using TRACER v.1.7 (Rambaut et al. 2018) for the effective sample size of model parameters. The results obtained from both calibration setting analyses were compared by estimating marginal likelihoods via harmonic mean estimator (Kass & Raftery 1993) using TRACER v.1.7 with 1000 bootstraps. The maximum clade credibility trees were built by discarding the initial 25% of the samples as burn-in using TREEANNOTATOR implemented in BEAST. Trees were visualized by FIGTREE v.1.4.3 (Rambaut 2014).

Results

Radiation history of Pholidopterini

The marginal likelihoods estimation by the harmonic mean provided strong support for the calibration by the fossil + Tortonian over the fossil + Pliocene with (BF = 55.557; for the interpretation of Bayes factor comparisons, see Kass & Raftery 1993). Thus, the results obtained by the fossil+Tortonian calibration setting were presented and considered in further accounts (Fig. 2, Table 2).

The BEAST chronogram produced from concatenated COI+ND2+VAL+ITS matrix largely recovered the topology given in Çıplak et al. (2021). The clade (C1) consisting of Pholidopterini and its sister group *Bolua* shared the last common ancestor 21.97 (95% HPD: 17.52–26.25) myr ago in Aquitanian in the beginning of Miocene. The ancestral node of Pholidopterini (clade C2) was dated to 17.91 (95% HPD 14.19–22.21) myr ago, falling in Burdigalian, in the Early Miocene. Pholidopterini consists of two sister clades. The first clade (C3) includes *Aparapholidoptera* + *Apholidoptera* and the second (C4) remaining all members of the tribe. The ancestral node of C4 was dated to 16.38 (95% HPD: 13.04–20.11) myr ago and that of C3 to 9.63 (95% HPD: 5.14–14.30) myr ago, and the first corresponds to the Burdigalian-Langhian transition and the second to the Tortonian. The subsequent node in C4 is a dichotomy consisting of the clades C5 and C6. The C5 comprising *Psorodonotus* + *Pholidoptera* and the C6 remaining four genera with the relationship of (*Eupholidoptera* + *Parapholidoptera*) + (*Uvarovistia* + *Spinopholidoptera*) with the ancestral ages of 13.74 (95% HPD: 9.67–17.81) and 14.30 (95% HPD: 11.10–17.70) myr respectively, both falling to the late Serravallian. The *Eupholidoptera* + *Parapholidoptera* clade (C7) share

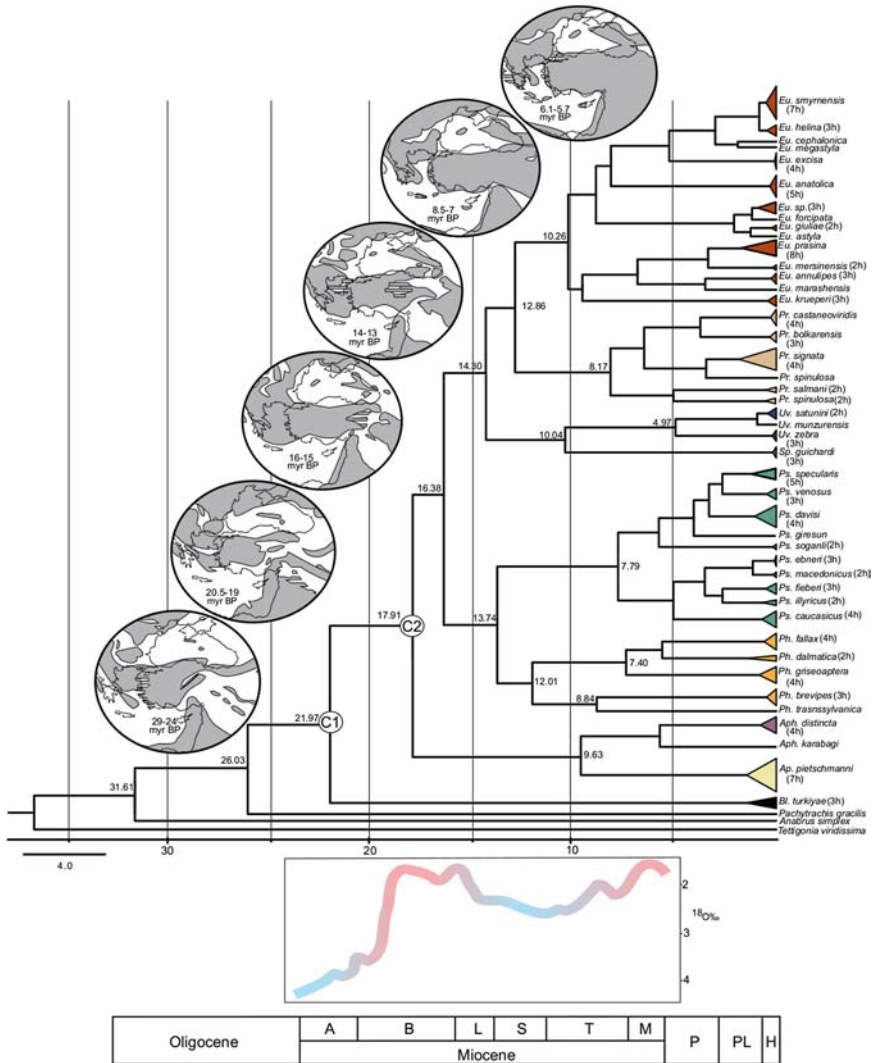


Fig. 2. The chronograms produced from the concatenated COI+ND2+VAL+ITS matrix. Change in temperature through Miocene is expressed by cold and warm colours below the chronogram. The number of unique haplotypes (h) per clade given along the species (generic names abbreviated as following: *Pr*, *Parapholidoptera*; *Uv*, *Uvarovistia*; *Ps*, *Psorodonotus*; *Ph*, *Pholidoptera*; *Eu*, *Eupholioptera*; *Aph*, *Aparapholidoptera*; *Ap*, *Apholidoptera*; *Sp*, *Spinopholidoptera*; *Bl*, *Bolua*). Stages of Miocene are indicated by their initial letters (A, Aquitanian; B, Burdigalian; L, Langhian; S, Serravallian; T, Tortonian; M, Messinian; P, Pliocene; PL, Pleistocene; H, Holocene). Small maps given along the nodes of the tree were modified from Popov et al. (2004).

the last common ancestor 12.86 (95% HPD: 10.48–16.07) myr ago and *Uvarovistia* + *Spinopholidoptera* (clade C8) 10.04 (95% HPD: 4.95–15.29) myr ago, corresponding to the Serravallian and the Tortonian, respectively.

The proximate generic ancestral nodes of *Spinopholidoptera* and *Apholidoptera* are not considered here as the former is monotypic and the latter is represented by

Table 2. Node ages and 95% HPD calculated by the Fossil + Tortonian and Fossil + Pliocene calibration setting (for details of calibration setting see Material and Method section).

Clades (according to Figure 2)	Calibration time			
	Fossil + Tortonian		Fossil + Pliocene	
	Nodes ages (myr)	95% HPD	Nodes ages (myr)	95% HPD
C1	21.97	17.52 – 26.25	18.19	13.04 – 23.48
C2	17.91	14.19 – 22.21	13.13	9.50 – 17.38
C3	9.63	5.14 – 14.30	6.85	3.61 – 10.68
C4	16.38	13.04 – 20.11	11.61	8.62 – 15.54
C5	13.74	9.67 – 17.81	9.70	6.32 – 13.22
C6	14.3	11.10 – 17.70	9.75	7.10 – 13.23
C7	10.04	4.95 – 15.29	NA	NA
C8	12.86	10.48 – 16.07	8.42	6.23 – 11.27
<i>Eupholidoptera</i> (Eu)	10.26	8.84 – 11.64	5.80	4.92 – 6.70
<i>Parapholidoptera</i> (Pr)	8.17	5.39 – 11.62	5.65	3.57 – 7.94
<i>Uvarovistia</i> (Uv)	4.97	1.82 – 9.58	3.49	1.45 – 6.25
<i>Psorodonotus</i> (Ps)	7.79	4.48 – 11.7	4.25	3.58 – 8.60
<i>Pholidoptera</i> (Ph)	12.01	8.14 – 16.35	5.32	2.88 – 8.69
<i>Aparapholidoptera</i> (Aph)	5.76	2.98 – 9.33	4.18	0.57 – 2.36

a single species in the chronogram. The proximate ancestral node of *Eupholidoptera* was used as the calibration point with the Tortonian regression of Aegean Trench as 10.0 ± 1.0 myr, and this node was dated to 10.26 (95% HPD: 11.10–17.70) myr. For the remaining five polytypic genera the chronogram suggested the age of proximate ancestral node as 12.01 (95% HPD: 8.14–16.35) myr for *Pholidoptera*, 8.17 (95% HPD: 5.39–11.62) myr for *Parapholidoptera*, 7.79 (95% HPD: 11.10–17.70) myr for *Psorodonotus*, 5.76 (95% HPD: 4.48–11.7) myr for *Aparapholidoptera* and 4.97 (95% HPD: 1.82–9.58) myr for *Uvarovistia*. The proximate ancestor of *Pholidoptera* is dated to the Serravallian, those of *Parapholidoptera* and *Psorodonotus* to the Late Tortonian, and those of *Aparapholidoptera* and *Uvarovistia* to the end of Messinian and Early Pliocene, respectively. The ultimate ancestors of all genera are older than 10 myr or the Middle Tortonian.

Substitution rate estimations

Results of substitution rate estimation obtained by the BEAST analysis with fossil + Tortonian calibration setting are presented in Table 3. The COI gene showed the highest substitution rate by 0.0187 ± 0.002315 s/s/myr. The second highest rate was observed in ND2 by the substitution rate as 0.018 ± 0.001269 s/s/myr. The substitution rate for the gene segment, including 12S - tRNA^{Val}-16S rRNA (the VAL data set) was 0.0141 ± 0.0016269 s/s/myr, the lowest value amongst the mitochondrial genes studied here. The lowest rate estimation was found in the nuclear gene segment (ITS1-5.8S rRNA-ITS2) with 0.0010207 ± 0.0007481 s/s/myr.

Table 3. Estimated gene-specific divergence rates for each of COI, ND2, VAL and ITS based on fossil + Tortonian calibration

Data matrix	UCLD		
	Mean	HPD 95%	Stdev
COI	0.0187	0.0145 – 0.023	0.002315
ND2	0.018	0.0137 – 0.0229	0.0024993
VAL	0.0141	0.0109 – 0.0171	0.0016269
ITS	0.0010207	0.0005036 – 0.0019091	0.0007481

Discussion

A total of 3656bp sequences provided a considerable amount of information and allowed us to draw conclusion on the phylogeography of the tribe and substitution rate estimations of the genes studied. Regarding the present results, a striking point to be mentioned is discordance/concordance among the topology of the chronograms that confirm Çıplak et al. (2021). The concatenated dataset of COI+ND2+VAL+ITS provided a better resolution for the relationships of the genera (see Çıplak et al. 2021 for the previous trees). The sister group relationships of *Bolua* and Pholidopterini, the monophyly of Pholidopterini, and the monophyly of four genera (*Eupholidoptera*, *Parapholidoptera*, *Uvarovistia* and *Spinopholidoptera*) clade are those to be noted. Additionally, the chronogram suggested the monophyly of each genus in the tribe with the following intergeneric relationships; (*Apholidoptera* + *Aparapholidoptera*) + ((*Psorodonotus* + *Pholidoptera*) + ((*Eupholidoptera*, *Parapholidoptera*) + (*Uvarovistia* and *Spinopholidoptera*))) (Fig. 2). Further, the BEAST analysis calibrated by the fossil + Tortonian received strong support suggesting that the Tortonian calibration is more plausible for the lineage split due to the terrestrial splitting of Anatolia and Crete/southern Aegean Islands.

Substitution rate estimations

The molecular clock approach provided a revolution in the history of evolutionary studies, but its bioinformatics is complicated and largely assumption-dependent (Broham et al. 2018). Calibrations by actual vicariant events allow us to estimate substitution rates to be used in other studies when geographic and/or fossil calibrations are unavailable. We benefitted from the splitting of Crete, thus that of the genus *Eupholidoptera* to estimate or verify the substitution rate for four genes studied here. This geographic calibration point was also supplied by a fossil calibration point to support the robustness of the estimations. The results suggest that the two protein-encoding segments more rapidly diverged than the RNA encoding segments of the mitochondrial genome (Table 3). We attribute a priority to COI as it has been the most frequently preferred marker. Our estimation for COI as 0.0187 s/s/myr was slightly higher than most of the earlier reported rates (Brower 1994; Papadopoulou et al. 2010; Allegrucci et al. 2011; Kaya & Çıplak 2016), but less than the uncorrected estimations by Shapiro et al. (2006). The first three publications based their estimation on mainland taxa while the last only on the island species. The disparity may arise from historical population

size as island populations are expected to be small and to diverge more rapidly (Ho et al. 2015). The rate estimated for ND2 as 0.018 s/s/myr is the first report and is much lower than the previous indirect estimation (0.02342 s/s/myr) by Chobanov et al. (2017). ND2 can be considered as a rapidly evolving segment of the mitochondrial genome, and therefore suitable for species level studies. Our rate estimation for the mitochondrial 12S rDNA-tDNAval-16S rDNA segment was 0.0141 s/s/myr, higher than the earlier estimation (0.0119 s/s/myr in Kaya & Çıplak, 2016), but show considerable difference from the estimations for 12S and 16S rDNAs alone (Papadopoulou et al. 2010; Allegrucci et al. 2011). These comparisons confirm that estimation per gene may differ from lineage to lineage among insects or even among the lineages belonging to the same order (Ho & Lo 2013), but differences are not as much as folds at least in Orthoptera.

Rate estimations of ITS1-5.8S rDNA-ITS2 have commonly been reported for plants (Villarreal & Renner 2014 and references therein). Bargues et al. (2006) reported 0.0052–0.0242 and 0.004–0.01 s/s/myr for ITS1 and ITS2, respectively, in dipterans, but these estimations were indirect by linking other genes. The present estimation is the first report for the total segment of ITS1-5.8S rDNA-ITS2 in insects and orthopterans based on a geographic calibration. Our results, as 0.0010207 ± 0.0007481 s/s/myr, prove a considerable departure from all earlier estimations by being lower than that reported for plants (Villarreal & Renner 2014) and insects (Bargues et al. 2006). Although addition of ITS sequences to mitochondrial sequences of COI+ND2+VAL considerably resolved the intergeneric relationships (see Çıplak et al. 2021) its variation and its evolutionary dynamics in Orthoptera indicated a considerable homoplasy (Uluar & Çıplak 2020), suggesting an unreliable marker in phylogenetic and time estimation studies.

The Mid-Aegean Trench as a biogeographic calibration point

Although it is not known for certain that how many times the Aegean Area regressed during Neogene, the Tortonian (12–9 myr) and the Messinian-Pliocene (app. 5.5 myr) regressions have been well documented (see Rögl 1998; Meulenkamp & Sissingh 2003; Popov et al. 2004 for pre-Messinian time and Dermitzakis 1990; Anastasakis et al. 2006; Loget et al. 2006; Krijgsman et al. 2019 for post-Messinian time). These regression times were used as calibration points to date phylogenies or to estimate substitution rates in earlier studies (see Poulakakis et al. 2015 and reference therein). Results of the present study support those using the first regression time (Parmakelis et al. 2006a, b; Papadopoulou et al. 2010; Kornilios et al. 2016) or in favour of the Tortonian calibration in the studies using both (Kasapidis et al. 2005; Cellinese et al. 2009; Simaiakis et al. 2012). The present results do not falsify those using the second (Bitkau & Comes 2005; Allegrucci et al. 2011; Kyriazi et al. 2013) but indicate that special caution must be taken when considered for this type of studies.

As mentioned earlier for insects, Papadopoulou et al. (2010) calibrated their analyses by Tortonian regression (10.5 ± 1.5 myr), while Allegrucci et al. (2011) by Pliocene regression (5–5.3 myr) and their rate estimations for the same genes segments, for example, for COI and 16S rDNA, were similar. We think both calibrations are valid

regarding the present results. Then, how can both estimations come to a compromise? Particularly from the branching pattern of *Eupholidoptera* and obtained nodal ages, we concluded that if the splitting was between Crete/Cyclades and Anatolia, then Tortonian calibration is accurate. However, if the splitting was between Crete/Sporades/Cyclades and mainland Greece, then the Pliocene calibration stands accurate. We assume that the study of Papadopoulou et al. (2010) represents the first case (see also the review by Poulakakis et al. 2015) while that of Allegrucci et al. (2011) represents the second. This also explains why they obtained similar rate estimations. Therefore, we think the scenario for *Dolichopoda* should be revised by assuming reverse dispersal routes (see fig. 5 in Allegrucci et al. 2011). In other words, the lineages dispersed from Anatolia to Europe using the Bosphorus/Balkan corridor and then distributed to Aegean area especially during Messinian exhibit the second pattern (confirmed clearly by Kornilios et al. 2019) and, the lineages radiated from ancestral stocks resident in Aegean area and split by Tortonian regression exhibit the first. This explanation well fits to our assumptions for ancestral splitting in Tortonian and for the extension of *Eu. megastyla* subgroup of *Eu. chabrieri* species group from Anatolia to the mainland Europe (also see Çıplak et al. 2010). Thus, the phylogeography studies on the Aegean lineages, especially dating phylogenies, require caution in this respect. The results also imply that Aegean biogeography is complex and accurate pattern definitions require considering lineage-specific aspects.

Early radiation of Pholidopterini

The chronogram suggested that the radiation of Pholidopterini began by the beginning of the Middle Miocene (Fig. 2, Table 2). The last common ancestor of *Bolua* + Pholidopterini dated back to ~22 myr, corresponding to Aquitanian in the Early Miocene. The ancestral node of Pholidopterini itself was dated as 18 myr, falling into Burdigalian again in the Early to Middle Miocene. The internal nodes between the tribal and proximate generic ancestral nodes are dated to the Langhian-Serravallian (16–10 myr before the present). We, therefore, suggest that radiation of Pholidopterini began by Langhian and the tectonic or climatic paleo-events during/after this period as possible evolutionary drivers.

Regarding present distributions (Fig. 1) and tectonic plates at origin time, splitting of *Bolua* and Pholidopterini occurred possibly within the Anatolio-Balkan plate, including Rhodope and Greek Arch (Fig. 2). This plate was relatively stable through the Late Oligocene-Early Miocene (Rögl 1998; Popov et al. 2004), thus, there is no obvious geographic splitting to assume a vicariant separation. Because of the same reasons, the same scenario seems to be valid for the basal radiation of Pholidopterini. The geography of the region in the origin time of the Pholidopterini was relatively stable, but fluctuations in the climate were prominent. The Aquitanian climate was reported as dry-cold due to Antarctic “Mi-1 glaciation”, but later it reverted to warming reaching to Mid-Miocene Climatic Optimum by the beginning of Langhian (Fauquette et al. 2007; Popescu 2008; Ivanov et al. 2011; Suc et al. 2018). From this combination of the paleo-events, it is more plausible to assume ecological speciation for the splitting of *Bolua* and Pholidopterini and for the early radiation of the tribe.

Tribal radiation requires further evaluations. At present, the main diversity of Pholidopterini is confined to Anatolia, including Lesser Caucasus and Levant (see Çıplak 2008 for biogeographic borders of Anatolia), Greece, and the Balkans, and the diversity outside of this area is roughly 10% of the total of species number (Fig. 1). Habitat preference of the extant species ranges from cold Euro-Siberian climate to warm Mediterranean in parallel with the climatic heterogeneity of the tribal core range. Although this is the case, changes in the habitat preference through the history of the tribe can be followed using radiation steps and historical climatic fluctuations. Especially, the correlation between early speciation steps in the tribe with the Middle Miocene Climatic Optimum indicated that warming facilitated the first radiation step. The second radiation step within Pholidopterini once more seems to be due to climatic fluctuations. Concordant branching events (Fig. 2) supported this assumption. One such event was the basal radiation of the clade, including *Eupholidoptera*, *Parapholidoptera*, *Uvarovistia*, and *Spinopholidoptera* corresponding around 13 myr ago. The first two genera (see Çıplak 2000; Çıplak et al. 2009, 2010) possibly represent the southern ancestral stocks, while the last two the northern ones. We should note that the first two genera are more diverse than the last two (Çıplak et al. 2021). Again, the splitting of *Apholidoptera* + *Aparapholidoptera*, *Pholidoptera* + *Psorodonotus*, and *Uvarovistia* + *Spinopholidoptera* genus pairs occurs around the same ages. All these branching steps correlate with the period of Serravallian/Tortonian following the climate cooling started in the Serravallian (Fig. 2). From radiation patterns of the first and second steps, it is plausible to assume that warming is the reason for the first and cooling is the reason for the second radiation step, possibly in correlation with the changes in the vegetation (Popescu 2008; Ivanov et al. 2011; Suc et al. 2018).

These assumptions raise some specific questions that need to be answered. The first one is related to vicariant speciation as expected because of the active tectonic history of the area. There was no obvious clue supporting vicariant speciation in the basal part of the tribe as almost all branching events occurred within the same tectonic plate. For example, *Spinopholidoptera*, *Uvarovistia*, *Parapholidoptera*, and *Eupholidoptera* historically and contemporarily shared the same plate, which was relatively stable during Langhian and Serravallian up to the Tortonian regression of the Mid-Aegean Trench (Rögl 1998; Meulenkamp & Sissingh 2003; Popov et al. 2004). Thus, we considered the absence of obvious clues for vicariance as further evidence for ecological speciation for the early radiation of the tribe. The second question is related to the absence of the tribal representatives in Africa and Iberia. Although there are inconsistencies or uncertainties between/in reconstructed paleogeographic maps, the candidate ancestral plate was distinguishable in whole Miocene and well isolated in Langhian. The terrestrial connections may provide dispersal to Africa, Iberia and also Zagros/Iran lands occurred during Langhian-Tortonian (Rögl 1998; Meulenkamp & Sissingh 2003; Popov et al. 2004). Thus, the absence of representatives in Africa and Iberia may be attributed to ecology because of differences in climate and vegetation of these adjacent regions (Fauquette et al. 2007; Popescu 2008; Suc et al. 2018). At present there are representatives of the tribe, two species of *Apholidoptera* (Yahyaoglu et al. 2022), and three species of *Uvarovistia* (Uluar et al. 2021), in the area originated from the Zagros plate. However, these genera are younger compared to the early radiation of the tribe.

The ancestral node of *Uvarovistia* dates back to around 4.5 myr following the Zagros uplift (Mouthereau et al. 2012).

Brief biogeography of each genus of Pholidopterini

Since each genus has peculiarities in generic range area and species-specific habitat preference, a detailed phylogeographic evaluation for each genus exceeds the content of the present study. Nine genera have currently been listed under Pholidopterini (Çıplak et al. 2021), and samples for the monotypic genera *Exopholidoptera* were not available to include in analyses. It occurs in Irano-Anatolian vegetation from a single locality in Central Anatolia. Another monotypic genus is *Spinopholidoptera*, which has distribution in a narrow part of Northern Anatolia with Euxinic vegetation and possibly originated from the Eastern Pontic satellite part of the Langhian Anatolian Plate. It diverged from the close relatives around Serravallian in parallel with cooling in climate and without subsequent speciation thenceforth. The genus *Uvarovistia* comprises five known species and is distributed in Zagros Mountain Belt or associated highlands in Eastern Turkey, Northern Iraq, and Western Iran (Çıplak et al. 2021; Uluar et al. 2021). The chronograms obtained here suggested the age of the ancestral node was around 4.97 myr. It possibly had radiated from an ancestor distributed along with topographic heterogeneity of the Zagros Mountains Belt after the terrestrial connection between Anatolia and Zagros plates was established around 5 myr ago (Mouthereau et al. 2012). The same evolutionary scenario seems acceptable for the two-species genus *Apholidoptera* (Yahyaoğlu et al. 2022). *Aparapholidoptera* consists of four species ranged along with the southern parts of Pontic Taurus, with vegetation showing transition characteristics between Euxinic and Irano-Anatolian phytogeographical provinces. This genus represents a lineage originating from an ancestral stock split from the base of the tribe around Langhian, but intra-generic radiation is after Messinian.

The remaining four are species-rich genera of the tribe with >15 species. The genus *Pholidoptera* comprises 18 described species, with poor support for its monophyly (Çıplak et al. 2021). The *Pholidoptera* clade, represented with five species in this study, arose from an ancestral stock split from the base of the tribe in Serravallian in parallel with climate cooling. Its species diversity is confined to the Balkans with the Euro-Siberian vegetation (Fig. 1). Although there are a few species extended out of the core range area, possibly it was originated from an ancestral stock existed in Rhodope-Balkan plate and much later, possibly during Pleistocene, dispersed to other peripheral area. The genus *Psorodonotus* shows similarities with *Pholidoptera*. It also radiated from an ancestral stock split from the base of the tribe around Serravallian in parallel with climate cooling, but intra-generic radiation was after Tortonian. Contrary to *Pholidoptera*, the origin place of ancestral stock of *Psorodonotus* is possibly the Eastern Pontic satellite part of the Langhian Anatolian plate. In the Late Pliocene/Pleistocene, the genus dispersed to Irano-Anatolian vegetation in East Anatolia and West Anatolia + the Balkans via Taurus Way (Kaya et al. 2013b, 2014, 2015; Kaya & Çıplak 2016, 2017).

Parapholidoptera is mainly an Anatolian genus with the total range of 18 species and its main diversity is confined to the Anatolian mountain belts, including Anatolian Diagonal and Taurus Way (see Çıplak 2000, 2008). Intra-generic radiation of the genus

began in the Messinian around 8 myr ago, but its ancestor split from the sister genus *Eupholidoptera* in Serravallian around 12 myr ago, from a likely ancestral stock in the old Anatolian plate. The species of the genus are Mediterranean or Irano-Anatolian in vegetation preference, but some species probably recently extended to Euro-Siberian vegetation in Lesser Caucasus and Balkans. A full examination of its phylogeography is needed to be conducted, especially regarding it as a model group to examine the role of the Taurus Way in the establishment of regional biodiversity.

Eupholidoptera is the most speciose genus of Pholidopterini, with the presence of 57 species (Çıplak et al. 2009, 2010, 2021). Its range starts from the Alps in the west and extends to Sinai in the east/southeast along the northern Mediterranean Basin. However, the core range, according to species number, is biogeographic Anatolia (see Çıplak 2008) and Greece, including Aegean islands, and a few species occur in the remaining peripheral range area (Çıplak et al. 2010; Allegrucci et al. 2014; Çıplak et al. 2021). The data specific to the genus in the present study (Figure 2) confirms Çıplak et al. (2010) in terms of the origin place of the ancestor as the southern part of the Langhian Anatolian plate, including Greek Arc (or Aegeid plate according to Dermitzakis 1990). Present results also confirm Çıplak et al. (2010) for the origin time of ancestor, which split from other related genera in Serravallian and for intra-generic radiation since Tortonian. Intra-generic radiation based on molecular data and its possible drivers requires further studies. However, a few brief statements may be conducted as follows. The order of the intra-generic branches suggested an origin in South Anatolia plus Crete, but not in mainland Greece because the species from the Greek mainland (e.g. *Eu. megastyla*, *Eu. chabrieri*) occur as terminal branches within Anatolian lineages. The ancestor was with a large range which split by development of the Aegean Sea in Tortonian and later extended to the Balkans and Greece as the basal branches were splitting almost contemporarily. A significant majority of the species is associated with maquis vegetation along the northern Mediterranean Basin with arid climate and vegetation. Occurrence of over 10 species (present data indicate further new species) in Crete (for which Çıplak et al. (2010) referred to as “wonderful life in Crete”) and a much higher number along the Mediterranean Anatolia plus the northern Levant (see Fig. 1) are a typical representation of these characteristics (Çıplak et al. 2009, 2010). Thus, we assume that the range of the genus in Northern Greece, Balkans, Italy and France (the range area of *Eu. megastyla* subgroup of *Eu. chabrieri* species group) was established much later in the Late Pliocene/Pleistocene as proposed by Çıplak et al. (2010).

Distribution and radiation of all genera together indicate to some general patterns. In parallel with ecological preference of the origin, *Pholidoptera*, *Aparapholidoptera*, *Psorodonotus*, *Spinopholidoptera*, *Apholiidoptera*, *Uvarovistia*, and *Exopholidoptera* occur in a relatively cold climate in Euro-Siberian or montane Irano-Anatolian vegetation. Ecological habitat conservatism assumption seems to be valid for *Eupholidoptera* as well, as a Mediterranean lineage. *Parapholidoptera* comprises elements distributed in Mediterranean and Irano-Anatolian provinces, thus indicating shifts in habitat preference. The evidence for habitat conservatism or shift are also coming from members of *Psorodonotus*. The most basal branches of the genus are strictly Euxinian and lived a

stasis while the branches extended to mountain meadows of Irano-Anatolian vegetation rapidly radiated (Kaya & Çıplak 2016, 2017). Therefore, they were considered as candidate model groups to examine the correlation between genotypic and phenotypic divergence (Çıplak et al. 2021). On the other hand, there is a considerable interval between ages of the ultimate and proximate ancestors of the genera. Splitting of the generic ancestors from the other relatives or main lineage of the tribe was in Serravallian, but all intrageneric radiations were in Tortonian and mainly after Messinian. This pattern also suggests radiation correlated with shifts in climate and vegetation (Favre et al. 2007; Vasiliev et al. 2017; Suc et al. 2018). We propose that the ancestor of the tribe probably constituted a large population in a wide range through the Middle Miocene Climatic Optimum, and when the climate cooling began in Serravallian, this large population split into many fragments these fragmented populations then formed ancestors of the present genera. Radiation of these fragmentary ancestral populations became available by the Late Tortonian, but especially by the Messinian onward warming. Thus, considerable radiation took place in the Pliocene/Pleistocene, though all species of the tribe did not include in the analyses.

Conclusions

Members of Pholidopterini share its most recent common ancestor at the beginning of Middle Miocene (approximately 18 myr ago). There appear to be three important waves of radiation in the history of the tribe, the first was 16–14 myr ago in Langhian, the second was 12–11 myr ago in Serravallian, and the third following the Messinian climatic crisis. Although the tectonic activity in the range area during the origin time was very high, there was no sign of vicariant speciation producing generic radiation of the tribe. Since the above speciation steps correlate with the climatic shifts of Miocene, we argue that ecological speciation is the likely process responsible for the diversification of the tribe.

Although regression of the Mid-Aegean Trench is an important geological event to calibrate the molecular clock but requires caution. The regression at Tortonian is reliable for the splitting between Crete and Anatolia, while Pliocene regression is reliable for the splitting between Crete and Greece. Rate estimations in this study confirmed previous ones for COI, suggesting it as a reliable marker to date phylogenies. We report rate estimation for ND2 for the first time, and our results suggest it as a reliable marker in dating phylogenies. The 12S rDNA-tDNAval-16S rDNA segment comprises parts from three, and our results show that mitochondrial RNA genes are less variable than the protein-coding genes, thus, less informative in phylogenetic studies, at least for Orthoptera. The ITS1-5.8S rDNA-ITS2 part of the ribosomal cistron shows insufficient variation and not reliable in phylogenetic studies, at least for Orthoptera.

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Supplementary material

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