

## Mitochondrial phylogeny of the subgenus *Hamatopoecilimon* Heller, 2011

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### Abstract

The present study focuses on the basal clade of the genus *Poecilimon* – subgenus *Hamatopoecilimon*, with a complex distribution in the eastern Aegean. We compare different mitochondrial gene trees, test the phylogenetic utility of the mitochondrial A+T-rich region and discuss mitochondrial phylogeny. We infer that the ancestral lineages of *Hamatopoecilimon* split rather simultaneously, affected by geological events during late Miocene.

### Zusammenfassung

Die vorliegende Arbeit konzentriert sich auf die basale Gruppe der Gattung *Poecilimon* mit der Untergattung *Hamatopoecilimon*, die eine komplexe Verbreitung in der Ostägäis aufweist. Wir vergleichen verschiedene Stammbäume, basierend auf Sequenzen mitochondrialer Gene, testen die mitochondriale A-T-reiche Region auf phylogenetische Aussagekraft und diskutieren die mitochondriale Phylogenie der Gruppe. Wir nehmen an, dass die ursprünglichen Linien von *Hamatopoecilimon* nahezu gleichzeitig entstanden sind, beeinflusst durch die geologischen Vorgänge im späten Miozän.

### Introduction

*Poecilimon*, Fischer 1853 is the most diverse bush-cricket genus in the Palearctic including more than 140 species (CIGLIANO et al. 2020). Despite the wide range of several species, diversity is concentrated in the Balkans, Asia Minor and the Caucasus where levels of endemism are noticeably high. This pattern is explained by rapid diversification, guided by geological events in the region during the Neogene and by the following Pleistocene climatic cycles (HEWITT 1996, LA GRECA 1999, ÇIPLAK 2004, 2010, KAYA 2015).

Variety of broad studies accumulated knowledge about the distribution, morphology, bioacoustics, karyology and systematics of the genus (RAMME 1933, BEYBIENKO 1954, HELLER 1984, 1988, WARCHAŁOWSKA-ŚLIVA et al. 2000, ULLRICH et al. 2010, ÜNAL 2010, GRZYWACS et al. 2014). Recently authors focus their research on separate species groups which share common morpho-acoustic traits and evolutionary history (HELLER et al. 2005, 2006, 2011, HELLER & SEVGILI 2005, CHOBANOV & HELLER 2010, KAYA 2018, KAYA et al. 2012, 2015, 2018, SEVGILI et al. 2018).

A phylogenetic study (ULLRICH et al. 2010) based on mitochondrial and nuclear markers suggested update to the relationships within *Poecilimon* with *Poecilimon hamatus* species group representing its basal branch.

A detailed research on morphology and acoustic behavior in the group followed (HELLER et al. 2011), raising the group rank to a subgenus level – *Hamatopoecilimon*. Six species are recognized within the subgenus – *P. deplanatus* Brunner von Wattenwyl, 1891, *P. hamatus* Brunner von Wattenwyl, 1878, *P. ikariensis* Willemse, 1982, *P. klausgerhardi* Fontana, 2004, *P. paros* Heller & Reinhold, 1992, and *P. unispinosus* Brunner von Wattenwyl, 1878. All are found over Aegean islands and the mainland of western Anatolia with three species endemic to single islands (see HELLER et al. 2011 for detailed distribution map). Distribution of the subgenus has been thus related to the paleogeographic evolution of the region – the Neogene disintegration of the Aegeid plate, which triggers speciation by vicariance, and the subsequent reconnection of landmasses, which could reestablish contact between populations (ÇIPLAK et al. 2010, POULAKAKIS et al. 2014).

Our study aims to reconstruct a robust phylogeny of *Hamatopoecilimon* as a stepping stone to further phylogeographic research. We selected two mitochondrial protein coding regions – nicotinamide adenine dinucleotide dehydrogenase subunit 2 (ND2) and cytochrome C oxidase subunit I (COI). These markers, especially COI, are widely used in phylogenetic studies of orthopterans (e.g. ALLEGRUCCI et al. 2017, KAYA et al. 2015, CHOBANOV et al. 2017). In addition, we test the phylogenetic utility of the mitochondrial control region (CR, A+T- rich region) in *Hamatopoecilimon*.

## Material and Methods

### DNA Sampling and molecular marker amplification

Material was collected during field trips across the Aegean islands in 2018-2019 and stored in absolute ethanol or was received from colleagues. All species in the subgenus *Hamatopoecilimon* were represented in our sample (Table 1). Total genomic DNA was extracted applying salt-ethanol protocol (ALJANABI & MARTINEZ 1997). A fragment from the 3'end of COI was amplified with forward primer C1-J1751- GGAGCTCCAGATATAGCTTTTCC and reverse TL2-N-3014 – TCTAA TGCATTAATCTGCCATCTTA (SIMON et al. 1994). ND2 was amplified with forward TM-J210 – AATTAAGCTAATGGGTTTCATACCC, paired with TW-N1284 – AYAGCTTTGAARGYTATTAGTTT (SIMON et al. 2006). CR was amplified with SR-J14610 – ATAATAGGGTATCTAATCCTAGT and T1-N18 – CTCTATCAARRT AAYCCTTT (SIMON et al. 2006). PCR was performed using Thermo Scientific™ DreamTaq™ Hot Start PCR Master Mix, Thermo Fisher Scientific Inc., following the protocol of the manufacturer. For COI and ND2 we applied thermal cycling suggested by CHOBANOV et al. (2017). Cycling protocol from ZHAO et al. (2011) was applied to obtain the A+T-rich region, together with a small fragment of 12S rDna. PCR product purification and Sanger sequencing was carried out by MacroGen Europe (MacroGen, inc., Amsterdam, The Netherlands) as an external service.

### Sequence preparation and phylogenetic analysis

Chronograms were visualized and assembled with CodonCode Aligner version 8.0.2 (CodonCode, Dedham, MA, USA). Sequences were aligned via MUSCLE algorithm (EDGAR 2004), implemented in MEGA X (KUMAR et al. 2018). Selection of unique haplotypes, scanning protein coding sequences for stop codons and substitution saturation tests were carried out in DAMBE ver. 7.2.43 (XIA et al. 2003,

XIA 2018). A total of four matrices were created – ND2, COI, CR, and ND2+COI+CR. Best-fit partitioning schemes and models of evolution were selected using PartitionFinder ver. 2.1.1 (LANFPEAR et al. 2016), run on the CIPRES Science Gateway webserver (MILLER et al. 2010). Results were later used to set phylogenetic analysis. Maximum likelihood (ML) analyses were conducted using RAxML ver. 8.2.12 (STAMATAKIS 2014) (on CIPRES Science Gateway). Node support was obtained through 1000 bootstrap resampling. Bayesian inference (BI) analyses were accomplished in Mr. Bayes 3.2.5 (RONQUIST & HUELSENBECK 2003, RONQUIST et al. 2005). Four simulations of Markov chains and  $1 \times 10^6$  generations were run, sampling 1 of every 100 trees. The MCMC parameters were monitored in Tracer ver. 1.7.1 (RAMBAUT et al. 2018). The first 25% of trees were discarded as burnin.

Table 1: Decimal coordinates of localities and GenBank accession numbers of sequences (ND2 - mitochondrial nicotinamide adenine dinucleotide dehydrogenase gene; COI - mitochondrially encoded cytochrome c oxidase I; CR – mitochondrial control region).

Species	Locality	N	E	ND2	COI	CR
<i>Poecilimon unispinosus</i>	Chios island, Olimpi	38.247022	25.955876	MT416239	MT416229	MT416251
<i>Poecilimon unispinosus</i>	Chios island, Olimpi	38.247022	25.955876	-	MT416228	MT416252
<i>Poecilimon ikariensis</i>	Ikaria island	37.63446	26.2887	MT416240	MT416231	MT416253
<i>Poecilimon ikariensis</i>	Ikaria island	37.63446	26.2887	MT416241	MT416230	MT416254
<i>Poecilimon klausgerhardi</i>	Andros island, Apikia	37.8678	24.920	MT416243	MT416234	MT416255
<i>Poecilimon klausgerhardi</i>	Andros island, Apikia	37.8678	24.920	MT416244	MT416233	MT416256
<i>Poecilimon hamatus</i>	Fournoi Island	37.574	26.4763	MT416242	MT416232	MT416257
<i>Poecilimon hamatus</i>	Kos Island, Kefalos	36.7619	26.9601	MN114185	MN114162	MT416258
<i>Poecilimon paros</i>	Naxos island	37.0577	25.51	MT416245	MT416235	MT416259
<i>Poecilimon paros</i>	Naxos island	37.0577	25.51	MT416246	MT416236	MT416260
<i>Poecilimon deplanatus</i>	Karpathos island, above Volada vill.	35.5591	27.159	MT416247	MT416237	MT416261
<i>Poecilimon deplanatus</i>	Karpathos island, above Volada vill.	35.5591	27.159	MT416248	-	MT416262

Species	Locality	N	E	ND2	COI	CR
<i>Poecilimon cretensis</i>	Crete island, Prases	35.37465	23.83878	MT416238	MT416227	MT416249
<i>Poecilimon cretensis</i>	Crete island, Skaloti	35.1969	24.2597	MN114198	MN114173	MT416250

## Results

### Characteristics of the datasets

The final alignment of ND2 consisted of 957 bp of which 375 variable and 331 parsimony informative sites. The COI fragment consisted of 1008 bp of which 287 variable and 258 parsimony informative sites. The final alignment of the control region was 976 bp including 15 indels, 295 variable sites and 284 parsimony informative sites. No stop codons were detected in protein coding sequences. The sequence saturation test (XIA et al. 2003) did not show any substantial saturation. The concatenated matrix resulted in a dataset of 2941 bp (ND2+COI+CR).

### Phylogenetic analysis

A total of eight trees were inferred, using different markers and methods. BI and ML methods showed agreement on the tree topology (Fig. 1), although ML analyses show generally lower support (bootstrap). All single gene trees and the concatenated dataset strongly support the early split of *P. unispinosus* + *P. ikariensis* branch. All trees support the monophyly of the other four species in *Hamatopoeilimon*. COI (Fig. 1a) and CR (Fig. 1b), separately, support (*P. hamatus*+*P. deplanatus*)+( *P. paros*+*P. klausgerhardi*) topology, which is in concordance with the results from the concatenated matrix (Fig. 1d). All trees support the *P. paros*+*P. klausgerhardi* clade. Most gene trees received poor support for a *P. hamatus*+*P. deplanatus* clade with the exception of BI analysis of the CR matrix – Bayesian posterior probability close to 1 (Fig 1b). The concatenated mitochondrial dataset (NAD+COI+CR) (fig. 1d) showed improved support for most nodes. The BI tree, inferred from the concatenated dataset, showed full support of all nodes presenting the topology (*P. unispinosus*+*P. ikariensis*) + ((*P.hamatus*+*P.deplanatus*)+( *P. paros*+*P. klausgerhardi*)). The ND2 tree (Fig. 1c) is the only one showing different topology. ND2 suggests *P. deplanatus* being a sister clade to *P. hamatus*+(*P.paros*+*P. klausgerhardi*) with highest support in comparison to the other single matrices, which corresponds to the nuclear and mitochondrial phylogenies by ULLRICH et al. (2010).

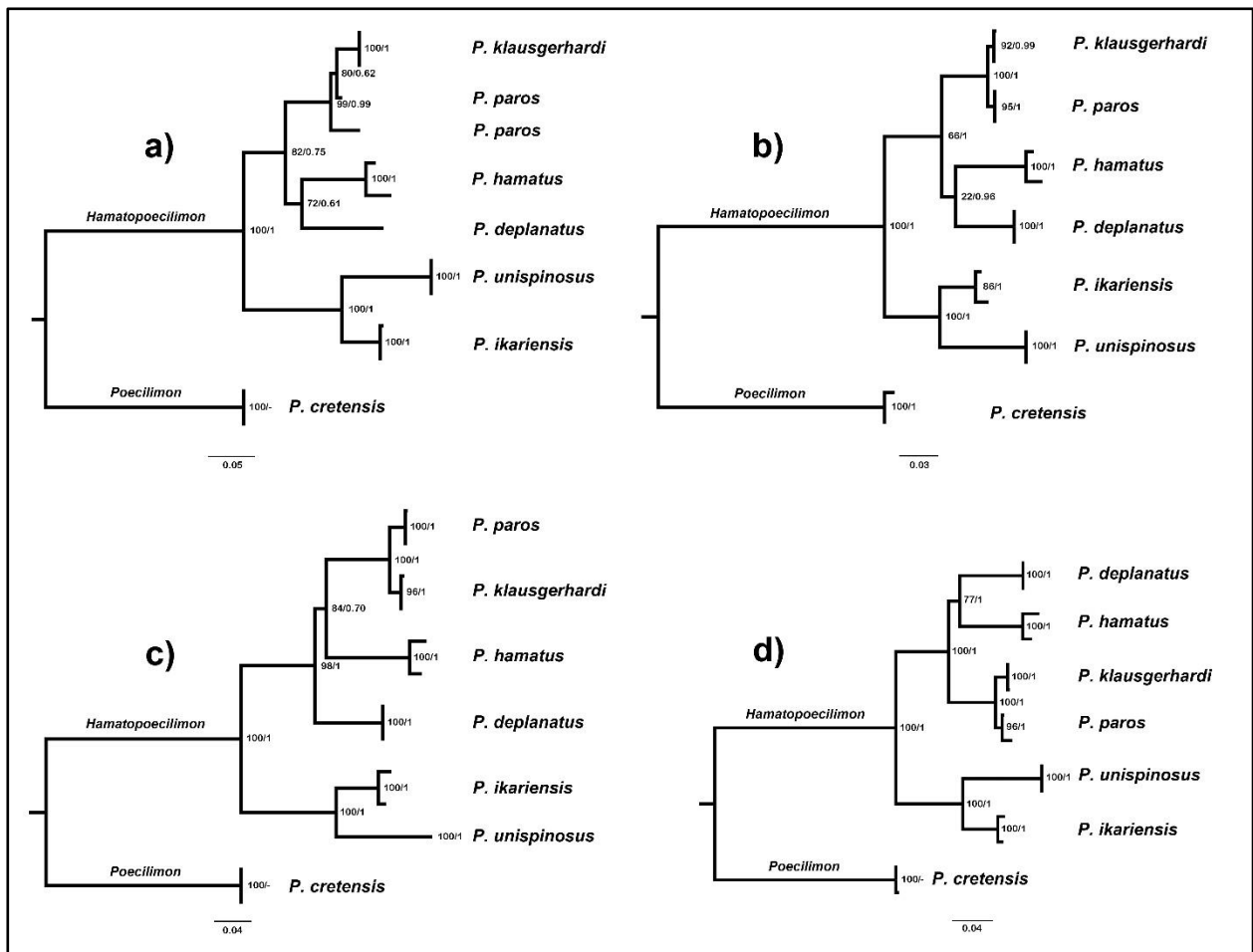


Figure 1: Mitochondrial phylogenetic trees of subgenus *Hamatopoecilimon*, based on ML and BI analyses of: a) 1008 bp of COI; b) 976 bp of the control region; c) 957 bp of ND2; d) concatenated dataset (ND2+COI+CR) with 2941 bp. Branch numbers show branch support (ML bootstrap/BI Bayesian posterior probability).

## Discussion

### Phylogenetic performance of molecular markers

COI gene is a widely used marker for inferring mitochondrial phylogeny, showing strong phylogenetic signal on a broad taxonomic scale (SPICER 1995, GOTO & KIMURA 2001, REMIGIO & HEBERT 2003, KJER et al. 2014, WANG et al. 2017). It is relatively long and easy to amplify due to availability of conserved primer sequences (FOLMER 1994, ZHANG & HEWITT 1997b, SIMON et al. 1994, 2006). ND2 performed better than other mitochondrial markers in phylogenetic analysis of Odonata due to the high proportion of variable and parsimony informative (PI) sites, low substitution rate heterogeneity and other performance criteria (CHENG et al. 2018). The situation in the genus *Isophya*, closely related to *Poecilimon*, is similar as ND2 shows about 20% more variable and PI sites than COI (CHOBANOV et al. 2017). Our study confirms that even with small taxon sampling and closely related species, ND2 provides more variable and PI sites than COI and overall higher branch support.

Results from the control region dataset are generally consistent with these from protein-coding genes. Both highly variable parts and conserved structural blocks are observed in the A+T-rich region, however, in Orthoptera they are not separated into distinct domains (compare CLARY & WOLSTENHOLME 1987, ZHANG & HEWITT 1997a, ZHAO et al. 2011). While variation of the control region above the genus level could be extremely high, restraining sequence comparison (MARDULYN et al. 2003, AMARAL et al. 2016), CR is a useful phylogenetic marker for closely related taxa, providing additional information (AMARAL et al. 2016, LI & LIANG 2018), thus, with the potential of providing better resolution than COI (VILA & BJÖRKLUND 2004, CHENG et al. 2018). CR was recently used to test speciation hypothesis and detect barriers to gene flow between *Poecilimon* populations (EWELEIT et al. 2015). In our study, CR produced a well-resolved phylogeny and provided additional phylogenetic signal in the concatenated mitochondrial dataset.

### Phylogeny and history of lineages

Mitochondrial phylogeny based on 16S rRNA, tRNA-Val and 12S rRNA fragment (ULLRICH et al. 2010) suggested early separation of the northern group (*P. unispinosus*+*P. ikariensis*) which was corroborated by our study. HELLER et al. (2011) points out that *P. hamatus*, *P. paros* and *P. klausgerhardi* share the unusual bifurcate shape of the cerci and form the southern group of *Hamatopoecilimon*. Our analyses confirm that *P. deplanatus*, endemic to the island of Karpathos, shares a common ancestor with the southern group. This species, which does not have bifid cerci, branches out first on the ND2 tree (Fig. 1c) as well as on the mitochondrial tree by ULLRICH et al. (2010), but surprisingly groups with *P. hamatus* in other analyses (Fig. 1a, b, d), although usually with low support. Failure to obtain strong node support and unambiguous tree topology is a common pattern, observed in phylogenetic studies of terrestrial organisms in the Aegean archipelago. This phenomenon is explained by simultaneous splits of many ancient lineages, thus, adding taxa or markers does not improve resolution (compare GANTENBEIN & KEIGHTLEY 2004, PARMAKELIS et al. 2005, KLOSSA-KILIA et al. 2006, ALLEGRUCCI et al. 2009).

### Phylogeographical implication

*Poecilimon* shares many phylogeographic traits with *Eupholidoptera* – another genus that originated on the Aegeid plate (ÇIPLAK 2004, 2010, HELLER et al 2011). One of the key geological events that affected the terrestrial lineages in the Aegean is the formation of the Mid-Aegean Trench 12-9 Mya (CREUTZBURG 1963, DERMITZAKIS & PAPANIKOLAU 1981), which isolated western and eastern lineages by sea. The following desiccation of the Mediterranean causing the Messinian salinity crisis (MSC) 5.9- 5.33 Mya (KRIJGSMAN et al. 2018) reestablished terrestrial connections. The only species of *Poecilimon* on Crete is *P. cretensis* Werner, 1903, which is distant from *Hamatopoecilimon* and diverged later (ULLRICH et al. 2010). Since *Hamatopoecilimon* is basal of *Poecilimon* and there are no older lineages on Crete or Peloponnese, *Poecilimon* possibly originated on the east side of the Aegean trench. The Aegean islands may thus have been colonized later after the appearance of suitable land bridges. Similar scenario is proposed for the biogeography of Greek species of *Dolichopoda* (ALLEGRUCCI et al. 2009).

Karpathos was connected to Rhodes and Anatolia during the early Pliocene until 3-3.5 Mya (DAAMS & VAN DER WEERD 1980). However, some Karpathos lineages are directly linked to Anatolia, while distant from these on Rhodes, which may be suggesting different age of vicariance events during Miocene (see VEITH & STEINFARTZ 2004, PARMAKELIS et al. 2006a, b). Situation is similar in *Eupholidoptera* as *E. uvarovi* (Karabag, 1952) from Karpathos groups with *E. akdeniz* Ünal & Naskrecki, 2002 from southeastern Turkey (ÇIPLAK et al. 2009, 2010). This might be explained by later re-colonization of Rhodes as during Late Pliocene it was fully submerged (POPOV et al. 2004) or at least partly covered with brakish waters (DERMITZAKIS 1990). The lineage of *P. deplanatus* was thus isolated from the 'southern' group during the Middle or Late Pliocene, while the 'northern' group (*P. unispinosus* and *P. ikariensis*) diverged earlier, possibly at the end of the Miocene.

Though mitochondrial phylogeny supports simultaneous divergence of *P. deplanatus*, *P. hamatus* and *P. paros/P. klausgerhardi*, morphological traits clearly place *P. deplanatus* in its own lineage. Early divergence of *P. deplanatus* (before Mid Pliocene) combined with polytomy at the tree root of the "southern group" may be linked with gene exchange between those lineages prior to the isolation of Karpathos. Based on genetic distances and phylogeny, dispersal of *P. paros+P. klausgerhardi* lineage to the Cyclades may have thus also occurred during the Pliocene as suggested for a few lineages (compare POULAKAKIS et al. 2014, KORNILIOS et al. 2018). This may explain the lack of *Hamatopoecilimon* on the Balkan mainland and on Crete. Therefore, we have a case of dispersal of the "southern group" followed by isolation contrary to the possible vicariance scenario in the case of *P. ikariensis* and *P. unispinosus*, *P. hamatus* and *P. deplanatus*. The split between *P. paros* and *P. klausgerhardi* may be quite recent as suggested by low genetic distances, morphological similarity and lack of acoustic differentiation (HELLER et al. 2011). The latter might have happened during Pleistocene as a result of repeated shifts in the sea level and thus connection-disconnection of the Southern and Northern Cyclades.

Less may be speculated about the "northern group". *P. ikariensis* is present only on Ikaria, while *P. unispinosus* has wider distribution on the Islands of Chios, Lesvos and the neighboring mainland Turkey. As Ikaria was recently connected to the mainland, its separation may have allowed the "last stand" for *P. ikariensis* escaping from possible competition with *P. unispinosus* on the mainland (Fig. 2).

*Hamatopoecilimon* represents a remnant from an ancient lineage that split comparatively early in the geological history of the Aegean area. This generally agrees with the results from the closely related genus *Isophya*, whose TMRCA was estimated at 8-9 Mya (CHOBANOV et al. 2017). Data from our study would be useful for testing further hypotheses regarding the origin and evolution of *Poecilimon* and other related genera in connection with the paleogeography of the Aegean region.

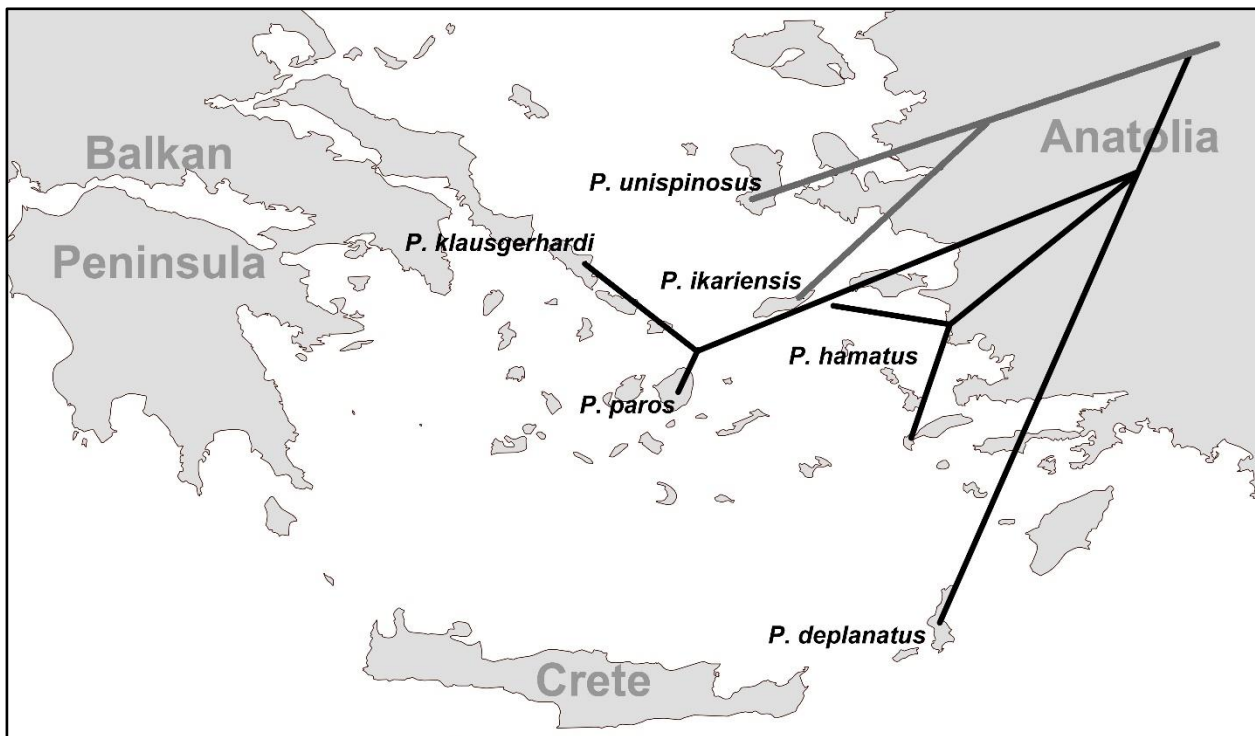


Figure 2: Spatial dimension of the mitochondrial phylogeny of *Hamatopoeecilimon*.

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