

## The Relationships within the *Poecilimon ornatus* Group (Orthoptera: Phaneropterinae) Based on the Cytochrome C Oxidase I Gene

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The genus *Poecilimon* includes 142 species divided into 18 groups. It is distributed throughout the Palearctic area. One of the groups is the *Poecilimon ornatus* group, in which many closely related taxa have been identified (13 species). Although several searches have been carried out, the phylogeny and systematics of *P. ornatus* are only partly resolved. The most dispersed taxon within the group is *Poecilimon affinis*, having numerous subspecies. Species from the *P. ornatus* group have been described mainly based on morphological characteristics, as well as type of song. The aim of this study is to clarify the relationships between species from the *P. ornatus* group by comparing partial sequences of the cytochrome c oxidase subunit I (COI) mitochondrial gene. The analyses were carried out on 84 specimens from 23 taxa. Bush-crickets from the *P. ornatus* group are monophyletic, in contrast to taxa within the *P. affinis* complex. Not all of the previously described divisions of the group based on morphology, bioacoustics, distribution, and ecology were confirmed.

Key words: bush-crickets, phylogeny, polymorphism, mitochondrial DNA.

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*Poecilimon* Fischer, 1853 is one of the largest genus in the subfamily Phaneropterinae Burmeister, 1838 with 142 species classified under 18 species groups (*P. ampliatus*, *P. armeniacus*, *P. bosporicus*, *P. celebi*, *P. concinnus*, *P. davisii*, *P. elegans*, *P. heroicus*, *P. inflatus*, *P. jonicus*, *P. luschani*, *P. minutus*, *P. ornatus*, *P. pergamicus*, *P. propinquus*, *P. sanctipauli*, *P. syriacus*, and *P. zonatus*) (CIGLIANO *et al.* 2019). These bush-crickets occur from the Apennines to Eastern Siberia and Central Tien-Schan (BEY-BIENKO 1954). *Poecilimon* consists of short-winged, sluggish, herbivorous bush-crickets that are characterized by complex acoustic communication. In Europe, *Poecilimon* is most diverse in the Balkan Peninsula, this area represents many taxa of recent origin (e.g. CHOBANOV *et al.* 2016). The Balkans have been considered an important refugium during the Quaternary glacial periods (HEWITT 2000). The complex geomorphology and climate of the Balkan Peninsula in combination with its long terrestrial history, having been isolated and reconnected to Anatolia and Europe multiple times, and the influence of alternating cold and warm stages during the Pleistocene may underlie its vast biological

diversity (SAVIĆ 2008). Although the speciation that occurred in the Tertiary period has been documented for well separated lineages, the diversification of within-species groups and complexes of closely related species is frequently confined to the Quaternary period and the latter lineages are frequently poorly phenetically and genetically separated, possibly due to incomplete lineage sorting or hybridization (e.g. CHOBANOV *et al.* 2016).

So far, a few complete or partial revisions of the genus have been carried out based on morphological, cytogenetic, and molecular studies (e.g. RAMME 1933; BEY-BIENKO 1954; WILLEMSE 1982; HELLER 1984; HELLER & LEHMANN 2004; HELLER & SEVGILI 2005; HELLER *et al.* 2006, 2008; CHOBANOV & HELLER 2010; ULLRICH *et al.* 2010; GRZYWACZ *et al.* 2014), but still, the phylogeny and systematics of *Poecilimon* is only partly resolved. One of the least known groups within the genus is the *Poecilimon ornatus* group (Schmidt, 1850). The species from this group were outlined and revised first by RAMME (1933) and subsequently by HELLER (1984) and CHOBANOV &

HELLER (2010). The latter authors considered the group to contain 14 taxa. However, since then, three new taxa have been described (INGRISCH & PAVIĆEVIĆ 2010) and the authors, though not considering the whole group, suggested a different species composition for the relatives of *Poecilimon affinis* (Fivaldszky, 1868) – which is the widest distributed species among the *P. ornatus* group. As a result, the group currently consists of 17 valid taxa (13 species) (CIGLIANO *et al.* 2019). *P. affinis* is found in the mountainous areas of northern Greece, through the central and western Balkans to the Carpathians in Romania and in an isolated spot in Ukraine. It currently consists of five subspecies: *P. affinis affinis* (Fivaldszky, 1868); *P. a. komareki* Cejchan, 1967; *P. a. dinaricus* Ingrisch & Pavićević, 2010; *P. a. hajlensis* Karaman, 1974; and *P. a. serbicus* Karaman, 1974 (CIGLIANO *et al.* 2019). In this study, the species *P. pseudornatus* Ingrisch & Pavićević, 2010 and *P. nonveileri* Ingrisch & Pavićević, 2010 as well as the subspecies of *P. affinis*, are categorized as the *Poecilimon affinis* complex due to their morphological similarity (CHOBANOV & HELLER 2010). This complex is an example of a diverse group of closely related taxa distributed in a comparatively small area. Its disputable systematics are largely based on morphological and, to some extent, acoustic traits. However, the phenetic distinction of populations is frequently difficult due to both the similarity between and the considerable variation within the taxa. Phylogenetic data are practically lacking and thus, relationships between taxa remain unclear.

This is the first insight into the relationships between the closely related species and subspecies of the *Poecilimon ornatus* group. The aims of the present study are (i) to evaluate the genetic diversity in the *Poecilimon ornatus* group and (ii) to clarify the taxonomic status of some taxa in the *Poecilimon affinis* complex. The study provides a new data set of the cytochrome c oxidase subunit I (COI) mitochondrial gene for 13 species belonging to the *P. ornatus* group. The primers of COI used in this study are highly variable (LUNT *et al.* 1996) and thus suitable for the phylogenetics of closely related species.

## Material and Methods

### Taxon sampling

For this study, 84 specimens of bush-cricket were selected from 27 localities/populations of the *Poecilimon ornatus* group and four taxa: *P. ampliatus* Brunner von Wattenwyl, 1878 (*P. ampliatus* group); *P. heroicus* Stshelkanovtzev, 1911 (*P. heroicus* group); *P. ukrainicus* Bey-Bienko, 1951; and *P. schmidtii* (Fieber, 1853). *Polysarcus denticauda* (Charpentier, 1825) was treated as an outgroup. Insects were collected in the Balkan Peninsula (Bulgaria, Serbia, Montenegro, Al-

bania, North Macedonia, and Greece) and in Romania and Ukraine between 2006 and 2018. The species included in this study and their sampling localities are presented in Table 1. Samples have been preliminarily identified using original descriptions and published reviews (CHOBANOV & HELLER 2010).

### DNA extraction, amplification and sequencing

Genomic DNA was extracted from one leg of each specimen using a NucleoSpin® tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Partial gene sequences were amplified by PCR using the following primers: UEA7 (5' TAC AGT TGG AAT AGA CGT TGA TAC 3') and reverse UEA10 (TCC AAT GCA CTA ATC TGC CAT ATT A) (LUNT *et al.* 1996).

Amplification was done in 20 µl reaction volumes containing 3 µl of DNA, 1.0 µl of each primer, 5 mM of each dNTP, 25 mM MgCl<sub>2</sub>, 2.0 µl 10xPCR buffer, 5 U/µl of Gold *Taq* DNA polymerase (Syngen, Wrocław, Poland), and sterile water. To amplify COI, the following PCR protocol was used: initial melting step of 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 48°C, 2 min at 72°C, and a final step of 7 min at 72°C. The total volume of the PCR product was run out by electrophoresis on a 1% agarose gel at 100 V for 35 min. The correct fragment at ~ 826 bp was removed from the gel and purified using a NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany). Primers were diluted to 2.0 µM for the sequencing reactions which were carried out in 10 µl reaction mixture containing: 1.5 µl of sequencing buffer, 1.0 µl of BrilliantDye (Nimagen, Nijmegen, The Netherlands), 1.0 µl of primer (forward or reverse), 3.0 µl of the purified DNA, and 3.5 µl of sterile water. The sequencing reaction was as follows: 3 min at 94°C, 25 cycles of 10 s at 96°C, 5 s at 55°C, and 90 s at 60°C.

The sequencing of amplified DNA fragments was executed as an external service by Genomed (Warsaw, Poland). Sixty genetic sequences were deposited and twenty-four sequences were acquired from GenBank ([www.ncbi.nlm.nih.gov/genbank](http://www.ncbi.nlm.nih.gov/genbank)) under the accession numbers provided in Table 1.

### Sequence alignment and phylogenetic analyses

DNA sequences were aligned using CodonCode Aligner 9.0 (<https://www.codoncode.com/aligner>) with default parameters. All sequences were checked for stop-codons in MEGA X (KUMAR *et al.* 2018), verified using BLAST of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Genetic distances were calculated using MEGA X (KUMAR *et al.* 2018). The substitution model of evolution was determined by using jModelTest2 (GUINDON & GASCUEL 2003; DARRIBA *et al.* 2013).

Table 1

Taxonomic information and GenBank accession numbers for taxa included in this study.  
Hyphen (-) means no data

Taxa	Species	Location	Geographical position	GenBank accession	Reference
<i>Poecilimon affinis</i> complex	<i>Poecilimon affinis affinis</i> (Frivaldszky, 1868)	Ukraine, Chereska Oblast	55.09285N 33.57554E	MH800893 MH800894 MH800895	This study This study This study
		Bulgaria, Rila Mts., Iliyana Reka	42.09874N 23.35717E	MH800896 MH800897 MH800898	This study This study This study
		Bulgaria, Pirin Mts., Yavorov Chalet	41.82365N 23.37846E	MH800899 MH800900 MH800901	This study This study This study
		Bulgaria, Osogovo Mts.	42.1884N 22.5804E	MH800902 MH800903 MH800904	This study This study This study
		Bulgaria, Rila Mts., Kirilova Polyana	42.15649N 23.39736E	MH800905 MH800906	This study This study
		Bulgaria, Sredna Gora Mts., Bratiya peak	42.59104N 24.15718E	MH800907 MH800908	This study This study
	<i>Poecilimon affinis komareki</i> Cejchan, 1957	Albania, Laç	41.63168 N 19.752 E	MH800867 MH800868 MH800869	This study This study This study
		Montenegro, Kolasin	42.79198N 19.42646E	MH800873 MH800874 MH800875	This study This study This study
	<i>Poecilimon affinis dinaricus</i> Ingrisch & Pavićević, 2010	Montenegro, Susica	43.1776N 19E	MH800856	This study
		Montenegro, Mratinje	43.2477N 18.817E	MH800857	This study
	<i>Poecilimon affinis serbicus</i> Karaman, 1974	North Macedonia, Shar Mts, Ljuboten Park	42.18481N 21.12973E	MH800861 MH800862 MH800863	This study This study This study
	<i>Poecilimon affinis hajlensis</i> Karaman, 1974	Montenegro, Hajla	42.80296N 20.22638E	MH800864 MH800865 MH800866	This study This study This study
	<i>Poecilimon affinis poecilus</i> Ramme, 1951	North Macedonia, Shar Mts., Popova Shapka	42.01265N 20.88399E	MH800890 MH800891 MH800892	This study This study This study
	<i>Poecilimon nonveilleri</i> Ingrisch & Pavićević, 2010	Montenegro, Susica	43.1776N 19E	MH800858 MH800859 MH800860	This study This study This study
	<i>Poecilimon pseudornatus</i> Ingrisch & Pavićević, 2010	Montenegro, Durmitor, Boricje	43.14251N 18.92046E	MH800870 MH800871 MH800872	This study This study This study
		Montenegro, Treshnievik	42.73849N 19.68358E	MH800876 MH800877 MH800878	This study This study This study
		Montenegro, Vusanje	42.5193N 19.86526E	MH800879 MH800880 MH800881	This study This study This study
		Montenegro, Hajla	42.81517N 20.18915E	MH800882 MH800883 MH800884	This study This study This study
		Serbia, Kamena Gora	43.32859N 19.578E	MH800885 MH800886 MH800887 MH800888 MH800889	This study This study This study This study This study
	<i>Poecilimon ornatus</i> group	<i>Poecilimon ornatus</i> (Schmidt, 1850)	North Macedonia, Jakupica Mts., Cheples Chalet	41.71163N 21.40915E	MH800911 MH800912
<i>Poecilimon hoelzeli</i> Harz, 1966		–	–	AM886726	ULLRICH <i>et al.</i> (unpublished)
<i>Poecilimon jablanicensis</i> Chobanov & Heller, 2010		North Macedonia, Jablanica Mt	41.2302N 20.5131E	MN737107 MN737108	This study This study
<i>Poecilimon nobilis</i> Brunner von Wattenwyl, 1878		–	–	AM886695	ULLRICH <i>et al.</i> (unpublished)
<i>Poecilimon obesus</i> Brunner von Wattenwyl, 1878		–	–	AM886773	ULLRICH <i>et al.</i> (unpublished)
<i>Poecilimon pindos</i> Willemse, 1982		–	–	AM886765	ULLRICH <i>et al.</i> (unpublished)
<i>Poecilimon artedentatus</i> Heller, 1984		–	–	AM886816	ULLRICH <i>et al.</i> (unpublished)
<i>Poecilimon gracilis</i> (Fieber, 1853)		Montenegro, Mratinje	43.25216 N 18.81014E	MH800909 MH800910	This study This study
<i>Poecilimon gracilioides</i> Willemse & Heller, 1992	–	–	AM886751	ULLRICH <i>et al.</i> (unpublished)	
<i>Poecilimon ampliatus</i> group	<i>Poecilimon ampliatus</i> Brunner von Wattenwyl, 1878	Montenegro, Durmitor	43.15107N 19.08135E	MH800913 MH800914	This study This study
<i>Poecilimon</i> genus	<i>Poecilimon ukrainicus</i> Bey-Bienko, 1951	–	–	AM886832	ULLRICH <i>et al.</i> (unpublished)
<i>Poecilimon heroicus</i> group	<i>Poecilimon heroicus</i> Stshelkanovtzev, 1911	–	–	AM886756	ULLRICH <i>et al.</i> (unpublished)
<i>Poecilimon</i> genus	<i>Poecilimon schmidti</i> (Fieber, 1853)	–	–	AM886810	ULLRICH <i>et al.</i> (unpublished)
subfamily <i>Phaneropterinae</i>	<i>Polysarcus denticauda</i> (Charpentier, 1825)	–	–	AM886784	ULLRICH <i>et al.</i> (unpublished)

Two different phylogenetic methods, Bayesian inference (BI) and maximum likelihood (ML) were used to infer evolutionary relationships. BI was performed with 6,000,000 generations, with a sampling of trees every 100 generations. Likelihood values were observed with Tracer v.1.5 (RAMBAUT & DRUMMOND 2003-2009). ML analysis was implemented in Phylml (GUINDON & GASCUEL 2003). 1,000 pseudoreplicates were generated for bootstrapping analyses. The trees were visualized by FigTree 1.4.4 (RAMBAUT & DRUMMOND 2002-2013).

## Results and Discussion

The final alignment of the COI gene used for phylogenetic analyses was ~ 826 bp. Of these sites, 303 were variable sites and 239 were parsimony-informative sites. The average base composition was 29.6% A, 38.0% T, 18.9% C, 13.5% G, with the A+T contents higher than those of G+C, which is a pattern that has been repeatedly seen in the mtDNA of insects. The evolution model, SYM+G (gamma distribution shape parameter  $G = 0.9910$ ), was determined to be the most justified. The Bayesian inference and maximum likelihood analyses showed similar trees. The difference between them was in the degree of statistical support for the recovered nodes (Fig. 1). ML bootstrap values (bv) were lower than BI posterior probabilities (pp). The genetic distances between the *Poecilimon affinis* complex and other representatives from the *Poecilimon ornatus* group are presented in Table 2. The genetic distance was greater between the *P. affinis* complex and the outgroup (4%) than that between *P. affinis* and *P. ornatus* (1%), which may indicate a variability within the complex.

The tree (Fig. 1) was divided into four clades (I, II, IV, V) and one paraphyletic group (III). Species from the outgroup were not considered as a clade in this study. The first clade consisted of *Poecilimon gracilis*. The second clade included six species from the *Poecilimon ornatus* group (*Poecilimon gracilioides*, *P. soulion*, *P. jablanicensis*, *P. obesus*, *P. nobilis*, and *P. artedentatus*). The group (III) contained one subspecies from the *P. affinis* complex (*P. affinis affinis*). The fourth clade was comprised of two species from the *P. ornatus* group (*P. hoelzeli* and *P. pindos*) and one subspecies from the *P. affinis* complex (*P. affinis*

*dinaricus*). The last, fifth clade included the other representatives of the *Poecilimon affinis* complex and two species from the *P. ornatus* group (*P. hoelzeli* and *P. ornatus*). The species that were initially identified as a *Poecilimon affinis* complex did not form a monophyletic group, two subspecies were present in group III and other representatives in clade V. The relationships within clade V were not well resolved with many polytomous nodes. Clade V includes 22 branches (ca. one third of all branches) with a single terminal taxon: two subspecies of *P. affinis* (*P. a. affinis*, *P. a. hajlensis* and *P. a. serbicus*) and two species of *Poecilimon* (*P. pseudornatus* and *P. nonveilleri*).

This study verifies the division of the *Poecilimon ornatus* group suggested by CHOBANOV & HELLER, 2010, taking into account various factors:

Factor (1) is based on the localities where the species occur: (i) Bulgaria and North Macedonia, (ii) Greece. The first group consists of large and bulky animals (*P. ornatus*, *P. affinis*, *P. hoelzeli* – clade V) or small and slender ones (*P. gracilis* – clade I, *P. jablanicensis* – clade II). The phylogenetic tree (Fig. 1) confirms a strong relationship between large and bulky species with high posterior probability (pp = 1.00). The second group contains species distributed in Greece: *P. pindos*, *P. obesus*, *P. artedentatus*, *P. nobilis*, *P. soulion*, and *P. gracilioides*. Results (Fig. 1) did not confirm a close relationship within this group. *Poecilimon pindos* (clade IV) is more closely related to *P. hoelzeli* (from Bulgaria) than to other representatives from Greece;

Factor (2) is a division of species according to the morphology of four groups: (I) *P. gracilis* appears to be a sister taxon to the hypothetical ancestor of the *P. ornatus* group. On the tree (Fig. 1), this species occupies the most distant position, which confirms the above assumptions (pp = 0.89); (II) The southern stem includes two subgroups: (A) *P. gracilioides* and *P. soulion* are morphologically similar to *P. gracilis* and are distributed south of its range; (B) *P. nobilis*, *P. obesus*, and *P. artedentatus* are morphologically similar to each other. This division is confirmed by molecular data (Fig. 1) with high statistical support (pp = 0.97 and pp = 1.00, respectively); (III) The northern stem consists of four sibling species: *P. pindos*, *P. hoelzeli*, *P. affinis*, and *P. ornatus*. *P. pindos* shows some similarity with two species from the southern stem A (*P. gracilioides* and *P. soulion*), but generally,

Table 2

Net mean genetic distances (%) between the *Poecilimon affinis* complex, other representatives from the *Poecilimon ornatus* group, and the outgroup

	<i>P. affinis</i> complex	<i>P. ornatus</i> group	outgroup
<i>P. affinis</i> complex	–	–	–
<i>P. ornatus</i> group	0.01	–	–
outgroup	0.04	0.01	–



the species in this stem have much more pronounced apomorphies (both species in clade V). The last (IV) group includes only one species *P. jablanicensis* which is morphologically closest to *P. gracilis*. However, due to many autapomorphies it is considered separately. Molecular analysis shows that *P. jablanicensis* is more associated with *P. gracilioides* and *P. soulion* than *P. gracilis* (pp = 0.91);

Factor (3) differentiates species by habitat and/or altitude preferences into three groups: (I) *P. affinis*, *P. ornatus*, and *P. gracilis*, the most widely distributed species in this group, and *P. hoelzeli* which has a restricted distribution. These species prefer high altitudes, except for *P. ornatus* which has less restricted distribution, occurring in the lowlands in Slovenia and from about 300-500 m a.s.l. in Bulgaria and North Macedonia up to 2400-2450 m a.s.l. in the Pirin Mts. The present study showed a strong relationship between *P. affinis*, *P. ornatus*, and *P. hoelzeli* (all occur in clade V) as opposed to *P. gracilis* which is in clade I (Fig. 1); (II) *P. pindos*, *P. soulion*, *P. gracilioides*, and *P. jablanicensis* are intermediate between the first and third group. They prefer to live at altitudes from 1500 to 2100 m a.s.l. However, *P. soulion* is closer to the third group occurring down to 1200 m. The phylogenetic tree (Fig. 1) shows that *P. pindos* is closely related to *P. hoelzeli* (pp = 1.00) which is located in the first group. The other species from the second group have a strong relationship with high statistical support (pp = 0.91); (III) The last group includes the southern species *B. Poecilimon nobilis* is found up to 2000 m a.s.l. *Poecilimon artedentatus* prefers lower altitudes from 500 to 1000 m a.s.l. *Poecilimon obesus* has a strong preference for lowlands. Present results confirm the affinity between these species with high posterior probability (pp = 1.00; Fig. 1);

Factor (4) is distinguished by bioacoustics. A close relationship between *P. obesus* and *P. nobilis*, *P. soulion*, and *P. gracilioides* as well as *P. pindos* and *P. hoelzeli* is shown on the phylogenetic tree (Fig. 1) which is partly consistent with previous bioacoustic data (CHOBANOV & HELLER, 2010).

ULLRICH *et al.* (2010) conducted an analysis on the *Poecilimon ornatus* group using ribosomal internal transcribed spacers (ITS 1 and 2). However, it did not provide conclusive information on the relationship between species in this group, either. Despite numerous polytomies, it can be said that the *P. ornatus* group is monophyletic, which is confirmed by the current study (Fig. 1).

In conclusion, the previous division described by CHOBANOV & HELLER (2010) was confirmed only in some parts. In the factor based on localities, only species from Bulgaria and North Macedonia are related. According to morphology, *P. gracilis* is the most distant species from the *P. ornatus* group. The preferences of altitude are not connected with relationships

between species. In the bioacoustics group, only species from type two have a strong affinity. To confirm the exact relationships between taxa from the *Poecilimon ornatus* group and *Poecilimon* genus, additional analysis based on mitochondrial and nuclear genes must be performed.

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## Author Contributions

Research concept and design, collection and/or assembly of data, data analysis and interpretation, writing the article, critical revision of the article, final approval of article – M.K.

## Conflict of Interest

The author declares no conflict of interest.

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