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

Maciej KOCIŃSKI

Accepted January 21, 2020Published online March 19, 2020Issue online March 31, 2020

Original article

KOCIŃSKI M. 2020. The relationships within the Poecilimon ornatus group (Orthoptera: Phaneropterinae) based on the cytochrome c oxidase I gene. Folia Biologica (Kraków) 68: 7-13.

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.

Maciej KOCIŃSKI, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Kraków, Poland.
E-mail: kocinski@isez.pan.krakow.pl

Poecilimon Fischer, 1853 is one of the largest genera in the subfamily Phaneropterinae Burmeister, 1838 with 142 species classified under 18 species groups (P. ampliatus, P. armentiacus, P. bosphoricus, P. celebi, P. concinnus, P. davisi, P. elegans, P. heroicus, P. inflatus, P. jonicus, P. hischani, P. minutus, P. ornatus, P. pergamicus, P. propinguus, 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 & SEVGLI 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 &
**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. ukrajinicus* Bey-Bienko, 1951; and *P. schmidti* (Fieber, 1853). *Polysarcus denticauda* (Charpentier, 1825) was treated as an outgroup. Insects were collected in the Balkan Peninsula (Bulgaria, Serbia, Montenegro, Albania, 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, Duren, 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).

A 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 MgCl₂, 2.0 µl 10xPCR buffer, 5 µl of Gold Taq DNA polymerase (Syngen, Wroclaw, 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, Duren, 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) 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 |
|------|---------|----------|-----------------------|-------------------|-----------|
| Poecilimon affinis complex | Poecilimon affinis (Frivaldszky, 1868) | Ukraine, Chereska Oblast | 55.09285N 33.57554E | MH800893 MH800894 MH800895 | This study |
| | | Bulgaria, Rila Mts., Iliyna Reka | 42.09874N 23.55717E | MH800896 MH800897 MH800898 | This study |
| | | Bulgaria, Pirin Mts., Yavorov Chalet | 41.82365N 23.37846E | MH800899 MH800900 MH800901 | This study |
| | | Bulgaria, Osogovo Mts. | 42.1884N 22.5804E | MH800902 MH800903 MH800904 | This study |
| | | Bulgaria, Rila Mts., Kirilova Polyana | 43.1593N 23.37846E | MH800905 MH800906 MH800907 | This study |
| | | Bulgaria, Sredna Gora Mts., Bratiya peak | 42.5910N 24.15718E | MH800908 MH800909 MH800910 | This study |
| Poecilimon affinis komareki | | | | | |
| | | Albania, Laç | 41.63168 N 19.752 E | MH800867 MH800868 MH800869 | This study |
| | | Montenegro, Kolasin | 42.79198N 19.42646E | MH800873 MH800874 MH800875 | This study |
| Poecilimon affinis dinaricus | | Montenegro, Susica | 43.1726N 19E | MH800856 | This study |
| | | Montenegro, Mratinje | 43.2477N 18.8317E | MH800876 | This study |
| Poecilimon affinis sericus | | North Macedonia, Shar Mts, Ljuboten Park | 41.9845N 21.0521E | MH800861 MH800862 MH800863 | This study |
| | | Montenegro, Hajla | 42.80296N 20.22638E | MH800864 MH800865 MH800866 | This study |
| Poecilimon affinis poecilus | | North Macedonia, Shar Mts., Popova Shapka | 42.0125N 20.83199E | MH800870 MH800871 MH800872 | This study |
| | | Montenegro, Susica | 43.1726N 19E | MH800873 MH800874 MH800875 | This study |
| Poecilimon nonveilleri | | Montenegro, Durmitor, Boriçe | 43.14251N 18.92046E | MH800876 MH800877 MH800878 | This study |
| | | Montenegro, Treshnievik | 42.72348N 19.68335E | MH800879 MH800880 MH800881 | This study |
| | | Montenegro, Vusanje | 42.5193N 19.86526E | MH800882 MH800883 MH800884 | This study |
| | | Montenegro, Hajla | 42.81517N 20.18815E | MH800885 MH800886 MH800887 | This study |
| | | Serbia, Kamena Gora | 43.32959N 19.578E | MH800888 MH800889 MH800890 | This study |
| Poecilimon ornatus group | | North Macedonia, Jakupica Mts., Cheples Chalet | 41.71163N 21.40915E | MH800901 MH800912 | This study |
| | Poecilimon hoelzeli | | – – | AM886726 | ULLRICH et al. (unpublished) |
| | Poecilimon javanicensis | | – – | AM886695 | ULLRICH et al. (unpublished) |
| | Poecilimon nobilitis | | – – | AM886773 | ULLRICH et al. (unpublished) |
| | Poecilimon ohebus | | – – | AM886765 | ULLRICH et al. (unpublished) |
| | Poecilimon pindos | | – – | AM886616 | ULLRICH et al. (unpublished) |
| | Poecilimon artedentatus | | – – | AM886675 | ULLRICH et al. (unpublished) |
| | Poecilimon gracilis | | – – | AM886751 | ULLRICH et al. (unpublished) |
| | Poecilimon graciloides | | – – | AM886832 | ULLRICH et al. (unpublished) |
| | Poecilimon hercogenus | | – – | AM886874 | ULLRICH et al. (unpublished) |
| | Poecilimon amplatus group | | – – | AM886875 | ULLRICH et al. (unpublished) |
| | Poecilimon schmidti | | – – | AM886874 | ULLRICH et al. (unpublished) |
| | Poecilimon客气 genus | | – – | AM886875 | ULLRICH et al. (unpublished) |
| | subfamily Phaneropterinae | | – – | AM886874 | ULLRICH et al. (unpublished) |

Relationships within the *Poecilimon ornatus* group

Poecilimon ornatus (Schmidt, 1850) North Macedonia, Jakupica Mts., Cheples Chalet 41.71163N 21.40915E MH800901 MH800912 This study
Poecilimon hoelzeli Harz, 1966 – – AM886726 ULLRICH et al. (unpublished)
Poecilimon javanicensis Chobanov & Heller, 2010 North Macedonia, Jablanica Mt 41.2302N 20.5131E MN737107 MN737108 This study
Poecilimon nobilitis Brunner von Wattenwyl, 1878 – – AM886695 ULLRICH et al. (unpublished)
Poecilimon ohebus Brunner von Wattenwyl, 1878 – – AM886773 ULLRICH et al. (unpublished)
Poecilimon pindos Willemsen, 1982 – – AM886765 ULLRICH et al. (unpublished)
Poecilimon artedentatus Heller, 1984 – – AM886616 ULLRICH et al. (unpublished)
Poecilimon gracilis (Fieber, 1853) Montenegro, Mratinje 43.25216N 18.81014E MH800909 MH800910 This study
Poecilimon graciloides Willemsen & Heller, 1992 – – AM886751 ULLRICH et al. (unpublished)
Poecilimon amplatus group Poecilimon amplatus Brunner von Wattenwyl, 1878 Montenegro, Durmitor 43.15107N 19.08135E MH800913 MH800914 This study
Poecilimon schmidti (Fieber, 1853) – – AM886832 ULLRICH et al. (unpublished)
Poecilimon hercogenus group Poecilimon hercogenus Stshelkanovtzev, 1911 – – AM886756 ULLRICH et al. (unpublished)
Poecilimon schmidti (Fieber, 1853) – – AM886810 ULLRICH et al. (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 Phyml (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, SY M +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. jablanciensis, P. obesus, P. nobilis, and P. artendentatus). 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. serbicu s) 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. jablanciensis - 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. artendentatus, 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. artendentatus 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 |
| | P. affinis complex | P. ornatus group | outgroup |
| P. affinis complex | – | – | – |
| P. ornatus group | 0.01 | – | – |
| outgroup | 0.04 | 0.01 | – |
Fig. 1. Bayesian tree of the *Poecilimon ornatus* group based on COI sequences. Node labels indicate BI posterior probability (pp) and maximum likelihood bootstrap values (bv) over 50% (pp/bv). Green lines show specimens from the *Poecilimon affinis* complex; blue lines – the *Poecilimon ornatus* group; red lines – specimens from the outgroup. Scale bar = 0.02 substitutions per position.
the species in this stem have much more pronounced apomorphies (both species in clade V). The last (IV) group includes only one species P. jablancensis which is morphologically closest to P. gracilis. However, due to many autapomorphies it is considered separately. Molecular analysis shows that P. jablancensis 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. jablancensis 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. A grouping 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.

Acknowledgements

I am grateful to Dragan Chobanov, Klaus-Gerhard Heller and Slobodan Ivković for providing material to this study.

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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