A taxonomic outline of the *Poecilimon affinis* complex (Orthoptera) using the geometric morphometric approach

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The genus *Poecilimon* contains 142 species, widely distributed in the Palaearctic, among which *Poecilimon ornatus* group has the greatest diversity in the Balkans. Despite several revisions of the genus, the systematics of the species group, and in particular of taxa associated with the species *Poecilimon affinis*, is still unsolved. Due to morphological similarity, *P. affinis* with its subspecies, *P. nonveilleri* and *P. pseudornatus* form *Poecilimon affinis* complex. The aim of this study is to test the hypotheses of an outlined species complex, namely *P. affinis* complex, within *P. ornatus* group using morphological data. Geometric analysis was conducted to explore variation in the structure of tegmen, ovipositor, cercus, and pronotum. Number of teeth and stridulatory file measurements provided additional information on morphological variation within the complex. A phylogenetic tree based on the cytochrome c oxidase subunit I gene (COI) was used for comparison with the morphological data. Canonical variate analysis showed that tegmen and cercus are good morphostructures to distinguish taxa belonging to *P. affinis* complex from other species in the *P. ornatus* group. This may confirm our assumptions for the designation of the *P. affinis* complex. Results of the principal component analysis of stridulatory file measurements, molecular data and CVA of ovipositor suggest adding to the complex two additional species: *P. ornatus* and *P. hoelzeli*.

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Abstract

The genus *Poecilimon* contains 142 species, widely distributed in the Palaearctic, among which *Poecilimon ornatus* group has the greatest diversity in the Balkans. Despite several revisions of the genus, the systematics of the species group, and in particular of taxa associated with the species *Poecilimon affinis*, is still unsolved. Due to morphological similarity, *P. affinis* with its subspecies, *P. nonveilleri* and *P. pseudornatus* form *Poecilimon affinis* complex. The aim of this study is to test the hypotheses of an outlined species complex, namely *P. affinis* complex, within *P. ornatus* group using morphological data. Geometric analysis was conducted to explore variation in the structure of tegmen, ovipositor, cercus, and pronotum. A number of teeth and stridulatory file measurements provided additional information on morphological variation within the complex. A phylogenetic tree based on the cytochrome c oxidase subunit I gene (COI) was used for comparison with the morphological data. Canonical variate analysis showed that tegmen and cercus are good morphostructures to distinguish taxa belonging to *P. affinis* complex from other species in the *P. ornatus* group. This may confirm our assumptions for the designation of the *P. affinis* complex. Results of the principal component analysis of stridulatory file measurements, molecular data and CVA of ovipositor suggest adding to the complex two additional species: *P. ornatus* and *P. hoelzeli*.

Introduction

Poecilimon Fischer, 1853 is the most species-rich genus within the Phaneropterinae subfamily. This genus comprises 142 species distributed in the Palearctic region (*Cigliano et al., 2021*). All species are short-winged and flightless herbivorous bush-crickets with complex acoustic behavior (*Heller, 1990*). *Poecilimon* is currently divided into 18 species groups based on molecular, morphological and bioacoustic data, while 16 species are not assigned to any of them (*Cigliano et al., 2021*). Similarity and variability of morphological characters make many *Poecilimon* species difficult to identify. *Poecilimon ornatus* group (13 species) (Fig. 1) is one of the groups for which the phylogenetic relationships between species remain unclear and the status of several taxa is under discussion. Due to the reduced wings and the influence of climatic and geomorphological factors, a rapid morphological evolution in this group took place (*Chobanov & Heller, 2010*).

The first revision of *Poecilimon* was conducted by *Ramme (1933)*, who included taxa from the currently recognized *Poecilimon ornatus* group in “Gruppe I.” In *1984, Heller* suggested dividing the group into eight taxa (*P. nobilis* Brunner von Wattenwyl, 1878; *P. obesus obesus* Brunner von Wattenwyl, 1878; *P. obesus artedentatus* Heller, 1984; *P. affinis affinis* (Frivaldszky, 1867); *P. affinis komareki* Cejchan, 1957; *P. affinis hoelzeli* Harz, 1966; *P. ornatus* (Schmidt, 1850) and *P. pancici* Karaman, 1958; distributed mainly in the Balkans). Later, *P. artedentatus* and *P. hoelzeli* were given species status (*Willemse, 1985; Willemse & Heller, 1992*), while *P. pancici* was synonymized (*Willemse, 1985*). Further, six new species were described *(P. pindos* F. Willemse, 1982; *P. soulion* L. Willemse, 1987; *P. gracilioides* F. Willemse & Heller, 1992; *P. jablanicensis* Chobanov & Heller, 2010; *P. pseudornatus* Ingrisch & Pavicevic, 2010; *P. nonveilleri* Ingrisch & Pavicevic, 2010).

Among the *P. ornatus* group, *P. affinis* has the widest geographic range. It is distributed from northern Greece to the Carpathians in Romania and an isolated spot in Ukraine. According to *Cigliano et al., (2021)*, *P. affinis* consists of five subspecies (*P. affinis affinis* (Frivaldszky, 1868); *P. a. dinaricus* Ingrisch & Pavicevic, 2010; *P. a. hajlensis* Karaman, 1974; *P. a. komareki* Cejchan, 1957 and *P. a. serbicus* Karaman, 1974). *Karaman (1974)* reduced the status of *P. poecilus* Ramme, 1951 to a subspecies of *P. affinis* and described two new subspecies: *P. a. serbicus* and *P. a. hajlensis*. In *1984, Heller* suggested that *P. poecilus* and *P. a. affinis* are synonymous. Due to doubts about the taxonomic status of *P. poecilus*, in the present study it will be treated separately. *P. komareki* was described by Cejchan (1957), but *Heller (1984)* regarded it as a subspecies of *P. affinis* because of their similarity. *Karaman (1972)* described *P. komareki rumijae* based on the shape of the pronotum and body size. Because of the lowering the status of *P. komareki* to a subspecies of *P. affinis*, *P. k. rumijae* became synonymous of *P. a. komareki* , which was confirmed by *Chobanov and Heller (2010)*. On the other hand, *Ingrisch & Pavicevic (2010)* suggested regarding *P. rumijae* as a separate species, differing distinctly from *P. affinis*. Morphological variability in these taxa was determined only based on minor differences. Also, there are almost no differences in the male song pattern (*Chobanov & Heller, 2010*). Due to the discrepancy between the authors, *P. rumijae* will also be treated separately in the present study. *P. pseudornatus*, *P. nonveilleri* and the subspecies of *P. affinis* are morphologically similar, although a recent molecular study based on cytochrome c oxidase I gene has shown that the above taxa do not form a monophyletic group (*Kociński, 2020*). The lack of clear boundaries between them and the unsolved phylogenetic relationship suggest that *P. pseudornatus*, *P. nonveilleri* and subspecies of *P. affinis* should be treated as *P. affinis* complex.

The ‘species complex’ is an informal taxonomic term showing the uncertainty of a taxonomic identification (*Sigovini et al., 2016*) and it is commonly used in insects (e.g., *Genier & Moretto, 2017; Manani et al., 2017; Elfekih et al., 2018; Selnekovič & Kodada, 2019*). It may be defined as a group of very closely-related taxa with similar morphology and hard to distinguish from another. Taxa from a complex require a critical revision in order to clarify the actual taxonomic position (*Sigovini et al., 2016*).

To circumscribe the morphological variation and to understand the relationships within the *Poecilimon ornatus* group, especially within the *Poecilimon affinis* complex, we used geometric morphometric methods based on the shape variation of four structures: pronotum, cercus, ovipositor, and tegmen (Fig. 2). Geometric morphometrics is an approach that applies the landmark coordinates, which are the correspondence points marked on a given morphostructure and are the same in all studied specimens or species (*Bookstein, 1991; Dryden & Mardia, 1998*). This method considers the spatial relationships between landmark variables, therefore providing more powerful statistical results. It is also possible to find and analyze shape variations in the species within and between populations (*Walker & Bell, 2000*). The geometric morphometrics method has been proved to be very useful to distinguish species in insects (*Nunes et al., 2012; Prado-Silva et al., 2016; da Silva et al., 2018*), especially in Orthoptera (*Romero, Rosetti & Remis, 2014; Barcebal et al., 2015; Kaya, Boztepe & Ciplak, 2015; Kaya et al., 2015; Mugleston et al., 2016; Bian & Shi, 2018; Pan, Hong & Jiang, 2018; Liu, Chen & Liu, 2020*). The aim of the present study is to assess the morphological diversity of species within *P. ornatus* group and reconstruct their relationship. We test the hypothesis of the existence of the *P. affinis* complex.

Materials & Methods

Specimen collection

Bush-crickets were collected in the Balkan Peninsula (Bulgaria, Serbia, Montenegro, Albania, North Macedonia, Greece) between 2017 and 2019 and stored in 96% ethanol (Table 1).

Geometric morphometrics

In total, 196 specimens belonging to 16 taxa of *Poecilimon ornatus* group were used for geometric morphometrics analyses. Four morphostructures (pronotum, cercus, ovipositor, and tegmen) were photographed using a stereomicroscope (Leica M165C) equipped with a digital camera (Leica DMC5400) under strictly maintained magnification and resolution and saved in jpg format. TPS files for each structure were created from the photographs with the software tpsUtil v.1.26 following *Rohlf (2004)*. To explore patterns of morphological variation, 8 landmarks of pronotum, 13 of cercus, 13 of tegmen, and 9 of ovipositor (Fig. 2) were plotted manually in tpsDIG2 v.2.17 (*Rohlf, 2015*). After plotting the landmarks, the intersections marked in TPS files were aligned using a Procrustes superimposition. Partial warp scores were studied using Canonical variate analysis (CVA) for each structure in MorphoJ v.1.06d (*Klingenberg, 2011*). The first two Canonical Variables (CVs) with the greatest power to distinguish groups were plotted in the same software. Mahalanobis distance was measured and statistically tested using 10,000 permutation repeats.

Stridulatory measurements

Length of the stridulatory file and number of stridulatory teeth were calculated for 154 specimens from *P. ornatus* group (9 specimens of *P. affinis*, 24 - P. *affinis affinis*, 1 - *P. affinis dinaricus*, 7 - *P. affinis hajlensis*, 5 - *P. affinis komareki*, 12 - *P. affinis serbicus*, 8 - *P. hoelzeli*, 3 - *P. jablanicensis*, 15 - *P. nobilis*, 10 - *P. nonveilleri*, 12 - *P. obesus*, 10 - *P. ornatus*, 29 - *P. pseudornatus*, 8 - *P. soulion*). Measurements were taken under stereomicroscope with the aid of an ocular micrometer. For measurement of the stridulatory file length, we used the distance from the first proximal (basal) to the last distal (apical) tooth. Total number of stridulatory teeth and number of teeth within 2 mm at the middle of the stridulatory file were counted. Measurements data were analyzed using Principal Component Analysis (PCA) in Past 4.03 (https://www.nhm.uio.no/english/research/infrastructure/past/).

Phylogenetic analyses

A fragment of the cytochrome c oxidase subunit I (COI) of mitochondrial DNA (mtDNA) was used for determining the phylogenetic relationship between taxa. We aimed to construct a phylogenetic tree focusing on the species of the *P. affinis* complex. A total of 71 sequences of 14 *Poecilimon* taxa were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). DNA sequences were aligned using CodonCode Aligner 9.0.2 (https://www.codoncode.com/aligner) with default parameters. The maximum likelihood (ML) and Bayesian inference (BI) analyses were used to infer the phylogenetic relationships. Best-fit model of nucleotide substitution was determined with jModelTest2 (*Guindon & Gascuel, 2003; Darriba et al., 2013*). ML was performed in IQ-TREE (*Nguyen et al., 2015*), whereas BI in MrBayes 3.2. (*Ronquist et al., 2012*). For bootstrap analyses, 1,000 pseudoreplicates were generated. BI was carried out with 10,000,000 generations, with a sampling of trees every 100 generations. Likelihood values were observed with Tracer v.1.7 (*Rambaut et al., 2018*). The tree was visualized in FigTree 1.4.4 (*Rambaut, 2018*).

Results

Morphology

As a result, 54 images of ovipositor, 130 of tegmen, 142 of pronotum, and 141 of cercus were used in the analyses. In some specimens, tegmen and cercus were damaged and not used for this study. The landmarks were chosen based on the shape and structure of the ovipositor (9 landmarks) (Fig. 2A), cercus (13 landmarks) (Fig. 2B), pronotum (8 landmarks) (Fig. 2C), and tegmen (13 landmarks) (Fig. 2D).

CV analysis of the tegmen (Fig. 3) revealed significant variation within the *P. ornatus* group and *P. affinis* complex. At the species group level, the first two CV analyses together accounted for 77.72% of the total variation (CV1=55.64%, CV2=22.08%). A combination of the results of the CV1 and CV2 analyses of the tegmen separated the species *P. hoelzeli*, *P. obesus*, *P. jablanicensis* and *P. nobilis* from the other species of the *Poecilimon ornatus* group and revealed an overlap between *P. pseudornatus*, *P. poecilus*, *P. nonveilleri*, and *P. affinis* (Fig. 3A). The Mahalanobis distance obtained by pairwise comparisons among the group revealed highly significant differences (10,000 permutation rounds; P<0.0001), ranging from 2.5030 (*P. affinis* and *P. pseudornatus*) to 19.6637 (*P. poecilus* and *P. obesus*). Procrustes distances also showed significant differences between groups (10,000 permutation rounds; P<0.0001) ranging from 0.0309 (*P. poecilus* and *P. pseudornatus*) to 0.2788 (*P. nobilis* and *P. obesus*) (Table S1).

At the species complex level, the first two CVs together accounted for 47.9% of the total variation of the tegmen (CV1=28.5% and CV2=19.4%). CV1 and CV2 analyses of the *Poecilimon affinis* complex did not indicate clear clusters representing each of the existing species/subspecies. However, specimens of *P. a. affinis* show differentiation in terms of their occurrence (Bratiya, Kirilova Polyana, Yavorow-Pirin, Osogovo, Rila) in contrast to *P. pseudornatus*, where specimens from different localities (Kamena Gora, Durmitor, Treschnievik, Vusanje) are grouped together (Fig. 3B). The Mahalanobis distances between taxa for tegmen are 2.7717 for *P. poecilus* and *P. pseudornatus*, and 8.1340 for *P. a. komareki* and *P. a. dinaricus* (10,000 permutation rounds; P<0.0001). Procrustes distances also showed significant differences (10,000 permutation rounds; P<0.001), ranging from 0.0251 (*P. a. serbicus* and *P. pseudornatus*) to 0.1161 (*P. rumijae* and *P. a. dinaricus*) (Table S2).

For the ovipositor, at the species group level, the first two CVs together accounted for 78.43% of the total variation (CV1=54.78%, CV2=23.65%) **(**Fig. 4A). The scatter plot from CV1 and CV2 shows that species from the *Poecilimon affinis* complex cannot be clearly separated from other species of the *Poecilimon ornatus* group **(**Fig. 4A). The Mahalanobis distances obtained by pairwise comparisons among group revealed highly significant differences (10,000 permutation rounds, P<0.0001), ranging from 2.7815 (*P. poecilus* and *P. hoelzeli*) to 15.7156 (*P. gracilis* and *P. nobilis*). Procrustes distances also showed significant differences between groups (10,000 permutation rounds, P<0.0001) ranging from 0.0351 (*P. affinis* and *P. hoelzeli*) to 0.1941 (*P. pseudornatus* and *P. gracilis*) (Table S3).

At the complex species level, the first two CVs together accounted for 83.92% of the total variation of the ovipositor (CV1=70.26% and CV2=13.66%) (Fig. 4B). The centroid size (the square root of the sum of the squared distances of all landmarks from their centroid) of CV1 and CV2 shows that species from the *Poecilimon affinis* complex can be clearly separated from each other (Fig. 4B). The Mahalanobis distances obtained by pairwise comparisons of the complex revealed highly significant differences (10,000 permutation rounds; P<0.0001), ranging from 2.6873 (*P. rumijae* and *P. a. affinis*) to 14.5025 (*P. pseudornatus* and *P. a. hajlensis*). Procrustes distances also showed highly significant differences (10,000 permutation rounds; P<0,005), ranging from 0.0319 (*P. a. serbicus* and *P. a. affinis*) to 0.1542 (*P. a. komareki* and *P. a. dinaricus*) (Table S4).

CV analysis of the cercus (Fig. 5) also revealed significant variation within the *P. ornatus* group and the *P. affinis* complex. At the group level, the first two CVs together accounted for 69.82% of the total variation (CV1=40.59%, CV2=29.23%). The scatter plot from CV1 and CV2 shows that species from the *Poecilimon affinis* complex can be clearly separated from other species of the *Poecilimon ornatus* group (Fig. 5A). The Mahalanobis distances obtained by pairwise comparisons among group revealed highly significant differences (10,000 permutation rounds; P<0.0001), ranging from 2.7073 (*P. pseudornatus* and *P. affinis*) to 12.2488 (*P. hoelzeli* and *P. jablanicensis*). Procrustes distances also showed significant differences between groups (10,000 permutation rounds; P<0.0001), ranging from 0.0339 (*P. affinis* and *P. pseudornatus*) to 0.1706 (*P. pseudornatus* and *P. nobilis*) (Table S5).

For the cercus, at the complex level, the first two CVs together accounted for 54.33% of the total variation (CV1=30.38% and CV2 =23.95%). The centroid size of CV1 and CV2 shows that only *P. a. affinis*, *P. rumijae*, *P. a. komareki*, and *P. nonveilleri* can be clearly separated from other members of the *P. affinis* complex (Fig. 5B). Mahalanobis distances obtained by pairwise comparisons of the complex revealed significant differences (10,000 permutation rounds; P<0.0001), ranging from 2.8732 (*P. pseudornatus* and *P. a. hajlensis*) to 8.6480 (*P. a. dinaricus* and *P. a. komareki*). Procrustes distances also showed significant differences (10,000 permutation rounds; P<0.0001), ranging from 0.0301(*P. a. affinis* and *P. poecilus*) to 0.0985 (*P. a. komareki* and *P. nonveilleri*) (Table S6).

For the pronotum, at the group level, the first two CVs together accounted for 75.84% of the total variation (CV1 =57.24%, CV2=18,60%) (Fig. 6). The scatter plot from CV1 and CV2 shows that species from the *Poecilimon affinis* complex cannot be clearly separated from other species of the *Poecilimon ornatus* group (Fig. 6A). The Mahalanobis distances obtained by pairwise comparisons among group revealed significant differences (10,000 permutation rounds; P<0.0001), ranging from 2.2038 (*P. poecilus* and *P. affinis*) to 12.8096 (*P. gracilis* and *P. obesus*). Procrustes distances also showed significant differences between groups (10,000 permutation rounds; P<0.0001), ranging from 0.0286 (*P. poecilus* and *P. affinis*) to 0.1591 (*P. gracilis* and *P. jablanicensis*) (Table S7).

At the complex level, the first two CVs together accounted for 72.01% of the total variation of the pronotum (CV1=46.56% and CV2 =25.45%). The centroid size of CV1 and CV2 shows that only *P. rumijae* can be clearly separated from other species from *P. affinis* complex (Fig. 6B). Mahalanobis distances obtained by pairwise comparisons of the complex revealed significant differences (10,000 permutation rounds; P<0.0001), ranging from 2.7308 (*P. a. hajlensis* and *P. a. affinis*) to 5.6766 (*P. rumijae* and *P. nonveilleri*). Procrustes distances also showed highly significant differences (10,000 permutation rounds; P<0.0001), ranging from 0.0369 (*P. poecilus* and *P. a. affinis*) to 0.1351 (*P. rumijae* and *P. nonveilleri*) (Table S8).

Stridulatory measurements

P. soulion and *P. jablanicensis* have the shortest stridulatory file of all studied species (2.74 – 3.17 and 2.96-3.04, respectively). In contrast, *P. affinis komareki* has the longest stridulatory file (5.34-5.88) and the greatest number of teeth on its structure (158-195). *P. obesus* has the lowest number of teeth, which proves that the length of the stridulatory file does not correlate with the number of teeth (Table 2). Principal Component Analysis of stridulatory file and number of teeth shows that *P. nonveilleri, P. ornatus, P. hoelzeli. P. pseudornatus, P. a. serbicus, P. a. hajlensis*, and *P. a. affinis* are overlapping. Moreover, we can conclude that *P. a. affinis* is the most diverse taxon within *P. ornatus* group, while *P. a. komareki* is the most distinct taxon of the studied group (Fig. 7).

Phylogenetic analyses

The final alignment consists of 607 bp, of which 450 were conservative, 157 variable and 83 parsimony-informative sites. HKY+G was selected as the best-fit evolution model for site substitution. The topologies obtained from BI and ML analyses were similar. Bootstrap values (ML) (>50%) and BI posterior probabilities (>0.5) are shown on the nodes of the tree presented on Fig. 8. To root the tree, *Poecilimon cervus* Karabag, 1950, belonging to the *Poecilimon bosphoricus* Brunner von Wattenwyl, 1878 species group, was chosen. The BI and ML trees based on COI data shows that the *P. affinis* complex forms a paraphyletic group. The most diverse taxon in the complex is *P. a. affinis*, occupying different nodes on the phylogenetic tree due to its place of occurrence. *Poecilimon a. affinis* from Kirilova Polyana (Bulgaria, Rila Mtns) occupies a basal position in the tree and seems to be a sister taxon to the remaining taxa of the complex. Two species of the *P. ornatus* group, preliminary left outside the *P. affinis* complex, *P. ornatus* and *P. hoelzeli,* were placed within the same clade (Fig. 8).

Discussion

Morphology

This work aimed to determine the morphological characteristics that separate bush-crickets belonging to the *P. affinis* complex from other species of the *P. ornatus* group through the geometric morphometrics approach. The morphology of the tegmen, ovipositor, cercus and pronotum were used successfully in morphological studies of *Poecilimon* (*Heller, 2004*; *Chobanov & Heller, 2010*; *Kaya et al., 2012*; *Kaya, Boztepe & Ciplak, 2015*; *Kaya et al., 2018*). The present work exhibited that the studied morphostructures can partly be used to separate taxa of the species rank in the *Poecilimon ornatus* group. *Chobanov & Heller (2010)* noticed that the pronotal shape and the size of the area of the tegmen covered by the pronotum vary between specimens from the same locality. Our results support the poor taxonomic utility of the shape of pronotum in this group in distinguishing species belonging to the *P. affinis* complex from other species in the group (Fig. 6A). However, based on the shape of the tegmen, *P. affinis* and its subspecies group with *P. nonveilleri*, *P. pseudornatus* in the same place, and this clearly separates them from other species (Fig. 3A). This may confirm our assumptions for the designation of the *P. affinis* complex including other species from the *Poecilimon ornatus* group. CV analysis of centroid sizes of the pronotum (Fig. 6B) shows that *P. rumijae* is the most distinct taxon among the *P. affinis* complex, and does not overlap with *P. a. komareki*. Probably, *P. rumijae* could be treated as a separate species of the *P. ornatus* group, differing distinctly from subspecies of *P. affinis (Ingrisch & Pavicevic, 2010)*, but further studies are required to confirm its taxonomic position. This supposition is also confirmed by the ovipositor's analysis, where *P. a. komareki* is more similar to *P. a. dinaricus* and *P. pseudornatus*, whereas *P. rumijae* is more similar to *P. a. affinis* (Fig. 4B). On the other hand, the results based on cercus (Fig. 5B) show that *P. a. komareki* and *P. rumijae* are overlapping, which proves high similarities within this morphostructure and may confirm the accuracy of lowering *P. rumijae* to the rank synonymous with *P. a. komareki* (*Chobanov & Heller, 2010*; *Cigliano et al., 2021*). *Ingrisch & Pavicevic (2010)* considered *P. rumijae* to be similar to *P. nonveilleri* and *P. affinis*. Our results confirm a close relationship between *P. rumijae* and *P. affinis*, but not between P. *rumijae* and *P. nonveilleri*, which, according to all morphostructures, are the most distant from each other (Fig. 3A, 4A, 5A, 6A).

The most distinct species in our sample is *P. nobilis* based on the analysis of the tegmen (Fig. 3A) and cercus (Fig. 5A), *P. gracilis* based on ovipositor (Fig. 4A), and *P. obesus* based on pronotum (Fig. 6A), which suggest not to include these species in the *P. affinis* complex. On the other hand, *P. affinis* is the most diffuse taxon in the group (Fig. 3A, 4A, 5A, 6A). The results suggest that the difference between specimens of *P. a. affinis* is related to the locality in which it occurs (Fig. 3B), and is generally connected with altitude (*Chobanov & Heller, 2010*). Specimens of *P. a. affinis* from Pirin are distant from individuals from Bratiya, Kirilowa Polyana, Osogovo, Rila and are more closely related to *P. poecilus*, *P. a. hajlensis* and *P. a. komareki* (Fig. 3B). On the other hand, the position of the centroid size of *P. pseudornatus* from different localities (Durmitor, Kamena Gora, Treshnievik, Vusanje) overlaps, which proves a lower morphological variability in terms of location than in the case of *P. a. affinis* (Fig. 3B). At the group level based on cercus (Fig. 5A), species from the *P. affinis* complex (*P. affinis* with its subspecies, *P. nonveilleri* and *P. pseudornatus*) overlap. Thus, this is the second morphostructure to confirm the existence of this complex. Additionally, *Chobanov & Heller (2010)* suggested that cercus may be a better feature for separating species in this group. Results of the CV analysis of centroid size of the ovipositor (Fig. 4A) shows the similarity between *P. affinis*, *P. hoelzeli*, *P. pseudornatus*, *P. poecilus*, and *P. nonveilleri*, which may indicate the extension of the *P. affinis* complex with *P. hoelzeli* species (Fig. 4A). *Poecilimon poecilus*, which we suggested to treat separately in this work, seems to be a subspecies of *P. a. affinis*. It is confirmed by all the morphostructures studied, where *P. poecilus* overlaps with other subspecies: *P. a. affinis, P. a. hajlensis, P. a. komareki* (Fig. 3A, 4A, 5A, 6A). However, to establish the taxonomic status of *P. poecilus*, additional research is needed.

Stridulatory measurements

Stridulatory file and number of teeth can be a good morphological feature for distinguishing taxa in *P. ornatus* group (*Heller, 1984; Willemse, 1985; Heller, 1988; Chobanov & Heller, 2010*). *Heller (1988)* reports that *P. ornatus* has fewer teeth than *P. affinis,* about 158-212, with some exceptions of large specimens having up to 220 teeth, as confirmed by our results (Table 2). The length of stridulatory file is the same in both species and averaged 4.08. Thus, this morphostructure and the number of teeth are not a good feature for distinguishing *P. affinis* from *P. ornatus. Heller (1984)* observed about 220-230 teeth in *P. affinis* species while *Chobanov & Heller (2010)* 180-240. They suggest that the number is generally more variable in southeastern populations (SW Bulgaria). The lowest number of teeth is found in small specimens from high altitudes. Principal Component Analysis (PCA) shows a similarity between three subspecies (*P. a. affinis, P. a. serbicus* and *P. a. hajlensis*) (Fig. 7). On the other hand, *P. a. komareki* is not overlapping with other subspecies, which may mean that it is the most distinct subspecies from all studied taxa of the *P. ornatus* group. *Poecilimon hoelzeli* and *P. pseudornatus* have a similar number of teeth and length of stridulatory file. *Poecilimon ornatus*, *P. nonveilleri*, *P. a. affinis, P. a. hajlensis*, *P. a. serbicus*, *P. pseudornatus* and *P. hoelzeli* overlap, which can suggest that *P. hoelzeli* and *P. ornatus* should be included in the designated *P. affinis* complex.

Phylogenetic data

The first genetic studies using ribosomal internal transcribed spacers (ITS1 and 2) involving some of the group's species were conducted by *Ullrich et al., (2010)*. However, it did not provide conclusive information on the relationship between species in this group. *Kocinski (2020)* performed a genetic analysis based on the cytochrome c oxidase I gene (COI) of the *P. ornatus* group, and confirmed the monophyly of this group. Our results, focusing on species from the *P. affinis* complex, show that it forms a paraphyletic group (Fig. 8). Two additional species, *P. hoelzeli* and *P. ornatus*, are distributed with the other taxa of the complex, thus they probably should be included in the *P. affinis* complex determined previously. This assumption is similar to the results of the CVA of the ovipositor, where taxa from the complex overlap with *P. hoelzeli* (Fig. 4A). Moreover, based on the phylogenetic tree (Fig. 8), *P. a. affinis* is the most diverse species in the complex, occupying different nodes, which is supported by the CVA results of the tegmen (Fig. 3B). The variability is related to the location (Bratiya, Kirilova Polyana, Rila, Yavorow) of the populations of *P. a. affinis*, and is connected with the altitude of occurrence (*Chobanov & Heller, 2010*). *Poecilimon a. komareki* and *P. rumijae* form different nodes, which may suggest treating them as separate species of the *P. ornatus* group or subspecies of *P. affinis*. This opinion is confirmed by the CVA results of pronotum and ovipositor (Fig. 4B, 6B). Specimens from *P. poecilus* also form different nodes than *P. a. affinis*, thus, it may be treated as its subspecies, which is supported by CVA analysis of the tegmen, cercus, ovipositor, and pronotum (Fig. 3B, 4B, 5B, 6B).

Conclusions

The geometric morphometrics method has proven to be useful in studying the morphological diversity of bush-crickets. Combined with the analysis of the stridulatory file and molecular phylogeny, it can give better insight into the relationship between species from the *Poecilimon ornatus* group and in particular the taxa of the *Poecilimon affinis* complex. Morphological analysis of selected morphostructures and molecular data showed the paraphyly of the *P. affinis* complex unless *P. ornatus* and *P. hoelzeli* are included. Additionally, the taxonomic status of the *P. rumijae* and *P. poecilus* species remains unclear. In order to reveal the evolutionary history and phylogenetic relationships within the *P. ornatus* group, a comprehensive biogeographic study based on phylogenetic analyses of mitochondrial and nuclear DNA, and species delimitation is needed.

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Figure 1

Representatives of the studied taxa from the *Poecilimon ornatus* group.

(A) *P. affinis hajlensis.* (B) *P. affinis affinis.* (C) *P. hoelzeli.* (D) *P. rumijae.* (E) *P. nonveilleri.* (F) *P. poecilus.* (G) *P. pseudornatus.* (H) *P. ornatus*. Photo D. Chobanov.

Figure 2

Position of the landmarks (red dots) on *Poecilimon* species used for geometric morphometrics.

(A) ovipositor. (B) cercus. (C) pronotum. (D) tegmen.

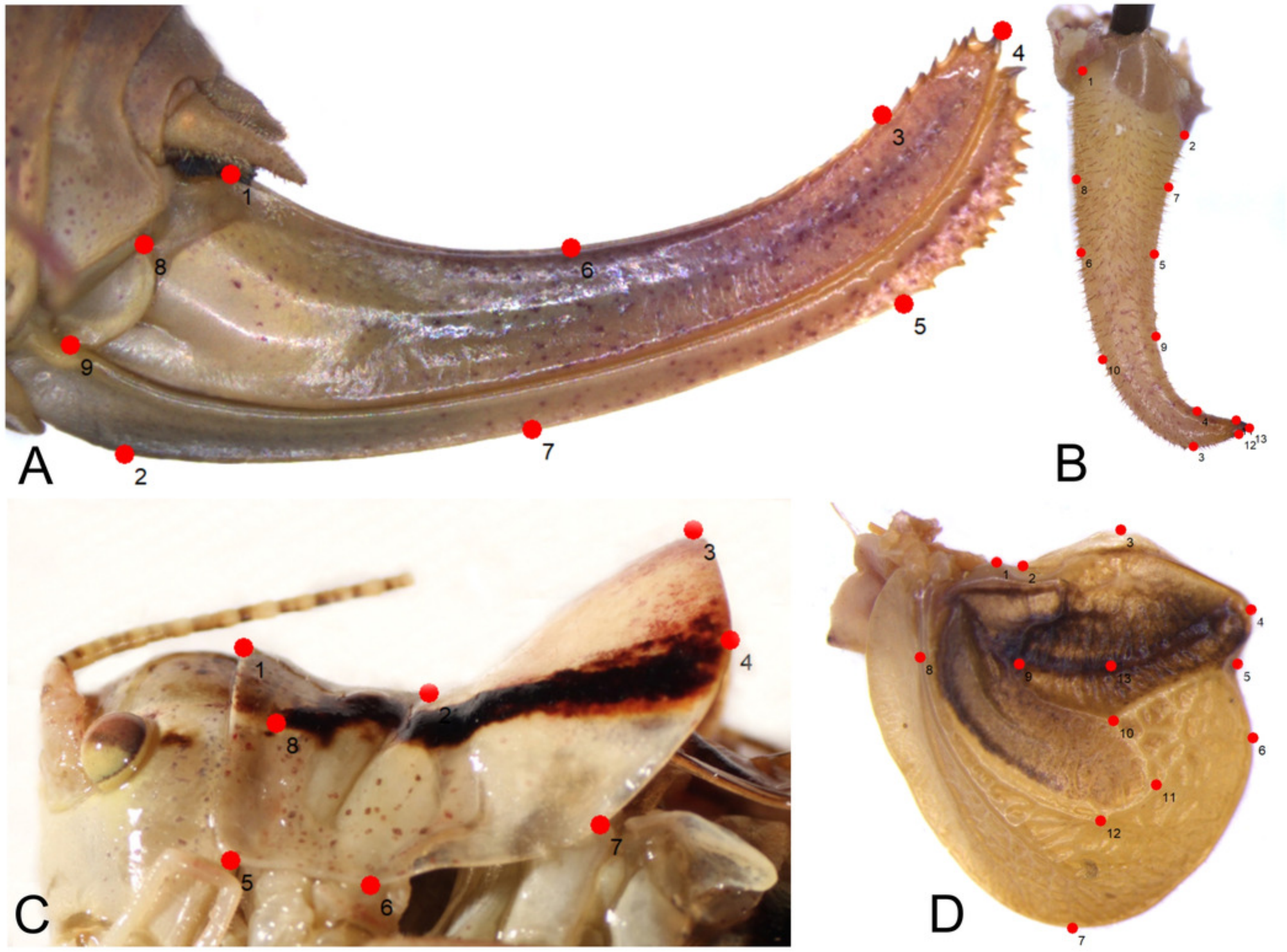

Figure 3

Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of tegmen: *P. ornatus* group (A) and *P. affinis* complex (B).

The different colors of the species *P. pseudornatus* and *P. a. affinis* indicate different locations from which the specimens were collected. The localities are indicated below taxa name (SR – Serbia, MN – Montenegro, BG – Bulgaria).

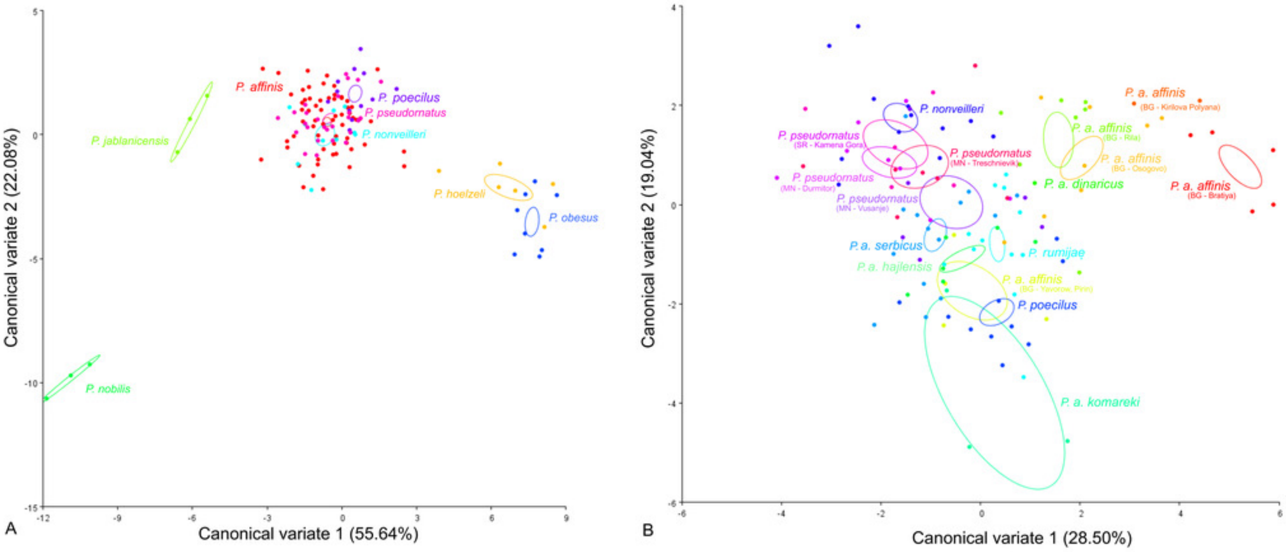

Figure 4

Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of ovipositor: *P. ornatus* group (A) and *P. affinis* complex (B).

The different colors indicate different species/subspecies of studied bush-crickets.

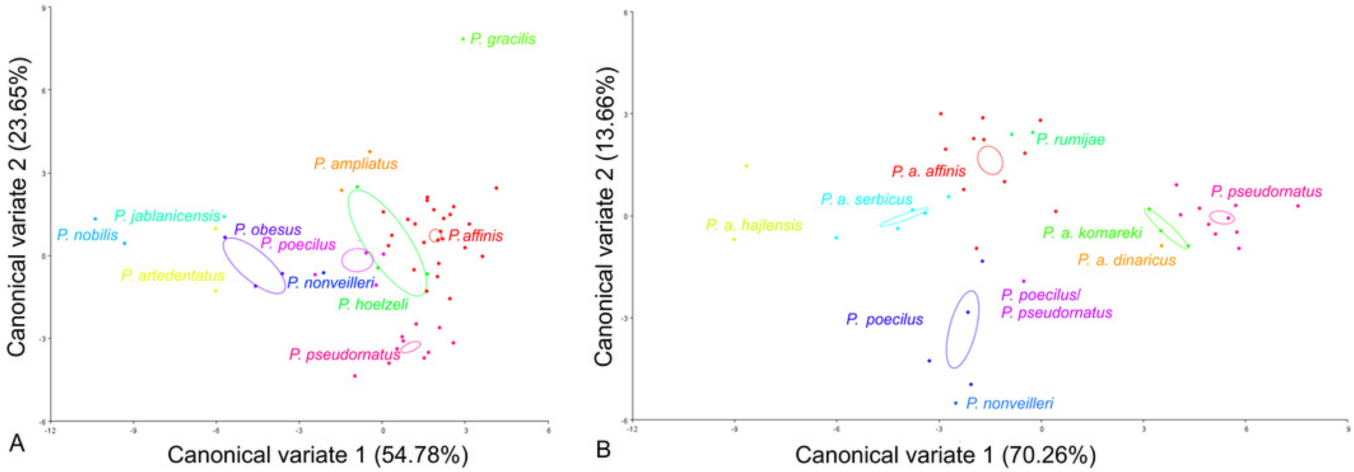

Figure 5

Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of cercus: *P. ornatus* group (A) and *P. affinis* complex (B).

The different colors indicate different species/subspecies of studied bush-crickets.

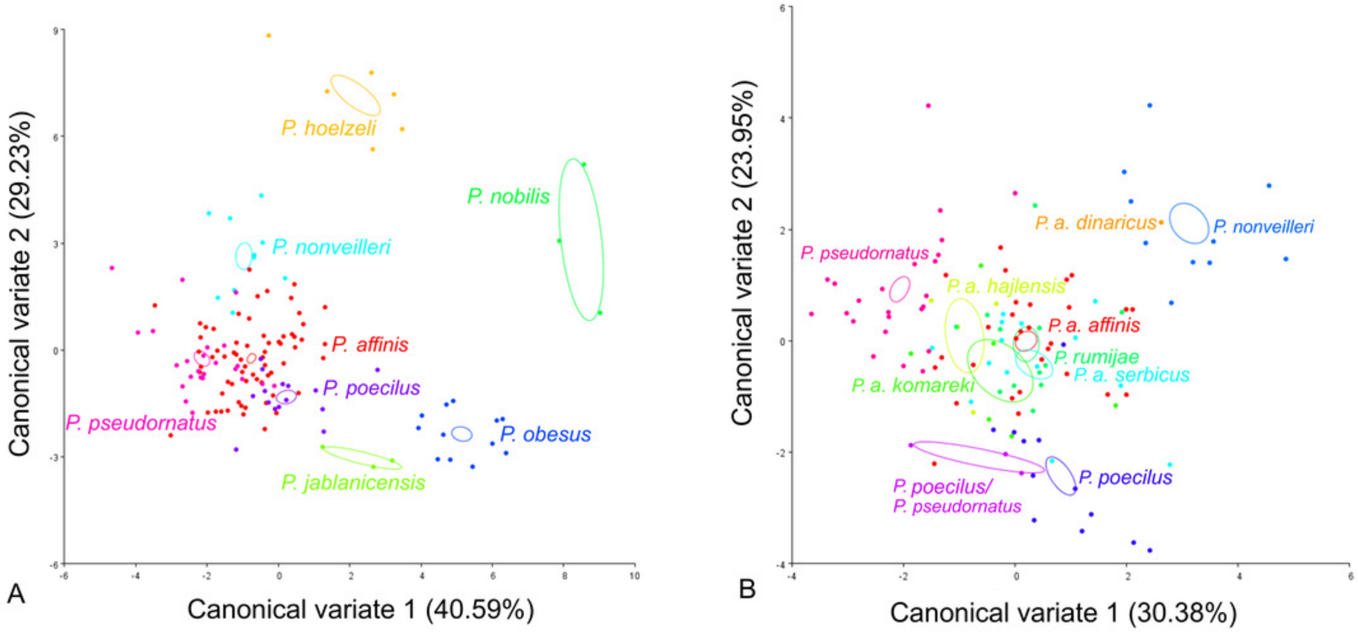

Figure 6

Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of pronotum: *P. ornatus* group (A) and *P. affinis* complex (B).

The different colors indicate different species/subspecies of studied bush-crickets.

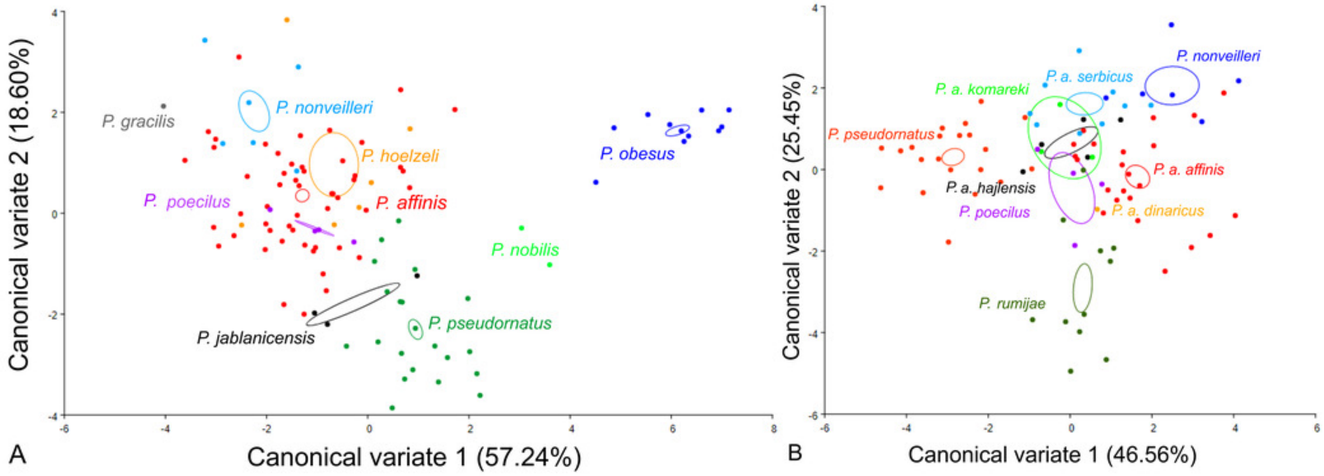

Figure 7

Principal Component Analysis (PCA) of stridulatory measurements and number of teeth: *P. ornatus* group.

The different colors indicate different species/subspecies of studied bush-crickets.

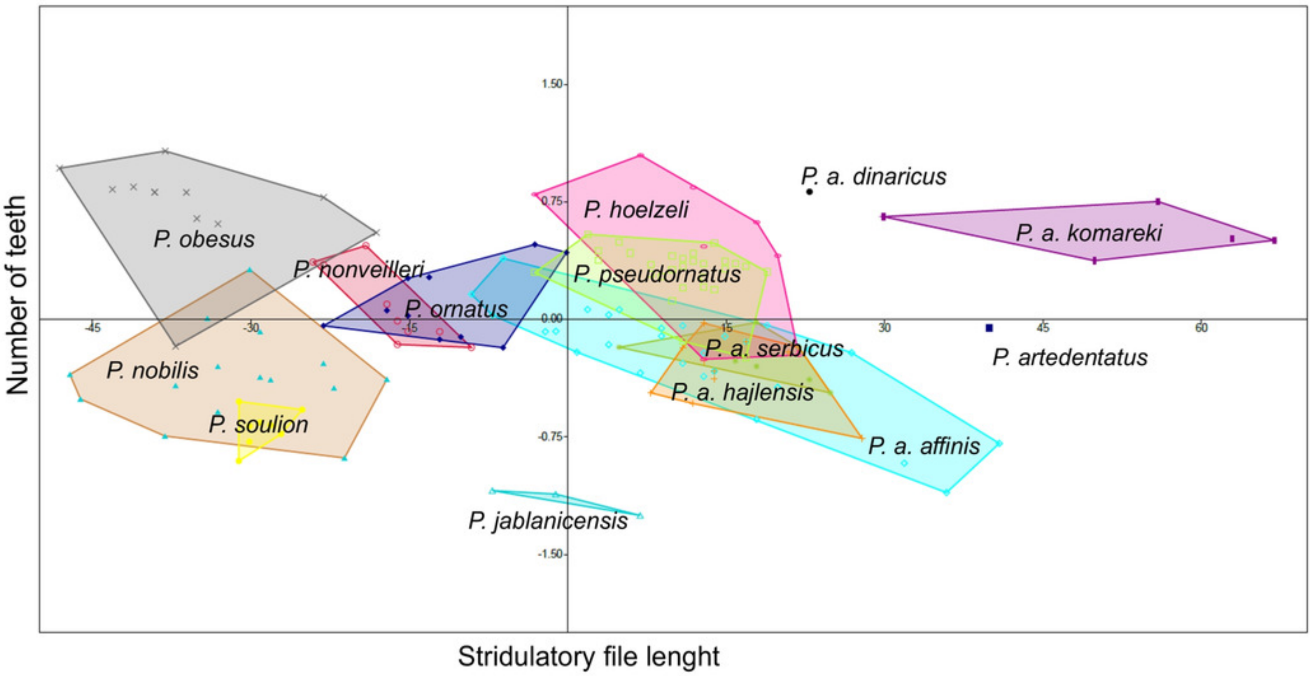

Figure 8

Phylogenetic tree based on Bayesian inference method including COI sequences of the *P. affinis* complex.

Bayesian Inference (BI) and Maximum Likelihood (ML) topologies were similar, so only one tree is shown. BI posterior probability (>0.5) and ML bootstrap values (>50) are shown on the nodes. Pink indicates species outside of the *P. affinis* complex; blue indicates taxa from the designated *P. affinis* complex.

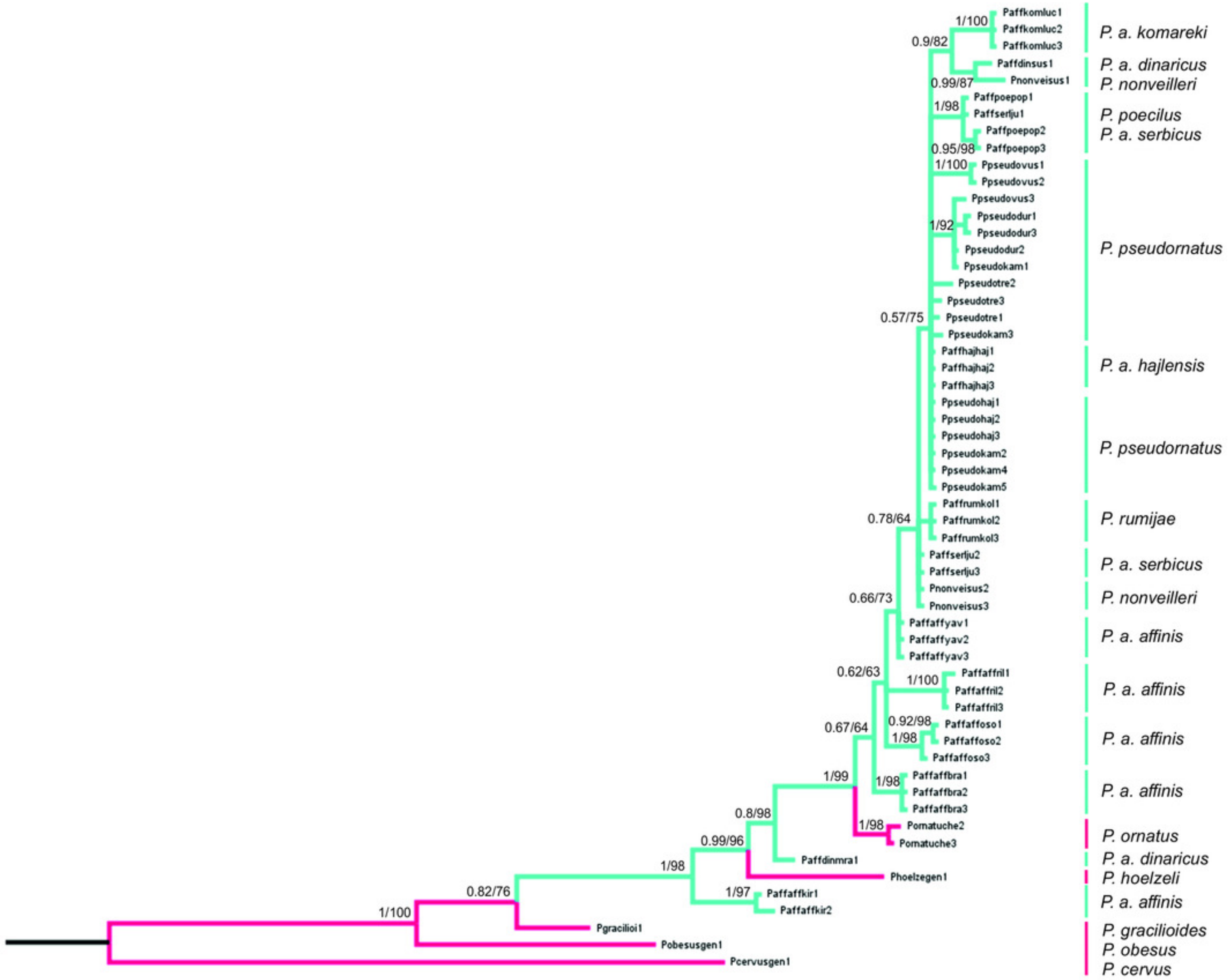

Table 1(on next page)

The number of specimens used for geometric morphometrics analysis.

Table 1:

The number of specimens used for geometric morphometrics analysis.

Species	Cercus	Tegmen	Ovipositor	Pronotum
*Poecilimon affinis affinis** (Frivaldszky, 1868)	29	26	11	23
*Poecilimon affinis komareki** Cejchan, 1957	6	3	3	3
*Poecilimon affinis dinaricus** Ingrisch & Pavićević, 2010	1	1	1	1
*Poecilimon affinis serbicus** Karaman, 1974	14	14	5	9
*Poecilimon affinis hajlensis** Karaman, 1974	4	6	2	5
*Poecilimon affinis poecilus** Ramme, 1951	15	12	5	4
*Poecilimon rumijae** Karaman, 1972	12	12	2	11
*Poecilimon nonveilleri** Ingrisch & Pavićević, 2010	10	10	1	6
*Poecilimon pseudornatus** Ingrisch & Pavićević, 2010	24	26	10	21
Poecilimon hoelzeli Harz, 1966	6	6	3	6
Poecilimon jablanicensis Chobanov & Heller, 2010	3	3	1	3
Poecilimon nobilis Brunner von Wattenwyl, 1878	3	3	2	2
Poecilimon obesus Brunner von Wattenwyl, 1878	12	8	3	11
Poecilimon gracilis (Fieber, 1853)	-	-	1	1
Poecilimon artedentatus Heller, 1984	-	-	2	-

*- *Poecilimon affinis* complex

Table 2(on next page)

Measurements for stridulatory files of *P. ornatus* group. Measurements are given in mm: first row – Min-Max values. in brackets – avarage ± Standard deviation.

Measurements are given in mm: first row – Min-Max values. in brackets – avarage ± Standard deviation.

Table 2:

Measurements for stridulatory files of *P. ornatus* group. Measurements are given in mm: first row – Min-Max values. in brackets – avarage ± Standard deviation.

Species	Number of specimens	Stridulatory length	Number of stridulatory teeth
P. affinis	9	3.68-4.46 (4.08)	122-169 (146)
P. affinis affinis	24	3.84-4.46 (4.17±0.19)	119-151 (138±12)
P. affinis hajlensis	7	4.08-4.46 (4.38±0.14)	133-153 (149±7)
P. affinis komareki	5	5.34-5.88 (5.64±0.25)	158-195 (181±15)
P. affinis serbicus	12	3.84-4.37 (4.14±0.21)	136-156 (144±6)
P. hoelzeli	8	4.14-5.34 (4.85±0.42)	125-150 (141±8)
P. jablanicensis	3	2.96-3.04 (3.01±0.05)	121-135 (128±7)
P. nobilis	15	2.78-3.98 (3.28±0.33)	81-111 (97±9)
P. nonveilleri	10	3.74-4.32 (3.97±0.18)	104-119 (111±5)
P. obesus	12	3.37-4.6 (4.28±0.31)	80-110 (92±8)
P. ornatus	10	3.74-4.6 (4.08±0.31)	105-128 (117±7)
P. pseudorantus	29	4.22-4.9 (4.66±0.16)	125-147 (139±5)
P. soulion	8	2.74-3.17 (2.99±0.13)	97-103 (99±2)
P. affinis dinaricus	1	5.38	149
P. artedentatus	1	4.8	168