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Filogeneza pasikoników z grupy *Poecilimon ornatus* (Orthoptera)

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Rozprawa doktorska

Promotor

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Phylogeny of the bush-crickets from the *Poecilimon ornatus* group (Orthoptera)

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Doctoral thesis

Supervisor

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Spis treści

Streszczenie

Rodzaj *Poecilimon* Fischer, 1853, należy do rzędu prostoskrzydłych (Orthoptera), rodziny Tettigoniidae Krauss, 1902 i występuje w Palearktyce. Obejmuje 145 gatunków, podzielonych na 17 grup i 16 gatunków nieprzypisanych do żadnej z nich. Jedną z grup gatunków jest *Poecilimon ornatus*, której największa różnorodność gatunkowa obserwowana jest na Półwyspie Bałkańskim. Pomimo kilku przeprowadzonych rewizji, systematyka i filogeneza tej grupy wciąż jest niejasna, a największy problem stanowi gatunek *P. affinis* i taksony blisko z nim spokrewnione. Dlatego w grupie *P. ornatus* wyróżniono kompleks *P. affinis* w oparciu o duże morfologiczne podobieństwo pięciu podgatunków *P. affinis* oraz dwóch gatunków *P. nonveilleri* i *P. pseudornatus*. Przedstawiona rozprawa doktorska w formie cyklu trzech artykułów (Kociński, 2020, *Folia Biologica (Kraków)*; Kociński i in., 2021, *PeerJ*; Kociński i in., 2022, *Arthropod Systematics & Phylogeny* – przyjęta do druku) weryfikuje relacje filogenetyczne gatunków z grupy *P. ornatus* przy zastosowaniu metod molekularnych i morfologicznych. W badaniach molekularnych użyto sekwencje trzech markerów mitochondrialnych - pierwszej podjednostki oksydazy cytochromowej (COI), dehydrogenazy NADH 2 (ND2), regionu kontrolnego mtDNA (CR) oraz jednego markeru jądrowego - niekodującego regionu jądrowego DNA (ITS1). Dokonano również pomiarów morfometrycznych czterech morfostruktur: przedplecza, przysadki odwłokowej, pokładełka oraz przedniego skrzydła. Dodatkowo przeprowadzono analizę aparatu strydulacyjnego. Badania molekularne i morfologiczne potwierdziły, że grupa *P. ornatus* jest monofiletyczna. Stwierdzono, że w obrębie grupy znajduje się kompleks *P. affinis*, do którego należy włączyć dwa dodatkowe gatunki: *P. ornatus* i *P. hoelzeli*. Przodek gatunków badanej grupy prawdopodobnie pochodził z południowych Bałkanów, a następnie rozszerzał zasięg występowania w kierunku północnej części Półwyspu Bałkańskiego. Proces różnicowania się gatunków poprzedzony został sześcioma wydarzeniami dyspersji i pięcioma wikariancjami, powiązanymi ze zmianami klimatycznymi i geologicznymi w Plejstocenie. Analiza wyznaczania granic gatunków wykazała dziewięć hipotetycznych gatunków w grupie *P. ornatus* oraz jeden gatunek w kompleksie *P. affinis*. Wyniki morfometrii geometrycznej wykazały, że przednie skrzydło i przysadka odwłokowa są odpowiednimi strukturami do rozróżnienia taksonów wchodzących w skład kompleksu od pozostałych taksonów z grupy *P. ornatus*. Z kolei potwierdzenie statusu taksonomicznego *P. poecilus* i *P. rumijae* wymaga dodatkowych badań opartych na analizie śpiewu pasikoników.

Summary

Poecilimon Fischer, 1853, is a genus of bush-crickets, that belongs to the order of the Orthoptera of the family Tettigoniidae Krauss, 1902 and is found in the Palearctic area. It includes 145 species, divided into 17 groups and 16 species not assigned to any of them. One of the species groups is *Poecilimon ornatus*, of which the greatest species diversity is observed on the Balkan Peninsula. Despite several reviews, the systematics and phylogeny of this group are still unclear, and the biggest problem is with *P. affinis* and the taxa closely related to it. Therefore, within the *P. ornatus* group, the *P. affinis* complex was designated based on the morphological similarity of five subspecies of *P. affinis* and two species: *P. nonveilleri* and *P. pseudornatus*. The presented doctoral dissertation in the form of a series of three articles (Kociński, 2020, *Folia Biologica (Kraków)*; Kociński et al., 2021, *PeerJ*; Kociński et al., 2022, *Arthropod Systematics & Phylogeny* - accepted for publication) verifies the phylogenetic relationships of species from the *P. ornatus* group using the molecular and morphological methods. For molecular studies, the sequences of three mitochondrial markers - the cytochrome c oxidase subunit I (COI), NADH dehydrogenase subunit 2 (ND2), the control region (CR), and one nuclear marker - the internal transcribed spacer 1 (ITS1) were used. The morphometric measurements of four morphostructures (pronotum, cercus, ovipositor, and tegmen) were also performed. Additionally, an analysis of the stridulatory file was conducted. The molecular and morphological studies confirmed that the *P. ornatus* group is monophyletic. It was found that within the group there is the *P. affinis* complex, into which two additional species should be included: *P. ornatus* and *P. hoelzeli*. The ancestor of the species in the studied group probably came from the southern Balkans, and then extended its range towards the northern part of the Balkan Peninsula. The process of species differentiation was preceded by six dispersals and five vicariance events linked to climate and geological events changes in the Pleistocene. Analysis of the species delimitation revealed nine hypothetical species within the *P. ornatus* group and one species within the *P. affinis* complex. The results of geometric morphometrics showed that the tegmen and the cercus are suitable structures to distinguish taxa composing the complex from other taxa of the *P. ornatus* group. The confirmation of the taxonomic status of *P. poecilus* and *P. rumijae* requires additional research based on the bioacoustic data of the bush-crickets.

Publikacje stanowiące przedmiot rozprawy doktorskiej

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.

Impact Factor: 0,432; 5-letni Impact Factor: 0,692; punkty MEiN: 100
Mój wkład w powstanie tej publikacji polegał na opracowaniu koncepcji badań, zaplanowaniu doświadczeń, współudziale w zebraniu materiału, wykonaniu prac laboratoryjnych i analiz filogenetycznych, interpretacji wyników, przygotowaniu tekstu manuskryptu i korespondencji z redakcją czasopisma. Mój udział szacuję na 100%.

Kociński M, Grzywacz B, Hristov G, Chobanov D (2021) A taxonomic outline of the *Poecilimon affinis* complex (Orthoptera) using the geometric morphometric approach. PeerJ 9:e12668.

Impact Factor: 2,984; 5-letni Impact Factor: 2,929; punkty MEiN: 100
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Kociński M, Chobanov D, Grzywacz B (2022) New insights into the genetic diversity of the Balkan bush-crickets of the *Poecilimon ornatus* group (Orthoptera). Arthropod Systematics & Phylogeny - w druku.

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1. Wstęp

Ogromna różnorodność grup blisko spokrewnionych taksonów w czasie specjacji stanowi wyzwanie dla systematyki. Jedną z takich grup są owady prostoskrzydłe, których systematyka do tej pory nie została ujednolicona. Proponowana współczesna klasyfikacja gatunków zawiera szereg niezgodności i niekiedy wywołuje pewne kontrowersje (np. niezgodność oznaczeń morfologicznych z molekularnymi). Owady prostoskrzydłe odznaczają się ogromnym bogactwem taksonów (ponad 20 tysięcy gatunków), dlatego też określenie relacji filogenetycznej tej grupy jest niezwykle istotne dla nauki.

Początkowo w analizach filogenetycznych posługiwano się wyłącznie cechami morfologicznymi. Zdarzało się, że organizmy klasyfikowane były na podstawie niewielu struktur, co prowadziło do nieprawidłowych wniosków. Wraz z rozwojem technologii badawczych, do prac taksonomicznych włączono analizy molekularne, dzięki którym z większą dokładnością można było określić pokrewieństwo między badanymi osobnikami. Dlatego klasyfikacja organizmów na podstawie danych molekularnych zaczęła uzupełniać tradycyjną „linneuszowską". W filogenezie molekularnej stosuje się zarówno markery mitochondrialne jak i jądrowe. Przypisanie osobników do gatunku wykonywane jest za pomocą „barkodowego DNA" (COI), który jest tzw. kodem kreskowym organizmu (Karmazina i in., 2020). Kolejnym powszechnie wykorzystywanym markerem jest ND2, który charakteryzuje się większą liczbą miejsc zmiennych i informatywnych niż COI, stosując kryterium parsymonii (ang. *parasimony informative*) (Cheng i in., 2018). Coraz większą popularność zyskuje marker CR, służący głównie do badania powiązań filogenetycznych u blisko spokrewnionych taksonów (Li i Liang, 2018), pomyślnie wykorzystany u owadów prostoskrzydłych z rodzaju *Poecilimon* (Borissov i Chobanov, 2020). Wśród markerów jądrowych, najbardziej popularny jest ITS1, charakteryzujący się wyższym tempem ewolucji, prowadzącej do większej zmienności nukleotydowej (Gu i in., 2020). Współczesna klasyfikacja organizmów opiera się zarówno na danych molekularnych, jak i morfologicznych.

Rodzaj *Poecilimon* Fischer, 1853 o polskiej nazwie pstrokaczek należy do rzędu prostoskrzydłych (Orthoptera), rodziny Tettigoniidae Krauss, 1902, podrodziny Phaneropterinae Burmeister, 1838, plemienia Barbitistini Jacobson, 1905. Owady tego rodzaju stanowią najliczniejszą grupę gatunków pasikoników (Tettigoniidae,

Phaneropterinae) występujących w Palearktyce od Apeninów po Zachodnią Syberię i Centralny Tienszan (Bey-Bienko, 1954). Najwięcej gatunków endemicznych jest rozmieszczonych w rejonach Morza Egejskiego oraz Półwyspu Bałkańskiego. Pasikoniki należące do tego rodzaju są zwykle krótkoskrzydłe, roślinożerne i charakteryzują się złożoną komunikacją akustyczną. *Poecilimon* obejmuje około 145 gatunków, które aktualnie są podzielone na 17 grup gatunków oraz 16 gatunków, które nie są przypisane do żadnej z grup (Cigliano i in., 2022). W ciągu ostatnich dwóch lat liczba grup zmieniła się (w 2020 r – 18 grup gatunkowych), co świadczy o wciąż istniejących problemach taksonomicznych w obrębie rodzaju. Filogeneza i systematyka *Poecilimon* jest tylko częściowo rozwiązana, pomimo kilku przeprowadzonych rewizji rodzaju w oparciu o dane morfologiczne, bioakustyczne, cytogenetyczne i molekularne (Ramme, 1933; Bey-Bienko, 1954; Heller, 1984; Heller i Lehmann, 2004; Heller i Sevgili, 2005; Heller i in., 2006, 2008; Chobanov i Heller, 2010; Ullrich i in., 2010; Grzywacz i in., 2014). Podobieństwo i zmienność cech morfologicznych sprawiają, że wiele gatunków *Poecilimon* jest trudnych do zidentyfikowania, a ich pozycja taksonomiczna wymaga dokładnego rozpoznania.

Obiektem prowadzonych badań do rozprawy doktorskiej były pasikoniki z grupy *Poecilimon ornatus* (Schmidt, 1850). Pierwszy przegląd systematyczny rodzaju *Poecilimon* został opublikowany przez Ramme (1933). Następną rewizję przeprowadził Heller (1984), który do grupy *P. ornatus* zaliczył osiem taksonów rozmieszczonych głównie na Półwyspie Bałkańskim: *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) i *P. pancici* Karaman, 1958. Kilka lat później, *P. artedentatus* i *P. hoelzeli* otrzymały status gatunku (Willemse, 1985; Willemse i Heller, 1992), podczas gdy *P. pancici* został zsynonimizowany z *P. ornatus* (Willemse, 1985). Ponadto, opisano sześć nowych gatunków: *P. pindos* F. Willemse, 1982; *P. soulion* L. Willemse, 1987; *P. gracilioides* F. Willemse i Heller, 1992; *P. jablanicensis* Chobanov i Heller, 2010; *P. pseudornatus* Ingrisch & Pavićević, 2010; *P. nonveilleri* Ingrisch i Pavićević, 2010.

W obrębie grupy *P. ornatus*, gatunkiem o najbardziej rozproszonym zasięgu jest *P. affinis*, występujący w górach północnej Grecji, aż do Karpat w Rumunii i na niewielkich obszarach Ukrainy. Aktualnie, *P. affinis* złożony jest z pięciu podgatunków: *P. affinis affinis* (Frivaldszky, 1868); *P. a. serbicus* Karaman, 1974; *P. a. dinaricus*

Ingrisch & Pavićević, 2010; *P. a. hajlensis* Karaman, 1974; *P. a. komareki* Cejchan, 1957. W 1974 roku Karaman obniżył status *P. poecilus* Ramme, 1951 do podgatunku *P. affinis,* a następnie opisał dwa nowe podgatunki: *P. a. serbicus* i *P. a. hajlensis*. Jednakże, już w 1984 roku Heller zasugerował, że *P. poecilus* i *P. a. affinis* mogą być synonimami. Z powodu wątpliwości co do statusu taksonomicznego, *P. poecilus* potraktowano w badaniach do rozprawy doktorskiej jako osobny gatunek. *Poecilimon komareki* został opisany przez Cejchana w 1957 r., natomiast w 1984 r. Heller obniżył jego status do podgatunku *P. affinis*. *Poecilimon komareki rumijae* został opisany przez Karamana w 1972 roku, ale ze względu na obniżenie statusu taksonomicznego *P. komareki* do podgatunku *P. affinis,* automatycznie *P. k. rumijae* stał się jego synonimem, co zostało potwierdzone przez Chobanova i Hellera (2010). Ingrisch i Pavićević (2010) zasugerowali, że *P. rumijae* może być osobnym gatunkiem, różniącym się od *P. affinis*. Różnice morfologiczne pomiędzy tymi dwoma taksonami są niewielkie i ograniczają się do kształtu przedplecza samca i wielkości ciała (Chobanov i Heller, 2010). W wyniku rozbieżności co do statusu taksonomicznego, *P. rumijae* był traktowany w badaniach do rozprawy doktorskiej jako osobny gatunek.

Duże podobieństwo morfologiczne oraz brak wyraźnych granic pomiędzy dwoma gatunkami *P. pseudornatus* i *P. nonveilleri* oraz pięcioma podgatunkami *P. affinis* sugerują by rozpatrywać je jako kompleks gatunkowy *P. affinis* (Chobanov i Heller, 2010; **Kociński, 2020**).

2. Cele i hipotezy badawcze

Celem przedstawionego cyklu artykułów była rekonstrukcja pokrewieństwa filogenetycznego taksonów grupy *P. ornatus* oraz określenie ich różnorodności genetycznej z wykorzystaniem stopnia zróżnicowania sekwencji nukleotydów fragmentów DNA mitochondrialnego (mtDNA) i jądrowego (rDNA), a także danych morfologicznych uzyskanych za pomocą morfometrii geometrycznej (ang. *geometric morphometrics*) i pomiaru aparatu strydulacyjnego.

Cel rozprawy doktorskiej podzielono na następujące zadania:

a) ocena różnorodności genetycznej pasikoników z grupy *P. ornatus* (**Kociński, 2020**; **Kociński i in., 2022**);

b) określenie pokrewieństwa filogenetycznego w grupie *P. ornatus* w oparciu o dane molekularne i morfologiczne (**Kociński, 2020**; **Kociński i in., 2021**; **Kociński i in., 2022**);

c) wyjaśnienie niejasnej pozycji taksonów należących do kompleksu *Poecilimon affinis* w oparciu o dane molekularne i morfologiczne (**Kociński, 2020**; **Kociński i in., 2021**; **Kociński i in., 2022**).

W publikacjach zweryfikowano następujące hipotezy:

I) Zróżnicowanie genetyczne pasikoników z grupy *Poecilimon ornatus* jest zgodne z ich zmiennością morfologiczną (**Kociński i in., 2021**; **Kociński i in., 2022**).

II) Pasikoniki z grupy *Poecilimon ornatus* tworzą grupę monofiletyczną (**Kociński, 2020**; **Kociński i in., 2021**; **Kociński i in., 2022**).

III) Dane molekularne i morfologiczne potwierdzają status kompleksu *Poecilimon affinis* i jego odrębność od pozostałych taksonów z grupy *Poecilimon ornatus* (**Kociński, 2020**; **Kociński i in., 2021**; **Kociński i in., 2022**).

3. Materiały i metody

3.1. Metody terenowe

Materiał do badań zebrano w latach 2006-2019 na terenie Półwyspu Bałkańskiego (Bułgaria, Serbia, Czarnogóra, Albania, Macedonia Północna, Grecja) oraz w Ukrainie (Tabela 1 w: **Kociński, 2020**; Tabela 1 i Rycina 1 w: **Kociński i in., 2022**). Owady odławiano za pomocą siatki entomologicznej. Pasikoniki utrwalano w alkoholu 96%, a następnie przechowywano w tempraturze -20°C. Zebrany materiał posłużył do badań genetycznych (**Kociński, 2020; Kociński i in., 2022**) oraz morfologicznych (**Kociński i in., 2021**).

3.2. Metody laboratoryjne

DNA wyizolowano z odnóży pasikoników dla 74 osobników reprezentujących 19 taksonów z grupy *P. ornatus* zebranych z 34 stanowisk. Izolacje przeprowadzono według standardowej procedury przy użyciu zestawu NucleoSpin tissue kit (Macherey–Nagel, Niemcy). Koncentrację i jakość uzyskanego DNA zmierzono przy użyciu spektrofotometru NanoDrop 2000. Następnie przeprowadzono amplifikację fragmentów trzech markerów mitochondrialnych - pierwszej podjednostki oksydazy cytochromowej (COI), dehydrogenazy NADH 2 (ND2), regionu kontrolnego mtDNA (CR) oraz jednego fragmentu markeru jądrowego - niekodującego regionu jądrowego DNA (ITS1) (**Kociński, 2020**; **Kociński i in., 2022**). Lista użytych starterów zawarta jest w Tabeli 2 w: **Kociński i in., 2022**; natomiast programy do amplifikacji w Tabeli 3 w: **Kociński i in., 2022**. Uzyskane produkty reakcji PCR zsekwencjonowano, a odczyty reakcji sekwencjonowania wykonano przy użyciu sekwenatora ABI3130xl. Otrzymane sekwencje DNA (ang. *forward* i *reverse*) porównano przy użyciu programu CodonCodeAligner 9.0 (https://www.codoncode.com/aligner). Odległości genetyczne pomiędzy taksonami z kompleksu *P. affinis*, a pozostałymi gatunkami z grupy *P. ornatus* obliczono przy użyciu programu MEGA 11 (Tamura i in., 2021) (Tabela 2 w: **Kociński, 2020**; Tabela 4 w: **Kociński i in., 2022**). Poziom nasycenia substytucji we fragmentach mtDNA (COI, CR, ND2) wykonano w programie DAMBE 7 (Xia, 2018) (Tabela 5 w: **Kociński i in., 2022**). Sprawdzono czy otrzymane sekwencje można analizować łącznie przeprowadzając test zgodności danych (ang. *partition homogeneity test*) (Farris i in., 1995) w programie PAUP 4.0a169 (Swofford, 2002).

3.3. Analiza filogenetyczna

W celu zbadania pokrewieństwa filogenetycznego zastosowano dwie metody: największej wiarygodności (ang. *Maximum Likelihood* - *ML*) oraz wnioskowania bayesowskiego (ang. *Bayesian Inference* - *BI*). Wybrano najlepszy model substytucji nukleotydów do dalszych analiz korzystając z programu MrModeltest 2.4 (Nylander, 2004). Analizę wnioskowania bayesowskiego wykonano w programie MrBayes 3.2.7a (Ronquist i in., 2012), a największej wiarygodności w programie IQ-TREE (Nguyen i in., 2015). Wnioskowanie bayesowskie przeprowadzono na 6 000 000 pokoleń, z zapisem drzew co 100 pokoleń. W metodzie największej wiarygodności zastosowano analizę próbkowania (ang. *bootstrap*) z 1000 powtórzeń. Jako grupy zewnętrzne wykorzystano sekwencje pozyskane z bazy danych GenBank dla gatunków: *Poecilimon ampliatus*, *P. ukrainicus*, *P. heroicus*, *P. schmidti*, *Polysarcus denticauda* (Tabela 1 w: **Kociński, 2020)**; *Poecilimon cretensis*, *P. turcicus*, *P. sureyanus*, *P. sanctipauli*, *Isophya speciosa*, *Leptophyes albovittata* (**Kociński i in., 2022**).

3.4. Analiza wyznaczania granic gatunków

Do wyznaczenia granic gatunków (ang. *species delimitation*) wykorzystano internetowe wersje programów ABGD (ang. *Automatic Barcode Gap Discovery*) (Puillandre i in., 2012), ASAP (ang. *Assemble Species by Automatic Partitioning*) (Puillandre i in., 2021), bPTP (ang. *Poisson Tree Processes*) (Zhang i in., 2013), GMYC (ang. *general mixed Yule-coalescent*) (Fujisawa i in., 2013). W powyższych analizach użyto drzewa filogenetycznego opartego na sekwencjach genu COI, pozbawionego grup zewnętrznych (**Kociński i in., 2022**).

3.5. Analiza zegara molekularnego

Oszacowanie czasu dywergencji pasikoników z grupy *P. ornatus* wykonano w programie BEAST v1.10.4 (Drummond i in., 2012), wykorzystując sekwencje genu COI. Punktem kalibracyjnym był czas izolacji endemicznego gatunku *Poecilimon cretensis* z Krety (Borissov i in., 2020). Wewnątrzgatunkowy podział *P. cretensis* na dwie grupy, wschodnią i zachodnią, oszacowano na 0,8 milionów lat temu (**Kociński i in., 2022**).

3.6. Analiza biogeograficzna

W celu przeprowadzenia analizy biogeograficznej wyznaczono na podstawie centrum występowania badanych taksonów cztery regiony biogeograficzne: A – południowy (południowa Grecja), B – centralny (północno-zachodnia Grecja, południowa część Macedonii Północnej, południowa Albania), C – północno-zachodni (północna część Macedonii Północnej, Czarnogóra, Kosowo, południowa Serbia, północna Albania), D – północno-wschodni (wschodnia część Macedonii Północnej, Bułgaria) (Ryc. 2, 3 w: **Kociński i in., 2022**). Rekonstrukcję biogeograficzną wykonano przy użyciu analizy S-DIVA 1.9 (Yu i in., 2010) w programie RASP (Yu i in., 2015) wykorzystując sekwencje genu COI. Sprawdzono związek między odległościami genetycznymi a geograficznymi taksonów z grupy *P. ornatus* przy użyciu testu Mantela w programie PAST 4.03 (Hammer i in., 2001).

3.7. Analiza morfologiczna

Do badań morfologicznych wykorzystano 196 osobników należących do 16 taksonów z grupy *Poecilimon ornatus* (Tabela 1 w: **Kociński i in., 2021**). Do analiz morfometrycznych wybrano cztery morfostruktury – przedplecze (*pronotum*), przysadkę odwłokową (*cercus*), pokładełko (*ovipositor*) i przednie skrzydło (*tegmen*). Dokumentację sporządzono w postaci zdjęć zrobionych z mikroskopu stereoskopowego (Leica M165C) wyposażonego w aparat cyfrowy (Leica DMC5400) przy zachowaniu stałych parametrów powiększenia (przedplecze i pokładełko – 0,8x; przysadka odwłokowa – 2,0x; przednie skrzydło – 1,0x). Do badań wykorzystano 54 zdjęcia pokładełka, 130 zdjęć przedniego skrzydła samców, 142 zdjęcia przedplecza samców oraz 141 zdjęć przysadki odwłokowej samców. Punkty orientacyjne (ang. *landmarks, semilandmarks*) naniesiono ręcznie w wybranych analogicznych miejscach (np. w miejscu przecięcia żyłek, wypustkach, dołkach) przy użyciu programu tpsDIG v.2.17 (Rohlf, 2015). Na przedplecze nałożono 8 punktów orientacyjnych, na przysadkę odwłokową i przednie skrzydło po 13 punktów orientacyjnych, na pokładełko 9 punktów orientacyjnych. Dokładne ich umiejscowienie zawarto w Tabeli 2 w: **Kociński i in., 2021**. Współrzędne wszystkich punktów podlegały superpozycji w programie MorphoJ 1.06d (Klingenberg, 2011), z wykorzystaniem tzw. nałożenia Procrusta (ang. *Procrustes superimposition*). Następnie przeprowadzono analizę zmiennych kanonicznych (ang. *Canonical Variate Analysis*, CVA) poszczególnych morfostruktur w celu zbadania zróżnicowania morfologicznego pomiędzy badanymi taksonami.

Odległość Mahalanobisa (ang. *Mahalanobis distance*) (wielowymiarowa miara zmienności cech morfologicznych pomiędzy badanymi taksonami) została obliczona i statystycznie przetestowana przy użyciu permutacji z 10 000 powtórzeń. Pomiar długości oraz liczby ząbków aparatu strydulacyjnego wykonany został na 154 osobnikach z grupy *P. ornatus* (Tabela 3 w: **Kociński i in., 2021**) przy użyciu mikroskopu stereoskopowego wyposażonego w mikrometr okularowy. Uzyskane dane posłużyły do przeprowadzenia Analizy Głównych Składowych (ang. *Principal Component Analysis*) w programie PAST 4.03 (Hammer i in., 2001) (Rycina 7 w: **Kociński i in., 2021**).

4. Wyniki

Wyniki z pierwszego etapu badań nad wstępnym określeniem pokrewieństwa filogenetycznego pasikoników z grupy *P. ornatus*, potwierdziły istnienie kompleksu *P. affinis* w obrębie grupy (**Kociński, 2020**). Stwierdzono, że grupa *P. ornatus* jest monofiletyczna, natomiast kompleks *P. affinis* tworzy grupę parafiletyczną. Do tego kompleksu zaliczono także dwa dodatkowe gatunki: *P. ornatus* i *P. hoelzeli* (Rycina 1 w: **Kociński, 2020**), znajdujące się w tym samym kladzie z pozostałymi taksonami z kompleksu. Poprzedni podział pasikoników ze względu na rozmieszczenie, ekologię, bioakustykę oraz morfologię, opisany przez Chobanova i Hellera w 2010 roku, został tylko częściowo potwierdzony (**Kociński, 2020**).

Wyniki z drugiego etapu oparte były na analizie morfometrycznej czterech morfostruktur (**Kociński i in., 2021**). Analiza CVA przedniego skrzydła samca wykazała znaczną zmienność (77,72%) wśród pasikoników z grupy *P. ornatus* i kompleksu *P. affinis* (Rycina 3 w: **Kociński i in., 2021**). *Poecilimon hoelzeli*, *P. obesus*, *P. jablanicensis* i *P. nobilis* są wyraźnie odseparowane na wykresie od pozostałych osobników z grupy, podczas gdy *P. pseudornatus*, *P. poecilus*, *P. nonveilleri* i *P. affinis* grupują się razem (Rycina 3A w: **Kociński i in., 2021**). Na poziomie kompleksu, wyniki nie wykazały wyraźnego oddzielenia poszczególnych taksonów z grupy. Jednakże, stwierdzono duże zróżnicowanie *P. a. affinis* ze względu na jego występowanie – osobniki z różnych lokalizacji tworzą osobne grupy na wykresie. Natomiast osobniki *P. pseudornatus* z różnych lokalizacji grupują się razem (Rycina 3B w: **Kociński i in., 2021**). W przypadku analizy przysadki odwłokowej pasikoniki z kompleksu można oddzielić od pozostałych taksonów *P. ornatus* (Rycina 5A w: **Kociński i in., 2021**). Analiza zmiennych kanonicznych pokładełka oraz przedplecza pokazała, że taksony z kompleksu *P. affinis* nie były wyraźnie oddzielone od innych gatunków z grupy *P. ornatus* (Rycina 4A, 6A w: **Kociński i in., 2021**), w przeciwieństwie do innych taksonów w obrębie kompleksu, które możemy wydzielić (Rycina 4B w: **Kociński i in., 2021**). W przypadku przedplecza na poziomie kompleksu, tylko *P. rumijae* nie grupuje się z resztą taksonów (Rycina 6B w: **Kociński i in., 2021**). Analiza liczby ząbków i długości aparatu strydulacyjnego wykazała, że *P. nonveilleri*, *P. ornatus*, *P. hoelzeli*, *P. pseudornatus*, *P. a. serbicus*, *P. a. hajlensis* i *P. a. affinis* są ze sobą blisko spokrewnione, co świadczy o istnieniu kompleksu *P. affinis*, jednak należy włączyć do niego *P. hoelzeli* i *P. ornatus* (Rycina 7 w: **Kociński i in., 2021**).

Wyniki trzeciego etapu badań oparte na analizie filogenetycznej z wykorzystaniem czterech markerów potwierdziły monofiletyzm grupy *P. ornatus* oraz parafiletyzm kompleksu *P. affinis. Poecilimon nobilis, P. obesus* i *P. artedentatus* tworzą grupę siostrzaną do wszystkich pozostałych taksonów z grupy *P. ornatus*. Ponadto, stwierdzono, że *P. rumijae* i *P. poecilus* tworzą osobną gałąź, co może świadczyć, że należy traktować je jako osobne gatunki lub podgatunki. Analiza wyznaczania granic gatunków na podstawie czterech testów (ASAP, GMYC, ABGD, bPTP) wykazała rozbieżne wyniki i nie jest zgodna z obecną klasyfikacją taksonomiczną. Metody ASAP, ABGD, bPTP sugerowały istnienie dziewięciu hipotetycznych gatunków w obrębie grupy *P. ornatus*, podczas gdy metoda GMYC aż 26-34 hipotetycznych gatunków. Analiza ASAP, ABGD i bPTP zgrupowała wszystkie taksony należące do kompleksu *P. affinis* wraz z *P. hoelzeli* i *P. ornatus* w jeden hipotetyczny gatunek, GMYC natomiast w 17 gatunków (Rycina 4 w: **Kociński i in., 2022**). Oszacowany czas dywergencji badanej grupy pasikoników przypada na środkowy Plejstocen, czyli 1,62 miliona lat temu, z podziałem linii rodowych estymowanym na 1,33 i 0,42 miliona lat temu podczas kalabryjskiego i chibańskiego etapu Plejstocenu. Rozejście się kompleksu *P. affinis* od *P. pindos* datuje się na ok. 0,71 miliona lat temu podczas Plejstocenu. Czas rozdziału taksonów z kompleksu przypada na okres od 0,42 do 0,02 miliona lat temu w późnym Plejstocenie (Rycina 2 w: **Kociński i in., 2022**). Wzorzec rozmieszczenia grupy *P. ornatus* oparty jest na sześciu wydarzeniach dyspersji (proces przemieszczania się organizmów poza obszar pierwotnie zajęty przez populację) i pięciu wikariancji (proces różnicowania się gatunków na skutek wystąpienia bariery geograficznej). Ostatni przodek grupy znajdował się w obszarze AB (południowy i centralny region), następnie ewoluował przez proces wikariancji i dyspersji do obszarów południowych (A) i centralnych (B), w których wystąpiły lokalne podziały linii. Region centralny (B) jest również głównym ośrodkiem specjacji i dyspersji grupy *P. ornatus*. Przodek kompleksu *P. affinis* ewoluował poprzez proces dyspersji w dwóch kierunkach – północno-zachodnim (C) i północno-wschodnim (D) (Rycina 2 i 3 w: **Kociński i in., 2022**). Test Mantela wykazał brak zależności między odległościami genetycznymi a geograficznym w obrębie grupy *P. ornatus* (R = 0,0469; p = 0,193).

5. Dyskusja

Niniejsze badania stanowią pierwszą kompleksową próbę rekonstrukcji filogenezy grupy *Poecilimon ornatus* na podstawie danych molekularnych (**Kociński, 2020**; **Kociński i in., 2022**) oraz morfologicznych (**Kociński i in., 2021**). Wyniki oparte na czterech markerach molekularnych (COI, CR, ND2, ITS1) potwierdzają, że grupa *P. ornatus* jest monofiletyczna, jak sugerowali Ullrich i in. (2010). Dane molekularne wykazały, że *P. gracilis* nie jest siostrzanym gatunkiem w stosunku do wszystkich pozostałych taksonów z grupy *P. ornatus*, jak proponowano na podstawie analizy morfometrycznej pokładełka (**Kociński i in., 2021**) oraz wcześniejszych badań morfologicznych i bioakustycznych (Chobanov i Heller, 2010). Analiza przedniego skrzydła i przysadki odwłokowej wskazała *P. nobilis* jako siostrzany gatunek do wszystkich innych taksonów z grupy, podczas gdy analiza przedplecza *P. obesus*. Wyniki oparte na analizie drzewa filogenetycznego (Ryc. 4 w: **Kociński i in., 2022**) wskazują, że *P. nobilis*, *P. obesus* i *P. artedentatus* są siostrzaną grupą do wszystkich pozostałych taksonów z grupy *P. ornatus*, co wraz z analizą morfometryczną potwierdza, że gatunki te nie należą do kompleksu *P. affinis*. Zarówno dane molekularne, jak i morfologiczne wykazały parafiletyzm taksonów należących do wyznaczonego wcześniej kompleksu *P. affinis*. Dwa dodatkowe gatunki, *P. hoelzeli* i *P. ornatus*, grupują się z pozostałymi taksonami należącymi do kompleksu na podstawie danych molekularnych, jak i analizy morfometrycznej pokładełka. Świadczy to o konieczności włączenia ich do wyznaczonego kompleksu *P. affinis*. Najbardziej zróżnicowanym taksonem w obrębie grupy *P. ornatus* jest podgatunek *P. a. affinis*. W zależności od występowania (Bułgaria: Góry Piryn, Bratiya, Osogovo, Kirilova Polyana, Góry Riła, Rilski Manastir) zajmuje on różne gałęzie na drzewie filogenetycznym (**Kociński i in., 2022**) oraz jest najbardziej rozproszony pod względem pomiarów morfologicznych przedniego skrzydła (**Kociński i in., 2021**). Sytuacja ta prawdopodobnie związana jest z wysokością nad poziomem morza, na której dana populacja występuje. *Poecilimon pseudornatus* zebrany z różnych lokalizacji (Czarnogóra: Durmitor, Treshnievik, Vusanje; Serbia: Kamena Gora), nie wykazuje takiego zróżnicowania genetycznego i morfologicznego, co może świadczyć o mniejszej różnorodności w obrębie samego gatunku. Wyniki oparte na analizach molekularnych pokazały zróżnicowanie genetyczne pomiędzy *P. a. komareki* a *P. rumijae* (**Kociński i in., 2022**), co jest sprzeczne z obecną systematyką, ponieważ *P. rumijae* jest traktowany jako synonim *P. a. komareki* (Cigliano i in., 2022). Ponadto, wyniki oparte na morfometrii geometrycznej przedplecza

i pokładełka potwierdziły, że *P. rumijae* i *P. a. komareki* mogą być osobnymi taksonami (**Kociński i in., 2021**). To stwierdzenie jest zgodne z Ingrisch i Pavićević (2010), którzy uznają *P. rumijae* jako osobny gatunek z grupy *P. ornatus*, porównując go do *P. nonveilleri*. Jednakże, kształt przysadki odwłokowej, przedniego skrzydła, a także długość oraz liczba ząbków aparatu strydulacyjnego *P. a. komareki* i *P. rumijae* wykazały duże podobieństwo, co świadczy o trudnościach z prawidłową klasyfikacją tych taksonów. Analiza wyznaczania granic gatunków trzema metodami (ASAP, ABGD, bPTP) wykazała dziewięć potencjalnych gatunków w obrębie grupy *P. ornatus*, co jest sprzeczne z danymi morfologicznymi i bioakustycznymi (Chobanov i Heller, 2010; Ingrisch i Pavićević, 2010; **Kociński i in., 2021**) oraz molekularnymi (**Kociński, 2020**; **Kociński i in., 2022**). Natomiast, analiza metodą GMYC ujawniła aż 26 potencjalnych gatunków w obrębie grupy, co prowadzi do niezgodności pomiędzy powyższymi metodami i może świadczyć o większej skuteczności ASAP, ABGD i bPTP (metody te pokazują najmniejszą liczbę możliwych gatunków w badanej grupie) niż GMYC (Magoga i in., 2021). Zastosowanie zegara molekularnego ujawniło, że specjacja w grupie *P. ornatus* nastąpiła pomiędzy środkowym Plejstocenem (ok. 1,62 milionów lat temu) a początkiem Holocenu (ok. 0,01 milionów lat temu). Rozdział taksonów z grupy *P. ornatus* od pozostałych osobników z rodzaju *Poecilimon* wystąpił ok. 1,62 miliona lat temu, co zbiega się znacząco z globalnym ochłodzeniem klimatu oraz ekspansją fauny przystosowanej do chłodniejszych terenów (Lisiecki i Raymo, 2005). Większość taksonów z grupy występuje w wilgotnych obszarach górskich o chłodnym klimacie, oprócz dwóch gatunków (*P. artedentatus*, *P. nobilis*) zasiedlających nisko położone obszary południowych i zachodnich Bałkanów oraz jednego gatunku (*P. obesus*) o dość szerokiej tolerancji temperaturowej (Chobanov i Heller, 2010). Pierwszy podział linii filogenetycznej w grupie mógł nastąpić w wyniku izolacji z powodu pogorszenia klimatu w centralnym (północno-zachodnia Grecja, południowa Macedonia Północna, południowa Albania) lub południowym regionie (południowa Grecja) Półwyspu Bałkańskiego i późniejszej adaptacji nowych linii filogenetycznych do chłodniejszego klimatu z rozmieszczeniem w północnej części Bałkanów. Kolejne podziały linii filogenetycznych przypadają w środkowym Plejstocenie, kiedy klimat stopniowo się zmieniał. W ramach nieregularnego powtarzania się okresów cieplejszych, zimniejszych, wilgotniejszych i suchszych o zmiennej amplitudzie temperatury i wilgotności występowały najpewniej zjawiska izolacji i wymierania populacji. Gatunki takie jak *P. jablanicensis* prawdopodobnie wyewoluowały od swojego przodka, *P. gracilis*, z małych populacji poddanych surowemu klimatowi, izolowanych na

grzbietach górskich przez gęsty pas lasów. Rozdział taksonów z kompleksu *P. affinis* od pozostałych gatunków z grupy przypada w Plejstocenie, ok. 0,71 milionów lat temu. Wyniki oparte na analizie zegara molekularnego potwierdzają konieczność rozszerzenia kompleksu *P. affinis* o dwa dodatkowe gatunki: *P. ornatus* i *P. hoelzeli*. Kompleks ten rozdzielił się na dwie linie ok. 0,42 miliona lat temu i jest częściowo zgodny z wyznaczonymi regionami biogeograficznymi (A, B, C, D) (Rycina 2 w: **Kociński i in., 2022**). Do pierwszej linii można zaliczyć gatunki z centralnego (B) i północno-zachodniego regionu Półwyspu Bałkańskiego (C), a do drugiej gatunki z północno-wschodniego (D) i północno-zachodniego regionu Bałkanów (C).

Prezentowane wyniki badań genetycznych w połączeniu z danymi mofologicznymi będą wykorzystane do rewizji rodzaju. Stanowią one punkt wyjściowy dla dalszych badań nad filogenezą, genetyką populacyjną i filogeografią taksonów rodzaju *Poecilimon*.

6. Podsumowanie

Uzyskane wyniki pozwalają na wysunięcie następujących wniosków na temat systematyki i filogenezy grupy gatunków *Poecilimon ornatus*:

1. Dotychczasowa systematyka grupy gatunków *Poecilimon ornatus* jest prawidłowa. Potwierdzono że grupa ta jest monofiletyczna w obrębie rodzaju *Poecilimon,* zarówno na podstawie danych molekularnych jak i morfologicznych. Stwierdzono również istnienie kompleksu gatunkowego *Poecilimon affinis*, do którego należy zaliczyć cztery gatunki: *P. nonveilleri, P. pseudornatus, P. hoelzeli, P. ornatus* oraz pięć podgatunków *P. affinis*: *P. a. affinis, P. a. serbicus, P. a. komareki, P. a. hajlensis, P. a. dinaricus*.
2. Gatunki z grupy *P. ornatus* oddzieliły się od innych gatunków z rodzaju *Poecilimon* w Plejstocenie, na skutek zmian klimatycznych. Ich przodek najprawdopodobniej pochodził z południowych Bałkanów (Grecja) skąd następnie migrował w kierunku północnej części Półwyspu Bałkańskiego.
3. Pozycja taksonomiczna dwóch taksonów: *P. rumijae* i *P. poecilus* pozostaje niejasna. Wyniki analiz molekularnych i częściowo morfometrycznych sugerują, że mogą być traktowane jako dwa taksony, odrębne gatunki lub podgatunki *P. affinis*.

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Artykuły

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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 Palaearctic 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. bosphoricus*, *P. celebi*, *P. concinnus*, *P. davisi*, *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* (Frivaldszky, 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* (Frivaldszky, 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. nonveilleri* 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. 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, 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 $MgCl_2$, 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) 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 affinis* (Frivaldszky, 1868)	Ukraine, Chereska Oblast	55.09285N 33.57554E	MH800893 MH800894 MH800895	This study This study This study
		Bulgaria, Rila Mts., Iliyna 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
	Poecilimon affinis komareki 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
	Poecilimon affinis dinaricus Ingrisch & Pavićević, 2010	Montenegro, Susica	43.1776N 19E	MH800856	This study
		Montenegro, Mratinje	43.2477N 18.817E	MH800857	This study
	Poecilimon affinis serbicus Karaman, 1974	North Macedonia, Shar Mts, Ljuboten Park	42.18481N 21.12973E	MH800861 MH800862 MH800863	This study This study This study
	Poecilimon affinis hajlensis Karaman, 1974	Montenegro, Hajla	42.80296N 20.22638E	MH800864 MH800865 MH800866	This study This study This study
	Poecilimon affinis poecilus Ramme, 1951	North Macedonia, Shar Mts., Popova Shapka	42.01265N 20.88399E	MH800890 MH800891 MH800892	This study This study This study
	Poecilimon nonveilleri Ingrisch & Pavićević, 2010	Montenegro, Susica	43.1776N 19E	MH800858 MH800859 MH800860	This study This study This study
	Poecilimon pseudornatus 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
Poecilimon ornatus group	*Poecilimon ornatus* (Schmidt, 1850)	North Macedonia, Jakupica Mts., Cheples Chalet	41.71163N 21.40915E	MH800911 MH800912	This study This study
	Poecilimon hoelzeli Harz, 1966	–	–	AM886726	ULLRICH *et al.* (unpublished)
	Poecilimon jablanicensis Chobanov & Heller, 2010	North Macedonia, Jablanica Mt	41.2302N 20.5131E	MN737107 MN737108	This study This study
	Poecilimon nobilis Brunner von Wattenwyl, 1878	–	–	AM886695	ULLRICH *et al.* (unpublished)
	Poecilimon obesus Brunner von Wattenwyl, 1878	–	–	AM886773	ULLRICH *et al.* (unpublished)
	Poecilimon pindos Willemse, 1982	–	–	AM886765	ULLRICH *et al.* (unpublished)
	Poecilimon artedentatus Heller, 1984	–	–	AM886816	ULLRICH *et al.* (unpublished)
	Poecilimon gracilis (Fieber, 1853)	Montenegro, Mratinje	43.25216 N 18.81014E	MH800909 MH800910	This study This study
	Poecilimon gracilioides Willemse & Heller, 1992	–	–	AM886751	ULLRICH *et al.* (unpublished)
Poecilimon ampliatus group	*Poecilimon ampliatus* Brunner von Wattenwyl, 1878	Montenegro, Durmitor	43.15107N 19.08135E	MH800913 MH800914	This study This study
Poecilimon genus	*Poecilimon ukrainicus* Bey-Bienko, 1951	–	–	AM886832	ULLRICH *et al.* (unpublished)
Poecilimon heroicus group	*Poecilimon heroicus* Stshelkanovtzev, 1911	–	–	AM886756	ULLRICH *et al.* (unpublished)
Poecilimon genus	*Poecilimon schmidti* (Fieber, 1853)	–	–	AM886810	ULLRICH *et al.* (unpublished)
subfamily *Phaneropterinae*	*Polysarcus denticauda* (Charpentier, 1825)	–	–	AM886784	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, 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. 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	–

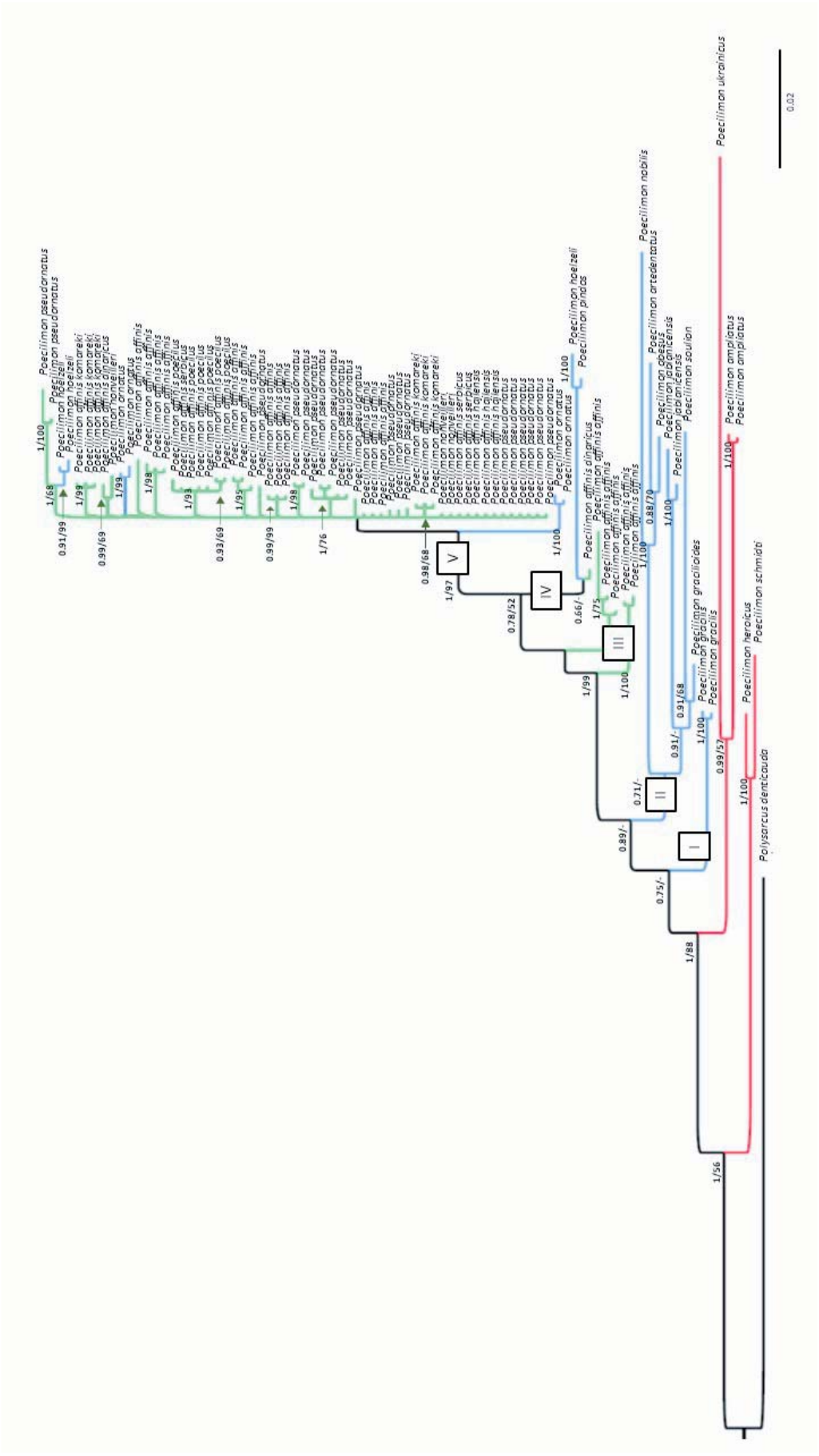

Fig. 1. Bayesian tree of the *Poecilimon ornatus* group based on COI sequences. Node labels indicate BI posterior probability (pp) and maximum likelihood bootstrap values (bv) over 50% (pp/bv). Green lines show specimens from the *Poecilimon affinis* complex; blue lines – the *Poecilimon ornatus* group; 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. 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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A taxonomic outline of the *Poecilimon affinis* complex (Orthoptera) using the geometric morphometric approach

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ABSTRACT

The genus *Poecilimon* contains 145 species, widely distributed in the Palaearctic, among which the *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 the 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 the *Poecilimon affinis* complex. The aim of this study is to test the hypotheses of an outlined species complex, namely the *P. affinis* complex, within the *P. ornatus* group using morphological data. Geometric analysis was conducted to explore variation in the structure of the male tegmen, ovipositor, male cercus, and male pronotum. The 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 male tegmen and male cercus are good morphostructures to distinguish the taxa belonging to the *P. affinis* complex from other species in the *P. ornatus* group. This may confirm our assumption for the designation of the *P. affinis* complex. The results of the principal component analysis of stridulatory file measurements, molecular data, and CVA of the ovipositor suggest adding two additional species to the complex: *P. ornatus* and *P. hoelzeli*.

Subjects Entomology, Taxonomy, Zoology
Keywords Systematics, Bush-crickets, Morphology, Phylogeny

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INTRODUCTION

Poecilimon Fischer, 1853 is one of the most species-rich genera within the Phaneropterinae subfamily. This genus comprises 145 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*). The similarity and variability of morphological characteristics make many *Poecilimon* species difficult to identify. The *Poecilimon ornatus* group (13 species and five subspecies) (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

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*. Photos: D. Chobanov.

Full-size DOI: 10.7717/peerj.12668/fig-1

and geomorphological factors, a rapid morphological evolution took place in this group (*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; *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. *Poecilimon 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 male pronotum and body size. Because of the lowering of 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 & 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 in the shape of the male pronotum and body size (*Chobanov & Heller, 2010*). Furthermore, song of *P. a. komareki* and *P. rumijae* resembles that of *P. pseudornatus* with a long silent beginning. Song of *P. nonveilleri* is short with a typical structure, whereas *P. a. affinis* has also short song and shows morphological differences to *P. nonveilleri* (own unpublished data). Due to the discrepancy between the authors, *P. rumijae* will also be treated separately in the present study. *Poecilimon pseudornatus*, *P. nonveilleri* and the subspecies of *P. affinis* are morphologically similar, although a recent molecular study based on the 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 the *P. affinis* complex.

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

To determine the morphological variation of the *Poecilimon ornatus* group, especially within the *Poecilimon affinis* complex, we used geometric morphometric methods based on the shape variation of four structures: male pronotum, male cercus, ovipositor, and male 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 morphometric method has been proved to be very useful for distinguishing 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 & Çiplak, 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 the species within the *P. ornatus* group, outline morpho-units and discuss the importance of morphological traits for the systematics of the group. 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). In Greece, field studies were approved by the Greek Ministry of the

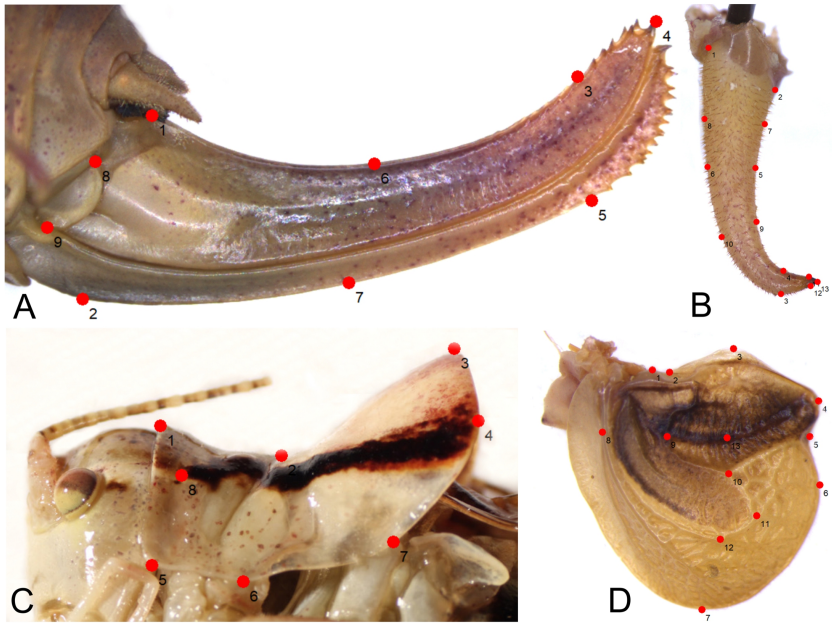

Figure 2 Position of the landmarks (red dots) on *Poecilimon* species used for geometric morphometrics. (A) Ovipositor. (B) Male cercus. (C) Male pronotum. (D) Male tegmen.
Full-size DOI: 10.7717/peerj.12668/fig-2

Environmental, Energy, and Climate Change (No 154812/951). In Bulgaria, we did not need a permit for collecting for scientific purposes because it was outside protected areas, and animals were not protected. The material was collected with scientific purpose through scientific activities of the Institute of Biodiversity and Ecosystem Research-BAS. In North Macedonia, the material was collected with collaboration with the Macedonian Ecological Society (https://mes.org.mk/en/) and the Biology Students' Research Society during their field studies with the respective permissions provided. In Montenegro, Serbia, and Albania we did not need a permit for collecting for scientific purposes because it was outside protected areas, and animals were not protected.

Geometric morphometrics

In total, 196 specimens belonging to 16 taxa of the *Poecilimon ornatus* group were used for geometric morphometric analyses. Four morphostructures (male pronotum, male cercus, ovipositor, and male 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 the patterns of morphological variation, 8 landmarks (including 1 semilandmark) of male pronotum, 13 (7 semilandmarks) of male cercus, 13 (1 semilandmark) of male tegmen, and 9 (2 semilandmarks) of ovipositor (Fig. 2) were plotted manually in tpsDIG2 v.2.17 (*Rohlf, 2015*). The list of landmarks and semilandmarks used in this study is included in Table 2. After plotting the landmarks, the intersections marked in the TPS files were aligned using a Procrustes superimposition. Partial warp scores were studied using Canonical variate

Table 1 The number of specimens used for the geometric morphometric analysis.

Species	Male cercus	Male tegmen	Ovipositor	Male 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 & Pavicevic, 2010	10	10	1	6
Poecilimon pseudornatus * Ingrisch & Pavicevic, 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	–

Notes.
**Poecilimon affinis* complex.

analysis (CVA) for each structure in MorphoJ v.1.06d (*Klingenberg, 2011*). The first two Canonical Variables (CVs) with the greatest power to distinguish the groups were plotted in the same software. The Mahalanobis distance was measured and statistically tested using 10,000 permutation repeats.

Stridulatory measurements

The length of the stridulatory file was measured and the number of stridulatory teeth was counted for 154 specimens from the *P. ornatus* group (9 specimens of *P. affinis ssp.*, 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)

Table 2 List of the landmarks and semilandmarks of the pronotum, male cercus, tegmen, and ovipositor used in the geometric morphometric analysis.

The landmark number	Pronotum	Male cercus	Tegmen	Ovipositor
1	upper frontal part	groove left at base	most distant point	highest point at the base
2	upper part of mid groove	groove right at base	upper concave point	lowest point of the base
3	upper posterior point	most distant point at apex	most distant point	begging of teeth at the upper valve
4	lateral posterior point	opposite to 3*	most distant point	tip of upper valve
5	lower frontal part	middle measured approximately between 4 and 2*	concave side point	begging of teeth at the lower valve
6	lowest middle part	opposite to 5*	most distant point	middle between 1 and 3*
7	mid point between 4 and 6*	approximetly middle between 2 and 5*	most distant point	middle between 2 and 5*
8	begging of dark band	approximetly middle between 1 and 6*	most distant point of the lateral vein	upper point of gonangulum
9		approximetly middle between 5 and 4*	bifurcation between veins	lower point of gonangulum
10		approximetly middle between 6 and 3*	bifurcation between veins	
11		upper end of black spine	bifurcation between veins	
12		lower end of black spine	bifurcation between veins	
13		tip of cercus	mark on the stridulatory vein between the points 3 and 10*	

Notes.
*semilandmarks.

tooth. The tegmen was placed upside down so that the stridulatory file could be viewed with its proximal and distal ends being at the same level. This way, the distance between the ends was measured along the imaginary line connecting those. The total number of stridulatory teeth and the number of teeth within 2 mm at the middle of the stridulatory file were counted. Measurement 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 to determine the phylogenetic relationship between the 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/). The 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. The 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

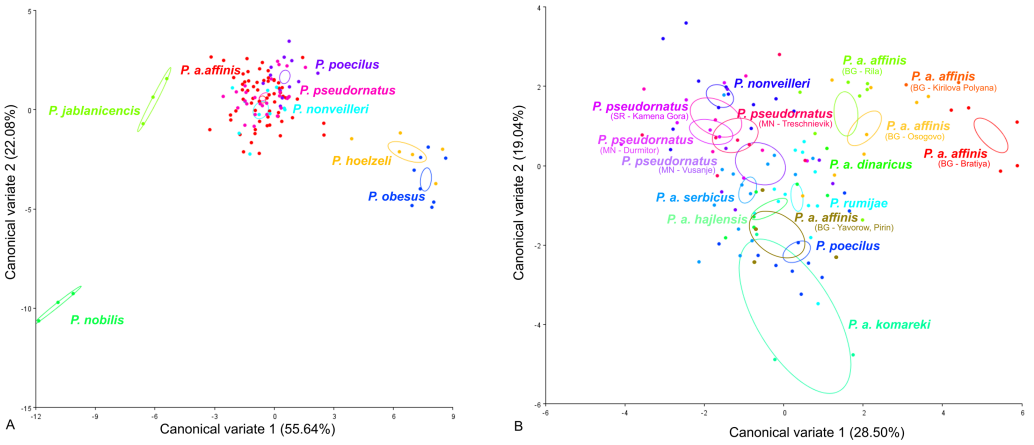

Figure 3 Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of male tegmen: *P. ornatus* group (A) and *P. affinis* complex (B). The different colors of the species *P. pseudornatus* and *P. a. affinis* indicate different locations from which the specimens were collected. The localities are indicated below taxa name (SR, Serbia; MN, Montenegro; BG, Bulgaria).

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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 male tegmen, 142 of male pronotum, and 141 of male 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 (seven landmarks, two semilandmarks) (Fig. 2A), male cercus (six landmarks, seven semilandmarks) (Fig. 2B), male pronotum (seven landmarks, one semilandmark) (Fig. 2C), and male tegmen (12 landmarks, one semilandmark) (Fig. 2D).

CV analysis of the male 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 male 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 through pairwise comparisons among the group revealed highly significant differences (10,000 permutation rounds; $P < 0.0001$), ranging from 2.50 (*P. affinis* and *P. pseudornatus*) to 19.66 (*P. poecilus* and *P. obesus*). The Procrustes distances also showed significant differences between groups (10,000 permutation rounds; $P < 0.0001$) ranging from 0.03 (*P. poecilus* and *P. pseudornatus*) to 0.28 (*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 male tegmen (CV1 = 28.5% and CV2 = 19.4%). CV1 and CV2 analyses

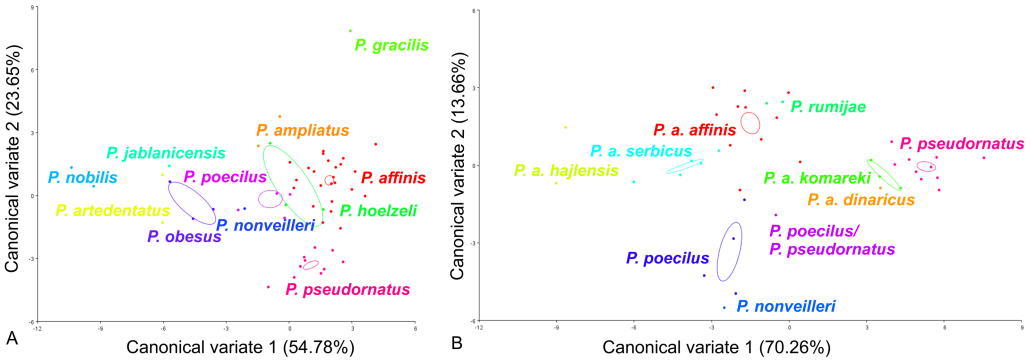

Figure 4 Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of ovipositor: *P. ornatus* group (A) and *P. affinis* complex (B). The different colors indicate different species/subspecies of studied bush-crickets.

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of the *Poecilimon affinis* complex did not indicate clear clusters representing each of the existing species/subspecies. However, the 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 male tegmen are 2.77 for *P. poecilus* and *P. pseudornatus*, and 8.13 for *P. a. komareki* and *P. a. dinaricus* (10,000 permutation rounds; $P < 0.0001$). The Procrustes distances also showed significant differences (10,000 permutation rounds; $P < 0.001$), ranging from 0.03 (*P. a. serbicus* and *P. pseudornatus*) to 0.12 (*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 2.78 (*P. poecilus* and *P. hoelzeli*) to 15.72 (*P. gracilis* and *P. nobilis*). The Procrustes distances also showed significant differences between groups (10,000 permutation rounds, $P < 0.0001$) ranging from 0.04 (*P. affinis* and *P. hoelzeli*) to 0.19 (*P. pseudornatus* and *P. gracilis*) (Table S3).

At the species complex 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 through pairwise comparisons of the complex revealed highly significant differences (10,000 permutation rounds; $P < 0.0001$), ranging from 2.69 (*P. rumijae* and *P. a. affinis*) to 14.50 (*P. pseudornatus* and *P. a. hajlensis*). The Procrustes distances also showed highly

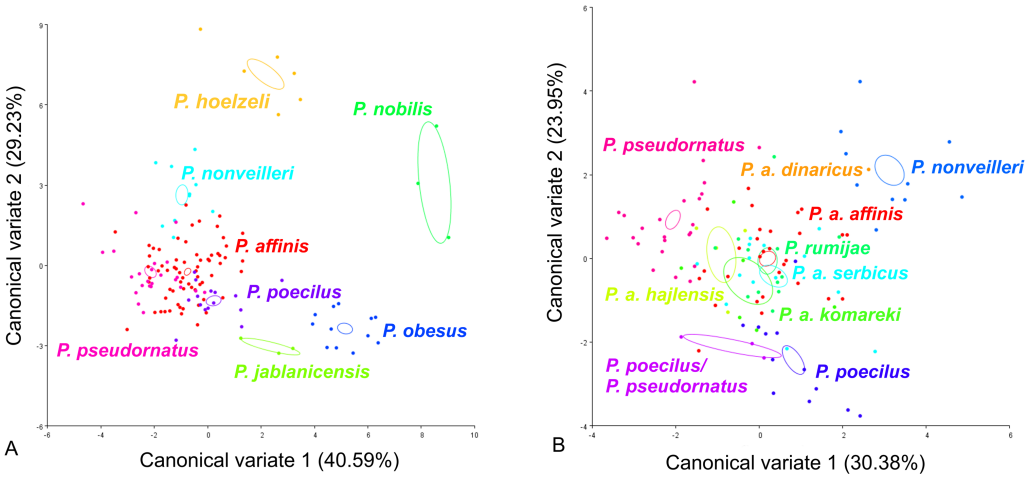

Figure 5 Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of male cercus: *P. ornatus* group (A) and *P. affinis* complex (B). The different colors indicate different species/subspecies of the studied bush-crickets.

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significant differences (10,000 permutation rounds; $P < 0,005$), ranging from 0.03 (*P. a. serbicus* and *P. a. affinis*) to 0.15 (*P. a. komareki* and *P. a. dinaricus*) (Table S4).

CV analysis of the male 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 through pairwise comparisons among group revealed highly significant differences (10,000 permutation rounds; $P < 0.0001$), ranging from 2.71 (*P. pseudornatus* and *P. affinis*) to 12.25 (*P. hoelzeli* and *P. jablanicensis*). The Procrustes distances also showed significant differences between groups (10,000 permutation rounds; $P < 0.0001$), ranging from 0.03 (*P. affinis* and *P. pseudornatus*) to 0.17 (*P. pseudornatus* and *P. nobilis*) (Table S5).

For the male 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). The Mahalanobis distances obtained through pairwise comparisons of the complex revealed significant differences (10,000 permutation rounds; $P < 0.0001$), ranging from 2.87 (*P. pseudornatus* and *P. a. hajlensis*) to 8.65 (*P. a. dinaricus* and *P. a. komareki*). The Procrustes distances also showed significant differences (10,000 permutation rounds; $P < 0.0001$), ranging from 0.03 (*P. a. affinis* and *P. poecilus*) to 0.10 (*P. a. komareki* and *P. nonveilleri*) (Table S6).

For the male 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

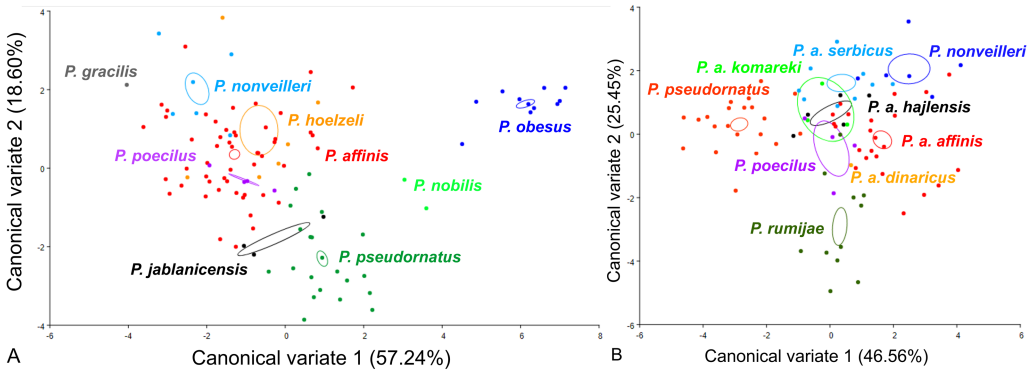

Figure 6 Scatter plot of the two first canonical variate axes (CV1 and CV2) analysis of centroid sizes of male pronotum: *P. ornatus* group (A) and *P. affinis* complex (B). The different colors indicate different species/subspecies of the studied bush-crickets.

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be clearly separated from other species of the *Poecilimon ornatus* group (Fig. 6A). The Mahalanobis distances obtained through pairwise comparisons among group revealed significant differences (10,000 permutation rounds; $P < 0.0001$), ranging from 2.20 (*P. poecilus* and *P. affinis*) to 12.81 (*P. gracilis* and *P. obesus*). The Procrustes distances also showed significant differences between groups (10,000 permutation rounds; $P < 0.0001$), ranging from 0.03 (*P. poecilus* and *P. affinis*) to 0.16 (*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 male 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 the *P. affinis* complex (Fig. 6B). The Mahalanobis distances obtained through pairwise comparisons of the complex revealed significant differences (10,000 permutation rounds; $P < 0.0001$), ranging from 2.73 (*P. a. hajlensis* and *P. a. affinis*) to 5.68 (*P. rumijae* and *P. nonveilleri*). The Procrustes distances also showed highly significant differences (10,000 permutation rounds; $P < 0.0001$), ranging from 0.04 (*P. poecilus* and *P. a. affinis*) to 0.14 (*P. rumijae* and *P. nonveilleri*) (Table S8).

Stridulatory measurements

Poecilimon 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). *Poecilimon 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 3). Principal Component Analysis of the stridulatory file and the number of teeth shows that *P. nonveilleri, P. ornatus, P. hoelzeli, P. pseudornatus, P. a. serbicus, P. a. hajlensis*, and *P. a. affinis* overlap. Moreover, we can conclude that *P. a. affinis* is the most diverse taxon within the *P. ornatus* group, while *P. a. komareki* is the most distinct taxon of the studied group (Fig. 7).

Table 3 Measurements for stridulatory files of the *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

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 the COI data show 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 grouping by geographic locality. *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).

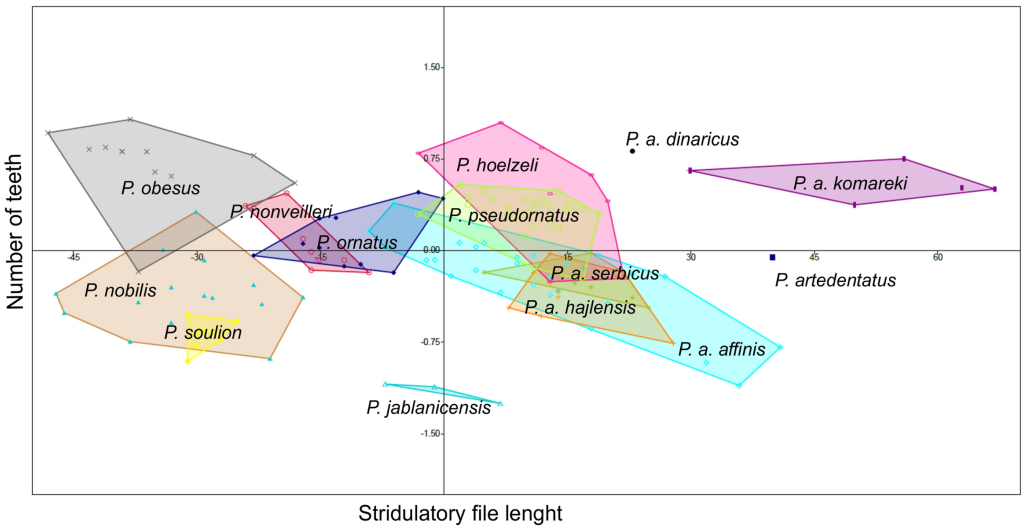

Figure 7 Principal Component Analysis (PCA) of stridulatory measurements and number of teeth: *P. ornatus* group. The different colors indicate different species/subspecies of studied bush-crickets.
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DISCUSSION

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 male tegmen, ovipositor, cercus and male pronotum were used successfully in morphological studies of *Poecilimon* (*Heller, 2004*; *Chobanov & Heller, 2010*; *Kaya et al., 2012*; *Kaya, Boztepe & Çiplak, 2015*; *Kaya et al., 2018*). The present work showed 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 male tegmen covered by the male pronotum vary between specimens from the same locality. Our results support the poor taxonomic utility of the shape of male pronotum in this group for distinguishing the species belonging to the *P. affinis* complex from other species in the group (Fig. 6A). However, based on the shape of the male tegmen, *P. affinis* and its subspecies group with *P. nonveilleri*, *P. pseudornatus* in the same place, which clearly separates them from other species (Fig. 3A). This may confirm our assumption for the designation of the *P. affinis* complex including other species from the *Poecilimon ornatus* group. CV analysis of centroid sizes of the male 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*. *Poecilimon rumijae* may likely 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 assumption is also confirmed by the analysis of the ovipositor, 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).

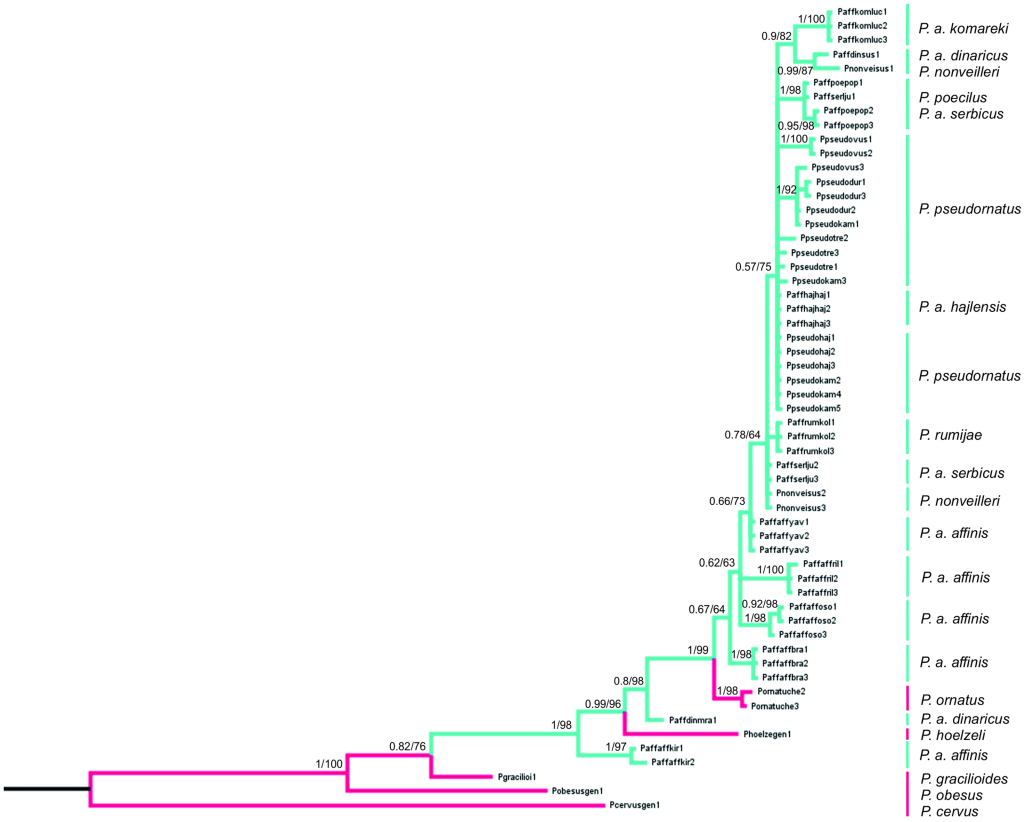

Figure 8 Phylogenetic tree based on Bayesian inference method including COI sequences of the *P. affinis* complex. Bayesian Inference (BI) and Maximum Likelihood (ML) topologies were similar, so only one tree is shown. BI posterior probability (<0.5) and ML bootstrap values (<50) are shown on the nodes. Pink indicates species outside of the *P. affinis* complex; green indicates taxa from the designated *P. affinis* complex.

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On the other hand, the results based on the male cercus (Fig. 5B) show that *P. a. komareki* and *P. rumijae* overlap, 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 (Figs. 3A, 4A, 5A, 6A).

The most distinct species in our sample is *P. nobilis* based on the analysis of the male tegmen (Fig. 3A) and male cercus (Fig. 5A), *P. gracilis* based on the ovipositor (Fig. 4A), and *P. obesus* based on the male 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 (Figs. 3A, 4A, 5A, 6A). The results suggest that the difference between specimens of *P. a. affinis* is related to the locality in which they occur (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 the male 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 the male cercus may be a better feature for separating species in this group. The results of the CV analysis of centroid size of the ovipositor (Fig. 4A) show 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* (Fig. 4A). *Poecilimon poecilus*, which we suggested to treat separately in this work, seems to fell within the variation 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* (Figs. 3A, 4A, 5A, 6A). However, to establish the taxonomic status of *P. poecilus*, additional research is needed.

Stridulatory structures measurements

The stridulatory file and the number of teeth can be a good morphological feature for distinguishing taxa in the *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 3). 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)* observed 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* does not overlap with other subspecies, which may mean that it is the most distinct taxon from all studied taxa of the *P. ornatus* group. *Poecilimon hoelzeli* and *P. pseudornatus* have a similar number of teeth and length of the 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) and the mitochondrial genes (16S rRNA, tRNA-Val, 12S rRNA) involving some of the group's species were conducted by *Ullrich et al. (2010)*. However, they did not provide conclusive information on the relationship between species in this group. *Kociński (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 male 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 taxa of the *P. ornatus* group. This opinion is confirmed by the CVA results of male pronotum and ovipositor (Figs. 4B, 6B). The specimens from *P. poecilus* also form different nodes compared to *P. a. affinis*, thus, it may be treated as a subspecies of *P. affinis*, which is supported by the CVA of the male tegmen, male cercus, ovipositor, and male pronotum (Figs. 3B, 4B, 5B, 6B).

CONCLUSIONS

The geometric morphometric 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 provides better insight into the relationships 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 *P. rumijae* and *P. poecilus* remains unclear. Our results show some discordances with previous studies and point to the need for a most thorough interdisciplinary phenetic and genetic study in order to solve the systematics of this particular group of bush-crickets.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Maciej Kociński conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Beata Grzywacz performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Georgi Hristov analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Dragan Chobanov conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

In Greece, field studies were approved by the Greek Ministry of the Environment, Energy, and Climate Change.

In Bulgaria, we did not need a permit for collecting for scientific purposes because it was outside protected areas, and animals were not protected. The material was collected with scientific purpose through scientific activities of the Institute of Biodiversity and Ecosystem Research-BAS. In North Macedonia, the material was collected with collaboration with the Macedonian Ecological Society (https://mes.org.mk/en/) and the Biology Students' Research Society during their field studies with the respective permissions provided. In Montenegro and Albania, we did not need a permit for collecting for scientific purposes because it was outside protected areas, and animals were not protected.

In Serbia, we also did not need a permit for collecting insects because it was outside protected areas, and animals were not protected.

Data Availability

The following information was supplied regarding data availability:

The 4 morphostructures (male tegmen, ovipositor, cercus, male pronotum) and the localization of each landmark of each specimen and the measurements of the stridulatory's file and the number of teeth of each specimen are available in the Supplementary Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.12668#supplemental-information.

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New insights into the genetic diversity of the Balkan bush-crickets of the *Poecilimon ornatus* group (Orthoptera: Tettigoniidae)

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Abstract

The Balkan Peninsula is treated as a hotspot of biodiversity with over 40% of European bush-crickets occurring there. *Poecilimon* Fischer, 1853 is one of the largest Palaearctic orthopteran genera containing several species groups. One of them is the *Poecilimon ornatus* group (Schmidt, 1850) with 13 species and 5 subspecies. Among the group, the *Poecilimon affinis* complex is designated as consisting of *P. pseudornatus* Ingrisch & Pavićević, 2010, *P. nonveilleri* Ingrisch & Pavićević, 2010, and five subspecies of *P. affinis* (Frivaldszky, 1868). The aim of this study is to reconstruct the phylogenetic relationships among taxa of the *P. ornatus* group and to elucidate the position of taxa related to the *P. affinis* complex. Molecular phylogeny supported the monophyly of the *P. ornatus* group and showed that their ancestor probably originated in the southern Balkans. The underlying processes are thought to be six dispersals and five vicariance events linked to geological events and climate changes in the Pleistocene. The species delimitation analysis showed mostly nine hypothetical species among the group.

Keywords

biogeography, evolution, phylogeny, *Poecilimon affinis* complex, taxonomy

1. Introduction

The Balkan Peninsula is considered one of the most important Mediterranean refugia during the Quaternary glacial periods (Hewitt 2000). Multiple isolations and reconnections to Anatolia and Europe during the Neogene may underlie the huge biodiversity of this area with high levels of species richness and endemism. The region of the Balkan Peninsula is treated as a hotspot of biodiversity (Blondel and Aronson 1999; Myers et al. 2000; Mittermeier et al. 2003). Several land connections and submergences during the Miocene (23-5.33 Mya) and Pliocene (5.33-2.58 Mya) influenced the later development of this region (Steininger and Rögl 1984; Dermitzakis 1990; Popov et al. 2004; Husemann et al. 2014; Previšić et al. 2014; Poulakakis et al. 2015; Simaiakis et al. 2017; Španiel et al. 2017; Gömöry et al. 2020).
The Balkan Peninsula is at the forefront of the orthopteran diversity in the Palaearctic with over 40% of all European bush-crickets recorded from this region and new species being constantly described (Heller et al. 1998; Hochkirch et al. 2016). With the present study, we focus on one of the largest Palaearctic orthopteran genera, *Poecilimon*, comprising 145 species divided into 18 species groups (Cigliano et al. 2022). Members of the genus are distributed from the Apennines to Western Siberia and Central Tian-Shan (Bey-Bienko 1954) with the highest number of endemic species concentrated in the Aegean and Pontic areas. All species of *Poecilimon* are short-winged and flightless with complex acoustic communication. Cyclic glaciations during the Pleistocene influenced the diversity of the genus causing rapid radiation and diversification (La Greca 1999; Kaya et al. 2015; Borissov and Chobanov 2020; Borissov et al. 2020, 2021).
The taxonomy and phylogenetic relationships within *Poecilimon* are mainly based on morphological and bioacoustic traits (e.g., Heller et al. 2006, 2011; Chobanov and Heller 2010; Ingrisch and Pavićević 2010; Kaya et al. 2012, 2018; Boztepe et al. 2013; Sevgili et al. 2018; Chobanov et al. 2020). Many species groups of this genus have been studied in terms of molecular phylogeny and biogeography (Boztepe et al. 2013; Kaya et al. 2015; Kaya 2018; Borissov et al. 2020, 2021) while one of the largest groups – the *Poecilimon ornatus* group, has only recently been considered (Kociński 2020; Kociński et al. 2021). This species group contains bush-crickets distributed mostly in mountainous areas from the South-Eastern Alps to the Carpathians and Peloponnese and an isolated spot in Ukraine. The latest findings using cytochrome c oxidase subunit I (COI) barcodes showed the monophyly of the *P. ornatus* group (Kociński 2020). However, there is still an unclear relationship among the taxa associated with the *Poecilimon affinis* complex in the *P. ornatus* group (Chobanov and Heller 2010; Kociński 2020; Kociński et al. 2021). Currently, the *P. affinis* complex includes *P. nonveilleri*, *P. pseudornatus* and five subspecies of *P. affinis* (*P. a. affinis*, *P. a. hajlensis* Karaman, 1974, *P. a. serbicus* Karaman, 1974, *P. a. komareki* Cejchan, 1957, *P. a. dinaricus* Ingrisch & Pavićević, 2010). Recent studies suggested extending this complex with *P. hoelzeli* Harz, 1966 and *P. ornatus* (Schmidt, 1850) (Kociński 2020; Kociński et al. 2021).
'Species complex' refers to a group of sibling species with similar morphology or identical populations that are reproductively isolated (Mayr 1963; Sigovini et al. 2016) or cryptic species, where the boundaries between taxa are morphologically indeterminate. 'Species complex' has also been defined as consisting of closely related taxa that are still waiting for critical revision to clarify their taxonomic status (Sigovini et al. 2016). Cryptic species were defined as "two or more distinct species that are erroneously classified (and hidden) under one species name" (Bickford et al. 2007). In this sense, the *P. ornatus* group constitutes one or more species complexes that need to be resolved using interdisciplinary research.
Molecular data and species delimitation methods have become very important tools to detect and delimit new species (Luo et al. 2018; Mendes et al. 2021). DNA sequence analysis has revolutionized the way of recognizing species (Hajibabaei et al. 2007; Taylor and Harris 2012) and helped to reveal the existence of cryptic species in many taxa (Knowlton 1993;

Bickford et al. 2007; Scheffers et al. 2012). The cytochrome c oxidase subunit I (COI) gene is a commonly used marker, easy to amplify due to the availability of conserved primers, with a strong phylogenetic signal, used in taxonomy (Folmer et al. 1994; Simon et al. 1994, 2006; Spicer 1995; Zhang and Hewitt 1997; Goto and Kimura 2001; Remigio and Hebert 2003; Kjer et al. 2014; Wang et al. 2017; Jafari et al. 2019; Karmazina et al. 2020). This marker is successfully used in Orthoptera and treated as a DNA barcode (Lehmann et al. 2017; Kaya and Çıplak 2018; Kundu et al. 2020; Liu and He 2021; Şirin et al. 2021; Warchałowska-Śliwa et al. 2021). NADH dehydrogenase subunit 2 (ND2) shows a higher proportion of variable and parsimony-informative sites (PI) and a lower heterogeneity of the substitution index than COI (Cheng et al. 2018), which was confirmed in *Isophya* - a closely related genus to *Poecilimon* (Chobanov et al. 2017), and in *Hematopoecilimon* (Borissov and Chobanov 2020). The control region (CR) is mainly used to study phylogenetic relationships in closely related taxa (Amaral et al. 2016; Li and Liang 2018), successfully tested in *Poecilimon* (Eweleit et al. 2015; Borissov and Chobanov 2020). The internal transcribed spacer 1 (ITS1) region represents a useful marker for the analysis of relationships in closely related species of Orthoptera and for recognition of new species because of higher evolutionary rates leading to greater variability in both, nucleotide sequence and length (Hillis and Dixon 1991; Gu et al. 2020). In this study, we perform molecular analyses of taxa in the *P. ornatus* group using a combined dataset (COI, ND2, CR, and ITS1).

Our study aims to reconstruct the phylogenetic relationships among taxa in the *P. ornatus* group and to elucidate the position of taxa related to the *P. affinis* complex. We test the hypothesis of a recent origin and divergence of the taxa in the *P. affinis* complex from the rest of the species in the *P. ornatus* group. The estimated divergence times were applied to test the correlation between the evolutionary history of this group and paleogeographic events in the Balkan Peninsula. Additionally, phylogeographical biogeographic tools were used to check if speciation was affected by vicariances, dispersal, and/or extinction events.

2. Material and methods

2.1 Taxon sampling

A total of 74 specimens from 34 populations representing 19 formerly recognized taxa of the *Poecilimon ornatus* group were used in this study (Table 1). Six outgroup species were selected representing three other species groups of *Poecilimon* (*P. sureyanus* Uvarov, 1930 and *P. turcicus* Karabag, 1950 from the *P. bosphoricus* group Brunner von Wattenwyl, 1878; *P. sanctipauli* Brunner von Wattenwyl, 1878 from the *P. sanctipauli* group Brunner von Wattenwyl, 1878; *P. cretensis* Werner, 1903 from the *P. jonicus* group (Fieber, 1853)), and two related genera of Barbitistini Jacobson, 1905 (*Isophya speciosa* (Frivaldszky, 1868), *Leptophyes albovittata* (Kollar, 1833)). Specimens from the *P. ornatus* group were collected in the Balkan Peninsula (Bulgaria, Serbia, Montenegro, Albania, North Macedonia, Greece) between 2006 and 2018 (Table 1, Fig. 1). Bush-crickets were collected by Maciej Kociński and Dragan Chobanov.

Table 1 should be placed here horizontally.

2.2 Molecular laboratory procedure

DNA was extracted from hind leg-muscle tissue using the NucleoSpin tissue kit (Macherey–Nagel, Germany) according to the manufacturer's protocol. Genomic DNA was used for the amplification of three mitochondrial markers (COI, ND2, CR) and one nuclear marker (ITS1). The Polymerase chain reaction (PCR) primer pairs used in this study are included in Table 2. The amplification was performed in 25 µl reaction volume containing 12.5 µl 2x Phanta Max

Master Mix (Vazyme, China), 10 mM dNTP mixture, 10 µM forward and reverse primers, 1-3 µl genomic DNA, and sterile deionized water. The PCR protocols used for amplification of COI, ND2, CR, and ITS1 are included in Table 3. All PCR products were purified using Exo-BAP Mix (EURx, Poland, following the standard protocol). The sequencing reaction was carried out in 10 µl reactions containing: 1.5 µl of sequencing buffer, 1.0 µl of BrilliantDye™ v3.1 Terminator Cycle Sequencing Kit (NimaGen, 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 protocol was as follows: the initial melting step of 3 min at 94°C followed by 25 cycles of 10 s at 96°C, 5 s at 55°C and a final step of 90 s at 60°C. The obtained sequences were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank) under the accession numbers provided in Table 1. Additionally, 85 DNA sequences were acquired from GenBank. The nucleotide sequences were edited and aligned in CodonCode Aligner 9.0 (CodonCode Corporation; https://www.codoncode.com/aligner) with default parameters. All sequences were checked for stop-codons in MEGA 11 (Tamura et al. 2021), verified using BLAST of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Genetic distances were calculated using MEGA 11 (Tamura et al. 2021). The saturation of the nucleotide substitution was checked for CR, ND2, and two separate partitions of COI (with codon positions 1 + 2 and codon position 3) (Xia et al. 2003) through the substitution saturation test in DAMBE (Xia 2013). The partition homogeneity test (Farris et al. 1995) was conducted in PAUP (Swofford 2002) with 1000 replicates to determine whether all regions (COI, ND2, CR, ITS1) could be combined in a unique data matrix.

Figure 1 should be place here vertically (half page).

2.3 Phylogenetic analyses

To infer evolutionary relationships, two methods were used – Bayesian inference (BI) and maximum likelihood (ML). The substitution model of evolution was estimated in MrModeltest software (Nylander 2004) using the Akaike Information Criterion (AIC). MrBayes (Ronquist et al. 2012) was used to obtain the Bayesian tree (BI). Posterior probabilities were based on two independent Markov chain Monte Carlo (MCMC) runs, each composed of four chains (three heated chains and one cold chain). BI was performed for 6,000,000 generations, with a sampling of trees every 100 generations. The convergence of the analyses was validated by monitoring the likelihood values using Tracer (Rambaut et al. 2018). Maximum likelihood (ML) estimates of the phylogeny were conducted using IQ-TREE (Nguyen et al. 2015). For bootstrap analyses, 1,000 pseudoreplicates were generated. BI and ML trees were visualized in FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 2 should be placed here vertically.

2.4 Sequence-based species delimitation test

To detect independently evolved lineages, three different DNA sequence-based species delimitation approaches were chosen. The first approach was the general mixed Yule-coalescent (GMYC) model. It uses the maximum likelihood approach based on the prediction that independent evolution leads to the appearance of distinct genetic clusters (Fujisawa and Barraclough 2013). This approach was successfully used for detecting cryptic lineages (e.g., Pons et al. 2006; Jörger et al. 2012; Chobanov et al. 2017). The next approaches were the Automatic Barcode Gap Discovery (ABGD) and Assemble Species by Automatic Partitioning (ASAP). These methods use pairwise distances to group sequences into potential species based on detecting gaps in the variation between supposed intra- and interspecies groups (barcode thresholds) (Puillandre et al. 2012, 2021). The last method was the Poisson Tree

Processes (bPTP), which is mainly intended for delimiting species in single-locus molecular phylogenies (Zhang et al. 2013).

Table 3 should be placed here vertically.

2.5 Estimation of divergence time and biogeographic analysis

To date the most recent common ancestor, the Bayesian approach with an MCMC integration was used in BEAST (Drummond et al. 2012) based on COI sequences. In order to follow the phylogenetic tree-topology, we have constrained monophyly for the well-supported clades of the *P. ornatus* group, while monophyly was not set for the branches within the *P. affinis* complex due to poor resolution. The analysis was run for 10,000,000 generations with sampling every 1,000 generations and a 10% burn-in. For time estimation analyses, an uncorrelated lognormal relaxed clock was applied (Drummond et al. 2006). The convergence to stationary distribution and the effective sample size of model parameters were checked using Tracer. The maximum clade credibility trees were built with TreeAnnotator (Drummond et al. 2012). In a recent study, divergence dates in *Poecilimon* were estimated based on the minimum time of isolation of *Poecilimon cretensis*, endemic to the island of Crete (Borissov et al. 2020). As a result, an intraspecific lineage split between the easternmost and the other lineages of *P. cretensis* was estimated at 0.8 Ma, possibly reflecting former vicariant events as a result of the former disconnection of the easternmost part of Crete. The latter dating is here used as a secondary calibration to date recent divergence times in the *P. ornatus* species group. *Poecilimon cretensis* was included in the analyses based on ND2 and the age of the eastern lineage (Kotsounari) was constrained at 0.8 Ma (SD=0.2) (see also Borissov et al. 2021). In order to infer the biogeographic history of the *Poecilimon ornatus* group, we first selected areas defined as centers of endemism. As most taxa concerned are regional endemics (occurring in a mountain range or a geographic outline of a few mountain ranges and/or valleys) and only one species (*Poecilimon jablanicensis* Chobanov & Heller, 2010) is strictly a local endemic, the regions selected cover the geographical extent of a few sympatric taxa. Thus, wider distributed species may occur in more than one region. As a result, five biogeographical regions (Fig. 2, 3; A- Southern, B- Central, C- North-Western, D-(North)-Eastern) (some bordering or isolated areas that are considered outliers and are not sampled here are omitted) related to species distribution were defined: Southern (S Greece) – *P. nobilis* Brunner von Wattenwyl, 1878, *P. artedentatus* Heller, 1984, *P. obesus* Brunner von Wattenwyl, 1878; Central (NW Greece, S North Macedonia, S Albania) – *P. jablanicensis*, *P. soulion* Willemse, 1987, *P. hoelzeli*, *P. pseudornatus*, *P. obesus*, *P. gracilioides* Willemse & Heller, 1992, *P. pindos* Willemse, 1982; North-Western (N North Macedonia, Montenegro, Kosovo, S Serbia, N Albania) – *P. pseudornatus*, *P. poecilus* Ramme, 1951, *P. a. dinaricus*, *P. a. hajlensis*, *P. a. serbicus*, *P. a. komareki*, *P. rumijae*, *P. nonveilleri*, *P. gracilis* (Fieber, 1853); (North-)Eastern (E North Macedonia, Bulgaria) – *P. ornatus*, *P. affinis* s. str. Biogeographic reconstruction was conducted in Statistical dispersal-vicariance analysis (S-DIVA; Yu et al. 2010) in RASP (Yu et al. 2015) using the maximum clade credibility tree and distribution file. The condensed tree was generated by BEAST. The number of maximum ancestral areas was set to four. The S-DIVA analysis was conducted with the default settings. The Mantel test was used to analyze the association between the genetic mean distance matrix based on four genes (COI, ITS1, ND2, CR) and the geographic distance matrix in Past 4.03 (https://www.nhm.uio.no/english/research/infrastructure/past/) with 10 000 permutations. The geographic distance matrix was prepared in Geographic Distance Matrix Generator v. 1.2.3 (https://biodiversityinformatics.amnh.org/open_source/gdmg/).

3. Results

The final alignment of the COI sequence results in 607 bp with 129 parsimony-informative sites and 196 variable sites. The CR (including the 12S rDNA gene containing A+T-rich region) consists of 446 bp with 188 parsimony-informative and 272 variable sites. ND2 sequences include 695 bp, among them 168 are parsimony-informative and 245 variable sites. The final alignment of ITS1 sequences consists of 465 bp with 70 parsimony-informative and 130 variable sites. The combined matrix data of COI, ND2, CR, ITS1 consists of 2213 bp and involved six outgroup species. The genetic mean distance for CO1 and ND2 among taxa from the *P. affinis* complex is 0.02, whereas among the rest of the species from the *P. ornatus* group – 0.1. For CR, the genetic mean distance among taxa from the *P. affinis* complex is 0.05, among the rest of the species from the *P. ornatus* group is 0.2. The genetic mean distance for ITS1 is 0.04 for taxa from the *P. affinis* complex, and 0.09 for the rest of the taxa from the *P. ornatus* group. The genetic distances between species from the *P. affinis* complex and the *P. ornatus* group for each marker (COI, ND2, CR, ITS1) are available in Table 4.

Table 4 should be placed here vertically.

The results of the substitution saturation test for COI, ND2, and CR alignments are summarized in Table 5. Calculated P-values were significant for all gene alignments and Iss (index of substitution saturation) values were lower than Iss.c (critical index of substitution saturation) in all cases. No saturation of the phylogenetic signal was observed for the COI, ND2, and CR datasets.

Table 5 should be placed here vertically.

The substitution one-parameter model Jukes–Cantor (JC) with Gamma Distribution (G) and Invariable site (I) was the best fit for the COI, ND2, CR and ITS1 data matrix.
The BI and ML phylogenetic trees showed the same topology (Fig. 4) and confirmed the monophyly of the *P. ornatus* group (posterior probability support, PP = 1.0; bootstrap support, BP = 100), whereas the *P. affinis* complex was paraphyletic as suggested in Kociński (2020). The first clade consists of *P. nobilis, P. artedentatus* and *P. obesus*. The second clade includes *P. gracilis, P. jablanicensis,* and *P. soulion*. *Poecilimon gracilioides* and *P. pindos* occupy the branches between the second and third clade. The third clade consists of the taxa from the *P. affinis* complex: *P. affinis affinis, P. a. dinaricus, P. poecilus, P. a. komareki, P. a. serbicus, P. nonveilleri, P. a. hajlensis, P. rumijae, P. pseudornatus*; and two additional species: *P. hoelzeli* and *P. ornatus*. *Poecilimon a. affinis* is the most diverse taxon among the complex, which supports recent studies (Kociński 2020; Kociński et al. 2021). *Poecilimon a. affinis,* from Rilski Manastir and the Rila Mtns, seems to be a sister taxon to the remaining representatives of the *P. affinis* complex. *Poecilimon rumijae* forms a separate branch among the third clade, as does *P. poecilus,* which is treated as a synonym of *P. a. affinis* according to the current systematics (Cigliano et al. 2022). Specimens of *P. pseudornatus* are grouped regardless of their location. Moreover, the phylogenetic relationship between taxa does not correlate with their place of occurrence (Fig. 4 – Locality).
Five species delineation tests revealed different taxonomic schemes that disagreed on some points with each other and with the current taxonomic classification. As a result of the ASAP analysis (Fig. 4 – ASAP), a barcoding gap of about 2-10% was estimated. The pairwise distance gap approach (Fig. 4 – ASAP) identified from 2 to 43 hypothetical species. We chose the fifth ASAP-score (6.50) which provides the best-fit scenario at the threshold distance of 2.68% (JC69) with 9 hypothetical species. The maximum-likelihood approach (Fig. 4 – GMYC) defined 34 species under a single threshold and 26 under multiple thresholds. The

pairwise distance gap approach (Fig. 4 – ABGD) with the default settings (X = 0.5) suggested 9 groups with prior intraspecific divergence (P) reaching 0.007, while 36 groups were defined with P ≤ 0.001. For bPTP (Fig. 4 – bPTP ML), we conducted two analyses based on BI and ML approaches. BI showed 52 species, whereas ML identified 9 groups or species. Thus, only ML was used in this study. ASAP, ABGD, and bPTP grouped species from the *P. affinis* complex, *P. hoelzeli* and *P. ornatus* into one species, whereas GMYC recognized 17 species among the complex.

The time estimation analysis dated the last common ancestor (LCA) of the *P. ornatus* group at 1.62 Mya with the following main lineage splits dated between 1.33 and 0.42 Mya (Fig. 2) during the Calabrian and Chibanian stage of the Pleistocene. The divergence of the *P. affinis* complex from *P. pindos* was dated at *ca.* 0.71 Mya during the Pleistocene (95% –confidence interval) based on the molecular clock analysis and *a priori* calibration. The LCA of the *P. affinis* complex was dated at *ca.* 0.42-0.02 Mya in the Late Pleistocene.

Figure 2 should be placed here vertically (half page).

The distribution pattern of the *P. ornatus* group results in six dispersal and five vicariance events (Fig. 2). The LCA of the group was positioned in the AB area and the group evolved by a vicariant event and subsequent dispersal within the Southern (A) and Central (B) areas where local lineage splits occurred. The Central region also represents the main speciation and dispersal centre of the *Poecilimon ornatus* group. From here, the *Poecilimon affinis* complex-ancestor evolved by dispersal in two main directions – North-West and (North-)East, where local dispersal and vicariant events contributed to the recent evolutionary history of the complex. Within the crown lineages, though poorly resolved, worth mentioning as stepping-stone - dispersal taxa are *Poecilimon hoelzeli* – distributed at the border of the Central with the (North-) Eastern lineage, and *Poecilimon pseudornatus*, having quite a wide distribution in the Central and North-Western regions. There was no correlation between genetic mean distance and geographic pattern in the *P. ornatus* group (Mantel Test, R = 0.0469; p = 0,193).

Figure 3 should be placed here vertically (half page).

4. Discussion

The present study represents the first comprehensive attempt to reconstruct the molecular phylogeny of the *Poecilimon ornatus* group. The molecular results support the monophyly of the *P. ornatus* group, as suggested in recent studies, based on ITS1, ITS2, 16S rRNA, tRNA-Val, 12S rRNA (Ullrich et al. 2010; part of the taxa), and the COI gene (Kociński 2020). The Control region is the most variable marker, as confirmed in the previous studies on *Poecilimon* (Eweleit et al. 2015; Borissov and Chobanov 2020). It shows the highest genetic mean distance between taxa from the *P. affinis* complex and the remaining species from the *P. ornatus* group. The Control region is a useful phylogenetic marker with the potential of providing better resolution than COI (Vila and Björklund 2004; Cheng et al. 2018). The number of variable and PI sites in ND2 is about 20% higher than in COI which is similar to the results provided for *Isophya* (Chobanov et al. 2017). However, the internal transcribed spacer 1 (ITS1) region contains the lowest number of variable and PI sites.

Figure 4 should be placed here horizontally (one page).

Poecilimon nobilis, *P. artedentatus*, and *P. obesus* form the sister clade to the remaining species of the group. The latter lineage is consistent with the morphological similarity of these three species (Chobanov and Heller 2010). The present data do not confirm that *P. gracilis* is

the sister species to the remaining taxa of the *P. ornatus* group, as suggested in previous studies based on morphology, bioacoustics (Chobanov and Heller 2010) and molecular data (Ullrich et al. 2010; Kociński 2020). *Poecilimon gracilis* is morphologically similar to *P. jablanicensis* and occurs parapatrically with the latter (Chobanov and Heller 2010) which is a prerequisite for close relationships as supported by our molecular results, where these species occupy the same subclade with *P. soulion* (Fig. 4). The sister clade to the latter includes the lineages of *P. gracilioides, P. pindos*, and the clade richest in taxa forming the *P. affinis* complex (Chobanov and Heller 2010; Kociński 2020; Kociński et al. 2021). *Poecilimon hoelzeli* and *P. ornatus* are placed among the taxa of the complex. Thus, the *P. affinis* complex is paraphyletic when these two species are not included. This finding is consistent with the previous studies (Kociński 2020; Kociński et al. 2021). *Poecilimon pseudornatus* occupies one subclade, regardless of where it occurs (North Macedonia (MK): Jablanica Mt.; Montenegro (MN): Durmitor, Treshnievik, Vusanje, Hajla; Serbia (SR): Kamena Gora) (Figs 1, 2), which corresponds to the low morphological variability of the species (Kociński et al. 2021). We can notice a distant genetic relationship between *P. a. komareki* and *P. rumijae*, which contradicts the current systematics where *P. rumijae* is treated as a synonym of *P. a. komareki* (Cigliano et al. 2022). Moreover, the results based on the geometric morphometric method of male pronotum and ovipositor confirmed that *P. rumijae* and *P. a. komareki* may be separate taxa (Kociński et al. 2021). This assumption is in line with the opinion of Ingrisch and Pavićević (2010), regarding *P. rumijae* as a species of the *P. ornatus* group, comparing it to *P. nonveilleri*. Nevertheless, as discussed by Kociński et al. (2021), *P. nonveilleri* does not seem to be closely related to *P. rumijae*, while the shape of the cercus and tegmen, length of the stridulatory row and number of stridulatory teeth in *P. affinis komareki* and *P. rumijae* show great similarity. In addition, the third clade (*P. affinis* complex) shows very low genetic structuring and low genetic variation, with poor resolution between groups of different taxonomic level. Specimens of *P. a. affinis* from different localities (Bulgaria (BG): Pirin Mtns, Bratiya, Osogovo, Kirilova Polyana, Rila Mtns, Rilski Manastir) form separate subclades (Figs 1, 4). Our results were confirmed by a geometric morphometric analysis of the male tegmen, cercus, pronotum, and ovipositor, where *P. a. affinis* was the most diffuse taxon among the group (Kociński et al. 2021). The above data suggest an infraspecific division of some local populations of *Poecilimon a. affinis* and contradict the assumption that the variability within this taxon depends mostly on the altitude of occurrence (Chobanov and Heller 2010). Despite the genetic variability in *P. a. affinis* from different localities, the Mantel test suggested no association between genetic and geographic distances in this group. Our results, based on three species delimitation methods (ASAP, ABGD, bPTP) (Fig. 4), suggest to divide the *P. ornatus* group into nine potential species, which contradicts the morphological, bioacoustics (Chobanov and Heller 2010; Ingrisch and Pavićević 2010; Kociński et al. 2021), and earlier molecular data (Kociński 2020). On the other hand, GMYC analysis reveals 26 hypothetical species among the group. The discrepancy in the results of species delimitation may indicate a greater conservatism of ASAP, ABGD, and bPTP over GMYC, which shows lower efficiency in data sets at the genus than at higher levels (Magoga et al. 2021). Though species delimitation has been defined as a method that sometimes causes confusion about almost every aspect of the definition of the 'species' level (Stanton et al. 2019), the problem with delineating species' boundaries at the tree top must be related to the low-level independent genetic differentiation of the third clade in our tree. Based on the recent lineage splits (Fig. 2) and the large number of taxa occurring over a significant geographic area (most of the central and northern part of the Balkan Peninsula reaching the Eastern Alps and Carpathians), we assume a recent contemporary allopatric origin of the taxa within the *Poecilimon affinis* complex. The latter may still be in the genetic "gray" zone of speciation, forming clines of a multitude of phenotypes with poor genetic structure (de Queiroz 1998). In

conclusion, our results confirmed the existence of the *P. affinis* complex, though they failed at separating species.

Poecilimon consists of groups of poorly morphologically distinguishable units/taxa that have been subjected to a rapid diversification following the set of the Miocene and especially during the Plio-Pleistocene climatic cycles (Borissov et al. 2020). According to our molecular clock (Fig. 2), most speciation processes in the *P. ornatus* group occurred between the middle Pleistocene (*ca.* 1.62 Mya) and the beginning of the Holocene (*ca.* 0.01 Mya). The dating of LCA of the *P. ornatus* group (1.62 Mya) coincides with a significant global climate cooling, which was also connected with the expansion of cold climate-adapted fauna in the North Atlantic (Lisiecki and Raymo 2005). Though most taxa of the group tend to occur in humid mountain areas with cool climates, the first clade of the group involves two species occurring in the lowland and middle-mountain belts in the Southern biogeographical region (in Peloponnesos) (*P. nobilis* and *P. artedentatus*) and one species with a narrower temperature tolerance (*P. obesus*) occurring in the lowlands of the Southern and southern part of the Central region (Chobanov and Heller 2010). Thus, the first lineage split in the group may have happened as a result of isolation due to climate deterioration in the Central or Southern region of distribution of the group (S and W Balkans) and subsequent adaptation of new lineage(s) with northern distribution to a cooler climate.

The following major lineage splits fall within the period called the Middle Pleistocene transition when climate cycles gradually changed from 41- to 100-Ka periods. This switch started *ca.* 1.25 Mya and after interruption continued after 0.9 Mya to be established *ca.* 0.7 Mya (Lisiecki and Raymo 2005; Clark et al. 2006). Within this irregular repetition of warmer, colder, wetter and dryer periods of variable temperature and humidity amplitude, multiple range shifts, accompanied by isolation and extinction events were driven. Thus, species like *Poecilimon jablanicensis* may have evolved from its ancestor, *P. gracilis*, from small populations subjected to the severe climate being isolated at mountain ridges by dense forest belt. The latter pattern may be applied to the origin of *P. pindos*, *P. gracilioides* and *P. soulion*, which possibly due to a wider ecological tolerance and/or eco-graphic factors have spread to a few or more mountain ranges.

The so-called Mid-Brunhes Transition *ca.* 430 ka ago marks a sharp increase in the temperature amplitude of the Pleistocene climate cycles (Barth et al. 2018). This time corresponds to a thermal minimum (l.c.), preceded by a minimum in the solar radiation in Europe (Boryczka and Stopa-Boryczka 2004) and concurs with the cold Marine Isotope Stage MIS 12 (478-424 ka ago) that was followed by Glacial Termination with a very large magnitude (Lisiecki and Raymo 2005). The time to LCA of the *Poecilimon affinis* complex (Fig. 2) corresponds well with the Mid-Brunhes Transition and interestingly – with the results for the two major lineage splits of the *Poecilimon ampliatus* complex (see Borissov et al. 2021). The larger temperature amplitudes with colder glacials and a larger decrease in humidity should be the main trigger for dispersal, isolation (vicariance), extinction, and ecological adaptation in the *Poecilimon affinis* complex, similarly to many other animals (Hewitt 1996, 2000; Taberlet et al. 1998; Wallis et al. 2016). As the multitude of geographic taxa within the *Poecilimon affinis* complex shows an overall low genetic differentiation of similar scale and a wider distribution than the ancestral lineages of the *Poecilimon ornatus* group, its evolution should have been ruled by fast spreading within comparatively short climatically favorable periods during the last two glacial periods. During this vast expansion accompanied by versatile morpho-acoustic diversification, distinct ecological forms evolved, including both mountain specialists (e.g., geographic forms of *P. affinis* s.str.), ecologically tolerant species (*P. ornatus, P. pseudornatus*), and early-seasonal Mediterranean species (*P. a. komareki*, *P. 'rumijae'* – synonym of *P. a. komareki*).

The ancestor(s) of the *Poecilimon affinis* complex splits off from the rest of the *P. ornatus* group in the Pleistocene (*ca.* 0.71 Mya). The results of the molecular clock confirmed the need to extend the complex with two species: *P. ornatus* and *P. hoelzeli*. The *P. affinis* complex diverged into two lineages *ca.* 0.42 Mya. The first lineage consists of *P. hoelzeli*, *P. pseudornatus*, *P. a. komareki*, *P. poecilus*, *P. rumijae*, *P. a. serbicus*, *P. nonveilleri*, *P. a. hajlensis*, which are partly consistent with their biogeographical regions (Central and North-Western). The second lineage includes species from the Eastern (*P. ornatus*, *P. a. affinis*), and North-Western regions (*P. a. dinaricus*).

5. Conclusion

The present study generated additional evidence for the relationships within the *P. ornatus* group. Our results indicate that COI, ND2, CR, and ITS1 markers can be successfully used for phylogenetic analyses, supporting the previous studies on the phylogeny of *Poecilimon*. The presented results confirmed the monophyly of the *P. ornatus* group and the existence of the *P. affinis* complex containing two additional species: *P. hoelzeli* and *P. ornatus*. Using phylogenetic and time estimation analyses, biogeographic reconstruction, and available paleoclimatic data, we reveal the origin and evolutionary patterns of the *Poecilimon ornatus* group and shed light on the climate-driven complex evolution of the *Poecilimon affinis* complex. The young taxa were formed by complex speciation modulated by dispersal, vicariance, and extinction events, and directed towards phenotypic and ecological diversification.

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Figure Legend

Figure 1. Map of collecting sites of analyzed specimens of the *Poecilimon ornatus* group. Triangle indicates the taxa from the *P. affinis* complex, circle indicates the rest of the taxa from the *P. ornatus* group.

Figure 2. The Beast tree shows the reconstructed geographic ranges and dated phylogeny of the *Poecilimon ornatus* group. The values indicated under the branches represent the mean ages of lineage divergence; acronyms on the nodes indicate geographic areas: [A] – Southern, [B] – Central, [C] – North-Western, [D] – Eastern. The different color rectangle on the branches close to the nodes represents different events: pink—vicariance, purple—dispersal. The red dot indicates the split of the *P. affinis* complex from the *P. ornatus* group.

Figure 3. The biogeographic reconstruction of the ranges of the *Poecilimon ornatus* group as shown on the BEAST tree (S-DIVA results). The values at nodes indicate the probability, acronyms on the nodes, and colors indicate geographic areas: [A] – Southern, [B] – Central, [C] – North-Western, [D] – Eastern.

Figure 4. *Poecilimon pseudornatus* (A), *P. gracilioides* (B), *P. a. affinis* (C), *P. a. hajlensis* (D), *P. gracilis* (E), *P. nobilis* (F), *P. rumijae* (G), *P. hoelzeli* (H), *P. ornatus* (I), Photos Dragan Chobanov. Bayesian inference tree from a dataset including COI, ND2, CR, and ITS1 sequences of the *Poecilimon ornatus* group. Bayesian (BI) and Maximum likelihood (ML) topologies were consistent, so only one tree is shown. I –the first clade, II – the second clade, III – the third clade. The right panel shows groupings from different species delimitation approaches, as follows: bPTP ML – the Poisson Tree Processes; ASAP – Assemble Species by Automatic Partitioning; GMYC – maximum-likelihood approach based on the general mixed Yule-coalescent model; ABGD – Automatic Barcode Gap Discovery. The last grouping is based on localities of the taxa studied (NM – North Macedonia, MN – Montenegro, SR – Serbia, BG – Bulgaria, AL – Albania, GR – Greece). Scale bar: number of substitutions per nucleotide position.

Table Legend

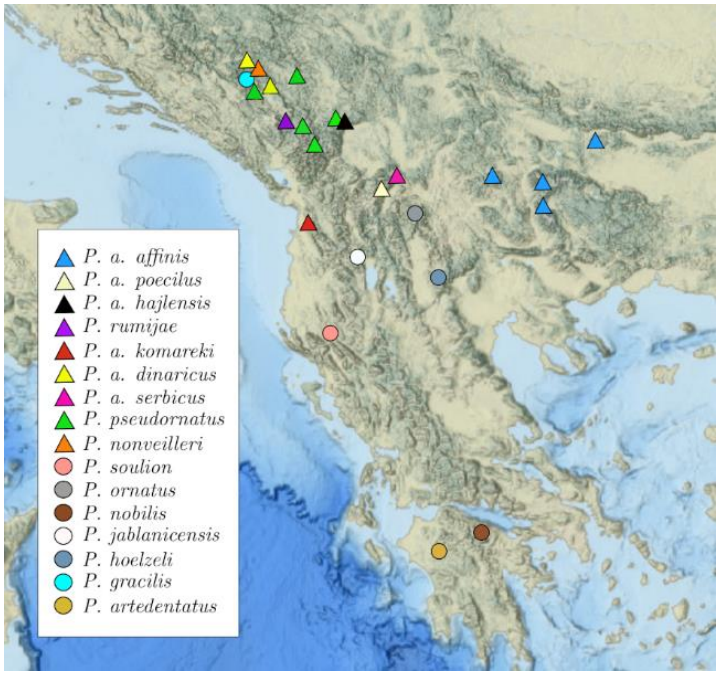
Table 1. Information on specimens and sequences included in this study.

Table 2. Primers used to amplify and sequence in this study.

Table 3. PCR protocol for COI, ND2, CR, and ITS1 were used in this study.

Table 4. The genetic distances for COI, ND2, CR, and ITS1.

Table 5. Results of the substitution saturation tests performed in DAMBE.



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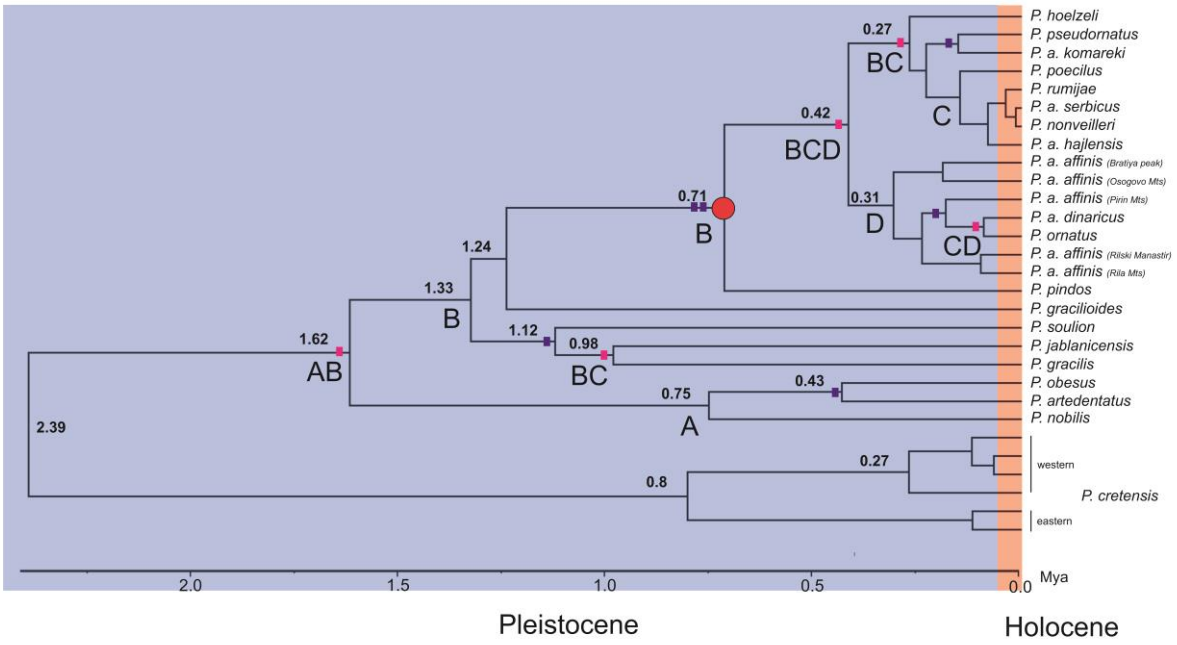

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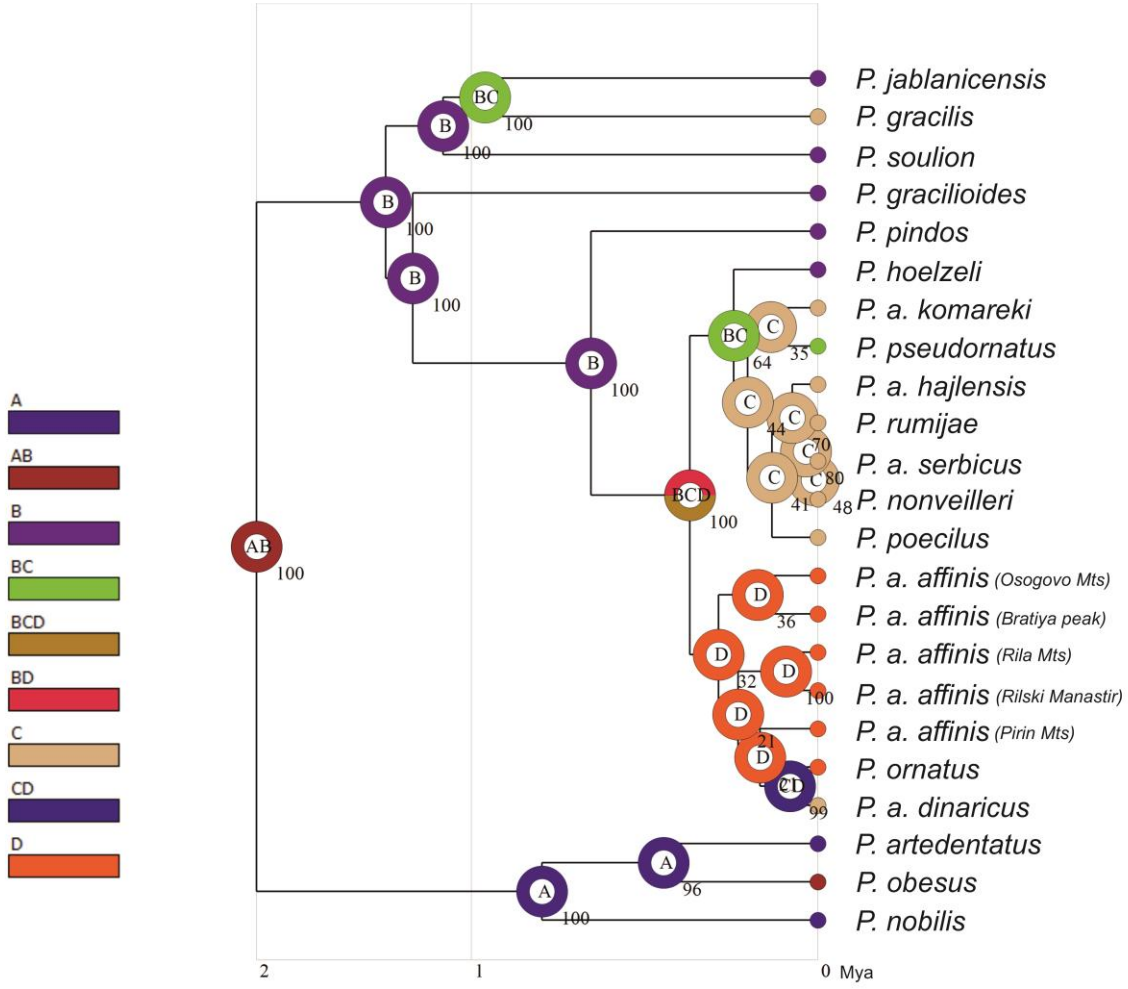

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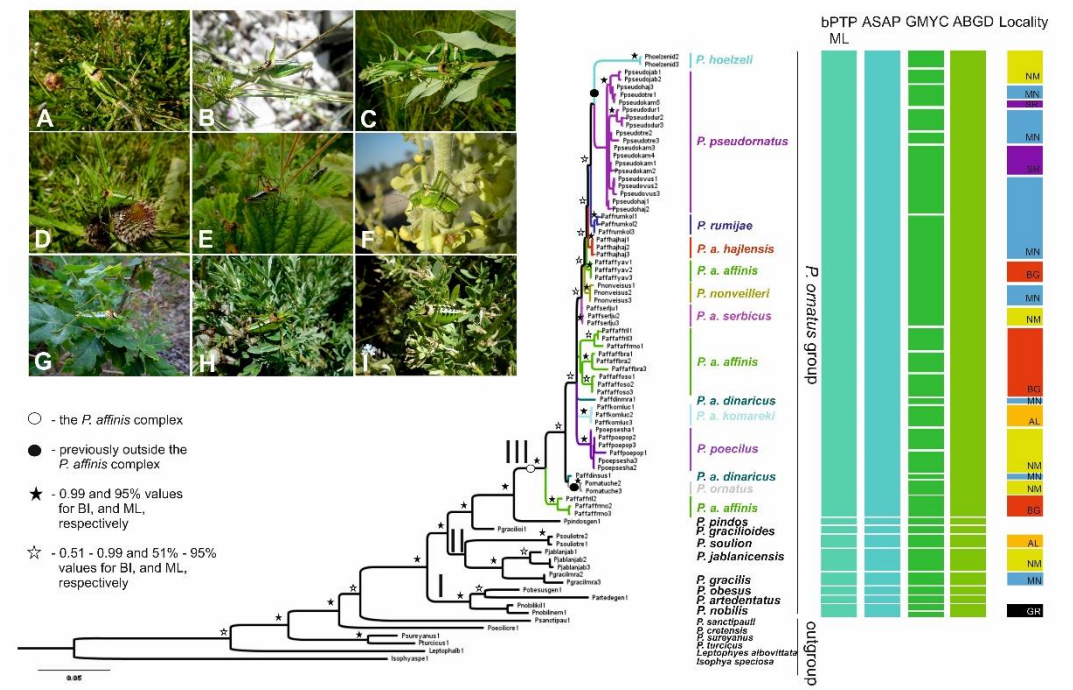

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Table 1. Information on specimens and sequences included in this study.

	Taxa	Locality and the date of collection	GenBank accession numbers			
			COI	**ND2**	**ITS1**	**CR+12S**
the *Poecilimon ornatus* group	*Poecilimon affinis affinis* (Frivaldszky, 1868)*****	Bulgaria, Rila Mts., Iliyna Reka 01.07.2017	MH800896	OM372375	ON181606	ON340858
			MH800897	-	ON181607	ON340859
			MH800898	OM372376	ON181608	ON340860
		Bulgaria, Pirin Mts., Yavorov Chalet 02.07.2017	MH800899	OM372378	ON181609	ON340852
			MH800900	OM372379	ON181610	ON340853
			MH800901	OM372380	ON181611	ON340854
		Bulgaria, Osogovo Mts. 01.07.2017	MH800902	OM372372	ON181587	ON340861
			MH800903	OM372373	ON181588	ON340862
			MH800904	OM372374	ON181589	ON340863
		Bulgaria, Sredna Gora Mts., Bratiya peak 30.06.2017	MH800907	OM372369	ON181590	ON340855
			MH800908	OM372370	ON181591	ON340856
			OM629176	OM372371	-	ON340857
		Bulgaria. Rilski Manastir 13.06.2006	OM629182	OM372377	ON181637	ON340879
			OM629183	-	ON181635	ON340880
			OM629184	-	ON181636	ON340881
	Poecilimon affinis komareki Cejchan, 1957*****	Albania, Laç 09.07.2017	MH800867	OM372386	ON181617	ON340910
			MH800868	OM372387	ON181618	ON340911
			MH800869	OM372388	ON181619	ON340912
	Poecilimon affinis dinaricus Ingrisch & Pavićević, 2010*****	Montenegro, Susica 06.07.2017	MH800856	OM372382	ON181613	-
		Montenegro, Mratinje 07.07.2017	MH800857	OM372381	ON181612	ON340909
	Poecilimon affinis serbicus Karaman, 1974*****	North Macedonia, Shar Mts, Ljuboten Park 13.07.2017	MH800861	OM372395	ON181632	ON340887
			MH800862	OM372396	ON181633	ON340888
			MH800863	OM372397	ON181634	ON340889
	Poecilimon affinis hajlensis Karaman, 1974*****	Montenegro, Hajla 08.07.2017	MH800864	OM372383	ON181614	ON340884
			MH800865	OM372384	ON181615	ON340885
			MH800866	OM372385	ON181616	ON340886
	Poecilimon poecilus Ramme, 1951*****	North Macedonia, Shar Mts., Popova Shapka 13.07.2017	MH800890	OM372389	ON181623	-
			MH800891	OM372390	ON181624	ON340916
			MH800892	OM372391	ON181625	ON340917
		North Macedonia, Shar Mt. Borislovee 24.08.2018	OM629177	OM372406	ON181626	ON340913
			OM629178	OM372407	ON181627	ON340914
			OM629179	OM372408	ON181628	ON340915

Poecilimon rumijae Karaman, 1972*****	Montenegro, Kolasin 07.07.2017	MH800873	OM372392	ON181629	ON340901
		MH800874	OM372393	ON181630	ON340902
		MH800875	OM372394	ON181631	ON340903
Poecilimon nonveilleri Ingrisch & Pavićević, 2010*****	Montenegro, Susica 06.07.2017	MH800858	OM372401	ON181640	ON340895
		MH800859	OM372402	ON181641	ON340896
		MH800860	OM372403	ON181642	ON340897
Poecilimon pseudornatus Ingrisch & Pavićević, 2010*****	Montenegro, Durmitor, Boricje 06.07.2017	MH800870	OM372409	ON181592	ON340869
		MH800871	OM372410	ON181593	ON340870
		MH800872	OM372411	ON181594	ON340871
	Montenegro, Treshnievik 08.07.2017	MH800876	OM372422	ON181600	ON340872
		MH800877	OM372423	ON181601	ON340873
		MH800878	OM372424	ON181602	-
	Montenegro, Vusanje 08.07.2017	MH800879	OM372425	ON181603	ON340874
		MH800880	OM372426	ON181604	ON340875
		MH800881	OM372427	ON181605	ON340876
	Montenegro, Hajla 08.07.2017	MH800882	OM372412	ON181643	ON340906
		MH800883	OM372413	ON181644	ON340907
		MH800884	OM372414	ON181645	ON340908
	Serbia, Kamena Gora 06.07.2017	MH800885	OM372417	ON181595	ON340864
		MH800886	OM372418	ON181596	ON340865
		MH800887	OM372419	ON181597	ON340866
		MH800888	OM372420	ON181598	ON340867
		MH800889	OM372421	ON181599	ON340868
	North Macedonia, Jablanica Mt. 31.07.2018	OM629180	OM372415	ON181646	ON340904
		OM629181	OM372416	ON181647	ON340905
Poecilimon ornatus (Schmidt, 1850)	North Macedonia, Jakupica Mts., Cheples 13.07.2017	MH800911	OM372404	ON181622	-
		MH800912	OM372405	-	-
Poecilimon hoelzeli Harz, 1966	North Macedonia, Nidzhe-Kopanki 18.06.2018	OM629185	OM372398	ON181648	ON340899
		OM629186	OM372399	ON181649	ON340900
Poecilimon jablanicensis Chobanov & Heller, 2010	North Macedonia, Jablanica Mt 31.07.2018	MN737107	OM372364	ON181650	ON340892
		MN737108	OM372365	ON181651	ON340893
			OM372366	ON181652	ON340894
Poecilimon nobilis Brunner von Wattenwyl, 1878	Greece, Kilini Mt 17.06.2015	-	-	ON181620	ON340883
	Greece, Nemea 18.05.2018	OM629187	OM372428	ON181621	ON340882
Poecilimon obesus	-	AM886773	-	AM888939	-

		Brunner von Wattenwyl, 1878					
		Poecilimon pindos Willemse, 1982	-	AM886765	-	AM888928	-
		Poecilimon artedentatus Heller, 1984	Greece, Natpantos 03.06.2018	AM886816	-	AM888983	-
		Poecilimon gracilis (Fieber, 1853)	Montenegro, Mratinje 07.07.2017	MH800910	OM372362 OM372363	ON181639	ON340890 ON340891
		Poecilimon soulion Willemse, 1987	Albania, Trebeshina 04.07.2015	-	OM372367 OM372368	ON181638	ON340877 ON340878
		Poecilimon gracilioides Willemse & Heller, 1992	-	AM886751	-	AM888914	-
outgroup	the *Poecilimon jonicus* group	*Poecilimon cretensis* Werner, 1903	-	MT416227 MW796385 MN114198 MW796384 MN114199 MN114200	MT416238 - - - - -	MN129804 - - - - -	MT416250 - - - - -
	the *Poecilimon bosphoricus* group	*Poecilimon turcicus* Karabag, 1950	-	AM886828	KX026727	AM888995	-
		Poecilimon sureyanus Uvarov, 1930	-	AM886823	KX026731	AM888990	-
	the *Poecilimon sanctipauli* group	*Poecilimon sanctipauli* Brunner von Wattenwyl, 1878	-	AM886779	KX026729	AM888946	-
		Isophya speciosa (Frivaldszky, 1868)	-	KX026710	KX026767	KX026810	-
		Leptophyes albovittata (Kollar, 1833)	-	MN114160	MN114183	MN129806	-

*-taxa from the *Poecilimon affinis* complex

Table 2. The primers used to amplify and sequence in this study.

Locus	Primer	5'-3' primer sequence	Reference
COI	UEA7 (Forward)	TAC AGT TGG AAT AGA CGT TGA TAC	Lunt et al. 1996
	UEA10 (Reverse)	TCC AAT GCA CTA ATC TGC CAT ATT A	
ND2	TM-J210 (F)	AATTAAGCTAATGGGTTCATACCC	Simon et al. 2006
	TW-N1284 (R)	AYAGCTTTGAARGYTATTAGTTT	
CR	SR-J14610 (F)	ATA ATM GGG TAT CWA ATC CTA GT	Simon et al. 2006
	T1-N18 (R)	CTCTATCAARRTAAYCCTTT	
ITS1	ITS1-F (F)	TCC GTA GGT GAA CCT GCG G	Weekers et al. 2001
	ITS2-R (R)	GCT GCG TTC TTC ATC GAT GC	

Table 3. PCR protocol for COI, ND2, CR, and ITS1 were used in this study.

Locus	Steps of PCR	PCR condition	
COI	Initial activation	3 min – 94°C	
	Denaturation	1 min – 94°C	36 cycles
	Annealing	1 min – 48°C	
	Elongation	2 min – 72°C	
	Final Elongation	7 min – 72°C	
ND2	Initial activation	3 min – 94°C	
	Denaturation	30 s – 95°C	36 cycles
	Annealing	1 min – 48°C	
	Elongation	2 min – 72°C	
	Final Elongation	10 min - 72°C	
CR	Initial activation	3 min – 94°C	
	Denaturation	20 s – 92°C	35 cycles
	Annealing	30 s – 52°C	
	Elongation	3 min – 60°C	
	Final Elongation	7 min - 72°C	
ITS1	Initial activation	5 min – 94°C	
	Denaturation	1 min – 95°C	25 cycles
	Annealing	110 s – 52°C	
	Elongation	2 min – 72°C	
	Final Elongation	10 min - 72°C	

Table 4. The genetic distance for COI, ND2, CR, and ITS1.

		***P. affinis* complex**
***P. ornatus* group**	COI	0,0740
	ND2	0,0583
	CR	0,163
	ITS1	0,0694

Table 5. Results of the substitution saturation tests performed in DAMBE.

Dataset	**ISS**	**ISS.c S**	**P**	**ISS.c A**	**P**
COI (1+2)	0.028	0.691	0	0.363	0
COI (3)	0.192	0.690	0	0.375	0
ND2	0.075	0.722	0	0.398	0
CR	0.144	0.696	0	0.369	0

Załączniki do publikacji

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Table S1:
Difference in tegmen shapes among species from *P. ornatus* group with canonical variate analysis (CVA). Mahalanobis distances (bold) and Procrustes distances (narrow).

Species	***affinis***	***hoelzeli***	***jablanicensis***	***nobilis***	***nonveilleri***	***obesus***	***poecilus***	***pseudornatus***
affinis	-	0.1243	0.0964	0.1790	0.0467	0.1347	0.0392	0.0323
hoelzeli	**8.0504**	-	0.1663	0.2703	0.1108	0.0705	0.1298	0.1341
jablanicensis	**8.2179**	**14.1805**	-	0.1857	0.0977	0.1774	0.1217	0.1091
nobilis	**14.5668**	**18.8282**	**14.0134**	-	0.1894	0.2788	0.1818	0.1745
nonveilleri	**2.9027**	**8.4726**	**7.6440**	**14.6725**	-	0.1267	0.0615	0.0515
obesus	**9.2912**	**5.0108**	**14.9755**	**19.6637**	**9.3176**	-	0.1369	0.1416
poecilus	**3.2060**	**7.9251**	**9.5758**	**15.9968**	**4.7456**	**9.3763**	-	0.0309
pseudornatus	**2.5030**	**8.5373**	**8.7091**	**14.5359**	**3.6143**	**9.5615**	**2.7984**	-

Table S2:
Difference in tegmen shapes among taxa from *P. affinis* complex with canonical variate analysis (CVA). Mahalanobis distances (bold) and Procrustes distances (narrow).

Species	***a.affinis***	***a.dinaricus***	***a.hajlensis***	***a.komareki***	***rumijae***	***a.serbicus***	***nonveilleri***	***poecilus***	***pseudornatus***
a.affinis	-	0.0848	0.0453	0.0884	0.0711	0.0537	0.0609	0.0547	0.0529
a.dinaricus	**5.9991**	-	0.0732	0.1100	0.1161	0.0880	0.0968	0.0878	0.0816
a.hajlensis	**4.0575**	**5.7681**	-	0.0739	0.0747	0.0513	0.0682	0.0485	0.0477
a.komareki	**6.2092**	**8.1340**	**5.6483**	-	0.0741	0.0629	0.0843	0.0750	0.0691
rumijae	**3.9873**	**7.0199**	**4.9784**	**5.3684**	-	0.0537	0.0601	0.0657	0.0575
a.serbicus	**3.8398**	**6.8326**	**4.6652**	**6.1018**	**4.6009**	-	0.0448	0.0361	0.0251
nonveilleri	**3.6498**	**7.1259**	**4.1816**	**5.6240**	**4.3468**	**3.9114**	-	0.0615	0.0515
poecilus	**3.8299**	**6.3236**	**4.5367**	**6.9142**	**4.7404**	**3.6537**	**4.7649**	-	0.0309
pseudornatus	**3.3300**	**6.72220**	**4.4654**	**6.4675**	**4.2757**	**3.0901**	**3.7281**	**2.7717**	-

Table S3:
Difference in ovipositor shapes among species from *P. ornatus* group with canonical variate analysis (CVA). Mahalanobis distances (bold) and Procrustes distances (narrow).

Species	***affinis***	***ampliatus***	***artedentatus***	***gracilis***	***hoelzeli***	***jablanicensis***	***nobilis***	***nonveilleri***	***obesus***	***poecilus***	***pseudornatus***
affinis	-	0.0949	0.1139	0.1473	0.0351	0.0969	0.1437	0.0720	0.1004	0.0472	0.0637
ampliatus	**6.0532**	-	0.0954	0.0956	0.0713	0.0797	0.1117	0.1072	0.1078	0.0881	0.1336
artedentatus	**8.4370**	**7.7347**	-	0.1169	0.1026	0.0917	0.0718	0.0795	0.0397	0.0748	0.1299
gracilis	**9.0042**	**8.9659**	**12.7381**	-	0.1235	0.1159	0.1108	0.1565	0.1387	0.1345	0.1941
hoelzeli	**3.0805**	**5.4878**	**7.0018**	**9.5072**	-	0.0789	0.1273	0.0711	0.0940	0.0467	0.0829
jablanicensis	**9.5259**	**10.4518**	**7.7488**	**14.1705**	**8.9512**	-	0.0932	0.0920	0.0899	0.0802	0.1315
nobilis	**11.9581**	**10.5757**	**5.4908**	**15.7156**	**10.7249**	**7.4292**	-	0.1230	0.0859	0.1110	0.1679
nonveilleri	**5.7586**	**6.5558**	**6.1878**	**11.7449**	**4.2352**	**8.1217**	**9.6513**	-	0.0495	0.0381	0.0704
obesus	**6.9948**	**6.7869**	**3.0230**	**12.0451**	**5.5767**	**7.1694**	**6.6624**	**3.6954**	-	0.0555	0.1068
poecilus	**3.5589**	**5.5663**	**5.7738**	**10.0441**	**2.7815**	**8.6736**	**9.6064**	**3.9132**	**4.0711**	-	0.0722
pseudornatus	**4.4070**	**7.7901**	**8.0642**	**12.0775**	**4.5740**	**10.4786**	**11.9396**	**5.7617**	**6.6893**	**4.1426**	-

Table S4:
Difference in ovipositor shapes among taxa from *P. affinis* complex with canonical variate analysis (CVA). Mahalanobis distances (bold) and Procrustes distances (narrow).

Species	*a.affinis*	*a.dinaricus*	*a.hajlensis*	*a.komareki*	*rumijae*	*a.serbicus*	*nonveilleri*	*poecilus*	*pseudornatus*
a.affinis	-	0.0656	0.0685	0.1349	0.0356	0.0319	0.0824	0.0603	0.0714
a.dinaricus	**6.8512**	-	0.1008	0.1542	0.0924	0.0745	0.1005	0.0928	0.0882
a.hajlensis	**8.9269**	**14.4906**	-	0.1523	0.0881	0.0460	0.0847	0.0596	0.1099
a.komareki	**6.3547**	**5.2708**	**13.9868**	-	0.1346	0.1441	0.0847	0.1311	0.1027
rumijae	**2.6873**	**7.4340**	**10.2852**	**5.7461**	-	0.0531	0.0860	0.0652	0.0678
a.serbicus	**3.2550**	**8.4123**	**7.1306**	**8.2658**	**4.9003**	-	0.0679	0.0419	0.0844
nonveilleri	**7.6163**	**8.9126**	**10.8068**	**9.1799**	**9.0290**	**6.2751**	-	0.0392	0.0705
poecilus	**5.2135**	**7.8453**	**8.8232**	**7.2700**	**6.2663**	**4.1432**	**4.1693**	-	0.0755
pseudornatus	**7.2647**	**5.3820**	**14.5025**	**4.4960**	**7.1256**	**9.6129**	**10.1917**	**6.4303**	-

Table S5:
Difference in cercus shapes among species from *P. ornatus* group with canonical variate analysis (CVA). Mahalanobis distances (bold) and Procrustes distances (narrow).

Species	***affinis***	***hoelzeli***	***jablanicensis***	***nobilis***	***nonveilleri***	***obesus***	***poecilus***	***pseudornatus***
affinis	-	0.1341	0.0886	0.1529	0.0563	0.0632	0.0350	0.0339
hoelzeli	**8.3755**	-	0.1349	0.0814	0.0928	0.1437	0.1373	0.1426
jablanicensis	**8.7027**	**12.2488**	-	0.1519	0.1064	0.1186	0.1001	0.0817
nobilis	**10.5209**	**9.0083**	**11.2681**	-	0.1129	0.1459	0.1565	0.1706
nonveilleri	**4.1064**	**7.2670**	**10.7513**	**10.7568**	-	0.0792	0.0671	0.0757
obesus	**6.5412**	**10.3968**	**9.3670**	**8.1348**	**8.5007**	-	0.0443	0.0898
poecilus	**3.1067**	**9.1822**	**7.8319**	**10.4994**	**5.1264**	**6.0240**	-	0.0587
pseudornatus	**2.7073**	**8.8268**	**8.9629**	**11.6876**	**5.3179**	**8.0193**	**4.1552**	-

Table S6:
Difference in cercus shapes among taxa from *P. affinis* complex with canonical variate analysis (CVA). Mahalanobis distances (bold) and Procrustes distances (narrow).

Species	***a.affinis***	***a.dinaricus***	***a.hajlensis***	***a.komareki***	***rumijae***	***a.serbicus***	***nonveilleri***	***poecilus***	***pseudornatus***
a.affinis	-	0.0634	0.0318	0.0534	0.0304	0.0376	0.0569	0.0301	0.0391
a.dinaricus	**7.2368**	-	0.0690	0.0977	0.0666	0.0682	0.0402	0.0711	0.0842
a.hajlensis	**3.3261**	**8.3160**	-	0.0696	0.0463	0.0323	0.0541	0.0481	0.0315
a.komareki	**4.6509**	**8.6480**	**5.4297**	-	0.0436	0.0749	0.0985	0.0555	0.0535
rumijae	**3.7141**	**6.9099**	**4.9815**	**3.4088**	-	0.0440	0.0680	0.0461	0.0416
a.serbicus	**3.1956**	**8.1597**	**3.1122**	**5.5398**	**4.9102**	-	0.0494	0.0508	0.0453
nonveilleri	**4.3693**	**5.7994**	**5.4986**	**6.7563**	**5.5340**	**5.0108**	-	0.0645	0.0756
poecilus	**3.4275**	**8.6212**	**3.7312**	**5.2959**	**5.0327**	**3.1874**	**5.4729**	-	0.0585
pseudornatus	**3.4136**	**8.3486**	**2.8732**	**4.7826**	**4.4681**	**4.2193**	**5.8261**	**4.6717**	-

Table S7:
Difference in pronotum shapes among species from *P. ornatus* group with canonical variate analysis (CVA). Mahalanobis distances (bold) and Procrustes distances (narrow).

Species	***affinis***	***gracilis***	***hoelzeli***	***jablanicensis***	***nobilis***	***nonveilleri***	***obesus***	***poecilus***	***pseudornatus***
affinis	**-**	0.1120	0.0400	0.0692	0.1110	0.0818	0.1398	0.0286	0.0754
gracilis	**8.7757**	**-**	0.1338	0.1591	0.1545	0.1011	0.0286	0.1140	0.1332
hoelzeli	**2.7954**	**10.3568**	**-**	0.0697	0.1030	0.0972	0.1320	0.0533	0.0815
jablanicensis	**4.1802**	**11.4995**	**4.9195**	**-**	0.1098	0.1365	0.1495	0.0800	0.0880
nobilis	**5.9322**	**12.2787**	**6.0601**	**5.5256**	**-**	0.1587	0.1135	0.1167	0.0953
nonveilleri	**3.5627**	**7.9683**	**4.1678**	**6.3727**	**7.6699**	**-**	0.1544	0.0762	0.1090
obesus	**7.5493**	**12.8096**	**7.3672**	**8.1186**	**5.2972**	**8.7641**	**-**	0.1386	0.0875
poecilus	**2.2038**	**9.7049**	**3.4030**	**4.1163**	**5.5951**	**4.2377**	**7.7369**	**-**	0.0727
pseudornatus	**3.7020**	**9.9918**	**4.5955**	**4.4239**	**5.0230**	**5.7472**	**6.5642**	**3.7766**	**-**

Table S8:
Difference in pronotum shapes among taxa from *P. affinis* complex with canonical variate analysis (CVA). Mahalanobis distances (bold) and Procrustes distances (narrow).

Species	***a.affinis***	***a.dinaricus***	***a.hajlensis***	***a.komareki***	***rumijae***	***a.serbicus***	***nonveilleri***	***poecilus***	***pseudornatus***
a.affinis	-	0,0884	0,0474	0,0445	0,0622	0,0784	0,0800	0,0369	0,0837
a.dinaricus	**4,3943**	-	0,0650	0,0954	0,1044	0,0886	0,1128	0,0825	0,0964
a.hajlensis	**2,7308**	**4,0142**	-	0,0487	0,0891	0,0609	0,0776	0,0470	0,0569
a.komareki	**4,1060**	**5,2404**	**3,4003**	-	0,0754	0,0899	0,0893	0,0518	0,0757
rumijae	**3,3874**	**4,0739**	**4,0658**	**5,1072**	-	0,1261	0,1351	0,0743	0,1122
a.serbicus	**3,1575**	**4,9320**	**3,3360**	**4,7335**	**4,8939**	-	0,0695	0,0613	0,0836
nonveilleri	**3,5199**	**5,0842**	**3,7839**	**4,7645**	**5,6766**	**4,0796**	-	0,0762	0,1091
poecilus	**3,1391**	**3,9590**	**3,6948**	**4,3128**	**3,2625**	**2,9688**	**4,6044**	-	0,0727
pseudornatus	**4,6006**	**5,3384**	**3,4472**	**4,4488**	**4,5959**	**4,1093**	**5,9363**	**3,8378**	-

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mój udział polegał na interpretacji wyników i konsultacji tekstu manuskryptu. Oceniam swój procentowy wkład w przygotowanie publikacji na 10%.

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mój udział polegał na pomocy w opracowaniu koncepcji badań, interpretacji wyników i konsultacji tekstu manuskryptu. Oceniam swój procentowy wkład w przygotowanie publikacji na 15%.

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DECLARATION OF AUTHOR CONTRIBUTION TO THE PAPER

As co-author of the paper
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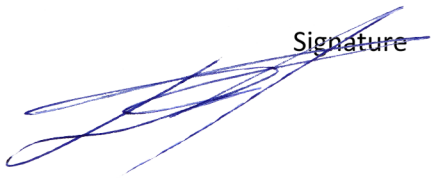
I confirm that my contributions were as follows: conception and idea of the study together with the first author, collecting samples of *Poecilimon* in the field, identification of species, interpretation of the results and writing comments on the manuscript. I estimate my participation in the formation of the publication at 15%.

Signature



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DECLARATION OF AUTHOR CONTRIBUTION TO THE PAPER

As co-author of the paper
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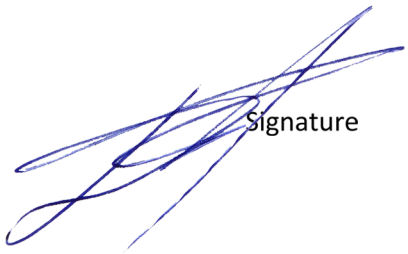
I confirm that my contributions were as follows: collecting samples of *Poecilimon* in the field, identification of species, interpretation of the results and writing comments on the manuscript. I estimate my participation in the formation of the publication at 15%.



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STATEMENT

I declare that in the work of **Kociński M., Grzywacz B., Hristov G., Chobanov D.2021. A taxonomic outline of the *Poecilimon affinis* complex (Orthoptera) using the geometric morphometric approach.PeerJ 9:e12668; DOI: 10.7717/peerj.12668**, my participation consisted in providing the measurements data of stridulatory's file. I estimate my participation in the formation of the publication at 5%.

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