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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

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

		Taxa	Locality and the date of collection	GenBank accession numbers			
				COI	**ND2**	**ITS1**	**CR**
	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

		Taxa	Locality and the date of collection	GenBank accession numbers			
				COI	**ND2**	**ITS1**	**CR**
		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

		Taxa	Locality and the date of collection	GenBank accession numbers			
				COI	**ND2**	**ITS1**	**CR**
		Poecilimon obesus Brunner von Wattenwyl, 1878	—	AM886773	—	AM888939	—
		Poecilimon pindos Willemse, 1982	—	AM886765	—	AM888928	—
		Poecilimon artedentatus Heller, 1984	Greece, Nafpaktos 03.06.2018	AM886816	—	AM888983	—
		Poecilimon gracilis (Fieber, 1853)	Montenegro, Mratinje 07.07.2017	MH800910	OM372362	ON181639	ON340890
				—	OM372363	—	ON340891
		Poecilimon soulion Willemse, 1987	Albania, Trebeshina 04.07.2015	—	OM372367	ON181638	ON340877
				—	OM372368	—	ON340878
		Poecilimon gracilioides Willemse & Heller, 1992	—	AM886751	—	AM888914	—
outgroup	the *Poecilimon jonicus* group	*Poecilimon cretensis* Werner, 1903	—	MT416227	MT416238	MN129804	MT416250
				MW796385	—	—	—
				MN114198	—	—	—
				MW796384	—	—	—
				MN114199	—	—	—
				MN114200	—	—	—
	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	—
	the Barbitistini genera	*Isophya speciosa* (Frivaldszky, 1868)	—	KX026710	KX026767	KX026810	—
		Leptophyes albovittata (Kollar, 1833)	—	MN114160	MN114183	MN129806	—
*-taxa from the *Poecilimon affinis* complex							

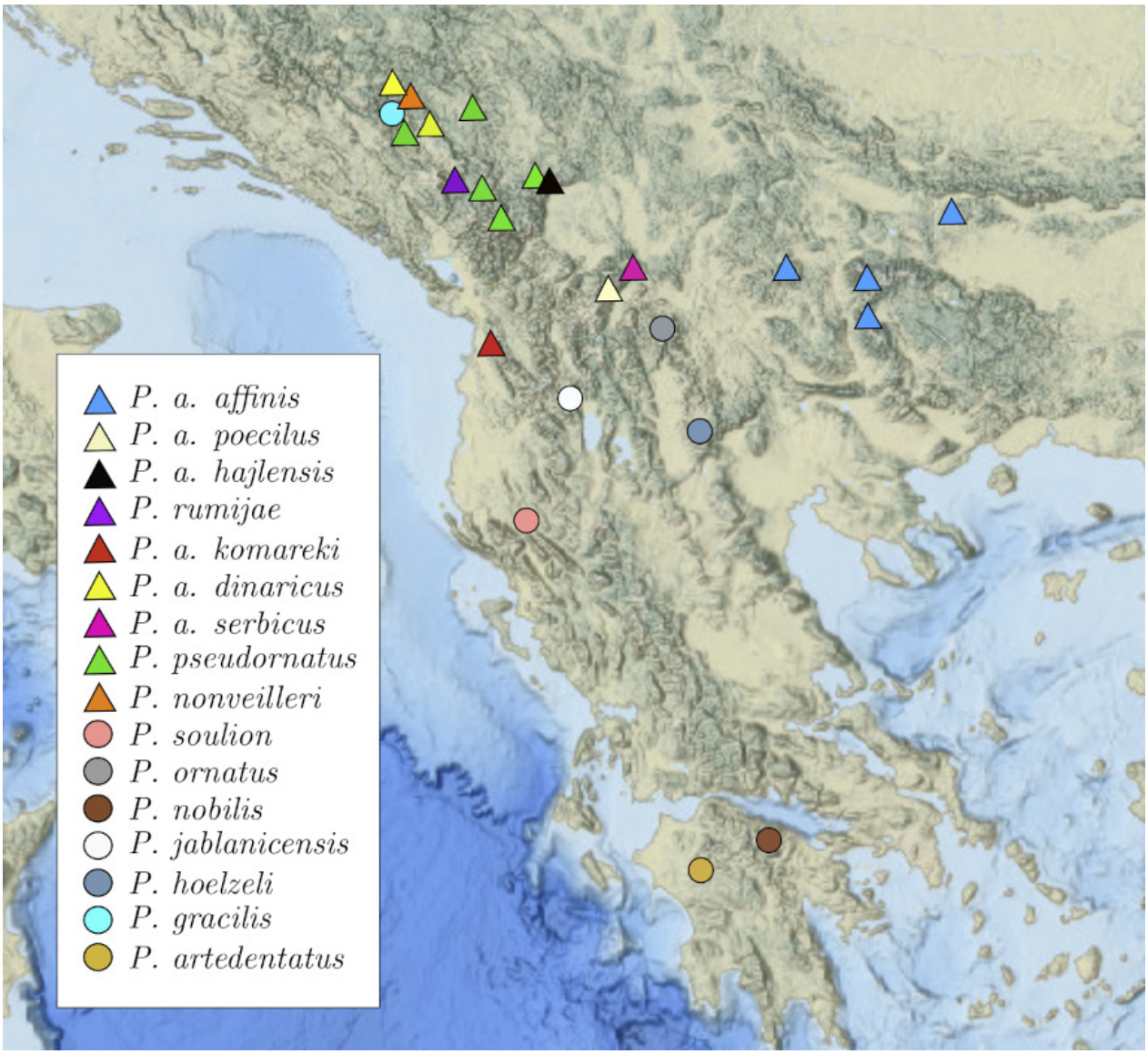

Figure 1. Map of collecting sites of analyzed specimens of the *Poecilimon ornatus* group. Triangle indicates the taxa from the *P. affinis* complex, circle indicates the rest of the taxa from the *P. ornatus* group.

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) by Maciej Kociński and Dragan Chobanov.

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.

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).

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).

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, four 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.*

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

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

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

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

Table 4. The genetic distances between the *P. affnis* complex and the *P. ornatus* group for COI, ND2, CR, and ITS1.

		the *P. affinis* complex
the *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

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 markers (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).

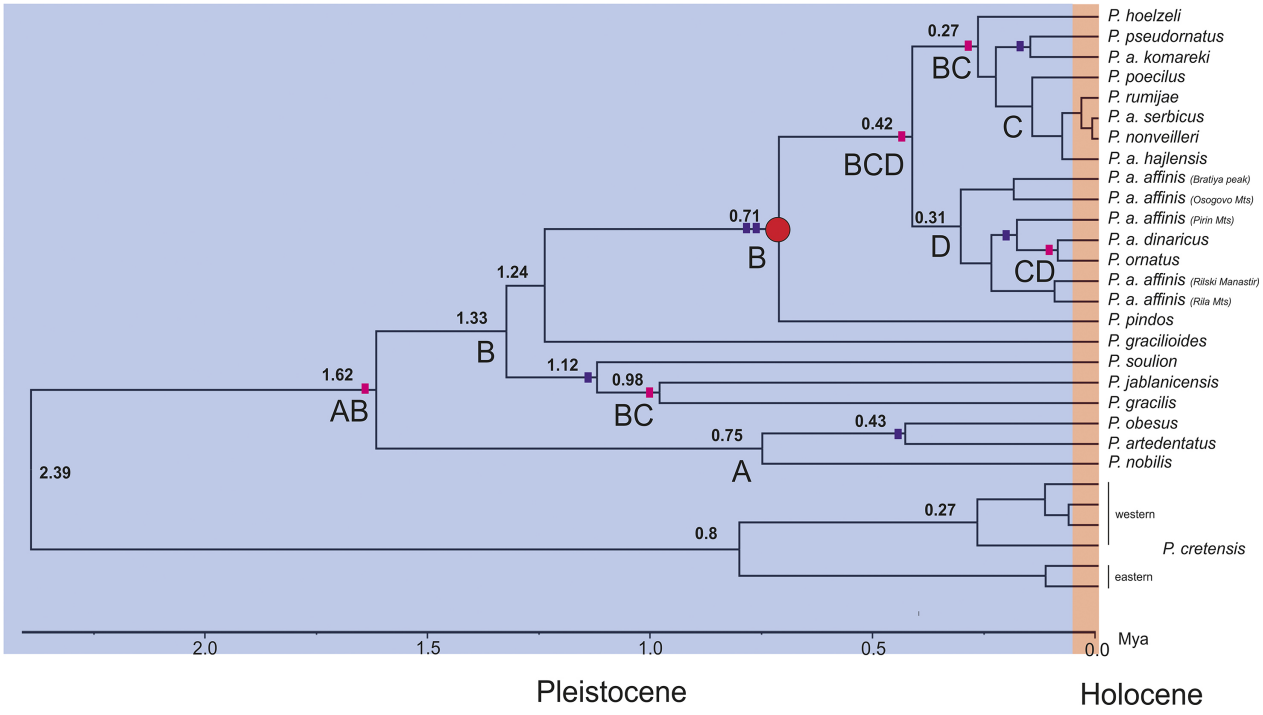

Figure 2. The Beast tree showing the reconstructed geographic ranges and dated phylogeny of the *Poecilimon ornatus* group. The values indicated under the branches represent the mean ages of lineage divergence; acronyms on the nodes indicate geographic areas: [A] – Southern, [B] – Central, [C] – North-Western, [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.

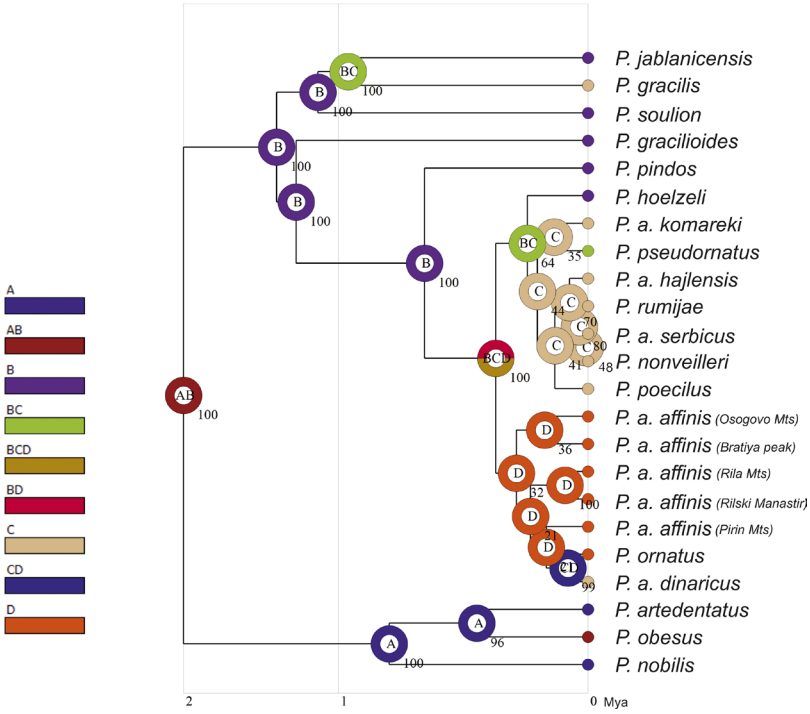

Figure 3. The biogeographic reconstruction of the ranges of the *Poecilimon ornatus* group as shown on the BEAST tree (S-DIVA results). The values at nodes indicate the probability, acronyms on the nodes, and colors indicate geographic areas: [A] – Southern, [B] – Central, [C] – North-Western, [D] –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

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 genet-

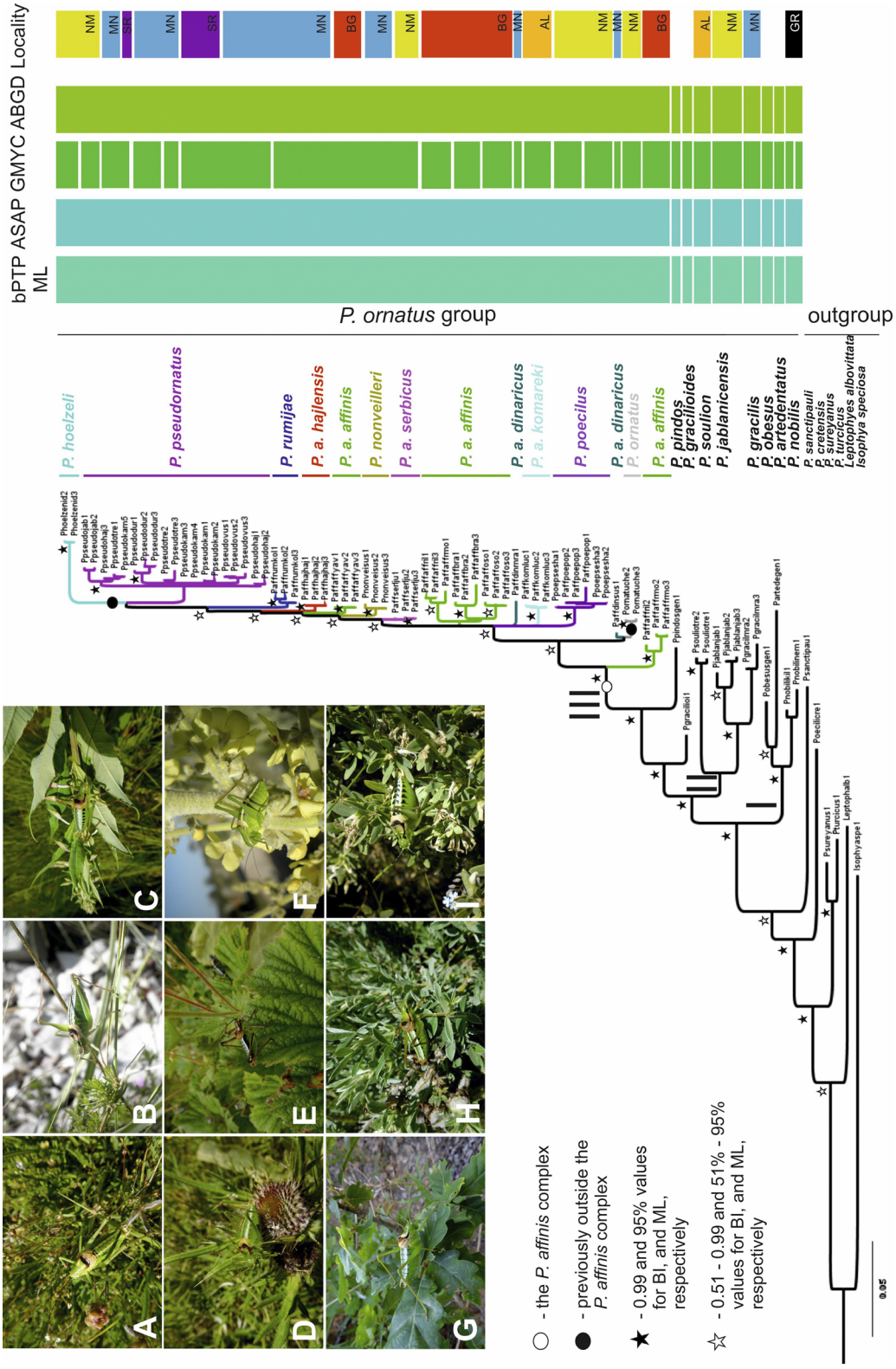

Figure 4. **A** *Poecilimon pseudornatus*, **B** *P. gracilioides*, **C** *P. a. affinis*, **D** *P. a. hajlensis*, **E** *P. gracilis*, **F** *P. nobilis*, **G** *P. rumijae*, **H** *P. hoelzeli*, **I** *P. ornatus*. Photos: Dragan Chobanov. Bayesian inference tree from a dataset including COI, ND2, 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.

ic 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.

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.

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 Mts., 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 $P \leq 0.001$. 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.

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).

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.

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 Mts., Bratiya, Osogovo, Kirilova Polyana, Rila Mts., 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. These young taxa were formed by speciation modulated by dispersal, vicariance, and extinction events, and directed towards phenotypic and ecological diversification.

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