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Increased geographic sampling suggests incomplete lineage sorting and recent introgression between *Pyrrhosoma nymphula* (Sulzer, 1776) and *P. elisabethae* Schmidt, 1948 in the Western Palearctic

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Abstract. We analysed COI and ITS sequences from a total of 69 European *Pyrrhosoma nymphula* (Sulzer, 1776) and three *P. elisabethae* Schmidt, 1948 to explore species boundaries and phylogeographic patterns in their Western Palearctic distributions. We found that phylogenetic and haplotype network analyses support previous results that the widespread *P. nymphula* and the endangered *P. elisabethae* are distinct species, and that *P. nymphula* in North Africa are distinct from *P. nymphula* in Europe. However, our results also suggest that neither diversification is complete as we found evidence for introgression of mitochondrial DNA between *P. elisabethae* and eastern Europe *P. nymphula*, as well as possible incomplete lineage sorting. Finally, our results indicate that while *P. nymphula* likely recolonized most of Europe from an Iberian Peninsula refugium following the Weichsel Glaciation, separate refugia possibly existed in North Africa, southern Italy, and for *P. elisabethae* in the southwestern Balkan.

Key words. Odonata, Zygoptera, distribution

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Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Introduction

Pyrrhosoma Charpentier, 1840 is a small genus of damselflies confined entirely to the Western Palearctic region (Guan et al., 2013; Kalkman & Jović, 2015; Kalkman et al., 2015). The genus was previously considered to be comprised by the Western Palearctic or 'western branch', and an 'eastern branch' found in the southern and southeastern parts of the Himalayan Mountains (e.g. Guan et al., 2013). However, Guan et al. (2013) demonstrated that *Pyrrhosoma* as then understood was non-monophyletic, as the monobasic Nearctic genus *Chromagrion* Needham, 1903 is the sister group to Western Palearctic *Pyrrhosoma*. In their study, they therefore erected the genus *Huosoma* Guan et al., 2013 for the two species *H. tinctipenne* (McLachlan, 1894) and *H. latiloba* (Yu et al., 2008) that comprised the 'eastern branch' of *Pyrrhosoma* s.l. The Western Palearctic *Pyrrhosoma* appears to be more complex (Guan et al., 2013). It was originally thought to comprise a single species, *P. nymphula* (Sultzer, 1776), with a current distribution which, except for northern Scandinavia, southern Greece, and southern Italy, includes most of Europe, and with isolated populations in Morocco (e.g., Askew, 2004). However, Kalkman & Lopau (2006) demonstrated, based on adult morphology, that populations in southern Albania, as well as costal Greece previously considered a separate sub-

species, *P. nymphula elisabethae* Schmidt, 1948, are sufficiently distinct to warrant full species status as *P. elisabethae*. This was later backed up by nymphal characters by Borchard & van der Ploeg (2013). *Pyrrhosoma elisabethae* must thus be considered one of the most threatened odonates in Europe (Kalkman, 2020; Kalkman & Jović, 2013), and resolving its exact relationships with *P. nymphula* should be a matter of urgency. Kalkman et al. (2015) revised the distribution of *P. nymphula* and *P. elisabethae* and showed that *P. nymphula* also occur in northern Anatolia, the Caucasus, and northern Iran (Fig. 1). As mentioned above, Guan et al. (2013), in a phylogenetic study of *Pyrrhosoma* s.l. based on the mitochondrial COI gene and the nuclear Internal Transcribed Spacer (ITS) region, showed that *Pyrrhosoma* s.l. is paraphyletic with respect to *Chromagrion.* They also showed that Moroccan *P. nymphula* populations are genetically highly distinct and may warrant specific taxonomic status. Finally, their results showed that *P. elisabethae* are genetically distinct and, together with the Moroccan populations, may comprise the sister group to the remaining *P. nymphula*. However, their results did not clearly resolve the relationships between these three groups in the *P. nymphula* complex, and as their focus was on the overall phylogenetic pattern in *Pyrrhosoma*, their geographical sampling was not aimed at resolving patterns within *P. nymphula* s.l.

Here we present a phylogeographic study of the *P. nymphula-P. elisabethae* complex with a considerably increased geographical sampling of *P. nymphula* compared to Guan et al. (2013) with the aim of further exploring the relationship between *P. nymphula, P. elisabethae* and Moroccan specimens. We take advantages of the barcode sequences already published in Guan et al. (2013), and target the same gene regions (COI and ITS) in additional specimens. We further take advantage of recent advances in barcoding of European Odonata (Galimberti et al., 2020; Geiger et al., 2021) and complement our dataset with publicly available barcode sequences.

Material and methods

Sampling

We sampled 36 specimens of *P. nymphula* with a focus on northern and southeastern Europe, either collected in the field for this study or obtained from various public or private collections. We complemented the samples with nine *P. nymphula* and three *P. elisabethae* samples from Guan et al. (2013), 13 *P. nymphula* from Geiger et al. (2021), four *P. nymphula* from Galimberti et al. (2020), and seven *P. nymphula* available on the public databases of Barcode of Life Database (boldsystems.org) and/or Genbank (ncbi.nlm.nih.gov/genbank/). Eight of the *P. nymphula* specimens and two *P. elisabethae* specimens from which COI was available also had ITS sequences available on Genbank from Guan et al. (2013). The total dataset thus comprises three *P. elisabethae*

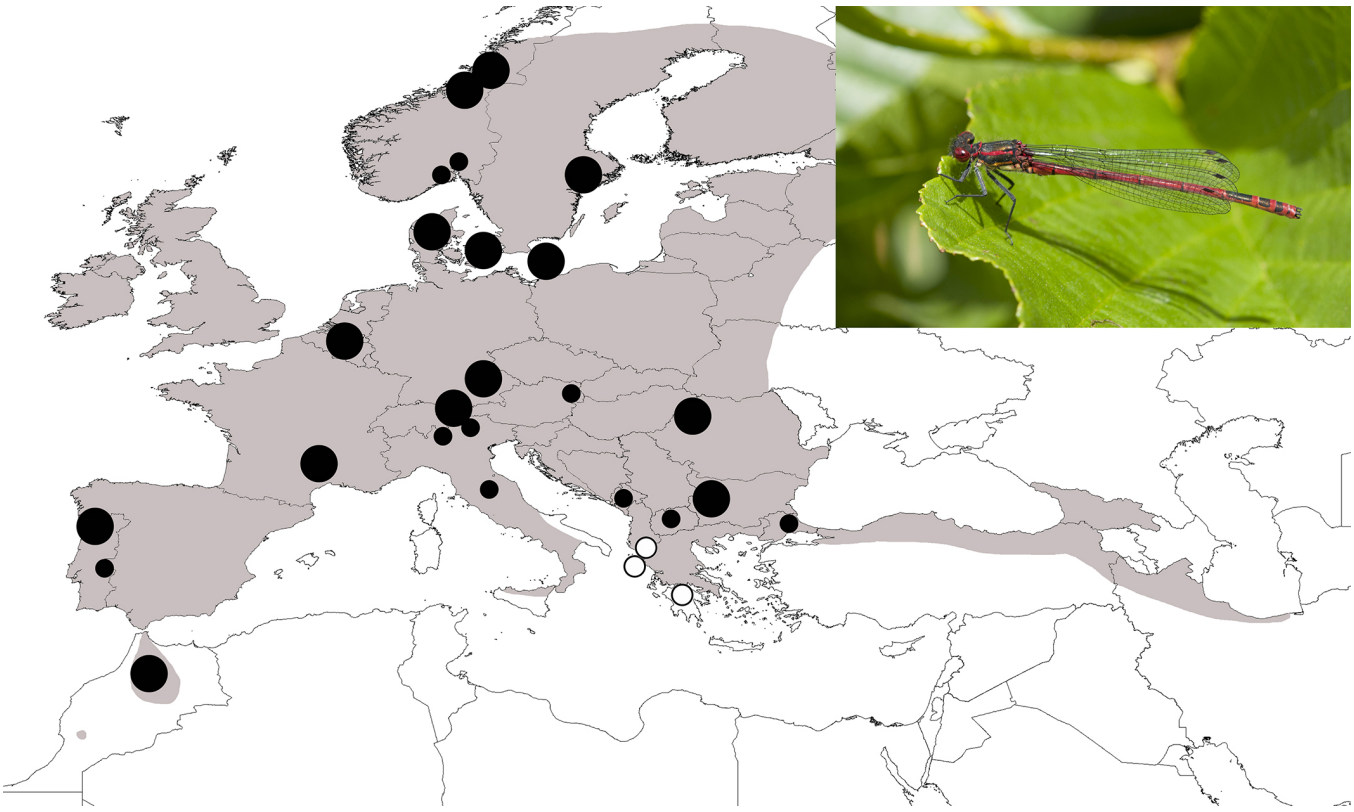

Figure 1. Range and sampling sites for *Pyrrhosoma nymphula* (photo) and *P. elisabethae*. Distribution for *P. nymphula* (based on Kalkman et al., 2015) is indicated in grey. The distribution of *P. elisabethae* (see Kalkman & Jović, 2015) is approximately covered by the white circles indicating sample sites. Black circles indicate *P. nymphula*, and white circles indicate *P. elisabethae*. Small circles indicate single specimens, whereas large circles indicate several specimens. See Table 1 for specimen details.

Table 1. *Pyrrahosoma nymphula* (n = 69) and *P. elisabethae* (n = 3) used in this study with localities, voucher designations, Genbank and BOLD accession numbers, and voucher deposits. KMO: Kjell-Magne Olsen collection; NHMA: Natural History Museum Aarhus; NHRM: Swedish Museum of Natural History.

Species	Country	Region	HT group	Voucher	Ref	Genbank (COI)	BOLD (COI)	Genbank (ITS)	Deposit (this study)
P. nymphula	Denmark	Northwest Jutland	1	ENT-DNA-33	New	MN913134	DANOD026-22	MN963375	NHMA
P. nymphula	Denmark	Bornholm	1	ENT-DNA-34	New	MN913135	DANOD027-22	MN963377	NHMA
P. nymphula	Denmark	West Jutland	1	ENT-DNA-35	New	MN913136	DANOD028-22	MN963378	NHMA
P. nymphula	Denmark	South Jutland	1	ENT-DNA-36	New	MN913137	DANOD029-22	MN963390	NHMA
P. nymphula	Denmark	East Jutland	1	ENT-DNA-105	New	MN913138	DANOD098-22	MN963364	NHMA
P. nymphula	Denmark	Bornholm	1	ENT-DNA-191	New	MN913139	DANOD169-22	MN963367	NHMA
P. nymphula	Denmark	Bornholm	1	ENT-DNA-193	New	MN913140	DANOD171-22	MN963369	NHMA
P. nymphula	Denmark	Northwest Jutland	1	ENT-DNA-32	New	MN913141	DANOD025-22	MN963374	NHMA
P. nymphula	Denmark	Bornholm	1	ENT-DNA-333	New	MN913142	DANOD305-22	MN963376	NHMA
P. nymphula	Norway	Skien	1	ENT-DNA-687	New	MN913143	DANOD578-22	–	KMO
P. nymphula	Sweden	Södermanland	1	ENT-DNA-883	New	MN913144	DANOD755-22	MN963384	NHRM
P. nymphula	Sweden	Södermanland	1	ENT-DNA-884	New	MN913145	MN913145	MN963385	NHRM
P. nymphula	Austria	Tyrol	1	ENT-DNA-889	New	MN913146	DANOD761-22	MN963387	NHMA
P. nymphula	Belgium	Bornem	1	ENT-DNA-893	New	MN913147	DANOD765-22	MN963389	NHMA
P. nymphula	Bulgaria	Sofia	1	ENT-DNA-1016	New	MN913148	DANOD822-22	MN963383	NHMA
P. nymphula	Bulgaria	Sofia	1	ENT-DNA-1017	New	MN913149	DANOD823-22	MN963361	NHMA
P. nymphula	Norway	Oslo	1	ENT-DNA-686	New	MN913150	DANOD577-22	–	KMO
P. nymphula	Denmark	Bornholm	1	ENT-DNA-192	New	MN913151	DANOD170-22	MN963368	NHMA
P. nymphula	Denmark	East Jutland	1	ENT-DNA-103	New	MN913152	DANOD096-22	MN963362	NHMA
P. nymphula	Denmark	South Zealand	1	ENT-DNA-263	New	MN913153	DANOD236-22	MN963372	NHMA
P. nymphula	Denmark	South Zealand	1	ENT-DNA-264	New	MN913154	DANOD237-22	MN963373	NHMA
P. nymphula	Denmark	East Jutland	1	ENT-DNA-104	New	MN913155	DANOD097-22	MN963363	NHMA
P. nymphula	Austria	Tyrol	1	ENT-DNA-890	New	MN913156	DANOD762-22	MN963388	NHMA
P. nymphula	Belgium	Leuven	1	ENT-DNA-892	New	MN913157	DANOD764-22	–	NHMA
P. nymphula	Belgium	Leuven	1	ENT-DNA-891	New	MN913158	DANOD763-22	–	NHMA
P. nymphula	Denmark	West Jutland	1	ENT-DNA-480	New	MN913159	DANOD417-22	MN963386	NHMA
P. nymphula	France	Mende	1	ENT-DNA-887	New	MN913160	DANOD759-22	MN963392	NHMA
P. nymphula	France	Mende	1	ENT-DNA-888	New	MN913161	DANOD760-22	MN963391	NHMA
P. nymphula	Denmark	Northeast Zealand	1	ENT-DNA-37	New	MN913162	DANOD030-22	MN963379	NHMA
P. nymphula	Denmark	South Zealand	1	ENT-DNA-261	New	MN913163	DANOD234-22	MN963370	NHMA
P. nymphula	Denmark	South Zealand	1	ENT-DNA-262	New	MN913164	DANOD235-22	MN963371	NHMA
P. nymphula	Sweden	Södermanland	1	ENT-DNA-885	New	MN913165	DANOD757-22	MN963380	NHMA
P. nymphula	Sweden	Södermanland	1	ENT-DNA-886	New	MN913166	DANOD758-22	MN963381	NHMA
P. nymphula	Sweden	Södermanland	1	ENT-DNA-928	New	MN913167	DANOD792-22	MN963382	NHMA
P. nymphula	Romania	Vadu	2	ENT-DNA-1174	New	MN913168	DANOD931-22	MN963365	NHMA
P. nymphula	Romania	Vadu	2	ENT-DNA-1175	New	MN913169	DANOD932-22	MN963366	NHMA
P. nymphula	Austria	Vienna	1	NOaS3-2019_ Odo0003	BOLD	-	AODON003-20	–	
P. nymphula	Germany	Bavaria	1	BC-ZSM-WI_333913	BOLD	GU682191	FBAQU202-09	–	
P. nymphula	Germany	Bavaria	1	BC-ZSM-WI_332828	BOLD	GU682172	FBAQU321-09	–	
P. nymphula	Germany	Bavaria	1	BC ZSM AQU 00414	BOLD	HM901874	FBAQU509-10	–	
P. nymphula	Germany	Bavaria	1	BC ZSM AQU 00415	BOLD	HM901875	FBAQU510-10	–	
P. nymphula	Germany	Bavaria	1	BC ZSM AQU 00416	BOLD	HM901876	FBAQU511-10	–	
P. nymphula	Germany	Bavaria	1	BC-ZSM-WI_338731	BOLD	HM901899	FBAQU547-10	–	
P. nymphula	Germany	Bavaria	1	GBOL05486	Geiger et al. (2021)	MW490341	GBEPT916-14	–	
P. nymphula	Germany	Bavaria	1	GBOL05487	Geiger et al. (2021)	MW490499	GBEPT917-14	–	
P. nymphula	Portugal	Bertinandos	1	P.ny_Portugal_ Bertiandos	Guan et al. (2013)	KU220884	GB-MIN88634-17	KU245333	
P. nymphula	Portugal	Escarei	1	P.ny_Portugal_ Escarei	Guan et al. (2013)	KU220879	GB-MIN88635-17	KU245328	

Table 1 continued

Species	Country	Region	HT group	Voucher	Ref	Genbank (COI)	BOLD (COI)	Genbank (ITS)	Deposit (this study)
P. nymphula	Turkey	Eur. Turkey	1	P.ny_Turkey	Guan et al. (2013)	KU220878	GB-MIN88638-17	KU245327	
P. nymphula	Morocco	Ifrane	3	P.ny_Morocco_1	Guan et al. (2013)	KU220882	GB-MIN88639-17	KU245332	
P. nymphula	Morocco	Ifrane	3	P.ny_Morocco_2	Guan et al. (2013)	KU220883	GB-MIN88640-17	KU245336	
P. nymphula	Macedonia		1	P.ny_Macedonia	Guan et al. (2013)	KU220885	GB-MIN88636-17	KU245334	
P. nymphula	Portugal	Marvão	1	P.ny_Portugal_Marvao	Guan et al. (2013)	KU220881	GB-MIN88637-17	KU245335	
P. nymphula	Portugal	Afonsin	1	P.ny_Portugal_Afonsin	Guan et al. (2013)	KU220880	GB-MIN88642-17	KU245329	
P. nymphula	Belgium	Affligem	1	P.ny_Belgium	Guan et al. (2013)	KU220874	GB-MIN88641-17	–	
P. nymphula	Germany	Bavaria	1	GBOL 20221	Geiger et al. (2021)	MW490308	GBODO098-18	–	
P. nymphula	Germany	Bavaria	1	GBOL 20275	Geiger et al. (2021)	MW490339	GBODO152-18	–	
P. nymphula	Germany	Bavaria	1	GBOL02853	Geiger et al. (2021)	MW490465	GBUPS203-14	–	
P. nymphula	Germany	Bavaria	1	GBOL02854	Geiger et al. (2021)	MW490106	GBUPS204-14	–	
P. nymphula	Germany	North Rhine-Westphalia	1	ZFMK-TIS-2010626	Geiger et al. (2021)	MW490401	GODO026-18	–	
P. nymphula	Norway	Nord-Trøndelag	1	49411	Geiger et al. (2021)	MW490373	ODTRI002-14	–	
P. nymphula	Norway	Nord-Trøndelag	1	49412	Geiger et al. (2021)	MW490089	ODTRI003-14	–	
P. nymphula	Norway	Sør-Trøndelag	1	49441	Geiger et al. (2021)	MW490357	TRDOD014-14	–	
P. nymphula	Norway	Sør-Trøndelag	1	49442	Geiger et al. (2021)	MW490431	TRDOD015-14	–	
P. nymphula	Norway	Nord-Trøndelag	1	49497	Geiger et al. (2021)	MW490504	TRDOD070-14	–	
P. nymphula	Norway	Nord-Trøndelag	1	49498	Geiger et al. (2021)	MW490345	TRDOD071-14	–	
P. nymphula	Italy	Trentino-Alto Adige	1	MIB:ZPL:08495	Galimberti et al. (2020)	MT298604	ZPLOD695-20	–	
P. nymphula	Italy	Umbria	2	MIB:ZPL:08496	Galimberti et al. (2020)	MT298605	ZPLOD696-20	–	
P. nymphula	Italy	Lombardy	1	MIB:ZPL:08498	Galimberti et al. (2020)	MT298603	ZPLOD698-20	–	
P. nymphula	Montenegro		1	MIB:ZPL:08635	Galimberti et al. (2020)	MT298606	ZPLOD835-20	–	
P. elisabethae	Albania	South Albania	2	P.el_Albania	Guan et al. (2013)	KU220875	GBM-HO1829-19	–	
P. elisabethae	Greece	Kalavrita	2	P.el_Greece	Guan et al. (2013)	KU220877	GB-MIN88626-17	KU245326	
P. elisabethae	Greece	Corfu	2	P.el_Corfu	Guan et al. (2013)	KU220876	GB-MIN88627-17	KU245325	

and 69 *P. nymphula* with good geographical coverage. See Table 1 for specimen details and Figure 1 for an overview of sample sites. The sample localities and distribution map in Figure 1 were constructed following the procedures outlined in Simonsen et al. (2020).

Laboratory procedures

Following Guan et al. (2013) we targeted the barcode region of the mitochondrial COI gene (Hebert et al., 2003) and the nuclear ITS region. This allowed us to

build directly on the earlier studies listed that have made numerous sequences for both *P. nymphula* and *P. elisabethae* available.

Genomic DNA was extracted at the Department of Biology, Aarhus University, Denmark using the E.Z.N.A. Tissue DNA kit (Omega BIO-TEK), modifying the manufacturers' instructions slightly: samples were incubated at 42°C for 18–23 hours, following Krosch & Cranston (2012), as well as skipping steps 5 and 6, as centrifugation and transfer of supernatant was not needed when extracting DNA from legs of odonates. We used the PCR protocols outlined in Simonsen et al. (2020, 2021) and the universal tail primers OdoF2 and OdoR3 for COI (Simonsen et al., 2020) and VRAIN2F and VRAIN2R for ITS (Félix et al., 2001). All samples were sequenced using the Sanger method at Macrogen Europe. Contigs and consensus sequences were obtained using DNA Baser Sequence Assembler v5.8.0 (Heracle BioSoft, Romania, 2018). We checked the identity of all sequences using BLAST on GenBank and/or BOLD (Barcode of Life Database) Identification System. Separate alignments of COI and ITS were constructed in Mega 11 (Tamura et al., 2021) using the MUSCLE algorithm. The alignments were subsequently checked and corrected manually in Mesquite 3.70 (Maddison & Maddison, 2021). The COI alignment was used separately for haplotype network and genetic diversity analyses, and a concatenated COI+ITS alignment was used for the phylogenetic analyses.

Phylogenetic analyses, haplotype network, and genetic diversity

We analysed phylogenetic patterns in MrBayes 3.2 (Ronquist et al., 2012) and Garli 2.01 (Zwickl, 2006) on Cipres XCEDE (Miller et al., 2010). Following Guan et al. (2013) we used sequences from *Chromagrion conditum* (accession numbers, COI: KU220873; ITS: KU245324), *Huosoma tinctipenne* (accession numbers, COI: KU220892; ITS: KU245344), *H. latiloba* (accession numbers, COI: KU220891; ITS: KU245340), and *Enallagma cyathigerum* Charpentier, 1840) (accession numbers, COI: MN934754; ITS: MN963487) as outgroups. We performed three analyses in MrBayes: one of the COI dataset only, one of the ITS dataset only, and one of the combined COI+ITS dataset. In the latter analysis the dataset was partitioned into genes. In all analyses we used model jumping with a gamma distribution (nst = mixed, rates = gamma). All analyses were run for 10 million generations with sampling every 1000 generation. The output files were examined in Tracer 1.7.2 (part of the BEAST package: Bouckaert et al., 2019), and the first 25% of the sampled trees were discarded as burnin. The resultant majority rule consensus trees were examined and visualized in FigTree 1.4.4 (Rambaut, 2018). In Garli we analysed the combined dataset partitioned into genes using a GTR model with a gamma distribution. We used subset specific rates, and the analyses were terminated after 20,000 generations without a significant change of topology. We did 10 independent runs of the analysis and constructed a majority rule consensus tree in Mesquite. To facilitate the construction of consensus trees, we specified *E. cyathigerum* as outgroup. We calculated bootstrap values in Mesquite based on 1000 bootstrap repetitions with one tree search repetition per bootstrap round. We constructed a minimum spanning haplotype network (Bandelt et al., 1999) in PopART (Leigh and Bryant, 2015) (available at popart.otago.ac.nz) of the COI dataset, following Kohli et al. (2018, 2021) and Simonsen et al. (2021, 2023a, b). As haplotype network analyses are sensitive to missing data, the alignment was trimmed to remove sections of missing data at the start and the end before analysis.

We identified COI haplotype groups based on the results of the haplotype network analysis and calculated genetic distances for COI within and between groups based on the Kimura-2 Parameter as well as overall mean distance (π) in Mega 11 (Tamura et al., 2021).

Results

We sequenced at least 622 bp COI from all 36 specimens, and 828 bp ITS from 32 specimens (Table 1). The 36 COI sequences available on Genbank and BOLD had at least 537 overlapping base pairs (Table 1). The final, combined COI+ITS alignment comprises 1293 characters including gaps in ITS (Supplementary Material S1). The COI alignment for the haplotype network analysis was trimmed to avoid missing data as haplotype network analyses are sensitive to such data, and the final alignment comprises 537 characters (Supplementary Material S2).

The haplotype network analysis (Fig. 2) shows that the *P. nymphula-P. elisabethae* complex can be divided into three groups in the Western Palearctic. One group (Group 1, n = 65) comprise almost all European *P. nymphula* with the exceptions of two Romanian specimens (ENT-DNA-1174 and ENT-DNA-1175) and one central Italian specimen (MIB:ZPL:08496); the second group (Group 2, n = 6) comprises the two Romanian specimens and the central Italian specimen listed above, and the three *P. elisabethae* specimens; the third group (Group 3, n = 2) comprise the two *P. nymphula* specimens from Morocco. Following the surprise inclusion of the Romanian specimens in Group 2, we double-checked their identity using the morphological characters in Kalkman & Lopau (2006), and both specimens were unequivocally *P. nymphula*. The central Italian and two Moroccan specimens were studied by others (Galimberti et al., 2020 and Guan et al., 2013, respectively) and we rely on their identification.

The phylogenetic analyses of the combined dataset (Fig. 3, Supplementary Material S3) and the COI dataset (Supplementary Material S4) show a pattern similar to the haplotype network analysis (Fig. 2). Group 2 and Group 3 are both monophyletic with strong Bayesian and weak Bootstrap support. Together Group 2 and Group 3 form a monophyletic group that is well sup-

Table 2. Average genetic distance (Kimura 2 parameter) calculated in MEGA 11.

	Group 1	Group 2	Group 3
Group 1	0.0024		
Group 2	0.0218	0.0025	
Group 3	0.0264	0.0196	0.0000

ported in the Bayesian analysis, but poorly supported in the Maximum Likelihood Bootstrap analysis. But the relationship between this combined group (Group 2 + Group 3) and Group 1 is not resolved, as nine individual specimens from Group 1 form an unresolved polytomy with (Group 2 + Group 3) and the remainder of Group 1, the latter being a monophyletic group of its own with strong support in the Bayesian analysis and moderate support in the Maximum Likelihood Bootstrap analysis. The tree from the Bayesian analysis of the ITS dataset is largely unresolved, but the two *P. elisabethae* for which we have ITS comprise a monophyletic group. Similarly, the two Moroccan samples comprise a monophyletic group, as do five *P. nymphula* (ENT-DNA-36, ENT-DNA-888, ENT-DNA-889, ENT-DNA-890, ENT-DNA-893).

The genetic diversity analysis supports the division of the *P. nymphula-P. elisabethae* complex into three haplotype groups (Table 2). The average K2P distance varies between 0.0196 between Group 2 and Group 3, to 0.0218 between Group 1 and Group 2, and 0.0264 between Group 1 and Group 3. The within group average K2P distance varies between 0 in Group 3, and 0.0024 in Group 1, and 0.0025 in Group 2. The overall nucleotide diversity (π) is 0.0078.

Discussion

Our results are in overall agreement with Guan et al. (2013) that *P. elisabethae* and Moroccan *P. nymphula* appear genetically isolated from European *P. nymphula*. But our increased geographic sampling also highlights that the situation is complex. Although the two Moroccan specimens indeed are isolated (Figs 2–3, Table 2), *P. elisabethae* is closely related to a specimen from central Italy and two specimens from Romania—indeed the two Romanian specimens and the two Greek *P. elisabethae* share an identical COI haplotype (Figs 2–3, Table 2). The average genetic distances between (the remaining) European *P. nymphula*, Moroccan *P. nymphula*, and *P. elisabethae* (including the three *P. nymphula* listed above) are generally high (Table 2) and an order of magnitude higher than intersubspecific variation in *Sympetrum vulgatum* (Hinojosa et al., 2017). They are more comparable to (but still higher than) intersubspecific variation in *Lestes virens* (Simonsen et al., 2023a) and infraspecific variation in *Aeshna cyanea* (Simonsen et al., 2020), *Orthetrum coerulescens*

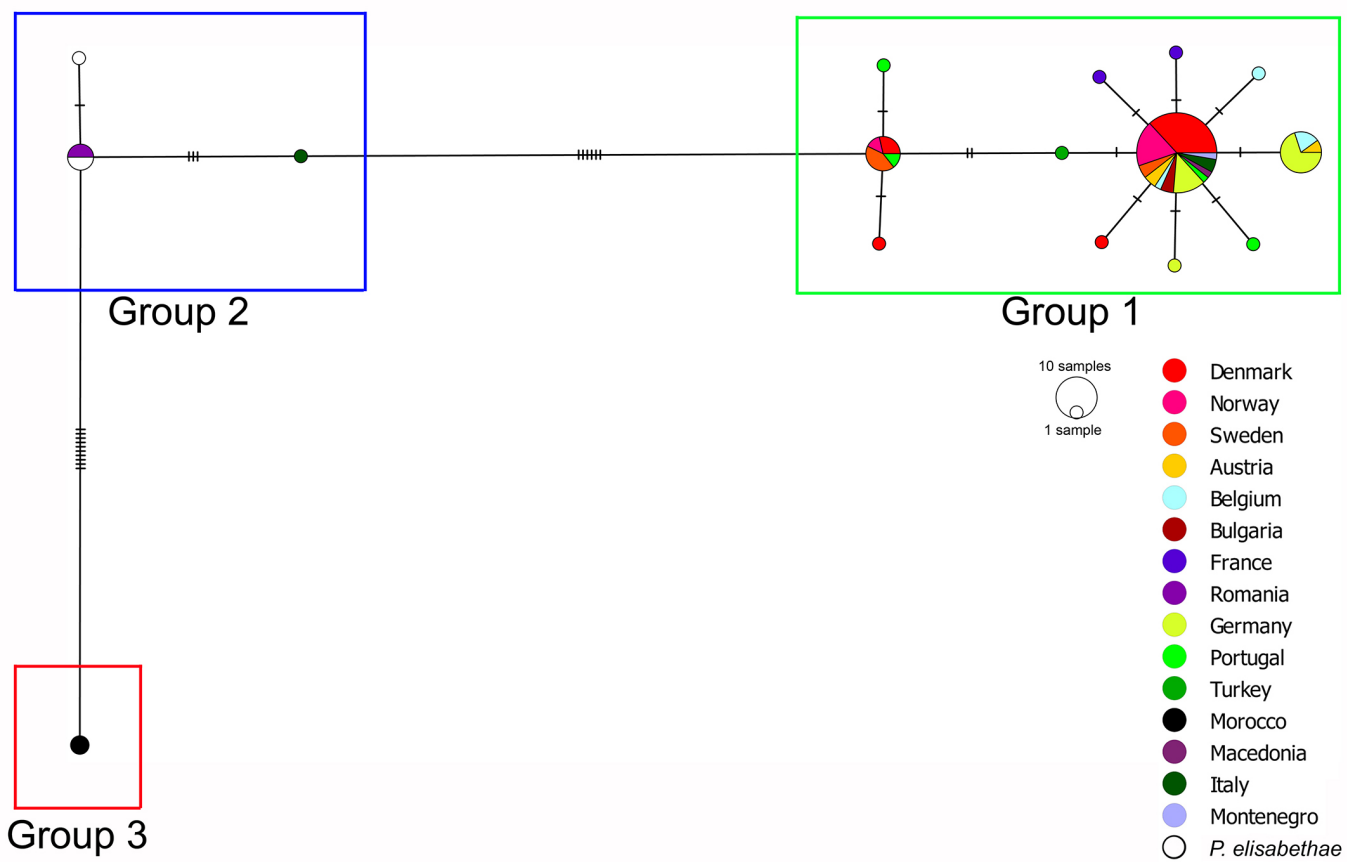

Figure 2. Minimum-spanning haplotype network based on the 537 bp COI dataset. The number of mutations between groups are indicated by bars. Groups are indicated as described in the text.

(Simonsen et al., 2021), and *Platycnemis pennipes* (Simonsen et al., 2023b). Overall, our results thus support Guan et al. (2013) that Western Palearctic *Pyrrhosoma* can be divided into three groups: *P. elisabethae*, European *P. nymphula*, and Moroccan *P. nymphula*.

The identical COI haplotypes found in Greek *P. elisabethae* and a few *P. nymphula* indicate a possible recent introgression event (e.g., Dowling & Secor, 1997; Grant et al., 2005; Harrison & Larson, 2014)—especially as *P. elisabethae* and European *P. nymphula* are generally very divergent, with an average genetic distance of 2.18% between Group 1 and Group 2, which is higher than the predicted hybridization threshold for Zygoptera (Sanchez-Guillen et al., 2014). This is supported by the results of the analysis of the ITS dataset, where the two *P. elisabethae* for which ITS was available are placed as sister taxa, whereas the two Romanian specimens are placed in an unresolved polytomy with most

Figure 3. Tree from the 10 million generation analysis of the combined COI+ITS dataset in MrBayes. Numbers above a branch indicate relevant ML bootstrap values, while numbers below a branch indicate the corresponding posterior probability values. Groups are indicated as discussed in the text and colors correspond to Figure 2. Far outgroup, *Enallagma cyathigerum*, is removed from the tree.

of the European *P. nymphula* and not closely related to *P. elisabethae* (Supplementary Material S5). As the COI haplotype found in the two Romanian *P. nymphula* is very different from other Balkan (and other European) *P. nymphula*, it seems more likely that the mitochondrial genome from *P. elisabethae* has introgressed into *P. nymphula* than the other way around. Although it is intriguing that *P. elisabethae* mtDNA is found in *P. nymphula* from northwestern Romania and not in populations much closer to the occurrence of *P. elisabethae*, this may be coincidental and/or an indication that the introgression is not adaptive (Harrison & Larson, 2014).

Although the occurrence of a central Italian specimen (MIB:ZPL:08496) in Group 2 as sister to the Romanian specimens and *P. elisabethae* (Figs 2–3) could likewise be due to introgression between an Italian *P. nymphula* population and *P. elisabethae*, we find this explanation unlikely. As the Italian specimen is not identical to any sampled *P. elisabethae* haplotype, the introgression would have had to come from an unsampled and quite divergent population. We therefore consider it most likely that the pattern represents a potential ancient *P. nymphula* population in central and southern Italy as recently demonstrated for *Platycnemis pennipes* (Simonsen et al., 2023b), which may show incomplete lineage sorting with respect to *P. elisabethae* (Holder et al., 2001; Maddison, 1997; Maddison & Knowles, 2006). Unfortunately, statistical tests that can distinguish between introgression and incomplete lineage sorting such as the ABBA-BABA test require genomic data and can thus not be used in this case (e.g., Joly et al., 2009; Martin et al., 2015).

If our interpretation above is correct and the central Italian specimen (MIB:ZPL:08496) represents an ancient Italian lineage, this potentially has some important implications for the phylogeography of the *P. nymphula* complex in the Western Palearctic. It is possible that *P. elisabethae*, central Italian *P. nymphula* (MIB:ZPL:08496), Moroccan *P. nymphula* and European *P. nymphula* represent four different glacial refugia. *Pyrrhosoma elisabethae* then probably represents descendants from a refugium in south-costal Balkan, MIB:ZPL:08496 represents an Italian refugium, and Moroccan *P. nymphula* represents a North African/Maghreb refugium. We agree with Guan et al., (2013) that *P. nymphula* in Morrocco could represent a separate species, but more data is needed to determine this, and the taxonomic status Moroccan populations should be the subject of a future study. The extent of the refugium of European *P. nymphula* is more difficult to establish. But the fact that the four Portuguese specimens show considerable variation despite being geographically adjacent indicate that the original refugium may have been in the Iberian Peninsula. Indeed, the average variation between the four Portuguese specimens (0.0069) is much higher than the average variation within Group 1 (0.0024). If *P. nymphula* has recolonized much of Europe from an Iberian refugium following the Weichsel Glaciation, the pattern would thus be rather similar to the pattern found in *Aeshna cyanea* by Simonsen et al. (2020). The northern Italian specimens would then belong to this recolonization, a likely scenario as *P. nymphula* occurs at altitudes above 2000 m in the Alps (Kalkman et al., 2015). The pattern is somewhat similar to the 'Bear Paradigm' illustrated by Hewitt (1999, 2004), where the brown bear (*Ursus arctos*) survived the last glaciation in three European refugia: an Iberian, an Italian, and a Balkan refugium. However, the brown bear colonized Eastern Europe and parts of Fennoscandinavia from the Balkan refugium, whereas *P. elisabethae* never expanded from its costal Balkan refugium judging from its current distribution. Furthermore, recolonization of Europe from an Iberian refugium would also explain why *P. nymphula* apparently occurs rather fragmented in the Balkans, Ukraine, and European Russia (Kalkman et al., 2015).

Our results highlight that the *P. nymphula-P. elisabethae* complex need further study to establish the exact boundaries between the different groups as well as elucidate phylogeographical patterns within the complex. Although our dataset comprise both mitochondrial and nuclear data, we must urge caution, as especially COI variation can be caused by external factors such as *Wolbachia* (e.g., Kodandaramaiah et al., 2013; Sazama et al., 2017). We therefore urge that the *P. nymphula-P. elisabethae* complex should be the subject for a genomics study in the future.

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Data availability statement

All new DNA sequences are available on Genbank and BOLD (see Table 1), all datasets are made available as Supplementary Online Material.

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Supplementary Material

Supplementary Material S1. MrBayes Nexus file of the combined COI and ITS dataset (with BOLD IDs as termina names).

Supplementary Material S2. PopART Nexus of the 537 bp COI dataset used for haplotype network and MEGA 11 (with BOLD IDs as termina names).

Supplementary Material S3. Tree from the Maximum Likelihood Bootstrap analysis of the combined COI+ITS dataset in Garli.

Supplementary Material S4. Tree from the 10M generation MrBayes analysis of the 622 bp COI dataset.

Supplementary Material S5. Tree from the 10M generation MrBayes analysis of the ITS dataset.