



The Grasshopper Paradigm in damselflies: evidence for phalanx-like postglacial recolonization of Europe from a Balkan refugium in *Platycnemis pennipes* Pallas (Odonata: Zygoptera: Platycnemidae)

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Abstract

We explore haplotype diversity, phylogeography and phylogenetic relationships of the damselfly *Platycnemis pennipes* in Europe based on 618 bp DNA from the mitochondrial gene COI. A haplotype network analysis shows that the species is divided into two haplotype groups. One is restricted to the Italian Peninsula, while the other is found from the Black Sea region across eastern and central Europe to Scandinavia, England, and southwestern France. This pattern is recovered in a Bayesian phylogenetic analysis. Genetic distance (K2P) between the two groups is approximately 1.5%, while within-group variation is an order of magnitude lower. An analysis of the molecular variance (AMOVA) shows that variation between the two groups account for more than 96% of the total variation within the dataset, adding to the evidence that they have been isolated for a considerable amount of time. The pattern we find is similar to the so-called Grasshopper Paradigm in European phylogeography, where a species has recolonized Europe after the last glaciation from a glacial refugium in the southeast, while other refugial populations in the Iberian and Italian peninsulas have remained isolated to this day. In *P. pennipes* there is only an isolated refugial population in Italy as the species does not have current populations in the Iberian Peninsula. By comparing the genetic distance between the two groups to a previously published divergence time analysis of European Odonata we estimate that they have likely been isolated since the onset of the Saale Glaciation *ca* 400 ky ago.

Key words

Phylogeography, Glacial refugia, expansion, Europe, Zygoptera

1. Introduction

The past decades have seen a number of studies on molecular diversity, systematics and phylogeography of European Odonata. Weekers et al. (2001) used the high-

ly variable nuclear ribosomal ITS region to explore the phylogeny and diversity of European *Calopteryx* species. They found an overall east-west division at the species

level, but also a clear separation between *C. splendens splendens* (Harris, 1780) and the Italian subspecies *C. splendens caprai* Conci, 1956. Sadeghi et al. (2010) in an AFLP based study of *C. splendens* found a complex pattern that only partially corresponds to the current subspecific taxonomy. They found indication of a post-glacial recolonization of southern Europe from the southeast. Guan et al. (2013) studied the phylogeny of the genus *Pyrrosoma* Charpentier, 1840 (red damselflies) based on the ITS region and the mitochondrial gene COI with special focus on the status and position of the endangered *P. elisabethae* Schmidt 1948. They found that while *P. elisabethae* was indeed monophyletic it grouped within the widespread species *P. nymphula* (Sulzer, 1776) and—surprisingly—closely related to *P. nymphula* specimens from Morocco, clearly indicating that more studies on this species complex are needed. Hinojosa et al. (2017) found a distinct pattern—albeit with low variation—in the ITS region and COI of the skimmer *Sympetrum vulgatum* (Linnaeus, 1758) with the three Western Palearctic subspecies *S. vulgatum vulgatum* (most of Europe), *S. vulgatum ibericum* Ocharan, 1985 (Iberian Peninsula), and *S. vulgatum decoloratum* Selys, 1884 (Anatolia) appearing separated and distinct. In that study, they suggested that the three subspecies represented three different glacial refugia in the Balkans, the Iberian Peninsula, and Anatolia, respectively. Schneider et al. (2015) and Simonsen et al. (2020) studied the *Aeshna cyanea* (Müller, 1794) and *A. vercanica* Schneider et al., 2015 species complex. Their results indicate that *A. cyanea* colonized Western and Central Europe from the Iberian Peninsula, which had previously been colonized from the Maghreb region in Northern Africa. In contrast to the studies above, which provide detailed information on the diversity and distributional patterns of Western Palearctic Odonata, other studies have found little or no variation within the Western Palearctic region. Bernard et al. (2011) found evidence for a rapid expansion from East Asia through western Siberia into West Palearctic in *Nehalennia speciosa* Charpentier, 1840 with little variation across its range. Kohli et al. (2018; 2021) found very little variation and no phylogeographic pattern in the circumpolar *Somatochlora sahlbergi* Trybom, 1889 across its range. While Kohli et al. (2021) generally found clear distinction between Nearctic and Palearctic populations in *Aeshna juncea* (Linnaeus, 1758), *Aeshna subarctica* Walker, 1908, *Libellula quadrimaculata* Linnaeus, 1758, and *Sympetrum danae* (Sulzer, 1776), as well as some internal Nearctic population structure and occasional distinction between Japan and the remaining Palearctic, they found little variation and no internal structure in the Western Palearctic for either four species.

The molecular diversity and phylogeography of Odonata in the Western Palearctic thus appear highly variable with the potential to illuminate a number of phylogeographic patterns. European phylogeography is currently dominated by four paradigms associated with expansion from the three peninsular refugia, on the Iberian, Italian and Balkan peninsulas (see Hewitt 1999; 2004; Schmitt 2007 for review and details): the Hedgehog Paradigm

with postglacial expansion from the Iberian, Italian and Balkan peninsulas; the Bear Paradigm with postglacial expansion from the Iberian Peninsula and an eastern refugium (not necessarily the Balkan Peninsula); the Butterfly Paradigm with postglacial expansion from the Italian and Balkan peninsulas; and the Grasshopper Paradigm with postglacial expansion from the Balkan Peninsula. Especially the Iberian and Italian peninsulas may show historic connections to the North African Maghreb regions (see Husemann et al. 2014; Simonsen et al. 2020 and references therein). In addition to the four paradigms and North African refugia, two other patterns appear to be important in European phylogeography (e.g., Schmitt 2007). First, there is a growing body of evidence for refugia in Europe north of the Pyrenees, the Alps, and the Balkans (e.g., Urusenbacher et al. 2006; Schmitt 2007; Schmitt et al. 2007; Gratton et al. 2008; Varga & Schmitt 2008; Hammouti et al. 2009; Simonsen & Huemer 2014). Second, it has been hypothesized that a considerable number of European species originate in the East Palearctic (de Lattin 1967). Even though the latter has been challenged (e.g., Schmitt 2007), some of the studies listed above (Bernard et al. 2011; Kohli et al. 2018; 2021) indicate an East Palearctic origin of some West Palearctic odonate species.

Here we present a phylogeographic study of the damselfly *Platycnemis pennipes* (Pallas, 1771) in Europe based on the mitochondrial gene COI. *P. pennipes* is a widespread species in continental Europe (Fig. 1) except for the Iberian Peninsula, and central and northern Scandinavia and Finland. It is also missing from Ireland, northern England and Scotland, and most Mediterranean islands. Outside Europe it occurs in Anatolia, the Caucasus, and extends eastwards into western Siberia and northern Kazakhstan as far east as the Yenisei River (Boudot et al. 2015). Generally, the taxonomy of *P. pennipes* is considered uncomplicated, but a separate subspecies, *P. pennipes nitidula* (Brullé, 1832), occurs in the coastal regions of Montenegro, Albania and Greece where it may hybridize with the nominal subspecies (Boudot et al. 2015). Throughout its range *P. pennipes* is found associated with a variety of slow flowing and standing waters with surface and edge vegetation (Nielsen 1998; Askew 2004; Boudot et al. 2015). At least in Denmark it is considered a species with low mobility.

While there are problems associated with using COI as a single genetic marker for diversity and phylogeography studies (e.g. Kondandaramaiah et al. 2013; Brunet et al. 2017; Roe et al. 2017), several of the studies mentioned above demonstrate that it can be used (albeit with some caution) in Odonata. Utilising COI also allow us to combine new data with the extensive, and rapidly growing, DNA barcode data that are already available online. Recently, two major studies of European Odonata (Galimberti et al. 2020; Geiger et al. 2021) have made a considerable number of European odonatan COI sequences available. By combining these and other publicly available sequences with several new sequences we are able to analyse an extensive geographical dataset of a European damselfly to address key question about European phylogeography.



Figure 1. Approximate distribution of *Platycnemis pennipes* (inserted photo) in Western Palearctic (based on Boudot et al. 2015), and approximate sample sites for specimens included in this study. Small circles indicate single specimens, while large circles indicate multiple specimens. For details see Table 1.

2. Material and Methods

2.1. Material

We sampled 43 specimens of *Platycnemis pennipes* from Europe and one additional specimen from Caucasus (Georgia). The new samples were augmented with 17 samples from Genbank or Barcode of Life (BOLD). Nine samples were from Galimberti et al. (2020), six were from Geiger et al. (2021), one was from the Darwin Tree of Life project (<https://www.darwintreeoflife.org>), and one was unpublished but available in BOLD's public database. All specimen data are provided in Table 1. All specimens sequenced for this study are deposited at the Natural History Museum Aarhus. Approximate sample localities are shown in the distribution map in Fig. 1. The sequence from the Darwin Tree of Life project is part of a full reference genome. The full mitochondrial genome for this specimen was downloaded from Genbank and the relevant part of COI was identified in Mega 11 (Tamura et al. 2021) and saved as a fasta file.

2.2. Methods

2.2.1. Laboratory procedures

DNA was extracted at Department of Biology, Aarhus University (AU), Denmark using either E.Z.N.A. Tissue DNA Kit (Omega BIO-TEK) or DNeasy Blood & Tissue Kit (Qiagen). The E.Z.N.A. Tissue DNA Kit protocol was followed with some modifications: samples were incubated at 42°C for 18–23 hours during lysis, steps 5 and 6 in

the protocol were skipped, and samples were incubated with Elution Buffer for 5–10 min at 70°C and eluted once in 200 µl. The DNeasy Blood & Tissue Kit protocol was applied with modification following Krosch & Cranston (2012) and using a lower lysis temperature combined with a longer lysis time: samples were incubated at 42°C for 20 hours during lysis, elution buffer AE was heated to 60°C prior to elution, samples were incubated with buffer AE for 10 min at 60°C and eluted once in 100 µl.

We used the following PCR protocol for COI: 95°C, 2 min; then 35–45 cycles of 95°C, 30 s; 45°C, 30 s; 72°C, 1 min and a final extension of 72°C for 5 min using the primers from Simonsen et al. (2020) OdoF2 (with universal tail, M13-FP): ***TGTA AACGACGGCCAGTTTTCT-ACAAAYCAYAARGATATTGG*** (tail in boldface italics); and OdoR3 (with universal tail, M13R-pUC): ***CAGGA AACAGCTATGACTAAACYTCTGGRTG-RCCAAARAATCA*** (tail in boldface italics). All samples were sequenced at MacroGen Europe using the Sanger Method. Contigs and consensus sequences were obtained using DNA Baser Sequence Assembler v5.8.0 (Heracle Biosoft, Romania). We checked the identity of all sequences using BLAST on GenBank and/or BOLD (Barcode of Life Data base) Identification System. GenBank and BOLD accession numbers are listed in Table 1.

2.2.2. Haplotype network and phylogenetic analyses

All obtained COI sequences were aligned in Mega 11 (Tamura et al. 2021) using the built in Muscle algorithm. The resulting alignment was checked in Mesquite v. 3.03 (Maddison & Maddison 2015). As haplotype network analyses are highly susceptible to missing data, we

Table 1. *Platycnemis pennipes* specimens used in this study with sampling localities, voucher designations, Genbank and BOLD accession numbers, and voucher deposits for all samples when available, and references for sequences not generated in this study, localities for such samples are from BOLD, Genbank or the Welcome Sanger Institute Darwin Tree of Life Projects webpage. The asterisks (*) indicated that the COI sequence used here was extracted from a full mitochondrial genome available at Genbank. WSI: Welcome Sanger Institute; NMW: Naturhistorisches Museum Wien; UL: University of Lodz; UMB: University of Milano Bicocca.

Country	Region	Voucher	Haplotype	Source	Genbank	BOLD	Deposit
Denmark	South Jutland	ENT-DNA-22	B2	New	MN913173	DANOD015-22	NHMA
Denmark	South Jutland	ENT-DNA-23	B2	New	MN913174	DANOD016-22	NHMA
Denmark	South Jutland	ENT-DNA-24	B1	New	MN913179	DANOD017-22	NHMA
Denmark	South Jutland	ENT-DNA-25	B1	New	MN913180	DANOD018-22	NHMA
Denmark	South Jutland	ENT-DNA-26	B1	New	MN913181	DANOD019-22	NHMA
Denmark	South Zealand	ENT-DNA-27	B2	New	MN913175	DANOD020-22	NHMA
Denmark	South Zealand	ENT-DNA-28	B2	New	MN913176	DANOD021-22	NHMA
Denmark	South Zealand	ENT-DNA-29	B2	New	MN913170	DANOD022-22	NHMA
Denmark	East Jutland	ENT-DNA-30	B1	New	MN913182	DANOD023-22	NHMA
Denmark	East Jutland	ENT-DNA-31	B2	New	MN913177	DANOD024-22	NHMA
Denmark	East Jutland	ENT-DNA-169	B2	New	MN913171	DANOD153-22	NHMA
Denmark	East Jutland	ENT-DNA-170	B2	New	MN913172	DANOD154-22	NHMA
Denmark	East Jutland	ENT-DNA-258	B1	New	MN913183	DANOD231-22	NHMA
Denmark	South Zealand	ENT-DNA-259	B1	New	MN913184	DANOD232-22	NHMA
Denmark	South Zealand	ENT-DNA-260	B2	New	MN913178	DANOD233-22	NHMA
Sweden	Södermanland	ENT-DNA-762	B1	New	MN913185	DANOD640-22	NHRM
Sweden	Södermanland	ENT-DNA-763	B1	New	MN913186	DANOD641-22	NHRM
Sweden	Skåne	ENT-DNA-764	B1	New	MN913187	DANOD642-22	NHMA
Sweden	Skåne	ENT-DNA-765	B1	New	MN913188	DANOD643-22	NHMA
Germany	Slesvig-Holstein	ENT-DNA-912	B1	New	MN913189	DANOD776-22	NHMA
Germany	Slesvig-Holstein	ENT-DNA-913	B1	New	MN913190	DANOD777-22	NHMA
Germany	Slesvig-Holstein	ENT-DNA-914	B1	New	MN913191	DANOD778-22	NHMA
Germany	Slesvig-Holstein	ENT-DNA-915	B1	New	MN913192	DANOD779-22	NHMA
Germany	Slesvig-Holstein	ENT-DNA-916	B1	New	MN913193	DANOD780-22	NHMA
Georgia	Batumi	ENT-DNA-917	B4	New	MN913210	DANOD781-22	NHMA
Romania	Voila	ENT-DNA-918	B1	New	MN913194	DANOD782-22	NHMA
Romania	Voila	ENT-DNA-919	B3	New	MN913207	DANOD783-22	NHMA
Romania	Voila	ENT-DNA-920	B3	New	MN913208	DANOD784-22	NHMA
Romania	Voila	ENT-DNA-921	B3	New	MN913209	DANOD785-22	NHMA
Romania	Voila	ENT-DNA-922	B6	New	MN913206	DANOD786-22	NHMA
France	Sorgues	ENT-DNA-923	B1	New	MN913195	DANOD787-22	NHMA
France	Sorgues	ENT-DNA-924	B1	New	MN913196	DANOD788-22	NHMA
France	Sorgues	ENT-DNA-925	B1	New	MN913197	DANOD789-22	NHMA
France	Sorgues	ENT-DNA-926	B1	New	MN913198	DANOD790-22	NHMA
France	Sorgues	ENT-DNA-927	B1	New	MN913199	DANOD791-22	NHMA
Bulgaria	Varna	ENT-DNA-1011	B7	New	MN913211	DANOD818-22	NHMA
Bulgaria	Varna	ENT-DNA-1013	B1	New	MN913200	DANOD819-22	NHMA
Bulgaria	Varna	ENT-DNA-1014	B1	New	MN913201	DANOD820-22	NHMA
Bulgaria	Varna	ENT-DNA-1015	B1	New	MN913202	DANOD821-22	NHMA
Romania	Vadu	ENT-DNA-1179	B1	New	MN983216	DANOD936-22	NHMA
Romania	Vadu	ENT-DNA-1180	B1	New	MN983217	DANOD937-22	NHMA
Germany	Baden-Württemberg	ENT-DNA-1222	B1	New	MN913203	DANOD949-22	NHMA
Germany	Baden-Württemberg	ENT-DNA-1223	B1	New	MN913204	DANOD950-22	NHMA
Germany	Baden-Württemberg	ENT-DNA-1224	B1	New	MN913205	DANOD951-22	NHMA
England	Kent	SAMEA9065986	B3	WSTLP	OW121859*		WSI
Austria	Vienna	Odo0016	B5	BOLD	—	AODON016-20	NMW
Germany	North Rhine-Westphalia	ZFMK-TIS-2010623	B1	Geiger et al. (2021)	MW490449	GODO023-18	ZFAK
Germany	North Rhine-Westphalia	ZFMK-TIS-2010635	B1	Geiger et al. (2021)	MW490180	GODO027-18	ZFAK

Country	Region	Voucher	Haplotype	Source	Genbank	BOLD	Deposit
Germany	North Rhine-Westphalia	ZFMK-TIS-2010638	B1	Geiger et al. (2021)	MW490514	GODO030-18	ZFAK
Netherlands	Drentsche Aa	RMNH.INS.228274	B1	Geiger et al. (2021)	KF369498	ODOPH289-13	RMNH
Poland	Wadolek Lake	ODOPL_149	B1	Geiger et al. (2021)	MW490351	PLSW015-20	UL
Poland	Wadolek Lake	ODOPL_184	B1	Geiger et al. (2021)	MW490494	PLSW050-20	UL
Italy	Taranto	MIB:ZPL:08479	A4	Galimberti et al. (2020)	MT298597	ZPLOG679-20	UMB
Italy	Trento	MIB:ZPL:08480	A1	Galimberti et al. (2020)	MW377872	ZPLOG680-20	UMB
Italy	Torino	MIB:ZPL:08481	A1	Galimberti et al. (2020)	MT298595	ZPLOG681-20	UMB
Italy	Vercelli	MIB:ZPL:08483	A1	Galimberti et al. (2020)	MT298594	ZPLOG683-20	UMB
Italy	Lecco	MIB:ZPL:08485	A2	Galimberti et al. (2020)	MT298600	ZPLOG685-20	UMB
Italy	Grosseto	MIB:ZPL:08487	A1	Galimberti et al. (2020)	MT298599	ZPLOG687-20	UMB
Italy	Campobasso	MIB:ZPL:08490	A3	Galimberti et al. (2020)	MT298602	ZPLOG690-20	UMB
Italy	Lazio	MIB:ZPL:08491	A1	Galimberti et al. (2020)	MT298601	ZPLOG691-20	UMB
Montenegro	—	MIB:ZPL:08634	B8	Galimberti et al. (2020)	MT298598	ZPLOG834-20	UMB

Table 2. Estimates of evolutionary divergence over sequence pairs within and between groups as indicated in the text based on the Kimura-2 Parameter.

	Europe	Italy
Europe	0.0015	
Italy	0.0150	0.0021

Table 3. Summary of molecular variance analysis (AMOVA). The percentage of molecular variance (%variation) is provided, together with appropriate ϕ -statistics. The statistical significance of each value is based on 1000 permutation.

Variation	df	Sigma ²	% variation	ϕ -statistics	p
Among groups	1	40.400	96.5	0.985	< 0.001
Among sample localities	13	0.836	2.0	0.566	= 0.012
Within sample localities	46	0.642	1.5	0.965	< 0.001

trimmed the dataset so that all sequences were the same length. We constructed a minimum spanning haplotype network (Blandelt et al. 1999) in PopART (Leight & Bryant 2015) following Kohli et al. (2021) and Simonsen et al. (2021).

We analyzed phylogenetic patterns in MrBayes 3.2 (Ronquist et al. 2012) using *Platycnemis acutipennis* Selys, 1841 (Genbank accession# GU644640) as outgroup. Based on the model finding function in Mega 11 (Tamura et al. 2021), we set the model for molecular evolution to HKY. The analysis was run for 50 million generations with sampling every 10,000 generations. After evaluation, the log files in Tracer v1.7.2 (part of the BEAST package: Bouckaert et al., 2019) the first 25% were discarded as burnin. The resultant tree was examined and visualised in FigTree 1.4.4 (Rambaut 2018).

2.2.3. Assessment of genetic diversity.

Based on the results from the haplotype network and phylogenetic analyses, we divided the dataset into two haplotype groups as indicated in Fig. 2 and calculated the genetic distance within and between the two groups based on the Kimura-2 parameter (K2P) (Kimura 1980) in Mega

11 (Tamura et al. 2021). The distance values are provided in Table 2. To assess genetic divergence between and within these groups and sample sites (countries or regions as indicated in Table 1), we carried out a nested AMOVA test (Excoffier et al. 1992) as implemented in PopART. The results are provided in Table 3.

3. Results

3.1. Phylogenetic and haplotype network analyses

The 61-specimen dataset of *P. pennipes* was trimmed to 618 bp COI to avoid missing or ambiguous data. The minimum spanning network analysis in PopART (Fig. 2) revealed a striking pattern with two well-separated and distinct groups. One group (haplotype group A, n = 8, four haplotypes, numbered A1–4) comprises all specimens from Italy, while the other group (haplotype group B, n = 53, eight haplotypes, numbered B1–8) (hereafter called the European group) comprises all other specimens from

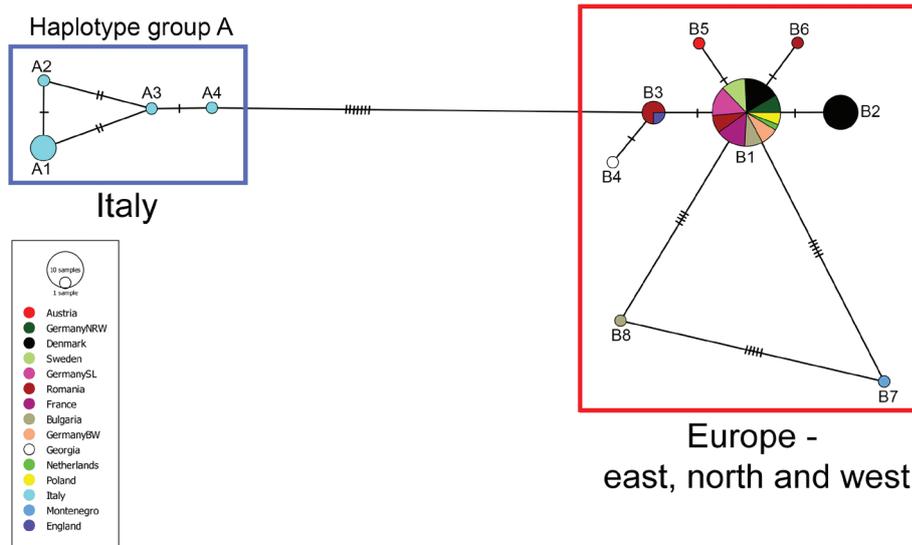


Figure 2. Minimum spanning haplotype network of *Platycnemis pennipes* COI sequences. The number of mutations between groups are indicated by bars. Groups and haplotypes are indicated as described in the text and Table 1.

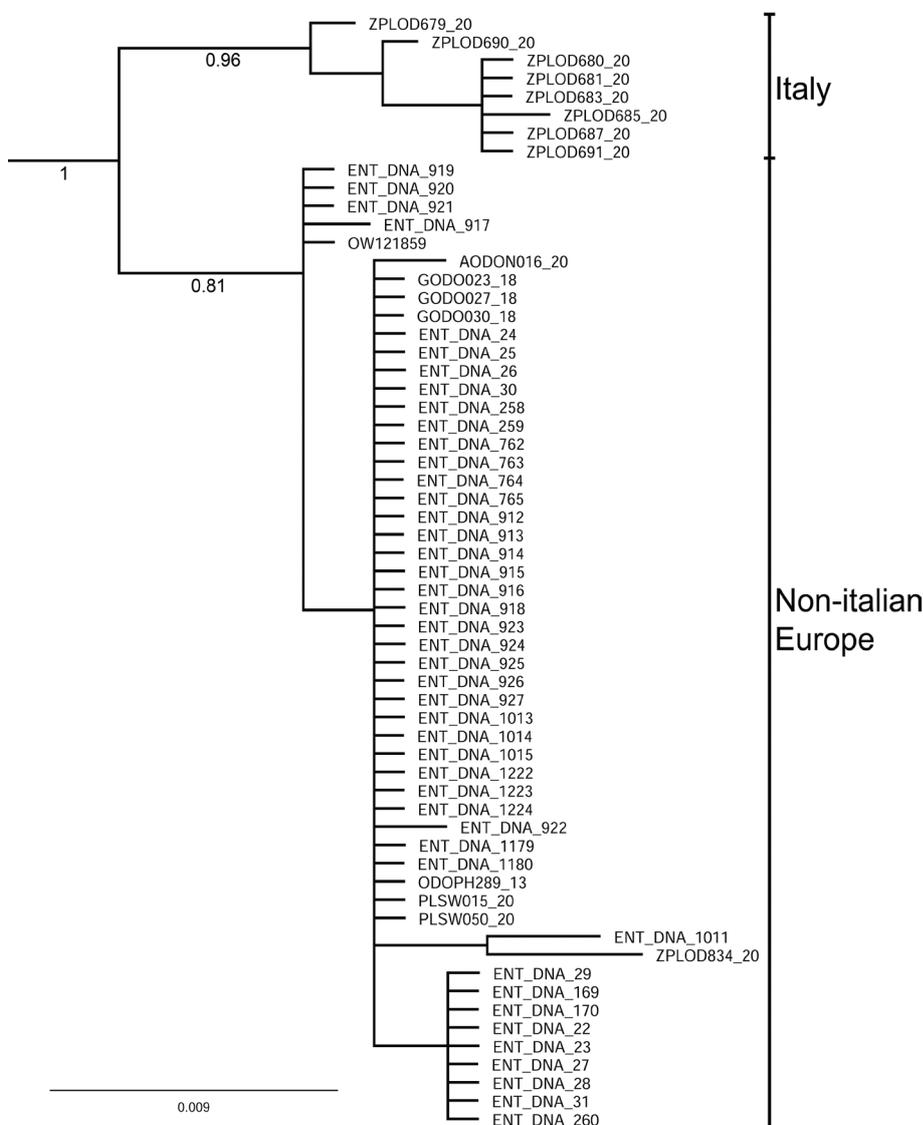


Figure 3. Tree from the 50 million generation analysis of *Platycnemis pennipes* COI sequences in MrBayes with the outgroup removed. Groups are indicated as discussed in the text. Relevant posterior probability values are shown below a branch.

Georgia in the east, to southern France and the UK in the west, and to southern Scandinavia in the north. The Italian group is overall homogenous with only a few base pair

differences between some of the specimens. The European group is somewhat more heterogenous, although the heterogeneity is skewed: most specimens form a homo-

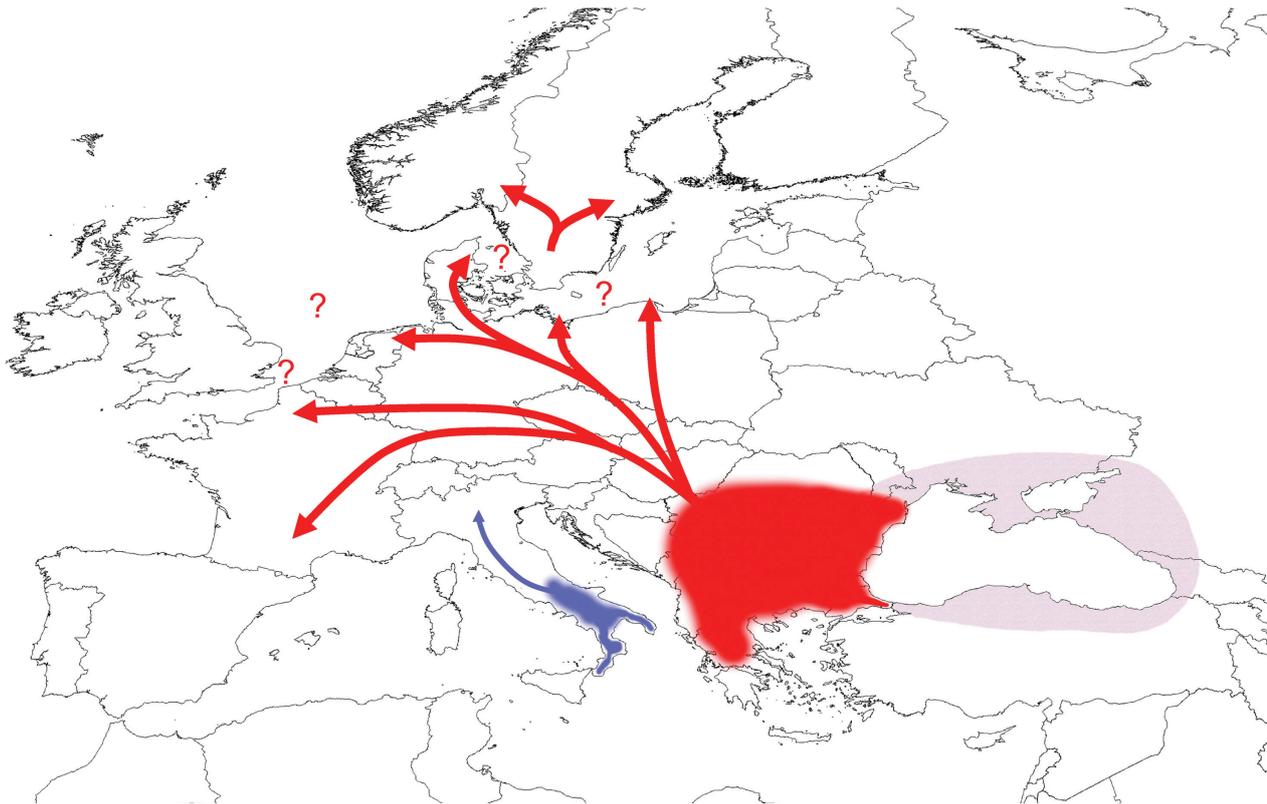


Figure 4. Potential glacial refugia and post-glacial dispersal route as discussed in the text. Blue indicates an Italian refugium and dispersal routes. Red indicates a southeastern refugium and dispersal routes. Question marks indicate uncertain dispersal routes as discussed in the text. The pink area indicates a potentially larger southeastern refugium that encircles the Black Sea.

genus group with little internal variation, one Bulgarian specimen (ENT-DNA-1011, haplotype B7) and a single specimen from Montenegro (ZPLOD834-20, haplotype B8) display considerable variation, both compared to each other and to the majority of the specimens in the group. The alignment is provided as File S1.

For the phylogenetic analysis in MrBayes, we added a COI barcode 648 pb sequence of *P. acutipennis* as out-group to the original 658 bp *P. pennipes* alignment. The phylogenetic tree (Fig. 3) displays a pattern very similar to the haplotype network. The Italian and European groups are both monophyletic although only the former receive strong support. The two outlying specimens in the European group (ENT-DNA-1011 and ZPLOD834-20) are deeply subordinate within that group but placed as sister taxa on a reasonably long branch. The alignment is provided as File S2.

3.2. Genetic diversity

The average Kimura-2 Parameter distance within each haplotype group (Table 2) is very low and ranges from 0.0015 in the European group to 0.0021 in the Italian group. The distance between the two groups is 0.015 and as such approximately an order of magnitude higher than within groups. The AMOVA-test (Table 3) shows that variation among the two major groups explains almost all the genetic variance (96.5%).

4. Discussion

Our results reveal a highly distinctive pattern with two clearly separated haplotype groups of *P. pennipes* in Europe—one restricted to Italy, and one found in the rest of the species' distribution area in Europe from Georgia to the UK and from southern France to Scandinavia. This clearly indicates that *P. pennipes* remained in at least two independent glacial refugia in Europe during the Weichsel Glaciation (Gibbard & Cohen 2008) (Fig. 4), and perhaps even before that. The genetic variation within and between the two haplotype groups in *P. pennipes* (Table 2) are much higher than in *S. vulgatum* (Hinojosa et al. 2017) or *N. speciosa* (Bernard et al. 2011), but similar to *A. cyanea* (Schneider et al. 2015; Simonsen et al. 2020), and *Orthetrum cancellatum* (Linnaeus, 1758) and *O. coeruleescens* (Fabricius, 1798) (Simonsen et al. 2021). If the rate of evolution in *P. pennipes* is comparable to that found in *A. cyanea* by Simonsen et al. (2020), the difference between the two haplotype groups in *P. pennipes* (0.015) corresponds roughly to the difference between North African and European *A. cyanea* (0.013—Simonsen et al. 2020, table 2). The split between the two latter groups was estimated to have occurred ca. 400 ky ago (Simonsen et al. 2020, fig. 3), meaning that the split between the two haplotype groups in *P. pennipes* could date back to the onset of the Saale Glaciation, rather than the onset of the Weichsel Glaciation (Gibbard & Cohen 2008).

One of these refugia was obviously in Italy as haplotype group A is restricted to that area. As the greatest variation in haplotype group B is found in the east and southeast, we infer that the second refugium was in the Balkan-Black Sea region of Europe. This pattern is directly comparable to the so-called Grasshopper Paradigm, so named because the pattern is found in the grasshopper *Pseudochorthippus parallelus* (Zetterstedt, 1821) (Hewitt 1999; 2004; Schmitt 2007). According to these authors *P. parallelus* recolonized Europe north of the Alps and the Pyrenees from a refugium in the northern Balkans with isolated populations still existing in the southern Balkans/Anatolia as well as in the Iberian Peninsula and the Italian Peninsula. *P. pennipes* does not occur in the Iberian Peninsula (Boudot et al. 2015), but the Italian population is isolated from the rest of Europe, and it does not appear to have crossed the Alps, possibly because it rarely occurs more than 1000 m above sea level (Boudot et al. 2015). Our regional sampling from the southern Balkans and Anatolia is too limited to allow us to infer the extent of the Balkan refugium for *P. pennipes* with certainty, but the presence of a specimen with a haplotype very similar to Romanian specimens in Georgia (ENT-DNA-917) could indicate that it extended to the eastern shores of the Black Sea (Fig. 4). While we cannot exclude that a single, large Balkan refugium existed during the Weichsel Glaciation, the presence of two genetically highly distinct specimens from Bulgaria (ENT-DNA-1011, haplotype A7) and Montenegro (ZPLOD834-20, haplotype A8) could indicate that several subrefugia existed in the region, possibly in the south as in *P. parallelus*. As mentioned earlier, the distinct subspecies *P. pennipes nitudula* occurs in coastal Montenegro as well as Albania and Greece. It is therefore also possible that ZPLOD834-20 represents this subspecies. However, neither the original publication (Galimberti et al. 2020) nor the record on BOLD mention subspecific status for the specimen. The low genetic variation in the European group—with the exception of the two Balkan specimens mentioned above—indicate a rapid, phalanx-like post-glacial expansion (Fig. 4) as seen in several common butterfly species (see Schmitt 2007 for a review). The low genetic variation in the European group does not allow us to assess how *P. pennipes* colonized southern Scandinavia or the British Isles (Fig. 4). To reach southern Scandinavia it may have used the area that is today Denmark as steppingstones, or it may have crossed the Baltic Sea directly, either by long-distance dispersal or at a time when the sea levels were lower. Similarly, it may have reached the British Isles via the ancient landmass Doggerland (Lambeck 1995) as hypothesized for the ghost moth *Hepialus humuli* (Linnaeus, 1758) by Simonsen & Huemer (2014), or it may have crossed the English Channel either by long-distance dispersal or reached what is today southern England before the English Channel formed 10,000-8000 yrs ago (Lambeck 1995).

In addition to *P. parallelus*, several European species show a similar post-glacial dispersal pattern, and Hewitt (2004) stated that the Balkans comprise the main refugium for many European species. The two tree species *Fagus*

sylvatica Linnaeus, 1753 and *Alnus glutinosa* (Linnaeus, 1790), and the water newt *Triturus cristatus* (Laurenti, 1768) all show a pattern with expansion across Europe from the Balkans, with populations remaining isolated in the Iberian Peninsula and the Italian Peninsula (Demesure et al. 1996; King & Ferris 1998; Wallis & Arntzen 1989; Hewitt 1999; 2004; Schmitt 2007). Ursenbacher et al. (2006) found that in the viper *Vipera berus* (Linnaeus, 1758) there is a distinct Italian refugium clearly separated from a Balkan/Northern European group, while Sztencal-Jablonka et al. (2015) found potential support for a southeastern refugium in the smooth snake *Coronella austriaca* Laurenti, 1768. In Lepidoptera, Louy et al. (2013) found a clear split between Italian and Balkan populations in the butterfly *Coenonympha rhodopensis* Elwes, 1900, while Simonsen & Huemer (2014) found deep division between Italian (and southern Austrian) and northern (including northern Austrian) populations in *H. humuli*. Simonsen & Huemer (2014) concluded that *H. humuli* likely survived the Weichsel Glaciation in peripheral alpine refugia to the south, northwest, north, and northeast of the Alps. They did, however, mention the possibility that the northeastern refugium could have extended further to the southeast.

Within Odonata several different patterns have been elucidated in the Western Palearctic region. Weekers et al. (2001) found in a study of the highly variable nuclear ITS in *C. splendens* that there was a clear gap between Italy and the rest of the Western Palearctic, indicating an isolated Italian refugium. In an AFLP study of the same species, Sadeghi et al. (2010) found indication of a southeastern refugium. The two studies thus point to a pattern similar to what we find here. Hinojosa et al. (2017) similarly found evidence for an isolated Iberian refugium, an Anatolian refugium, and a Balkan refugium in *S. vulgatum* with post-glacial expansion to the rest of Europe from the latter. In contrast to these studies, Guan et al. (2013) found in a phylogenetic study of the genus *Pyrrhosoma* with focus on the relationship of the widespread *P. nymphula* and the endangered south Balkan endemic *P. elisabethae* that *P. elisabethae* is closer related to *P. nymphula* from Morocco than either are to *P. nymphula* for the Balkans or western Europe. Schneider et al. (2015) and Simonsen et al. (2020) found a complex pattern in *A. cyanea* with sequential glacial refugia in the Caucasus, the Maghreb region in Northern Africa, and the Iberian Peninsula, all related to different glacial events in the Quaternary. Simonsen et al. (2021) found complex and admixed patterns in *O. cancellatum* and *O. coerulescens* in Europe. However, they did find evidence for post-glacial expansion from an Italian refugium in both species, as well as evidence for post-glacial expansion from a Balkan refugium in *O. cancellatum*. In *O. coerulescens* they found some evidence of contact between the Italian refugium and the Balkan region, as well as evidence for east-west Mediterranean contact. Simonsen et al. (2023) found evidence in COI for the current division of the small emerald spreadwing *Lestes virens* (Charpentier, 1825) into the subspecies *L. virens virens* and *L. virens vestalis* Rambur, 1842, probably originating

from a southwestern and a combined Italian/Balkan refugium respectively. However, they also found evidence for a separate Sicilian/Mediterranean refugium. Finally, Bernard et al. (2011), and Kohli et al. (2018; 2021) found no internal pattern in *N. speciosa*, and *A. juncea*, *A. subarctica*, *S. sahlbergi*, *L. quadrimaculata* and *S. danae*, respectively, but rather evidence for colonization of the Western Palearctic region from the east.

5. Authors' contributions

T.J.S. and K.O. designed the study, secured funding and collected material. M.D. carried out laboratory work, data mining and the initial analyses, and drafted parts of the text. O.F.N. provided information on biology and natural history, and collected material. T.J.S. carried out the bulk of the analyses and drafted the text. All authors contributed to the Discussion and the final version of the paper.

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7. Competing interests

The authors have declared that no competing interests exist.

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9. References

- Askw RR (2004) The Dragonflies of Europe (revised edition). Harley Books, Colchester. 308 pp. <https://doi.org/10.1111/j.1096-3642.2006.00265.x>
- Bandelt H, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37–48. <https://doi.org/10.1093/oxfordjournals.molbev.a026036>
- Bernard R, Heiser M, Hochkirch S, Schmitt T (2011) Genetic homogeneity of the Sedgling *Nehalennia speciosa* (Odonata: Coenagrionidae) indicates a single Würm glacial refugium and trans-Palaearctic postglacial expansion. *Journal of Zoological Systematics and Evolutionary Research* 49: 292–297. <https://doi.org/10.1111/j.1439-0469.2011.00630.x>
- Bouckaert R, Vaughan TG, Barido-Sottani J, Duchêne S, Fourment M, Gavryushkina A, Heled J et al. (2019) BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. *PLoS computational biology* 15: e1006650. <https://doi.org/10.1371/journal.pcbi.1006650>
- Boudot J-P, Rimšaitė J, Bernard R (2015) *Platycnemis pennipes*. In: Boudot J-P, Kalkman VJ (Eds) Atlas of the European Dragonflies and Damselflies. KNNV Publishing, The Netherlands. 85–86.
- Brunet BMT, Blackburn GS, Muirhead K, Lumley LM, Boyle B, Lévesque RC, Cusson M, Sperling FAH (2017) Two's company, three's a crowd: new insights on spruce budworm species boundaries using genotyping-by-sequencing in an integrative species assessment (Lepidoptera: Tortricidae). *Systematic Entomology* 42: 317–328. <https://doi.org/10.1111/syen.12211>
- de Lattin G (1967) Grundriss der Zoogeographie. Verlag Gustav Fischer, Stuttgart, Germany. 602 pp.
- Demesure B, Comps B, Petit RJ (1996) Chloroplast DNA phylogeography of the common beech (*Fagus sylvatica* L.) in Europe. *Evolution* 50: 2515–2520.
- Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 479–491.
- Galimberti A, Assandri G, Maggioni D, Ramazzotti F, Baroni D, Bazzi G, ... Casiraghi M (2021) Italian odonates in the Pandora's box: A comprehensive DNA barcoding inventory shows taxonomic warnings at the Holarctic scale. *Molecular Ecology Resources* 21: 183–200. <https://doi.org/10.1111/1755-0998.13235>
- Geiger MF, Kolbmüller S, Assandri G, Chovanec A, Ekrem T, Fischer I, ... Moriniere J (2021) Coverage and quality of DNA barcode references for Central and Northern Europe Odonata. *PeerJ* 9: e11192. <https://doi.org/10.7717/peerj.11192>
- Gratton P, Konopiński MK, Sbordoni V (2008) Pleistocene evolutionary history of the Clouded Apollo (*Parnassius mnemosyne*): genetic signatures of climate cycles and a 'time-dependent' mitochondrial substitution rate. *Molecular Ecology* 17: 4248–4262. <https://doi.org/10.1111/j.1365-294X.2008.03901.x>
- Gibbard P, Cohen KM (2008) Global chronostratigraphical correlation table for the last 2.7 million years. *Episodes* 31: 243–247. <https://doi.org/10.18814/epiiugs/2008/v31i2/011>
- Guan Z, Dumont HJ, Yu X, Han B-P, Vierstraete A (2013) *Pyrrhosoma* and its relatives: a phylogenetic study (Odonata: Zygoptera). *International Journal of Odonatology* 16: 247–257. <https://doi.org/10.180/13887890.2013.821358>
- Hammouti N, Schmitt T, Seitz A, Kousch J, Veith M (2010) Combining mitochondrial and nuclear evidences: a refined evolutionary history of *Erebia medusa* (Lepidoptera: Nymphalidae: Satyrinae) in Central Europe based on the COI gene. *Journal of Zoological Systematics and Evolutionary Research* 48: 115–125. <https://doi.org/10.1111/j.1439-0469.2009.00544.x>
- Hewitt GM (1999) Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society* 68: 87–112. <https://doi.org/10.1111/j.1095-8312.1999.tb01160.x>
- Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences* 359: 183–195. <https://doi.org/10.1098/rstb.2003.1388>

- Hinojosa JC, Martin R, Maynou X, Vila R (2017) Molecular taxonomy of the *Sympetrum vulgatum* (Odonata: Libellulidae) complex in the West Palaearctic. *European Journal of Entomology* 114: 373–378. <https://doi.org/10.14411/eje.2017.048>
- Husemann M, Schmitt T, Zachos FE, Uirich W, Habel JC (2014) Palaearctic biogeography revisited: evidence for the existence of a North African refugium for Western Palaearctic biota. *Journal of Biogeography* 41: 81–94. <https://doi.org/10.1111/jbi.12180>
- King RA, Ferris C (1998) Chloroplast DNA phylogeography of *Alnus glutinosa* (L.) Gaertn. *Molecular Ecology* 7: 1157–1161.
- Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16:111–120.
- Kodandaramaiah U, Simonsen TJ, Bromilow S, Wahlberg N, Sperling FAH (2013) Deceptive single-locus taxonomy and phylogeography: *Wolbachia* mediated discordance between morphology, mitochondria and nuclear markers in a butterfly species. *Ecology and Evolution* 3: 5167–5176. <https://doi.org/10.1002/ece3.886>
- Kohli M, Sahlén G, Kuhn WR, Ware JL (2018) Extremely low genetic diversity in a circumpolar dragonfly species, *Somatochlora sahlbergi* (Insecta: Odonata: Anisoptera). *Scientific Reports* 8: 15114. <https://doi.org/10.1038/s41598-018-32365-7>
- Kohli M, Djernæs M, Sanchez Herrera M, Sahlén G, Pilgrim E, Simonsen TJ, Olsen K, Ware J (2021) Comparative phylogeography uncovers evolutionary past of Holarctic dragonflies. *PeerJ* 9: e11338. <https://doi.org/10.7717/peerj.11>
- Krosch MN, Cranston PS (2012) Non-destructive DNA extraction from Chironomidae, including of fragile pupal exuviae, extends analysable collections and enhances vouchering. *CHIRONOMUS Newsletter on Chironomidae Research* 25: 22–27. <https://doi.org/10.5324/cjcr.v0i25.1532>
- Lambeck K (1995) Late Devensian and Holocene shorelines of the British Isles and North-Sea from models of glacio-hydro-isostatic rebound. *Journal of the Geological Society* 152: 437–448.
- Leigh JW, Bryant D (2015) POPART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* 6: 1110–1116. <https://doi.org/10.1111/2041-210x.12410>
- Louy D, Habel JC, Abadjiev S, Schmitt T (2013) Genetic legacy from past panmixia: high genetic variability and low differentiation in disjunct populations of the Eastern Large Heath butterfly. *Biological Journal of the Linnean Society* 110: 281–290. <https://doi.org/10.1111/bij.12144>
- Maddison WP, Maddison DR (2015) Mesquite: A modular system for evolutionary analysis. Version 3.03. <http://mesquiteproject.org>
- Nielsen OF (1998) *De Danske Guldsmede*. Danmarks Dyreliv 8. Steenstrup, Denmark. 280 pp.
- Rambaut A (2018) FigTree Version 1.4.4. University of Edinburgh, Edinburgh. <http://tree.bio.ed.ac.uk/software/figtree>
- Roe A, Dupuis J, Sperling FAH (2017) Molecular dimensions of insect taxonomy in the genomics era. Pp. 547–573 in: *Insect Biodiversity: Science and Society* Vol. 1, 2nd ed (eds R. G. Footitt, P. H. Adler). John Wiley & Sons, Ltd, Chichester, UK. <https://doi.org/10.1002/9781118945568.ch16>
- Ronquist F, Huelsenbeck J, Teslenko M (2012) Draft MrBayes version 3.2 Manual: Tutorials and Model Summaries. Online publication available from the authors.
- Sadeghi S, Kyndt T, Dumont HJ (2010) Genetic diversity, population structure and taxonomy of *Calopteryx splendens* (Odonata: Calopterygidae). *European Journal of Entomology* 107: 137–146. <https://doi.org/10.14411/eje.2010.019>
- Schmitt T (2007) Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. *Frontiers in Zoology* 4: 11. <https://doi.org/10.1186/1742-9994-4-11>
- Schmitt T, Rakosy L, Abadjiev S, Mueller P (2007) Multiple differentiation centres of a non-Mediterranean butterfly species in south-eastern Europe. *Journal of Biogeography* 34: 939–950. <https://doi.org/10.1111/j.1365-2699.2006.01684.x>
- Schneider T, Schneider E, Schneider J, Vierstraete A, Dumont HJ (2015) *Aeshna vercanica* sp. nov. from Iran with a new insight into the *Aeshna cyanea* group (Odonata: Aeshnidae). *Odonatologica* 44: 81–106. <http://hdl.handle.net/1854/LU-7225496>
- Simonsen TJ, Huemer P (2014) Phylogeography of *Hepialus humuli* (L.) (Lepidoptera: Hepialidae) in Europe: short distance vs. large scale postglacial expansions from multiple Alpine refugia and taxonomic implications. *Insect Systematics and Evolution* 45: 209–249. <https://doi.org/10.1163/1876312X-44032104>
- Simonsen TJ, Olsen K, Djernæs M (2020) The African-Iberian connection in Odonata: mtDNA and ncDNA based phylogeography of *Aeshna cyanea* (Müller, 1764) (Odonata: Aeshnidae) in Western Palaearctic. *Arthropod Systematics and Phylogeny* 78: 309–320. <https://doi.org/10.26049/ASP78-2-2020-06>
- Simonsen TJ, Djernæs M, Nielsen OF, Olsen K (2021) A tale of two Skimmers: complex relationships between DNA barcoding, distributions and taxonomy in European *Orthetrum cancellatum* and *O. coerulescens*. *International Journal of Odonatology* 24: 316–331. https://doi.org/10.23797/2159-6719_24_23
- Simonsen TJ, Djernæs M, Nielsen OF, Olsen K, (2023) COI diversity supports subspecific diversion in Western European *Lestes virens* (Zygoptera: Lestidae), but hints at further Mediterranean complexity. *International Journal of Odonatology* 26: 18–26. <https://doi.org/10.48156/1388.2023.1917196>
- Sztencel-Jablonka A, Mazgajski TD, Bury S, Najbar B, Rybacki M, Bogdanowicz W, Mazgajska J (2015) Phylogeography of the smooth snake *Coronella austriaca* (Serpentes: Colubridae): evidence for a reduced gene pool and a genetic discontinuity in Central Europe. *Biological Journal of the Linnean Society* 115: 195–210. <https://doi.org/10.1111/bij.12496>
- Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution* 38: 3022–3027. <https://doi.org/10.1093/molbev/msab120>
- Ursenbacher S, Carlsson M, Helfer V, Tegelstrom H, Fumagalli L (2006) Phylogeography and Pleistocene refugia of the adder (*Vipera berus*) as inferred from mitochondrial DNA sequence data. *Molecular Ecology* 15: 3425–3439. <https://doi.org/10.1111/j.1365-294X.2006.03031.x>
- Varga ZS, Schmitt T (2008) Types of orcal and oreotundral disjunctions in the western Palearctic. *Biological Journal of the Linnean Society* 93: 415–430. <https://doi.org/10.1111/j.1095-8312.2007.00934.x>
- Wallis GP, Arntzen JW (1989) Mitochondrial-DNA variation in the crested newt superspecies: limited cytoplasmic gene flow among species. *Evolution* 43: 88–104.
- Weekers PHH, de Jonckheere J, Dumont H (2001) Phylogenetic relationships inferred from ribosomal ITS sequences and biogeographical patterns in representatives of the genus *Calopteryx* (Insecta: Odonata) of the West Mediterranean and adjacent West European zone. *Molecular Phylogenetics and Evolution* 20: 89–99. <https://doi.org/10.1006/mpev.2001.0947>

Supplementary Material 1

File S1

Authors: Simonsen TJ, Djernæs M, Nielsen OF, Olsen K (2023)

Data type: .nex

Explanation note: DNA alignment as a NEXUS file used for the PopART analyses.

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Supplementary Material 2

File S2

Authors: Simonsen TJ, Djernæs M, Nielsen OF, Olsen K (2023)

Data type: .nex

Explanation note: DNA alignment as a NEXUS file used for the MrBayes analysis.

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