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A tale of two Skimmers: complex relationships between DNA barcodes, distributions and taxonomy in European *Orthetrum cancellatum* and *O. coerulescens*

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Abstract: We explore the genetic diversity and phylogeography of the dragonflies *Orthetrum cancellatum* and *O. coerulescens* in Europe based on mitochondrial and nuclear DNA. *Orthetrum cancellatum* has a clear division between a group comprising Maltese, Italian, and central and northern European populations, and a group comprising mainly populations from southwestern and southeastern Europe, as well as some northern European specimens. We propose that the two groups represent two different Glacial refugia, one in the Italian Peninsula and one in the Balkans where the species survived during the Weichsel Glaciation. *Orthetrum coerulescens* shows a more complex pattern, although it too can be divided into two groups. One group comprise all the specimens we have identified as *O. coerulescens anceps* from their phenotype as well as specimens from Spain, Montenegro, and Pakistan, and some specimens from Italiy, Poland and Bulgaria. The other group comprise all other specimens from central and northern Europe, almost all specimens from Italy and Bulgaria, and all specimens from Malta. We propose that the latter group represents an Italian Glacial refugium from which the species spread to both central Europe, Malta and southern Balkan (Bulgaria) after the end of the Weichsel Glaciation. As specimens from Spain and Bulgaria, which were identified as *O. coerulescens coerulescens* group with specimens identified as *O. coerulescens anceps* we conclude that the two subspecies mix more or less freely across the Mediterranean and question the validity of two subspecies.

Introduction

Orthetrum Newman 1833 is a large genus of medium sized dragonflies, comprising more than 60 species distributed throughout the Old World and Australia; from Australia and East Asia to Africa and Western Europe (Silsby, 2001; Askew, 2004; Yong et al., 2014). The genus is closely associated with the genera *Libellula* Linnaeus 1758, *Ladona* Needham 1897; and *Plathemis* Hagen 1861 from which it can be distinguished by differences in wing morphology (Askew, 2004; Ware et al., 2007). Compared to the other genera, adult *Orthetrum* specimens have a narrow abdomen. Males of several—but not all—species have a pruinose pale-blue abdomen, which in combination with the narrow appearance has given rise to the genus' vernacular name in several European languages: e.g. "Blaupfeil" (German) and "Blåpil" (Danish) both meaning "blue arrow". The European *Orthetrum* fauna is rather poor compared to the Asian and African faunas as it comprises only seven species, with a further three species occurring in the adjacent Western Palaearctic areas Asia Minor and North Africa (Askew, 2004; Boudot & Kalkman, 2015). Among the European species, only *O. cancellatum* Linnaeus 1758 and *O. coerulescens* Fabricius 1798 occur as far north as the British Isles and southern Scandinavia (Figure 1-2), although *O. brunneum* Fonscolombe 1834 is likely to expand into these northern regions in the near future (Kalkman & Ambrus, 2015a). Both species have responded to rising temperatures by expanding their northern range over the past decades (Kalkman & Ambrus, 2015b; c). However, *O. coerulescens* has expanded its range much further than *O. cancellatum*. Denmark lies at the north-western range limit of both species, but *O. cancellatum* has been common in the country since records began, while *O. coerulescens* was registered at a few localities in the 1930s (Nielsen, 1998), but then disappeared from Denmark before recolonising the country in the early 2000s where it is now secure and stable in the western part. Both species have extended their range further north on the British Isles and in southern Scandinavia since 2000 (Kalkman & Ambrus, 2015b; c). This northwards expansion is part of a larger trend, which also include *O. brunneum*,

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where European species spread northward as the global temperature rises (Boudot & Kalkman, 2015; Kalkman & Ambrus, 2015a; b; c).

Ecologically, *O. cancellatum* and *O. coerulescens* utilise different habitats in northern Europe. *Orthetrum cancellatum* is an opportunist that occurs in most standing and slow-flowing fresh water and even brackish water in the Baltic Sea at the north-eastern limit of its range (Kalkman & Ambrus, 2015c). It is also considered a pioneer species, as it is among the first Odonata species to colonize newly established waterholes. *Orthetrum coerulescens,* on the other hand, is very much a habitat specialist of smaller streams and ditches including cold springs, preferably without too much edge vegetation and water plants. It is apparently also less tolerant of salt or pollution than *O. cancellatum* (Kalkman & Ambrus, 2015b; Nielsen, 1998). These differences in ecology reflect the fact that the two species are likely not closely related within the genus, and our preliminary analyses indeed indicate that both are closer related to other Palaearctic species (Supplementary Material S2–S5).

Orthetrum cancellatum is considered to belong to the same subspecies throughout its range without any taxonomic questions pending. In contrast, the situation is more complex for *O. coerulescens*, which is currently split into the two subspecies: *O. c. coerulescens* and *O. c. anceps* (Schneider 1845), the latter is found in North Africa, the southern Balkans and parts of Asia (Kalkman & Ambrus, 2015b). The latter form has previously been considered a separate species (e.g. Askew, 2004), *O. anceps*. Intermediate forms of the two subspecies occur in a broad zone in Iberia, Sicily and large parts of south-eastern Europe (Dijkstra & Lewington, 2006; Klingenberger & Martens, 1996; Mauersberger, 1994). Kalkman & Ambrus (2015b) therefore suggested that further molecular studies were needed to determine the taxonomic rank of the two forms.

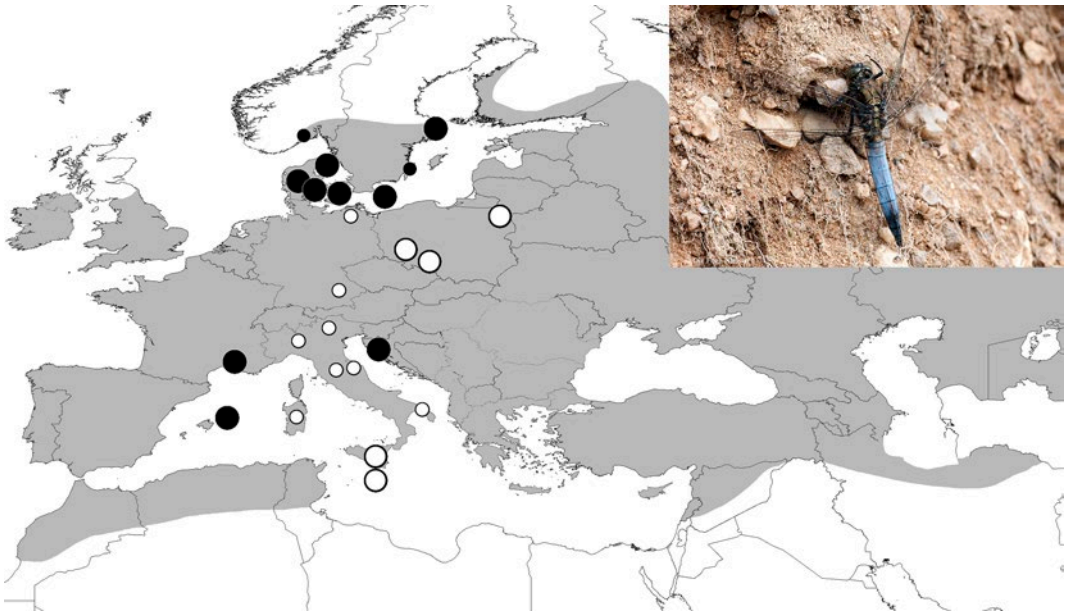

Figure 1. Western Palaearctic geographic range and sampling sites for *Orthetrum cancellatum* (photo). Overall distribution is shown in grey (based on Boudot & Kalkman 2015). Open circles indicate the approximate sampling sites for the specimens in Genbank and BOLD, while filled circles indicate our sampling sites. Small circles indicate a single specimen, while large circles indicate several specimens.

The two species' well documented and very different recent distribution history in Europe combined with their differences in ecology and habitat choice, makes them ideal to explore as to whether differences in ecology and current distribution are reflected in their historical biogeography. Or, on the contrary, whether their similar responses to recent climate change are reflected in their historical biogeography, and thus indicate similar responses to past climate changes as well. Finally, a molecular study of *O. coerulescens* in Europe could also explore the taxonomic questions related to this species. More specifically, it will be possible to address the taxonomic status of the eastern Mediterranean *O. c. anceps* populations. To address these questions we sequence the mitochon-

drial COI gene and the nuclear Internal Transcribed Spacer region (ITS-region), which comprises the genes ITS1, 5.8S, and ITS2, from a number of specimens from both species. We combine the obtained sequences with others available in public databases. Recently, two major Odonata DNA barcode studies with a strong Mediterranean focus have been published: Galimberti et al. (2020) on the Italian fauna, and Rewicz et al. (2020) on the Maltese fauna, making available a number of relevant COI sequences from geographical hotspots such as Sicily, Sardinia and Malta. Additionally, Geiger et al.'s (2021) study on central European Odonata have made available a number of more northern sequences. While the usage of single genes, and especially mitochondrial genes, in phylogeography and species delimitation studies has been criticised recently (e.g. Dupuis et al., 2012; Kodandaramaiah et al., 2013; Brunet et al., 2017; Roe et al., 2017; Simonsen et al., 2019), other studies have demonstrated their usefulness in Odonata (e.g. Bernard et al., 2011; Schneider et al., 2015; Hinojosa et al., 2017; Kohli et al., 2018, 2021; Simonsen et al., 2020). Furthermore, using the COI barcode gene allow us to combine our new data with the extensive recent DNA barcode data listed above, thereby considerably increasing the geographical sampling in the study. We use the combined results to analyse the molecular diversity and historical phylogeography of both species. We compare the patterns recovered for *O. cancellatum* and *O. coerulescens* to each other, and to recently published results for other Western Palaearctic Odonata such as *Nehalennia speciosa* Carpentier 1840*, Aeshna cyanea* Müller, 1764*, Somatochlora sahlbergi* Trybom 1889, and *Sympetrum vulgatum* (Linneaus 1758).

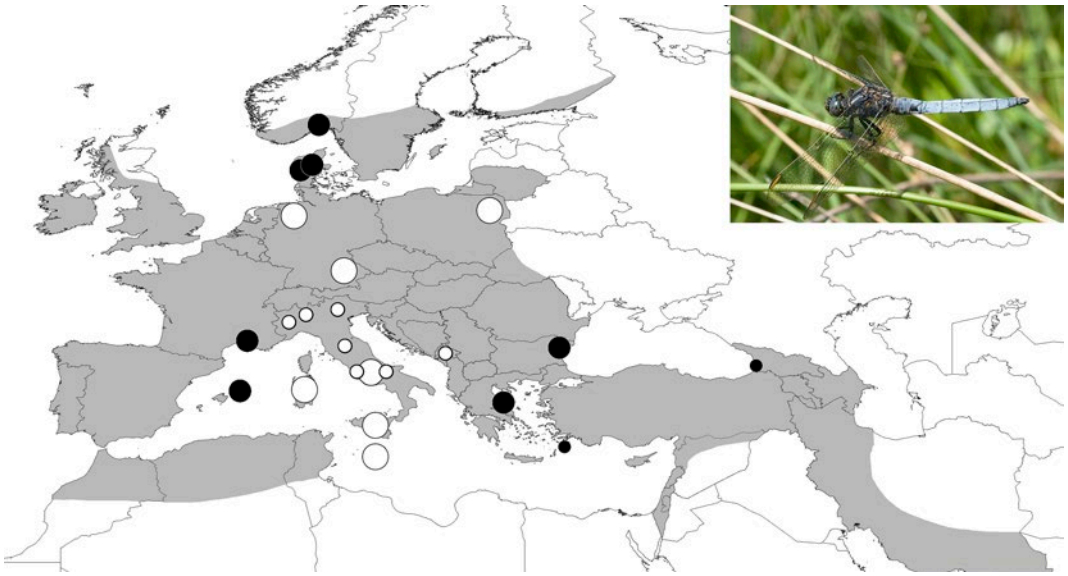

Figure 2. Western Palaearctic geographic range (samples from Pakistan not shown) and sampling sites for *Orthetrum coerulescens* (photo). Overall distribution shown in grey (based on Boudot & Kalkman 2015). Open circles indicate the approximate sampling sites for the specimens in Genbank and BOLD, while filled circles indicate our sampling sites. Small circles indicate a single specimen, while large circles indicate several specimens.

Materials and methods

Taxon and gene sampling

We sampled 36 specimens of *O. cancellatum* and 37 specimens of *O. coerulescens* from Europe, and one further specimen of *O. coerulescens* from Caucasus (Georgia). While most specimens were collected in the field for the study, specimens from Norway, Bulgaria, Greece and Sweden were older collection specimens. The single specimen from Georgia and the four specimens from Greece were all identified as *O. coerulescens anceps*, all other specimens were identified as *O. coerulescens coerulescens*. The *O. cancellatum* dataset was augmented with Genbank and BOLD sequences from 22

specimens from Europe, while the *O. coerulescens* dataset was augmented with GenBank and BOLD sequences from 43 specimens from Europe and Pakistan. We were not able to determine the subspecific status of any samples from Genbank or BOLD. The full *O. cancellatum* dataset thus comprise 58 samples, while the full *O. coerulescens* dataset comprise 82 samples. All specimen data is given in Table 1 for *O. cancellatum* and Table 2 for *O. coerulescens*.

Table 1. *Orthetrum cancellatum* specimens used in this study with localities, voucher designations, Genbank accession numbers, and voucher deposits for newly sequenced specimens provided. *= group 1 in Kimura 2 distance analyses (MEGA), all other specimens were group 2. KMO: Kjell Mange Olsen collection; NHMA: Natural History Museum Aarhus; NHRM: Swedish Museum of Natural History.

Country	Region	Voucher# (this study)	Ref	Genbank#/ BOLD# COI	Genbank# ITS	Voucher deposit
Denmark*	EJ	ENT-DNA-427	new	MN959414	MN963717	NHMA
Denmark	EJ	ENT-DNA-428	new	MN959427	MN963727	NHMA
Denmark	NWJ	ENT-DNA-500	new	MN959439	MN963745	NHMA
Denmark	NEJ	ENT-DNA-501	new	MN959430	MN963721	NHMA
Denmark	EJ	ENT-DNA-502	new	MN959440	MN963731	NHMA
Denmark	B	ENT-DNA-466	new	MN959437	MN963728	NHMA
Denmark	EJ	ENT-DNA-467	new	MN959428	MN963751	NHMA
Denmark	NEZ	ENT-DNA-468	new	MN959446	MN963729	NHMA
Denmark	NEZ	ENT-DNA-469	new	MN959431	MN963743	NHMA
Denmark	NEZ	ENT-DNA-470	new	MN959432	MN963730	NHMA
Denmark	B	ENT-DNA-471	new	MN959438	MN963718	NHMA
Denmark	F	ENT-DNA-472	new	MN959433	MN963744	NHMA
Denmark	F	ENT-DNA-473	new	MN959429	MN963723	NHMA
Denmark	WJ	ENT-DNA-504	new	MN959441	MN963746	NHMA
Denmark	WJ	ENT-DNA-505	new	MN959434	MN963732	NHMA
Denmark	SZ	ENT-DNA-506	new	MN959442	MN963733	NHMA
Denmark	SZ	ENT-DNA-507	new	MN959447	MN963734	NHMA
Norway	Bamble	ENT-DNA-712	new	MN959449	MN963726	KMO
Croatia*	Cres	ENT-DNA-905	new	MN959423	MN963735	NHMA
Croatia*	Cres	ENT-DNA-906	new	MN959426	MN963736	NHMA
Croatia*	Cres	ENT-DNA-907	new	MN959422	MN963749	NHMA
Sweden	Öland	ENT-DNA-931	new	MN959443	MN963719	NHRM
Sweden	Uppland	ENT-DNA-932	new	MN959444	MN963720	NHRM
Sweden	Söder-manland	ENT-DNA-933	new	MN959445	MN963722	NHRM
Spain*	Menorca	ENT-DNA-934	new	MN959416	MN963737	NHMA
Spain*	Menorca	ENT-DNA-935	new	MN959417	MN963738	NHMA
Spain*	Menorca	ENT-DNA-936	new	MN959418	MN963739	NHMA
France*	France	ENT-DNA-937	new	MN959419	MN963748	NHMA
France	France	ENT-DNA-938	new	MN959435	MN963750	NHMA
France*	Bouches-du-Rhône	ENT-DNA-939	new	MN959415	MN963740	NHMA

Country	Region	Voucher# (this study)	Ref	Genbank#/ BOLD# COI	Genbank# ITS	Voucher deposit
France	Bouches-du-Rhône	ENT-DNA-940	new	MN959448	MN963747	NHMA
France	Bouches-du-Rhône	ENT-DNA-941	new	MN959436	MN963741	NHMA
France*	Bouches-du-Rhône	ENT-DNA-942	new	MN959420	MN963742	NHMA
France*	Bouches-du-Rhône	ENT-DNA-943	new	MN959421	MN963724	NHMA
Croatia*	Cres	ENT-DNA-944	new	MN959424	MN963725	NHMA
Croatia*	Cres	ENT-DNA-945	new	MN959425	MN963752	NHMA
Germany	Bavaria		BOLD	FBAQU505-10	-	-
Germany	Mecklnb-Vorpom.		Geiger et al. (2021)	GODO038-18	-	-
Malta	Malta Island		Rewicz et al. (2020)	MTODO027-19	-	-
Malta	Malta Island		Rewicz et al. (2020)	MTODO028-19	-	-
Malta	Malta Island		Rewicz et al. (2020)	MTODO029-19	-	-
Malta	Malta Island		Rewicz et al. (2020)	MTODO030-19	-	-
Poland	Zdzieszo-wice		Geiger et al. (2021)	ODOPL051-19	-	-
Poland	Zdzieszo-wice		Geiger et al. (2021)	ODOPL052-19	-	-
Poland*	Barycz		Geiger et al. (2021)	ODOPL127-19	-	-
Poland	Barycz		Geiger et al. (2021)	ODOPL128-19	-	-
Poland*	Suwalki		Geiger et al. (2021)	PLSW027-20	-	-
Poland*	Suwalki		Geiger et al. (2021)	PLSW029-20	-	-
Poland*	Suwalki		Geiger et al. (2021)	PLSW028-20	-	-
Italy	Sardinia		Galimberti et al. (2021)	ZPLOD613-20	-	-
Italy	Marches		Galimberti et al. (2021)	ZPLOD614-20	-	-
Italy*	Trento		Galimberti et al. (2021)	ZPLOD616-20	-	-
Italy	Perugia		Galimberti et al. (2021)	ZPLOD618-20	-	-
Italy	Sicily		Galimberti et al. (2021)	ZPLOD619-20	-	-
Italy	Apulia		Galimberti et al. (2021)	ZPLOD620-20	-	-

Country	Region	Voucher# (this study)	Ref	Genbank#/ BOLD# COI	Genbank# ITS	Voucher deposit
Italy	Lombardy		Galimberti et al. (2021)	ZPLOD623-20	-	-
Italy	Sicily		Galimberti et al. (2021)	ZPLOD625-20	-	-
Montenegro			Galimberti et al. (2021)	ZPLOD832-20	-	-

Table 2. *Orthetrum coerulescens* specimens used in this study with localities, voucher designations, Genbank accession numbers, and voucher deposits for newly sequenced specimens provided. †= identified as *O. coerulescens anceps*. *= group 1 in K2 distance analysis in MEGA, all other specimens were group 2.

Country	Region	Voucher#	Ref	Genbank#/ BOLD# COI	Genbank# ITS	Voucher deposit
Denmark	WJ	ENT-DNA-378	new	MN957911	MN963695	NHMA
Denmark	EJ	ENT-DNA-451	new	MN957912	-	NHMA
Denmark	EJ	ENT-DNA-452	new	MN957913	MN963702	NHMA
Denmark	WJ	ENT-DNA-508	new	MN957914	MN963707	NHMA
Denmark	WJ	ENT-DNA-509	new	MN957915	MN963701	NHMA
Denmark	WJ	ENT-DNA-510	new	MN957923	MN963696	NHMA
Denmark	WJ	ENT-DNA-511	new	MN957916	MN963700	NHMA
Denmark	WJ	ENT-DNA-512	new	MN957919	-	NHMA
Denmark	WJ	ENT-DNA-513	new	MN957920	MN963697	NHMA
Denmark	WJ	ENT-DNA-514	new	MN957921	MN963703	NHMA
Denmark	EJ	ENT-DNA-515	new	MN957917	MN963704	NHMA
Denmark	WJ	ENT-DNA-516	new	MN957918	-	NHMA
Denmark	WJ	ENT-DNA-675	new	MN957924	MN963699	NHMA
Denmark	WJ	ENT-DNA-676	new	MN957922	MN963708	NHMA
Denmark	WJ	ENT-DNA-677	new	MN957935	MN963706	NHMA
Norway	Skien	ENT-DNA-710	new	MN957937	-	KMO
Norway	Dragendal	ENT-DNA-711	new	MN957928	MN963709	KMO
France	Bouches-du-Rhône	ENT-DNA-900	new	MN957929	-	NHMA
France	Bouches-du-Rhône	ENT-DNA-901	new	MN957925	-	NHMA
France	Bouches-du-Rhône	ENT-DNA-902	new	MN957926	MN963710	NHMA
France	Bouches-du-Rhône	ENT-DNA-903	new	MN957927	MN963698	NHMA
France	Bouches-du-Rhône	ENT-DNA-904	new	MN957930	MN963705	NHMA
Georgia*	Batumi	ENT-DNA-946	new	MN957938	MN963716	NHMA
Spain*	Menorca	ENT-DNA-967	new	MN957940	MN963711	NHMA
Spain*	Menorca	ENT-DNA-968	new	MN957945	MN963715	NHMA
Spain*	Menorca	ENT-DNA-969	new	MN957941	MN963714	NHMA
Spain*	Menorca	ENT-DNA-970	new	MN957942	MN963712	NHMA
Spain*	Menorca	ENT-DNA-971	new	MN957943	MN963713	NHMA

Country	Region	Voucher#	Ref	Genbank#/ BOLD# COI	Genbank# ITS	Voucher deposit
Greece*	Skiathos	ENT-DNA-977	new	MN957946	-	NHMA
Greece*	Skiathos	ENT-DNA-978	new	MN957947	-	NHMA
Greece*	Rhodos	ENT-DNA-979	new	MN957948	-	NHMA
Greece*	Skiathos	ENT-DNA-1001	new	MN957939	-	NHMA
Bulgaria*	Varna	ENT-DNA-1018	new	MN957944	-	NHMA
Bulgaria	Varna	ENT-DNA-1019	new	MN957931	-	NHMA
Bulgaria	Varna	ENT-DNA-1020	new	MN957932	-	NHMA
Bulgaria	Varna	ENT-DNA-1021	new	MN957933	-	NHMA
Bulgaria	Varna	ENT-DNA-1022	new	MN957934	-	NHMA
Denmark	WJ	ENT-DNA-1202	new	MN957936	-	NHMA
Germany	Bavaria		BOLD	FBAQU1439-13	-	-
Germany	Bavaria		BOLD	FBAQU1440-13	-	-
Germany	Bavaria		BOLD	FBAQU1442-13	-	-
Germany	Bavaria		BOLD	FBAQU320-09	-	-
Germany	Bavaria		BOLD	FBAQU506-10	-	-
Germany	Niedersachsen		Bergmann et al. 2013	KC912266	-	-
Germany	Niedersachsen		Bergmann et al. 2013	KC912267	-	-
Germany	Niedersachsen		Bergmann et al. 2013	KC912268	-	-
Germany	Niedersachsen		Bergmann et al. 2013	KC912269	-	-
Germany	Niedersachsen		Bergmann et al. 2013	KC912270	-	-
Germany	Niedersachsen		Bergmann et al. 2013	KC912271	-	-
Norway	Hordaland		BOLD	ZMBN329-16	-	-
Italy	Pontecorvo		Bergmann et al. 2013	KC912263	-	-
Italy	Pontecorvo		Bergmann et al. 2013	KC912264	-	-
Italy	Pontecorvo		Bergmann et al. 2013	KC912265	-	-
Germany	Barvaria		BOLD	GBEPT937-14	-	-
Germany	Barvaria		BOLD	GBEPT941-14	-	-
Germany	Barvaria		BOLD	GBODO033-18	-	-
Germany	Barvaria		BOLD	GBODO057-18	-	-
Pakistan*	Faisalabad		BOLD	MAODO020-10	-	-
Pakistan*	Rawalkot		BOLD	MAODO264-11	-	-
Pakistan*	Rawalkot		BOLD	MAODO265-11	-	-
Pakistan*	Rawalkot		BOLD	MAODO266-11	-	-
Malta	Malta Island		Rewicz et al. (2020)	MTODO025-19	-	-
Malta	Malta Island		Rewicz et al. (2020)	MTODO034-19	-	-
Malta	Gozo Island		Rewicz et al. (2020)	MTODO061-19	-	-
Poland*	Suwalki		Geiger et al. (2021)	PLSW047-20	-	-

Country	Region	Voucher#	Ref	Genbank#/ BOLD# COI	Genbank# ITS	Voucher deposit
Poland	Suwalki		Geiger et al. (2021)	PLSW048-20	-	-
Poland	Suwalki		Geiger et al. (2021)	PLSW049-20	-	-
Italy	Sicily		Galimberti et al. (2021)	ZPLOD626-20	-	-
Italy	Sicily		Galimberti et al. (2021)	ZPLOD627-20	-	-
Italy	Sicily		Galimberti et al. (2021)	ZPLOD628-20	-	-
Italy	Sicily		Galimberti et al. (2021)	ZPLOD632-20	-	-
Italy*	Trento		Galimberti et al. (2021)	ZPLOD639-20	-	-
Italy	Torino		Galimberti et al. (2021)	ZPLOD640-20	-	-
Italy	Perugia		Galimberti et al. (2021)	ZPLOD642-20	-	-
Italy	Sicily		Galimberti et al. (2021)	ZPLOD644-20	-	-
Italy	Campobasso		Galimberti et al. (2021)	ZPLOD648-20	-	-
Italy	Como		Galimberti et al. (2021)	ZPLOD649-20	-	-
Italy*	Lazio		Galimberti et al. (2021)	ZPLOD651-20	-	-
Italy	Sardinia		Galimberti et al. (2021)	ZPLOD658-20	-	-
Italy	Sardinia		Galimberti et al. (2021)	ZPLOD659-20	-	-
Italy	Sardinia		Galimberti et al. (2021)	ZPLOD833-20	-	-
Montenegro*			Galimberti et al. (2021)	ZPLOD833-20	-	-

Following previous studies on population and species level diversity of European Odonata (Gyulavári et al., 2011; Schneider et al., 2015; Hinojosa et al., 2017; Simonsen et al., 2020; Galimberti et al., 2021; Rewicz et al., 2020), we targeted the barcode region of the mitochondrial COI gene (Hebert et al., 2003), and the nuclear Internal Transcribed Spacer region (ITS-region), which comprises the genes ITS1, 5.8S, and ITS2.

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 followed with some modifications, as we follow Krosch & Cranston (2012) and use a lower lysis temperature combined with a longer lysis times: 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 OdoF2 (with universal tail, M13-FP): *TGTAAAACGACGGCCAGT*TTTCTACAAAYCAYAARGATATTGG (tail in boldface italics); and OdoR3 (with universal tail, M13R-pUC): *CAGGAAACAGCTATGAC*TAAACYTCTGGRTGRCCAAARAATCA (tail in boldface italics). We used the following PCR protocol for ITS: 95° C, 2 min; then 35–45 cycles of 95° C, 30 s; 50° C, 30 s; 72° C, 1 min and a final extension of 72° C for 5 min using the primers VRAIN2F (with universal tail, M13-FP): *TGTAAAACGACGGCCAGT*CTTTGTACACACCGCCCGTCGCT (tail in boldface italics); and VRAIN2R (with universal tail, M13R-pUC): *CAGGAAACAGCTATGAC*TTTCACTCGCCGTTACTAAGGGAATC (tail in boldface italics). The COI

primers were from Simonsen et al. (2020), while the ITS region primers were from Félix et al. (2001). 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 Tables 1 and 2.

Haplotype network and phylogenetic analyses

We constructed minimum-spanning haplotype networks (Bandelt et al. 1999) following Kohli et al. (2018, 2021) for the individual COI datasets for each species in PopART (Leigh & Bryant, 2015) (available at http://popart.otago.ca.nz). As haplotype networks are highly sensitive to missing data, we trimmed the alignments to remove sections at the start and end that contained missing bases in some specimens. We did not use the ITS datasets for haplotype networks as the main difference within species in the ITS region were gaps that are treated as missing data in haplotype network analyses.

For both species, we analysed phylogenetic patterns based on the combined COI + ITS datasets as well as the COI dataset alone in MrBayes 3.2 (Ronquist et al. 2012). For both datasets we used *Libellula fulva* to root the tree, and included the species *Orthetrum glaucum*, *O. chrysis*, *O. Sabina*, *O. testacum*, *O. melania*, *O. pruinosum*, *O. albistylum* as additional outgroup taxa. In the analyses of *O. cancellatum* we also included a specimen of *O. coerulescens*, and in the analyses of *O. coerulescens* we included a specimen of *O. cancellatum* as outgroup taxa. A full list of outgroup taxa is given in Supplementary Material S1. In the analyses of the combined COI + ITS datasets the data was partitioned into COI and the ITS-region. In all analyses we allowed the program to estimate the best model for molecular evolution (nst=mixed) with a gamma distribution. All analyses were run for 10 million generations with sampling every 1000 generations. The output files were assessed in Tracer and the first 25% of the sampled trees were used as burnin. We subsequently examined and visualised the trees in FigTree 1.4.4 (Rambaut, 2018).

Assessment of genetic variation

Based on the obtained haplotype networks and phylogenetic trees we divided the COI dataset for both species into haplotype groups and calculated the genetic distance within and between the groups as well as the overall mean distance for each dataset based on the Kimura-2 parameter (K2P) in MEGA (Kumar et al. 2018). The haplotype groups for each species are indicated in Tables 1 and 2, and Supplementary Material S2 and S4. As we had only ITS data for a minority of the samples we did not calculate distance values for these dataset.

Results

Phylogenetic and haplotype network analyses

We successfully sequenced 658 bp COI for all 36 *O. cancellatum* specimens and all 38 *O. coerulescens* specimens. We successfully sequenced up to 918 bp of the ITS region for all 36 specimens of *O. cancellatum*, and 951 bp of the ITS regions for 22 specimens of *O. coerulescens*. The 22 *O. cancellatum* COI sequences available from BOLD and Genbank were between 590 and 658 bp long, while the 43 *O. coerulescens* COI sequences were between 541 bp and 658 bp. The final datasets for the COI haplotype network analyses in PopART were therefore 58 specimens/590 bp for *O. cancellatum*, and 81 specimens/541 bp for *O. coerulescens*. The combined COI-ITS dataset for the Bayesian analyses in MrBayes (including outgroups and specimens with only COI) were 54 specimens/1610 bp for *O. coerulescens*, and 38 specimens/1649 bp for *O. cancellatum* as one *O. cancellatum* specimen was used as outgroup in the analysis of *O. coerulescens*, and one *O. coerulescens* specimen was used as outgroup in the analysis of *O. cancellatum*. All sequence alignments used for the PopART and MrBayes analyses are available as Supplementary Material in Nexus format (Supplementary Material S6–S9).

The haplotype network analysis of *O. cancellatum* (Figure 3) shows that the species can be divided into two well-defined haplotype groups that are only marginally consistent with the geographic distribution. The first group comprises all specimens from the Balearic Islands and Croatia, as well as most specimens from southern France, several specimens from Poland, one specimen from Italy, and one specimen from Denmark. The second group comprises all remaining specimens from northern and central Europe (Sweden, Norway, Denmark, Poland, and Germany), the vast majority of specimens from Italy, all specimens from Malta, as well as a single specimen from Montenegro, and three specimens from southern France.

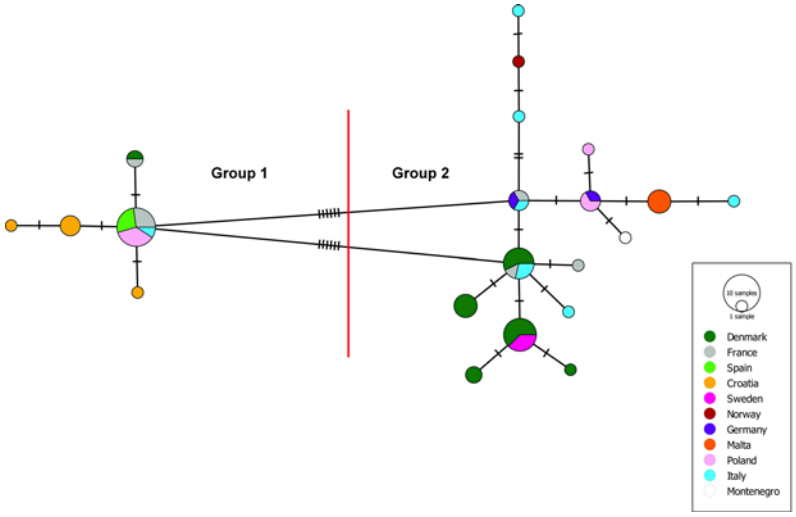

Figure 3. Median-joining haplotype network of *Orthetrum cancellatum* based on COI barcodes. The number of mutations between groups are indicated by bars. Groups are indicated as described in the text.

The haplotype network analysis of *O. coerulescens* (Figure 4) shows that the species can be divided into two haplotype groups, although the distinction is not as clear as in *O. cancellatum*. The first group comprises all specimens from the Balearic Islands, the single specimen from Montenegro, a single Bulgarian specimen, two Italian specimens, a single specimen from Poland, the four specimens from Pakistan, and all specimens identified as belonging to the subspecies *O. coerulescens anceps* (specimens from Georgia and Greece). The second group comprises all remaining specimens from northern and central Europe (Denmark, Germany, Poland and Norway), all specimens from southern France, all remaining specimens from Italy and Bulgaria, and all specimens from Malta. Within the second group, the majority of the Italian specimens, the specimens from Malta and the Bulgarian specimens appear to form a distinct subgroup.

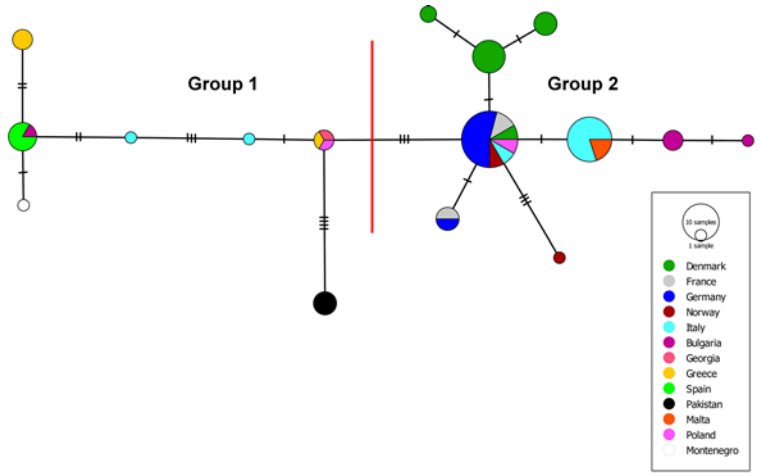

Figure 4. Median-joining haplotype network of *Orthetrum coerulescens* based on COI barcodes. The number of mutations between groups are indicated by bars. Groups are indicated as described in the text.

The Bayesian analyses in MrBayes overall confirm the patterns from the haplotype network analyses. The analysis of *O. cancellatum* (COI: Supplementary Material S2; combined data: Supplementary Material S3) supports the same pattern as the haplotype network, with the first group being monophyletic and well supported (pp = 0.9999 in the COI analysis and pp = 0.999 in the combined analysis). However, the second group is not recovered as monophyletic, but comprise a paraphyletic grade at the base of the tree. The analyses of *O. coerulescens* (COI: Supplementary Material S4; combined data: Supplementary Material S5) also confirm the patterns from the haplotype network analyses, albeit less clearly than for *O. cancellatum*. In the analysis of COI data only the first group is recovered as monophyletic with good support (pp = 0.9898), while the second group is comprising a paraphyletic grade at the base of the tree. Within the second, paraphyletic group, the majority of the Italian specimens, the specimens from Malta, and the Bulgarian specimens form a monophyletic group, albeit with poor support (pp = 0.7549). In the analysis of the combined data the first group is no longer monophyletic. Instead the Pakistani specimens, the specimen from Georgia, a single Greek specimen, a specimen from Italy and a specimen from Poland form a well-supported monophyletic group (pp = 0.9777) and the specimens from Spain, the remaining Greek specimens, the specimen from Montenegro, a single specimen from Italy, and a single specimen from Bulgaria form another well-supported monophyletic group (pp = 1). Within the paraphyletic second group, the group comprising the majority of the specimens from Italy, Bulgaria and the specimens from Malta is no longer monophyletic.

Genetic diversity

The K2P distance values for *O. cancellatum* COI reflects the pattern revealed by the haplotype network analysis. The average K2P distance between the two major groups in the network is 0.015, while the average K2P distances within the groups are 0.0018 and 0.0046, respectively (Table 3).

The K2P distance values for *O. coerulescens* COI reflect both the pattern revealed by the haplotype network analysis and the complex situation with respect to the subspecies *O. coerulescens anceps*. The average K2P distance between the two major groups in the network is 0.0159, while the average K2P distances within the groups are 0.0107 and 0.0024, respectively (Table 3). The average KP2 distance within group 2 that comprise *O. coerulescens anceps* is thus only marginally smaller than the average KP2 distance between the two groups.

Table 3. Average genetic distance (Kimura 2 parameter) calculated in MEGA X.

O. cancellatum		***O. coerulescens***	
Overall	0.0088	Overall	0.0077
Within groups		Within groups	
Group 1	0.0018	Group 1	0.0107
Group 2	0.0046	Group 2	0.0024
Between groups	Group 2	Between groups	Group 2
Group 1	0.015	Group 1	0.0159

Discussion

Phylogeography and genetic divergence

The haplotype network pattern recovered for *O. cancellatum* (Figure 3) shows that the species apparently is divided into two distinct groups in Europe. One group is found in southwestern Europe (Spain, southwest France), northern most Italy (Trento), Croatia, and in low numbers in northern Europe (Den-

mark, Poland). The second group is found widespread in the Italian peninsula and Malta, central and northern Europe (Germany, Poland, Denmark, Sweden, and Norway), as well as Montenegro, and in low numbers in southern France. This mixed pattern is puzzling, but the presence of numerous group 2 haplotypes in Italy indicate that the group may represent an Italian glacial refugium from which the species spread to northern Europe following in end of the Weichsel Glaciation *ca* 12Kya (Ehlers & Gibbard, 2008; Gibbard & Cohen, 2008; Houmark-Nielsen et al., 2012). A scenario that is similar to that reported for the European Hedgehog (*Erinaceus europaeus*) (Hewitt, 1999, 2004). The fact that the only Italian specimen in Group 1 is from northern Italy indicate that this group is not associated with an Italian glacial refugium. Instead the group may represent a different European glacial refugium. However, the fact that the group is comprised by specimens from both southwestern Europe (France and the Balearic Islands) and southeastern Europe makes it difficult to determine where that refugium was. The single Italian specimen from northern Italy found in this group, could indicate that the group may have originated in a peripheral refugium north of the Alps—as suggested for the moth *Hepialus humuli* by Simonsen & Huemer (2014), and subsequently spread to the east, west and north. However, given the marginally greater variation among the Croatian samples compared to specimens from Spain, France, Poland and Denmark in this group, a more likely explanation is that this group may represent a south eastern refugium from which it spread across Europe in a pattern similar to the grasshopper *Chorthippus parallelus* (Hewitt, 1999, 2004), or the Adder *Vipera berus* (Ursenbacher et al., 2006)—although in the latter two cases a relict population remained isolated in the Italian Penninsula.

The haplotype network pattern recovered for *O. coerulescens* (Figure 4) is even more mixed than that recovered for *O. cancellatum*. Still, some interesting aspects deserve to be highlighted. The species can be divided into two groups. Group one comprise all specimens we could identify as *O. coerulescens anceps*, all specimens from the Balearic Island and one from Bulgaria, which we confirmed were *O. coerulescens coerulescens*, a specimen from Montenegro, a specimen from Poland, two specimens from Italy, and four specimens from Pakistan, none of which we had access to and thus were unable to identify to subspecies. The internal genetic distance within the group is relatively high (K2P = 0.01), and the group it thus neither genetically, morphologically nor geographically homogenous. Group 2 comprise the remaining specimens from Italy and Bulgaria, all specimens from Malta, and almost all specimens from mainland western, central and northern Europe (France, Germany, Poland, Denmark, and Norway). Within the group most Italian specimens share a single haplotype with the specimens from Malta indicating that there is a closer connection between Italy and Malta for *O. coerulescens* than for *O. cancellatum*, and Malta may have been colonized by *O. coerulescens* quite recently. Given the strong Italian presence in Group 2 it is possible that this group, like Group 2 in *O. cancellatum* represents an Italian glacial refugium from which the species spread to the rest of Europe following the end of the Weichsel Glaciation. This is further supported by the fact that several Italian specimens share a haplotype found only in Italy and on Malta. The Bulgarian specimens, which are distinct yet closely related to the Italian/Maltese specimens, may represent a colonisation event from Italy to Bulgaria in a warming period during the Weichsel Glaciation. A similar connection, albeit in the opposite direction was recently shown in Cave Crickets (*Troglophilus*) (Allegrucci et al., 2017).

The genetic divergences we find between different haplotype groups in both *Orthetrum* species are considerably higher than what has generally been reported for mtDNA in European Odonata. In a study of the European subspecies of *Sympetrum vulgatum*, Hinojosa et al. (2017) found an uncorrected *p* distance of 0.3% between the Iberian Peninsula subspecies *S. v. ibericum* Ocharan 1985 and the European nominate subspecies *S. v. vulgatum*, and an uncorrected *p* distance of 0.1% between *S. v. vulgatum* and *S. v. decoloratum* (Selys 1884) from Caucasus and Anatolia. Bernard et al. (2011) found only a single nucleotide differing in *Nehalennia speciosa* from Western Siberia to Central Europe, although their results were based on two different mitochondrial genes (16S and COII). Kohli et al. (2018) found no variation in COI in Palaearctic populations of the arctic species *Somatochlora sahlbergi*. However, Schneider et al. (2015), and Simonsen et al. (2020) found similar divergences in *Aeshna cyanea*, where the uncorrected *p* distances between geographically separated population groups were between 0.044 and 0.013, with the lowest value being the distance between Northern Africa and Western Europe. In the two recently published major DNA barcode studies of European Odonata Rewicz et al. (2020) did not report any genetic difference in either species, while Galimberti et al. (2021) did report a potential barcode gap in both species.

Taxonomic consequences for O. coerulescens

Orthetrum coerulescens populations found in North Africa, the southeast Mediterranean, and Asia are often considered a separate subspecies, *O. ceorulescens anceps*, or even a full species (*O. anceps*), while populations in western and northern Europe are considered belonging to the nominate subspecies *O. c. coerulescens* (e.g. see Askew, 2004; Kalkman & Ambus, 2015b for an overview). However, Mauersberger (1994) and Klingenberg & Martens (1996) demonstrated that intermediates between the two forms occur widespread in the Mediterranean, and Kalkman & Ambus (2015b) suggested that the two forms were isolated in an eastern and a western group respectively during the last glaciation, and that they have come into contact allowing gene flow during the Holocene. Our results support this hypothesis, but also indicate that the situation is more complex. All the specimens included in our analysis from Greece and Georgia were identified as *O. c. anceps* based on morphology, while all other specimens we had access to are *O. c. coerulescens*. Yet, most Greek *O. c. anceps* group with Balearic *O. c. coerulescens* in all molecular analyses showing that they do indeed interbreed. The only two specimens that are different are one specimen from the Greek island of Skiathos (ENT-DNA-1001) and the single specimen from Georgia (ENT-DNA-946) in the Caucasus. These two specimens form an isolated group together with a single specimen from Poland indicating that the genetic admixture is widespread if rare in Europe, and extends considerably north of *O. coerulescens anceps* range. As the Balearic and most Greek specimens are very similar genetically, the hybridisation zone between the two populations probably spans the entire Mediterranean. The two Italian specimens in Group 1 are both from the Italian mainland (Lazio and Trento), which support the hypothesis that the hybridisation zone probably spans the Mediterranean. However, none of the specimens from the Mediterranean island of Sardinia, Sicily or Malta are found in Group 1. This does not rule out the possibility of a trans-Mediterranean hybridisation zone as some of them could represent *O. coerulescens anceps* morphotypes with *O. cerulescens coerulescens* haplotypes, or they may be back crosses that cannot be determined with certainty. Unfortunately, we did not have access to specimens from the Iberian Peninsula or North Africa, and we therefore cannot assess their status in this complex.

The widespread hybridisation in the Mediterranean between *O. c. coerulescens* and *O. c. anceps* does show that the latter should not be considered a separate species, but the morphological distinction, and potential ncDNA distinction still evident between the two groups also indicate that they should probably retain their subspecific status. We also note that especially *O. coerulescens* appear to be an excellent system to employ genomics to study postglacial hybridisation zones in European insects, and that these species together with other Western Palaearctic *Orthetrum* are an excellent system for comparative genomics-based phylogeography of the effects of Quaternary glaciation cycles on the European fauna. Applying a genomic approach will also address the weaknesses in the current single-gene approach noted above.

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Supplementary Materials

Supplementary Material S1: List of outgroup taxa and Genbank Association numbers for the Bayesian analyses.

Supplementary Material S2: Tree from the 10 million generation analysis Mr Bayes analysis of *O. cancellatum* based on COI.

Supplementary Material S3: Tree from the 10 million generation analysis Mr Bayes analysis of *O. cancellatum* based on COI and ITS.

Supplementary Material S4: Tree from the 10 million generation analysis Mr Bayes analysis of *O. coerulescens* based on COI.

Supplementary Material S5: Tree from the 10 million generation analysis Mr Bayes analysis of *O. cancellatum* based on COI and ITS.

Supplementary Material S6: The aligned *O. cancellatum* COI + ITS dataset for Mr Bayes as nexus file.

Supplementary Material S7: The aligned *O. coerulescens* COI + ITS dataset for Mr Bayes as nexus file.

Supplementary Material S8: The aligned *O. cancellatum* COI dataset for PopART as nexus file.

Supplementary Material S8: The aligned *O. coerulescens* COI dataset for PopART as nexus file.

Declarations

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Availability of data and material: All sequences have been uploaded to Genbank. Data files are available as Supplementary Material.

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. 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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