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COI diversity supports subspecific division in Western European *Lestes virens* (Charpentier, 1825) (Zygoptera: Lestidae), but hints at further Mediterranean complexity

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Abstract. We analyse COI sequences of 48 specimens of European *Lestes virens* (Charpentier) to explore patterns in genetic diversity including subspecific boundaries and potential glacial refugia. Our haplotype network and phylogenetic analyses reveal three distinct groups in Western and Northern Europe. One group corresponding to the nominate subspecies *L. virens virens* is confined to the Iberian Peninsula and southwestern France, and one group corresponding to the subspecies *L. virens vestalis* is found in the rest of western Europe including southern Scandinavia, mainland Italy and the Mediterranean island Sardinia. Surprisingly three specimens from the Mediterranean island Sicily form a highly distinct group in all our analyses. An analysis of molecular variance (AMOVA) confirms that almost all observed genetic variance is explained by variation between these three groups rather than by variation between sample areas or between individuals. We conclude that the subspecific division into *L. virens virens* and *L. virens vestalis* is justified, but further studies are needed to determine the status of the populations in Sicily, southeastern Europe, and North Africa. The genetic pattern we find may reflect different glacial refugia: an Iberian/North African refugium for *L. virens virens*; a potential Italian refugium for *L. virens vestalis*; and a Sicilian/North African refugium for the Sicilian populations.

Key words. Odonata, damselfly, Europe, glacial refugia, subspecies.

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

Introduction

Lestes virens (Charpentier, 1825) (Fig. 1) is a small emerald damselfly (Lestidae), which occurs widespread throughout Europe except for Great Britain and Ireland, Finland, and Scandinavia north of southern Sweden. From eastern Europe it continues into Central Asia, the Caucasus, and northern Iran, in the Mediterranean it extends into the North African Maghreb region, the Levant, and Turkey (Boudot & Willigalla, 2015). The subspecific taxonomy and distribution of *L. virens* in the Western Palearctic is not fully understood (Jödicke, 1997; Samraoui et al., 2003; Boudot & Willigalla, 2015). Jödicke (1997) in their treatment of Lestidae in Europe listed three potential subspecies: *L. virens virens* (Charpentier, 1825), *L. virens vestalis* (Rambur, 1840), and a potential, undescribed subspecies. They noted that *L. virens virens* is probably ['vermutlich'] limited to the western Mediterranean region, while the potential undescribed subspecies is present in Anatolia and the southwestern Black Sea region, and *L. virens vestalis* is present in the rest of the species' European range, including most of France and mainland Italy. According to Jödicke's (1997, p. 82, fig. 48) map the populations on the Mediterranean islands Sicily, Sardinia, and Corsica probably belong to *L. virens virens* (Fig. 1). However, they stated that both subspecies delimitation and distributions are uncertain (Jödicke, 1997). This

view was upheld by Boudot & Willigalla (2015), who stated that the identification of subspecies in their contact zones is often not possible. They further stated that while some southeastern European populations resemble *L. virens markovskii* Belyshev, 1961 it is uncertain if this subspecies occur in southeastern Europe and southwestern Asia (Boudot & Willigalla, 2015) as suggested by Samraoui et al. (2003). Samraoui et al. (2003) described *Lestes numidicus* Samraoui, Weekers & Dumont, 2003 from Algeria based on DNA sequence data from the nuclear markers 18S, ITS1, 5.8S, ITS2, and 28S (partial), and adult morphology including coloration. They showed that *L. numidicus* and *L. virens virens* co-exist in Algeria, separated by different mating seasons. Boudot & Willigalla (2015) suggested that further molecular studies are needed to clarify the taxonomic status and distributional limits of the *L. virens* complex.

Recently, two DNA barcoding studies on European Odonata species (Galimberti et al., 2021; Geiger et al., 2021) have made available several COI barcode sequences from Italian and central European *L. virens* populations, respectively. Galimberti et al. (2021, fig. 2) showed that depending on method *L. virens* in Italy could be divided into two groups. Here we combine these online available sequences with COI barcode sequences from *L. virens virens* from southwestern France and Spain, and *L. virens vestalis* from Denmark and Sweden to explore COI diversity and phylogenetic relationships of *L. virens* in Western Europe, specifically to address the difference between *L. virens virens* in southwestern and *L. virens vestalis* in north and central Europe, and to examine whether specimens from the Mediterranean islands Sicily and Sardinia belong to either COI group or are different altogether.

Material and methods

Sampling

We sampled 28 specimens of *L. virens* from Europe. Eighteen of the specimens were subspecies *L. virens vestalis* from Denmark and Sweden, and ten of the specimens were *L. virens virens* from southwestern France and Spain. We augmented the dataset with Barcode of Life Database (BOLD) and Genbank sequences from 20 specimens from Europe. We consider 16 of these to belong to *L. virens vestalis* based either on original determination in BOLD or distribution, while we consider four from Sicily and Sardinia to be of uncertain subspecific affinity. Five sequences were considerably shorter than the others and they were only used in one haplotype network analysis (see below). The full *L. virens* dataset thus comprises 48 samples, but the dataset used for most analyses comprises 43 samples. All

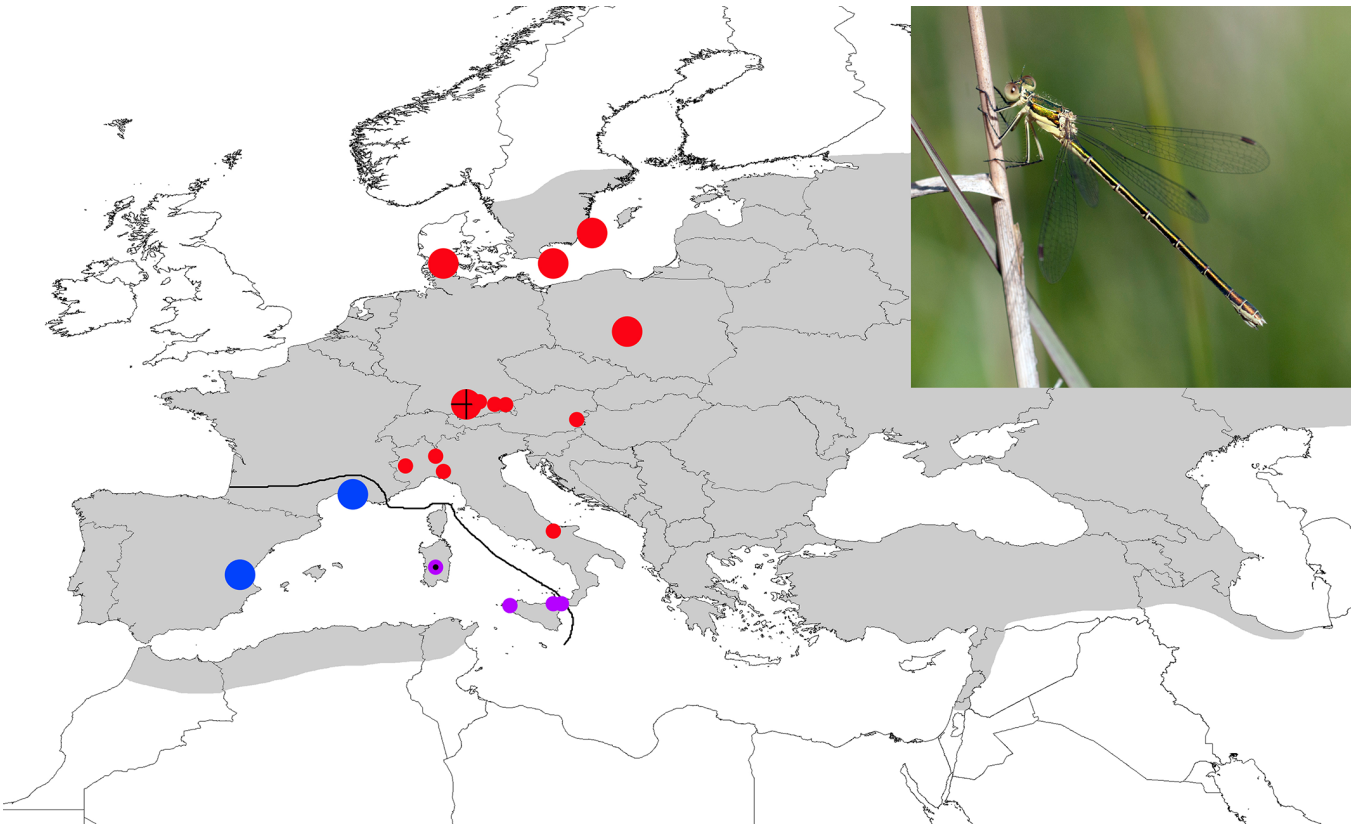

Figure 1. Western Palearctic range and sampling sites for *Lestes virens* (photo). Distribution (based on Boudot & Willigalla, 2015) is indicated in grey. Blue circles indicate *L. virens virens*, red circles indicate *L. virens vestalis*, and purple circles indicate uncertain subspecific affinity. The black-cross red circle indicates samples with 318 bp COI available. The black-dot purple circle indicates the Sardinian specimen, which groups with *L. virens vestalis* in the analyses. Small circles indicate single specimens, while large circles indicate several specimens. The black line indicates the possible boundary between *L. virens virens* and *L. virens vestalis* as indicated by Jödicke (1997, fig. 48). For specimen details see Table 1.

Table 1. *Lestes virens* ssp. (n = 48) used in this study with localities, subspecies affiliations, voucher designations, Genbank and BOLD accession numbers, and voucher deposits. NHMA: Natural History Museum Aarhus. *The Sardinian specimen is assigned to *L. virens vestalis* based on the results in our study. The five Bavarian specimens marked with an asterisks (*) were only included in the 48 samples/318 bp analysis.

Country	Region	Sub-species	Voucher	Ref	Genbank	BOLD	Deposit
Denmark	Bornholm	*vestalis*	ENT-DNA-157	New	MN912983	DANOD143-22	NHMA
Denmark	Bornholm	*vestalis*	ENT-DNA-156	New	MN912975	DANOD142-22	NHMA
Denmark	Bornholm	*vestalis*	ENT-DNA-155	New	MN912974	DANOD141-22	NHMA
Denmark	Bornholm	*vestalis*	ENT-DNA-154	New	MN912973	DANOD140-22	NHMA
Denmark	Bornholm	*vestalis*	ENT-DNA-153	New	MN912972	DANOD139-22	NHMA
Denmark	Bornholm	*vestalis*	ENT-DNA-152	New	MN912971	DANOD138-22	NHMA
Denmark	Bornholm	*vestalis*	ENT-DNA-151	New	MN912982	DANOD137-22	NHMA
Denmark	Bornholm	*vestalis*	ENT-DNA-150	New	MN912970	DANOD136-22	NHMA
Denmark	South Jutland	*vestalis*	ENT-DNA-11	New	MN912981	DANOD005-22	NHMA
Denmark	South Jutland	*vestalis*	ENT-DNA-10	New	MN912969	DANOD004-22	NHMA
Denmark	South Jutland	*vestalis*	ENT-DNA-9	New	MN912968	DANOD003-22	NHMA
Denmark	South Jutland	*vestalis*	ENT-DNA-8	New	MN912967	DANOD002-22	NHMA
Denmark	South Jutland	*vestalis*	ENT-DNA-7	Simonsen et al. (2022)	MN912966	DANOD001-22	NHMA
Sweden	Oeland	*vestalis*	ENT-DNA-756	New	MN912980	DANOD634-22	NHMA
Sweden	Oeland	*vestalis*	ENT-DNA-754	New	MN912979	DANOD633-22	NHMA
Sweden	Oeland	*vestalis*	ENT-DNA-753	New	MN912978	DANOD632-22	NHMA
Sweden	Oeland	*vestalis*	ENT-DNA-752	New	MN912977	DANOD631-22	NHMA
Sweden	Oeland	*vestalis*	ENT-DNA-751	New	MN912976	DANOD630-22	NHMA
France	Bouches-du-Rhône	*virens*	ENT-DNA-750	New	MN912993	DANOD629-22	NHMA
France	Bouches-du-Rhône	*virens*	ENT-DNA-749	New	MN912992	DANOD628-22	NHMA
France	Bouches-du-Rhône	*virens*	ENT-DNA-748	New	MN912991	DANOD627-22	NHMA
France	Bouches-du-Rhône	*virens*	ENT-DNA-747	New	MN912990	DANOD626-22	NHMA
France	Bouches-du-Rhône	*virens*	ENT-DNA-746	New	MN912989	DANOD625-22	NHMA
Spain	Valencia	*virens*	ENT-DNA-745	New	MN912988	DANOD624-22	NHMA
Spain	Valencia	*virens*	ENT-DNA-744	New	MN912987	DANOD623-22	NHMA
Spain	Valencia	*virens*	ENT-DNA-743	New	MN912986	DANOD622-22	NHMA
Spain	Valencia	*virens*	ENT-DNA-742	New	MN912985	DANOD621-22	NHMA
Spain	Valencia	*virens*	ENT-DNA-741	Simonsen et al. (2022)	MN912984	DANOD620-22	NHMA
Austria	Styria	*vestalis*	Odon164	BOLD		AODON330-20	Karl-Franzens University of Graz
Germany	Barvaria	*vestalis*	GBOL00271	Geiger et al. (2021)	MW490566	FBAQU1427-13	Zoologische Staatssammlung München
Germany	Barvaria	*vestalis*	GBOL05483	Geiger et al. (2021)	MW490326	GBEPT913-14	Zoologische Staatssammlung München
Germany	Barvaria	*vestalis*	GBOL05484	Geiger et al. (2021)	MW490561	GBEPT914-14	Zoologische Staatssammlung München
Germany	Barvaria	*vestalis*	GBOL 20162	Geiger et al. (2021)	MW490092	GBODO039-18*	Zoologische Staatssammlung München
Germany	Barvaria	*vestalis*	GBOL 20176	Geiger et al. (2021)	MW490144	GBODO053-18*	Zoologische Staatssammlung München
Germany	Barvaria	*vestalis*	GBOL 20196	Geiger et al. (2021)	MW490519	GBODO073-18*	Zoologische Staatssammlung München
Germany	Barvaria	*vestalis*	GBOL 20198	Geiger et al. (2021)	MW490098	GBODO075-18*	Zoologische Staatssammlung München
Germany	Barvaria	*vestalis*	GBOL 20213	Geiger et al. (2021)	MW490391	GBODO090-18*	Zoologische Staatssammlung München
Poland	Lodz	*vestalis*	OdoPL17	Geiger et al. (2021)	MW490552	ODOPL017-19	University of Lodz
Poland	Lodz	*vestalis*	OdoPL18	Geiger et al. (2021)	MW490534	ODOPL018-19	University of Lodz
Poland	Lodz	*vestalis*	OdoPL19	Geiger et al. (2021)	MW490556	ODOPL019-19	University of Lodz
Italy	Sicily	unknown	MIB:ZPL:08299	Galimberti et al. (2021)	MT298497	ZPLOD499-20	University of Milano Bicocca

Country	Region	Sub-species	Voucher	Ref	Genbank	BOLD	Deposit
Italy	Sicily	unknown	MIB:ZPL:08300	Galimberti et al. (2021)	MT298496	ZPLOD500-20	University of Milano Bicocca
Italy	Sicily	unknown	MIB:ZPL:08301	Galimberti et al. (2021)	MT298495	ZPLOD501-20	University of Milano Bicocca
Italy	Piedmont	*vestalis*	MIB:ZPL:08303	Galimberti et al. (2021)	MT298501	ZPLOD503-20	University of Milano Bicocca
Italy	Lombardy	*vestalis*	MIB:ZPL:08304	Galimberti et al. (2021)	MT298494	ZPLOD504-20	University of Milano Bicocca
Italy	Emilia-Romagna	*vestalis*	MIB:ZPL:08305	Galimberti et al. (2021)	MT298500	ZPLOD505-20	University of Milano Bicocca
Italy	Molise	*vestalis*	MIB:ZPL:08309	Galimberti et al. (2021)	MT298499	ZPLOD509-20	University of Milano Bicocca
Italy	Sardinia	*vestalis**	MIB:ZPL:08312	Galimberti et al. (2021)	MT298498	ZPLOD512-20	University of Milano Bicocca

data are provided in Table 1 and a graphic overview of sample sites is shown in Figure 1.

We targeted the barcode region of the mitochondrial COI gene (Hebert et al., 2003) following earlier studies on European Odonata (Gyulavári et al., 2011; Schneider et al., 2015; Hinojosa et al., 2017; Simonsen et al., 2020; 2021; Kohli et al., 2021; Galimberti et al., 2021; Geiger et al., 2021), which also allow us to explore sequences published in recent Odonata studies (Galimberti et al., 2021; Geiger et al., 2021; Simonsen et al., 2022).

Laboratory procedures

Genomic DNA was extracted using one or two legs from each sample at the Department of Biology, Aarhus University (AU), Denmark using E.Z.N.A. Tissue DNA Kit (Omega BIO-TEK) following manufacturer's instructions with the following modifications: We followed Krosch & Cranston (2012) and incubated samples at a lower temperature (here 42°C) for 18–23 hours during lysis, steps 5–6 were omitted, and samples were incubated for 5–10 min with elution buffer in the last step and eluted once in 200 µl.

We used the PCR protocol in Simonsen et al. (2021) and the universal tail COI primers (OdoF2 and OdoR3) developed for Odonata by Simonsen et al. (2020). PCR products were sequenced by Macrogen Europe using the universal primer tails for direct Sanger sequencing. Contigs and consensus sequences were obtained in DNA Baser Sequence Assembler v5.8.0 (Heracle Biosoft, Romania). All sequences were checked on Genbank and/or Barcode of Life using BLAST and BOLD Identification System. Genbank and BOLD accession numbers are provided in Table 1.

Haplotype network and phylogenetic analyses

We constructed minimum-spanning haplotype networks (Bandelt et al., 1999) following Simonsen et al. (2021) and Kohli et al. (2018, 2021) in PopART (Leigh & Bryant, 2015) (available at http://popart.otago.ac.nz).

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

	virens	*vestalis*	Sicily
virens	0.0004		
vestalis	0.0120	0.0015	
Sicily	0.0168	0.0173	0.0000

We trimmed the alignments and removed sections with missing bases at the start and end in some sampled sequences, as haplotype network analyses are sensitive to missing data.

We analysed phylogenetic patterns in MrBayes 3.2 (Ronquist et al., 2012). We used sequences from *Lestes barbarus* (Genbank accession number MN912942), *L. sponsa* (accession number MN912910), and *Chalcolestes viridis* (accession number MN912867) as outgroups, and the tree was rooted on *Chalcolestes viridis* based on Simonsen et al. (2022). We allowed MrBayes to estimate the best model for molecular evolution (nst=mixed) with a gamma distribution. The analysis ran for 10 million generations with sampling every 1,000 generation. After examining the output files in Tracer 1.7.2 (part of the BEAST package: Bouckaert et al., 2019), we used the first 25% of the sampled trees as burnin and examined and visualized the resulting tree in FigTree 1.4.4 (Rambaut, 2018).

Assessment of genetic variation

We divided the COI dataset into haplotype groups based on the results from the haplotype network and phylogenetic analyses and calculated the genetic distance within and between groups based on the Kimura-2 parameter (K2P) (Kimura, 1980) in MEGA 11 (Tamura et al., 2021). The results are provided in Table 2. We carried out a nested AMOVA test (Excoffier et al., 1992) as implemented in PopART to assess genetic divergence between and within these groups and sample areas

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 1,000 permutation.

Variation	df	Sigma²	%variation	ϕ-statistics	*p*
Among groups	2	27.320	97.469	0.981	< 0.001
Among sample sites	7	0.185	0.659	0.260	0.156
Within sample sites	33	0.525	1.872	0.975	< 0.001
Total	42	28.029	100.000		

(countries or regions) as illustrated in Figure 2. The results are provided in Table 3. Overall nucleotide diversity (π) was similarly calculated in PopART.

Results

Haplotype network and phylogenetic analyses

We sequenced 658 bp COI for all 28 *L. virens* specimens. There were 16 sequences available on BOLD and Genbank with at least 550 overlapping base pairs in the barcode region and a further five sequences with 318 overlapping base pairs. One sequence (Genbank accession number KF369424) did not have locality information and was therefore omitted. We constructed two different datasets: one dataset comprising the 43 sequences that were at least 550 base pairs long, and one that comprised all 48 sequences. The 43-sequence dataset was thus 550 base pairs long after trimming, and the 48-sequence dataset was 318 base pairs long after trimming. The 43-sequence dataset was used for haplotype network and phylogenetic analyses and assessment of genetic variation, while the 48-sequence dataset was only used for a haplotype network analysis. The

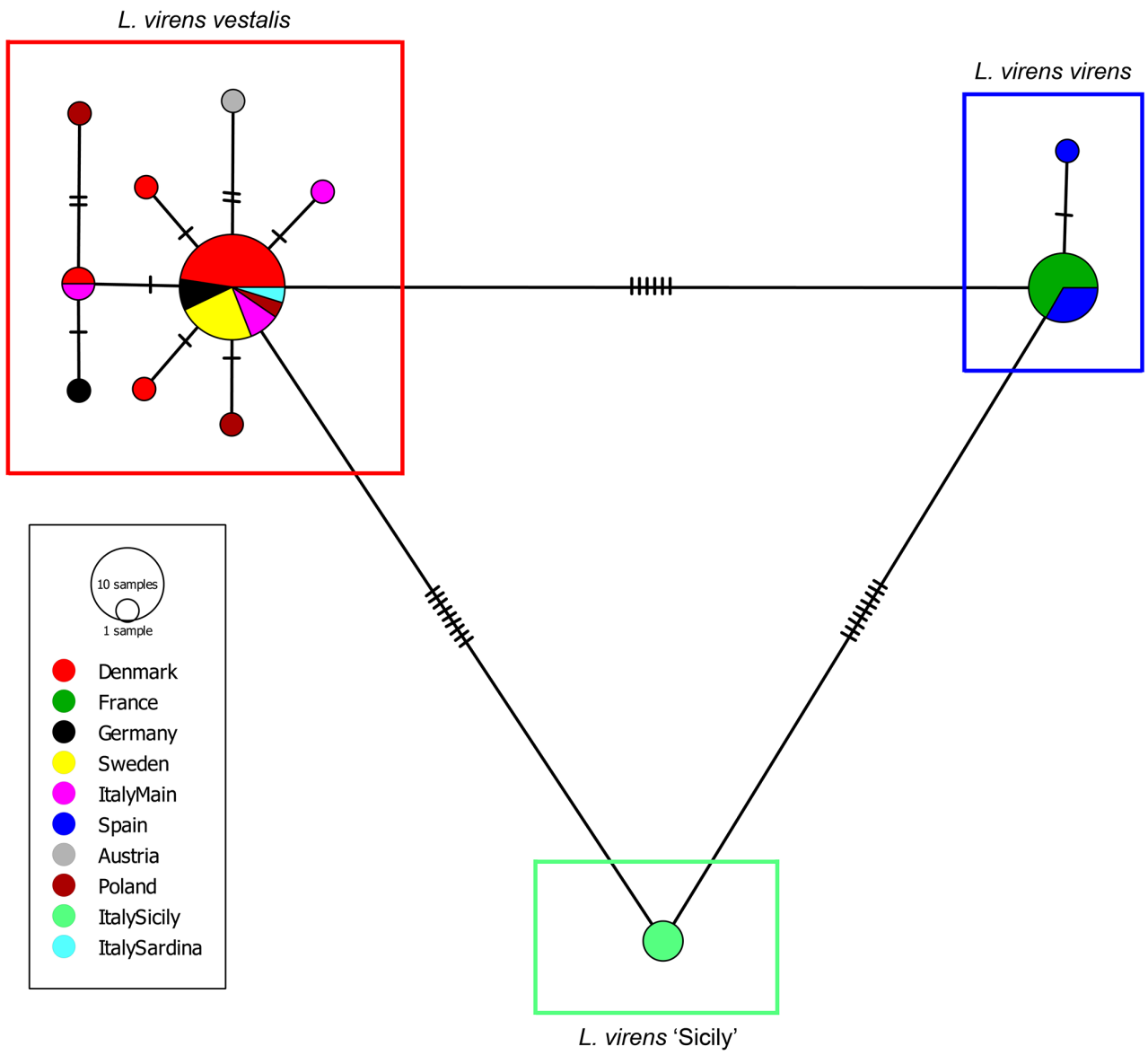

Figure 2. Minimum-spanning haplotype network based on the 550 bp COI dataset. The number of mutations between groups are indicated by bars. Groups are indicated as described in the text.

sequence alignments used for the PopArt and MrBayes analyses are available as Supplementary Material in the Nexus format (Supplementary Material S1–S3).

The haplotype network analysis of the 43-sequence dataset (Fig. 2) shows that *L. virens* can be divided into three well-defined haplotype groups in Western Europe that correspond well to the current geographic distribution and taxonomy. The specimens from southwestern Europe, all belonging to the current subspecies *L. virens virens*, form a well-delimited group (n = 10), defined by three unique nucleotide changes, with little internal variation. The specimens from northern and central Europe including mainland Italy, all belonging to the subspecies *L. virens vestalis*, form another well-delimited group (n = 30), defined by three unique nucleotide changes, albeit with greater internal variation. Interestingly, the single specimen from the Italian Mediterranean island of Sardinia is placed in this group. Finally, the three specimens from the Italian Mediterranean island of Sicily form a distinct and isolated group (n = 3), defined by five unique nucleotide changes, with no internal variation. The 48-sequence dataset with much shorter sequences confirms the division into three well-defined groups (Supplementary Figure S4)—the five additional specimens, all from Bavaria in southern Germany, only add to the internal variation of *L. virens vestalis*.

The Bayesian analysis in MrBayes (Fig. 3) confirms the results from the haplotype network analyses. *Lestes virens virens*, *L. virens vestalis*, and the specimens from Sicily all form distinct clades. But while the two latter clades are well supported with pp values of 99 and 96, respectively, *L. virens virens* only receives moderate support with a pp value of 84 despite appearing as a highly distinct group in the haplotype network—possibly because it shares homoplasious or plesiomorphic nucleotide changes with both the other groups. Interestingly, the specimens from Sicily form the sister group to a clade comprising *L. virens virens* and *L. virens vestalis*, although the latter is only poorly supported with a pp value of 63.

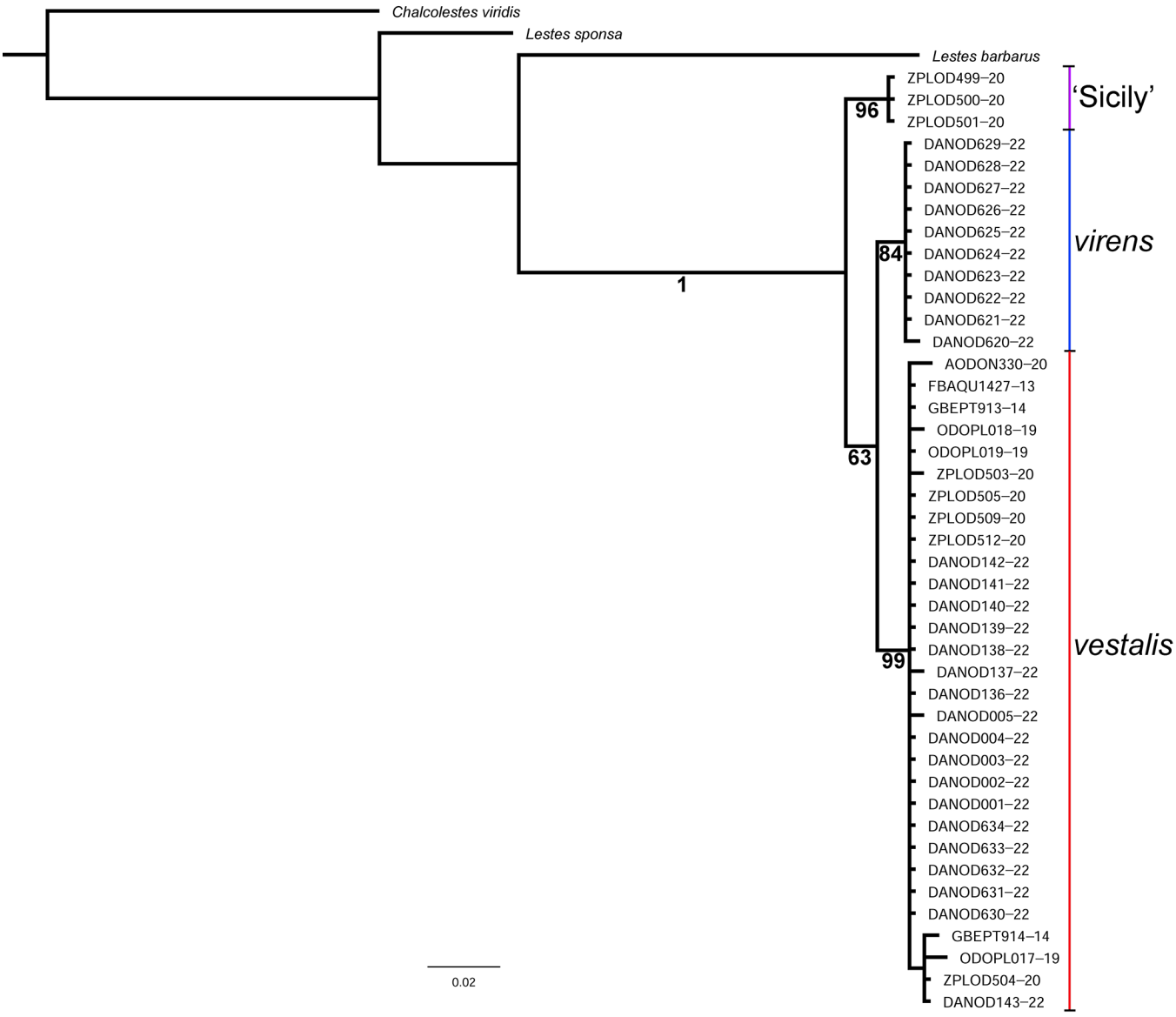

Figure 3. Tree from the 10 million generation analysis in MrBayes. Numbers indicate relevant posterior probability values. Groups are indicates as discussed in the text and colors correspond to Figure 1. For specimen details see Table 1.

Genetic diversity

The K2P distance values (Table 2) confirm the results from the haplotype network analyses. The average K2P distance between the groups varies from 0.0120 between *L. virens virens* and *L. virens vestalis* to 0.0173 between *L. virens vestalis* and the specimens from Sicily. The difference between *L. virens virens* and Sicily is 0.0163. The average K2P distance within groups varies between 0 in Sicilian specimens, 0.0004 in *L. virens virens*, and 0.0015 in *L. virens vestalis*. The average between groups K2P distance is thus approximately an order of magnitude higher than the highest within group K2P distance. The AMOVA-test (Table 3) shows that more than 97% of the genetic variance is explained by variation among the three major groups. The overall nucleotide diversity (π) is 0.0072.

Discussion

Both the haplotype network analyses (Fig. 2, Supplementary Fig. S4) and the Bayesian phylogenetic analysis (Fig. 3) show that *L. virens* in western Europe can be divided into three separate and well-defined groups: one comprising *L. virens virens*, one comprising *L. virens vestalis*, and one comprising only specimens from Sicily. Although relationships between the groups are poorly supported, the Bayesian analysis indicates that the Sicily group is the sister to a clade comprising *L. virens virens* and *L. virens vestalis*. While the Sicily group and *L. virens virens* appear homogenous comprising one and two haplotypes respectively, *L. virens vestalis* is more heterogenous comprising 9 different haplotypes. The K2P distance values support this (Table 2) with only *L. virens vestalis* displaying a non-neglible internal average distance, while the distance between the groups varies between 1.2% and 1.7%, with the distances between the Sicily group and the other two being higher than the distance between *L. virens virens* and *L. virens vestalis*. The AMOVA confirms that almost all genetic variance is explained by variation between the three groups (97.5%). The average distance values are much higher than the corresponding values within and between subspecies of *Sympetrum vulgatum* (Linnaeus, 1758) in West Palearctic (Hinojosa et al., 2017), within *Aeshna juncea* (Linnaeus, 1758), *Aeshna subarctica* Walker, 1908, *Libellula quadrimaculata* Linnaeus, 1758, and *Sympetrum danae* (Sulzer, 1776) in Europe (Kohli et al., 2021), and within *Nehalennia speciosa* (Charpentier, 1840) or *Somatochlora sahlbergi* Trybom, 1889 across the Palaearctic (Bernard et al., 2011; Kohli et al., 2018). However, the within and between population values are comparable to—or slightly lower—than what has been reported for *Aeshna cyanea* (Müller, 1764), *Orthetrum cancellatum* (Linneaus, 1758), and *Orthetrum coerulescens* (Frabricius, 1798) in the Western Palearctic (Simonsen et al., 2020, 2021). The differences between the three groups are relatively high, but below the upper hybridization threshold limit (1.78%) predicted for Zygoptera by Sanchez-Guillen et al. (2014). The results therefore clearly support the status of *L. virens virens* and *L. virens vestalis* as separate subspecies, but also show that the situation is more complex. Jödicke (1997) considered it likely that *L. virens virens* is present on the western Mediterranean islands Sicily, Sardinia, and Corsica. However, our results show that this is likely not the case. The single specimen from Sardinia we have been able to include groups with *L. virens vestalis* in our analyses and shares its haplotype with the majority (n = 20) of the specimens in that group. In addition, the three specimens from Sicily form an isolated group that is not closer to *L. virens virens* than either are to *L. virens vestalis*. It therefore seems likely that in Europe *L. virens virens* is restricted to the Iberian Peninsula and adjacent southwestern France. Unfortunately, we do not have access to material or COI sequences from North Africa, eastern or southeast Europe, or Asia Minor. We therefore cannot assess *L. numidicus* or the taxonomic status of southeastern populations of *L. virens* and evaluate whether the Sicilian population is closely related to either, or if they are indeed as isolated from other European populations as they appear in our results. Our overall results are quite similar to Hinojosa et al. (2017), who showed that the subspecific divisions of *S. vulgatum* in the Western Palearctic are consistent with differences in COI—albeit with much lower diversity. Contrary to this Simonsen et al. (2021) showed that COI variation in *O. coerulescens* in the Western Palearctic does not follow subspecific variation but indicates Mediterranean-wide genetic admixture between *O. coerulescens coerulescens* in the west and *O. coerulescens anceps* in the east.

The clear separation between *L. virens virens* and *L. virens vestalis* not only supports the subspecific division, but indicates that they were likely separated during the last glaciation—and perhaps longer than that. The widespread distribution of *L. virens vestalis* in Italy (Boudot & Willigalla, 2015) and the fact that Italian specimens are quite variable and group with several different Western European specimens (Fig. 2), could indicate that *L. virens vestalis* survived the last glaciation in a 'classical' Italian refugium (e.g. de Lattin, 1967; Hewitt, 2004). However, the greater complexity of *L. virens vestalis* and not least the fact that adding five Bavarian specimens with only 318 bp COI fragment apparently increase the variation in *L. virens vestalis* (Supplementary Fig. S4) show that the situation is complex and *L. virens vestalis* may be an admixture of an Italian lineage and a southeastern/eastern lineage. Without material from eastern and southeastern Western Palearctic we cannot determine this. *Lestes virens virens* likely survived the last glaciation in a southwestern refugium. Samraoui et al. (2003) and Samraoui (2009) showed that *L. numidicus* and *L. virens* exist geographically sympatric but seasonally segregated in Algeria. They further stated that *L. virens* in Algeria corresponds to *L. virens virens*, but there may be—or have

been historical— introgression between that taxon and *L. numidicus* in Algeria. The presence of *L. virens virens* in the Maghreb region of Northwest Africa as well as in the Iberian Peninsula could indicate an African-Iberian connection (e.g. Husemann et al., 2014) as recently shown for *Aeshna cyanea* by Simonsen et al. (2020). However, without having access to North African material we cannot test this hypothesis, nor can we determine the direction of a potential connection. The presence of a genetically distinct group in Sicily adds to the complexity of the situation in the Mediterranean. We cannot determine if the Sicilian population belongs to an African lineage, a southeastern West Palearctic lineage, or if the group is truly distinct—perhaps having survived the last glaciation in a Sicilian refugium. Alternatively, the distinctiveness in the mitochondrial gene COI in this population could have been caused by more recent, external factors such as an infection with a unique *Wolbachia* strain (e.g., Kodandaramaiah et al., 2013) as *Wolbachia* may be widespread in Odonata (Sazama et al., 2017). We suggest that future studies into the *L. virens* complex in the Western Palearctic should not only aim to include samples from North African, southeastern European, and Asian population, but also include nuclear molecular markers such as microsatellites or genome sequencing.

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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 Matrial S1: PopART file for the 43 sample. 550 bp haplotype network analysis in the nexus format

Supplementary Matrial S2: PopART file for the 48 sample. 318 bp haplotype network analysis in the nexus format

Supplementary Matrial S3: MrBayes file for the 46 sample. 550 bp haplotype phylogenetic analysis in the nexus format

Supplementary Figure S4: Minimum-spanning haplotype network based on the 318 bp COI dataset.