Italian Odonates in the Pandora's Box: A Comprehensive DNA Barcoding Inventory Shows Taxonomic Warnings at the Holarctic Scale

Running Title: DNA barcoding and taxonomic warnings in Odonata

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Abstract

Freshwater macroinvertebrates, and specifically Odonata, are considered among the most endangered faunal groups. Their biomonitoring has been progressively supported by DNA-based tools whose performance and accuracy rely on validated reference datasets that, in some cases, are lacking or do not cover important biogeographical centres of diversification.

This study reports the results of a DNA barcoding campaign on Odonata, involving the collection of 812 specimens (448 of which barcoded) from Italy and its main islands (328 localities), belonging to the 88 species (31 Zygoptera and 57 Anisoptera) inhabiting the country. Additional BOLD and GenBank data from Holarctic samples of the same taxa expanded the dataset to 1294 DNA barcodes. An integrative species delimitation analysis involving two distance (OT and ABGD) and four tree-based (PTP, MPTP, GMYC, bGMYC) approaches identified warnings of possible taxonomic relevance. Of the 88 investigated species, 85% could be successfully identified by their DNA barcodes, with damselflies showing a percentage of warnings (29%) higher than dragonflies (7%), contrasting with the other available DNA barcoding studies on Odonata (showing up to 95% of identification success). The species causing warnings were grouped in three categories depending on if they showed low, high or mixed genetic divergence patterns. Moreover, for the second class of warnings, the analysis of haplotypes revealed unexpected structure at the Italian, Palearctic and Holarctic scale. Overall, the DNA barcoding inventory assembled in this study will provide valuable insights into the systematics and conservation of many odonate species with implications for future DNA and eDNA monitoring-based studies.

Keywords: Anisoptera, BOLD, Cryptic species, Odonata, Species delimitation, Zygoptera

Introduction

Modern conservation efforts rely on the availability of comprehensive species inventories, particularly when implemented at the regional and local fine-scale (Morinière et al., 2019; Weigand et al., 2019; Altermatt et al., 2020). However, in most cases, morphology alone makes it hard to complete such inventories, so that improved taxonomic methods, based on technological and analytical advances, have become fundamental to support and integrate the study of biodiversity (Padial et al., 2010; De Salle & Goldstein, 2019; Schmid-Egger et al., 2019). In this context, since the early 2000s, DNA barcoding has become a reliable basis for assembling the reference sequence libraries necessary to identify specimens of known species, also enhancing species discovery in neglected or poorly investigated taxonomic groups (e.g., Galimberti et al., 2012; Dapporto et al., 2019; Morinière et al., 2019). Curated and comprehensive DNA barcode reference libraries such as the Barcode of Life Data System BOLD (Ratnasingham & Hebert, 2007; 2013) often allow fast and reliable species identification with a consistent saving in terms of time and resources when personnel and taxonomic expertise are limited. These reference databases constitute a keystone element for large biodiversity investigations associated with DNA and environmental DNA (eDNA) metabarcoding characterization of bulk or environmental samples (Porter & Hajibabaei, 2018; Creedy et al., 2020; Piper et al., 2019). Freshwater, in particular, is gaining more and more attention (Pawloski et al., 2018; Bush et al., 2019; Weigand et al., 2019) since it includes several of the most threatened ecosystems on Earth and there is an increasing need to improve the monitoring of its biodiversity and changes over time (Collen et al., 2014; Darwall et al., 2018).

Freshwater macroinvertebrates are among the most endangered faunal groups, which is even more worrying considering that they provide invaluable ecosystem functions (Borgwardt et al., 2019; May et al., 2019). Besides that, they are quickly reactive to habitat change and

deeply influenced by global phenomena (e.g., climate change, land-use change, pollution, and introduction of alien species; Strayer, 2006; Batzer & Boix, 2016; Kalkman et al., 2018, Termaat et al., 2019). For these reasons, they have been considered as an essential future addition to Europe's water monitoring programme (Termaat et al., 2019; Weigand et al., 2019). Among freshwater macroinvertebrates, a group of growing social and scientific interest is that of Odonata. It is a small order, by insect standards, counting about 6300 species worldwide (Schorr & Paulson, 2019) of which 144 were reported for Europe, with 51 and 93 species respectively belonging to the suborders Zygoptera (damselflies) and Anisoptera (dragonflies) (Boudot & Kalkman, 2015; Viganò et al., 2017). Although their moderate overall taxonomic diversity, odonates show a wide range of phenotypic and ecological adaptations and therefore constitute excellent models for ecological and evolutionary studies (Bybee et al., 2016). Due to their complex aquatic/terrestrial life cycle, high variability in life-history traits across species, and fully predatory habits, odonates are a group of primary scientific and historical importance as indicators of the quality and integrity of aquatic ecosystems (Flenner & Sahlén, 2008; Hassall, 2015). The European Red List reports 21 species considered to be threatened (plus 15 near threatened) and other 16 are listed in the Annexes II and/or IV of the European Union's Habitat Directive (Council Directive 92/43/EEC), thus making odonates one of the most relevant bioindicators to address conservation issues in freshwater ecosystems (Sahlén et al., 2010; Boudot & Kalkman 2015; Kalkman et al., 2018; Weigand et al., 2019). Traditionally, odonate taxonomy has relied on morphological data that could be biased by the seasonality, rarity or scattered occurrence of some species, as well as by problems of detectability, difficulties in larval/exuviae identification, the occurrence of cryptic species, and even high introgression rates (Marinov 2001; Bried, D'Amico, & Samways, 2012; Solano et al., 2018; Bried & Hinchliffe, 2019). In this context, the integration of traditional field protocols with

alternative strategies, such as DNA and eDNA-based approaches, could support fast and accurate species identification and monitoring.

Given the high relevance of molecular tools in freshwater surveys and the suitability of the mtDNA COI barcode region in these studies (Elbrecht & Leese 2017; Andùjar et al., 2018; Hajibabaei et al., 2019), international projects have been launched to fill the gaps in molecular species inventories (e.g., the EU COST Action DNAqua-NET https://dnaqua.net/). Odonata, together with Trichoptera, Hemiptera, and crustaceans are the best covered taxa in the BOLD System database, with ≥80% of European species barcoded (Weigand et al., 2019). However, such estimates do not take into account possible biases due to the absence of representatives from different European refugia (e.g., the Italian and the Balkan peninsulas) or areas of biogeographical/speciation interest, and the absence of genetic information (including DNA barcoding data) for some species of conservation interest.

Europe can be considered the cradle of odonate systematics, but the evolutionary history and radiation of many genera, especially in refugia areas, is still unresolved (e.g., *Calopteryx*, *Ischnura* and *Onychogomphus*, see Dijkstra & Kalkman, 2012). Moreover, we are still far from having a reliable reference DNA barcoding database for European odonate species, as some regions have been poorly sampled (e.g. Italy and the Balkan peninsula). Like the other southern European peninsulas, Italy (including its main islands, Sardinia and Sicily) is a major biodiversity hotspot for odonates with 89 breeding species (Riservato et al., 2014a; http://www.odonata.it/libe-italiane/), of which 19 are listed as threatened or near threatened in the Italian Red List (Riservato et al. 2014b) and 10 included in the European "Habitat" Directive. The peculiar biogeographic history of Italy and its high diversity of habitats and altitudinal gradients, make this peninsula particularly rich in terms of speciation and intraspecific diversification events for many animal groups, often with narrow distribution and

high conservation importance (e.g., Ghielmi et al., 2016; Wauters et al., 2017; Zuccon et al., 2020). However, almost no genetic information is available in public databases for Italian odonates with less than 100 COI barcode sequences available there, mostly belonging to a few *Cordulegaster* and *Coenagrion* species.

In order to increase the availability of genetic information on European Odonata, in this study we aimed at *i*) creating the first comprehensive and well-curated reference DNA barcoding library of Italian species, based on intense national sampling encompassing the main regions of biogeographical interest; *ii*) investigating the diversity and distribution of mitochondrial lineages of Italian Odonata, using a multiple species delimitation approach based on both character-explicit and distance-based methods (*sensu* De Salle and Goldstein, 2019). Moreover, since almost all the species occurring in Italy are more widely distributed in Europe, Asia and North America, the same approach has also been performed at the Holarctic scale by creating a second comprehensive dataset including a mixture of Italian and non-Italian publicly available sequences.

Materials and Methods

Specimen collection, identification and archiving

During 2018-2019, a network of 20 professional and voluntary taxonomists (including the authors), involved in the "DNA barcoding Italian odonates" project, collected odonates in six macro-regions of Italy (see Figure 1). Such regions correspond to the principal cradles of intraspecific genetic divergence previously identified in other taxa (e.g., Dapporto et al., 2010; Bernini et al., 2016; Wauters et al., 2017; Dufresnes et al., 2019; Scalercio et al., 2020).

Sampling sites included 328 localities scattered in most of the Italian administrative regions and were mostly wet ecosystems belonging to protected or not protected areas from the

Alpine (up to 2300 m a.s.l.), peninsular and insular (i.e., Sardinia and Sicily) regions. For the sampling of protected species, the Italian Ministry of the Environment issued a special permit (Prot. DPN 0031783.20-11-2019).

Only adults were sampled by hand netting and identified according to Dijkstra & Lewington (2006). In some cases, especially for damselflies belonging to *Coenagrion* and *Chalcolestes* genera, fine characters diagnostic for species identification (e.g., pronotum, cerci and ovipositor) were checked using a stereomicroscope (Olympus SZX7). The nomenclature used in this study follows the last update of the World Odonata List (Schorr and Paulson, 2019). For each individual, one or two middle legs were stored in 96% EtOH before DNA extraction. All samples and most specimens were vouchered and stored in the MIB:ZPL collection (University of Milano - Bicocca, Milan, Italy) whereas the remaining specimens are conserved in GA private collection.

A total of 812 specimens belonging to 88 morphospecies (31 Zygoptera and 57 Anisoptera) were collected. Sampling localities and details are available in BOLD (http://www.boldsystems.org/index.php) under the project code ZPLOD and are also reported in Appendix S1. Sampling location coordinates were given in decimal degrees (EPSG: 4326) and approximated at the first decimal unit (i.e., ≈ 11 km). Since many species (or biotopes) are of conservation concern, we chose not to give details on exact locations (Lunghi et al., 2019).

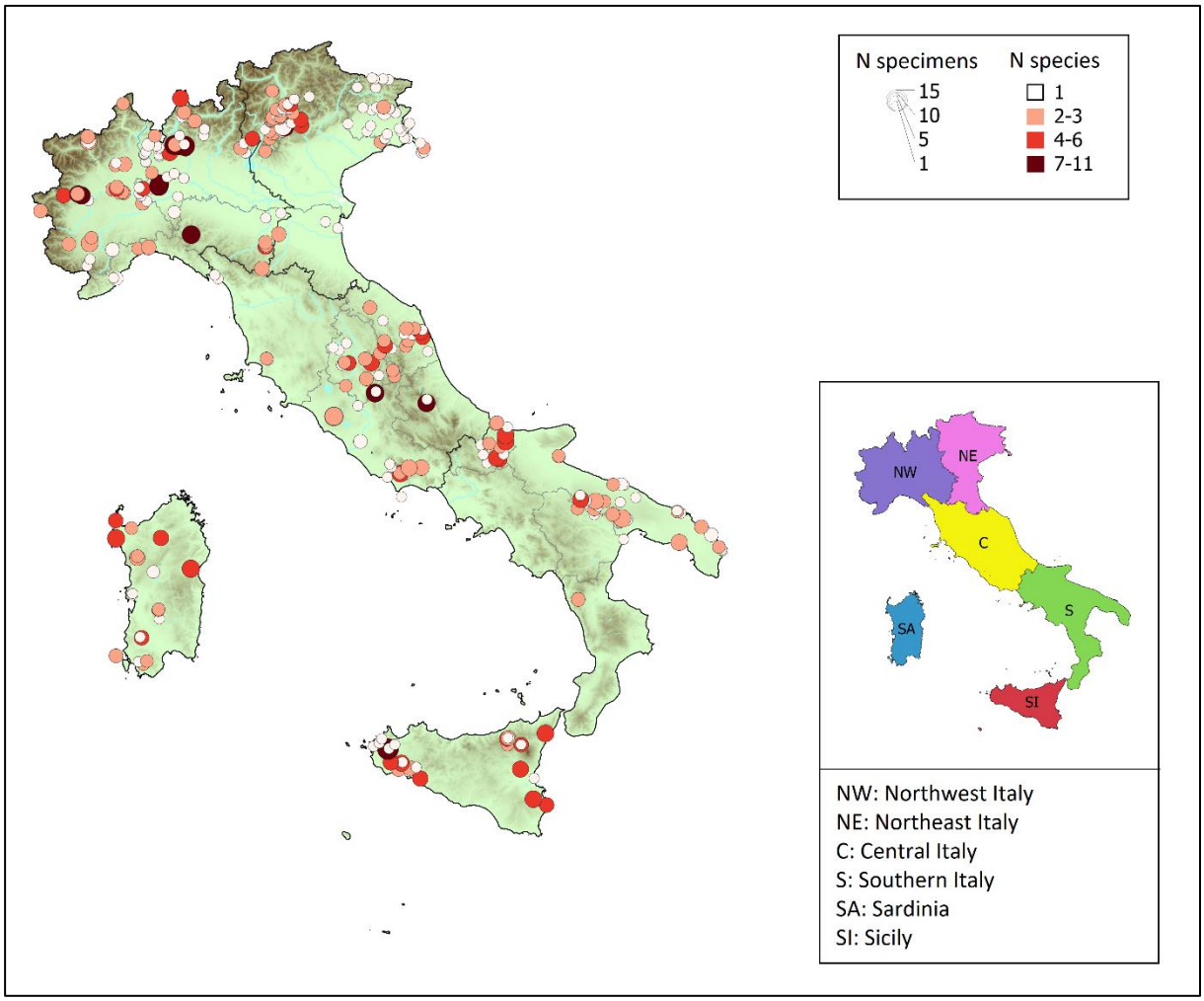

FIGURE 1: Collection sites for the sampled 88 odonate species included in the present study. The left map shows the number of specimens and the number of collected species per site indicated by circle size and colour respectively. The right map shows the borders of the macro-regions used for the analysis of genetic distances and structure of each species. Details of the 328 sites and collected and DNA barcoded specimens are provided in Appendix1. This map was created in QGIS version 3.4.13 (https://www.qgis.org/it/site/).

DNA extraction, amplification and sequencing

For each morphospecies, samples for genetic analysis were selected from distant sites in order to maximize the chance of observing intraspecific geographic variation. Overall, 409 specimens were selected for DNA barcoding characterization. DNA was extracted from a middle leg (Zygoptera) or a coxa (Anisoptera) using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Milan, Italy) following the manufacturer's instructions.

For each sample, the standard DNA barcode 5'-end region of the mitochondrial COI gene (658 bp) was amplified with the M13-tailed primers ODOF1_t1 and ODOR1_t1 (Semotok, unpublished, Source: BOLD Systems primer database), previously used by Koroiva and co-workers (2017). In case of unsuccessful amplification, the alternative COI primers ODO_LCO1490d/ ODO_HCO2198d (Dijkstra et al., 2014) were adopted to amplify the selected region. In both cases, PCR amplification conditions were as follows: 94°C for 5 min, 35 cycles at 94°C for 60 s, 50°C for 90s and 72°C for 60s, and a final extension at 72°C for 7 min. PCRs were conducted as in Mazzamuto et al. (2016) and sequencing was performed bi-directionally at Eurofins Genomics (Milan, Italy), with primers M13F and M13R for the ODOF1-ODOR1_tl fragment and with the same PCR primers in the other case. Consensus sequences were obtained by editing the electropherograms with Bioedit 7.2 (Hall, 1999). After primer trimming, the presence of open reading frame was verified for the obtained consensus sequences by using the on-line tool EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/), then sequences were aligned with MAFFT 7.110 (Katoh & Standley, 2013) using the *E-INS-i* option. Consensus sequences were deposited in the BOLD Systems (project code ZPLOD) and in GenBank (accession numbers reported in Appendix S1 and Appendix S2).

Sequence mining and datasets assembling

Orthologous COI barcode sequences, belonging to the species included in the Italian odonate list (Riservato et al., 2014a) were downloaded from public repositories (i.e., BOLD and GenBank). Sequences showing insertions/deletions, > 1% of missing sites or overlapping less than 500 bp with the COI region amplified and sequenced for the Italian samples, were discarded. Overall, a total of 885 COI barcode sequences were retrieved from public

repositories. These nucleotide sequences and those obtained in the present study were grouped in two datasets: (i) dataset DS1, composed by the DNA barcoding sequences produced in this study on Italian samples (i.e., 409) plus 69 sequences from Italian samples of 10 odonate species analysed in previous studies (see Appendix S1); (ii) dataset DS2, composed by the sequences mined from online databases plus dataset DS1 and other COI barcode sequences obtained from additional samples retrieved by the authors in other western Palearctic countries (See Appendix S2). The two datasets were kept separated in order to evaluate first the efficiency of the multi-approach species delimitation and to detect taxonomic warnings (e.g., reduced interspecific genetic variability or high intraspecific structure at the COI barcode locus) among Italian reference specimens only (DS1). Then, to better investigate such warnings on a wider geographic scale and find new cases of possible taxonomic relevance, the same species delimitation strategy was adopted on sequence data from across the Holarctic range of the taxa occurring in Italy (DS2).

Species delimitation analysis

Phylogenetic trees were reconstructed separately for Zygoptera and Anisoptera, because of the putative differences in rates of genetic evolution in some families (Bybee et al., 2016; Koroiva & Kvist, 2017). First, identical sequences were collapsed into unique haplotypes (See Appendix S1 and Appendix S2) and appropriate substitution models were determined using JModelTest 2 (Darriba et al., 2012) using the Akaike Information Criterion (AIC), the corrected Akaike Information Criterion (AICc), and the Bayesian Information Criterion (BIC). Phylogenetic inference analyses were performed using two optimality criteria: Bayesian inference (BI) and maximum likelihood (ML). BI analyses were performed using BEAST 1.8.2 (Drummond et al., 2012). A coalescent tree prior and the heterogeneity of the mutation rate

across lineages were set under an uncorrelated lognormal relaxed clock. Three replicate analyses were run for 10^8 million generations with a sampling frequency of 10,000 and were combined using LogCombiner 1.8.2 (Drummond et al., 2012) with a burn-in set to 25%, after checking the stationarity for effective sampling size and unimodal posterior distribution using Tracer v.1.6 (Rambaut et al., 2014). Maximum clade credibility trees were computed using TreeAnnotator 1.8.2 (Drummond et al., 2012). ML analyses were performed using RAxML 8.2.9 (Stamatakis, 2014) with 1000 bootstrap replicates. Each sub-order dataset was rooted using two sequences belonging to the other dataset (*Nehalennia speciosa* (Charpentier, 1840) and *Pyrrhosoma nymphula* (Sulzer, 1776) for Anisoptera, *Libellula quadrimaculata* Linnaeus, 1758 and *Lindenia tetraphylla* (Vander Linden, 1825) for Zygoptera). The obtained trees were visualised using FigTree v.1.4.3 (Rambaut, 2012) and customized using CorelDRAW X7. All analyses were run on CIPRES server (Miller et al., 2010).

Distance-based and tree-based species delimitation approaches (*sensu* De Salle & Goldstein, 2019) were employed to explore species boundaries in DS1, after removing the outgroups.

Distance-based methods rely on genetic distances to explore the presence of barcoding gaps and allow for species non-monophyly (Fontaneto et al., 2015). In this work, we used the Automatic Barcoding Gap Discovery (ABGD; Puillandre et al., 2012) and the Optimal Threshold (OT) (Brown et al., 2012). ABGD delimitations were run on the website https://bioinfo.mnhn.fr/abi/public/abgd/. Parameters were set as follows: Pmin = 0.001, Pmax = 0.1, Steps = 100, X = 1, Nb bins = 20, and all the three available models were tested (*p*-distance, Kimura 2-parameter, and Jukes Cantor). OT was calculated from the K2P genetic distance matrices with the 'LocalMinima' and 'treshOpt' functions of the SPIDER package (Brown et al., 2012) in R (R Core Team, 2019). In particular, OT is the genetic distance value

that minimizes the cumulative identification error, that is the sum of false positive (no conspecific matches within threshold of query) and false negative (sequences from multiple species within threshold) cases (Galimberti et al., 2012).

Tree-based methods require species monophyly and are based on the analysis of branching rates (Fontaneto et al., 2015). Here, the Poisson Tree Process (PTP; Zhang et al., 2013) and Generalized Mixed Yule-Coalescent (GMYC; Pons et al., 2006; Fujisawa & Barraclough, 2013) models were used. Single-threshold PTP analyses (Zhang et al., 2013) were performed on the website http://species.h-its.org/ptp, using both ML and BI phylogenetic trees, and running analyses for 400,000 MCMC generations, with thinning value = 100 and burn-in = 0.25, whereas multiple-threshold PTP (MPTP) analyses (Kapli et al., 2017) were run on the website https://mptp.h-its.org. Single-threshold GMYC (Pons et al., 2006) and bGMYC (Reid & Carstens, 2012) analyses were run using BI ultrametric trees and were performed in R (R Core Team, 2019) using the packages SPLITS (Ezard et al., 2009), APE (Paradis et al., 2004), and bGMYC (Reid & Carstens, 2012). bGMYC analyses were performed on a subset of 100 trees retrieved from the 10,000 trees obtained with each BI analysis.

Apart from OT, all the other species delimitation approaches were independent, requiring no *a priori* information on the existing morphospecies. Overall, we considered a delimitation to be reliable only when a minimum of four methods out of six were in agreement, and when the delimitation was supported by at least one distance-based and two tree-based analyses.

The same pipeline was applied to the Zygoptera and Anisoptera COI sequences alignments of DS2. Conversely, OT values remained the same inferred from DS1 because reliable a priori information on morphospecies was available only for the reference samples collected in this study. When mismatches occurred between delimitation based on DNA

barcoding data and morphospecies assignment based on samples collected in this study (DS1) and/or inferred from public data (DS2), we identified a 'warning'.

Warnings investigation

To better support the main categories of warnings resulting from species delimitation and to explore patterns of intraspecific geographic genetic variation, multi-locus genetic distance comparisons and/or haplotype networks were generated for selected species groups. In particular, public nucleotide sequences of the mitochondrial 16s rDNA and the nuclear AgT, PRMT and MLC genes were retrieved from GenBank for *Coenagrion mercuriale* (Charpentier, 1840), *C. puella* (Linnaeus, 1758), *C. pulchellum* (Vander Linden, 1825) and *C. ornatum* (Sélys, 1850). These nuclear markers were selected among the others as they were found to show a higher phylogeographic resolution in damselflies (Ferreira et al., 2014a). Genetic distances among species or lineages were computed using MEGA X (Kumar et al., 2018), and intra- and inter-clade uncorrected p-distances were calculated with 1000 bootstrap replicates. In case of intraspecific delimitation, and when at least 20 COI barcode sequences were available from specimens collected in Italy and across the species Holarctic range, unrooted minimum spanning COI networks were obtained using the median-joining algorithm implemented in PopART 1.7 (Leigh & Bryant 2015).

Results

A reference DNA barcoding database of Italian odonates

We obtained high-quality full-length COI DNA barcode sequences (658 bp) for all the 409 selected specimens (see Appendix S1 for BOLD Process IDs and GenBank accession

numbers), representing 88 morphospecies belonging to 36 genera and 10 families. No sequences contained insertions/deletions (indels), stop codons, or were biased by NUMT interference, with the only exception of one *Platycnemis pennipes* (Pallas, 1771) specimen (ZPLOD687-20) and one *Orthetrum cancellatum* (Linnaeus, 1758) specimen (ZPLOD613-20) with the primer pair ODOF1_t1/ODOR1_t1. Re-amplification and sequencing with the other primer set (i.e., ODO_LCO1490d/ODO_HCO2198d) allowed obtaining the correct DNA barcode sequence for these samples.

The number of newly barcoded specimens per species ranged from 1 to 34 (mean ± SD = 4.6 ± 4.6); 8 morphospecies were represented by a single accession (Appendix S1). The obtained DNA barcode sequences allowed to add two Italian (and Palearctic) species (i.e., *Orthetrum nitidinerve* (Sélys, 1841) and *Somatochlora meridionalis* Nielsen, 1935) not represented at all in the BOLD and GenBank databases, and novel haplotypes for almost all the investigated taxa. To date, the DNA barcoding reference dataset presented in this study ranks first in terms of the number of specimens, species and produced barcode data at the European level (BOLD taxonomy statistics, Accessed April 1st, 2020).

Considering the 69 Italian DNA barcode records mined from GenBank, a DNA barcoding reference library of 478 COI sequences from 88 species was established (i.e, DS1, see Appendix S1). The analysis of this dataset showed that genetic K2P-distance variation within morphospecies ranged

from 0% to 9.17% (mean ± SD= 0.48 ± 0.62 %) in Zygoptera and from 0% to 2.64% (mean ± SD = 0.33 ± 0.29 %) in Anisoptera. Interspecific K2P-distance values ranged from 0% to 27.29% (mean ± SD= 19.41 ± 3.71%) and from 0% to 25.28% (mean ± SD= 18.25 ± 3.18 %) in Zygoptera and Anisoptera, respectively.

The distributions of intraspecific and interspecific K2P distances overlap, thus resulting in the absence of a complete barcode gap in DS1. Both ABGD and OT approaches identified a number of groups quite lower than the collected morphospecies (Table 1). In particular, the optimal threshold that minimises the number of false positive and false negative identifications resulted in 3.16% and 1.96% of K2P distance for Zygoptera and Anisoptera respectively, with associated cumulative errors 24.1% (57 sequences out of 237) and 9.96% (24 sequences out of 241) and mainly due to false negative cases. Overall, the Maximum intraspecific vs. Nearest Neighbour and Mean intraspecific vs. Nearest Neighbour analyses conducted in BOLD, confirmed the occurrence of a barcode gap for the vast majority of investigated species groups (Appendix S3).

We obtained further 816 DNA barcode records of the same species included in DS1 from BOLD and GenBank referred to Holarctic specimens. These were assembled in a comprehensive DNA barcoding inventory of 1294 COI sequences (i.e, DS2, see Appendix S2). Other available COI sequences were discarded because they were shorter than 500 bp or containing >1% of missing positions or stop codons. In DS2, Genetic K2P-distance variation within morphospecies ranged from 0% to 8.81% (mean ± SD = 0.69% ± 0.78%) in Zygoptera and from 0% to 6.72% (mean ± SD = 0.59% ± 0.63%) in Anisoptera. Interspecific K2P-distance values ranged from 0% to 27.29% (mean ± SD = 19.51 ± 3.67%) and from 0% to 26.69% (mean ± SD = 18.09% ± 3.17%) in Zygoptera and Anisoptera, respectively. In contrast to DS1, the two genetic distance-based approaches (i.e., ABGD and OT) identified in DS2 a number of groups equal or quite higher than the collected morphospecies (Table 1). To better understand this unexpected result, a multi-approach species delimitation was conducted on both datasets.

Species delimitation

Species delimitation analysis was conducted first on the reference DNA barcoding DS1 dataset and was based on separate phylogenetic trees encompassing, respectively, the damselfly and dragonfly morphospecies occurring in Italy (see Figure 2 Zygoptera and Figure 3 Anisoptera). As highlighted in the trees and reported in Table 1, the overall number of groups delimited upon the criteria explained in Material and Methods, was quite lower than the number of recognized morphospecies (i.e., 28/31 in Zygoptera and 56/57 in Anisoptera). In general, the congruence between the delimited groups and the morphology-based identifications ('no warnings' in Table 1) was higher in Anisoptera with 52 out of 57 (91.2%) cases than in Zygoptera, with 21 out of 31 (67.7%) cases. Most of the warnings were due to reduced or no interspecific genetic diversity with seven and four morphospecies involved in Zygoptera and Anisoptera, respectively. Conversely, one case in both suborders was related to high genetic divergence at the intraspecific level (i.e., *Erythromma lindenii* (Sélys, 1840) and *Onychogomphus forcipatus* (Linnaeus, 1758)). Finally, in two damselfly species (i.e., *Chalcolestes viridis* (Vander Linden, 1825) and *C. parvidens* Artobolevskii, 1929), the pattern of genetic divergence was a mix of the other two warning categories with one sample (ZPLOD168-20), morphologically identified as *C. viridis*, included in the group of *C. parvidens* (see Figure 2, Figure 3 and Table 1).

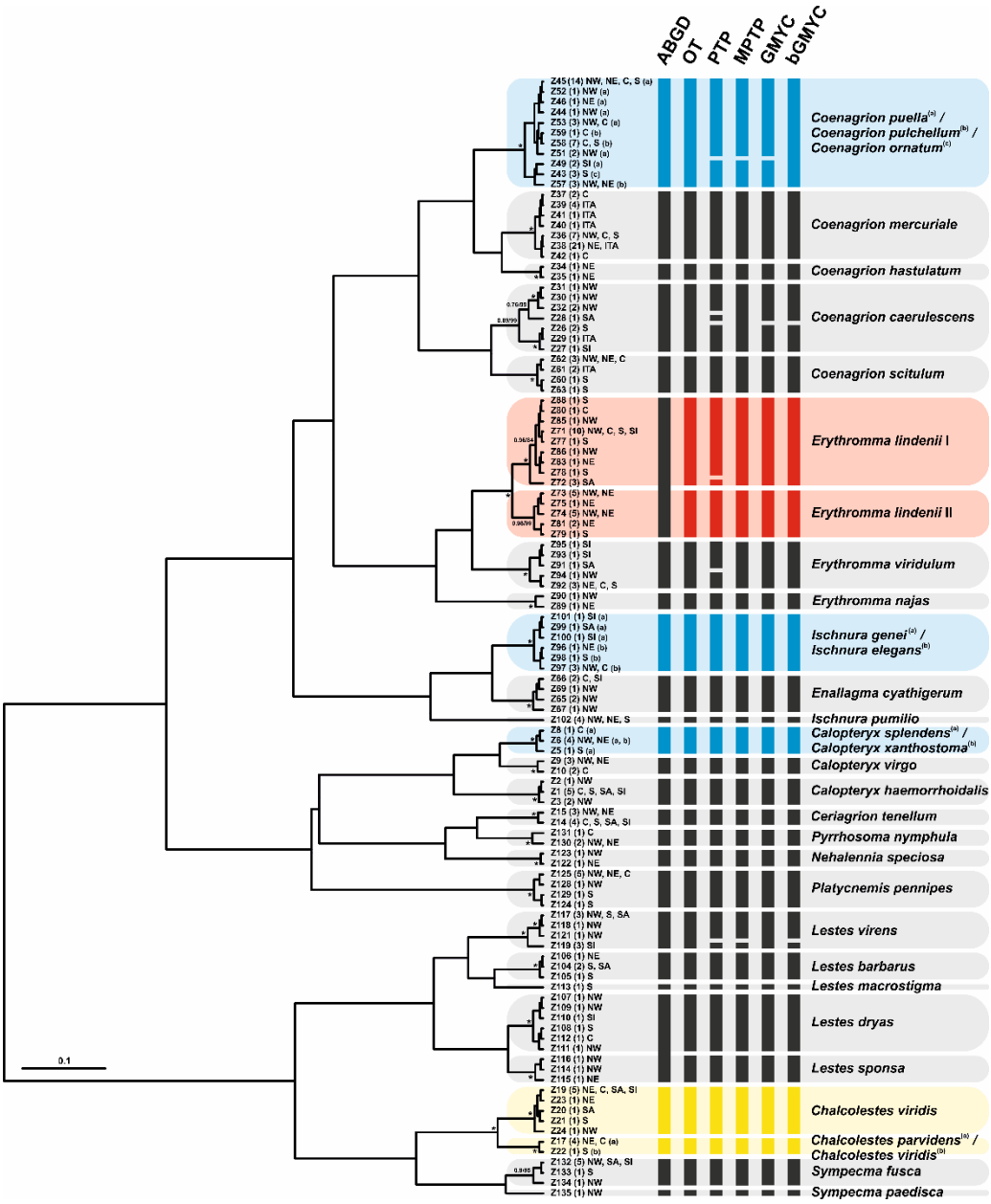

FIGURE 2: Multi-approach species delimitation of the 31 Italian Zygoptera morphospecies analyzed in this study, based on COI DNA barcode sequences from DS1. A Bayesian tree is used as a base to summarize the two threshold-based (OT and ABGD) and four character explicit-based (PTP, MPTP, GMYC, bGMYC) approaches. Specimen haplotype identifiers are reported on tips (see Appendix S1 for further details), the number of specimens sharing each haplotype is reported within brackets and abbreviations NW, NE, C, S, SI and SA correspond to the geographic haplotype origin as schematized in Figure 1 (ITA corresponds to unknown locality in Italy). Numbers above nodes represent BPP and BS, respectively, whereas asterisks correspond to maximal node support (BPP $\geq$ 0.99 and BS $\geq$ 95). Vertical colored solid boxes delimit putative species identified by the different approaches. Black: delimitation congruent with the identified morphospecies; Red: intraspecific delimitation; Light Blue: no interspecific delimitation; Yellow: Mixed species delimitation. In the case of intraspecific delimitation, each lineage has been numbered with Roman numerals. This tree was created with BEAST 1.8.2.

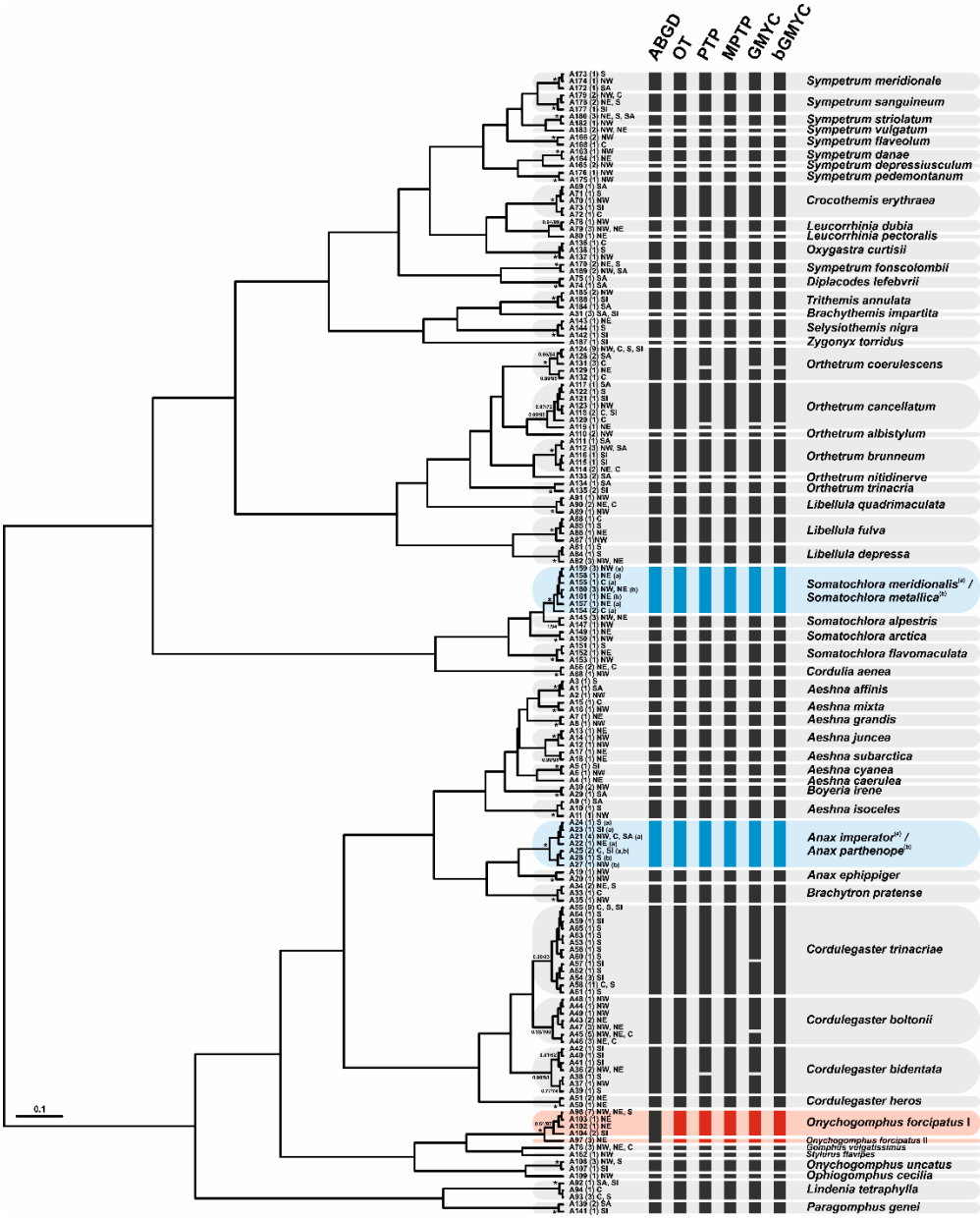

FIGURE 3: Multi-approach species delimitation of the 57 Italian Anisoptera morphospecies analyzed in this study, based on COI DNA barcode sequences from DS1. A Bayesian tree is used as a base to summarize the two threshold-based (OT and ABGD) and four character explicit-based (PTP, MPTP, GMYC, bGMYC) approaches. Specimen haplotype identifiers are reported on tips (see Appendix S1 for further details), the number of specimens sharing each haplotype is reported within brackets and abbreviations NW, NE, C, S, SI and SA correspond to the geographic haplotype origin as schematized in Figure 1. Numbers above nodes represent Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap values (BS), respectively, whereas asterisks correspond to maximal node support (BPP $\geq$ 0.99 and BS $\geq$ 95). Vertical colored solid boxes delimit putative species identified by the different approaches. Black: delimitation congruent with the identified morphospecies; Red: intraspecific delimitation; Light Blue: no interspecific delimitation. In the case of intraspecific delimitation, each lineage has been numbered with Roman numerals. This tree was created with BEAST 1.8.2.

TABLE 1: Species delimitation results. The table provides the number of groups delimited by each distance-based (ABGD and OT) and tree-based (PTP, MPTP, GMYC and bGMYC) approach conducted on the DNA barcode sequences of Italian odonate morphospecies grouped by suborder (Zygoptera and Anisoptera) and by DNA barcoding dataset (DS1 and DS2). The number of overall delimited groups, groups for each warning category and involved species (within brackets) are also reported.

			Species delimitation approaches and n of delimited groups							Warnings: n of cases (n of involved species)			
		n morphospecies	ABGD	OT	PTP	MPTP	GMYC	bGMYC	Overall n of groups	no warnings	no interspecific delimitation	intraspecific delimitation	mixed species delimitation
Zygoptera	DS1	31	26	28	34	30	30	30	28	21	3(7)	2(1)	2(2)
	DS2	31	32	31	33	32	36	40	32	19	2(4)	7(3)	4(5)
Anisoptera	DS1	57	55	56	59	55	61	58	56	52	2(4)	2(1)	0
	DS2	57	57	62	63	55	59	63	61	48	2(4)	11(5)	0

In contrast to DS1, the overall number of delimited groups in DS2 was higher than the number of investigated species (i.e., 32/31 in Zygoptera and 61/57 in Anisoptera). Overall, the warnings found in DS1 were almost all confirmed in DS2 (Table 1 and Appendix S4) and new 17 cases, belonging to 7 species (3 damselflies and 4 dragonflies) were found due to the new BOLD and GenBank DNA barcode sequences included in this dataset. Table 2 provides a list of the warning cases outlined in DS2.

In Zygoptera, several changes occurred in species delimitation between DS1 and DS2 involving all the three categories of warnings (Table 1, Table 2 and Appendix S4). Warnings related to a reduced interspecific genetic divergence slightly decreased because *Coenagrion puella*, which in DS1 was delimited in a single group with *C. pulchellum* and *C. ornatum*, in DS2 showed a mixed delimitation pattern due to the inclusion of some North African highly divergent DNA barcode sequences, already reported by a previous study (Ferreira et al., 2014). The remaining two cases belonging to this category involved the species pairs *Ischnura elegans* (Vander Linden, 1820) / *I. genei* (Rambur, 1842) and *Calopteryx splendens* (Harris, 1780) / *C. xanthostoma* (Charpentier, 1825). Conversely, the warning cases (and species) of intraspecific delimitation increased with *Coenagrion mercuriale* and *Lestes dryas* Kirby, 1890 showing three

and two lineages respectively, whereas the same two lineages of *Erythromma lindenii* found in DS1 were confirmed. Finally, apart from the already cited case of *C. puella* and close congenerics, *Chalcolestes viridis* and *C. parvidens* showed a mixed delimitation pattern, similarly to DS1. In Anisoptera, the groups of species not delimited remained the same of DS1 (i.e., *Anax imperator* Leach, 1815 / *A. parthenope* (Sélys, 1839) and *Somatochlora metallica* (Vander Linden, 1825) / *S. meridionalis*), whereas the number of warnings due to high intraspecific delimitation increased from two (one species) to 11 (five species). Concerning these lasts, two lineages were delimited in *Aeshna juncea* (Linnaeus, 1758), *Onychogomphus forcipatus*, *Stylurus flavipes* (Charpentier, 1825) and *Sympetrum danae* (Sulzer, 1776), while three groups were delimited from *Orthetrum albistylum* (Sélys, 1848) DNA barcode sequences.

TABLE 2: List of odonate species showing warnings as a result of species delimitation on DS2. The warning category (as in Table 1), the number of delimited groups and the geographic overall provenance of DNA barcode sequences included in DS2 for each species group are also reported.

Suborder	Involved morphospecies	warning category	n of delimited groups	Geographic scale	Also occurring in DS1?
Zygoptera	*Coenagrion mercuriale*	intraspecific delimitation	3	Palearctic	no
Zygoptera	*Coenagrion puella / Coenagrion pulchellum / Coenagrion ornatum*	mixed species delimitation	2	Palearctic	no
Zygoptera	*Erythromma lindenii*	intraspecific delimitation	2	Palearctic	yes
Zygoptera	*Ischnura genei/ Ischnura elegans*	no interspecific delimitation	1	Palearctic	yes
Zygoptera	*Calopteryx splendens / Calopteryx xanthostoma*	no interspecific delimitation	1	Palearctic	yes
Zygoptera	*Lestes dryas*	intraspecific delimitation	2	Holarctic	no
Zygoptera	*Chalcolestes viridis / Calcholestes parvidens*	mixed species delimitation	2	Palearctic	yes
Anisoptera	*Aeshna juncea*	intraspecific delimitation	2	Holarctic	no
Anisoptera	*Anax imperator / Anax parthenope*	no interspecific delimitation	1	Palearctic	yes
Anisoptera	*Onychogomphus forcipatus*	intraspecific delimitation	2	Palearctic	yes
Anisoptera	*Stylurus flavipes*	intraspecific delimitation	2	Palearctic	no
Anisoptera	*Orthetrum albistylum*	intraspecific delimitation	3	Palearctic	no
Anisoptera	*Sympetrum danae*	intraspecific delimitation	2	Holarctic	no
Anisoptera	*Somatochlora metallica / Somatochlora meridionalis*	no interspecific delimitation	1	Palearctic	yes

Warnings investigation

Some warning cases, resulting from the species delimitation of DS2, involved odonate species represented by a high number of publicly available DNA barcode sequences from different geographic regions or by the availability of multi-locus data deposited in GenBank. This data allowed to better investigate the geographic distribution of genetic diversity at the COI barcode region for at least four Italian species, also distributed at the Palearctic or Holarctic scale (two Zygoptera, *Coenagrion mercuriale* and *Lestes dryas* and two Anisoptera, *Aeshna juncea* and *Sympetrum danae*, see Figure 4). The three groups of *C. mercuriale* DNA barcodes delimited in DS2 correspond precisely to three distinct haplogroups that are exclusive to Europe (except for Italy), North Africa and Italy, showing no shared haplotypes and high values of K2P and uncorrected *p*-distance among them (Figure 4, Appendix S5). Such isolation and genetic distinctiveness are also supported by uncorrected genetic *p*-distance values at another mitochondrial (16s rRNA) and three nuclear (AgT, PRMT, MLC) loci sequences retrieved in GenBank (see Appendix S5).

In the other three species depicted in Figure 4, the European (and Italian) haplotypes were clearly separated from the North American ones, supporting the delimitation inferred from DS2. The number of substitutions characterizing this Holarctic disjunction was more than double in *S. danae* with respect to *A. juncea* and *L. dryas* delimited groups, with the only exception of one *S. danae* haplotype from Canada (A388, see Appendix S2) that is much closer to the Western Palearctic haplogroup.

Additional nuclear DNA sequences retrieved in GenBank allowed to better investigate the case of absent interspecific delimitation among *Coenagrion puella*, *C. pulchellum* and *C. ornatum*. However, these species were found to be clearly distinct at the three nuclear loci with no shared

haplotypes, thus indicating a mito-nuclear discordance pattern of genetic divergence (Appendix S5).

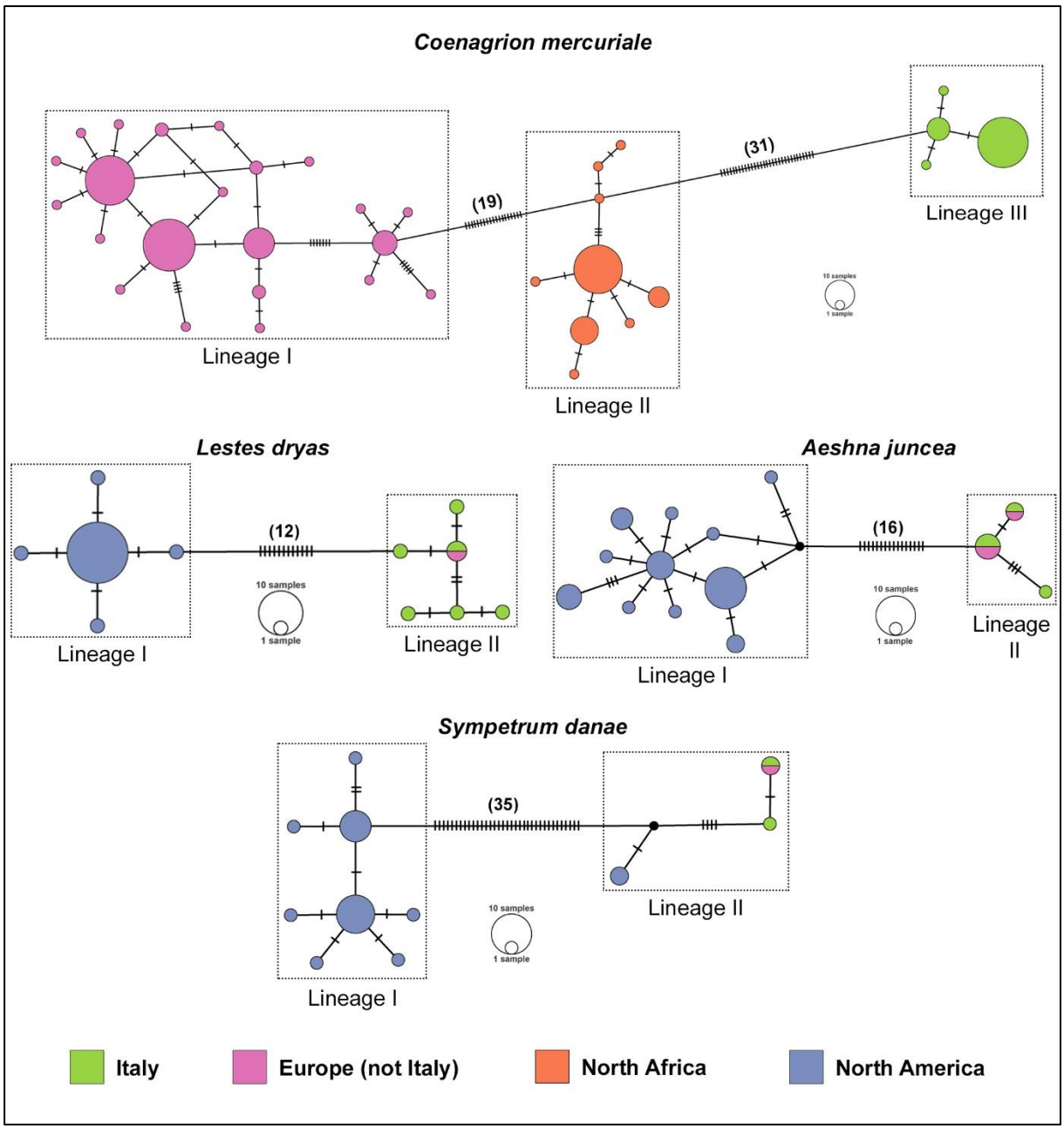

FIGURE 4: Median-joining network of COI DNA barcode haplotypes of four warning cases found by species delimitation of DS2 (*Coenagrion mercuriale*, *Lestes dryas*, *Aeshna juncea* and *Sympetrum danae*; see Appendix S2 and S4 for haplotypes and lineages subdivision). Each circle represents a haplotype and circle size is proportional to haplotype frequency. Colours indicate different sampling regions. Small black traits represent possible median vectors, while dashes represent substitutions (also indicated within brackets when > 10). Networks have been created with PopArt 1.7.

Discussion

DNA barcoding performance and reference dataset

The present study summarises the results of a DNA barcoding campaign on Italian Odonata. To the best of our knowledge, it is one of the most comprehensive DNA barcoding-based surveys on this insect order conducted on a national scale in the northern hemisphere. The performed sampling and sequencing, together with sequence data retrieved from public genetic databases, allowed to assemble a virtually complete inventory of the odonates for which breeding populations occur in Italy, with 88 of the 89 species successfully characterized by DNA barcoding. The only species not collected was *Ischnura fountaineae* Morton, 1905, for which only one isolated population is known to occur on Pantelleria island (Corso *et al.*, 2012). These data increase the available reference molecular data by adding novel sequences from Italian populations of the more widespread species, most of which distributed at the European, Palearctic, or Holarctic scales (Figure 4, Table 2). Overall, this work proved the effectiveness of DNA barcoding as a tool for identifying, and possibly discovering or questioning odonate species.

The multi-approach species delimitation showed high congruence between molecular groups and the Linnaean taxa with COI DNA barcodes, delimiting 85% of species in the reference Italian database as separate entities (71% and 93% in Zygoptera and Anisoptera respectively). Although most of the morphospecies are diagnosable, the identification performance of this study is lower with respect to other odonate DNA barcoding surveys, which have reported up to a 95% success rate (Bergmann et al., 2013). However, all the previous DNA barcoding studies regarding odonates were based on a lower number of specimens and morphospecies than the dataset considered in this study (e.g., Bergmann et al., 2013; Kim et al., 2014; Koroiva et al., 2017). Concerning sequence variation within and among

morphospecies, the average values obtained in this study are in line with those obtained by Koroiva and Kvist (2017) in their global overview of DNA barcoding of odonates, with the only exception of the mean intraspecific genetic variation in Italian Anisoptera (i.e., 0.33 DS1, 0.59 DS2), which is 4.8 (DS1) - 2.7 (DS2) times lower than the global average (i.e., 1.60). No more than a slight variation was observed in the average genetic distance values between DS1 and DS2. This was likely due to the higher completeness of DS2, where intraspecific variation was the result of the inclusion of samples from the whole Palearctic or Holarctic regions (Bergsten et al., 2012).

In the remaining cases (Table 1 and Table 2), the multi-approach species delimitation lumped the morphospecies or split them into additional molecular groups which, however, were not incongruent with the Linnaean species. This situation, and the finding that many of the species included in our dataset have been flagged as warnings, indicates that DNA barcoding could support the discovery, or the revision, of genetic entities that could eventually gain recognition as biological species or at least deserve taxonomic revision.

On the whole, the comprehensiveness of our DNA barcoding dataset and the many new haplotypes released on the BOLD public data portal, may add a great contribution to Odonata species identification for both theoretical (e.g., taxonomy, evolution, and biogeography) and applicative (e.g., eDNA-based biomonitoring) international research. Although their charismatic role and scientific relevance in biomonitoring and conservation issues, the use of odonates in eDNA-based studies is almost null, while a few studies of freshwater macrobenthos treated these insects marginally (e.g., Sun et al., 2019). The new molecular resources here provided will contribute to enhance the use of High Throughput Sequencing and improve the reliability of eDNA metabarcoding reads taxonomic assessment to monitor diversity and changes in odonate communities.

Species delimitation and warnings

Even considering the possible pitfalls in using a single genetic marker to infer species diversity and geographic structure, the use of a variety of sequences analysis methods and parameters can be adopted to detect warnings of possible taxonomic relevance (De Salle & Goldstein, 2019; Matos-Maravi et al., 2019). Warnings could be then used to plan subsequent researches aimed at assessing presumptive new species detected with these methods (Carstens et al., 2013). Sequence haplotypes, whose delimitation is supported by various character or distance-based approaches, could be considered as cases to better investigate. For example, by integrating morphological, multi-locus genetic assessment, ecological or other information, the molecularly-defined species could be either confirmed or rejected (Kajitoch et al., 2018; Dufresnes et al., 2019). On the other hand, morphological species that are inconsistently delimited at the molecular level could be designated for further analyses, such as geometric morphometrics (Solano et al., 2018) or population genomics (Dufresnes et al., 2020).

In this study, the multi-approach species delimitation conducted on DS1 showed that 32.3% of Zygoptera and 8.8% of Anisoptera species were interested by warnings (Table 1) and these percentages increased in DS2 (38.7% and 15.8%). While the discrepancy between the two datasets was due to the more geographic widespread sampling of DS2 (see also Bergsten et al., 2012), the higher warning rate in Zygoptera is more challenging to explain. In both datasets, damselflies were involved in all the three possible groups of warnings, that even occur simultaneously within the genus *Coenagrion* (Table 1, Figure 2, Appendix S4). The situation of Italian Zygoptera could confirm previous findings that recorded a wide variety of global biogeographical patterns among damselflies (e.g., Sánchez-Guillén et al., 2014a; Swaegers et al., 2015; Ferreira et al., 2016; Wellenreuther & Sánchez-Guillén, 2016). Such a situation could be partly due to the weaker dispersal ability of damselflies as compared to dragonflies (Heiser

& Schmitt 2010; Pinkert et al., 2018), which could have been more frequently resulted in isolation and diversification events. Besides, since dispersal ability is also driven by climate factors, it could have more accentuated consequences in the future due to the ongoing climate change. However, other studies (reviewed in Wellenreuther & Sánchez-Guillén 2016) point to sexual selection, and not ecological constraints, as playing the most important role in damselflies diversification over time and space.

Warnings identified by species delimitation of DS1 and DS2 provide evidence for different scenarios regarding Italian odonates taxonomy and conservation, with possible influence also at the Holarctic scale.

No interspecific delimitation

This category of warning encompasses four cases in DS2 that were already confirmed in the reference Italian sampling of DS1. The missing mitochondrial diversification in the damselflies species pairs *Ischnura elegans/I. genei* and *Calopteryx splendens/C. xanthostoma*, even though poorly investigated so far for Italian populations, was expected. The reproductive isolation of both species pairs in Mediterranean areas is indeed highly debated by entomologists with morphological and genetic evidence of introgressive hybridization, largely documented in *Ischnura* (Sánchez-Guillén et al., 2014a; 2014b) and less clear in *Calopteryx* (Dumont et al. 1993; Weekers et al., 2001). Conversely, the missing delimitation in *Somatochlora metallica/S. meridionalis* and *Anax imperator*/*A. parthenope* was unprecedented, also because no Italian samples and no COI barcode sequences at all for *S. meridionalis* were available before this study. This taxon was originally described as a subspecies of *S. metallica* (Nielsen, 1935) and elevated at species rank more recently (Schmidt, 1957); however, this status is still debated as intermediate specimens have been recorded in the contact areas (Fleck et al., 2007). *S.*

meridionalis replaces *S. metallica* in southern Europe; in Italy, both the morphospecies occur with the former distributed in central and southern portions of the peninsula (and in two disjunct and isolated populations in the North) and the latter mainly spread in northern regions. Both taxa are sometimes found syntopically (Boudot & Kalkman, 2015; Dijkstra & Kalkman, 2012) and lack clear structural differences apart from subtle, though stable, differences in colouration (Boudot & Kalkman, 2015) in the adults and minor differences at the larval stage (Seidenbusch, 1996). Species delimitation based on COI barcode sequences from specimens coming from all the three disjunct Italian ranges of *S. meridionalis* suggests that the two morphospecies in Italy are not distinguishable according to mitochondrial DNA. More challenging is the case of the two *Anax* species, which, contrary to *Somatochlora*, are well separated morphologically (but also according to their ecology and life-history traits), although they may share the same mitochondrial haplotypes. This scenario is not exclusive to Italian populations, as the same pattern was confirmed by DS2 European and Palearctic populations.

Intraspecific delimitation

Our results showed three subgroups of intraspecific delimitation warnings that can be classified based on their geographic extent.

The first group includes morphospecies that showed a marked genetic divergence within the Italian sampling (DS1), *Erythromma lindenii* and *Onychogomphus forcipatus*. Concerning the former taxon, it was initially described as *Agrion lindenii*, placed in the new genus *Cercion* by Navas (1907) and then recently assigned to its current genus by Weekeers & Dumont (2004). Across its vast range, it shows remarkably little morphological variation, but the eastern populations were described as *E. l. zernyi* Schmidt, 1938. This taxon occurs from Turkey eastward in isolated populations, surrounded by, and/or overlapping with the nominal subspecies; thus, a longitudinal introgression generating hybrid populations was hypothesized, although no genetic data has ever

confirmed this (Dumont et al., 1995). Unexpectedly, our Italian sampling, and the comparison with the few available European COI barcode sequences, provide evidence for the occurrence of two supported mitochondrial lineages within western Palearctic (Figure 2, Appendix S4), one of which completely new, and, based on these preliminary data, mainly occurring in Montenegro and north-eastern Italy.

Onychogomphus forcipatus has three recognized subspecies showing structural differentiation (both in adults and nymphs) and occurring in Europe, with discrete and mostly non-overlapping ranges. Of these, two subspecies are recorded in Italy, *O. f. forcipatus* being distributed only in the northeast and allegedly in Sicily, and *O. f. unguiculatus* (Vander Linden, 1823) being distributed in north-western and peninsular Italy (Boudot et al., 1990, Boudot & Kalkman, 2015). Our species delimitation approach identifies two distinct lineages as well (also supported in DS2); however, these do not show clear geographic separation, with a group including Sicilian, central, southern, and north-western Italy samples, but also several samples from north-eastern Italy and another encompassing a few samples from north-eastern Italy collected at several localities where the other lineage also occurs. In this sense, our study, as that by Ferreira et al. (2014b), is still not resolutive, and further integrated studies are needed to possibly solve this long-lasting taxonomic enigma.

The second group of intraspecific warnings emerged from the species delimitation of DS2 and encompasses lineages diverging at the Palearctic scale. The most interesting case is *Coenagrion mercuriale* that forms three delimited haplogroups, one of which exclusive to the Italian peninsula. In 1948, *C. castellani* was described from central Italy (Roberts, 1948), and in 1949 it was discovered that *C. mercuriale* male specimens from Italy invariably showed clear morphological differences (e.g., male appendages) with respect to specimens from central Europe, suggesting to consider the Italian ones as *C. m. castellanii* (Conci, 1949). Notably, the Italian populations show a geographic disjunction from the range of *C. mercuriale*, as also supported by genetic distance values at other mitochondrial and nuclear loci (Figure 4,

Appendix S5). Similarly, the populations from the Maghreb were often referred to as *C. m. hermeticum* Selys 1872, since they appeared slightly morphologically distinct from the nominal subspecies (Ben Azzouz et al., 1989). The validity of *hermeticum* was subsequently questioned (Lieftinck, 1966), although the necessity of further comparative investigations was advocated (Jacquemin & Boudot 1990; 1999). This long-awaited study did not come to light until a recent multi-locus characterization of North African populations (Ferreira et al., 2014a) and the data here provided for *castellanii*. Although Boudot & Kalkman (2015) stated that at present no subspecies of *C. mercuriale* are recognized, our results suggest that three clearly distinct lineages occur within its range, one of which possibly endemic to Italy. Further research including morphometric investigations is definitely needed to clarify the matter, also considering the high conservation measures involving *C. mercuriale* in Europe, which is listed in the Annex II of Council Directive 92/43/EEC.

Other two intraspecific delimitation warnings emerging from DS2 concerned *Orthetrum albistylum* and *Stylurus flavipes*. These showed two and one delimited Asiatic lineages respectively, in addition to the Italian/European one. Both taxa have a huge Eurasian range and while some (maybe isolated) subspecies have been described for *O. albistylum* (e.g., *O. a. speciosum* (Uhler, 1858), see Schorr & Paulson, 2019), *S. flavipes* has no currently accepted subspecies (Steinmann, 2013; Schorr & Paulson, 2019). Unfortunately, only a few DNA barcode sequences are available for these taxa and this does not permit to further clarify the observed warnings.

The last and unexpected group of intraspecific delimitation warnings included taxa having a Holarctic distribution, namely *Lestes dryas* (Zygoptera), *Aeshna juncea*, and *Sympetrum danae* (Anisoptera). The COI DS2 haplotypes of these three species showed a marked geographic structure with Italian/European haplotypes diverging from the North American ones (Figure 4, Appendix S4). Regarding *A. juncea*, Bartenef described the subspecies *A. j. americana* based on material

from Canada (Steinmann, 2013). Subsequently, Boudot & Kalkman (2015) stated that none of the various subspecies described are currently valid, while admitting that the specimens from North America are clearly morphologically different from those found in Europe. *Lestes dryas* and *S. danae* were described based on European specimens and no intraspecific taxa are currently recognized (Bridges 1994, Steinmann 2013), making the warnings here presented and their possible taxonomic significance an element of primary importance to be clarified.

Mixed species delimitation

This warning category involves two morphospecies groups of damselflies which DNA barcoded individuals showed either marked genetic divergence or complete sequence identity with congenerics. The first case, also occurring in DS1, concerns *Chalcolestes viridis* and *C. parvidens*. The latter was originally described as a subspecies of *C. viridis* and was subsequently elevated to the species rank (Dell'Anna et al., 1996; Gyulavari et al., 2011). Both species can occur syntopically in the Italian peninsula and in other parts of Europe, with rare hybridization events (Olias et al., 2007). Our DNA barcoding data confirm the genetic differentiation between *C. viridis* and *C. parvidens*, even if one specimen from Apulia, morphologically identified as *C. viridis*, was genetically assigned to *C. parvidens*. Several *Chalcolestes* populations from southern Italy are often difficult to be morphologically identified (authors pers. obs.), and intermediate morphological traits could be due to the close relationship and relatively recent divergence between the two taxa (Gyulavari et al., 2011).

Surprisingly, a mixed species delimitation warning was found among *Coenagrion puella*, *C. pulchellum* and *C. ornatum* in DS2, with *C. puella* showing a delimited diverging lineage corresponding to North African populations and the other Italian/western Palearctic representatives of the three species delimited in a single group and even sharing the same

haplotypes (as already found in DS1). These taxa can be found in syntopic conditions but can be clearly distinguished by means of morphology, ecological requirements, life-history traits, and sporadic hybridization is known to occur among them. The marked divergence at the investigated nuclear markers (Appendix S5) indicates that a mito-nuclear discordance pattern occurs throughout their overall distribution with the only exception of northern African populations, as already described by Ferreira et al. (2016). Also in this case, further investigation is needed to better address such discordance, also considering the high conservation value of *C. ornatum*, which, together with *C. mercuriale* is included in the Annex II of Council Directive 92/43/EEC.

Our comprehensive DNA barcoding characterization of Italian Odonata sheds light on several novel and other long-time debated taxonomic aspects of this group, with an unexpected geographical resonance well beyond Italy. The newly detected lineages may lead to the discovery of separate taxonomic entities within the Linnaean species and may provide fertile ground for future studies. Conversely, the reduced or missing delimitation between some known species challenges the current taxonomic knowledge on this presumed "well-explored" group of insects (at least in Europe). The warnings resulting from our species delimitation approach could indicate Italy as an important biogeographical and evolutionary centre for odonates diversification. A wide array of possible scenarios could explain the warnings found by our DNA barcoding-based species delimitation, such as different forms of mito-nuclear discordance due to more or less ancient introgression phenomena (e.g., Solano et al., 2018; Zhang et al., 2019). Further sampling campaigns, in Italy and throughout the whole range of the warning species groups, the integration of multiple genetic (or even whole genomes) markers and accurate morphometrics analyses will be necessary to correctly address each possible case of taxonomic (and conservation) interest.

Finally, this thorough DNA barcoding investigation of Italian Odonata and related warnings will allow to better support and interpret HTS eDNA metabarcoding data from European and even Holarctic freshwater environments and, at the same time, to design species-specific (or lineage-specific) probes to reliably address conservation studies on the whole range of the investigated species.

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Data Accessibility:

All specimen data are accessible on BOLD (www.boldsystems.org) under the project name 'ZPLOD - DNA barcoding Italian Odonata'. The data include collection locality, geographic coordinates, collector, identifier, voucher depository and other sampling details. Sequence data are available on BOLD and sequences have been deposited also in GenBank (Accession numbers MT298232 - MT298679).

Author contributions

AG and GA conceived the idea and designed the sampling protocol, which was then developed by all authors. AG, GA, DB, GB, IC, AC, VF, MG, LI, GLP, LL, FL, FM, SR, RS, GS, SS conducted the field sampling. FR with the help of AG stored the biological tissues, performed the lab analyses, and mined data from BOLD and GenBank. AG, GA, and DM ran the statistical analyses and all authors contributed to analyses checking/development and check the final model outcomes. AG, GA and DM lead the writing of a first draft of the manuscript and all authors critically contributed revising the final version.

Supporting information

APPENDIX S1: dataset DS1, composed by the DNA barcoding sequences produced in this study on Italian samples (409) plus 69 GenBank sequences from other Italian specimens. Species taxonomy, sex, specimen IDs, depositories accessions, sampling geographic details and COI haplotype are reported for each DNA barcoded sample.

APPENDIX S2: dataset DS2, composed by the sequences mined from BOLD and GenBank plus dataset DS1 and other COI barcode sequences obtained from additional samples retrieved by authors in other western Palearctic countries. Species taxonomy, DS1 specimen IDs, depositories accessions, are reported for each COI haplotype.

APPENDIX S3: Barcode Gap Analysis of DS1 for Anisoptera and Zygoptera respectively, generated by BOLD. Three scatterplots are provided to confirm the existence and magnitude of the Barcode Gap. For each suborder, the first two scatterplots show the overlap of the max and mean intra-specific distances vs the inter-specific (nearest neighbour) distances. The third scatterplot plots the number of individuals in each species against their max intra-specific distances, as a test for sampling bias. Distance Model: Kimura 2 Parameter, Deletion Method: Pairwise Deletion, Alignment: BOLD Aligner (Amino Acid based HMM), Filters Applied: ≥ 500 bp only.

APPENDIX S4: Multi-approach species delimitation of the 57 Anisoptera and 31 Zygoptera species investigated in this study based on Holarctic COI DNA barcode sequences from DS2. A Bayesian tree is used as a base to summarize the two threshold-based (OT and ABGD) and four character explicit-based (PTP, MPTP, GMYC, bGMYC) approaches. Specimen haplotype identifiers are reported on tips (see Appendix S1 and S2 for further details) and the number of specimens sharing each haplotype is reported within brackets. Numbers above nodes represent BPP and BS, respectively, whereas asterisks correspond to maximal node support (BPP ≥ 0.99 and BS ≥ 95). Vertical colored solid boxes delimit putative species identified by the different approaches. Black: delimitation congruent with the identified morphospecies; Red: intraspecific delimitation; Light Blue: no interspecific delimitation; Yellow: Mixed species delimitation. In case of intraspecific delimitation, each lineage has been numbered with Roman numerals. This tree was created with BEAST 1.8.2.

APPENDIX S5: average genetic p-distance divergence and Standard Deviation values among the geographic population of *Coenagrion mercuriale* (ITA: Italy, EUR: Europe excluding Italy, NAF: North Africa) and among three species belonging to *Coenagrion* (ORN: *ornatum*, PUL: *pulchellum*, PUE: *puella*) calculated at two mitochondrial (16s rDNA and COI) and three nuclear (AgT, PRMT and MLC) markers. Sequence data were retrieved from GenBank. Concerning COI, also the sequences produced in this study were used to calculate the genetic distances. The exclusiveness of haplotypes belonging to the Italian populations of *C. mercuriale* and to the other three *Coenagrion* species is indicated for each genetic marker. Genetic distance values have been calculated using MEGA X (Kumar et al., 2018).