# Intraspecific genetic diversity in selected widespread dragonfly species (Insecta: Odonata)

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Dragonflies inhabit a wide range of habitats, with different species having different species-specific habitat requirements and corresponding niches. Therefore, they are very well suited as indicator species for habitat quality and conservation. Especially in the fields of taxonomy and phylogenetics new insights in odonatology have been gained through molecular genetic methods. Using "DNA barcodes" (DNA sequences of a standardised gene segment, in this case the *cytochrome c oxidase 1* gene (*COI*)), the intraspecific genetic distances of 14 dragonfly species from 18 countries were analysed. We investigated whether there are general differences in intraspecific genetic variability between common species of Zygoptera and Anisoptera. In addition, we examined how DNA sequences with missing data and the different handling of such data in genetic analyses can affect the calculated distances and thus the interpretation of results. Our data show that the dragonfly species studied here generally show very little genetic variation over very large geographical distances, regardless of their assignment to Zygoptera or Anisoptera. The presumably lower active dispersal potential of Zygoptera obviously does not lead to an increased genetic structure. Although these results were consistent across all calculation methods, there were sometimes considerable differences in the way incomplete data were handled. For better comparability, we recommend documenting all parameters in detail in similar studies.

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Libellen bewohnen zum Teil sehr unterschiedliche Lebensräume und weisen infolgedessen auch unterschiedliche Ansprüche an ihre bevorzugten Habitate auf. Sie eignen sich daher sehr gut als Indikatoren für den Artenschutz und die Qualität von Lebensräumen. Neue Erkenntnisse in der Odonatologie konnten durch das Aufkommen und die Weiterentwicklungen genetischer Untersuchungsmethoden vor allem in den Bereichen der Taxonomie und Phylogenetik gewonnen werden. Anhand sogenannter „DNA-Barcodes" (DNA-Sequenzen eines standardisierten Genabschnittes, in diesem Fall des Cytochrom-c-Oxidase-1-Gens (*COI*)), wurden die intraspezifischen genetischen Distanzen von 14 Libellenarten aus 18 Ländern ermittelt, um zu untersuchen, ob es generell Unterschiede in der innerartlichen genetischen Variabilität zwischen weit verbreiteten Arten von Groß- und Kleinlibellen gibt und ob diese Variation mit der allgemeinen Anatomie und der aktiven Ausbreitungsfähigkeit zusammenhängt. Zusätzlich dazu wurde überprüft, wie sich unvollständige Sequenzen und die unterschiedlichen Settings für „missing data" in genetischen Analysen auf die errechneten Distanzen und somit auf die Interpretation der Ergebnisse auswirken können. Unsere Daten zeigen, dass die hier untersuchten Libellenarten, ungeachtet ihrer Zuordnung zu Groß- oder Kleinlibellen, im Allgemeinen sehr geringe genetische Variation über sehr große geographische Distanzen aufweisen. Das vermeintlich geringere aktive Ausbreitungspotential von Kleinlibellen führt bei den untersuchten Arten offensichtlich nicht zu einer erhöhten genetischen Strukturierung. Diese Ergebnisse waren zwar bei allen Berechnungsmethoden einheitlich, jedoch ergaben sich teilweise beträchtliche Unter-

1) We dedicate this article to Fritz Schiemer (Emeritus Professor at the University of Vienna) on the occasion of his 80th birthday. His interest in and commitment to the biodiversity of the Balkan region has always been inspiring. His special focus on the conservation of the wild rivers of the Balkans has been a key promoter for a lot of international research expeditions and environmental campaigns that have played a very important role in significantly increasing our knowledge on all aspects of these rivers, as well as in making the conservation of the Balkans' wild rivers an environmental issue of global concern.

schiede beim unterschiedlichen Umgang mit unvollständigen Daten. Zur besseren Vergleichbarkeit appellieren wir daher, solche Analyseparameter in ähnlichen Studien immer genauestens zur dokumentieren.

**Keywords:** DNA barcoding, *COI*, dragonflies, damselflies, genetic distance, geographic distribution range, missing data treatment.

## Introduction

Dragonflies and damselflies (which we here collectively refer to as dragonflies) inhabit a large variety of water bodies. Due to their species-specific habitat requirements regarding morphology, hydrological dynamics and plant communities of breeding waters (Corbet 1999, Schindler et al. 2003, Kadoya et al. 2004) they are important bioindicators used for characterizing aquatic systems as well as for assessing their ecological status (Samways 1993, Chovanec & Waringer 2001, Oertli 2008, Simaika & Samways 2009, Silva et al. 2010, Miguel et al. 2017, Chovanec 2018). Hence, knowledge on distribution and abundance of species is of relevance for both water management and conservation issues.

Molecular genetic methods fostered odonatological research regarding taxonomy and phylogenetic relationships of species (Ware et al. 2007, Dijkstra & Kalkman 2012, Min Jee et al. 2014, Koroiva et al. 2017). Besides phylogenetic relationships, information on intraspecific genetic variation is important for our understanding of the biogeographic history, for example, of Palearctic dragonflies. This may help to build hypotheses on population bottlenecks, refugia and postglacial range expansions or even speciation (Brockhaus 2018). Yet, for the assessment of past and recent colonisation or intra-habitat displacement, it has to be kept in mind that in many dragonfly species, migration is an important part in the life-history of most adults and distances may range from tens to thousands of kilometres (Corbet 1999, May & Matthews 2008).

In the past two decades, DNA barcoding has been established as an important tool to characterize species by standardized analysis of specific genes, e.g., the mitochondrial (mt) *cytochrome c oxidase subunit 1* (*COI*) gene, of which a ca. 650 bp fragment (the so-called „Folmer region") is used as the standard marker for animal taxa (Pentinsaari et al. 2016). With the increased availability of reference data in public data bases, DNA barcoding may add valuable ecological and biological information (Weigand et al. 2019). The advantages of diagnostic PCR and metabarcoding in detecting threatened, rare, or invasive species or pathogens are evident (De Ventura et al. 2017, Chovanec et al. 2018, Cowart et al. 2018). Moreover, DNA barcoding projects contribute essentially to the knowledge on intraspecific diversity, which is of crucial for addressing systematic and evolutionary questions as well as for assessing applicability and limitations of this method in a specific group (Mamos et al. 2016, Berchi et al. 2018).

In the course of the national DNA barcoding project "Austrian Barcode of Life" (ABOL; www.abol.ac.at) a complete DNA reference database of Austrian dragonfly species is presently being built up. Relative to its size, Austria has a large variety of landscape types and climate zones offering habitats for Mediterranean as well as boreo-alpine species (Chovanec 2015, Kalkman et al. 2018). A large portion of the species occurring in Austria has a Palaearctic distribution. At the end of 2019, the Austrian inventory of Odonata listed 78 species (Chovanec et al. 2017).

The Austrian dragonfly barcoding project in the frame of ABOL generally aims at Austrian reference specimens and their DNA barcodes. For some of those species analysed in the ABOL initiative, DNA barcodes were also obtained from specimens collected in south-eastern European countries (Albania, Bosnia and Herzegovina, Croatia, and Montenegro) in the course of recent excursions. We took the opportunity to use this data and, including publicly available data of those species, to compare intraspecific diversities in widely distributed species of Odonata. These species representing six of the nine families of dragonflies native to Austria (Zygoptera and Anisoptera) constitute the set of taxa investigated in the present study. We present data on intraspecific genetic diversity in the *COI* gene of 14 dragonfly species from 18 countries (including DNA barcodes from the public data bases BOLD and GenBank). Furthermore, we investigate the possible distortion of calculated genetic distances resulting from the application of different treatments of missing data and discuss its effect on the interpretation of results. Finally, we look into whether the supposed generally smaller dispersal distances in Zygoptera (compared to Anisoptera, Corbet 1999) are reflected by increased genetic variation between conspecifics and if geographic sub-structuring is detectable.

## Material and Methods

Altogether, *COI* sequences of 54 individuals (44 adults, 10 larvae) representing 14 dragonfly species (11 genera, 6 families) from five countries were newly generated for this study (Tab. 1). In addition, 49 published sequences were included, resulting in a total of 103 sequences from 18 countries. In the following we use the ISO 3166-1 alpha-3 three-letter country codes for countries mentioned in tables and in parentheses in the text. For better readability, full country names are used in the running text. Voucher specimens were deposited in the scientific collection of the Natural History Museum Vienna.

Tissue samples (approx. 2 × 2x2 mm) from individuals (imago and larvae) stored in 96 % ethanol were taken from muscle tissue of the femur. Extraction was performed with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) using the standard protocol specified by the company, eluting the DNA in a final volume of 60 µl.

For most samples, it was possible to amplify major parts or even the complete *COI* gene, as well as short sequences flanking the *COI* gene using primers binding within the flanking tRNA genes (*tRNA Tyr* gene, *tRNA Leu* gene). We pursued this strategy in the course of the ABOL project to obtain as much sequence information as possible for designing alternative primers employed in the various taxa. Lengths of readable sequences ranged from 1288-1608 bp and contained the (~650 bp) standard DNA barcoding region (the so-called Folmer region) which was the basis of the analysis in the present study. Primers used for PCR amplification are listed in Table 2. Of some samples, only smaller *COI* sections could be amplified (539-803 bp), which, however, still covered at least part of the DNA barcoding region. For the subsequent phylogenetic tree inference and distance calculations the long sequences were trimmed to 657 bp, corresponding to the standard DNA barcoding region. One sample of *Sympetrum striolatum* (OdoMon11) yielded DNA of very poor quality allowing to amplify a sequence of only 178 bp. Due to its shortness and since it hardly overlapped with the Folmer region, this sequence was excluded from the analyses (calculations of genetic distances and tree calculation). Yet we provide information concerning this sequence in the results section.

Tab. 1: Specimens analysed in the present study. Primers for PCR amplification and sequencing as well as BOLD IDs and GenBank (GB) accession numbers of *COI* sequences isolated from the specimens are given. For primer IDs see Table 2. Length = Amplicon length in base pairs. Countries are indicated by the following acronyms: ALB (Albania), AUT (Austria), BIH (Bosnia and Hercegovina), HRV (Croatia), MNE (Montenegro). – Tab. 1: Untersuchte Individuen. Verwendete Primer für PCR und Sequenzierung sowie BOLD-IDs und GenBank Nummern (GB) der *COI*-Sequenzen sind angegeben. Primer-IDs entsprechen den in Tabelle 2 angegebenen. Length = Länge des PCR-Produktes in Basenpaaren. Folgende Länderabkürzungen wurden verwendet: ALB (Albanien), AUT (Österreich), BIH (Bosnien und Herzegowina), HRV (Kroatien), MNE (Montenegro).

| Family / Species | Sample ID | Country | Water body | PCR primer | Length | Sequencing primer | BOLD ID | GenBank Acc. No. |
|---|---|---|---|---|---|---|---|---|
| **Lestidae** | | | | | | | | |
| *Sympecma fusca* | Odo_Alb_01 | ALB / Drilon National Park | Drilon | 1 / 4 | 1514 | 1 / 4 / 9 / 10 | AODON789-20 | MW208367 |
| *Sympecma fusca* | Odo_Alb_02 | ALB / Drilon National Park | Drilon | 1 / 4 | 1514 | 1 / 4 / 9 / 10 | AODON790-20 | MW208368 |
| *Sympecma fusca* | Odo0035 | AUT / Vienna, Lobau Nationalpark | Kühwörterwasser | 2 / 5 | 1608 | 2 / 5 / 9 / 10 | AODON034-20 | MW208369 |
| *Chalcolestes viridis* | Odo_Cro_08 | HRV / Karlovac | Dobra | 2 / 4 | 1523 | 2 / 4 / 9 / 10 | AODON776-20 | MW208370 |
| *Chalcolestes viridis* | Odo_BIH_06 | BIH / Čapljina | Hutuvo Blato | 2 / 4 | 1561 | 2 / 5 / 9 / 10 | AODON782-20 | MW208373 |
| *Chalcolestes viridis* | Odo_Mon_09 | MNE / Virpazar | Lake Shkoder/Skadar | 2 / 4 | 1502 | 2 / 5 / 9 / 10 | AODON785-20 | MW208374 |
| *Chalcolestes viridis* | Odo0206 | AUT / Steiermark, Frauenberg | Ennsauen | 2 / 5 | 1608 | 2 / 5 / 6 / 11 | AODON460-20 | MW208371 |
| *Chalcolestes viridis* | Odo0137 | AUT / Vienna, Lobau Nationalpark | Großenzersdorfer Arm | 2 / 5 | 1608 | 2 / 5 / 9 / 10 | AODON135-20 | MW208372 |
| *Chalcolestes parvidens* | Odo_BIH_03 | BIH / Bosanska Gradiška | Vrbaška | 2 / 5 | 1576 | 2 / 5 / 9 / 10 | AODON779-20 | MW208375 |
| *Chalcolestes parvidens* | Odo_BIH_04 | BIH / Bosanska Gradiška | Vrbaška | 2 / 5 | 1546 | 2 / 5 / 9 / 10 | AODON780-20 | MW208376 |
| *Chalcolestes parvidens* | Odon454 | AUT / Burgenland, Pamhagen | Drainage chanel | 6 / 13 | 657 | 6 / 13 | AODON454-20 | MW208377 |
| | | | | | | | | |
| **Libellulidae** | | | | | | | | |
| *Orthetrum cancellatum* | Odo_Alb_03 | ALB / Udënisht | Lake Ohrid | 2 / 5 | 803 | 2 / 5 / 9 / 10 | AODON791-20 | MW208378 |
| *Orthetrum cancellatum* | Odo_Alb_12 | ALB / Lin | Lake Ohrid | 3 / 6 | 538 | 3 / 6 | AODON800-20 | MW208379 |
| *Orthetrum cancellatum* | Odo0068 | AUT / Vienna, Lobau Nationalpark | Eberschüttwasser | 2 / 5 | 1608 | 2 / 5 / 9 / 10 | AODON066-20 | MW208380 |
| *Sympetrum sanguineum* | Odo_Cro_04 | HVR / Turopoljski lug | Odre | 2 / 5 | 1608 | 2 / 5 / 9 / 10 | AODON772-20 | MW208383 |
| *Sympetrum sanguineum* | Odo0163 | AUT / Vienna, Lobau Nationalpark | Kühwörterwasser | 2 / 5 | 1608 | 2 / 5 / 9 / 10 | AODON161-20 | MW208381 |
| *Sympetrum sanguineum* | Odon392 | AUT / Styria | Miesbodensee | 2 / 5 | 1581 | 2 / 5 / 9 / 10 | AODON588-20 | MW208382 |
| *Sympetrum striolatum* | Odo_Mon_11 | MNE / Virpazar | Lake Shkoder/Skadar | 7 / 8 | 178 | 7 / 8 | AODON787-20 | - |
| *Sympetrum striolatum* | Odo0141 | AUT / Vienna, Lobau Nationalpark | Mühlwasser | 2 / 5 | 1608 | 1 / 4 / 10 / 11 | AODON139-20 | MW208384 |
| *Sympetrum striolatum* | Odon444 | AUT / Styria | Gamperlacke | 2 / 5 | 1608 | 2 / 5 / 10 / 12 | AODON638-20 | MW208385 |

| Family / Species | Sample ID | Country | Water body | PCR primer | Length | Sequencing primer | BOLD ID | GenBank Acc. No. |
|---|---|---|---|---|---|---|---|---|
| *Sympetrum meridionale* | Odo_BIH_01 | BIH / Bosanska Gradiška | Vrbaška | 2 / 5 | 1608 | 2 / 5 / 12 / 10 | AODON777-20 | MW208388 |
| *Sympetrum meridionale* | Odo_BIH_02 | BIH / Bosanska Gradiška | Vrbaška | 2 / 5 | 1608 | 2 / 5 / 12 / 10 | AODON778-20 | MW208389 |
| *Sympetrum meridionale* | Odo_Mon_10 | MNE / Virpazar | Lake Shkoder/Skadar | 2 / 5 | 1608 | 2 / 5 / 12 / 10 | AODON786-20 | MW208390 |
| *Sympetrum meridionale* | Odon212 | AUT / Burgenland, Illmitz | - | 2 / 5 | 1608 | 2 / 5 / 10 / 12 | AODON374-20 | MW208386 |
| *Sympetrum meridionale* | Odon367 | AUT / Upper Austria, Pucking | Traunau | 2 / 5 | 1608 | 2 / 5 / 10 / 12 | AODON563-20 | MW208387 |
| | | | | | | | | |
| **Coenagrionidae** | | | | | | | | |
| *Ischnura elegans* | Odo_Alb_04 | ALB / Memëlisht | Lake Ohrid | 1 /4 | 1333 | 1 / 4 / 9 / 10 | AODON792-20 | MW208393 |
| *Ischnura elegans* | Odo_Alb_05 | ALB / Memëlisht | Lake Ohrid | 1 / 4 | 1514 | 1 / 4 / 10 / 11 | AODON793-20 | MW208397 |
| *Ischnura elegans* | Odo_Alb_06 | ALB / Memëlisht | Lake Ohrid | 1 / 4 | 1288 | 1 / 4 / 9 / 10 | AODON794-20 | MW208394 |
| *Ischnura elegans* | Odo_Alb_09 | ALB / Drilon National Park | Drilon | 3 / 6 | 538 | 3 / 6 | AODON797-20 | MW208395 |
| *Ischnura elegans* | Odo_Alb_10 | ALB / Drilon National Park | Drilon | 1 / 4 | 1514 | 1 / 4 / 10 / 11 | AODON798-20 | MW208396 |
| *Ischnura elegans* | Odo_Cro_03 | HRV/ Turopoljski lug | Odre | 1 / 4 | 1514 | 1 / 4 / 10 /11 | AODON771-20 | MW208391 |
| *Ischnura elegans* | Odo_BIH_08 | BIH / Čapljina | Hutuvo Blato | 1 / 4 | 1514 | 1 / 4 / 11 / 10 | AODON784-20 | MW208392 |
| *Ischnura elegans* | Odo0056 | AUT/ Vienna, Lobau Nationalpark | Kreuzgrund Traverse | 1 / 4 | 1514 | 1 / 4 / 10 / 11 | AODON054-20 | MW208398 |
| *Ischnura elegans* | Odo0202 | AUT / Upper Austria | Naarn | 1 / 4 | 1514 | 1 / 4 / 10 / 11 | AODON456-20 | MW208399 |
| *Erythromma lindenii* | Odon537 | AUT / Upper Austria | Großer Weikerlsee | 2 / 6 | 657 | 2 / 6 | AODON730-20 | MW208400 |
| *Erythromma lindenii* | Odo_Cro_05 | HRV/ Straza | Dobra | 2 / 5 | 1599 | 2 / 4 / 6 / 11 | AODON773-20 | MW208401 |
| *Enallagma cyathigerum* | Odo_Cro_06 | HRV/ Straza | Dobra | 1 / 4 | 1514 | 1 / 4 / 9 / 10 | AODON774-20 | MW208402 |
| *Enallagma cyathigerum* | Odo_BIH_05 | BIH / Jajce | Plivsko jezero | 1 / 4 | 1514 | 1 / 4 / 9 / 10 | AODON781-20 | MW208403 |
| *Enallagma cyathigerum* | Odo0178 | AUT / Vienna | - | 1 / 4 | 1514 | 1 / 4 / 9 / 10 | AODON439-20 | MW208404 |
| *Enallagma cyathigerum* | Odo0151 | AUT / Styria, Johnsbach | - | 1 / 4 | 1514 | 1 / 4 / 9 / 10 | AODON149-20 | MW208405 |
| | | | | | | | | |
| **Gomphidae** | | | | | | | | |
| *Gomphus vulgatissimus* | Odo_Alb_07 | ALB / Tushemisht | Lake Ohrid | 3 / 6 | 538 | 3 / 6 | AODON795-20 | MW208406 |
| *Gomphus vulgatissimus* | Odo_Alb_08 | ALB / Tushemisht | Lake Ohrid | 3 / 6 | 538 | 3 / 6 | AODON796-20 | MW208407 |
| *Gomphus vulgatissimus* | Odon256 | AUT / Styria, Thal | Thalbach | 2 / 5 | 1601 | 2 / 4 / 9 / 10 | AODON416-20 | MW208408 |
| *Gomphus vulgatissimus* | Odo0175 | AUT / Vienna | Wienfluss | 2 / 5 | 1608 | 2 / 4 / 9 / 10 | AODON436-20 | MW208409 |
| *Onychogomphus forcipatus* | Odo_Alb_11 | ALB / Lin | Lake Ohrid | 2 / 5 | 1574 | 2 / 5 / 7 / 10 | AODON799-20 | MW208412 |

| Family / Species | Sample ID | Country | Water body | PCR primer | Length | Sequencing primer | BOLD ID | GenBank Acc. No. |
|---|---|---|---|---|---|---|---|---|
| *Onychogomphus forcipatus* | Odon306 | AUT / Carinthia, Otrouza | Waidischbach | 2 / 5 | 1608 | 2 / 5 / 7 / 10 | AODON502-20 | MW208410 |
| *Onychogomphus forcipatus* | Odo0195 | AUT / Vienna | Wienfluss | 2 / 5 | 1608 | 2 / 5 / 7 / 10 | AODON450-20 | MW208411 |
| | | | | | | | | |
| **Calopterygidae** | | | | | | | | |
| *Calopteryx splendens* | Odo_Cro_01 | HVR / Turopoljski lug | Odre | 2 / 4 | 1600 | 2 / 4 / 9 / 10 | AODON769-20 | MW208413 |
| *Calopteryx splendens* | Odo0203 | AUT / Upper Austria | Naarn | 2 / 5 | 1608 | 13 / 14 / 9 / 10 | AODON457-20 | MW208415 |
| *Calopteryx splendens* | Odo0196 | AUT / Vienna | Wienfluss | 2 / 5 | 1608 | 13 / 14 / 9 / 10 | AODON451-20 | MW208414 |
| | | | | | | | | |
| **Aeshnidae** | | | | | | | | |
| *Aeshna mixta* | Odo_BIH_07 | BIH / Čapljina | Hutuvo Blato | 2 / 5 | 1558 | 2 / 5 / 9 / 10 | AODON783-20 | MW208416 |
| *Aeshna mixta* | Odo_Mon_12 | MNE / Petrovac | - | 2 / 5 | 1593 | 2 / 5 / 11 / 10 | AODON788-20 | MW208417 |
| *Aeshna mixta* | Odo0167 | AUT / Vienna, Lobau Nationalpark | Schwarzes Loch | 2 / 5 | 1608 | 2 / 5 / 9 / 10 | AODON165-20 | MW208418 |
| *Aeshna mixta* | Odon446 | AUT / Burgenland, Illmitz | Sandeck | 2 / 5 | 1608 | 2 / 5 / 10 /11 | AODON640-20 | MW208419 |

PCR was performed with the Taq DNA Polymerase (Qiagen, Hilden, Germany) in a volume of 50 µl containing 5 µl of 10x Taq Buffer, 10 µl of 5x Q-Solution, 0.5 µl Taq polymerase (5 units/µl), 1.5 mM MgCl2, 2.5 mM dNTP Mix, 0.5 µM of each primer, and 1 µl DNA template. The PCR cycling protocol included an initial denaturation at 94° C for 3 min, followed by 35 cycles of denaturation at 94° C for 1 min, annealing for 30 s, extension at 72° C for 30 s. The final step was an extension at 72° C for 10 min. The following annealing temperatures were used for the various primer combinations: COI-Odo-F5 /COI-Odo-R6: 47° C; CO1-Zyg-F1 / ODO_HCO2198d: 57° C; Tyr-Odo-F / Leu-Odo-R: 54° C; Tyr-Odo-F / COI-Odo-R6: 54° C (Tab. 2).

The success of the PCR amplification was checked by agarose gel electrophoresis. Samples with positive PCR bands were purified with the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol and sequenced in both directions at Microsynth (Balgach, Switzerland) using the PCR primers as well as two internal primers (Tab. 2). For sequencing of the small *COI* fragments, only the PCR primers were used.

Sequences were edited with the software Geneious 10.2.6 (Biomatters, Auckland, New Zealand; https://www.geneious.com) and final sequences were aligned in BioEdit (Hall 1999). Taxonomic sequence identification was controlled and confirmed through BLAST searches and through translation into protein sequences we checked for intact reading frames, to detect NUMT sequences (nuclear copies of mitochondrial genes). The authenticity of the sequences as being derived from the mt *COI* gene was further confirmed in the phylogenetic tree. In addition to the newly generated sequences, 49 published sequences downloaded from BOLD (http://www.

Tab. 2: PCR primers used in the present study for PCR and sequencing. – Tab. 2: PCR-Primer, die in der Analyse für PCR und Sequenzierung verwendet wurden.

| ID | Primer | Sequence 5′–3′ | Reference |
|---|---|---|---|
| 1 | CO1-Odo-F5 | TGCGACRATGRCTGTTTTC | present study |
| 2 | Tyr-Odo-F | CTCCTATATAGATTTACAGTCT | present study |
| 3 | CO1-Zyg-F1 | TTGGAGATGAYCAAATTTATAAYGT | present study |
| 4 | CO1-Odo-R6 | TGCACTTTTCTGCCACATTAAA | present study |
| 5 | Leu-Odo-R | CTTAAATCCATTGCACTTTTCTGCC | present study |
| 6 | ODO_HCO2198d | TAAACTTCWGGRTGTCCAAARAATCA | Dijkstra et al. 2014 |
| 7 | CO1-Lib-F1 | TTAACAGAYCGAAATATTAATAC | present study |
| 8 | CO1-Lib-R1 | CCTARAATACCAATTGCTACTAT | present study |
| 9 | CO1-Odo-F3 | GATTCTTTGGACAYCCHGAAG | present study |
| 10 | CO1-Odo-R3 | GTTTCCTTTTTACCTCTTTCTTG | present study |
| 11 | CO1-Odo-F1 | GGWATAATTTCACATATTATTGC | present study |
| 12 | CO1-Sym-F1 | TTAACTGAYCGAAATATTAATACATC | present study |
| 13 | ODOLCO1490d | TAAACTTCWGGRTGTCCAAARAATCA | Dijkstra et al. 2014 |
| 14 | CO1-Odo-R8 | GTARTTTTTGATATCATTCRAT | present study |
| 15 | CO1-Odo-R1 | TAATATGTGAAATTATWCCAA | present study |

boldsystems.org) or GenBank (https://www.ncbi.nlm.nih.gov/genbank) were included for comparison (listed in Tab. 3). For each species, representative sequences were selected under the rationale that they should cover the species' distribution ranges to the largest extent possible (i.e., available in the public data bases). If species were represented in the data bases by several sequences from a certain country, two or three sequences were selected that showed the largest genetic distances. If only identical sequences (or below 0.2 % sequence divergence) were present from a given country, a maximum of two sequences were included. Sequences from countries without published records of the corresponding species (potential misidentifications) were omitted. Sequences without any geographic information were also not included. The final data set included 102 *COI* sequences (one sequence, OdoMon11, was excluded; see above) from 18 countries with sequence lengths ranging from 407 to 657 base pairs. All sequences generated in the course of the present study were deposited on BOLD as well as GenBank (Tab. 1).

Differences between DNA sequences (uncorrected pairwise distances (p distances) in %) were calculated with MEGA5 (Tamura et al. 2011) using differently sized datasets and different treatments of missing data. Due to the various lengths of the sequences downloaded from GenBank and to some extent of our own sequences, settings for calculations had to be considered carefully since the treatment of missing data influences the distance values considerably. Therefore, we compiled three different versions of our dataset. The first version comprised a data set of all 102 sequences. For the second data set sequences < 550 bp were excluded, resulting in an alignment containing only 86 sequences. For the third data set, sequences were trimmed to a 177-bp section of complete data for almost all sequences (no missing data, except for two Ns in one sequence of *S. fusca*). Each version of the data set was used for the calculation of genetic distances once with the 'pairwise deletion' and once with the 'complete deletion' option for missing data treatment. The 'pairwise deletion' option retains the maximum information possible but uses sequences of different lengths (and consequently parts of the gene) while the 'complete deletion' option

Tab. 3: Sequences retrieved from BOLD and GenBank. Countries are indicated by the following acronyms: AUT (Austria), BEL (Belgium), CHN (China), CYP (Cyprus), ESP (Spain), FRA (France), GER (Germany), Greece (GRC), JPN (Japan), KOR (South Korea), MAR (Morocco), MLT (Malta), NLD (The Netherlands), and NOR (Norway). a = Dijkstra et al. 2013, b = Zhang et al. 2017, c = Bergmann et al. 2013, d = Sanchez-Guillen et al. 2013, e = Iserbyt et al. 2010, f = Corse et al. 2017, g = Karube et al. 2012, h = Froufe et al. 2014, i = Futahashi 2014, j = Kim et al. 2014. – Tab. 3: Sequenzen, die von BOLD bzw. GenBank heruntergeladen wurden. Folgende Länderabkürzungen wurden verwendet: AUT (Österreich), BEL (Belgien), CHN (China), CYP (Cypern), ESP (Spanien), FRA (Frankreich), GER (Deutschland), Greece (Griechenland), JPN (Japan), KOR (Südkorea), MAR (Marokko), MLT (Malta), NLD (Niederlande), and NOR (Norwegen). a = Dijkstra et al. 2013, b = Zhang et al. 2017, c = Bergmann et al. 2013, d = Sanchez-Guillen et al. 2013, e = Iserbyt et al. 2010, f = Corse et al. 2017, g = Karube et al. 2012, h = Froufe et al. 2014, i = Futahashi 2014, j = Kim et al.

| **Species** | **Geographic origin** | **BOLD ID** | **GenBank Acc. No.** | **References** |
|---|---|---|---|---|
| *Sympecma fusca* | GER, Bavaria, Münchener Ebene | FBAQU322-09 | HM422052 | - |
| *Sympecma fusca* | GER, München | FBAQU515-10 | HM901877 | - |
| *Sympecma fusca* | NLD, Leiden | ODOPH209-13 | KF369553 | a |
| *Chalcolestes viridis* | GER, Mittelfränkisches Becken | FBAQU200-09 | GU682190 | - |
| *Chalcolestes viridis* | GER, Ammer-Loisach-Hügelland | FBAQU201-09 | GU682188 | - |
| *Enallagma cyathigerum* | CHN | GBMHO3774-19 | MF716899 | b |
| *Enallagma cyathigerum* | CHN, Xinjiang | XJDQD086-18 | - | - |
| *Enallagma cyathigerum* | NOR, Hordaland | ZMBN960-17 | - | - |
| *Enallagma cyathigerum* | NOR, Finnmark | HETFI049-11 | - | - |
| *Enallagma cyathigerum* | ESP | GBMHO177-13 | KC912312 | c |
| *Enallagma cyathigerum* | ESP | GBMHO179-13 | KC912310 | c |
| *Enallagma cyathigerum* | GER, Brandenburg | FBAQU1504-13 | - | - |
| *Enallagma cyathigerum* | GER, Bavaria | FBAQU490-10 | HQ563103 | - |
| *Enallagma cyathigerum* | GER, Donaumoos | FBAQU532-10 | HQ563105 | - |
| *Ischnura elegans* | ESP | GBA8517-12 | HQ834803 | d |
| *Ischnura elegans* | BEL | GBMH7973-10 | GQ256032 | e |
| *Ischnura elegans* | MLT | GBMHO1617-19 | KX399383 | - |
| *Ischnura elegans* | FRA | GBMHO3410-19 | MF458738 | f |
| *Ischnura elegans* | JPN, Hokkaido | GBMIN24952-13 | AB708504 | g |
| *Ischnura elegans* | CYP | GBMIN88604-17 | KY127438 | - |
| *Ischnura elegans* | CYP | GBMIN88605-17 | KY127440 | - |
| *Ischnura elegans* | NLD | ODOPH118-13 | KF369415 | a |
| *Ischnura elegans* | CHN | XJDQD083-18 | - | - |
| *Ischnura elegans* | CHN | XJDQD091-18 | - | - |
| *Ischnura elegans* | GER, Brandenburg | FBAQU1611-13 | - | - |
| *Ischnura elegans* | GER, Steigerwald | FBAQU172-09 | HM376192 | - |
| *Ischnura elegans* | GER, Inn-Chiemsee-Hügelland | FBAQU536-10 | HM901892 | - |
| *Erythromma lindenii* | GER, Südliche Frankenalb | FBAQU562-10 | - | - |
| *Erythromma lindenii* | FRA | GBMHO3409-19 | MF458702 | f |
| *Aeshna mixta* | GER, Bavaria | FBAQU480-10 | - | - |
| *Aeshna mixta* | GER, Inn-Chiemsee-Hügelland | FBAQU522-10 | HM901884 | - |
| *Aeshna mixta* | JPN, Toyama | GBMIN24908-13 | AB708592 | g |
| *Onychogomphus forcipatus* | GER, Falkensteiner Vorwald | FBAQU542-10 | HM901896 | - |

| Species | Geographic origin | BOLD ID | GenBank Acc. No. | References |
|---|---|---|---|---|
| *Onychogomphus forcipatus* | MAR | GBMHO415-14 | KF584975 | h |
| *Onychogomphus forcipatus* | AUT | GBMHO983-15 | KJ873220 | - |
| *Onychogomphus forcipatus* | GER | GODO002-18 | - | - |
| *Gomphus vulgatissimus* | GER | FBAQU1445-13 | - | - |
| *Gomphus vulgatissimus* | GER, Falkensteiner Vorwald | FBAQU534-10 | HQ563106 | - |
| *Orthetrum cancellatum* | GER | FBAQU505-10 | HM901872 | - |
| *Sympetrum striolatum* | KOR | GBMHO2999-19 | LC366706 | i |
| *Sympetrum striolatum* | GRC | GBMHO3066-19 | LC366773 | i |
| *Sympetrum striolatum* | HRV | GBMHO3145-19 | LC366852 | i |
| *Sympetrum striolatum* | KOR | GBMHO565-14 | KF257086 | j |
| *Sympetrum striolatum* | JPN, Hokkaido | GBMHO2956-19 | LC366663 | i |
| *Sympetrum striolatum* | JPN, Toyama | GBMIN24126-13 | AB709190 | i |
| *Sympetrum striolatum* | NOR, Hordaland | ZMBN328-16 | - | - |
| *Sympetrum striolatum* | GER, Bavaria | FBAQU519-10 | HM901881 | - |
| *Sympetrum sanguineum* | GER, Inn-Chiemsee-Hügelland | FBAQU554-10 | - | - |
| *Sympetrum sanguineum* | GER, Bavaria | FBAQU518-10 | HM901880 | - |

rigorously excludes all sites with missing data from the analysis and might thus, depending on the completeness of the dataset, use only a very limited amount of the information present in the data. MEGA5 was also used to calculate Neighbour-Joining (NJ) trees based on K2P distances (standard for barcoding studies) and pairwise deletion of missing data. BIN composition was checked with the BIN details summary and the BIN discordance tool implemented on BOLD.

## Results

Of the 54 *COI* sequences generated in the present study, all but one were included in the final data set for tree calculation. Only the short sequence of Odo-Mon11 (178 bp) was not included, which is discussed below. In order to verify and display the unambiguous clustering of conspecific sequences as well as to visualize general sequence similarities, a NJ tree calculated from the final data set comprising 102 *COI* sequences is shown in Fig. 1 (length of alignment 657 bp). Conspecific sequences clustered together in the NJ tree with high statistical support and branch lengths within species were short (Fig. 1).

Table 4 shows, for the three data sets (407-657 bp, 550-657 bp, 177 bp), average ($I_{mean}$) and maximum ($I_{max}$) intraspecific p distances among the *COI* sequences as well as minimum interspecific distances (distance to the nearest neighbour, DNN). Albeit in the same range, the distances calculated from the three data sets vary considerably, in particular the DNN values. Minimum interspecific distances exceeded intraspecific variation by far in all species studied, consistent with the barcode gap hypothesis.

Among the 14 species analysed, 13 showed mean intraspecific p distances below 1 %, despite huge geographic distances between sampling localities. Maximum genetic distances between conspecifics were also relatively low in general, exceeding 1 % in only a few species (here the ranges obtained with the various settings are given in parentheses): *I. elegans* ($I_{max}$: 1.8-4.3 %), *A. mixta* ($I_{max}$: 0.6-2.4 %), *S. striolatum* ($I_{max}$: 0.5-2.3 %) and *O. forcipatus*

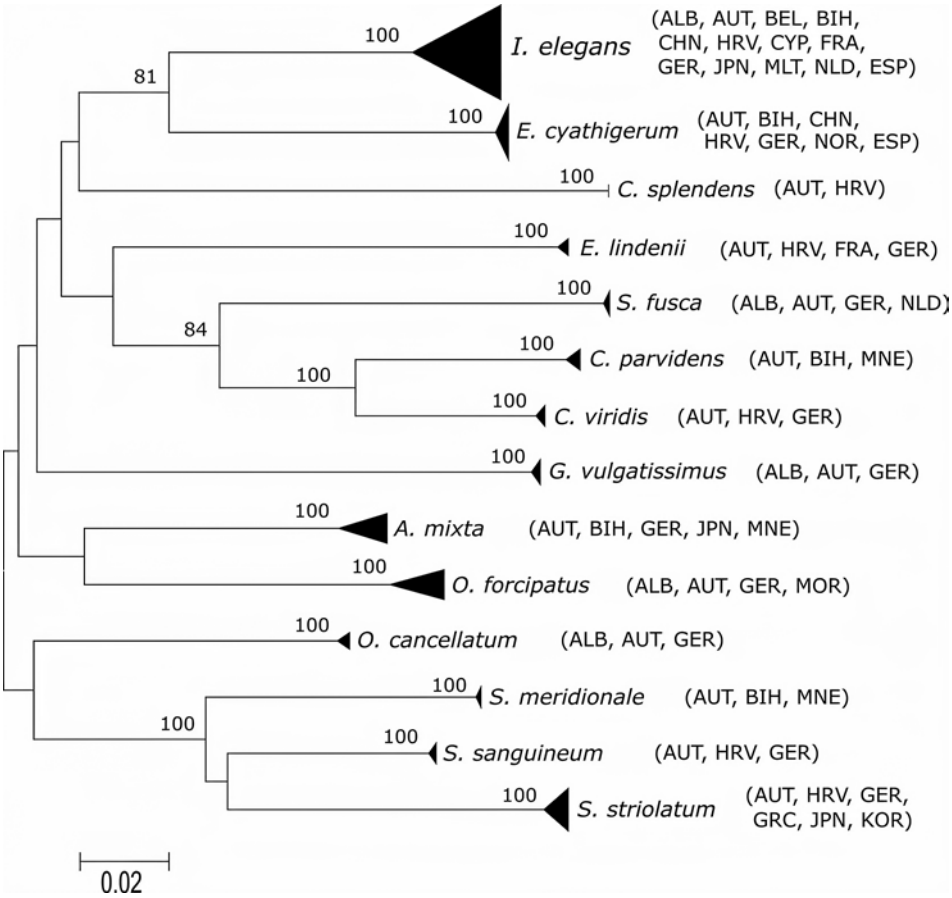


Fig. 1: Neighbor-joining tree based on 102 *COI* sequences. Bootstrap values (1000 replicates) are indicated at the branches. Clades are collapsed for improved visualization. Country acronyms apply as mentioned in Tables 1 and 2. – Abb. 1: Neighbor-joining-Baum berechnet aus 102 *COI* Sequenzen. Bootstrapwerte (1000 Wiederholungen) sind eingezeichnet. Die Clades sind zur übersichtlicheren Visualisierung kollabiert dargestellt. Länderabkürzungen wie in Tabellen 1 und 2.

($I_{max}$: 2.8-5.1 %). The largest deviation was observed in *I. elegans* between the initial dataset (407-657 bp, pairwise deletion of missing data) and the minimum barcode length dataset (550-657 bp, complete deletion of missing data) amounting to 2.5 percentage points (4.3-1.8 % respectively). In general, intraspecific distances only slightly increased when including sequences from broader geographic ranges.

## Geographic variation - intraspecific distances

Nine species represented by individuals from Western, Central and Southeastern European countries had very low maximum intraspecific distances (0-1.1 %; Tab. 4). *Calopteryx splendens* (AUT, HRV), *Erythromma lindenii* (France, GER, AUT, HRV), *Sympecma fusca* (ALB, AUT, GER, NDL), *Chalcolestes parvidens* (BIH, MNE, AUT), *Chalcolestes viridis* (AUT, GER, HRV), *Gomphus vulgatissimus* (ALB, AUT, GER), *Orthetrum cancellatum*

Tab. 4: P distances (in %) calculated from *COI* sequences of three different lengths. Species mean (Imean) and maximum intraspecific (Imax) distances as well as the distance to the nearest neighbour (DNN) are given for different treatments of missing data. For species where DNN differed between the three fragments, species names are indicated as *E. lin.* (*Erythromma lindenii*), *O. can.* (*Orthetrum cancellatum*), *E. cya.* (*Enallagma cyathigerum*), *S. fus.* (*Sympetrum fusca*). – Tab. 4: P-Distanzen (in %) zwischen *COI*-Sequenzen der drei unterschiedlich langen Datensätze. Durchschnittliche innerartliche Distanzen (Imean) und maximale zwischenartliche Distanzen (Imax) sowie die Distanzen zur nächstverwandten Art im Datensatz („nearest neighbour", DNN) wurden jeweils für die zwei unterschiedlichen Einstellungen für fehlende Daten (pairwise deletion, complete deletion) berechnet. Bei Arten, wo DNN für die drei Fragmentlängen unterschiedlich war, sind die jeweiligen Artnamen abgekürzt angegeben: *E. lin.* (*Erythromma lindenii*), *O. can.* (*Orthetrum cancellatum*), *E. cya.* (*Enallagma cyathigerum*), *S. fus.* (*Sympetrum fusca*).

| | 102x 407-657 bp | | | | | | 86x 550-657 bp | | | | | | 102x 177 bp | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pairwise deletion | | | Complete deletion | | | Pairwise deletion | | | Complete deletion | | | No missing data (PD) | | | |
| **Species** | $I_{max}$ | $I_{mean}$ | **DNN** | $I_{max}$ | $I_{mean}$ | **DNN** | $I_{max}$ | $I_{mean}$ | **DNN** | $I_{max}$ | $I_{mean}$ | **DNN** | $I_{max}$ | $I_{mean}$ | **DNN** | **Nearest neighbour** |
| *Sympecma fusca* | 0.5 | 0.2 | 14.5 | 0.9 | 0.3 | 15.9 (*E. lin.*) | 0.5 | 0.2 | 14.5 | 0.6 | 0.2 | 15.4 | 0.6 | 0.3 | 12.6 | *C. parvidens* |
| *Chalcolestes parvidens* | 0.6 | 0.3 | 8.2 | 0.6 | 0.3 | 10.1 | 0.6 | 0.3 | 8.2 | 0.6 | 0.3 | 9.7 | 0.6 | 0.2 | 12.6 (*S. fus.*) | *C. viridis* |
| *Chalcolestes viridis* | 0.6 | 0.4 | 8.2 | 0.9 | 0.3 | 10.1 | 0.6 | 0.4 | 8.2 | 0.8 | 0.3 | 9.7 | 1.1 | 0.5 | 13.0 | *C. parvidens* |
| *Calopteryx splendens* | 0 | 0 | 17.1 | 0 | 0 | 19.4 | 0 | 0 | 17.1 | 0 | 0 | 17.9 | 0 | 0 | 16.9 | *I. elegans* |
| *Enallagma cyathigerum* | 1.0 | 0.5 | 12.7 | 0.9 | 0.4 | 14.2 | 1.0 | 0.5 | 12.7 | 0.8 | 0.4 | 13.1 | 0.6 | 0.3 | 14.1 | *I. elegans* |
| *Ischnura elegans* | 4.3 | 0.8 | 12.7 | 4.1 | 0.7 | 14.2 | 2.0 | 0.6 | 12.7 | 1.8 | 0.4 | 13.1 | 3.4 | 0.8 | 14.1 | *E. cyathigerum* |
| *Erythromma lindenii* | 0.5 | 0.2 | 15.8 | 0.6 | 0.3 | 15.9 | 0.5 | 0.2 | 15.8 | 0.4 | 0.2 | 16.0 (*E. cya.*) | 1.1 | 0.6 | 15.3 (*O. can.*) | *S. fusca* |
| *Aeshna mixta* | 2.4 | 0.8 | 12.1 | 2.3 | 0.9 | 13.6 | 0.6 | 0.3 | 12.1 | 0.6 | 0.4 | 12.7 | 2.3 | 0.9 | 13.6 | *O. forcipatus* |
| *Onychogomphus forcipatus* | 3.5 | 1.8 | 12.1 | 3.5 | 2.0 | 12.8 (*O. can.*) | 2.8 | 1.4 | 12.1 | 2.9 | 1.5 | 12.7 | 5.1 | 2.6 | 13.6 | *A. mixta* |
| *Gomphus vulgatissimus* | 0.7 | 0.3 | 15.5 | 0.3 | 0.2 | 16.5 | 0.4 | 0.2 | 15.5 | 0.2 | 0.1 | 13.3 | 0.6 | 0.3 | 13.6 | *O. forcipatus* |
| *Orthetrum cancellatum* | 0.7 | 0.4 | 13.0 | 0.3 | 0.2 | 12.8 | 0.5 | 0.3 | 13.0 | 0.6 | 0.4 | 13.1 | 0.6 | 0.4 | 14.1 | *O. forcipatus* |
| *Sympetrum striolatum* | 1.5 | 0.5 | 10.5 | 1.4 | 0.4 | 11.9 | 0.5 | 0.3 | 10.5 | 0.6 | 0.2 | 10.5 | 2.3 | 0.7 | 13.0 | *S. sanguineum* |
| *Sympetrum sanguineum* | 0.3 | 0.1 | 9.8 | 0 | 0 | 10.1 | 0.3 | 0.1 | 9.8 | 0 | 0 | 9.9 | 0 | 0 | 12.4 | *S. meridionale* |
| *Sympetrum meridionale* | 0.3 | 0.2 | 9.8 | 0 | 0 | 10.1 | 0.3 | 0.2 | 9.8 | 0.2 | 0.1 | 9.9 | 0 | 0 | 12.4 | *S. sanguineum* |

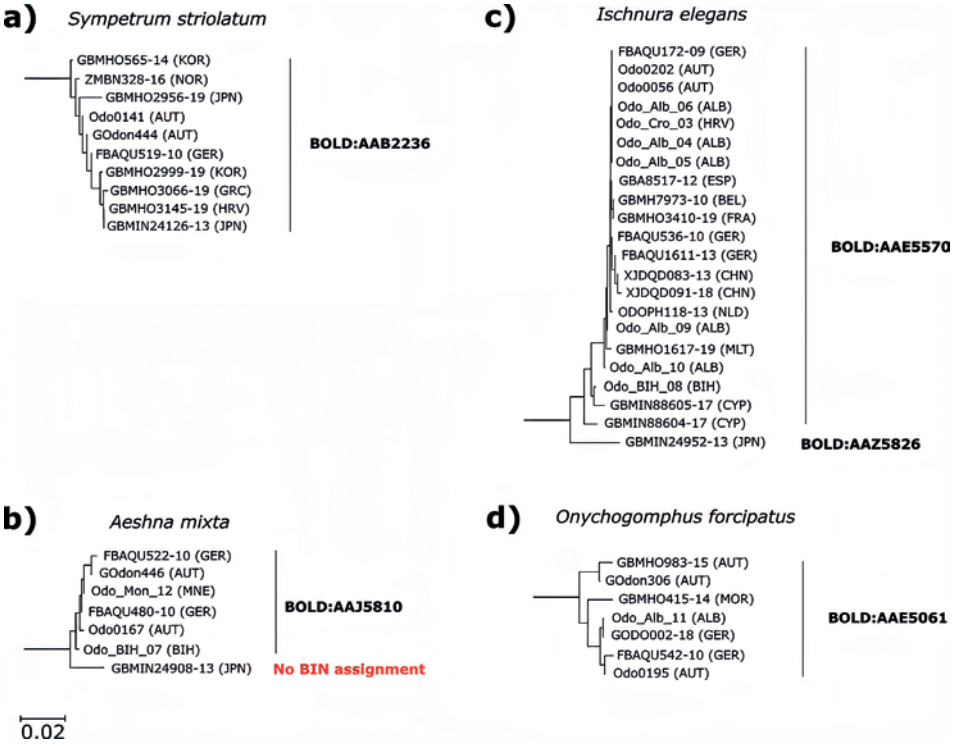


Fig. 2: Partial NJ trees calculated with *COI* sequences of *Sympetrum striolatum, Ischnura elegans, Aeshna mixta*, and *Onychogomphus forcipatus* with BIN assignment. – Abb. 2: NJ-Bäume und entsprechende BIN-Zuordnungen berechnet aus den *COI*-Sequenzen von *Sympetrum striolatum, Ischnura elegans, Aeshna mixta* und *Onychogomphus forcipatus*.

(GER, AUT, ALB), *Sympetrum meridionale* (AUT, MNE, BIH) and *Sympetrum sanguineum* (GER, AUT, HRV).

In *Enallagma cyathigerum*, the maximum p distance was 0.6-1.0 % which is insofar remarkable as the clade comprises not only individuals from Northern, Central, Southern and Southeastern Europe (NOR, GER, AUT, BIH, ESP, HRV) but also two sequences from China, one of which has an identical sequence as a specimen from Germany. The low intraspecific genetic distances are reflected by only a single BIN (BOLD:AAA2218) containing all analysed sequences (Fig. 2).

The remaining four species showed intraspecific distances >1 %: (1) *Sympetrum striolatum* is represented by Northern, Central and Southeastern European countries (NOR, AUT, GER, HRV, GRC) as well as Asia (JPN, KOR). In respect of the huge geographic range, the intraspecific distances (max. 2.3 %; Tab. 4) are very low and no geographic pattern is visible. The grouping into one single BIN (BOLD:AAB2236) further corroborates these findings. In particular, a sequence from South Korea is placed in the middle of the clade, close to sequences from Greece and Croatia and one sequence from Japan is identical to a sequence from Croatia (Fig. 2a). The 178 bp-sequence of *S. striolatum* (OdoMNE_11), which was not included into the calculations (tree and distances) due to its short length,

was identical within the part of the comparable (178 bp) sequence with that of the individual GOdon444 from Austria, Styria. Thus, it fits into the variation of this group of closely related sequences. (2) In the clade representing *Aeshna mixta* there is one quite distinct sequence from Japan which is separated from a sequence from Germany by the maximum distance (2.4 %) found within this clade (Fig. 2b). In the remaining clade (not considering the Japanese sequence) the maximum distance is 0.6 % and comprises sequences from Central and Southeastern Europe (GER, AUT, BIH, MNE). This is also reflected by the BIN analysis, as all but the sequence from Japan share the same BIN (BOLD:AAJ5810). However, the Japanese sample was not assigned to any BIN by the BOLD system. (3) Intraspecific distances within *Ischnura elegans* range up to 4.3 % and again (as in *A. mixta*) the highest distance separates a sequence from Japan. In this case, the sequence from Japan represents a unique BIN (BOLD:AAZ5826) and stands in contrast to BOLD:AAE5570, which encompasses all other sequences of *I. elegans* included in this study. In contrast to *A. mixta*, the remaining clade is still quite variable (mean 0.4-0.8 %) comprising sequences from Southern, Western, Central and Southeastern Europe (ESP, MLT, BEL, FRA, NLD, GER, AUT, HRV, BIH, ALB, CYP) as well as China. Interestingly, the two sequences from China are very similar to German sequences, while sequences from Cyprus and Bosnia are somewhat divergent from the remaining clade (Fig. 2c). (4) In *Onychogomphus forcipatus* the seven sequences form three lineages separated by mean distances ranging from 1.4-2.6 %. The maximum distance within the clade is 5.1 %. One specimen from Carinthia (AUT) and one sequence downloaded from BOLD (GBMHO983-15) from an unspecified locality in Austria differ significantly from the rest of the samples (mean p distance 2.9 %, range 2.8-5.1 %). The second lineage is a single sequence from Morocco and the third one comprises sequences from Central and Southeastern Europe (AUT, GER, ALB). Thus, compared to the other species, *O. forcipatus* displays a considerable variation in our data set, with Austrian sequences present in two of the three lineages (Fig. 2d). Since the Austrian sequence downloaded from BOLD is not provided with more detailed geographical information, drawing conclusions about a geographical pattern of this species within Austria remains speculative. However, despite the increased intraspecific genetic distances all sequences included in the present study share the same BIN assignment (BOLD:AAE5061).

## Discussion

The present study provides insights into intraspecific genetic variation of selected species of dragonflies and damselflies across large geographic distances. All but one species of six different families (Lestidae, Calopterygidae, Coenagrionidae, Aeshnidae, Gomphidae, Libellulidae) of both Anisoptera and Zygoptera displayed maximum genetic distances to conspecifics <1 % (depending on the set of analysis parameters). Furthermore, we exemplified how the inclusion of sequences available in public data bases may bias the calculations and comparisons of genetic distances. In the present study, the complete *COI* sequence was not obtained from all samples and sequences derived from GenBank were of various lengths. Despite the fact that there is a defined standard DNA barcoding region, the sequences available in the data bases, even in BOLD, are of various lengths. The fact that several studies have used different primers targeting different genetic regions of the *COI* gene led to inconsistent coverage of the Folmer region in the various studies, resulting in a heterogeneous data set of differently sized sequences (Iserbyt et al. 2010, Karube et al. 2012, Bergmann et al. 2013, Dijkstra et al. 2013, Sanchez-Guillenet al. 2013, Futa-

hashi 2014, Froufe et al. 2014, Kim et al. 2014, Corse et al. 2017, Zhang et al. 2017). Therefore, we analyzed our data using different treatments of missing or ambiguous data, which mathematically leads to reduced datasets in terms of length (number of base pairs) and compared them to actively shortened datasets based on different criteria (minimum barcode length, no missing data at all; Tab. 4). The differences in calculated distances, of course, reflect the amount of available data, the number of substituted sites and the ratio thereof. The amount of missing data can be viewed as a proxy for alignment length, as positions including missing data (at the beginning or end of the dataset) are excluded from the analysis. The 'pairwise deletion' option retains the maximum information possible but uses sequences of different lengths (and consequently parts of the gene). This option is in general rather useful for tree inference, allowing to use incomplete data sets comprising sequences of various lengths and still arriving at reasonable phylogenetic reconstructions. The 'complete deletion' option on the other hand rigorously excludes all sites with missing data from the analysis and might thus, depending on the completeness of the dataset, use only a very limited amount of the information present in the data. Hence, position and amount of missing data as well as its treatment in subsequent analyses may have substantial impact on the results, exceeding the magnitude of decimal digits (Tab. 4). This circumstance should be kept in mind considering suggested species delimitation ratios and thresholds (Bergmann et al. 2013 and references therein). Hence, we recommend that barcoding studies should provide detailed information on how missing sequence data have been dealt with in the various analyses.

Nonetheless, in our selection of dragonfly species, intraspecific genetic distances are relatively low, despite considerable variation depending on the size of the dataset and the treatment of missing data, which is in line with previous studies on dragonfly dispersal and genetics (Keller & Holderegger 2013 and references therein). Rapid population expansion after a genetic bottleneck and/or high levels of gene flow due to high dispersal capacity might be the straightforward explanations for such a homogeneity. Yet, the results are still preliminary and each of the species requires further investigation and a more comprehensive sampling, both in terms of individuals and molecular markers, to allow for the robust reconstruction of past demographic events and phylogeographic/population genetic structure. From the present data set it becomes apparent, though, that, while there are specimens with (almost) identical haplotypes from regions separated by extremely large distances (e.g., Europe to China), populations on certain islands tend to be more divergent (e.g., Cyprus, Japan).

Still, the extremely low intraspecific distances found in all our study species are surprising. Whereas Anisoptera are known to be very good active dispersers that actively disperse over large geographic distances (Corbet 1999, May & Matthews 2008), dispersal distances are generally smaller in Zygoptera (e.g. Rouquette & Thompson 2005, Harabiš & Dolný 2011, Hassall & Thompson 2012, Kellller & Holderegger 2013). Both taxa, however, are known to passively travel large distances with seasonal winds (Corbet 1999, May & Matthews 2008). Consequently, it appears as if morphological prerequisites and active dispersal ability do not constrain geographical large-scale migrations, resulting in similarly low intraspecific genetic distances and a general lack of geographic structuring. This is also consistent with findings by May & Matthews (2008), who suggested one large population for *Anax junius* in Northeast America due to long distance dispersal, rather than many small populations connected by occasional single individual migrations.

In conclusion, dragonflies, at least the species under study here, show relatively low intraspecific genetic diversity despite their often vast distribution ranges. Judging from our results, the general differences in anatomy inherent with the distinction into Anisoptera and Zygoptera are not reflected by their long-distance dispersal abilities.

Our study further illustrates how comparisons of genetic distances might suffer from different treatment of missing data and variation in sequence lengths. Thus, we advocate to clearly state analysis parameters in publications or even strive for a common standard.

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