Journal of Insect Conservation (2023) 27:435–450
https://doi.org/10.1007/s10841-023-00467-x

ORIGINAL PAPER

DNA barcoding of exuviae for species identification of Central European damselflies and dragonflies (Insecta: Odonata)

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Received: 17 August 2022 / Accepted: 9 February 2023 / Published online: 14 March 2023

Abstract

Monitoring of odonates has become an important instrument for ecological status assessment of (semi-)aquatic habitats. Besides information on presence and abundance, knowledge about a species´ autochthony at the surveyed waterbody is a significant information within the assessment process. Here, the finding of exuviae represents the ultimate proof of successful reproduction. Although feasible for most odonate species, morphological identification of exuviae is often time consuming, as it relies on small, fragile structures. To facilitate species identification of exuviae, a DNA barcoding approach was developed, including (1) non-destructive extraction of DNA using whole exuviae or their tracheal tubes, and (2) primer systems for long (< 600 bp) and short (< 200 bp) *CO1* fragments. A total of 85 exuviae from 33 species were analysed and compared to results of morphological identification. Additionally, factors potentially influencing DNA quality and quantity, as well as PCR and sequencing success were investigated. Eighty-two exuviae matched the morphologically identified genus, and 60 matched at species level. Of the 33 species present in the data set, 82% could be identified to species level via DNA barcoding. The results show how DNA-based approaches can support fast and accurate species identification and therefore enhance monitoring of an ecologically important taxonomic group, with high relevance for conservation and habitat restoration. Moreover, the use of exuviae as DNA resource once more shows that non-invasive sampling offers great potential for molecular species identification, which is essential when studying rare and endangered species.

Implications for insect conservation Our results show how molecular tools, here DNA barcoding of odonate exuviae, can support species monitoring without the need of catching individuals, harming, or even killing them. Obtaining DNA from non-invasive sources can thus be a direct advantage to the conservation of insects, especially when dealing with rare and endangered species and/or populations. Using the example of odonates as bioindicator organisms for aquatic and semi-aquatic habitats, we highlight the importance of non-invasive genetic approaches for population studies and monitoring of insect species and/or species communities for ecosystem assessments and conservation management.

Keywords DNA barcoding · Odonates · Insect skin · Non-invasive sampling · Species identification

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Introduction

For biological monitoring and the assessment of the ecological state of aquatic habitats (e.g. within the European Water Framework Directive 2000/60/EC by the European Union), benthic macroinvertebrates are commonly used as they show high species diversity and distinct sensitivity to water and habitat quality (Hering et al. 2004; Birk et al. 2012; Macher et al. 2016; Elbrecht and Leese 2017; Kuntke et al. 2020). Within the group of benthic macroinvertebrates, odonates, comprising dragonflies and damselflies are very suitable as bioindicator organisms, due to their well-known species-specific habitat preferences. Furthermore, in contrast to other benthic invertebrates, species identification in odonates is comparatively straight forward. Hence, surveying odonate species communities or single species as proxies for habitat quality parameters has become an important instrument in monitoring, classifying, and assessing aquatic ecosystems (Chovanec and Waringer 2001; Oertli 2008; Silva de paiva et al. 2010; Chovanec 2018, 2022; Vorster et al. 2020; Gómez-Tolosa et al. 2022). Dragonflies and damselflies have an amphibiotic lifecycle and colonize all kinds of aquatic and semi-aquatic ecosystems but exhibit species-specific habitat requirements regarding hydrology and structural heterogeneity of the waterbody itself and the water-land ecotone (e.g. type of sediment, riparian morphology, aquatic and riparian vegetation). The latter one comprises the most sensitive part of the life cycle, the emergence of the adult (Corbet 1999). Besides their relevance in biological monitoring, the presence and absence of certain odonate species is also of conservation concern: on the European scale, of the 143 species occurring in Europe, 13% (19 species) are listed in one of the three "threatened" categories according to the IUCN Red List criteria, and another 12% (17 species) are classified as "near threatened". Furthermore, 16 species are listed in the Annexes II and/or IV of the Habitats Directive 92/43/EEC by the European Union, and 16 species are endemic to Europe (Boudot and Kalkman 2015; Kalkman et al. 2018).

Traditional field methods used to monitor odonate species comprise observation of adult specimens (including capture and release approaches), complemented by the collection and identification of exuviae (Foster and Soluk 2004; Raebel et al. 2010; Bried et al. 2015). Exuviae represent the exoskeletons shed by the adults during final moulting. They withstand a variety of environmental conditions over longer periods (up to several weeks) after the moulting process and can be easily found at riparian vegetation and other structures (Roe 2018). Exuviae are a source of information on the presence of a species at a specific locality and provide evidence about its reproductive success (Corbet 1999). As many damsel- and dragonflies have a high dispersal capacity, studying only adults may lead to an overestimation of the number of reproducing species at a given locality. Sampling of exuviae may help to reduce such a bias in estimated species numbers towards adult records and an overestimation of resident species diversity (Raebel et al. 2010; Bried et al. 2012). Furthermore, sampling of exuviae is completely non-invasive—unlike so-called non-lethal methods (e.g., wing clips or leg sampling in larval stages) when aiming for material to be used for genetic analyses. Such non-invasive approaches are essential when studying rare and/or endangered species, and species with high dispersal activity, low philopatry or short flight periods. In these cases, the observation of adult specimens and the assessment of autochthony based on observation of reproductive behaviour is often not feasible. Here, a targeted search for exuviae represents an important approach for unambiguous species records and evidence for autochthony (Boda et al. 2015). However, morphological identification of exuviae to species level is often time consuming, depends on the availability of taxonomical expertise, and is not feasible in case of damaged or fragmented specimens. Furthermore, for several closely related species it is difficult or even impossible to distinguish their exuviae morphologically, thus impeding species identification (for damsel- and dragonflies e.g. *Sympetrum striolatum/sanguineum/meridionale, Coenagrion puella/pulchellum*).

Here, the combination of non-invasive sampling of exuviae and molecular genetic methods offers great potential and is essential when studying rare and endangered species (Beja-Pereira et al. 2009; Lefort et al. 2022). In general, DNA barcoding provides a reliable, inexpensive genetic tool for identification of species. This method employs short DNA sequences of specific genes selected as "DNA barcoding genes" (Hebert et al. 2003). For animals, the mitochondrial (mt) *cytochrome c oxidase subunit 1* (*CO1*) gene is most commonly used and has also proven successful for discriminating species in Odonata (Galimberti et al. 2021; Geiger et al. 2021; Maggioni et al. 2021). In addition to species identification, information on intra- and interspecific variation can be obtained from barcode sequence data (for odonates see for example Haring et al. 2020; Geiger et al. 2021).

DNA extracted from exuviae was already used for species identification in butterflies (Feinstein 2004), chironomids (Krosch and Cranston 2012; Kranzfelder et al. 2016, 2017), beetles (Lefort et al. 2012; Inoda et al. 2015) and tarantulas (Petersen et al. 2007), and further to answer questions on species distribution, genetic variation and population structure, dispersal or monitoring of reintroduction programmes (e.g. Keller et al. 2010; Lefort et al. 2012; Dolný et al. 2018). For DNA analyses of dragonfly and damselfly exuviae,

mostly species-specific microsatellites were used as genetic markers to study population diversity, genetic structure, and dispersal (Watts et al. 2005; Keller et al. 2010; Monroe et al. 2010; Dolný et al. 2018). To our knowledge no study exists using DNA barcoding methods for species identification from odonate exuviae.

While invasive genetic sampling could negatively affect populations, especially small ones, non-invasive genetic approaches allow to study wildlife populations without harming them (Lefort et al. 2022). However, despite the tempting advantages of non-invasive genetic approaches and continuous technological advances (Beja-Pereira et al. 2009; De Barba et al. 2017; Carroll et al. 2018; von Thaden et al. 2020), certain limitations regarding the utilization of left-behind animal material (collected in the field) as DNA source remain. DNA isolated from such samples is typically characterized by low DNA quantity and quality (degraded DNA), and the presence of inhibitors (Taberlet et al. 1999), which pose a risk of high sample dropout rates and erroneous results. This also applies to DNA isolated from exuviae. As exuviae consist of extracellular chitin, the only source of genomic DNA are cells randomly attached to the inner surface of the larval skin during the moulting process. In principle, this small amount of starting material can be sufficient for successful amplification and sequencing of target marker genes. However, various environmental factors impact DNA quality already in the field (e.g. ambient temperature, humidity, sun exposure) and the duration of exposure of exuviae to these environmental conditions, causing degradation of DNA, is usually unknown (Watts et al. 2005). Also, after collection, additional factors like preservation method, storage time, and extraction chemicals may influence DNA yield (Nguyen et al. 2017). Moreover, even if isolation of DNA traces was successful, contamination with non-target DNA (originating from organisms feeding on or living in the exuvia) as well as the presence of potential PCR inhibiting substances (e.g. humic acids) may decrease success rates (Petersen et al. 2007; Nguyen et al. 2017).

Most protocols on isolation of DNA from exuviae (odonates and other taxa) published so far included grinding up (i.e. destroying and homogenizing) the exuviae prior to digestion (e.g. Gregory and Rinderer 2004; Watts et al. 2005; Keller et al. 2009, 2010; Lefort et al. 2012; Nguyen et al. 2017; Ali et al. 2019), which probably may increase the concentration of potential inhibitors. Furthermore, after extraction of DNA, the exuvia no longer exists and may not serve as voucher specimen in a reference collection. Therefore, a non-destructive approach of DNA isolation leaving the exuviae intact for future morphological studies and reducing the amount of inhibiting substances in the final DNA solution would be preferable.

The main objective of the present study was to establish a DNA barcoding approach covering all nine Central European odonate families to test DNA-based species identification using *CO1* sequences. This included two tasks: (1) the increase of amplification success rate for degraded DNA samples and (2) the avoidance/minimisation of co-amplification of contaminating non-target taxa. Therefore, our strategy was to aim at long (< 600 bp; LF) as well as short (< 200 bp; SF) *CO1* fragments, and to develop primer systems specifically designed for certain taxonomic groups of odonates (e.g., families, genera). We combined "rough" morphological assignment (achievable with even low experience and skills in morphological identification of odonate exuviae) with subsequent selection of the most suitable primers.

For extraction of DNA from exuviae we applied a non-destructive approach aiming to (1) minimize the amount of potential inhibitor substances in the DNA extract for downstream analysis, and (2) preserve the morphological characteristics of exuviae for optimal handling of voucher specimens in reference collections. Furthermore, we tested whether concentration and degradation of the isolated DNA from exuviae, and subsequent PCR and sequencing success are impacted by preservation method (dry vs. ethanol), storage time, source of DNA (two types of tissue), fragment length (short vs. long), and size of exuviae (Anisoptera vs. Zygoptera).

Materials and methods

Exuviae collection and sample preservation

A total of 85 exuviae was collected in the years 2014–2019 along different waterbodies located in Austria and stored either in > 96% ethanol (71 specimens) or dry with silica gel (14 specimens) until isolation of DNA (see Table 1). A detailed specimen list and additional information on the collected and analysed exuviae can be found in Online Resource 1.

Morphological identification and documentation

The collected exuviae were morphologically identified using a dissecting microscope and taxonomic keys (Heidemann and Seidenbusch 2002; Brochard et al. 2012). For documentation, voucher specimens were photographed and deposited in the scientific collection of the Natural History Museum Vienna, Austria.

To evaluate the non-destructiveness of DNA extraction of exuviae, we repeated the morphological identification after DNA extraction for a subset of 36 specimens in a blind test, i.e., without knowing the predefined species. This was to test whether the species-specific morphological key characteristics still existed after the extraction procedure.

Table 1 Dragonfly and damselfly exuviae collected in Austria in the years 2014–2019. Consensus species identification based on morphological characteristics and DNA barcoding using *CO1*.

Suborder/family	Species	Number of exuviae	Preservation
Zygoptera			
Calopterygidae	*Calopteryx splendens*	4	Ethanol
Coenagrionidae	*Coenagrion puella/pulchellum/ornatum**	8	Ethanol
	Erythromma najas	2	Ethanol
	Erythromma viridulum	1	Ethanol
	Ischnura elegans	3	Ethanol
	Ischnura pumilio	3	Ethanol
Lestidae	*Chalcolestes viridis*	1	Dry
	Sympecma fusca	2	Ethanol
Platycnemididae	*Platycnemis pennipes*	4	Ethanol
Anisoptera			
Aeshnidae	*Aeshna cyanea*	4	Ethanol (3); dry (1)
	Aeshna isoceles	1	Dry
	Aeshna juncea	1	Dry
	Aeshna mixta	1	Ethanol
	Anax imperator	6	Ethanol (5); dry (1)
	Anax parthenope	3	Ethanol (2); dry (1)
Cordulegastridae	*Cordulegaster bidentata*	1	Dry
	Cordulegaster heros	5	Ethanol
Corduliidae	*Cordulia aenea*	1	Ethanol
	Epitheca bimaculata	1	Ethanol
	Somatochlora alpestris	1	Dry
	Somatochlora flavomaculata	1	Ethanol
	Somatochlora metallica	1	Ethanol
Gomphidae	*Gomphus vulgatissimus*	1	Ethanol
	Onychogomphus forcipatus	4	Ethanol
Libellulidae	*Crocothemis erythraea*	3	Ethanol (1); dry (2)
	Leucorrhinia albifrons	5	Ethanol (4); dry (1)
	Leucorrhinia caudalis	1	Ethanol
	Libellula depressa	4	Ethanol (3); dry (1)
	Libellula quadrimaculata	1	Ethanol
	Orthetrum brunneum	1	Dry
	Sympetrum sanguineum	1	Ethanol
	Sympetrum striolatum	7	Ethanol (6); dry (1)
	Sympetrum vulgatum	2	Ethanol
Total	33 (35)[§]	85	Ethanol (71); dry (14)

**Coenagrion puella/pulchellum/ornatum* cannot be distinguished via DNA barcoding using *CO1*, and, furthermore, exuviae of *Coenagrion puella/pulchellum* cannot be distinguished based on morphological characteristics

[§]Minimum and maximum number of 33 and 35 species, respectively, due to limitations in species assignment in the *Coenagrion puella/pulchellum/ornatum* species complex

DNA extraction of exuviae

We applied two different approaches for DNA extraction: (1) using the whole larval skin and (2) using only the tracheal tubes of an exuvia (white, threadlike structures at the moulting opening representing the moulted cuticula of the respiratory system; Fig. 1). For both DNA extraction approaches, the DNeasy Blood and Tissue Kit was used (with slight modifications of the manufacturer's protocol; see below) together with the QIAvac 24 Plus manifold and a vacuum pump (Qiagen, Germany) for better handling of larger volumes (see below). In a first step, exuviae stored in ethanol were put on a tissue paper for at least 30 min to let the residual ethanol evaporate before lysis.

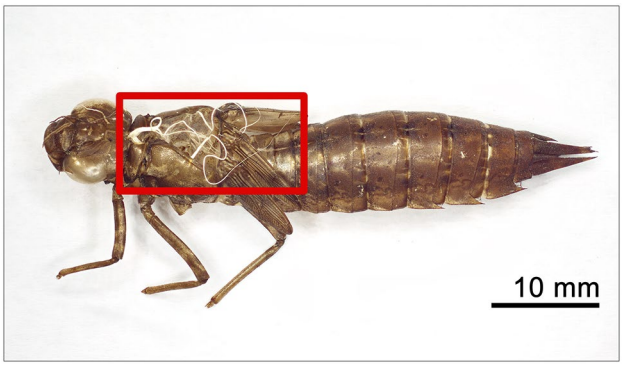

Fig. 1 Exuvia of *Anax imperator*. Red rectangle shows the location of the tracheal tubes (white, threadlike structures at the moulting opening representing the cuticular part of the moulted respiratory system) which were used for extraction of DNA. (Color figure online)

For the first approach, i.e. using the whole exuviae for extraction, we adjusted the applied volumes of extraction reagents according to the size of the exuviae: for exuviae of the suborder Zygoptera (excl. Calopterygidae) and the subfamily Sympetrinae (Anisoptera) we applied the fivefold of the original volume of the manufacturer´s protocol. For exuviae of all other families of the suborder Anisoptera and the family Calopterygidae (Zygoptera), we increased the volume up to tenfold of the original volumes. Lysis was carried out overnight in an incubator at 37 °C, with soft rotation in a tube rotator. After the lysis step, the exuviae were removed carefully and transferred to > 96% ethanol for long-term voucher storage and extractions proceeded with the remaining solution. Tubes were never vortexed while the exuviae remained in the tube and only centrifuged shortly at minimum speed (to prevent damaging of morphological characteristics relevant for species identification of exuviae). Final elution of DNA was carried out with 40 µl of AE buffer after an incubation time of 5 min at room temperature.

In a second, entirely non-destructive approach, we tested DNA extraction for a subset of eight exuviae using the tracheal system of the exuviae as DNA source material. The tracheal tubes were cut off without destroying the exuviae and put into the tube containing the lysis buffer. The subsequent extraction steps followed the manufacturer´s protocol. For final elution we used 30 µl of AE buffer with an incubation time of 5 min at room temperature, before the columns were centrifuged to elute the DNA.

Quantity and quality of DNA samples

DNA yield, quality, and integrity for the 85 DNA extracts was measured with a 4150 TapeStation System using the Genomic DNA ScreenTape Analysis (Agilent, USA). In addition to DNA concentration (ng/µl), the TapeStation System provides an objective, numerical assessment of DNA integrity (using an algorithm; TapeStation Software 4.1.1), referred to as the "DNA integrity number" (DIN). This value ranges from 1 to 10, where 1 indicates highly degraded DNA and 10 represents highly intact DNA.

Primer design and evaluation

Primers were manually designed based on a *CO1* sequence reference database established in the course of the Austrian Barcode of Life initiative (ABOL; www.abol.ac.at) and consisting of 68 species (800 specimens) (Zangl et al., in prep.; partial release of corresponding BOLD data: data set name "DS-AODOEX"). In addition, *CO1* sequences of 10 additional odonate species accessed from BOLD and NCBI GenBank were included and considered for primer design. Altogether, the data set represented 91% of the Central European odonate fauna. Primers were designed for the two different fragment lengths (long fragment: < 600 bp, LF and short fragment: < 200 bp, SF) (Table 2; Fig. 2). Short fragments facilitate amplification also in highly degraded DNA samples, which are common when samples are collected non-invasively in the field from remains of individuals (as it is the case for exuviae) and not from living individuals. For the LF, one primer pair covers all families tested, except for Aeshnidae (Table 2) for which a separate primer pair was designed. Thus, for the selection of the correct LF primers, it is only necessary to distinguish between the exuviae belonging to the family Aeshnidae or other families. For the SF, six different group-specific primer pairs were designed to ensure both optimal primer binding sites as well as high taxonomic resolution. Nevertheless, primer selection in both cases is based on a few morphological characteristics easily identifiable by eye—also by non-entomologists. In Fig. 4, we provide a simple guide for the correct primer selection. All fragments (long and short) were designed to lie within the standard barcoding region of *CO1*, i.e. 5′-end of *CO1* (Hebert et al. 2003). The SFs were positioned either entirely within the LFs or overlapping with them. Locations and array of the primers in the *CO1* gene is shown in Fig. 2.

Candidate primers were tested in silico with the software Amplify4 (Engels 2015) to control for specificity and quality of the primers, primer dimers, multiple binding sites, amplicon length and quality.

Selected primer pairs were tested by PCR with DNA samples from exuviae (for those species collected as exuviae within our study) as well as from tissue of collected imagines (ABOL reference specimens). Hence, altogether, the primers were validated by PCR for a total of 65 species (76%), representing 25 genera (83%) and 9 families (100%) occurring in Central Europe. The complete list of species tested can be found as supporting information in Online Resource 2.

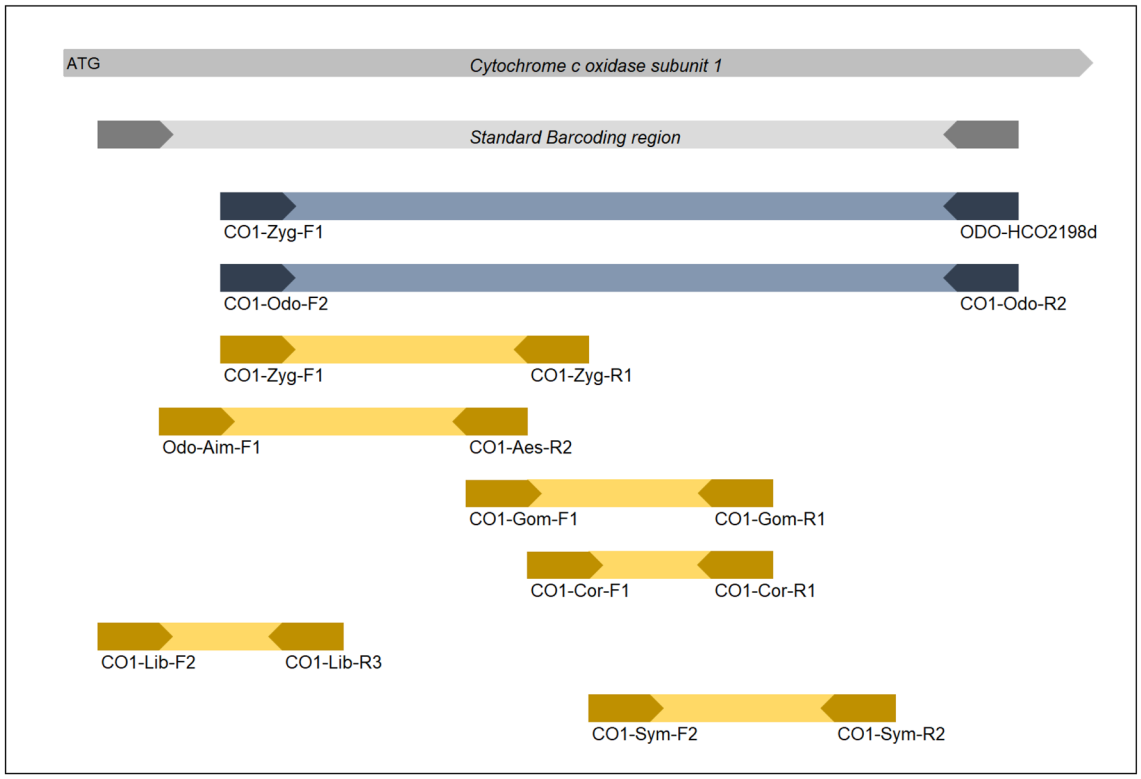

Fig. 2 Location of primers and amplicons on the *cytochrome c oxidase subunit 1* gene for species identification via DNA barcoding of dragonfly and damselfly exuviae. Blue bars indicate amplicons (together with primers) for the < 600 bp long fragments (LF), yellow bars indicate amplicons for various shorter fragments (< 200 bp; SF). *ATG* indicates the start codon of *CO1*. (Color figure online)

Table 2 Primers for species identification in taxonomic groups via DNA barcoding (*CO1* gene) of dragonfly and damselfly exuviae. Some primers are applicable in suborders, some in certain families, genera or even species

For use in odonate taxa	P_{fwd}	P_{fwd} sequence (5′–3′)	P_{rev}	P_{rev} sequence (5′–3′)	AL	SL	T_{ann} (°C)
Zygoptera, Gomphidae, Cordulegastridae, Corduliidae, Libellulidae	CO1-Zyg-F1*	ttggagatgaycaaatttataaygt	ODO_HCO2198d**	taaacttcwggrtgtccaaaraatca	538	588	57
Aeshnidae	CO1-Odo-F2	ggagatgaycaaatttataatgt	CO1-Odo-R2	cttcdggrtgtccaaagaatc	539	580	51
Zygoptera	CO1-Zyg-F1*	ttggagatgaycaaatttataaygt	CO1-Zyg-R1	cctgttcctgctcctctttctacta	182	231	60
Aeshnidae	Odo-Aim-F1	gtaggaactgctctaagagttt	CO1-Aes-R2	ggtggtaataatcaaaatcttat	191	235	54
Gomphidae	CO1-Gom-F1	acaggatgaactgtttatcc	CO1-Gom-R1	catacaaatagaggtatttgatcta	143	187	54
Cordulegastridae	CO1-Cor-F1	ggagcttccgtagatttaactatt	CO1-Cor-R1	acagcagtaatagcaactgctc	115	160	60
Corduliidae, Crocothemis, Libellula,Orthetrum, *Sympetrum fonscolombii*	CO1-Lib-F2	gatggctattttckactaatcat	CO1-Lib-R3	ttataaatttgatcatctccaat	105	150	52
Sympetrum***, Leucorrhinia	CO1-Sym-F2	catcttgcaggagtatcmtcaat	CO1-Sym-R2	gatgtattaatatttcgrtcagtta	155	202	58

P_{fwd} forward primer, P_{rev} reverse primer, *AL* Amplicon length (bp), *SL* sequence length (bp), T_{ann} annealing temperature

*Haring et al. (2020)

**Dijkstra et al. (2014)

***Exceptional case: *Sympetrum fonscolombii*

PCR amplification and conditions

PCR reactions were performed with the Multiplex PCR Kit (Qiagen, Germany) in a total volume of 25 µl, containing 12.5 µl Multiplex PCR Master Mix, 0.5 µM of each primer, and 1 µl template DNA. The PCR cycling protocol included an initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 90 s, extension at 72 °C for 60 s. The final step was an extension at 72 °C for 10 min. Annealing temperatures are given in Table 2. The "PCR success" of a sample was indicated by the presence of a band of the expected size in an agarose gel after electrophoresis.

Sanger sequencing and species identification

Samples with visible PCR bands were purified and sequenced with PCR primers in both directions using Sanger sequencing technology (Microsynth, Switzerland). Sequences were edited using the software Geneious 10.2.6 (Biomatters, New Zealand) and final consensus sequences were aligned with BioEdit 7.2 (Hall 1999). Sequences were compared with reference sequences in (1) our own reference database (Zangl et al. in prep), (2) the BOLD Identification Engine, and (3) via BLAST search in NCBI GenBank. The yield of a high-quality sequence from an exuvia sample was classified as "sequencing success". Species/genus "identification success" was defined as a consistent species/genus match between morphological identification and the identification of the sequence via reference data bases.

Sequences of LFs generated from exuviae were uploaded to BOLD/GenBank (BOLD ID see Online Resource 1) together with a picture and all metadata of the specimen. Sequences of the SFs are provided in Online Resource 3 and 4.

Statistical analysis

We tested for differences in DNA concentration and DIN value of DNA extracts from exuviae concerning source of DNA (whole exuvia vs. tracheal tubes), preservation method (ethanol vs. dry) and taxonomic suborder (Anisoptera vs. Zygoptera). As data for DNA concentration and DIN values was not normally distributed (Shapiro–Wilk Test), we used the non-parametric Mann–Whitney-U test. We further tested for relationship between DNA concentration and storage time (i.e. the time span between collection and DNA extraction of an exuvia), as well as DIN value and storage time using a Spearman correlation.

To determine whether there were differences in PCR success (for both, short and long fragments) in relation to storage time, DNA concentration and DIN value, we applied Mann-Whitney-U tests. The chi-squared test of independence was used to test for differences in PCR success, sequencing success, and species/genus identification success between fragment length (short vs. long), extraction method, preservation method and taxonomic group (Anisoptera vs. Zygoptera).

All statistical analyses were performed using the software R 4.0.3 (R Core Team 2022).

Results

Morphological identification and non-destructiveness of DNA extraction protocol

As revealed by morphological analysis, the exuviae samples collected within this study covered 33 odonate species (but see comments on *Coenagrion* spp. in Table 1) from 20 genera, which represent the nine families reported for Central Europe. Table 1 and Online Resource 1 provide an overview of the specimens analysed. The morphological re-analysis after DNA extraction of a subset of 36 exuviae (where the whole exuvia was used) revealed a successful re-identification of all 36 specimens to family level, and of 30 exuviae to species level.

DNA amplification and sequencing success

While for the amplification of short fragments (SF) PCR failed only once, PCR failed in 12 out of 85 cases for the long fragment (LF) ($\chi^2 = 10.1$, df = 1, $p < 0.01$). From all the successfully amplified PCR products, sequences were not analysable in three cases for the SF and in 21 cases for the LF. Hence, there was a significant difference in sequencing success ($\chi^2 = 19.1$, df = 1, $p < 0.001$) between SF and LF. However, there were no significant differences in genus and species identification success ($\chi^2 = 2.2$, df = 1, $p > 0.05$; $\chi^2 = 2.7$, df = 1, $p > 0.05$) between the fragment length categories.

Concerning PCR success between suborders, the difference of 79% for Anisoptera compared to 100% for Zygoptera was significant only for LF ($\chi^2 = 6.9$, df = 1, $p < 0.01$) (Fig. 3). The two suborders did not differ significantly concerning source of DNA ($\chi^2 = 0.02$, df = 1, $p > 0.05$) and preservation method ($\chi^2 = 0.004$, df = 1, $p > 0.05$).

Identification success and taxonomic resolution

In all of the 85 exuviae analysed, at least one of the desired fragments (SF and/or LF) could be amplified. Out of these, a total of 83 (98%) resulted in high-quality DNA sequences allowing unambiguous base calling for the whole sequence. Of the 83 sequences, 82 (96%) matched the morphologically identified genus, and 60 sequences (71%) matched at species

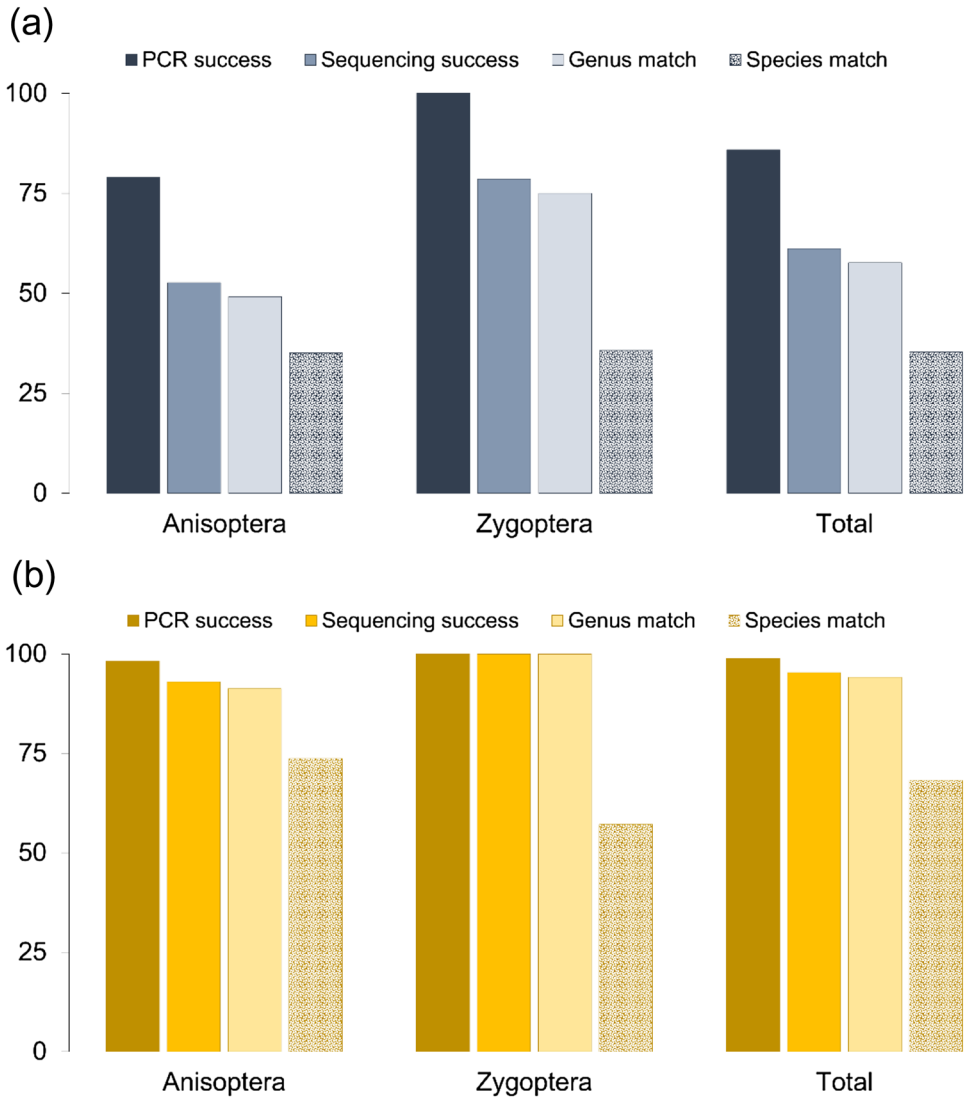

Fig. 3 Success rates for DNA amplification, high-quality sequences, genus, and species identification for barcoding of 85 odonate exuviae. Grouped barplots show success rates for odonate suborders (Anisoptera, Zygoptera) for **a** the long fragment (LF; < 600 bp), and **b** the short fragment (SF; < 200 bp)

level (Table 3). In terms of species, this means that of the 33 species present in our sample set, 27 (82%) could be identified to species level based on our DNA barcoding approach.

From 72 exuviae both LF and SF were amplified, and in 50 of them high-quality DNA sequences were obtained for both fragment lengths. Of these, 47 could be identified at genus level and 28 at species level (for both fragments; Table 3).

Reduced identification success was observed with SF as well as LF to the same extent, as it was mainly due to lack of taxonomic resolution at species level of some species pairs or groups that can be regarded as problematic (see discussion): (1) *Somatochlora* spp. (*S. metallica*, *S. alpestris*, *S. meridionalis*); (2) *Anax* spp. (*A. imperator*, *A. parthenope*); (3) *Platycnemis* spp. (*P. pennipes*, *P. latipes*); (4) *Coenagrion* spp. (*C. puella*, *C. pulchellum*, *C. ornatum*). In our sample set, 23 of 85 exuviae belonged to one of these groups (Table 1).

Another factor reducing identification success (at both genus and species level) was contamination of exuviae. Besides unreadable sequences as one consequence of contamination (causing overlay of sequence variants and thus reduction in sequencing success), there were some clearly readable DNA barcode sequences allowing to identify the contaminating taxon: this happened in three cases for the LF, when sequence analysis revealed contamination of exuviae with Chironomidae (*Cricotopus festivellus*; *Polypedilum cultellatum*). In one case for the SF, comparison with reference databases did not find a match for the contaminating sequence.

Impact on DNA concentration and integrity of exuviae DNA samples

DNA concentration ranged from 0.9 to 140.0 ng/µl with a mean value of 18.8 ng/µl (Table 3). We found no significant

Table 3 Summary statistics for DNA quantity and quality (DNA concentration and DIN values), and success rates (PCR, sequencing, genus, and species match) for DNA extracted from dragonfly and damselfly exuviae. For further details at individual level see Online Resource 1

Group	N	DNA (ng/µl)		DIN		At least one fragment (SF or LF)								Both fragments (SF AND LF)					
						PCR success		Sequencing success		Genus match		Species match		PCR success		Sequencing success		Genus match		Species match	
		Mean	SD	Mean	SD	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Family																					
Calopterygidae	4	46.5	62.6	3.7	1.7	4	100.0	4	100.0	4	100.0	4	100.0	4	100.0	1	25.0	0	0.0	0	0.0
Coenagrionidae	17	15.2	17.5	2.7	2.0	17	100.0	17	100.0	17	100.0	9	52.9	17	100.0	15	88.2	15	88.2	8	47.1
Lestidae	3	8.4	4.8	4.3	1.3	3	100.0	3	100.0	3	100.0	3	100.0	3	100.0	2	88.7	2	88.7	2	66.7
Platycnemididae	4	11.1	5.7	6.2	0.1	4	100.0	4	100.0	4	100.0	0	0.0	4	100.0	4	100.0	4	100.0	0.0	0.0
Aeshnidae	16	22.6	29.8	3.8	2.0	16	100.0	16	100.0	16	100.0	7	43.7	16	100.0	10	62.5	10	62.5	3	18.7
Cordulegastridae	6	16.7	6.8	2.6	1.0	6	100.0	6	100.0	6	100.0	6	100.0	3	50.0	0	0.0	0	0.0	0	0.0
Corduliidae	5	19.5	9.7	3.4	1.3	5	100.0	4	80.0	3	60.0	2	40.0	3	60.0	3	60.0	3	60.0	2	40.0
Gomphidae	5	18.7	10.3	2.9	0.9	5	100.0	4	80.0	4	80.0	4	80.0	3	100.0	3	60.0	3	60.0	3	60.0
Libellulidae	25	17.2	17.3	3.8	1.6	25	100.0	25	100.0	25	100.0	25	100.0	5	68.0	12	48.0	10	40.0	10	40.0
Suborder																					
Zygoptera	28	18.4	27.7	3.6	2.1	28	100.0	28	100.0	28	100.0	16	57.1	28	100.0	22	78.6	21	75.0	10	35.7
Anisoptera	57	19.0	19.8	3.6	1.6	57	100.0	55	96.5	54	94.7	44	77.2	44	77.2	28	49.1	26	45.6	18	31.6
Preservation																					
Ethanol	71	20.8	24.0	3.7	1.8	71	100.0	69	97.2	68	95.8	48	67.6	60	84.5	44	62.0	41	57.7	23	32.4
Dry	14	8.4	5.0	3.0	1.4	14	100.0	14	100.0	14	100.0	12	85.7	12	85.7	6	42.9	6	42.9	5	35.7
Source of DNA																					
Tracheal tubes	8	1.5	0.3	–	–	8	100.0	8	100.0	8	100.0	4	50.0	7	87.5	7	87.5	7	87.5	3	37.5
Whole exuviae	77	20.6	22.9	–	–	77	100.0	75	97.4	74	96.1	56	72.7	65	84.4	43	55.8	40	51.9	25	32.5
Overall	85	18.8	22.5	3.6	1.8	85	100.0	83	97.6	82	96.5	60	70.6	72	84.7	50	58.8	47	55.3	28	32.9

differences in DNA concentration between Anisoptera and Zygoptera ($W = 908$, $p = 0.31$). However, DNA concentration differed significantly between sources of DNA ($W = 616$, $p < 0.001$) with DNA extracts of tracheal tubes showing lower values (mean ± SD: 1.5 ± 0.3 ng/µl) than those of whole exuviae in the non-destructive extraction approach (mean ± SD: 20.6 ± 22.9 ng/µl). DNA concentration also varied between preservation method ($W = 308$, $p = 0.020$), with higher values measured for exuviae stored in ethanol compared to storage with silica gel (mean ± SD: 20.8 ± 24.0). Yet, concerning DIN values we found no significant differences between preservation method ($W = 322$, $p = 0.260$) and suborders ($W = 655.5$, $p = 0.740$). DIN values ranged from 1.0 to 7.0 (mean ± SD: 3.6 ± 1.8) (Table 3). A comparison between the sources of DNA (whole exuviae vs. tracheal tubes), however, has to be taken with caution, since for 10 specimens including all eight DNA samples from tracheal tubes, the DNA concentration was too low to provide a reliable DIN value.

There was no significant correlation between storage time (ranging from 1 to 1707 days) and DNA concentration ($r^2 = -0.19$, $p = 0.084$), but we did find a significant, though negative, correlation ($r^2 = -0.30$, $p = 0.009$) between storage time and DIN (Figs. S1, S2 in Online Resource 5). Storage time had no effect on PCR success (SF: $W = 4$, $p = 0.126$; LF: $W = 552$, $p = 0.152$; Figs. S3, S4 in Online Resource 5), and no differences in PCR success were found in relation to DNA concentration (SF: $W = 43$, $p = 0.984$; LF: $W = 514$, $p = 0.341$). Nevertheless, although not statistically significant, samples with higher DIN values showed higher PCR success rates, at least for the LF ($W = 263.5$, $p = 0.187$).

Discussion

In this study, we demonstrated successful isolation of DNA from dragonfly and damselfly exuviae and application of a specially tailored DNA barcoding approach for odonate species identification using DNA of material collected non-invasively in the field.

Our two-way approach offers primer systems for a longer (< 600 bp, LF) and a shorter *CO1* fragment (< 200 bp, SF) applicable according to quality and quantity of the DNA extracted. For correct primer choice of LF, it is only necessary to assign the exuvia to either the family Aeshnidae or the other families. To increase PCR and sequencing success rates and to accommodate low DNA concentrations and quality, primers for SF were also developed. For the selection of the appropriate SF primer pair, a rough assignment of the exuvia is necessary (mostly to family level). This can be easily done based on a few morphological characteristics, requiring neither prior taxonomic experience, nor the use of a dissecting microscope. Here, the number of successfully sequenced odonate exuviae was 96% when both fragment lengths were considered, compared to only 58% when restricted to LF alone.

Although DNA concentration and DIN values varied a lot among samples (mean ± SD: 18.8 ± 22.5 ng/µl; 3.6 ± 1.8), in most cases there was sufficient DNA for PCR amplification and subsequent sequencing (at least for a short fragment of ~ 200 bp). Moreover, for both fragment lengths, success rates (at all stages of the DNA barcoding pipeline: PCR, sequencing, genus/species identification) were relatively high, compared to other studies using insect skin as DNA source material for genetic analyses (e.g. Krosch and Cranston 2012: 46% for mitochondrial DNA and 29% for nuclear DNA; Ožana et al. 2020: 56% for mitochondrial and nuclear DNA). This may be explained by a combination of several reasons, which are discussed hereafter.

Advantages of non-destructive extraction

Concerning the optimization of DNA extraction, in our non-destructive approach, instead of homogenizing the whole larval skin (e.g. with mortar and pestle or in a ball mill as applied in other insect studies (Monroe et al. 2010; Nguyen et al. 2017; Ožana et al. 2020), we removed the intact exuviae after lysis or—as shown in our second approach—used only the tracheal tubes as starting material for DNA extraction. To our knowledge, the use of tracheal tubes as starting material for DNA extraction was tested in the present study for the first time. Although DNA extraction of tracheal tubes yielded less DNA compared to using whole exuviae, DNA concentration and quality was appropriate to generate high-quality DNA barcodes. In line with our high PCR success rates, our non-destructive extraction approach (either using whole exuviae or tracheal tubes only) may indeed have increased the quality of the extract by reducing the input of potential inhibitors (e.g. humic acids from soil), which are known to impede a successful downstream analysis (Nguyen et al. 2017). Furthermore, for DNA extraction we used a silica-membrane binding method, which is known to provide good quality genomic DNA from insect skins (Watts et al. 2005; Keller et al. 2009; Krosch and Cranston 2012; Kranzfelder et al. 2016, 2017). In terms of non-destructiveness of our extraction method, the repeated morphological identification of a small subset of 36 exuviae revealed slight damages in some exuviae after DNA extraction (e.g. the loss of tracheae in Zygoptera; a compressed abdomen).

However, morphological key characteristics relevant for species identification were generally well preserved, still enabling morphological species identification. Nevertheless, for voucher specimens(i.e., exuviae that will be kept as reference and stored in a scientific collection), we recommend isolation of DNA from tracheal tubes instead of the whole exuviae. Cutting off the tracheal tubes does not damage the

exuviae. However, one must keep in mind that DNA yield is reduced compared to using the whole exuviae for DNA extraction.

Particularities of non-invasively collected samples

As expected from samples collected non-invasively, DNA quality and quantity of exuviae samples in general was lower compared to DNA extracted from fresh tissue material (e.g. femur; own unpublished data). Also, Ožana et al. (2020) advised against the use of odonate exuviae for DNA sampling for both mitochondrial and nuclear gene applications, if the conservation status of the respective species allows non-lethal sampling (e.g. wing clip, leg of larvae).

However, depending on the research question of interest and the DNA extraction method used, exuviae can provide sufficient DNA for species identification via DNA barcoding based on mitochondrial markers, as shown by our study and others (Petersen et al. 2007; Krosch and Cranston 2012; Kranzfelder et al. 2016). We advocate non-invasive approaches, as exemplified here, as the number of threatened species is increasing and thus, from a conservation point of view, they appear generally preferable to invasive alternatives, even if they are non-lethal. Monroe et al. (2010) failed to obtain high-quality DNA from odonate exuviae that could be used for microsatellite genotyping. As multiple copies of the mitochondrial genome are present in a cell, mitochondrial markers are assumed to perform better than nuclear markers when working with degraded DNA samples (Krosch and Cranston 2012). Nevertheless, DNA from exuviae was also successfully applied in population genetic studies on insects using nuclear DNA, namely microsatellites (Gregory and Rinderer 2004; Watts et al. 2005; Keller et al. 2010; Dolný et al. 2018). Microsatellite markers are usually small in length and acknowledged to be promising markers when working with degraded DNA (e.g., forensics). When working with sample material collected non-invasively from remains animals left in the field, usually higher PCR dropout rates can be expected for longer fragments, as was also the case in this study.

Another factor for successful amplification of difficult, low-quality DNA samples may be the application of high-quality polymerases and high-end chemicals for PCR setup. Here, we used the Multiplex PCR Kit (Qiagen, Germany), which provided better PCR amplification in preliminary tests, resulting in stronger bands on the agarose gel compared to cheaper, standard PCR chemicals (own unpublished data).

Sample preservation and storage

We hypothesised that DNA quality and quantity of non-invasively collected exuviae may be affected by the size of exuviae (suborders Zygoptera and Anisoptera), the preservation method, and the time span between preservation and DNA extraction. Interestingly, we did not find differences in DNA concentration between suborders Zygoptera and Anisoptera, which was unexpected as we assumed larger specimens (Anisoptera) to leave higher amounts of cells during the moulting process in the larval skin (but see Ožana et al. 2020). Although DIN values did not differ between suborders, we observed higher PCR success rates for Zygoptera. This may be due to the fact that larvae of many species of Anisoptera (especially representatives of the families Cordulegastridae and Libellulidae also present in our data set) are buried in fine sediments and organic detritus and are covered with algae (Lang et al. 2001; Wildermuth and Martens 2019). Consequently, their exuviae are covered by mud/soil, which potentially contains more inhibiting substances for downstream analysis than other species.

Our tests of preservation method (ethanol vs. dry) revealed equal PCR success rates for both methods, but higher DNA concentrations were measured in specimens stored in ethanol. Consequently, although genetic identification via DNA barcoding also worked with dry exuviae (which is the common way for odonatologists to collect and store them), for future studies, in which genetic analyses of collected material might be applied, we recommend collecting and storing exuviae in ethanol. Not surprisingly, integrity of DNA (expressed as DIN value) negatively correlated with storage time. However, as the effect of storage time may also depend on how samples have been preserved, the effects of storage time and preservation method may be interdependent (discussed by Sittenthaler et al. 2021). Supporting this assumption, if planning for longer storage time (more than a few days) Ožana et al. (2020) recommend preserving exuviae in ethanol. Unfortunately, due to unbalanced sampling with our limited data set we were not able to incorporate all factors and interactions in a single statistical model. However, it is known that also for well-preserved collection material degradation of DNA is proceeding with time, even when all attempts are made to slow down these processes to a minimum (Blom 2021).

One factor that may explain the high variability of DNA concentrations in our DNA extracts, is the (mostly unknown) time span between the moulting of the individual and the collection of the exuviae (Watts et al. 2005; Keller et al. 2010). During this time span, the exuvia is exposed to abiotic and biotic environmental conditions and processes in the field, which promote DNA degradation (e.g. UV light exposure, humidity, temperature amplitudes, microbial activity) or increase the presence of inhibiting substances (e.g. soil, salinity; underground substrate) (Barnes et al. 2014; Strickler et al. 2015; Eichmiller et al. 2016). However, these factors are difficult to control in field experiments. It should be kept in mind, the longer the exuviae are exposed to them,

the farther DNA degradation may have progressed (Watts et al. 2005; Keller et al. 2010). Other studies reporting similar, or even higher, PCR amplification success rates, did not sample exuviae under field conditions as we did, but used exuviae of different insect species reared under very controlled (experimental) conditions, allowing for the collection of exuviae within a few hours (Feinstein 2004; Keller et al. 2010; Lefort et al. 2012; Inoda et al. 2015; Ali et al. 2019). Apart from optimized lab protocols, this highlights the high dependence of success rates from environmental conditions affecting exuviae between moulting and sampling. Nevertheless, regardless of such unknown factors and the duration the exuviae were exposed to them in our sample set (consisting of exuviae collected non-systematically), we achieved high success rates.

Contamination with non-target taxa

In the next step of the barcoding pipeline, the generation of high-quality sequences, we observed a relatively high drop-out rate of 5% (SF) and 28% (LF), which means that these sequences were generally of too low quality, or not analysable unambiguously due to double peaks. The latter may be related to accidental primer binding and co-amplification of DNA from non-target organisms, which either use the interior of the exuviae for housing (e.g. Chironomidae) or are attached to the surface of the exuviae. This kind of natural contamination already takes place in the field and cannot be effectively avoided. Despite the aim to design specific primers, the possibility of binding to a non-target species cannot be ruled out completely. The chance for such a false binding increases with low quality and concentration of the target DNA. In the present study, in four cases it even happened that we amplified only the contaminant DNA (three Chironomidae and one unknown sequence) and obtained high-quality non-target sequences. Reduced sequencing success of exuviae due to contamination with non-target organisms was also reported by Ožana et al. (2020) and Kranzfelder et al. (2016, 2017). In the study of Kranzfelder et al. (2016), PCR success rate of chironomid exuviae was quite high (83–99%), followed by comparatively low sequencing success rates (36% including non-target sequences) and even lower target sequencing success (13.7%). Such a high difference between PCR success and target sequencing success may be due to unspecific primers, covering many other taxa.

Hence, especially when using exuviae, it is essential to use primers designed specifically for the organism group of interest and tested for potential cross-contaminations. Although contamination with non-target organisms also occurred in our study, due to the use of primers optimized for certain odonate groups (especially for SF), we prevented co-amplification of non-target DNA to a higher extent, which probably would have been the case if we had used universal DNA barcoding primers. In our sample, the problem of contamination was not as severe as in the above-mentioned study. It was observed predominantly for the LF in Anisoptera. Anisopteran exuviae potentially provide more space for other organisms, which use the larval skin for housing, than the small exuviae of Zygoptera, probably increasing the risk of contamination. To further prevent co-extracted DNA from non-target organisms beforehand, extracting DNA from tracheal tubes may be a promising approach. Furthermore, in case of co-amplification of non-target DNA, next generation techniques may be the method of choice, as they enable to filter out non-target sequences deriving from natural contaminations.

Applicability and taxonomic resolution of *CO1* in odonates

Unlike in other organism groups (e.g. chironomids; Ekrem et al. 2007; Kranzfelder et al. 2017), for odonates, a quite comprehensive DNA barcode reference library for *CO1* is available (e.g. 71% of European odonate species are represented on BOLD; see Geiger et al. 2021). This is one of the most important prerequisites for successful establishment and application of DNA barcoding approaches to identify unknown material, material which is impossible to identify based on morphological traits, or in metabarcoding studies (e.g. based on eDNA sampling methods).

Concerning the taxonomic resolution, within our data set both *CO1* fragments (long and short) had the same power and provided congruent results on both, genus, and species level. Identification success at the genus level was high (96%). In contrast, due to a somewhat unbalanced sampling—23 of 85 (27%) specimens analysed within our study belonged to one of the "problematic" groups—species identification success (71%) was relatively low in our data set. It should be emphasized, however, that species identification failed exclusively in these four problematic species groups consisting of morphologically differentiated but very closely related species which are not discriminated by a so-called barcode gap: (1) three *Somatochlora* species (*S. metallica*, *S. alpestris*, *S. meridionalis*), (2) two *Anax* species (*A. imperator*, *A. parthenope*), (3) two *Platycnemis* species (*P. pennipes*, *P. latipes*), and (4) three *Coenagrion* species (*C. puella*, *C. pulchellum*, *C. ornatum*). Excluding these species from our data set, species identification success increased to 98%.

Most of these haplotype sharing groups of species are already well known and have been discussed by other barcoding studies on odonates, using the 5´-region of *CO1* (Galimberti et al. 2021; Geiger et al. 2021) and additionally the 3´-region of *CO1* (Maggioni et al. 2021). Unfortunately, except for *Platycnemis* spp. which are largely allopatric, the members of all other species-groups are known to co-occur.

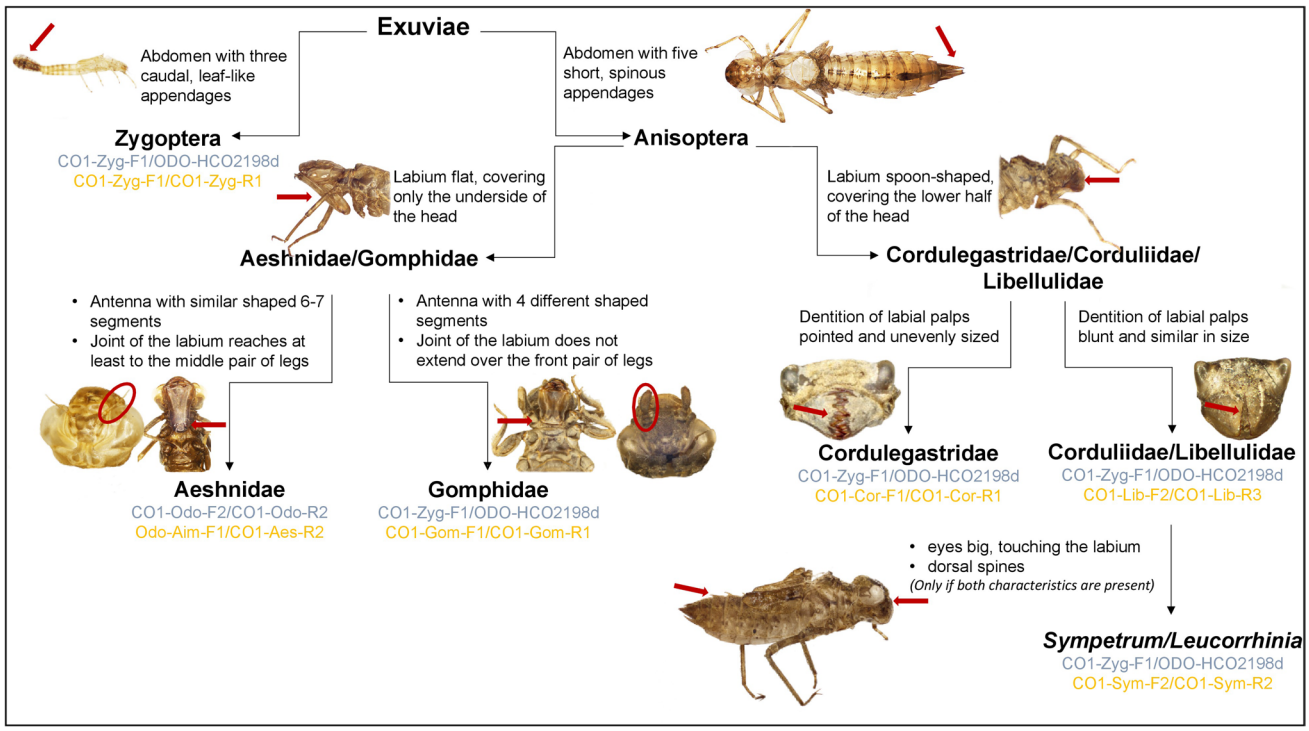

Fig. 4 Decision diagram based on easy recognizable morphological characteristics to choose the appropriate primer pairs (blue ≤ 600 bp fragment, yellow ≤ 200 bp fragment) for DNA-based species identification of dragonfly and damselfly exuviae. Red symbols mark the location of the relevant morphological key characteristics.(Color figure online)

Furthermore, for two of the *Coenagrion* species (*C. puella* and *C. pulchellum*) exuviae can neither be identified based on morphological characteristics, nor with barcoding approaches using mitochondrial markers. In this light, one should distinguish between the methodological success of obtaining DNA barcodes from exuviae, as addressed in the present study, and the limitations of DNA barcoding itself when it comes to differentiating closely related and/or potentially hybridising taxa. In such problematic cases, it should be tested whether nuclear markers may increase taxonomic resolution.

Conclusion

Here, we show that exuviae are a suitable DNA source for species identification based on DNA barcoding and give a promising perspective for using DNA extracted from exuviae for other applications in odonatological research. Yet, it should be considered that for genomic applications (e.g. ddRAD, whole genome sequencing) and long-read sequencing technologies (e.g. Oxford Nanopore Technology, PacBio) DNA yield might be insufficient, but a comparative study testing different tissue types from non-invasive, non-lethal and invasive insect sampling for modern technologies is still not available. However, in general, protocols for degraded and/or ancient DNA are constantly being developed and improving quickly (e.g. hyRAD; Suchan et al. 2016; Raxworthy and Smith 2021).

Constantly improving methods in the field of degraded or ancient DNA is essential when dealing with endangered, threatened, and protected species, or with small declining populations that are vulnerable to extinction. Most studies applying non-invasive genetic methods focus on vertebrates. Here we broaden the picture and show that obtaining DNA from non-invasive sources can also be a direct advantage to the conservation of insects.

Appendix 1

See Fig. 4.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10841-023-00467-x.

Acknowledgements We thank Victoria Pail for providing several exuviae samples. For collecting exuviae in the field, we thank Michael Duda, Sandra Kirchner, Luise Kruckenhauser, Julia Schindelar,

Michaela Sonnleitner, Barbara Tautscher and Paul Wolf. Samples and sequencing data were collected and generated within a project funded by the Municipal Department 22 – Environmental Protection (MA22) and the European Agricultural Fund for Rural Development 2014–2020 (Project Number W-UNS-01/17). Additional financial support was provided by the Austrian Federal Ministry of Science, Research and Economy in the frame of a project within the framework of the "Hochschulraum-Strukturmittel" Funds. The study is associated to the Austrian Barcode of Life (ABOL) initiative (www.abol.ac.at).

Author contributions IF, MS and EH contributed to the study conception and design. Sample collection was performed by IF, MS, AC, SK, LK, NK, OM, HS, EH. Samples were analysed by MS and IF. The first draft of the manuscript was written by MS and IF with equal contribution. All other authors edited subsequent versions of the manuscript and approved the final version.

Funding Open access funding provided by University of Vienna.

Declarations

Competing interests The authors declare no competing interests.

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