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RESOURCE ARTICLE

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A DNA barcode library for Germany's mayflies, stoneflies and caddisflies (Ephemeroptera, Plecoptera and Trichoptera)

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Abstract

Mayflies, stoneflies and caddisflies (Ephemeroptera, Plecoptera and Trichoptera) are prominent representatives of aquatic macroinvertebrates, commonly used as indicator organisms for water quality and ecosystem assessments. However, unambiguous morphological identification of EPT species, especially their immature life stages, is a challenging, yet fundamental task. A comprehensive DNA barcode library based upon taxonomically well-curated specimens is needed to overcome the problematic identification. Once available, this library will support the implementation of fast, cost-efficient and reliable DNA-based identifications and assessments of ecological status. This study represents a major step towards a DNA barcode reference library as it covers for two-thirds of Germany's EPT species including 2,613 individuals belonging to 363 identified species. As such, it provides coverage for 38 of 44 families (86%) and practically all major bioindicator species. DNA barcode compliant sequences (≥500 bp) were recovered from 98.74% of the analysed specimens. Whereas most species (325, i.e., 89.53%) were unambiguously assigned to a single Barcode Index Number (BIN) by its COI sequence, 38 species (18 Ephemeroptera, nine Plecoptera and 11 Trichoptera) were assigned to a total of 89 BINs. Most of these additional BINs formed nearest neighbour clusters, reflecting the discrimination of geographical subclades of a currently recognized species. BIN sharing was uncommon, involving only two species pairs of Ephemeroptera. Interestingly, both maximum pairwise and nearest neighbour distances were substantially higher for Ephemeroptera compared to Plecoptera and Trichoptera, possibly indicating older speciation events, stronger positive selection or faster rate of molecular evolution.

KEYWORDS
barcode library, bioindicators, COI, cryptic diversity, DNA barcoding, Ephemeroptera, Germany, mitochondrial DNA, Plecoptera, Trichoptera, water quality

1 | INTRODUCTION

Three aquatic insect orders, the mayflies (Ephemeroptera), stoneflies (Plecoptera) and caddisflies (Trichoptera), also known as EPT, are often used as biological indicators in freshwater quality assessments (Baird & Sweeney, 2011; Hering et al., 2004; Illies & Schmitz, 1980; Kolkwitz & Marsson, 1909; Liebmann, 1951; Meier et al., 2006; Pantle & Buck, 1955; Rolauffs, Hering, Sommerhäuser, Jähnig, & Rödiger, 2003; Sweeney, Battle, Jackson, & Dapkey, 2011; Zhou et al., 2011) and ecological studies (Böhmer, Rawer-Jost, & Zenker, 2003;

Böhmer et al., 1999; Braukmann & Biss, 2004; Lorenz, Hering, Feld, & Rolauffs, 2004; Schmedtje & Colling, 1996; Schöll, Haybach, & König, 2005; Vannote, Minshall, Cummins, Sedell, & Cushing, 1980). Bioindicator species are often used to survey the health of ecosystems, as they exhibit strong responses to pollution, mining, fracking or climate change (Álvarez-Troncoso, Benetti, Sarr, Pérez-Bilbao, & Garrido, 2015; Burton et al., 2014; Dedieu, Rhone, Vigouroux, & Céréghino, 2015; Wallace, Grubaugh, & Whiles, 1996; Wiederholm, 1984; Zhou et al., 2011). Therefore, an unambiguous identification of EPT species is a crucial step for investigations of freshwater quality, ecology and possible change or loss of biodiversity (Macher et al., 2016).

Despite their importance for the assessment of freshwater ecosystems, accurate morphological delineation of most EPT species, especially females and immatures, remains a challenging task, even for experts, as some traits necessary for reliable identification are only present in one sex or at a certain stage of development (Zhou, Adamowicz, Jacobus, DeWalt, & Hebert, 2009; Zhou et al., 2011). Morphological identifications of these freshwater invertebrates are therefore not only extremely time-consuming and hence costly, but often result in misidentifications, making any assessments of impact highly problematic (Haase, Pauls, Schindehütte, & Sundermann, 2010; Haase et al., 2006; Pfrender et al., 2010). Moreover, despite the growing need for taxonomic expertise to support ecosystem assessments, the number of well-trained taxonomists is decreasing (New, 1996; Stribling, Moulton, & Lester, 2003; Wheeler, 2014). In this context, DNA barcoding provides an effective way to overcome the difficulties in morphological identifications, as this technology delivers fast, efficient and reliable species identification (Hausmann, Parisi, & Sciarretta, 2015; Hausmann et al., 2016; Hebert, Cywinska, Ball, & DeWaard, 2003b; Hebert, Ratnasingham, & de Waard, 2003a; Miller, Hausmann, Hallwachs, & Janzen, 2016; Vane-Wright, Smith, & Kitching, 1994), even in areas where only little information on the benthic macroinvertebrate fauna is present (Geraci, Al-Saffar, & Zhou, 2011; Ibrahimi, Kučinić, Gashi, & Kotori, 2012). A well-curated, comprehensive DNA barcode library based upon voucher species is the foundation for such applications (Ball, Hebert, Burian, & Webb, 2005; Boumans & Brittain, 2012; Gattolliat, Cavallo, Vuataz, & Sartori, 2015; Kjaerstad, Webb, & Ekrem, 2012; Ruiter, Boyle, & Zhou, 2013; Salokannel, Rantala, & Wahlberg, 2010; Vuataz, Sartori, Wagner, & Monaghan, 2011; Webb et al., 2012; Zhou et al., 2009, 2011, 2016), as it also enables promising future applications such as environmental DNA barcoding (Baird & Hajibabaei, 2012; Carew, Pettigrove, Metzeling, & Hoffmann, 2013; Hajibabaei, Shokralla, Zhou, Singer, & Baird, 2011; Hajibabaei, Spall, Shokralla, & van Konynenburg, 2012; Shokralla, Spall, & Gibson, 2012) and metabarcoding (Gibson et al., 2014; Leray & Knowlton, 2015; Morinière et al., 2016; Yu et al., 2012), based on high-throughput sequencing (HTS).

This study documents the assembly of a DNA barcode reference library which provides coverage for 363 taxonomically well-curated EPT species, approximately two-thirds of the known German fauna (Haybach, 2013; Haybach & Malzacher, 2003; Koch, 2014, 2016; Neu, 2013; Reusch & Weinzierl, 1999). The corresponding data are the result of the DNA barcoding projects overseen by the Bavarian State Collection of Zoology in Munich (SNSB-ZSM—www.barcoding.zsm.de) through the "Barcoding Fauna Bavarica project" (BFB—www.faunabavarica.de—Haszprunar, 2009) launched in 2009, and by the "German Barcode of Life project" (GBOL—www.bolgermany.de) launched in 2012 (Geiger et al., 2015). Within the framework of the International Barcode of Life (iBOL) project, and in close cooperation with the Biodiversity Institute of Ontario (BIO, Guelph, Canada), the German barcoding projects are assembling a DNA barcode library for all animal species present in this country. Both projects together have accumulated records for more than 20,000 animal species to the Barcode of Life Database (BOLD—www.boldsystems.org; Ratnasingham & Hebert, 2007), with a special focus on the insect orders Coleoptera (Hendrich et al., 2015), Heteroptera (Raupach et al., 2014), Hymenoptera (Schmidt, Schmid-Egger, Morinière, Haszprunar, & Hebert, 2015), Lepidoptera (Hausmann, Haszprunar, & Hebert, 2011a; Hausmann et al., 2011b), Neuroptera (Morinière et al., 2014) and Orthoptera (Hawlitschek et al., 2016). Aside from extending parameterization of the barcode reference library, work is now being directed towards developing the methods and techniques, such as high-throughput DNA metabarcoding (Morinière et al., 2016), to allow large-scale identification of bioindicator species within bulk samples of benthic organisms.

2 | MATERIALS AND METHODS

2.1 | Fieldwork

A network of researchers and professional taxonomists, mainly working as consultants for water quality assessment studies, and a number of citizen scientists collected specimens of EPTs from throughout Germany, with a focus on southern Germany. Field work permits were issued by the responsible state environmental offices in Bavaria [Bayerisches Staatsministerium für Umwelt und Gesundheit, for the project: "Barcoding Fauna Bavarica"]. The study sites included more than 573 localities in state forests, peatlands, lakes and rivers, and also protected areas such as the national parks "Bayerischer Wald" and "Berchtesgadener Land" (Appendix S1).

2.2 | Specimen sampling

Most voucher specimens were collected from Germany (2,564), but a few specimens were derived from nearby nations including Austria (18), Croatia (1), France (28) and northern Italy (2). Specimens are deposited in SNSB-ZSM, Zoologisches Forschungsmuseum Alexander Koenig (ZFMK, Bonn, Germany) and University of Duisburg-Essen (UDE, Duisburg, Germany), with the exception of a few specimens which are stored in private collections. Although in total 3,624 EPT were collected since 2009 and submitted for sequence analysis to the Canadian Centre for DNA Barcoding (CCDB, Guelph, Canada), only 2,613 specimens were used for molecular analysis in this study. A priori species identification was performed by the co-authors and

professional taxonomists, who were involved in the assembly of a reference library for aquatic invertebrates within the BFB and GBOL framework, usually basing on valuable identification literature [e.g., Ephemeroptera: Bauernfeind & Humpesch (2001); Eiseler (2005), Elliott, Humpesch, & Macan (1988), Studemann, Landolt, Sartori, Hefti, & Tomka (1992), Macan (1955), Malzacher (1986); Trichoptera: Higler (2005), Neu & Tobias (2004), Waringer & Graf (1997), Edington & Hildrew (1995), Malicky (2012); Wallace, Wallace, & Philipson (1990), Lepneva (1966); Plecoptera: Hynes (1977), Lillehammer (1988), Rauser (1980), Zwick (2004)]. When species needed to be re-identified, we submitted the questionable specimens to a specialized taxonomist. Less than 5% of the specimens (mostly larvae derived from macrozoobenthos collections) were identified using reverse taxonomy and BIN comparisons after COI sequence recovery. Less than 20 of the studied specimens were dried and pinned (mostly adults of Trichoptera); the rest were stored in 80% or 96% EOH. The specimen ages at the time of sequence analysis ranged from 1 year (2015) to 42 years (1974)1 year (2015) to 42 years (1974). The number of specimens examined per species ranged from 1 to 91 (*Baetis vernus* Curtis, 1834; see Appendices S1 and S2). Most of the specimens analysed were immature states (84% of Ephemeroptera, 47% of Plecoptera and 58% of Trichoptera), and 80% of the species were represented by adults.

2.3 | Laboratory protocols

A single leg, a leg segment or muscle tissue from the thorax was removed from each specimen and transferred into 96-well plates at the SNSB-ZSM for subsequent DNA extraction. UD samples were extracted using a modified DNA salt extraction (Weiss & Leese, 2016). In other cases (vouchers from Malaise traps within the Global Malaise Trap Project—www.globalmalaise.org), DNA was extracted from the whole voucher at the CCDB (Guelph, Canada) using voucher recovery and allowing specimens to be repatriated to the SNSB-ZSM and ZFMK for identification and curation. Voucher information such as locality data, habitat, altitude, collector, identifier, taxonomic classifications, habitus images, DNA barcode sequences, primer pairs and trace files are publicly accessible in the "German EPT" data set in BOLD (http://www.boldsystems.org—DS-GEREPT data set DOI: dx.doi.org/https://doi.org/10.5883/DS-GEREPT). Once tissue samples were added, the plates were shipped to the CCDB where they were processed using standard barcoding protocols.

Samples from the SNSB-ZSM and ZFMK were PCR-amplified with a cocktail of modified Folmer primers CLepFolF and CLepFolR for the barcoding fragment (COI-5′, Hernández-Triana et al., 2014), whereas the UD samples were processed using HCO2198, LCO1490 (Folmer, Hoeh, Black, & Vrijenhoek, 1994) and a modified version of these primers FL_rueck1 and LCO_mod (Leese, 2004). The same primers were employed for subsequent Sanger sequencing reactions (see also Ivanova, Dewaard, & Hebert, 2006; deWaard, Ivanova, Hajibabaei, & Hebert, 2008). The sequence data and trace files were uploaded to BOLD and subsequently also to GenBank (accession numbers are available in Appendix S1).

2.4 | Data analysis

Sequence divergences for the barcode region (mean and maximum pairwise distances for intraspecific variation, and minimum genetic distance to the nearest neighbour species) were ascertained using the "*Barcode Gap Analysis*" tool on BOLD (Ratnasingham & Hebert, 2007). The software MUSCLE was employed to align sequences (Edgar, 2004). A neighbour-joining (NJ) tree was constructed for each family and colorized based on the assignment of specimens to Barcode Index Numbers (BINs) (Figure 1a,b). Further analysis was restricted to sequences with a minimum length of 500 bp, employing the Kimura 2-parameter model (K2P) (Kimura, 1980). Within BOLD, the BIN system groups sequences into clusters of closely similar COI barcode sequences that are assigned a globally unique identifier, termed a "*Barcode Index Number*" or BIN (Ratnasingham & Hebert, 2013). BINs can be used for provisional species assignments when taxonomic information (i.e., validated binomial) is lacking. BINs for species clusters also enable the delineation of geographical clades, which might reflect local environmental features (Figure 1c, Supporting Information). Members of a BIN usually belong to a single species recognized by traditional taxonomy (Hausmann et al., 2013; Hendrich et al., 2015). Every case of disagreement/conflict is the starting point for reevaluation of both molecular and morphological data. We follow the concept of an integrative taxonomic approach (Fujita, Leaché, Burbrink, McGuire, & Moritz, 2012; Padial, Miralles, De la Riva, & Vences, 2010; Schlick-Steiner, Arthofer, & Steiner, 2014; Schlick-Steiner et al., 2010) to infer whether there are previously overlooked species in the sample or whether barcode divergence between species is large enough to enable delineation of species using the usual partial COI sequence.

2.5 | OTU delineation based on DNA barcodes using Automatic Barcode Gap Discovery (ABGD)

To compare the results of the BIN-based "*Barcode Gap Analysis*," we also applied the Automatic Barcode Gap Discovery (ABGD) analysis (Puillandre, Lambert, Brouillet, & Achaz, 2012) to our data. ABGD analysis was carried out on 30 March 2017 using the web interface (http://wwwabi.snv.jussieu.fr/public/abgd/). ABGD partitions sequences into groups based on comparisons of pairwise distances. As ABGD and the BIN system are both distance-based methods, they can be prone to the same source of error. However, compared to tree-based delineation methods such as the Generalized Mixed Yule Coalecent (GMYC) approach, which is suspected to oversplit (Esselstyn, Evans, Sedlock, Khan, & Heaney, 2012; Paz & Crawford, 2012; Pentinsaari, Vos, & Mutanen, 2016; Sauer & Hausdorf, 2012; Talavera, Dinca, & Vila, 2013), ABGD provides a more conservative approach to estimate the number of species given comprehensive sampling. For analysis of the whole data set (including all three orders), we used the default maximum intraspecific distance values (*Pmax* of .1), typically producing a range of OTU counts. We applied X = 1.0 for relative barcode gap width, as the default value of X = 1.5 did not produce a result for our data set. As distance correction, we used the K2P

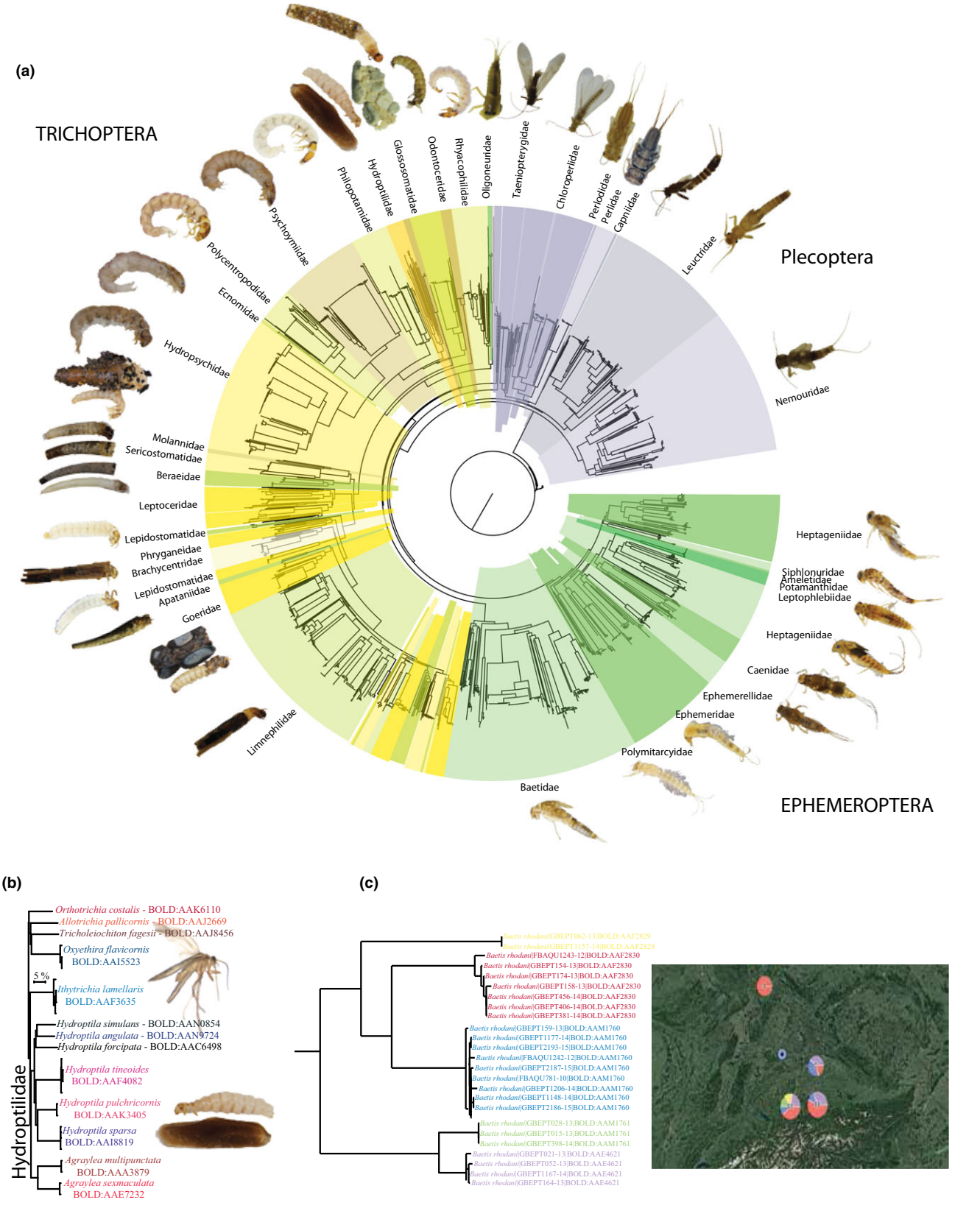

FIGURE 1 (a) Circular neighbour-joining (NJ) tree from analysis of the whole data set (performed on BOLD)—different colours indicate the orders; Ephemeroptera (green), Plecoptera (blue), Trichoptera (yellow). Sequence records (sequences >500 bp) from 2,613 specimens submitted for analysis representing 363 species, covering 38 of 44 families (86%) recorded from Germany. (b) NJ tree for the family Hydroptilidae (Trichoptera). All remaining families are provided within Appendix S3. (c) Geomap for the *Baetis rhodani* clade containing five BINs. Each BIN is indicated as a different colour, and representation of each BIN within southern Germany is displayed on a map provided by batchgeo (http://batchgeo.com/). [Colour figure can be viewed at wileyonlinelibrary.com]

model as above. We also analysed each order in a separate analysis, where we applied X = 1.5 for relative barcode gap width. Default settings were employed for all remaining parameters.

3 | RESULTS

A full-length barcode with a length of 658 base pairs (bp) was obtained from 57.8% of the available 3,624 specimens, representing 2,095 specimens from a priori identified species with representatives from 38 of 44 families (86%, Table 1) known from Germany. The success rate increased to 77.81% for sequences ≥300 bp vs. 75.99% for sequences ≥500 bp. For 21 specimens (0.58%), a barcode sequence of <300 bp was generated while for the other 990 specimens (27.32%) we failed to generate a sequence. Barcode recovery ranged from 61.5% (Ephemeroptera) to 79.8% (Trichoptera) and 87.9% (Plecoptera). Success in barcode recovery was highest in EtOH-preserved samples which were not older than 5 years.

All subsequent analyses are restricted to sequences ≥500 bp. These 2,613 barcode sequences provide coverage for 363 species (87 of 113 Ephemeroptera, 199 of 316 Trichoptera and 76 of 123 Plecoptera) occurring in Germany that were assigned to 410 BINs (113 Ephemeroptera, 85 Plecoptera, 212 Trichoptera BINs) (Figure 1a,b, Figs S1, S2 and S3). In total, 132 new BINs were added to BOLD, most representing new species entries for the system. Inspection of the sequence clusters in the NJ tree revealed high congruence with morphology-based identifications. In fact, 89.53% of the species (325) could be unambiguously identified to a species with a Linnaean name by their COI sequence. However, 38 species (10.47%), representing 22.9% of all specimens (582 individuals), were assigned to more than one BIN, resulting in a total of 89 BINs for these taxa (Table 2). Most of these BINs unequivocally correlate with unambiguous species identification, but occasionally also enabled the delineation of geographical subclades (Figure 1c, Supporting Information). Two species pairs, both Ephemeroptera, showed BIN sharing, reflecting an identical or overlapping COI barcode sequence that prevented their discrimination. The analysis also revealed two ephemeropteran species (89 specimens of *B. vernus* Curtis, 1834 and 11 specimens of *Ephoron virgo* Olivier, 1791), each represented by four and two BINs, giving some evidence for the existence of cryptic species. Appendix S3 provides NJ trees for all covered families. Besides the species name, a voucher number, region and country of origin are given for each specimen (available under DOI: dx.doi.org/https://doi.org/10.5883/DS-GEREPT).

3.1 | BIN sharing

3.1.1 | Ephemeroptera

The ephemeropteran species *Rhithrogena picteti* Sowa, 1971, *R. semicolorata* Curtis, 1834 and *R. carpatoalpina* Klonowska, Olechowska, Sartori & Weichselbaumer, 1987 share the BIN [AAN0425], whereas *R. carpatoalpina* also shares a BIN assignment [ACB4789] with

TABLE 1 Overall success rate in barcode sequence recovery covering 38 of 44 families of Ephemeroptera, Plecoptera and Trichoptera recorded from Germany

Order	Family	Species in Germany	Species with CO1 barcodes	% of German species
Ephemeroptera	Ameletidae	2	2	100
	Ametropodidae	1	0	0
	Athropleidae	1	0	0
	Baetidae	28	20	71
	Caenidae	10	10	100
	Ephemerellidae	5	4	80
	Ephemeridae	4	3	75
	Heptageniidae	44	34	77
	Isonychiidae	1	0	0
	Leptophlebiidae	11	8	73
	Oligoneuriidae	1	1	100
	Palingeniidae	1	0	0
	Polymitarcyidae	1	1	100
	Potamanthidae	1	1	100
	Prosopistomatidae	1	0	0
	Siphlonuridae	5	3	60
Plecoptera	Capniidae	6	5	83
	Chloroperlidae	9	3	33
	Leuctridae	28	19	68
	Nemouridae	33	22	66
	Perlidae	9	4	44
	Perlodidae	22	10	45
	Taeniopterygidae	16	9	56
Trichoptera	Apataniidae	4	2	50
	Beraeidae	5	4	80
	Brachycentridae	7	7	100
	Ecnomidae	2	1	50
	Glossosomatidae	12	8	66
	Goeridae	6	6	100
	Hydropsychidae	19	12	63
	Hydroptilidae	35	13	37
	Lepidostomatidae	4	3	75
	Leptoceridae	41	25	61
	Limnephilidae	96	62	64
	Molannidae	4	3	75
	Odontoceridae	1	1	100
	Philopotamidae	11	6	54
	Phryganeidae	10	6	60
	Polycentropodidae	17	12	70
	Psychomyiidae	14	9	64
	Ptilocolepidae	1	1	100
	Rhyacophilidae	24	14	58
	Sericostomatidae	4	4	100
	Uenoidae	1	0	0

TABLE 2 Total of 38 cases in which high intraspecific divergence (ISD) led to the assignment of multiple BINs for conspecific individuals (582 individuals)

	Family	Species	Author, year	BIN	*n*	Country	Max ISD
Ephemeroptera							
	Baetidae	*Baetis alpinus*	Pictet, 1843-45	BOLD:AAM4710	26	DE, FR	21.78
				BOLD:ACQ2018	9	DE	
				BOLD:ACY4576	1	DE	
		Baetis fuscatus	Linnaeus, 1761	BOLD:AAM4708	3	DE	3.31
				BOLD:ACF6196	5	DE	
		Baetis muticus	Linnaeus, 1758	BOLD:AAJ9345	7	DE	23.96
				BOLD:AAE4622	1	DE	
		Baetis rhodani	Pictet, 1843-45	BOLD:AAE4621	30	DE	18.29
				BOLD:AAM1760	14	DE	
				BOLD:AAM1761	3	DE	
				BOLD:AAF2829	2	DE	
				BOLD:AAF2830	11	DE	
		Baetis vernus	Curtis, 1834	BOLD:AAM1832	4	DE	13.94
				BOLD:ACF8572	3	DE	
				BOLD:ACB1757	1	DE	
				BOLD:ACP8603	81	DE	
		Centroptilum luteolum	Müller, 1776	BOLD:AAK0606	6	DE	21.6
				BOLD:AAU1007	6	DE	
		Cloeon dipterum	Linnaeus, 1761	BOLD:AAJ4113	2	DE	8.51
				BOLD:AAM7076	8	DE	
				BOLD:ACB0671	3	DE	
		Procloeon bifidum	Bengtsson, 1912	BOLD:AAM9815	2	DE	5.63
				BOLD:ACB0495	2	DE	
				BOLD:ACL7780	1	DE	
	Ephemerellidae	*Ephemerella ignita*	Poda, 1761	BOLD:AAB3693	33	DE	9.45
				BOLD:ACB0418	4	DE	
				BOLD:AAJ7442	2	DE	
		Torleya major	Klapalek, 1905	BOLD:ACB1851	9	DE	3.78
				BOLD:ACF8616	1	DE	
	Ephemeridae	*Ephemera glaucops*	Pictet, 1843-45	BOLD:ACL7388	3	DE	8.2
				BOLD:AAN1308	1	DE	

(Continues)

TABLE 2 (Continued)

	Family	Species	Author, year	BIN	*n*	Country	Max ISD
	Heptageniidae	*Rhithrogena beskidensis*	Alba-Tercedor & Sowa, 1987	BOLD:AAX8968	6	DE	6.62
				BOLD:ACB6074	2	DE	
		Rhithrogena carpatoalpina	Klonowska, Olechowska, Sartori & Weichselbaumer, 1987	BOLD:ACB4789	1	DE	7.7
				BOLD:AAN0425	1	DE	
		Rhithrogena picteti	Sowa, 1971	BOLD:ACB4518	2	DE	7.66
				BOLD:AAN0425	1	DE	
		Rhithrogena semicolorata	Curtis, 1834	BOLD:AAN0425	13	DE	7.67
				BOLD:ACB5605	1	DE	
	Leptophlebiidae	*Habroleptoides confusa*	Sartori & Jacob, 1986	BOLD:AAK5034	4	DE	5.87
				BOLD:AAM2063	2	DE	
				BOLD:AAM2064	5	DE	
				BOLD:ACF7718	1	DE	
	Oligoneuriidae	*Oligoneuriella rhenana*	Imhoff, 1852	BOLD:ACB1816	10	DE	11.75
				BOLD:AAY5558	1	DE	
	Polymitarcyidae	*Ephoron virgo*	Olivier, 1791	BOLD:ACB0688	10	DE	20.57
				BOLD:AAN0647	1	DE	
Plecoptera							
	Leuctridae	*Leuctra alpina*	Kühtreiber, 1934	BOLD:AAN1941	1	DE	3.45
				BOLD:ACB1932	1	DE	
		Leuctra cingulata	Kempny, 1899	BOLD:AAK9204	3	DE	1.08
				BOLD:ACY4626	2		
		Leuctra hippopus	Kempny, 1899	BOLD:AAK8670	3	DE	3.81
				BOLD:ACL7184	1	DE	
	Nemouridae	*Amphinemura sulcicollis*	Stephens, 1836	BOLD:AAM5074	2	DE	5.27
				BOLD:AAK1251	1	DE	
		Nemoura cambrica	Stephens, 1836	BOLD:ACL9207	20	DE	3.95
				BOLD:ACX3978	4	DE	
		Nemoura marginata	Pictet, 1836	BOLD:AAN1631	3	DE	9.51
				BOLD:ACD2281	5	DE	
		Protonemura auberti	Illies, 1954	BOLD:AAH7652	15	DE	7.47

(Continues)

TABLE 2 (Continued)

	Family	Species	Author, year	BIN	*n*	Country	Max ISD
				BOLD:AAH7653	22	DE	
		Protonemura nimborum	Ris, 1902	BOLD:AAK9861	1	DE	2.82
				BOLD:ACI6385	1	FR	
	Perlodidae	*Perlodes microcephalus*	Pictet, 1833	BOLD:ACD2609	2	DE	5.78
				BOLD:AAL2343	1	DE	
Trichoptera							
	Goeridae	*Silo pallipes*	Fabricius, 1781	BOLD:AAD0972	1	DE	3.81
				BOLD:ABY8958	21	DE	
				BOLD:ACF6524	1	DE	
	Hydropsychidae	*Cheumatopsyche lepida*	Pictet, 1834	BOLD:ACH0431	8	DE	1.26
				BOLD:AAD1893	19	DE	
		Hydropsyche instabilis	Curtis, 1834	BOLD:AAB1966	13	DE	6.42
				BOLD:ABZ1867	8	DE	
				BOLD:AAB1967	1	DE	
		Halesus tessellatus	Rambur, 1842	BOLD:ABY8874	9	DE	2.14
				BOLD:AAF7714	7	DE	
		Limnephilus rhombicus	Linnaeus, 1758	BOLD:AAB1517	3	DE	3.32
				BOLD:AAB1515	1	DE	
		Potamophylax nigricornis	Pictet, 1834	BOLD:AAF7557	1	DE	1.71
				BOLD:ACE7362	1	DE	
	Odontoceridae	*Odontocerum albicorne*	Scopoli, 1763	BOLD:AAB5624	12	DE	2.83
				BOLD:ACE9892	4	DE	
	Philopotamidae	*Wormaldia occipitalis*	Pictet, 1834	BOLD:AAE2350	1	DE	9.88
				BOLD:AAE2351	15		
	Rhyacophilidae	*Rhyacophila fasciata*	Hagen, 1859	BOLD:AAJ3443	2	DE	3.86
				BOLD:AAD5716	14		
		Rhyacophila obliterata	McLachlan, 1863	BOLD:ACO6145	4	DE	3.64
				BOLD:ACO5356	1		
		Rhyacophila vulgaris	Pictet, 1834	BOLD:AAF8099	1	DE	3.15
				BOLD:ACE4583	1		

R. puytoraci Sowa & Degrange, 1987. Otherwise, *R. semicolorata* and *R. picteti* are represented as singleton clades with their own BIN (Appendix S3).

3.2 | Paraphyletic species

3.2.1 | Ephemeroptera

Specimens of *B. vernus* showed deep COI divergence (13.94%) and were assigned to four BINs [ACB1757, AAM1832, ACP8603 and ACF8572] that form a cluster together with *Baetis liebenauae* Keffermueller, 1974 appearing paraphyletic in the NJ analysis (Appendix S3). Specimens of *E. virgo* showed deep sequence divergence (20.57%) leading to their assignment to two BINs [ACB0688 and AAN0647] that were in two neighbouring clades, the first including *Ephemerella notata* Eaton, 1887 and *E. mucronata* Bengtsson, 1909, while the other one contains *Potamanthus luteus* Linnaeus, 1767, *Kageronia fuscogrisea* Retuzius, 1783 and *Metreletus balcanicus* Ulmer, 1920 (Appendix S3).

3.3 | Species without identification

3.3.1 | Ephemeroptera

Although a larva of *Electrogena* sp. BOLDACP4735 from Croatia could not be morphologically identified to a species level, it was closely related to *E. ujhelyii* Sowa, 1981. Bauernfeind and Soldán (2012) noted that several species of *Electrogena* occur in southern Europe. As the larvae of some species are not described, adults are needed for their identification.

3.3.2 | Plecoptera

Three BINs (*Nemoura* sp. BOLDACK0198 from France; *Nemoura sp.* BOLDACP8086 from Bavaria and Austria; and *Protonemura sp.* BOLDACP7617 from Germany) could not be assigned to a species because they were only represented by immatures.

3.3.3 | Trichoptera

Annitella sp. BOLDACQ6161 found in Bavaria and France could not be identified to a species level, because it was only represented by immatures.

TABLE 3 Comparison of identified species, detected BINs within the "Barcode Gap Analysis" on BOLD and recovered groups within ABGD

	Ephemeroptera	Plecoptera	Trichoptera	Sum
Species	87	76	200	363
BINs (BOLD)	113	85	212	410
Groups (ABGD)	101	80	197	378

BOLD, Barcode of Life Database; ABGD, Automatic Barcode Gap Discovery.

3.4 | High genetic divergences within species and cases of cryptic diversity

3.4.1 | Ephemeroptera

A total of 345 specimens in 18 species were assigned to two or more BINs, because of their relatively high intraspecific genetic divergences with maximum pairwise K2P (max ISD) distance ranging from 3.31% [*Baetis fuscatus* Linnaeus, 1761 represented by two BINs] to 23.96% [*Baetis muticus* Linnaeus, 1758 represented by two BINs]. In total, 18 species were assigned to 47 BINs (11 species were each partitioned into two BINs, four species were each partitioned into three BINs, two species were partitioned into clusters with four BINs, and one species was assigned to five BINs) (Table 2).

3.4.2 | Plecoptera

A total of 88 specimens in nine species were assigned to multiple BINs because of their relatively high intraspecific genetic divergences (max ISD) which ranged from 2.82% [in *Protonemura nimborum* Ris 1902 represented by two BINs] to 9.51% [in *Nemoura marginata* Pictet, 1836]. Each of the nine species was assigned to two BINs, resulting in a total of 18 BINs (Table 2).

3.4.3 | Trichoptera

Eleven species represented by a total of 149 specimens were assigned to two or more BINs due to their relatively high intraspecific genetic divergences. Maximum pairwise K2P distances ranged from 1.26% in *Cheumatopsyche lepida* Pictet, 1834 to 9.88% in *Wormaldia occipitalis* Pictet, 1834. In nine cases, the members of a species were assigned to two BINs while the other two were assigned to three, resulting in 24 BINs (Table 2).

3.5 | Analysis of maximum pairwise distance and distances to nearest neighbours

The analysis of mean intra- and interspecific distances (to the nearest neighbour species—NN) varied strongly among the three orders, being highest for Ephemeroptera (1.2% mean ISD/14.73% to NN) but substantially for Plecoptera (0.53% mean ISD/10.76% to NN) and Trichoptera (0.36% mean ISD/12.3% to NN) (Appendix S2).

3.6 | OTU delineation based on DNA barcodes using Automatic Barcode Gap Discovery (ABGD)

ABGD analysis of the whole data set (EPT combined) recognized a total of 378 OTUs with a prior intraspecific divergence of *Pmax* = .0359. All OTUs were generated by the initial partition (See Table S1, File S1). Comparison of the BIN-based "Barcode Gap Analysis" approach and the ABGD analysis revealed that most closely related species groups as well as neighbouring species with an interspecific distance of approximately 5%, are displayed as distinct BIN

clusters within the "Barcode Gap Analysis", whereas they are merged into a single group within the ABGD analysis (Table 3, Table S2, Appendix S1). We additionally tested each order in a separate experiment. ABGD analysis recognized a total of 99 OTUs for Ephemeroptera (*Pmax* = .0379), 78 OTUs for Plecoptera (*Pmax* = .0483) and 196 OTUs for Trichoptera (*Pmax* = .0379) (Supporting Information).

4 | DISCUSSION

In this study, we report the construction of a DNA barcode reference library which provides coverage for two-thirds of the EPT fauna known for Germany (77% of the Ephemeroptera, 63% of the Trichoptera, 63% of the Plecoptera) (Figure 1a). Although one-third of the fauna awaits analysis, the present library includes nearly all of the dominant species encountered in environmental assessment studies (Haybach, 2013; Haybach & Malzacher, 2003; Koch, 2014; Neu, 2013; Reusch & Weinzierl, 1999). To enable the practical application of DNA barcoding for species identification, an essential prerequisite is the establishment of a high-quality reference database which is based on specimens that have been curated by taxonomists or highly trained experts. Even though the utility of a single mitochondrial gene fragment in species delineation has now been proved to be of great value in various applications such as metabarcoding, the identification of imaginal life stages or even in the discovery of cryptic and new species, several potential pitfalls have been suggested. One drawback is posed by introgression of mitochondrial DNA (mtDNA) due to hybridization and another is incomplete lineage sorting of mitochondrial haplotypes (Hebert et al., 2003a,b; Lukhtanov, Sourakov, Zakharov, & Hebert, 2009; Moritz & Cicero, 2004; Whitworth, Dawson, Magalon, & Baudry, 2007), which can both lead to an absence of the barcoding gap and thus cause misidentifications (Ermakov et al., 2015). Further challenges can occur when nuclear mitochondrial pseudogenes (NUMTs), nonfunctional copies of mtDNA within the nuclear DNA, are co-amplified by universal primers (Hawlitschek et al., 2016; Hebert, Penton, Burns, Janzen, & Hallwachs, 2004; Moulton, Song, & Whiting, 2010; Song, Buhay, Whiting, & Crandall, 2008). Endosymbiotic bacteria, such as *Wolbachia*, can lead to insufficient variation within the barcode region within insects, hampering their identification (Leite, 2012; Raupach, Hannig, Moriniere, & Hendrich, 2016; Smith et al., 2012). In addition, the coexistence of multiple mitochondrial haplotypes can lead to sequence variability in a single organism (heteroplasmy) and can impede correct species identification (Kang, Kim, Park, Kim, & Kim, 2016; Magnacca & Brown, 2010). Finally, other demographic processes, for example, migration driven by major climate changes such as glaciation events or bottleneck events, can have a huge impact on the variability of the mtDNA, which was reported among other orders for mayflies, caddisflies and stoneflies (Williams, Ormerod, & Bruford, 2006; Lehrian, Pauls, & Haase, 2009; Kubow, Robinson, Shama, & Jokela, 2010; Bálint et al., 2011; Vuataz et al., 2011; Theissinger et al., 2013; Bisconti et al., 2016; Gattolliat et al., 2015). Despite such potential complications, there is evidence from many large-scale studies that DNA barcoding is a highly effective tool for species identification.

This study represents an important step towards a completion of a DNA barcode reference library for German species, and enables a variety of approaches of molecular data assessments, linked to other recent publications focusing mainly on terrestrial arthropods (Astrin et al., 2016; Hausmann et al., 2011a,b; Hawlitschek et al., 2016; Hendrich et al., 2015; Morinière et al., 2014; Raupach et al., 2014; Schmidt et al., 2015, 2016; Spelda, Reip, Biener, & Melzer, 2011; Wesener et al., 2015). Although our research is constructing a reference library for German species, it will also be a valuable resource for DNA barcoding and metabarcoding applications at sites across Central Europe. Most of the species (89.5%) examined in this study could be unambiguously identified based on their COI sequence, as they were assigned to a single BIN that was not shared with any other taxa. Most of the remaining species possessed deep intraspecific divergences (≥2% ISD) that led their specimens to be assigned to two or more BINs. Because these BINs were not shared by other species, they also allowed species identification, but as discussed below, these cases reflect taxa that require further study. As just four of the 363 species examined in this study shared barcodes with another species, the present study indicates that DNA barcodes deliver nearly 99% species resolution. Interestingly, analysis of the nucleotide divergences between EPT species revealed that mean intra- and interspecific distances among Ephemeroptera were higher than in Plecoptera and Trichoptera. High genetic distances have already been reported in earlier studies on North American Ephemeroptera (Ball et al., 2005; Webb et al., 2012).

The species assigned to multiple BINs might indicate the existence of overlooked cryptic species or the presence of regional mitochondrial variation in a single species. The latter situation might explain the fact that *Baetis rhodani* (Pictet, 1843) is represented by seven lineages, one that is dominant and six less common ones (Lucentini et al., 2011; Williams et al., 2006). Our analysis assigned specimens of this species to five BINs, some of which correspond to haplogroups reported by Williams et al. (2006) (Appendix S4). However, because several former species in this genus have been synonymized with *B. rhodani* (Gattolliat & Sartori, 2008; Müller-Liebenau, 1969), there is a strong possibility that there may be more than one valid species (Bauernfeind & Soldán, 2012; Bisconti et al., 2016; Rutschmann, Gattolliat, Hughes, Sartori, & Monaghan, 2014). Another species with apparently difficult taxonomy is *B. vernus* Curtis, 1834, which was represented by four clusters in a paraphyletic group with *B. liebenauae* Keffemuller, 1974 that was previously considered as belonging to the *B. vernus* species group (Savolainen, Drotz, Hoffsten, & Saura, 2007; Ståhls & Savolainen, 2008) based on allozyme electrophoresis. An additional candidate for cryptic species is the *Cloeon dipterum* species group as defined by Sowa (1975). The latter author separated its larvae into three groups corresponding to *C. dipterum*, *C. cognatum* Stephens, 1836 and *C. inscriptum* Bengtsson, 1914. Although his conclusions were rejected by several authors, who consider *C. dipterum* as a polymorphic species (for synopsis, see Bauernfeind & Soldán, 2012, p. 187), it has to be further

investigated whether the three identified BINs for *Cloeon dipterum* represent the three *Cloeon* species recognized by Sowa (1975).

Although we did not test for it, the assignment of multiple BINs in some EPT species might reflect variation accumulated during historical isolation in separate refugia throughout the Pleistocene (Figure 1c, File S2). Mayfly species can, for example, be assigned to their refugial origins with some species deriving from the Mediterranean region, and others from different locations in Europe or Siberia. Still other taxa survived in ice-free locations in the European high mountains (nunataks, see Haybach, 2003; Haybach & Jacob, 2010). An example for multiple BINs in stoneflies possibly separated during the Pleistocene is *Dinocras cephalotes* Curtis, 1827: individuals belonging to two BINs are known to interbreed, so they are viewed as members of a single species (Elbrecht et al., 2014).

Members of the *R. semicolorata* group represented the sole case of BIN and barcode sharing detected in this study. One case involved three species (*carpatoalpina* + *picteti* + *semicolorata*), while the other involved two (*carpatoalpina* + *puytoraci)*. The situation is further complicated because certain specimens of *R. picteti* and *R. semicolorata* were assigned to additional private BINs. Similar results for the *R. semicolorata* group were discovered by recent work of Vuataz et al. (2011) and Vuataz Rutschmann, Monaghan, & Sartori, (2016). The morphologies of these four species are very similar to each other, a fact that may cause difficulties in their discrimination. However, the specimens in question were carefully identified. Male imagines are separable by the shape of their genitalia (Bauernfeind & Humpesch, 2001), but female imagines and mature female larvae can only be distinguished by structures on the surface of their eggs (Haybach, 2002). Immature stages hardly show suitable features for taxonomy. Only larvae of *R. semicolorata* can be easily separated by the shape of the plica on the first gill.

It is far beyond the scope of the present study to solve these cases of conflict. Accordingly, we invite the EPT research community to join forces to clarify the status of the possible cryptic species recognized by this study. We also encourage colleagues in the fields of environmental and freshwater studies to aid the progress of the Barcode of Life Initiative, by providing specimens of species that still lack barcode coverage, by aiding with identifications and by completing the descriptions of new species, with the goal to speed the completion of the DNA barcode reference library for EPT species that will support a wide range of important applications relying on identified species composition within freshwater ecosystems.

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AUTHOR CONTRIBUTIONS

GH, AH, MB and PDNH obtained funding; AJB, MH, SK, RM, FL and CDS collected the samples; JM, LH and MB conceived and designed the experiments; JM, LH and TK analysed the data; JM, LH, AJB and FL wrote the manuscript; AJB, PDNH, AH, FL, MH, SK, RM, GH and CDS contributed (additions/corrections) to the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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