Received: 23 November 2021 | Revised: 8 February 2022 | Accepted: 11 February 2022

DOI: 10.1111/ddi.13504

RESEARCH ARTICLE

Diversity and Distributions WILEY

DNA barcoding of Chironomidae from the Lake Skadar region: Reference library and a comparative analysis of the European fauna

Piotr Gadawski[1] | Matteo Montagna[2,3] | Bruno Rossaro[4] | Wojciech Giłka[5] | Vladimir Pešić[6] | Michał Grabowski[1] | Giulia Magoga[4]

[1]Department of Invertebrate Zoology and Hydrobiology, University of Lodz, Lodz, Poland

[2]Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy

[3]BAT Center - Interuniversity Center for Studies on Bioinspired Agro-environmental Technology, University of Napoli Federico II, Portici, Italy

[4]Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, University of Milan, Milan, Italy

[5]Laboratory of Systematic Zoology, Department of Invertebrate Zoology and Parasitology, Faculty of Biology, University of Gdańsk, Gdańsk, Poland

[6]Department of Biology, Faculty of Science, University of Montenegro, Podgorica, Montenegro

Correspondence
Giulia Magoga, Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, University of Milan, Via Celoria 2, Milan 20133, Italy.
Email: giulia.magoga@unimi.it

Funding information
This research was supported by grants from the Polish National Science Center grants [2016/23/NZ8/02123, 2014/15/B/NZ8/00266] and by the funds of the Polish National Agency for International Exchange (NAWA) [PPN/IWA/2018/1/00021/DEC/2], as the well as "DNA-Eco" project financed by the Montenegrin Ministry of Sciences. The funders had no role in study design, data collection and analysis, decision to publish or manuscript preparation

Editor: Zhonge Hou

Abstract

Aims: The main aim of this study was to fill a gap in barcoding data of the European Chironomidae of the Balkan region, developing and testing the efficiency of a reference DNA barcode library for ancient Lake Skadar basin species (Montenegro/Albania), a region of Europe never before subjected to barcoding studies on Chironomidae. Another aim was to test the efficiency of DNA barcoding for the identification of European Chironomidae, including the estimation of optimal identification thresholds, using >12,000 barcodes.

Location: Lake Skadar basin and adjacent area (Montenegro/Albania).

Methods: Through this study, 770 individuals of Chironomidae from the Lake Skadar region were barcoded, both at adult and pre-imaginal stages. Adults were morphologically identified, while larvae were assigned to species by molecular identification, using different methods, of which the efficiency was tested, for a total of 97 different barcoded species.

Results: The identification efficiency of the reference dataset developed for the Lake Skadar region was 98.6%, a value in line with that obtained when the identification efficiency for European Chironomidae was evaluated (95.8%), which confirms the accuracy of DNA barcoding for the identification of these insects. Moreover, we found that the optimal threshold for the molecular identification of the family is 1.6% nucleotide distance, though more specific thresholds are suggested for the identification of species belonging to Chironomidae subfamilies, since they are related to lower identification errors than to the use of a general threshold. The analysis of inconsistency between molecular and morphological identification shed light on taxonomic issues within European Chironomidae. Previously postulated species synonyms were confirmed, and also further cases requiring deeper investigation were detected.

Main conclusions: Our de novo DNA barcode library was shown to have a high identification efficiency. Taxon-specific thresholds increase the efficacy of molecular identification. Hypothesized species synonyms could be validated through molecular techniques.

KEYWORDS
DNA barcoding efficiency, Europe, integrative taxonomy, Lake Skadar, non-biting midges

1 | INTRODUCTION

Chironomidae, also known as non-biting midges, is a flagship taxon in freshwater ecology. Approximately 7500 species of Chironomidae grouped into 550 genera have been described globally (Pape et al., 2011). This estimate partly includes synonyms and doubtful names, and additional species have been described and synonymized in recent years. The actual number of species included in this family could be close to the above-mentioned number, but still it has been not precisely defined. Anyway, Chironomidae are known as one of the most species-rich dipteran families (Ashe & O'Connor, 2009; Ashe &O'Connor, 2012; Giłka et al., 2021), continually attracting considerable interest from researchers worldwide (Whitmore et al., 2021). At the larval stage, chironomids inhabit various freshwater ecosystems, such as streams, rivers, ponds, lakes, reservoirs and, to a much lesser extent, brackish waters and soil. Characteristic behavioural and physiological adaptations, such as the presence of heat shock proteins and haemoglobin as the haemolymph oxygen carrier, have enabled chironomids to colonize extreme environments. They are able to inhabit hot springs, glacial rivers, closed supply systems for potable water and even highly polluted waters (Armitage et al., 1995; Rossaro et al., 2016). The mechanisms of cellular and molecular protection enable some chironomids to endure severe osmotic stress, ultraviolet radiation and long-term dehydration to survive in the most arid areas. Other species live actively on snow, as is the case of *Belgica antarctica* Jacobs, 1900, one of the few species of terrestrial animals permanently inhabiting Antarctica, which has the smallest genome of any sequenced insect species so far (Bernabò et al., 2011; Gusev et al., 2010; Ha & Choi, 2008; Kelley et al., 2014; Kreiling et al., 2018; Lee et al., 2006; Mazin et al., 2018; Panis et al., 1995; Soszyńska-Maj et al., 2016). Furthermore, due to the high habitat specificity and the rapid response of larvae to environmental changes, chironomids are effective biological indicators of water and sediment quality (Free et al., 2009; Lindegaard, 1995; Skoulikidis et al., 2009). However, most studies on chironomid communities have been hampered by the challenge of accurate identification to species, especially for adult females, and even more so for pre-imaginal stages (Rossaro et al., 2022). For this reason, identification of these insects is usually restricted to higher taxonomic levels, that is, genera, species groups (Nyman et al., 2005) or to "larval types" (Real et al., 2000).

Molecular taxonomy techniques, such as DNA barcoding, are therefore particularly useful not only for the identification of Chironomidae, especially when subtle variation in morphological features is present between closely related species, but also for identifying pre-imaginal stages, or when a researcher lacks the skill to identify species reliably on the basis of their morphology (Andersen et al., 2013; Giłka et al., 2018; Lin et al., 2018; Montagna, Mereghetti, et al., 2016; Stur, 2015; Stur & Ekrem, 2015; Wiedenbrug et al., 2009). Furthermore, molecular identification is favourable for its speed and low cost (Baloğlu et al., 2018; Meier et al., 2015). This can be considered a major advantage in the case of Chironomidae, where identification using the morphological approach is time-consuming, since it usually requires dissection and mounting of specimens onto microscope slides (at least 15–20 min *per* processed specimen) and also time-consuming for the development of the necessary taxonomic expertise (Carew et al., 2007; Cranston et al., 2013; Epler, 2001; Wong et al., 2014).

The use of DNA-based taxonomy for Chironomidae has been demonstrated to be: (i) effective for species identification (Brodin et al., 2013; Ekrem & Stur, 2007; Ekrem et al., 2007, 2010; Failla et al., 2016; Kondo et al., 2016; Krosch & Cranston, 2012; Lin et al., 2015; Silva et al., 2013; Sinclair & Gresens, 2008; Song et al., 2016); (ii) suitable to link the different life stages of a species (Carew et al., 2005; Silva & Wiedenbrug, 2014; Stur & Ekrem, 2011); (iii) useful for resolving taxonomic relationships within species groups (Cranston et al., 2010; Demin et al., 2011; Ekrem et al., 2007; Krosch et al., 2020; Lin et al., 2017; Montagna, Mereghetti, et al., 2016; Montagna, Urbanelli, et al., 2016; Sari et al., 2015; Stur et al., 2019); and (iv) facilitate the incorporation of Chironomidae into biodiversity surveys and biomonitoring (Brodin et al., 2013; Carew et al., 2013; Carew and Hoffmann, 2015; Cranston et al., 2013; Sharley et al., 2004). Many Chironomidae COI barcode sequences are already available from major reference databases (e.g. the Barcode Of Life Database (BOLD) and NCBI; Ratnasingham & Hebert, 2007; https://www.ncbi.nlm.nih.gov/), though a considerable amount of effort is still required to provide a more complete representation of the diversity of this family worldwide (sequences for nearly 2800 species are currently available).

For the Balkan region, only 271 COI barcodes of Chironomidae (3 from Croatia and 268 from Bulgaria) belonging to 40 taxa have been registered in BOLD prior to our study. In this region, among the most suitable areas for Chironomidae, we found the Lake Skadar basin, the largest lake in the Balkan Peninsula, located in the Zeta-Skadar valley (south-west part of the Balkan Peninsula) (Pešić et al., 2019). This is a shallow lacustrine ecosystem, of which two thirds (229 km^2) of its surface belongs to Montenegro and about one third (142 km^2) to Albania. The lake is approximately 44 km long and 14 km wide, with a surface area that fluctuates seasonally between 370 and 530 km^2. The Lake Skadar basin contains a very young lake that originated in the mid-sub-Atlantic stage of the Holocene, fed by a geologically old spring system, which has its origins in the Pliocene (Grabowski et al., 2018; Pešić et al., 2019).

The main aim of this study is to develop a comprehensive barcode reference library for the molecular identification of aquatic and terrestrial Chironomidae from the Lake Skadar area (Albania and Montenegro), through a DNA barcoding approach, targeting a fragment of ~658 bp of the COI gene. Moreover, taking advantage of publicly available COI sequences of European Chironomidae, we aim to estimate the general efficiency of DNA barcoding for the molecular identification of species within this family and to develop nucleotide distance thresholds

to improve molecular identification for the family and for the different subfamilies of non-biting midges present in Europe.

2 | METHODS

2.1 | Sample collection and identification

Chironomidae specimens were collected from 72 sampling sites located in the Lake Skadar area (Montenegro and Albania) during four sampling campaigns: spring and fall 2014, summer 2015 and summer 2018 (Figure 1). Aquatic immature stages were collected from streams and rivers using a standard kick sampling procedure, from the bottom of the lake and from macrophytes, using a benthic dredge. Imagines were collected by sweeping through littoral vegetation using an entomological hand net and through light trapping (in open areas, a 500W white light was placed in front of a white screen of 2 m × 3 m, ensuring that the light was visible for a long distance above the lake surface). Sampling sites were selected to maximize the representativeness of habitats present in the area (i.e. open lake, shallow lake, springs, sublacustrine springs, river inflow and terrestrial parts). The collected specimens were removed from bulk samples and placed into absolute ethanol, then stored at −20°C. Morphological identification of specimens took place prior to DNA extraction. Adult males were selected from each vial and morphologically identified using appropriate keys (Giłka, 2011; Langton & Pinder, 2007; Widerholm, 1989). The larvae and pupae were not morphologically identified; only molecular identification was performed after the development of the adult barcode library.

2.2 | DNA extraction, PCR and sequencing

For most specimens, DNA was extracted using the GeneMATRIX Tissue DNA Purification Kit, provided by EURx (Gdańsk, Poland), following the manufacturer's protocol. In the case of larvae, DNA was extracted from the whole body after tissue homogenization using sterile pestles. For adults, DNA was extracted from one/two legs (specimens >2 mm) or from the entire body (specimens <2 mm). The DNA extract was then used as a template for amplification through PCR, using the standard 658 bp barcode region of COI and the primer pairs LCO1490/HCO2198 (Folmer et al., 1994) and C_LepFolF/C_LepFolR (Folmer et al., 1994; Hebert et al., 2004). PCR was carried out in a final volume of 11 µl, containing DreamTaq reaction buffer (DreamTaq DNA polymerase, 2× DreamTaq buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each and 4 mM $MgCl_2$; Thermo Scientific Inc.), 5 µmol each primer, ultrapure water and the DNA template. The following PCR thermal protocol was adopted: 94°C for 1 min followed by five cycles of 30 s at 94°C, 1 min 30 s at 45°C and 1 min at 72°C; 36 cycles of 94°C for 30 s, 51°C for 1 min 30 s and 72°C for 1 min; with the final extension of 5 min at 72°C. Successful amplification was verified by agarose gel electrophoresis. The PCR products were purified using a mixture of FastAP (1 U/µl, ThermoFisher Scientific) and Exonuclease I (20 U/µL ThermoFisher Scientific). Positive amplicons were directly sequenced using the Sanger method and the marker-specific primers by Macrogen Europe (Amsterdam, the Netherlands) (Sanger & Coulson, 1975). For a further 85 specimens, DNA was extracted, and the barcode region was amplified and sequenced at the Centre for Biodiversity Genomics, Guelph, Canada, following the standard protocols of the centre (Porco et al., 2010). All electropherograms obtained were edited and primers removed using Geneious Pro 11 (Biomatters Ltd., Auckland, New Zealand; Kearse et al., 2012). The presence of an open reading frame was verified using Geneious software. Consensus sequences were deposited in BOLD (Dataset "DS-CHBAL—Chironomidae of the Skadar Lake basin," https://doi.org/10.5883/DS-CHBAL) and are available on GenBank (BankIt2349074, Accession numbers MT534631 to MT535400). Adult voucher specimens are deposited at the University of Lodz, Poland, and at the University of Milan, Italy, under P. Gadawski's and B. Rossaro's curation respectively.

2.3 | DNA barcode identification efficiency for Chironomidae

The reference library for the molecular identification of Chironomidae from the Lake Skadar area developed in this study was first examined using BOLD tools (Ratnasingham & Hebert, 2013). The distribution of barcode clusters [Barcode Index Numbers (BINs)] was performed

FIGURE 1 Sampling sites in the Lake Skadar area. Sampling sites are represented with green circles, the number of individuals from which DNA barcodes were developed is reported in the circles. Map source: OpenStreetMap contributors based on the Open Data Commons Open Database License

by the Barcode of Life Data System v4. BIN was considered homogeneous when consisting of records of one species and heterogeneous when there was more than one. The number of haplotypes was estimated using the BOLD data systems' accumulation curve tool (Ratnasingham & Hebert, 2007; Villesen, 2007). The Refined Single Linkage (RESL) algorithm, a staged clustering process that employs single linkage clustering implemented in BOLD, was used to assign Chironomidae barcodes to operational taxonomic units.

Subsequently, newly developed sequences were aligned at the codon level using MUSCLE (Edgar, 2004) and a pairwise nucleotide distance matrix calculated using the library *ape* (Paradis & Schliep, 2018) in R version 3.5.2 (R Core Team, 2020), adopting Kimura's two-parameter (K2P) as a nucleotide substitution model (Kimura, 1980). This model is routinely used to calculate the distances between DNA barcodes (Nishimaki & Sato, 2019). All of the following analyses were performed only on the barcode sequences obtained from adult Chironomidae. A K2P nucleotide distance matrix was developed and used for the calculation of the optimal threshold for the molecular identification of species present in the dataset using *threshold optimization analysis* of the R library *spider* v1.4-2 (Brown et al., 2012). This method, firstly proposed by Meyer and Paulay (2005) allows the estimation of a threshold value that minimizes the cumulative identification error (the number of false positives (FP), conspecifics with a value of nucleotide divergence higher than the threshold value, plus the number of false negatives (FN), i.e., heterospecifics with a value of nucleotide divergence lower than the threshold value). The optimal threshold (OT) obtained was then used in the *Best Close Match function* of the R library *spider* v1.4–2 (Brown et al., 2012) to evaluate the efficiency of the newly developed dataset for the identification of Chironomidae in the Lake Skadar area. The *Best Close Match* method compares each sequence of the dataset with the others included in the same dataset and checks if the best matches (pairs of sequences with the lowest values of nucleotide distance) are between sequences of organisms morphologically identified as the same species. The sum of the correct matches obtained thus allowed us to estimate the efficiency of DNA barcoding regarding our dataset.

To test the general efficiency of barcoding for Chironomidae, all publicly available COI-5P sequences of European Chironomidae available in BOLD (Ratnasingham & Hebert, 2007) were retrieved (date of download: 13.04.2021) and filtered to create an accurate species-level dataset, excluding all sequences missing species-level identification or proper scientific names and shorter than 350 base pairs (bp). This procedure was performed using an *ad hoc* R script. This dataset, which also included the barcodes for the Chironomidae of Lake Skadar developed in this study, was analysed with the same procedure as previously described, to estimate the barcoding efficiency and the OT. Furthermore, the dataset was used to estimate and compare barcoding optimal thresholds and the efficiency of barcoding for the molecular identification of European Chironomidae subfamilies. For this purpose, the whole set of sequences was arranged into sub-datasets, each including barcodes of one of the following subfamilies: Chironominae, Diamesinae, Orthocladiinae, Podonominae, Prodiamesinae, Tanypodinae and Telmatogetoninae. Minimum spanning haplotype networks (Bandelt et al., 1999) were reconstructed using PopART (Leigh & Bryant, 2015) for genera including species that posed difficulties during morphological identification, and for those for which incongruences between morphological and molecular species identification were recovered.

2.4 | Identification of Chironomidae larvae

To perform the molecular identification of barcodes generated from larvae and pupae, two different molecular identification methods were applied and compared (adults' barcodes developed in this study were deposited in BOLD before the molecular identification of larvae). (i) The BOLD taxonomy browser was used, and the congruence of BIN assignment was evaluated. Sequences were matched against barcodes registered in the BOLD COI database (already including barcodes of adults developed in this study), each one was assigned to a BIN which was first inspected for concordance (all the barcodes composing it had to belong to a single morphospecies). In the case of concordance, the respective species identification was assigned to the developed barcode. When the BIN consisted of sequences of two or more species within the same genus, only genus-level identification was given to the barcode. Moreover, when the newly developed barcode formed a new BIN, identification was given to the family level. (ii) The optimal threshold for the identification of Chironomidae estimated in this study was used as a clustering threshold. A dataset comprising unidentified larval sequences plus all identified sequences at the species level present in BOLD was assembled and those sequences were clustered at the nucleotide distance value corresponding to the optimal threshold. When the derived clusters were composed of one or more unidentified larval sequences plus one or more sequences retrieved from BOLD belonging to the same species, the identification of the latter was assigned to the larval sequences. When the clusters included sequences of two or more species of the same genus, only genus-level identification was assigned to the larvae. Finally, when the clusters comprised only larval sequences, taxonomic assignment to the family level was given to those sequences. Clustering analysis was performed using the R library *spider* v1.4-2 (Brown et al., 2012).

3 | RESULTS

3.1 | DNA barcode library for Lake Skadar Chironomidae

In this study, a total of 770 COI barcode sequences of Chironomidae were recorded from the Lake Skadar area (744 specimens collected in Montenegro and 26 in Albania), representing the first barcodes of Chironomidae from these two countries available on BOLD. Sequences had a median length of 622 bp [range: 325–658], mean base composition G = 16%, C = 17.9%, A = 27.5% and T = 38.6%.

TABLE 1 Results of the barcoding efficiency analysis

	Skadar Chironomidae	European Chironomidae	European Chironomidae subfamilies						
			Podonominae	Telmatogetoninae	Prodiamesinae	Diamesinae	Chironominae	Orthocladiinae	Tanypodinae
N of individuals	330	12165	17	5	40	564	3330	7769	440
Incorrect	-	328	-	-	-	25	71	219	-
No id	4	173	0	0	1	45	89	72	21
Correct	326	11664	17	5	39	494	3170	7478	419
OT	2.4%	1.6%	4.4%	1.4%	3.2%	0.3%	1.6%	1%	0.7%
Cumulative error	4	1079	0	0	1	98	325	566	21
Barcoding efficiency	98.7%	95.8%	100.0%	100.0%	97.5%	87.5%	95.1%	96.2%	95.1%

Note: N of individuals—number of individuals whose barcode sequences were included in the analysed dataset; Incorrect—incorrect identifications detected by Best Close Match analysis; No id—number of sequences having no match with conspecifics, detected by Best Close Match analysis, due to a pairwise nucleotide distance higher than the adopted OT; Correct—correct identifications detected by Best Close Match analysis, plus singletons; OT—optimal threshold value estimated for the dataset; Cumulative error—cumulative error related to the OT estimation; Barcoding efficiency—barcoding efficiency estimated for the dataset, values are reported as a percentage.

Almost half of the sequences were obtained from larvae (403 sequences), the rest were from adult males (367 sequences). In this study, we tested two approaches to the molecular identification of Chironomidae larvae from Lake Skadar that gave concordant results. Specifically, the identification of 93.5% of the sequences (377/403 sequences) provided identical results using both approaches, but using the BIN congruence method, 22 more sequences were assigned to the species level (Table S1). According to the results of the latter method, 263 out of 403 barcodes were assigned to 52 species; 74 barcodes were identified only to the genus level, were assigned to the following seven genera: *Chironomus* (17), *Cricotopus* (1), *Microtendipes* (14), *Paracladopelma* (3), *Polypedilum* (8), *Procladius* (30) and *Tanypus* (1), while another 66 remained unidentified.

Adult males were morphologically identified as belonging to 77 species, 37 individuals were identified only to the genus level or to a higher taxonomic rank (23 and 14 individuals respectively). The BIN and RESL (for operational taxonomic units) analyses of adult males assigned sequences to 98 BINs and 102 OTUs. Some species were collected only at the adult or pre-imaginal stages; therefore, the total dataset obtained for the Chironomidae of Lake Skadar includes barcodes from 97 different species (Table S2). Analysing the entire dataset (barcodes of adults and larvae) through BIN and RESL clustering analyses, the 770 barcodes were assigned to 159 different BINs and 167 OTUs. During the dataset publishing process, 65 BINs were new for BOLD (39.1%, 201 sequences), 100 of the remaining were concordant (60.2%) and one was discordant (0.6%). Seventy-one BINs were each represented by a single sequence (42.7%). One hundred and one BINs (60.8%) were already present in the database.

3.2 | Chironomidae diversity of Lake Skadar

Within the Lake Skadar area, 97 species, assigned to 44 genera, were recorded, with a mean number of collected specimens per species of 6 (1–29). The most common genus was *Chironomus*, for which 114 barcode sequences were generated (95 barcodes identified as belonging to 11 species), followed by *Polypedilum* (60 barcodes, 51 identified at the species level to nine species), and *Cricotopus* and *Procladius* (50 and 49 barcodes, eight and two species respectively). Ten genera were represented, each by a single specimen. The highest number of specimens was recorded for *Paratendipes albimanus* (29), *Chironomus luridus* (26), *Kiefferulus tendipediformis* (26) and *Cladopelma edwardsi* (22), while 33 species were represented, each by a single specimen. The mean intrageneric nucleotide distance was 10.6% [SD = 7.3%], while the mean intergeneric distance was 19.3% [range 3.8%–30.7%]. Intraspecific and interspecific nucleotide distances, estimated from adult male barcodes only, resulted in a mean intraspecific nucleotide distance of 1.5% (0%–17.5%) and of 19.5% (3.3%–30.2%) respectively (Table S3). On average, 3.29 haplotypes (SD = 3.1) per species were found. Due to the presence of species having unusually high intraspecific nucleotide distance values, for example *Limnophyes minimus* (17.4%), a clear barcode gap was not observed within the Lake Skadar barcode dataset.

3.3 | Optimal thresholds for molecular identification and barcoding efficiency

In this study, the identification efficiency of the de novo barcode reference dataset from adult males of the Lake Skadar lake area (330 sequences identified to species level) was estimated, and the optimal threshold for the identification of species within this dataset was calculated. The OT of 2.4% K2P nucleotide distance was associated with cumulative error (CE) of four sequences (FP = 4). The *Best Close Match* analysis performed on this dataset, implementing the estimated OT of 2.4%, resulted in 326 correctly identified sequences out of 330, thus demonstrating a 98.6% efficiency of the dataset (Table 1). The same analyses were also performed on all the barcodes of European Chironomidae available in BOLD (including also the sequences developed in this work) identified to the species level (12,165 sequences; 599 species) in order to evaluate the general efficiency of DNA barcoding for the identification of European Chironomidae species and the optimal threshold value for their molecular identification. The OT resulted in 1.6% nucleotide distance (associated CE = 1079; FP = 173, FN = 906) (Table 1; Figure 2). For the dataset containing 12,165 sequences, the *Best Close Match* analysis using this threshold resulted in 11,664 correctly identified sequences and 328 sequences were incorrectly identified (Table 1). A total of 173 sequences had no match with conspecifics due to their having a pairwise nucleotide distance higher than the adopted OT (1.6%). Thus, the final efficiency of DNA barcoding on European Chironomidae, based on publicly available barcodes, was estimated to be 95.8%.

Sequences were also used to estimate the efficiency of DNA barcoding on the seven Chironomidae subfamilies present in Europe (Chironominae, Diamesinae, Orthocladiinae, Podonominae, Prodiamesinae, Tanypodinae and Telmatogetoninae) and the OT for the molecular identification of each of them. The subfamilies were differentially represented within the dataset (median of 440 sequences per subfamily), accordingly also to the number of taxa they encompass within Europe. Thus, Chironominae and Orthocladiinae were the most highly represented subfamilies (3330 and 7769 sequences respectively), while Telmatogetoninae were the least represented, including only five sequences (Table 1). A clear barcode gap in distribution of nucleotide distances was not found for some of these subfamilies. This pattern is strikingly evident for Tanypodinae, where particularly high intraspecific distance values were estimated (4.1% mean intraspecific distance, Figure 2).

The estimated OT values ranged from 0.3% (Diamesinae) to 4.4% (Podonominae) (Figure 2). The OT of Orthocladiinae (1%) was related with the highest cumulative error (CE = 566, total number of sequences = 7769) (Figure 2), while Podonominae, Telmatogetoninae (OT = 1.4%) and Prodiamesinae (OT = 3.2%) with the lowest (CE = 0, 0 and 1 sequences respectively). It is notable that the sum of all cumulative errors associated with the OT values estimated for each subfamily (total CE = 1011) was lower than the CE related to the OT estimated for the entire family (Table 1; Figure 2). The DNA barcoding efficiency values obtained for the different Chironomidae subfamilies ranged from 87.5% to 100%. The efficiency analysis for the Chironominae subfamily (represented by 3330 barcode sequences, clustered in 356 OTUs and 352 BINs by BOLD analyses) confirmed 3170 correctly identified sequences (71 incorrect and 89 missing identifications), thus a 95.1% efficiency was estimated for this subfamily (Figure 2; Table S4). The Orthocladiinae, represented by 7769 barcode sequences (clustered in 395 OTUs and 395 BINs by BOLD), resulted in 7478 correctly identified sequences (96.2% efficiency; Figure 2, Table S4). Even Diamesinae, although represented by considerably fewer records than the previously mentioned subfamilies (564 sequences, 27 OTUs and 27 BINs), resulted in 25 incorrect and 45 missing identifications during the efficiency test (Table S4). For the latter subfamily, the lowest barcoding efficiency within the Chironomidae was thus 87.5%. An efficiency of 100% was obtained for the smallest datasets, that is, Telmatogetoninae and Podonominae.

3.4 | Haplotype network analysis

A haplotype network was inferred for each of the following genera: *Cricotopus*, *Chironomus*, *Diamesa*, *Micropsectra* and *Polypedilum*.

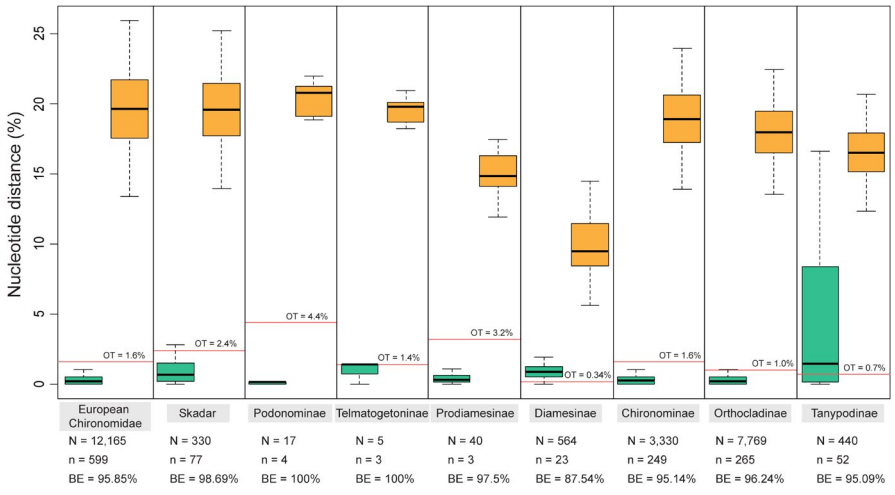

FIGURE 2 Inter- and intraspecific K2P distances for the developed datasets: European Chironomidae, Lake Skadar adult males, seven Chironomidae subfamiles. Green—intraspecific distance, orange—interspecific distance, OT—optimal threshold; *N*—number of sequences; *n*—number of species; BE—barcoding efficiency. Summary statistics on intra and interspecific distances (Table S5)

Based on the haplotype network of *Cricotopus*, the sequences of this genus were clustered into two groups—the first consisting of four species and the other of seven species (Figure 3; Table S5). Only *C. sylvestris* and *C. glacialis* presented shared haplotypes (Figure 3). The genus *Chironomus* was represented by 24 species in the haplotype network (Table S5). Two groups of species—*C. pseudothummi–C. riparius* and *C. heteropilicornis–C. pilicornis*—were found to share haplotypes (Figure 4). The haplotype network for *Diamesa* consisted of 15 species (Table S5), and also within this genus, two groups of species had shared haplotypes—*D. bohemani–D. zernyi* and *D. tonsa–D. hyperborea* (Figure 5). In the haplotype network of the *Micropsectra* (25 species; Table S5) (Figure 6), no shared haplotypes were observed. Finally, the *Polypedilum* haplotype network obtained from 19 species (Table S5) included only one group sharing haplotypes—*P. bicrenatum–P. tridens* (Figure 7).

4 | DISCUSSION

4.1 | DNA barcoding efficiency and molecular identification thresholds

The present work led to the development of the first comprehensive barcode library for the molecular identification of Chironomidae of the Lake Skadar region, including 97 species, that is 45.3% of the regional Chironomidae fauna (Gadawski et al., 2022). The resulting barcode dataset (770 barcode sequences assigned to 159 BINs), currently available in BOLD, represents 12.4% of the total number of barcode clusters from European non-biting midges (1,280 BINs) and strongly supplements the barcode library for the Balkan region that was previously represented by just 271 records from Bulgaria and Croatia. Good correspondence was found between the morphological identification of adults used as vouchers to develop the dataset and the molecular entities (BINs) delineated by BOLD analyses of their barcodes. In particular, 74 of the 77 morphospecies precisely matched the BINs defined from BOLD. The total dataset obtained for the Chironomidae of the Lake Skadar region, validated through *Best Close Match* analysis, demonstrated a high identification efficiency (98.6%) using the 2.4% of nucleotide distance as the identification threshold. This strong level of efficiency concurs with that obtained for estimation of the general efficiency of DNA barcoding for the identification of European Chironomidae (i.e. 95.8%) on the more consistent dataset composed of about 12,165 barcodes identified to the species level. The values obtained confirm the efficiency of DNA barcoding as a tool for the identification of Chironomidae species (Ekrem et al., 2010, 2018; Lin et al., 2015). The potential value of this method is also supported by the number of sequences obtained from unidentified larvae that were successfully identified to the species level (>65%). Interestingly, both the approaches adopted in this study for the molecular identification of larvae (assessing the congruence of BINs to which sequences were assigned using the BOLD taxonomy browser and the clustering analysis using the optimal threshold for Chironomidae) gave highly similar results. Few inconsistencies were found, for example the BIN congruence method was able to reach species-level identification for 22 more sequences. According to these results, both methods could be considered valuable molecular identification approaches in the presence of an accurate barcode reference. In fact, beyond the efficiency of the DNA barcoding method, the results obtained are also related to the coverage of the BOLD database in terms of the European Chironomidae species diversity. However, we also conclude that the large proportion of larvae that were not assigned with species names (35%) indicates that a greater sampling effort should be applied to further expansion of the reference library for the identification of Chironomidae from the Lake Skadar region. To cover all the species diversity present in the region, particular efforts should be made for sampling adult males, to allow the development of a comprehensive, species-level reference library. This could be achieved through the implementation of sampling in diverse habitats in other periods of the year than those considered in this study. Identification of the immature stages of European Chironomidae at the species level, with a good level of efficiency, is a goal for the taxonomy of these insects, and also a very important prerequisite for the feasibility of the monitoring of European freshwater quality through DNA metabarcoding using Chironomidae as bioindicator. Chironomidae are well known and have historically been used as bioindicator of freshwater quality across Europe (Free et al., 2009; Nicacio & Juen, 2015; Skoulikidis et al., 2009). The development of resources for species-level identification is fundamental in this case, as only some species of Chironomidae, more sensitive to changes in the environment, are good bioindicators of freshwater quality (Lencioni et al., 2012; Theissinger et al., 2018).

The OT value inferred from the barcodes of European Chironomidae available in BOLD (1.6% of the nucleotide distance) is consistent with those estimated for other taxa, for example Bavarian moths (OT = 1.8%; Ratnasingham & Hebert, 2013); Euro-Mediterranean leaf beetles (OT = 1%; Magoga et al., 2018); mirid heteropterans (OT = 2.2%; Raupach et al., 2014) as well as other Arthropoda, for example *Gammarus* amphipods (OT = 4%; Delić et al., 2017). Interestingly, it is slightly higher than the identification threshold proposed for Alpine Chironomidae by Montagna, Mereghetti, et al. (2016) (OT = 0.7%–1.4%) but comparable with the maximum intraspecific distances estimated for *Diamesa* species by Lencioni et al. (2021) (OT = 1.9%). The reported threshold value, also compared to the threshold optimized on the more restricted dataset of the Lake Skadar region (OT = 2.4%), could result from a good coverage of the intraspecific diversity of Chironomidae in the BOLD database. In fact, having a good representation of haplotype diversity for the species included in a reference dataset may allow reduction of the distance of query sequences to the nearest neighbour sequence within the dataset and, consequently, also the optimal threshold value for the correct identification of these queries.

We also conclude that using specific thresholds for the identification of species belonging to different Chironomidae subfamilies is associated with lower identification errors than using a general threshold for the identification of all species within the family.

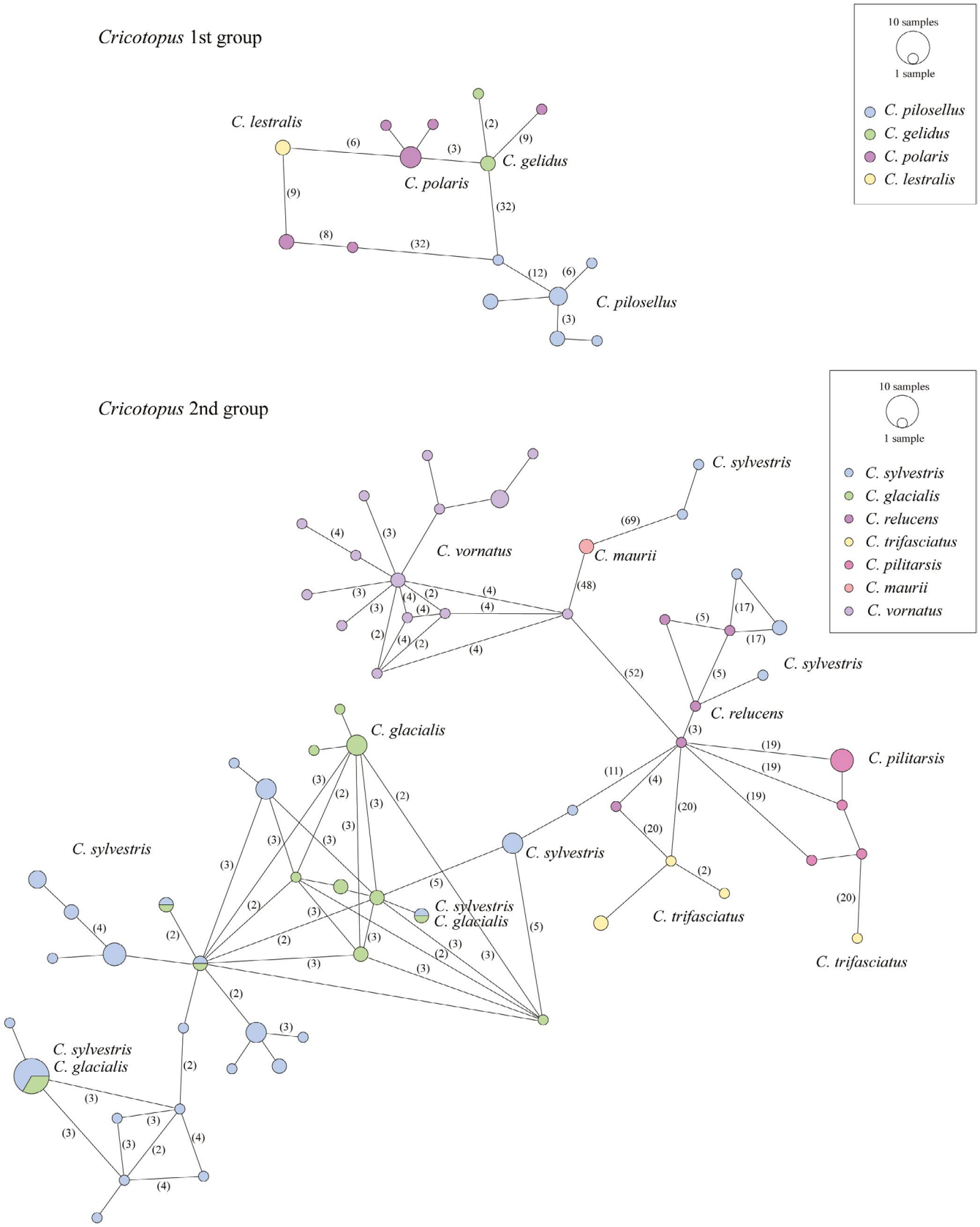

FIGURE 3 Minimum spanning haplotype network for the genus *Cricotopus*. Each colour represents one species. The diameter of the circle is proportional to the abundance of the haplotypes. Numbers of substitutions between haplotypes are reported within parentheses

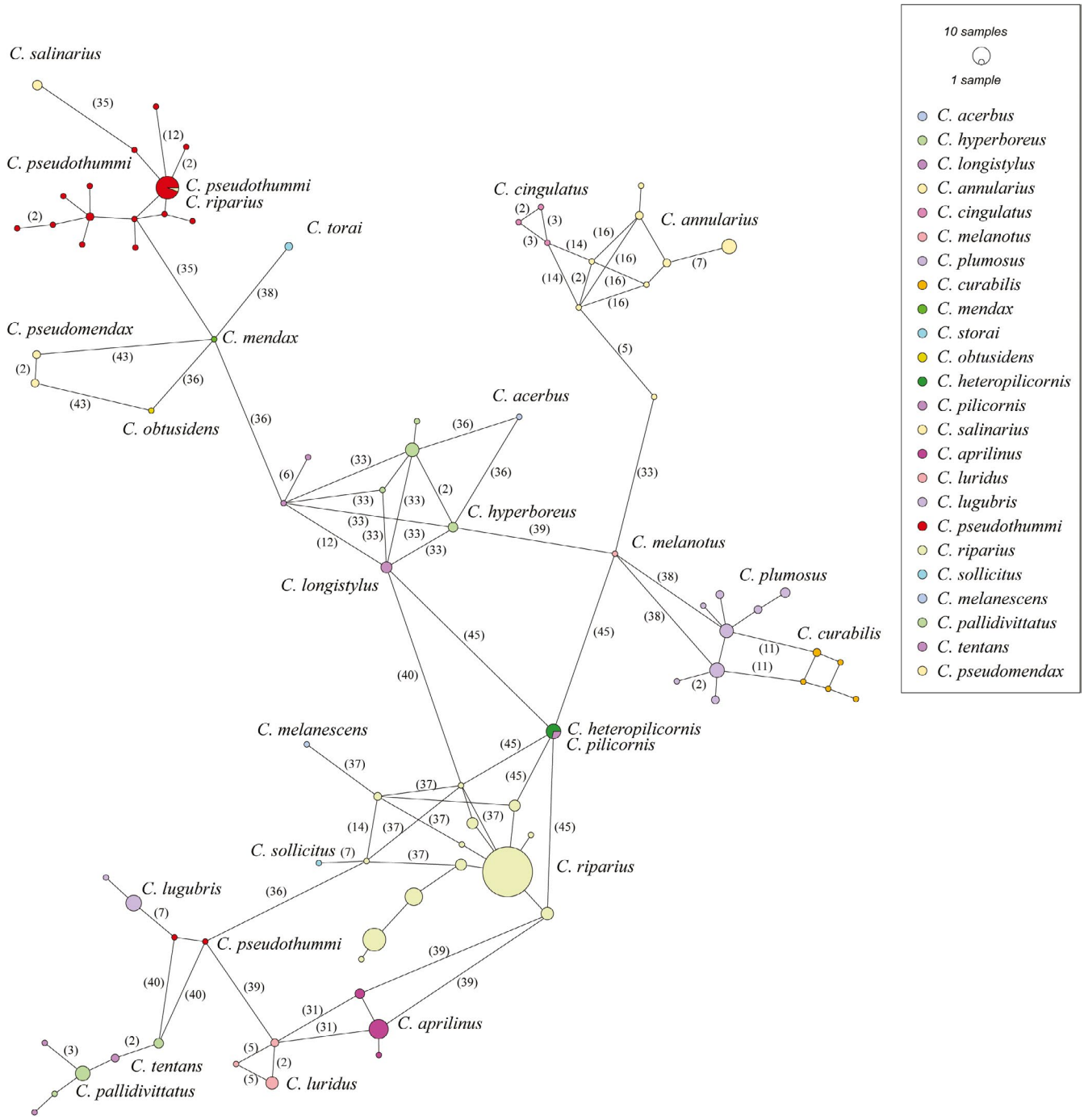

FIGURE 4 Minimum spanning haplotype network for the genus *Chironomus*. Each colour represents one species. The diameter of the circle is proportional to the abundance of the haplotypes. Numbers of substitutions between haplotypes are reported within parentheses

Specifically, the optimal thresholds estimated for each European Chironomidae subfamily resulted in a total cumulative error lower than that related to the more general family threshold (total CE for subfamilies = 1011 with respect to family CE = 1079). This result suggests that using taxon-specific identification thresholds improves molecular identification results (Lin et al., 2015; Magoga et al., 2018). Interestingly, when we analysed the intraspecific variability of species belonging to the different Chironomidae subfamilies, we found that some Tanypodinae species are characterized by unusually high intraspecific distances (e.g., *Monopelopia tenuicalcar*, mean intraspecific distance of 9.8%; *Conchapelopia pallidula* mean intraspecific distance of 8.1%; *Guttipelopia guttipennis* mean intraspecific distance of 7.6%; *Natarsia punctata*, mean intraspecific distance of 5.8%; and *Ablabesmyia longistyla*, mean intraspecific distance of 5.8%), which suggests the possible presence of cryptic diversity within these species. These high intraspecific distances also explain the lack of a barcoding gap in the distribution of nucleotide distances of this subfamily (Table S3). It is generally well known that DNA barcoding

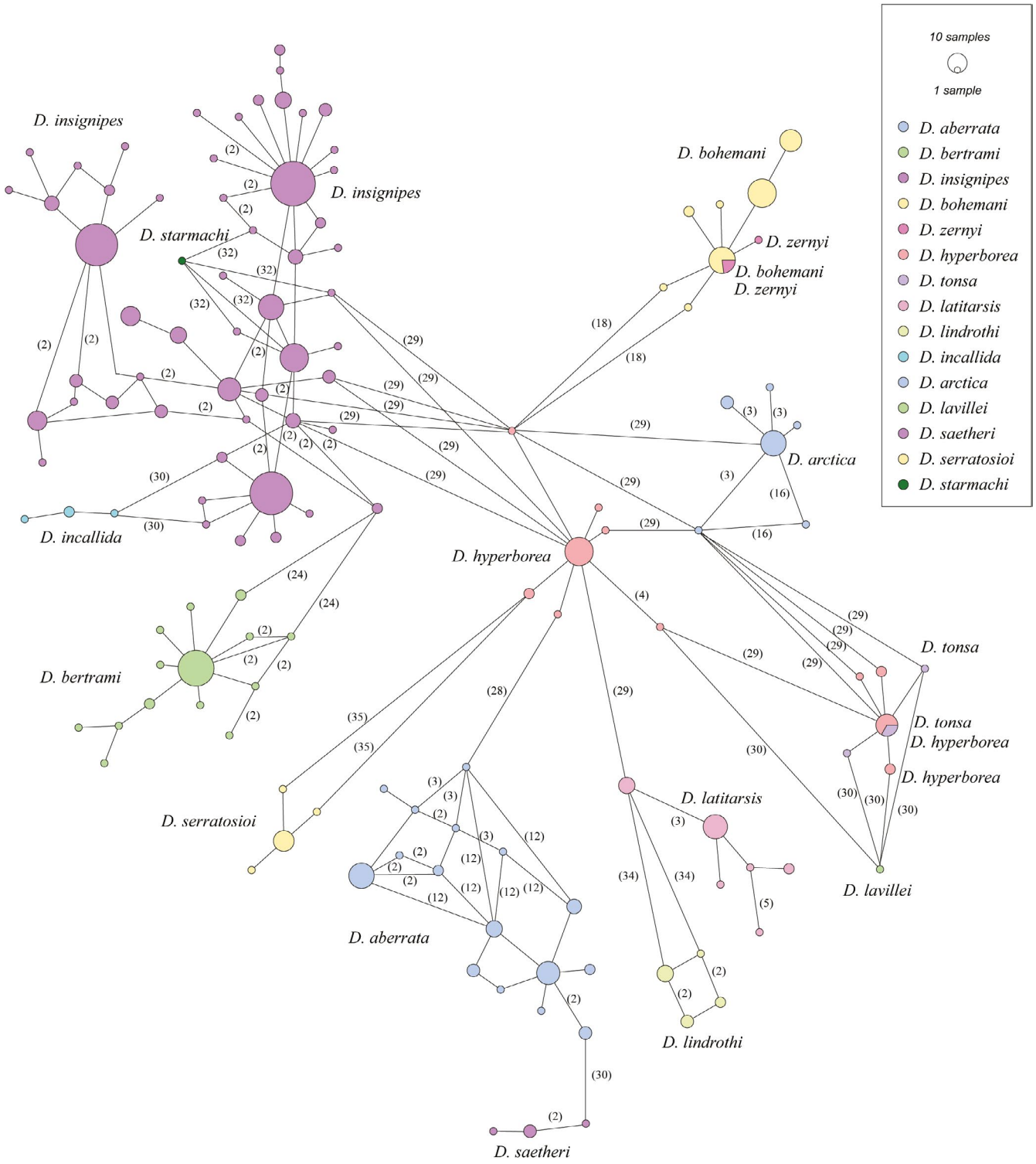

FIGURE 5 Minimum spanning haplotype network for the genus *Diamesa*. Each colour represents one species. The diameter of the circle is proportional to the abundance of the haplotypes. Numbers of substitutions between haplotypes are reported within parentheses

efficiency can be influenced by many factors, for example existence of superspecies or cryptic species within the studied group, occasional interspecific hybridization, introgression and intervention of bacterial endosymbionts changing pathways of mtDNA inheritance (Magoga et al., 2018). In addition to biological reasons, also human errors are frequent. Our analyses of BOLD public barcodes suggest that the most likely explanation of some cases of incongruences between morphological identification and molecular variability are morphological misidentifications of specimens, for example *Cladotanytarsus atridosum* GBMIN55141-17 or *Chironomus plumosus* BATFP249-11 are misidentified, the first record belonging to the genus *Chironomus* (BOLD: AAU2239) and the second to species

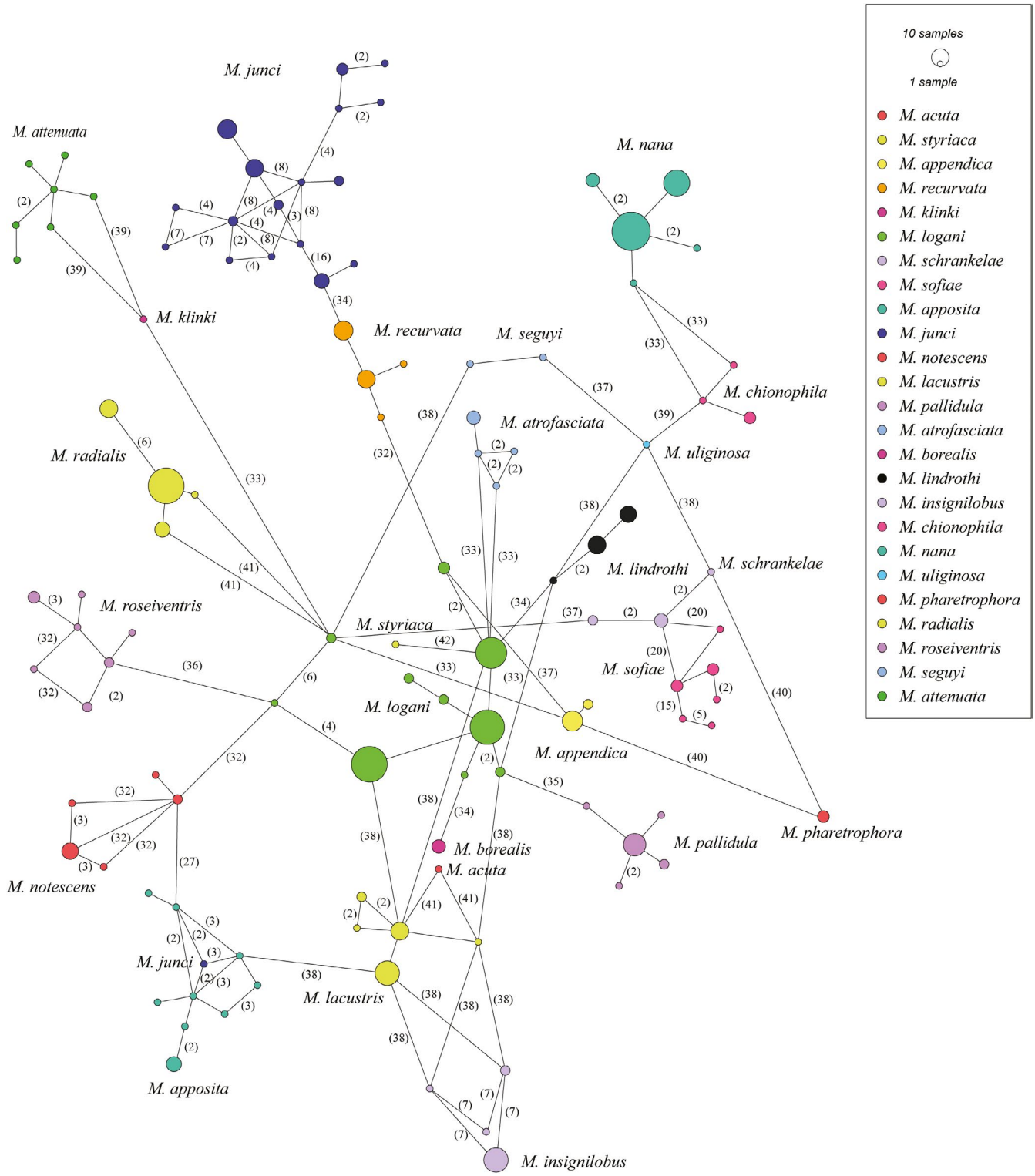

FIGURE 6 Minimum spanning haplotype network for the genus *Micropsectra*. Each colour represents one species. The diameter of the circle is proportional to the abundance of the haplotypes. Numbers of substitutions between haplotypes are reported within parentheses

Paratanytarus inopertus (BOLD: ACB4812) (Table S4). Moreover, frequent use of synonyms or invalid species names in association with public barcodes is another common source of incongruences (*Gymnometriocnemus volitans–Gymnometriocnemus kamimegavirgus*, Stur & Ekrem, 2015; Table S4).

4.2 | Chironomidae of the Lake Skadar region

All the specimens collected in the Lake Skadar region and morphologically identified were assigned to species already listed in the European fauna. Further in-depth studies, based on molecular

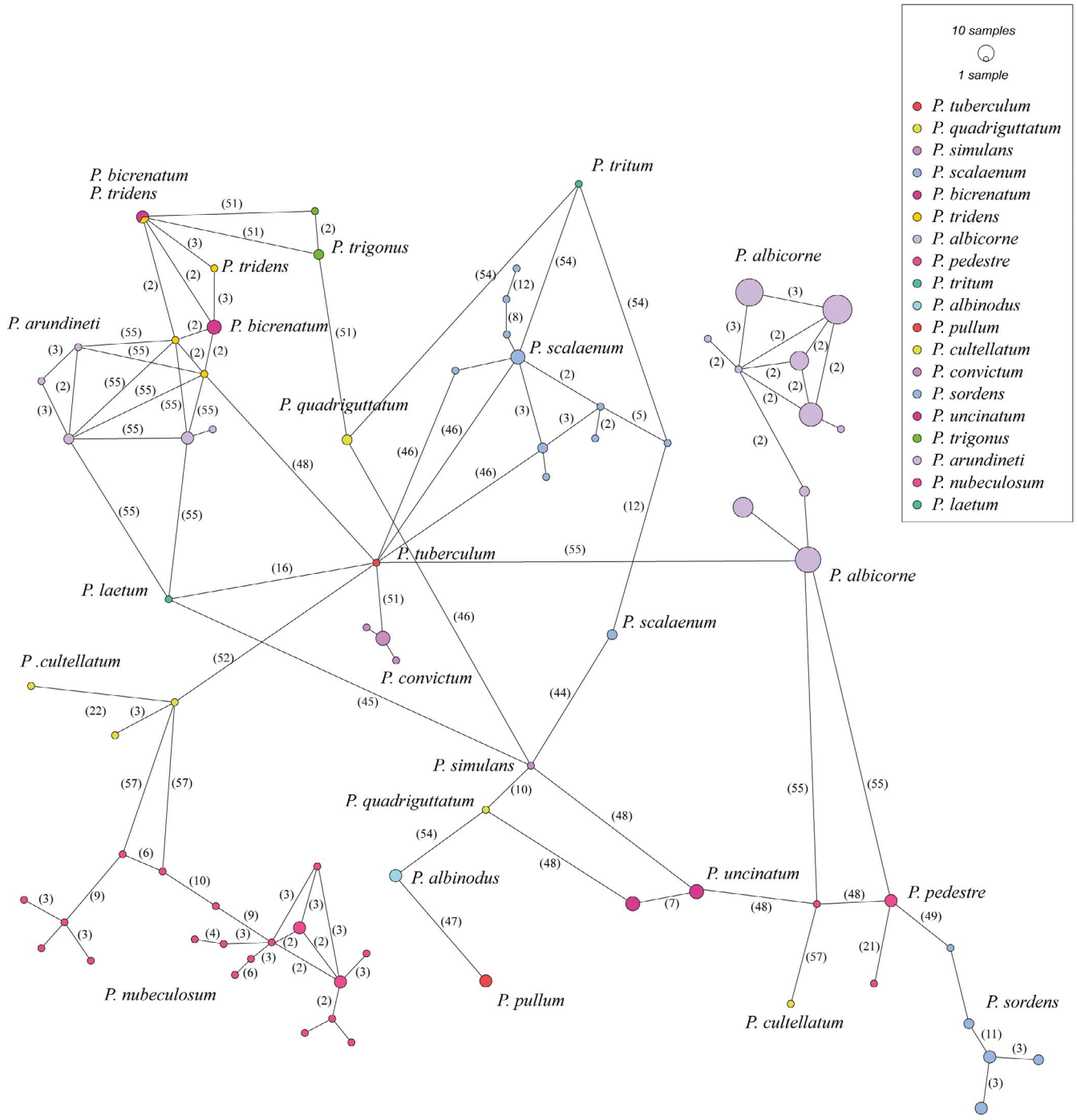

FIGURE 7 Minimum spanning haplotype network for the genus *Polypedilum*. Each colour represents one species. The diameter of the circle is proportional to the abundance of the haplotypes. Numbers of substitutions between haplotypes are reported within parentheses

data, should focus on the taxonomy of the local chironomid fauna to reveal the actual level of endemism, which could be high, even if that is not indicated by the most recent species checklist from the Lake Skadar basin (Gadawski et al., 2022). The progress of knowledge on chironomids suggests that many species have large distribution ranges. Thus, the presence of endemic cryptic species in the Lake Skadar area cannot be excluded (Giłka et al., 2013; Rossaro et al., 2019). Quite recently, an endemic chironomid species was described from Croatia (*Micropsectra uva* Giłka et al., 2013) and its presence in Lake Skadar cannot be excluded. The Lake Skadar basin, together with its large karst spring system, is definitely ancient, having originated more than 2.5 million years ago, and having been isolated for most of that time (Grabowski et al., 2018). Some of the karst spring connections are likely to be more than 100 Ka old and to have remained in prolonged isolation, increasing the probability of the emergence of divergent and locally endemic phylogenetic lineages or even species (Jabłońska et al., 2020). Since some larvae collected in this study were not

identified to the species level and their sequences were clustered in OTUs and BIN unassigned to species of BOLD, it is expected that undescribed or currently non-barcoded species may be present within the sampled material, possibly including endemics. For the barcode dataset developed using Lake Skadar adults Chironomidae as vouchers, the 1.5% mean intraspecific nucleotide distance was estimated. This value is in line with those found in previous barcoding studies on Chironomidae (Song et al., 2018) and lower than those usually estimated for species sampled on a larger geographical scale (Lin et al., 2015). Anyway, some species having an unusually high intraspecific diversity were recorded in this study, for example, *Limnophyes minimus*, for which a 17.4% maximum intraspecific distance was estimated. These distance values are similar to those found for Chironomidae species potentially hiding cryptic diversity (Lin et al., 2015; Song et al., 2018), further suggesting undescribed species could be present within the Lake Skadar area.

Among the 97 species recorded in the Lake Skadar area, 57 species were previously reported in the literature for the region (Gadawski et al., 2022). The seven records discussed hereafter are doubtful and need to be thoroughly investigated. *Bryophaenocladius nigrus* Albu, 1964, is known so far only from Romania, Finland and Germany, and this record would be the southernmost occurrence of this species. *Chironomus (Chironomus) curabilis* is currently known only from Russia (Belyanina et al., 1990). *Chironomus (Lobochironomus) pseudomendax* was initially identified as *C. mendax* by Wülker in Ryser et al. (1985). However, specimens from Sweden and Russia were subsequently identified as *C. mendax*, Storå, and this name was finally replaced by *C. pseudomendax* by Wülker (1999), based on a difference in chromosome number. *Cricotopus mauri* Spies and Sæther, 2004 is a *nomen novum* for *Cricotopus (Isocladius) polychaetus* Hirvenoja, 1989, recorded only in Finland. *Cricotopus relucens* Hirvenoja, 1973, has only been recorded from Finland, Germany and the Netherlands. Species identification of *Macropelopia johnseni* is probably incorrect, since this specific name is *nomen nudum*. Of the five specimens assigned to this BIN (BOLD: ADL2054), two were collected and identified in China and three were collected in the Lake Skadar basin. The specimens reported in this study were identified based on taxonomy match using BIN and *Macropelopia nebulosa* appeared as the closest species. The record of *Polypedilum absensilobum* is doubtful, since it is known only from oriental China (Zhang et al., 2016).

Considering general chironomid diversity of Europe, we can observe that ~1300 species have been recorded so far, 599 species barcoded and publicly available in BOLD represent 46% of the European chironomid fauna (Sæther & Spies, 2013). The most frequently recorded European species are *Limnophyes minimus* (Meigen, 1818) (1136 sequences) and *Chironomus riparius* (Meigen, 1804) (749 sequences), also represented by the different barcode clusters (5 and 3 respectively). *Cladotanytarsus mancus* included the highest number of BINs (10), which may indicate incorrect use of the name within the *mancus* group, which includes several species that are highly variable morphologically. Such high diversity may be explained by numerous aggregations of morphospecies within the C. *mancus* group.

5 | TAXONOMIC REMARKS ON EUROPEAN CHIRONOMIDAE DERIVED FROM MORPHOLOGICAL AND MOLECULAR INFORMATION

5.1 | The genus Cricotopus

In this study, some individuals of *Cricotopus (Isocladius) glacialis* Edwards, 1922, and others of *Cricotopus (Isocladius) sylvestris* (Fabricius, 1794) were found to share the same COI haplotype. Both species are very similar in morphology and can be easily misidentified. They differ in body colour, in fact *C. glacialis* has a darker colour morph than *C. sylvestris*. In the BOLD system, all barcodes of *C. glacialis* are clustered in one BIN, including some *C. sylvestris* barcodes (BOLD: AAA5299). Hirvenoja (1973) and Brodin et al. (2013) hypothesized that *C. glacialis* could be a junior synonym of *C. sylvestris*. Our findings support this hypothesis, but also a further scenario should be considered, that is, these two species could be recently evolved and maybe have not yet accumulated sufficient mutations to be separated by DNA barcodes. *Cricotopus glacialis* is restricted to northern Europe (Gresens et al., 2012), and in BOLD it is reported from continental Norway (seven sequences), 11 sequences from the Svalbard archipelago, and three individuals from Iceland. Moreover, some Chironomidae individuals collected in the Italian Alps were assigned provisionally to *C. glacialis*, but a comprehensive analysis of their morphological characters could not fully confirm this identification (Bruno Rossaro det.). For this reason, the species was not included in the Italian fauna (Rossaro et al., 2019). Individuals of *C. sylvestris*, whose barcodes are published in the BOLD system, were collected in Norway (21 individuals), Sweden (17 individuals), Czech Republic (six individuals), Finland (five individuals), Germany (four individuals), Denmark (both, Zealand and Bornholm, two individuals) and Poland (one individual). *Cricotopus sylvestris* is now confirmed also from the Lake Skadar basin, as well as 15 barcodes of individuals collected in Montenegro.

The *Cricotopus* haplotype network analysis provided interesting information and highlighted some possible mismatches in species identifications that are hereafter analysed (Figure 3). For example, some haplotypes of *Cricotopus sylvestris* (process ID: PGBAL250-19, PGBAL197-19) were found to group with others of *Cricotopus relucens* Hirvenoja, 1973 (PGBAL249-19). The individuals of both species were morphologically identified in this study framework and were grouped in one BIN (BOLD: AAA5299). It could be hypothesized that these individuals belong to a species of *Cricotopus* new to BOLD, which was not yet been barcoded, or possibly even to a species new to science. Furthermore, the BOLD data are ambiguous, because both *C. relucens* specimens (PGBAL030-19 and PGBAL249-19) are clustered close to *C. sylvestris* from Sweden (BSCHI628-17) and all

assigned together to the barcode cluster BOLD: AAA5299, which consists of the above-mentioned species, as well as *Cricotopus glacialis*, *Cricotopus sp. 3ES*, *Cricotopus laetus* Hirvenoja, 1973 and *Cricotopus sp*. This may result from confusion of *C. relucens* with *C. sylvestris*, since it is challenging to distinguish these two species based on morphological characters alone.

5.2 | The genus Psectrocladius

Within the genus *Psectrocladius*, incongruences between morphological and molecular identification were recorded for the species *Psectrocladius octomaculatus* Wülker (Kieffer, 1956) (two sequences of individuals collected in Sweden) and *Psectrocladius psilopterus* (Kieffer, 1906) (four sequences belonging to individuals collected in Finland, Norway and Iceland). These species were found to share haplotypes and their sequences are assigned to the same BIN (BOLD: AAX8186). Morphologically, they can be separated both as adults and pupae (Wülker, 1956), but a high level of taxonomic expertise is required. The detected inconsistency could thus be indicative of species misidentifications, or, on the other hand, the lack of genetic distinctiveness could suggest these taxa are the same species and that they should be synonymized. Another possible scenario may involve the hybridization/introgression phenomena.

5.3 | The genus Chironomus

The analyses performed in this study identified two groups of species: *Chironomus pseudothummi–C. riparius* and *C. heteropilicornis–C. pilicornis* that share haplotypes (Figure 4). *Chironomus pseudothummi*, *C. (Chironomus) lugubris* and *C. riparius* Meigen, 1808 are difficult to separate on the basis of the morphology of adult males (Lindeberg & Wiederholm, 1979). Recently, morphological and molecular characters allowing accurate identification of *C. lugubris* were presented by Stur and Ekrem (2020). *Chironomus pseudothummi* and *C. riparius* can indeed be identified on the basis of pupal exuviae (Langton & Visser, 2003). *Chironomus. lugubris* has a North-Central European distribution, even though Rossaro et al. (2019) preliminary identified some exuviae found in Italy as belonging to this species. The species was also reported from Switzerland and Hungary (Rossaro et al., 2019). The nine specimens whose barcodes are present in BOLD were collected from Spitsbergen (Norway) and assigned to one BIN (BOLD: AAB4581). *Chironomus pseudothummi* Strenzke, 1959 is distributed in Central and Northern Europe. It was not previously reported from the Mediterranean area, but it has been collected from the River Adda (Italy) (Rossaro et al., 2019). The species has been now reported also from the Lake Skadar basin and its occurrence is supported by molecular data (barcodes of 21 specimens). Other records for the species in BOLD are 14 specimens that were collected from Sweden and two from Norway. *Chironomus riparius*, a widespread species in Europe, was found to be well represented by public barcode data. Currently, 141 sequences are in BOLD from France (39 specimens), Germany (27), Italy (54, of which 44 from Sardinia), Bulgaria (5) and Sweden (3). A further eight barcodes from Montenegro were added thanks to this study. The *Chironomus* haplotype network indicates that the currently available barcodes of *Chironomus riparius* represent 46 haplotypes with a high nucleotide divergence (average 37 SNPs). The sequences of this species are assigned to three BINs in BOLD (BOLD: AAA7263 including 140 records, and BOLD: AAU4044 and BOLD: AAI4307 having one and two records respectively). This situation could be explained by a high level of intraspecific variability due to the wide species distribution.

Chironomus heteropilicornis Wülker (1996) was identified on the basis of the karyotype and it is restricted to Fennoscandia. *Chironomus pilicornis* (Fabricius, 1787) has a similar north-eastern distribution. *Chironomus pilicornis* can be identified according to the shape of the genitalia, which are very similar to those of *Chironomus (Chironomus) plumosus* (Linnaeus, 1758) (Martin, 2017; Wülker, 1996). The barcode data available in BOLD for *C. heteropilicornis* were developed from specimens collected from Norway (five barcodes) and those of *C. pilicornis* from Sweden (two barcodes). The sequences of *C. heteropilicornis* and *C. pilicornis* are assigned to one BIN (BOLD: ACX5781), which could indicate species misidentification or that both species are in fact one species and should be synonymized. Another possible scenario suggests the involvement of hybridization/introgression processes. Both species can be easily confused with *C. plumosus* in morphological identification, but they are clearly separated on the basis of molecular data.

Another species morphologically very similar to *C. plumosus* is *Chironomus (Chironomus) curabilis* Belyanina et al. (1990). Both molecular information and karyotype analysis (Polukonova et al., 2009) facilitate easier determination of these two species than using morphological characters. *Chironomus plumosus* is usually recognized as a widely distributed species in Europe, but is not well represented in the BOLD database. A total of 27 barcodes are currently available, 14 of which belong to specimens collected in Montenegro. Among the others, 11 were collected in Sweden and one in Poland. All these sequences are grouped within 18 haplotypes and assigned to one BIN—BOLD: AAU2239—but it is not homogeneous and includes also sequences of *C. usenicus* (8), *C. tentans* (two sequences) and *Cladotanytarsus atridorsum* (one sequence), which are probably the result of misidentification.

Beyond the two groups of *Chironomus* species mentioned at the beginning of this paragraph, other interesting information on species of this genus was derived from barcode data analyses. *Chironomus (Chironomus) sollicitus* Hirvenoja (1973), is reported from Finland only, but according to BOLD it was collected in Norway (a single sequence in the BIN: BOLD: AAI4306). The taxonomic status of *Chironomus pallidivittatus* and *Chironomus tentans* Fabricius, 1805, is currently being debated (Spies & Sæther, 2004). *Chironomus (Chironomus) pallidivittatus* sensu Edwards, 1929 (not *Ch. pallidivittatus* sensu Malloch, 1915 which is distributed in the Nearctic), in BOLD is reported from Europe and represented by 11 barcodes from Sweden and four from Finland,

though it does not have a clear taxonomic status. Four sequences of *C. tentans* are also available and these were obtained from specimens collected in Norway. Both species are grouped into one BIN (BOLD: AAE9024), which confirms their unclear taxonomic status.

5.4 | The genus Diamesa

Our results also reveal two groups of species sharing COI haplotypes: *Diamesa bohemani–D. zernyi* and *D. tonsa–D. hyperborea*. It has previously been suggested that *Diamesa zernyi* Edwards, 1933, and *Diamesa bohemani*, Goetghebuer, 1932, are the same taxon and should be regarded as synonyms (Lencioni et al., 2021) and our results support that conclusion.

The distribution of *Diamesa hyperborea* Holmgren, 1869 is restricted to Northern Europe, and it is very often confused with *Diamesa tonsa* (Haliday, 1856), due to their morphological similarity. According to Montagna, Urbanelli, et al. (2016), *D. tonsa* cannot be distinguished also from *D. cinerella* using COI barcode data and, on the basis of mitochondrial and nuclear markers, Lencioni et al. (2021) have confirmed that *Diamesa tonsa* and *Diamesa cinerella* Meigen in Gisti, 1835, represent the same species. However, it was hypothesized that both *Diamesa zernyi* and *Diamesa cinerella* species groups include species that have been separated from each other recently and for which complete lineage sorting has not yet been achieved (Lencioni et al., 2021).

Among the other *Diamesa* species analysed in this study, an interesting case is represented by *Diamesa insignipes* Kieffer, 1908, for which 238 sequences were analysed, including 79 unique haplotypes. All these barcodes form one BIN (BOLD: ACR0046), and cluster together in the haplotype network of *Diamesa* genus (Figure 5), with an average haplotype divergence of two nucleotide substitutions. According to BOLD metadata, all the specimens from which these barcodes were developed were collected at a single sampling site in Germany (between Bonn and Koblenz).

5.5 | The genus Micropsectra

In this study, the species *Micropsectra apposita* (Walker, 1856) and *Micropsectra contracta*, Reiss, 1965 were treated as synonyms (the latter as a junior synonym), following Sæther and Spies (2013) and earlier literature (e.g. Giłka, 2011) which report a lack of morphological characters to reliably separate these two species. Our results, as well as the data on ecological preferences (the present authors' datasets), support their synonymy. *Micropsectra junci* is morphologically very similar to *M. apposita* (Walker, 1856), but relatively easily separable using the key of Giłka (2011), as both species occur in the same type of habitat (Giłka, 2001). Among the barcodes mined from BOLD, *M. apposita (=M. contracta)* is represented by specimens collected from Norway (nine specimens) and from the South of Germany (four specimens). Forty barcodes of *M. junci* from Norway (34 barcodes), Finland (two barcodes), Luxembourg (two barcodes), Sweden (one barcode) and Germany (one barcode) were also available. The sequences of *M. junci* were assigned to five different BINs. Records of *M. apposita* (LEFIJ3845-16, LEFIJ3846-16, LEFIJ3861-16) and *M. junci* (BSCHI366-17) were merged into one BIN (BOLD: AAC7823), which probably indicates species misidentification. Another possible interpretation is the effect of hybridization/introgression in these sympatric species.

5.6 | The genus Polypedilum

From analyses of the barcode data of *Polypedilum* (*Tripodura*) *tridens* Freeman, 1955, and *Polypedilum* (*Tripodura*) *bicrenatum* Kieffer, 1921, shared haplotypes between these species were found. These are considered as distinct species, but they are very similar morphologically. In the literature, *P. tridens* is reported only from Africa. Within the barcodes we analysed, four *P. tridens* from Europe were present (three specimens from Germany and one from Norway), while *P. bicrenatum* was represented by six barcodes, all from Sweden. All these sequences were assigned to one BIN (BOLD: ACV3478). It let us hypothesize about these species' synonymy, but further taxonomic investigation is necessary to confirm it.

6 | CONCLUSIONS

- Our study provides a reference library including 770 COI DNA barcodes (97 species) of Chironomidae from the Lake Skadar basin area. This represents 45.3% of the chironomid species recorded so far from Montenegro. Chironomids of this region have never been barcoded before.
- Some of the species detected in this study had not been previously recorded in the Lake Skadar area. Since only one individual was collected for each of them, further investigations in the area are needed to confirm the status of these species.
- The de novo DNA barcode dataset resulted in a high identification efficiency (98.6%).
- In this study, the usefulness of DNA barcoding for the molecular identification of Chironomidae was reaffirmed. The general identification efficiency estimated for European Chironomidae resulted in 95.8%, when a threshold of 1.6% was used as a limit between intra/interspecific levels. Moreover, the method enabled us to identify >65% of the larvae included in this study to species level. This highlights the value of this method as a water bioindicator monitoring tool.
- Comparisons between optimal thresholds estimated at different taxonomic levels on Chironomids, *viz*. family and subfamily, have underlined the importance of using taxon-specific thresholds to increase the efficacy of molecular identification.
- The integration of morphological and molecular evidence supported five cases of synonymy representing genera: *Cricotopus*, *Psectrocladius*, *Chironomus*, *Diamesa* and *Micropsectra* within the Chironomidae family.
- Some cases of barcoding failure were observed for groups of closely related species or for species whose identification require

a high level of taxonomic expertise, such as those belonging to *Chironomus*, *Cricotopus*, *Polypedilum*, *Diamesa* and *Micropsectra* genera.

ACKNOWLEDGMENTS

The authors are grateful to all those involved in the initiative who helped during sample collections in Montenegro and Albania. The authors thank Sumer Alali, Aleksandra Bańkowska, Katarzyna Gadawska, Aleksandra Jabłońska, Małgorzata Kolicka, Tomasz Mamos, Marek Michalski, Grzegorz Michoński, Magdalena Kłosowska, Mateusz Puchalski, Tomasz Rewicz, Ewa Sarnacka, Przemysław Śmietana, Agnieszka Szlauer-Łukaszewska and Andrzej Zawal for their support in the field sampling. The authors are especially grateful to Stephen Venn for the linguistic proofreading.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ddi.13504.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in BOLD [Dataset "DS-CHBAL - Chironomidae of the Skadar Lake basin"] at https://doi.org/10.5883/DS-CHBAL and GenBank [BankIt2349074, Accession numbers MT534631 to MT535400].

ORCID

Piotr Gadawski https://orcid.org/0000-0001-9334-1960
Matteo Montagna https://orcid.org/0000-0003-4465-7146
Bruno Rossaro https://orcid.org/0000-0001-6154-1191
Wojciech Giłka https://orcid.org/0000-0002-8403-5432
Vladimir Pešić https://orcid.org/0000-0002-9724-345X
Michał Grabowski https://orcid.org/0000-0002-4551-3454
Giulia Magoga https://orcid.org/0000-0002-0662-5840

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BIOSKETCH

Piotr Gadawski currently works as an Assistant Professor in the Department of Invertebrate Zoology and Hydrobiology, University of Lodz (Poland). He is interested in the diversity and distribution of aquatic invertebrates in Europe. His main research interests are focused on integrative taxonomy, biology, ecology and biogeography of Chironomidae from the Balkan Peninsula and Central Europe. The research team is an interdisciplinary group of scientists with expertise in biogeography, taxonomy, phylogeography and ecology of terrestrial and aquatic invertebrates.

Author contributions: Conceptualization: PG, GM, MG, MM; Data Curation: PG, GM; Formal Analysis: PG, GM; Funding Acquisition: PG, MG, VP; Investigation: PG, GM, MM, MG, WG, BR; Methodology: PG, GM, MM, MG, BR, WG; Software Programming: PG, GM; Writing -Original Draft Preparation: PG, GM, MG, MM, WG; Writing -Review & Editing: PG, GM, MM, MG, BR, WG.

SUPPORTING INFORMATION

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How to cite this article: Gadawski, P., Montagna, M., Rossaro, B., Giłka, W., Pešić, V., Grabowski, M., & Magoga, G. (2022). DNA barcoding of Chironomidae from the Lake Skadar region: Reference library and a comparative analysis of the European fauna. *Diversity and Distributions*, *00*, 1–20. https://doi.org/10.1111/ddi.13504