DNA barcoding for biodiversity assessment: the stoneflies of Croatia (#69224)

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DNA barcoding for biodiversity assessment: the stoneflies of Croatia

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Background. The hemi-metabolous aquatic order Plecoptera (stoneflies) with their specificity of life cycle constitute as an indispensable part of terrestrial and aquatic food webs. For a reason of diminishing ~~populations~~ and abundance, stoneflies are considered as the most sensitive ~~group~~ to ~~the~~ environmental changes in freshwater ecosystems ~~compared with the other two orders from the EPT group: Ephemeroptera (mayflies) and Trichoptera (caddisflies) and are typically used for aquatic biomonitoring~~. Given the critical threat ~~of~~ stoneflies, the study of their distribution, morphological variability and genetic diversity should be one of the priorities in conservation biology. However, some aspects about stoneflies, especially a fully resolved phylogeny and phylogeographic structure are not well known. A study that includes comprehensive field research and combines morphological and molecular identification of stoneflies species has not been conducted in Croatia so far. Thus, the major aim of this study was to regenerate a comprehensive and taxonomically well-curated DNA barcode database for Croatian stoneflies, to highlight the morphological variability obtained for several species and to elucidate results in light of recent taxonomy.

Methods. Morphological examination of adult specimens was made using basic characteristics for distinguishing species: terminalia in males and females, head and pronotum patterns, penial morphology, and egg structures. ~~For the phylogenetic reconstruction, genetic intra- and interspecific distance calculations, potential observation of cryptic or yet undescribed species, as well as for additional species delineation methods, DNA barcoding was applied~~.

Results. Sequences (658 bp in length) of 74 morphospecies from all families in Croatia were recovered from 87% of the analysed specimens (355 of 410), with one partial sequence of 605 bp in length for *Capnopsis schilleri balcanica.* Species delineation methods confirmed the existence of six deeply divergent genetic lineages, with monophyletic origin, which also differ morphologically from their congeners and represent distinct entities. BIN (Barcode Index Number) assignment and species delineation methods clustered *COI* sequences into different numbers of operational taxonomic units (OTUs). ASAP delimited 76 putative species and achieved a maximum match score with morphology (97%). ABGD resulted in 62 and mPTP in 61 OTUs representing, thus a more conservative approach. Most BINs were congruent with traditionally recognized species. Deep intraspecific genetic divergences in some clades highlighted the need for taxonomic revision in several species ~~complex~~ and species groups. Research has yielded the first molecular characterization of 9 species, mostly having ~~the~~ restricted

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Abstract

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Introduction

With 3800 described species across ~~16~~ families (*Fochetti & Tierno de Figueroa, 2008*; *DeWalt et al., 2021*), ancient hemi-metabolou~~s~~ insects order Plecoptera, commonly known as stoneflies, represent an important component of freshwater ecological systems and terrestrial and aquatic food webs (*Fochetti & Tierno de Figueroa, 2008; South et al., 2020*). Stoneflies are widely

distributed on all continents, except Antarctica (*Zwick, 2000*) and their range and abundance have declined rapidly in the last 30 years in Central Europe (*Fochetti and Tierno de Figueroa, 2006)*, as well as in the territory of the well-studied Slovenia (*D Hlebec & I Sivec, 2021, unpublished data*), mainly due to the anthropogenic influences (i.e., destruction of habitats and pollution) (*Fochetti and Tierno de Figueroa, 2008; Bálint et al., 2011*). Typical stonefly habitat is a lotic system characterized by cold, fast flowing, and well-oxygenated water (*Sivec & Yule, 2004*) in the mountains of the temperate region. By the appearance of the wings, they are typically macropterous or brachypterous, but several species, especially males at higher altitudes are apterous or micropterous (*Illies, 1966*). Due to poor dispersal capacity and frequent retention on vegetation and rocks, stoneflies represent great study organisms for biogeographical studies (*Fochetti & Tierno de Figueroa, 2008; Murányi, 2011; Graf et al., 2012; Gamboa et al., 2019; Stevens, Bishop & Picker, 2018*). Since Plecoptera exhibit high degrees of endemism (*Fochetti & Tierno de Figueroa, 2006; Murányi, 2011; Murányi, Kovács & Orci, 2016*), as well as morphological diversity, regional field research has both local and global value. Until 2009 and systematic field research, only 28 species of stoneflies were recorded in Croatia (*Sivec, 1980; 1985*), a surprisingly low number considering the many suitable habitats (*Illies, 1978*) and compared to other neighboring countries: Slovenia with 123 species (*I Sivec, 2021, unpublished data*), Bosnia and Herzegovina with 73 species and subspecies (*Kaćanski, 1976*), Montenegro with 57 species (*Murányi*, 2008), Hungary with 61 species (*Andrikovics & Murányi, 2001*) and Serbia with 90 documented species (*Petrović et al., 2014*). Greater efforts in stonefly faunal research resulted in recording 50 species (Popijač, 2008; Popijač & Sivec, 2009a), but still studies were limited to narrow areas such as Plitvice Lakes National Park (*Popijač & Sivec, 2009b; Ridl et al., 2018*), Cetina River (*Popijač & Sivec, 2009b*), Čabranka and Gerovčica Rivers (*Popijač & Sivec, 2009c*) and lower reach of the Una River and its tributaries (*Popijač & Sivec, 2011*). In Plitvice Lakes National Park during the above-mentioned studies, some specimens were recorded that could not be asigned with certainty to a known species: *Perlodes* cf. *intricatus, Isoperla* cf. *lugens*, *Leuctra* cf. *pusilla*, *Leuctra* sp., *Nemoura* sp., *Protonemura* sp., *Isoperla* sp. and *Perlodes* sp. Also, during these studies, several remarkable species were documented: *Marthamea vitripennis* Burmeister, 1839, which was re-discovered again after one century (*Sivec, 1985; Popijač & Sivec, 2011*), *Perla abdominalis* Burmeister, 1839 (*Popijač & Sivec, 2009a)*, *Besdolus imhoffi* Pictet, 1841 (*Popijač & Sivec, 2010)* and *Protonemura julia* Nicolai, 1983 (*Popijač & Sivec, 2009c*).

Currently, the most widely accepted system of stoneflies classification is by *Zwick (2000)*, with two recognized suborders (Arctoperlaria and Antarctoperlaria) and 16 families. To resolve deeper phylogenetic relationships, research has highlighted the need for molecular data, which in the last years, at least in part, has helped overcome morphology-based identification limitations. Allopatric diversification driven mostly by glaciation and orogenesis affected extant diversity patterns of Plecoptera (*Zwick, 2000; Weiss, Stradner & Graf, 2011; Theissinger et al., 2013*). The most complete molecular phylogenetic study of stoneflies was made by *South et al. (2020)*, based on the North American fauna, after several studies with limited taxon sampling (*Thomas et al., 2000; Chen et al., 2018; Wang et al., 2018; Ding et al., 2019*). DNA barcoding uses sequence diversity in the standardized 658-bp region of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene to aid in species identification, link different life stages and to identify

cryptic species. Such an approach is extremely important for biodiversity assessment~~, especially for taxonomically understudied groups and most endangered species due to habitat destruction~~ (*Hebert et al., 2003a, 2003b; Hebert et al., 2004; Valentini, Pompanon & Taberlet, 2009; Morinière et al., 2017*). Application of DNA barcoding aided species delimitation in various groups of organisms and pointed to divergent haplotypes and hybridization (*Van Velzen et al., 2012; Szivák et al., 2017; Zangl et al. 2019, 2021*), and once a set of barcodes for a group of organisms is established, examination of previously unidentified specimens is greatly facilitated (*DeSalle, 2006; Ratnasingham & Hebert, 2007*). However, it has been observed that the results of DNA barcoding could be confounded (*Havemann et al., 2018*) due to~~,~~ *Wolbachia* infections (*Werren, Zhang & Guo, 1995*), incomplete lineage sorting (*Petit & Excoffier, 2009*), pseudogenes (*Ribeiro Leite, 2012*), introgressive hybridization (*Raupach et al., 2014*), and recent speciation (*Raupach et al., 2014*). DNA barcoding has proved to be a great tool for identification of species from the EPT group (*Gill et al.; 2014; Ball et al., 2005; Webb et al., 2012; Morinière et al. 2017; Kučinić et al. 2020*). Furthermore, the analysis of DNA barcoding results is often difficult due to deposited barcode sequences without scientific species names, known as "dark taxa", which represent groups of organisms characterized with a lack of taxonomic expertise or undescribed species (*Page, 2016; Ryberg & Nilsson, 2018*). Additionally, occasional incompatibilities between DNA barcoding and traditionally taxonomic approach are not surprising and follow the process of evolution itself (*Hendrich et al. 2010; Hendrich et al. 2014*). Therefore, for efficient species delimitation it is necessary to analyze multiple character systems and use integrative taxonomy (*Vitecek et al. 2017; Zhang et al. 2013*).

Conducted in the framework of the project DNA barcoding of Croatian faunal biodiversity, the present study aims at (i) developing a DNA reference barcode library for the Croatian stonefly fauna with macrophotographs of 26 species, (ii) getting first insight into inter- and intraspecific genetic diversity, (iii) distinguishing morphological variability of stoneflies in Croatia, (iv) highlighting localities with high biodiversity, especially in the isolated habitats in area of the Dinaric Karst (caves, pits, underground and temporary rivers and streams) to assist conservation planning and strategies for protecting the genetic diversity of stoneflies, and (v) filling the gaps in the Barcode of Life Data System (BOLD) database. Furthermore, the study will contribute to knowledge about the distribution of species, genetic lineages within species and systematic and phylogenetic relationships.

Materials & Methods

Taxon sampling

The specimen collection was conducted in concordance with the approval of the Ministry of Economy and Sustainable Development of the Republic of Croatia (UP/I-612-07/21-48/73). A total of 337 stoneflies specimens (Data S1, Table S1) from 95 different localities in Croatia and additionally 18 specimens from 17 localities in Slovenia (Fig. S1) were used in phylogenetic analysis. Information regarding species determinations and details about sampling sites can be found in Table S1. Adult specimens were collected using sweep nets and beating sheets, while larval specimens were collected by handpicking. The aedeagus was everted in the field and specimens were fixed and stored in 96% ethanol for morphological and molecular analysis. Morphological characteristics of male terminalia were examined after ~~the~~ KOH treatment.

Comparative study on the morphology was made using the specimens kept in the Collection of stoneflies in the Slovenian Museum of Natural History, Ljubljana, Slovenia (PMSL). Newly collected specimens are deposited in the Croatian Natural History Museum, Zagreb, Croatia (CNHM), under the Collection of Plecoptera Sivec & Hlebec (CPSH). Voucher information for individuals used in phylogenetic analysis are publicly accessible in the "CRO Plecoptera" data set in BOLD and GenBank (under the accession Nos. OK316149 - OK316486 and MW907977 - MW907993) (Table S1).

Individuals were sorted and identified under a Leica Wild M3Z stereomicroscope. Macrophotographs were taken using Canon EOS 5D Mark II. Morphological examination was made using descriptions and identification keys: *Illies, 1955*; *Kaćanski & Zwick, 1970*; *Raušer, 1980*; *Ravizza, 2002*; *Sivec & Stark, 2002*, *Graf & Schmidt-Kloiber, 2003*, *Zwick, 2004* and *Murányi, 2011*. The most reliable discriminating diagnostic characters for species determination were: terminalia in males and females, head and pronotum patterns, penial armatures, and egg structures for species within the genus *Perla*.

DNA extraction, gene amplification and sequencing

The number of specimens per species selected for *COI* marker amplification ranged from 1 to 24 (*Isoperla inermis;* see Table S1). Total genomic DNA was extracted from the single leg of specimens with the Sigma GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, US) for contemporary samples and QIAamp DNA Micro Kit (Qiagen, Germany) for the specimens older than 10 years, following the manufacturer's protocol, eluted in 55 µl of elution buffer and stored at -20 °C. The partial region of the mitochondrial cytochrome c oxidase subunit I gene (*COI,* the DNA barcode region, *Hebert et al., 2003a*) was amplified using two sets of primers: (a) LCO-1490/HCO-2198 (*Folmer et al., 1994*) or (b) C_LepFolF/C_LepFolR (*Hebert et al., 2003b*). For samples older than 10 years, the DNA barcode region was amplified as shorter overlapping fragments with primer sets: (c) MLepF1/LepR1 and MLepR1/LepF1 (*Hajibabaei et al., 2006*). All polymerase chain reactions (PCRs) were carried out in a total volume of 20 µl using: 1 x DreamTaq™ reaction buffer with 2 mM $MgCl_2$ (Thermo Fischer Scientific, Inc., US), 0.2 mM each of dNTPs, 0.4 µM of each primer, 0.025 U/µL of DreamTaq polymerase (Thermo Fischer Scientific, Inc., US) and 1 µl of eluted DNA. Thermocycling conditions are given in Table S2. PCR products were purified using Exonuclease I (0.05 U/µL) and FastAP Thermosensitive Alkaline Phosphatase (0.025 U/µL) enzymatic system (Thermo Fischer Scientific, Inc., US). The reaction was carried out using the following protocol: 1 h at 37 °C followed by 20 min at 80 °C. The sequencing of purified PCR products was performed by Macrogen Inc. (Amsterdam, Netherlands), using the amplification primers.

Data analysis

In total, 355 obtained sequences were checked, edited, assembled from both directions, and inspected manually for base pair ambiguities, indels and stop codons in Geneious Prime 2022.0.1 (Biomatters, Auckland, New Zealand). Sequences were aligned using MAFFT v.7. (*Katoh & Standley, 2013*). The final alignment for the *COI* gene fragment was 658 bp in length (Data S2). Sequences were collapsed into 268 *COI* haplotypes using the online tool FaBox v.1.5 (*Villesen, 2007*) and used for further phylogenetic analyses. Evolutionary divergence was estimated using uncorrected pairwise genetic distances (*p*-distances) in MEGA-X (*Kumar et al., 2018*) (mean

values are shown in Table S3). Phylogenetic relationships ~~with use of 268 obtained~~ ~~*COI* haplotypes~~ were estimated by using Maximum-likelihood (ML) optimality criteria in IQ-TREE2 (*Minh et al., 2020*) under a GTR+I+G optimal model of nucleotide evolution (as determined by jModelTest (*Darriba et al., 2012*) under the Bayesian information criterion (BIC)) and bootstrapping with 2000 ultrafast bootstrap replicates (*Hoang et al. 2018*). ABMAY005-09 (Heptageniidae) and ABMAY015-09 (*Stenacron interpunctatum*) were selected as outgroups. Additionally, phylogenetic relationships between obtained haplotypes were inferred using the Neighbour-Joining (NJ) method in MEGA-X (*Kumar et al., 2018*) (Fig. S2) and Bayesian analysis (BA) in MrBayes 3.2.7. (*Ronquist et al., 2012*) through the CIPRES Science Gateway web server (*Miller, Pfeiffer W & Schwartz T, 2010*) (Fig. S3). NJ was ~~made~~ using the Kimura-2-parameter (K2P) model with the pairwise deletion option. Bootstrap support was inferred using the fast bootstrap algorithm, based on 5000 replicates. For the BA, two separate runs with four Metropolis-coupled Monte Carlo Markov chains (MMCM) were performed for 10 million generations, while trees were sampled every 1,000 generations and 25% of the initially sampled trees were discarded as burn-in. Remaining trees were used to create a 50% majority rule consensus tree. TRACER v.1.7.1 (*Rambaut et al. 2018*) was used to check the convergence between the two runs. Phylogenetic trees were visualized using FigTree v.1.4.3. (*Rambaut, 2009*) and iTOL v.5 (*Letunic & Bork, 2021*). Existence of a barcoding gap (distance between the mean intraspecific sequence variability and interspecific variability for congeneric *COI* sequences) were ascertained using the "Barcode Gap Analysis" tool, provided on BOLD, using the Kimura-2-Parameter (K2P) distance metric (*Puillandre et al., 2012*) (Table S4). To visualize phylogeographic relationships among specimens in one of the newly observed distinct lineages with the largest number of specimens, median joining (MJ) network among 12 haplotypes (*Bandelt, Forster & Röhl, 1999*) (Fig. 3, Data S3) was generated using the program PopART v.1.7 (*Leigh & Bryant, 2015*) with default setting. In several cases ~~obtained~~ *COI* sequences were used in additional phylogenetic (ML in IQ-TREE2 with settings as above, under the optimal model of nucleotide evolution as is listed in Table S5) and species delineation analysis in order to interpret the results and to check the plausibility. ~~The above applied to:~~ (a) species with high level of intraspecific morphological variability (*Perlodes intricatus* and *Isoperla inermis*), (b) specimens which ~~differ~~ ~~mainly~~ ~~in taxonomic features~~ from the described morphospecies (*Isoperla* cf. *lugens*, *Protonemura hrabei* and *Taeniopteryx sp.*) and (c) species interesting from taxonomic point of view (*Besdolus imhofii* and *B. illyricus)*. Analys~~is~~ were performed with ~~retrieved~~ sequences of these species and their closely related congeners from BOLD (*Ratnasingham & Hebert, 2007,* http://www.boldsystems.org) or NCBI GenBank (https://www.ncbi.nlm.nih.gov/) (accessed 20/08/2021). The accession numbers of all sequences used in additional analysis are listed in Data sets 1-6, Table S5. Molecular species delineation was achieved through four different methods: the BIN (Barcode Index Number) assignment tool on the BOLD server using the refined single linkage (RESL) algorithm (*Ratnasingham & Hebert, 2013*), ABGD (Automatic Barcode Gap Discovery) (*Puillandre et al., 2012*), ASAP (Assemble Species by Automatic Partitioning) (*Puillandre, Brouillet & Achaz, 2021*) and mPTP (Multi-rate Poisson Tree Processes) method (*Kapli et al., 2017*). All methods clustered *COI* sequences into Operational Taxonomic Units (OTUs) based on sequence similarity. ASAP, a new advancement of the ABGD method, does not include a priori definition of a distance

threshold and uses ranked pairwise distances to cluster sequences into groups (*Puillandre, Brouillet & Achaz, 2021*) Members of a BIN usually belongs to a species recognized using traditional morphological analysis and taxonomy (*Hendrich et al., 2014*) and species assignment is based on a universal upper threshold for intraspecific distances (*e.g.*, 2.2%) (*Ratnasingham & Hebert, 2013*). BIN counts are usually used for species richness (*Hebert et al., 2016*), but single-locus delineation methods tend to oversplit by mistaking different lineages within populations as putative species (*Muster & Michalik, 2020; Meier et al., 2021*). Use of the "BIN Discordance" tool on BOLD, provides insight into the concordance between barcode sequence clusters and species designations.

ABGD analysis was carried out via the web version (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) applying the K2P model, using default parameters, except for the relative gap width, which was set as X = 1.0. The ASAP web server (https://bioinfo.mnhn.fr/abi/publi c/asap/asapw eb.html, accessed 10 September 2021) was performed using *p*-distances with default settings. The mPTP method was run on the web server at http://mptp.h-its.org/, using default settings. ML tree, inferred in IQ-TREE2 (*Minh et al., 2020*), was used as the input tree for the mPTP analysis.
Validity and reliability of the generated DNA barcode library was evaluated by comparing classical taxonomy and the counts and groups of OTUs with species delineation methods.

Results

Sequencing of the standardized fragment of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene was successfully carried out for 355 individuals from 410 selected (87%). All sequences were 658 bp in length, except one sequence of *Capnopsis schilleri balcanica*, which was 605 bp in length, representing 74 species and 19 genera (*Agnetina* (*n* = 1 species), *Amphinemura* (*n* = 3 species), *Besdolus* (*n* = 1 species), *Brachyptera* (*n* = 4 species), *Capnopsis* (*n* = 1 species), *Dinocras* (*n* = 1 species), *Isoperla* (*n* = 10 species), *Leuctra* (*n* = 16 species), *Marthamea* (*n* = 1 species), *Nemoura* (*n* = 10 species), *Nemurella* (*n* = 1 species), *Perla* (*n* = 6 species), *Perlodes* (*n* = 3 species), *Protonemura* (*n* = 6 species), *Rhabdiopteryx* (*n* = 1 species), *Siphonoperla* (*n* = 2 species), *Taeniopteryx* (*n* = 4 species), *Xanthoperla* (*n* = 1 species) and *Zwicknia* (*n* = 2 species)).
The morphological assessment and the present molecular data set support delineation of morphologically distinct individuals. In fact, 64 of the 74 species (86%) could be unambiguously identified by their *COI* sequence. The barcode recovery was high across different species when appropriate amounts of tissue were loaded and when specimens were well fixed and stored. This is especially applicable for freshly collected material compared to older specimens where the success rate was lower and whose sequences were flagged as contaminations. The median number of barcodes per species was four, and 17, predominantly rare species, were known only by single specimens.
The average confamilial *p*-distance was 0.208 (ranging from 0.182 to 0.234) whilst the average congeneric distance was 0.156 (ranging from 0.121 to 0.192). The mean intraspecific p-distance was 0.013 (ranging from 0 to 0.068) (Fig. 1A). A maximum intraspecific distance of 0.068 was obtained for *Isoperla illyrica*. Most individuals were above the line that represents the existence

of a barcoding gap, meaning that for each individual the difference between the distance to the NN (Nearest Neighbour) and the distance to the furthest conspecific is above zero (Figs. 1B–C).

Figure 1

The mean intraspecific *p*-distance distribution is partially overlapping with distance to the nearest neighbour distribution (Fig. 1C), but for most species, nearest-neighbour distances were on average several times higher than maximum intraspecific distances (Fig. 1B). Maximum intraspecific p-distance was ~~low~~ correlated with the number of individuals per species (Fig. 1D) (the correlation coefficient (ρ) = 0.447). For several neighbour species pairs, maximum intraspecific *p*-distance values were higher than their nearest-neighbour distance: *Leuctra albida*/*Leuctra mortoni*, *Leuctra fusca*/*Leuctra albida*, *Perla pallida*/*Perla marginata* and *Isoperla illyrica*/*Isoperla tripartita*. For listed neighbour species pairs, *p*-distance to the nearest neighbour was below <2%: *Leuctra albida*/*Leuctra mortoni*, *Nemoura* cf. *rivorum*/*Nemoura flexuosa*, *Perla illiesi*/*Perla abdominalis*, *Perla* sp./*Perla marginata*, *Perla pallida*/*Perla marginata* and *Isoperla illyrica*/*Isoperla tripartita*.
This study resulted in five entities which morphologically differ from their congeners and genetically appeared as separate lineage. These species therefore represent candidates for new, previously undescribed species: *Leuctra* cf. *prima* (clade No. 7 in Fig. 2, distance to NN = 0.093), *Leuctra* cf. *inermis* (clade No. 4 in Fig. 2, distance to NN = 0.036), *Protonemura* cf. *autumnalis* (clade No. 72 in Fig. 2, distance to NN = 0.087), *Isoperla* cf. *lugens* (clade No. 37 in Fig. 2, distance to NN = 0.067) and *Taeniopteryx* sp. (clade No. 47 in Fig. 2, distance to NN = 0.071) (Fig. 2, Table S4). Another separate lineage was recently described *Isoperla popijaci* (clade No. 35 in Fig. 2, distance to NN = 0.067) (*Hlebec et al., 2021*).
All methods used for phylogenetic reconstruction (ML (Fig. 2), NJ and BA (Fig. S3 and Fig. S4)) recovered the same, well-supported topology ~~of phylogenetic trees~~. All methods grouped phenotypically defined species in distinct, highly supported monophyletic species clusters (ultrafast bootstrap support > 99). Phylogenetic relationships above the species level are in concordance with morphology-based hypotheses, and all genera form monophyletic clusters.

Figure 2

Obtained sequences were allocated to 85 BINs (of which 29 were comprasing the specimens collected in specific localities or sequences reported for the first time in the present study), and delimited OTUs were mostly consistent with the clustering pattern observed in the ML tree, which was also concordant with morphological identification. Twenty-six BINs were represented by a single individual (singletons).
One BIN often belongs to a single species delineated by traditional taxonomy (Hausmann *et al.*, 2013), and every case can be an incentive for re-evaluation of morphological and molecular data, respectively (*Hendrich et al., 2014*). Specimens of several species showed deep *COI* divergence resulting in multiple BINs within a species: *Protonemura praecox* (BOLD:AEH4111 and BOLD:AEH7722), *Perlodes microcephalus* (BOLD:AAL2343 and BOLD:AEH5507), *Nemoura marginata* (BOLD:AAN1631, BOLD:AEH3564 and BOLD:AEK9273), *Isoperla illyrica* (BOLD:AEH3875 and BOLD:AEH7030), *Leuctra fusca* (BOLD:AAE6442 and

BOLD:ACY3863), *Leuctra albida* (BOLD:AAM4011 and BOLD:AEH5504), *Leuctra hippopus* (BOLD:ACL7184 and BOLD:AEH4770), *Isoperla tripartita* (BOLD:AEH3875, BOLD:AEH3876, BOLD:AEG6510 and BOLD:AEH7030), *Isoperla grammatica* (BOLD:AEH6396 and BOLD: AEG4373) and *Isoperla inermis* (BOLD:ACS6073, BOLD:AAZ7905 and BOLD:AEH8653). Intraspecific *p*-distances were as follows for the following species: *P. praecox* (0 – 0.032), *P. microcephalus* (0 – 0.042), *N. marginata* (0 – 0.048), *Isoperla illyrica* (0.002 – 0.068), *Leuctra fusca* (0 – 0.058), *Isoperla tripartita* (0 – 0.066), *Isoperla grammatica* (0 – 0.050) and *Isoperla inermis* (0 – 0.040).
A shared BIN assignment was obtained within three genera: *Isoperla, Perla* and *Nemoura,* and species *Isoperla illyrica/Isoperla tripartita, Nemoura flexuosa/Nemoura* cf. *rivorum* and *Perla pallida/Perla* sp./*Perla marginata.*
Overall, use of different species delineation algorithms resulted in a different number of putative species. ASAP (with the best ASAP-score (6.00) which was achieved at a distance threshold of 2.5% (*p*-distance) delimited 76 putative species and achieved a maximum match score with morphology (97%). The partition with the second-best ASAP-score (8.50, distance threshold 3,6%) delimits 70 species and the third partition (9.00, distance threshold 5.0%) delimits 64 species.
The ABGD method ~~was utilized to~~ delineate 62 species in the recursive approach (61 species in the initial approach) for the majority of prior intraspecific divergence values (P). mPTP ~~resulted in delineation of~~ 61 putative species, ~~thus they represent a more conservative estimate~~ (Fig. 2).
MJ network depicted relatedness and distribution within the newly obtained divergent lineage named as *Isoperla* cf. *lugens*. The MJ network among 12 unique haplotypes which were separated by a different number of mutational steps is shown in Fig. 3A-B. The MJ network also revealed ~~the very~~ low haplotype sharing among sampling sites. Haplotypes CROPL311-21, CROPL344-21, CROPL127-21, CROPL310-21, CROPL312-21 and CROPL352-21 grouped together in a well-supported subclade (Fig. 2, Fig. 3B). Haplotype CROPL352-21 (discovered in Vitunjčica River) was separated by five mutational steps from the closest haplotype that was recorded in Drakulić rijeka, Crna rijeka i Čabranka River. The remaining 7 haplotypes also grouped together in a well-supported subclade (Fig. 2, Fig. 3B).

Figure 3

Discussion

The present study represents the first comprehensive research combining morphological and molecular identification of stoneflies species in Croatia and establishes DNA barcoding as an effective tool for reliable species identification. Such an approach enhances taxonomic resolution and assists in the quality of faunal research and can be used in discovering cryptic diversity and species complexes (*Previšić et al., 2014a; Zangl et al., 2021*). Other studies with specimens from Croatia that focus on Mollusks (*Buršić et al., 2021*), Mosquitos (*Bušić et al., 2021*) and Trichoptera (*Kučinić et al., 2013; Valladolid et al., 2020*) have obtained similar results, which support the efficacy of DNA barcoding for species discrimination.
So far, sequencing of the *COI* gene fragment has been used to elucidate the systematics and phylogeography of Plecoptera (*Fochetti et al., 2009; 2011; Weiss et al., 2011*), in the description of new species (*Boumans & Murányi, 2014; Graf, Pauls & Vitecek, 2018; Pelingen & Freitag,*

2020), as part of DNA barcoding initiatives (*Morinière et al., 2017; Gattolliat et al., 2016; Ferreira et al., 2020*) and in the partial revision of the genus (*Fochetti et al., 2011*). ~~The obtained results of the molecular approach~~ are not always congruent with the morphological ~~arrangement~~ (*Fochetti et al., 2011*). Distinct DNA lineages obtained within morphospecies indicate the need for re-examination of morphological characters (*Muster & Michalik, 2020; Wachter et al., 2015*). Within this study, all methods for phylogenetic reconstructions show that ~~all~~ species can be distinguishable through *COI* (exception are *Isoperla illyrica*, *Isoperla tripartita*, *Perla pallida*, *Perla sp*., *Perla marginata*, *Leuctra albida*, *Leuctra mortoni*, *Leuctra fusca*, *Nemoura* cf. *rivorum* and *N. flexuosa*). All mentioned species pairs possessed identical or overlapping *COI* barcode sequences. For the above-mentioned species, identification is sometimes difficult due to the high level of intraspecific morphological variability between closely related species (*Ravizza & Ravizza Dematteis, 1995; Murányi, 2011; I Sivec, 2021, ~~unpublished data~~*) and often ~~with the unavailability of males/females, which have principal morphological features for species diagnosis.~~

High numbers of intraspecific BINs can be explained by the existence of geographical subclades of a currently identified species (*Morinière et al., 2017*), so the number of 85 BINs cannot be a proxy for the total number of stonefly species in Croatia. Nevertheless, the underlying RESL algorithm is based on a distance threshold of 2.2%, so it was to be expected to have a larger number of BINs in the data set. *Meier et al., 2021* made a claim that the cytochrome oxidase I (*COI*) barcode region cannot be used as the only/main data source for describing or delimitating species (*Sharkey et al. 2021*) and stressed the need for use of additional species delimitation methods as well as examination of morphological characters for justifying the validity of the species. *COI* barcode clusters ("BINs") as a basis for species descriptions, under the assumption that a BIN equals species, is often not consistent with the results of other species delineation methods (*Meier et al., 2021*)~~,~~ due to theoretical and empirical reasons (*Puillandre, Brouillet & Achaz, 2021*)*; Zhang et al., 2013*). BIN sharing reported in present study within three genera: *Isoperla, Perla* and *Nemoura* emphasizes the need for further study and the potential observation of cryptic species, introgression, hybridization or the conclusion that BIN divergence represents regional variation within specimens, as is mentioned before (*Hawlitschek et al., 2017*). The species assigned to multiple BINs (obtained for ten species) can indicate mitochondrial variation accumulated during historical isolation in separate refugia or overlooked cryptic species (*Morinière et al., 2017*) ~~and such case has already been recorded in previous research for the species~~ *Dinocras cephalotes* (*Elbrecht et al., 2014*).

Intra- and interspecific distances ~~were partially~~ overlapped (Fig. 1). Existence of a barcoding gap allows the use of DNA barcoding for the identification of species (*Meyer & Paulay, 2005*). Overlapping ~~between~~ intra- and interspecific *p*-distances can be represented as ~~a failure to demarcate~~ a universal cut-off value~~, as is mentioned before~~ (*Collins & Cruickshank, 2013*). Also, they can be a consequence of inaccurate taxonomy, indicating oversplit or cryptic species especially for species within the genera *Isoperla* and *Perla* (Fig. 1).

In the present study, the distance to the NN is predominantly higher than the maximum intraspecific distance, confirming the clear local barcoding gap, ~~which enables~~ successful use of DNA barcoding for Croatian stonefly identification (Table S4). Furthermore, the congeneric average distance among stoneflies species was five times higher than the average distance within

species.
Nevertheless, the observed overlap between intra- and interspecific *p*-distances may be related to the presence of cryptic species or species complexes, which would not be surprising given the results of the morphological study. Conflicts within traditional taxonomy (*Ciprandi Pires & Marioni, 2010; Morinière et al. 2017*) should be used as incentive for further sampling.

Geographic morphological variation

Perlodes intricatus
Previous research of the stonefly fauna in Croatia, based exclusively on morphological analysis, have established the presence of specimens which could not be identified with certainty to known species (*Popijač & Sivec, 2009b*). *Perlodes intricatus* (clade No. 32, Fig. 2) from Plitvice Lakes, in which morphological differences were observed with respect to the typical *Perlodes intricatus*, was also found during this study. Molecular methods confirmed identification, whereas DNA barcoded specimens were grouped with sequences of *P. intricatus* retrieved from BOLD, into a highly supported monophyletic clade (Data set 1, Table S5), but with high intraspecific *p*-distances (0.052 – 0.056) indicating the need for further field research in the entire range of the species.

Genus *Isoperla*
The genus *Isoperla* is characterized by ~~the not well distinguishable~~ West Palearctic species (*Zwick, 2004*) and the need for taxonomic revision ~~has already been emphasized~~ (*Murányi, 2011*). Within ~~the specimens determined as~~ *Isoperla inermis* (clade No. 34, Fig. 2), ~~which were collected in source areas,~~ great morphological variability~~, was also~~ observed in previous research (*Popijač & Sivec, 2009b*). Individuals from Plitvice Lakes are almost double in size compared to specimens from Cetina River (*Popijač & Sivec, 2009b*), which may be a result of different climates and a longitudinal gradient. ~~Besides size, morphological variation is visible in coloration of the abdomen, and the head and pronotum, in which coloration varies from brownish to almost completely black. In additional~~ phylogenetic analysis with sequences of all *Isoperla* species obtained within present study and ~~species~~ *I. difformis*, ~~DNA barcoded individuals of *I. inermis* grouped together with specimens~~ of the ~~Central~~ European ~~species *I. difformis* in~~ highly supported monophyletic clade, which could ultimately~~, by using other molecular markers,~~ result in the synonymy of these species (Data set 2, Table S5).
Isoperla cf. *lugens* (clade No. 37, Fig. 2) was recorded in the area of Plitvice Lakes (*Popijač & Sivec, 2009b*), and determined based on similarity of the penial armature. During comprehensive field research in this study, specimens were found associated with several sources of karst rivers. The species differs morphologically from the alpine species, *I. lugens*, by the lighter colored head and pronotum and different penial armatures. In addition to morphological characteristics, the species is also characterized by exceptional genetic distinctiveness. The lowest interspecific *p*-distance value from *I.* cf. *lugens* compared to other congeners from *I. tripartita* and *I. rivulorum* species group is 6.73% and represents a separate genetic lineage within the clade consisting of typical *I. lugens* and *I. popijaci,* closely related to the source of the intermittent river Krasulja (*Isoperla popijaci*, clade No. 37, Fig. 2) (Data set 3, Table S5). Due to the above-mentioned characteristics *Isoperla* cf. *lugens* most probably represents a new species. MJ

network (Fig. 3B) for species *Isoperla* cf. *lugens* revealed ~~the very~~ low haplotype sharing among sampling sites~~.~~ which may be due to the small number of specimens per sampling sites~~, where more comprehensive field research must be ensured for future analyses~~.

Genus *Protonemura*

Protonemura hrabei (clade No. 71, Fig. 2) from Cetina and Zrmanja rivers ~~has the time of emergence from the~~ beginning of summer, mostly due to climatic conditions, in contrast to ~~individuals from~~ Central Europe ~~(autumn)~~ (*Popijač & Sivec, 2009b*). Molecular analysis of obtained sequences and sequences of closely related *Protonemura* species, confirmed morphological identification and individuals from the Cetina River form a highly supported monophyletic clade with *Protonemura hrabei* from Central Europe with a intraspecific p-distance 0.025 (Data set 4, Table S5).

Genus *Nemoura*

High morphological variability has been observed within specimens of *Nemoura marginata* (clade No. 64, Fig. 2) (*Popijač & Sivec, 2009b*). The mean intraspecific p-distance was 3.05%. Future research should assess whether *Nemoura marginata* represents a single species with large intraspecific distances and high morphological variability or a species complex, as ~~is as~~ stated earlier (*Ravizza & Ravizza Dematteis, 1995*). For two species within genus *Nemoura*, *N.* cf. *rivorum* and *N. flexuosa*, BIN sharing (BOLD:AEH8297) was observed. Therefore, for the reconstruction of phylogenetic relationships within *Nemoura*, it is necessary to analyse the specimens across its whole distributional range.

Genus *Taeniopteryx*

In a comprehensive study of the genus *Taeniopteryx* in the framework of this study, morphological differences were determined between newly collected individuals *Taeniopteryx* sp. (clade No. 47, Fig. 2), *Taeniopteryx hubaulti* (clade No. 46, Fig. 2) and *Taeniopteryx auberti*, as well as specimens from ~~museum collection collected in~~ Croatia, Slovenia, Bosnia and Herzegovina, Montenegro and Germany. These morphological differences of species *Taeniopteryx* sp. are accompanied by genetic distinctiveness, which ~~certainly may~~ represent a candidate for a new species (interspecific *p*-distances ranged from 7.8 to 9.5~~-~~% in ~~comparison with other *Taeniopteryx* species), as already pointed out in~~ *Popijač & Sivec, 2009b*. Morphological analysis of individuals of the species *T. hubaulti* established the variability of the femoral thorn on the hind legs, present in some individuals and different sizes. According to the first description of the species it should be completely absent (*Aubert, 1946*), while this morphological characteristic should be clearly visible in *T. auberti* (*Kis & Sowa, 1964*). Genetic analysis with obtained sequences, as well as sequences retrieved from BOLD and GenBank databases (Data set 5, Table S5), has resulted in an unclear taxonomic status of *T. hubaulti* and *T. auberti*, so there is the suspicion that *T. hubaulti* may be synonym of *T. auberti*, and morphological variability is a consequence of geographical distribution. This is also similar for the species, *T. stankovitchi* and *T. schoenemundi*, for which it has already been pointed out that additional research is needed to clarify their distinction (*Fochetti & Nicolai, 1996*). As has been noted, the genus *Taeniopteryx* is, from the taxonomic point of view, complicated, and oftentimes only females show reliable characters, so the whole genus needs revision.

Genus *Leuctra*

Within the *Leuctra inermis* species group congruence of morphospecies concepts and phylogenetic relationships of taxa was not studied until 2017 (*Vitecek et al., 2017*). However, after that, relationships among species remained unresolved, suggesting sister taxon relationships between morphologically similar species and potential subspecies-level diversity (*Vitecek et al., 2017*). Morphological variability was observed within geographically isolated populations (*Vitecek et al., 2017*), and the same was confirmed by this research. As many species from the *L. inermis* species group have overlapping geographical ranges, appearance of morphological variability within several species is expected (*Fochetti et al., 2011*) and mitochondrial introgression ~~have been~~ already confirmed within the species, *L. fusca* and *L. digitata* (*Boumans & Tierno de Figueroa, 2016*). Thus, it can be assumed that assessment of drumming call variations could be helpful in resolving taxonomic relationships within this species group (*Vitecek et al., 2017*).

Few individuals collected in this study appeared as a distinct lineage (Fig. 2) with morphological features that only resemble already known species: *L.* cf. *inermis (*CROPL130-21) collected in the Plitvice Lakes National Park, *Leuctra sp.* (marked as *Leuctra sp.* Z, CROPL248-21) collected at Žumberak Hills and *L.* cf. *prima* (CROPL282-21, CROPL325-21 and CROPL326-21) collected at Papuk Mountain and near Plitvice Lakes National Park. Due to the unavailability of specimens of *L. carphatica*, widespread in southeastern and eastern Europe (*DeWalt et al. 2021*), the comparison of collected individuals and this species was omitted. Differences in morphological characteristics ~~was~~ also observed in individuals of *L. mortoni* and *L. fusca* which require~~s~~ further research with a more comprehensive sampling, which would contribute to a more precise taxonomic ~~assessment~~ of these ~~taxa~~.

Dinaric karst as a biodiversity hotspot

The dinaric karst area~~, a type of landscape~~ of the Dinaric Mountains, represents one of the most ~~dynamic~~ European freshwater habitats in terms of biological, geological and hydrological interplay, including many available microhabitats, which has resulted in speciation and endemism (*Bonacci, 2009*). Considering the results of earlier studies, high genetic diversity could be the result of habitat requirements and biological characteristics of individual taxa which promoted speciation and have important role in the genetic differentiation of freshwater taxa (*Previšić et al., 2009, 2014a, 2014b; Klobučar et al., 2013; Jelić et al., 2016; Szivák et al., 2017*). The same goes for taxa related to specific habitats which abounds in Dinaric karst (*Bilandžija et al., 2013; Bedek et al., 2019; Pavlek & Mammola, 2020*), which in general, can be considered as refugia from which taxa re-colonise Europe following glacial periods (*Hewitt, 2000*), often showing a pattern “refugia within refugia” (*Kryštufek et al., 2007; Ursenbacher et al., 2008; Previšić et al., 2009; Jug-Dujaković et al., 2020*).

The diversity of the stonefly fauna in Croatia is a probable consequence of the different climatic conditions in a variety of regions that can be divided into: continental, mountainous, submediterranean and mediterranean, the large number of unsuitable habitats, a substantial altitudinal gradient, and an immense number of preserved habitats. Within this study, ~~identification of local~~ hotspots of elevated species richness ~~was made using stoneflies as models~~. Highest level of species richness ~~was~~ primarily located in the northwest Dinarides (Fig. 4): the

border rivers: Kupa and Čabranka, Plitvice Lakes National Park, Cetina and Una Rivers, and the Mt Papuk where several localities have five or more species. Hotspots of species richness is ~~for stoneflies, mainly~~ coincident with protected areas (*e.g.*, Plitvice Lakes National Park, Mt Papuk, Mt Medvednica), which is not surprising, given that these areas have suitable conditions and mostly include fast streams with high saturation. Present research shows a significant decrease in the number of species from the northern part of the Dinaric karst (Gorski kotar and Lika) to the Cetina River and the source part of the Una River, which is consistent with the biological features of stoneflies. Great species richness is also confirmed by ~~research~~ *Ridl et al. 2018* where they recorded from 7 to 18 species at different study sites (in total 31 species) in the Plitvice Lakes National Park ~~during two years of research using pyramid type emergence traps (*Ridl et al. 2018*)~~. Furthermore, significant species richness (14) ~~has been observed~~ within the Cetina River ~~during three years of field research and at 13 locations (*Popijač & Sivec, 2010*)~~. Croatian fauna shows great species richness, not only for stoneflies but also for other aquatic insects (*Ivković & Plant, 2015; Vilenica et al., 2015; Mičetić Stanković, Jäch & Kučinić, 2015; Vilenica et al., 2016; Kučinić et al., 2017; Vilenica, Ternjej & Mihaljević, 2021*). ~~In research of other two orders from EPT group: Ephemeroptera and Trichoptera, more sampling effort has been put into it, which also contributed to a much better knowledge of biodiversity (*Vučković et al., 2021; Cerjanec et al., 2020; Vilenica et al., 2014, 2016, 2017; Kučinić et al., 2017; Vilenica, Ternjej & Mihaljević, 2021*). Thus, as many parts of the~~ Dinaric karst are ~~still not very well researched~~, due to the size of area and inaccessibility of the habitats, especially from the aspect of stoneflies fauna, species richness patterns are likely incomplete.

Figure 4

Database's enrichment and systematic implications

This study provides the first molecular characterization of 9 species: *Brachyptera tristis, Perlodes dispar, Leuctra bronislawi, Isoperla bosnica, Isoperla illyrica, Isoperla albanica, Perla carantana, Perla illiesi* and *Agnetina elegantula*.

Brachyptera tristis

Brachyptera tristis (clade No. 53, Fig. 2) is strictly related to source areas. Related species, *B. braueri* was not recorded during ~~field research implemented in this study, and previously it was recorded on~~ the Mura River (on the border of Slovenia and Austria), together with *Dinocras cephalotes* (also not recorded).

Perlodes dispar

~~The first molecular characterization is provided for~~ *Perlodes dispar* (clade No. 31, Fig. 2) ~~also~~ a rare species, ~~which has been recorded in~~ 3 localities on ~~the~~ Papuk Mountain. The increase in intraspecific *p*-distance values is reported for another species within genus *Perlodes*, *P. microcephalus,* and it is accompanied by increasing morphological variability from type specimens of *P. microcephalus* (Data set 1, Table S5).

Genus *Protonemura*

~~M~~olecular characterization of ~~*Protonemura* species (~~*P. auberti*, *P. hrabei*, *P. intricata*, *P. nitida* and *P. praecox*~~)~~ ~~can~~ be a part of potential future revision of the genus, whose need has already been emphasized (*Wagner et al., 2011; Vinçon, Reding & Ravizza, 2021*). ~~This is mostly caused by not always concordant and precise descriptions and illustrations in some identification publications (*Kühtreiber, 1934; Illies, 1955; Aubert, 1959; Kis, 1965; Kis, 1974*).~~ The finding of specimens from the *P. auberti* species subgroup (determined as *P.* cf. *autumnalis*, clade No. 72, Fig. 2) at the Plitvice Lakes National Park with morphological characteristics ~~which are very~~ similar to *P. aestiva*, which emerges throughout the spring and seems to be restricted to the Carpathian Mountains (*Kis, 1974; Graf et al., 2009; ~~DeWalt et al., 2020~~*), suggests a hybrid of these two species, with the spread of the species *P. aestiva* (*Vinçon, Reding & Ravizza, 2021*). Nevertheless, the production of hybrids with intermediate morphological characters has already been observed in the newly described *P. bispina* (*Vinçon, Reding & Ravizza, 2021*) often vicariant with specimens of *P. auberti*. Therefore, it is necessary to pay additional attention to the genus also from that point of view. DNA barcoded specimens of *P. auberti* (CROPL257-21, CROPL258-21, CROPL281-21) collected during the spring at the Una River appeared as a separate clade within other specimens of *P. auberti* (clade No. 74, Fig. 2). Therefore, establishing phylogenetic relationships with a multi-gene approach is necessary to unravel the taxonomy of this group.

Genus *Dinocras*

DNA barcoding of the species~~,~~ *Dinocras megacephala* (clade No. 23, Fig. 2) the only *Dinocras* species in Croatia, widely distributed in its northern and central part, emphasizes the need for revision of the BOLD database due to the noticeable number of erroneous determinations of species ~~(often mistaken for *D. cephalotes*).~~ Males of *D. cephalotes* differ from *D. megacephala* by having patches of stronger sensilla basiconica on the ventral side of the abdomen. Furthermore, brachypterous males of *D. megacephala* ~~appear~~ at higher altitudes (*Illies, 1966*), so ~~this~~ cannot be a characteristic to distinguish it from *D. cephalotes* (usually at higher altitudes), and it is precisely characteristic that is often used to discriminate those species (*D Hlebec & I Sivec, 2021, unpublished data*).

Genus *Zwicknia*

Morphological examination of individuals from the *Capnia bifrons* species group following *Murányi, Gamboa & Orci, 2014*, determined the presence of two species in Croatia: *Zwicknia bifrons* (clade No. 51, Fig. 2) and *Zwicknia rupprechti* (clade No. 50, Fig. 2). For the family Capniidae, from only one locality is the surprising find of the species *Capnopsis schilleri balcanica* (CROPL319-21) one of the smallest European species, after 17 years since the first finding (*Murányi, 2004*).

Genus *Nemoura*

Within the genus *Nemoura*, ten species were DNA barcoded (clades Nos. 60-68, Fig. 2): *N. avicularis*, *N. cinerea*, *N. dubitans*, *N. marginata*, *N. minima*, *N. sciurus*, *N. flexuosa*, *N.* cf. *rivorum*, *N. mortoni* and *N. uncinata*. ~~In the genus *Nemoura*, the~~ *Nemoura flexuosa-marginata* complex is one of the most enigmatic assemblages of species within the European stoneflies and requires revision (*Ravizza & Ravizza Dematteis, 1995*).

The *flexuosa-marginata* species group is composed of widely distributed European species (*N. flexuosa* Aubert, 1949, *N. marginata* Pictet, 1836, *N. uncinata* Despax, 1934) and 6 endemic species *N. hesperiae* Consiglio, 1960, *N. lucana* Nicolai & Fochetti, 1991, *N. oropensis* Ravizza & Ravizza Dematteis, 1980, *N. pesarinii* Ravizza & Ravizza Dematteis, 1979 (all occurring in Italy), *N. palliventris* Aubert, 1953 and *N. rivorum* Ravizza & Ravizza Dematteis, 1995 (occurring in Italy and further north, in French Alps) (*Fochetti & Vinçon, 2009*). Description of the last two species within that complex: *Nemoura rivorum* C. Ravizza & Ravizza Dematteis, 1995 and *Nemoura sabina* Fochetti & Vinçon, 2009 helped in understanding the morphology within specimens of this species complex. *N. rivorum* described as localized in the northern section of the Apennines with variable apical and arched sclerite of the epiproct, can often be erroneously identified as *N. flexuosa*, especially if only females are available for morphological analysis. Pregenital plate shape is similar in all species belonging to the *N. flexuosa-N. marginata* species group and separation based on ~~the mature~~ nymph is also almost impossible. Specimens collected as part of this study (CROPL075-21, CROPL076-21 and CROPL162-21) have morphological characteristics similar to *N. rivorum* (determined as *N.* cf. *rivorum*). However, based on the similarity of the sequences, these specimens are clustered with *N. flexuosa* (CROPL070-21, CROPL095-21, CROPL190-21) which further emphasizes the importance of species group revision, and revision of the sequences in BOLD and GenBank databases, respectively.

Leuctra bronislawi

This study also provides the first molecular characterization of *Leuctra bronislawi* (CROPL131-21 and CROPL132-21). This autumnal species, which is relatively rare and a relict species with disjunct~~ive~~ distribution in the Balkan and the Carpathians, was recently found in the Czech Republic (*Kroča, 2010*) and the first early spring records were reported from the Republic of Macedonia (*Murányi, Kovács & Orci, 2014*). Considering the limited knowledge of the stonefly fauna in countries which can include potential distribution areas of *L. bronislawi*, it is anticipated that many more populations remain to be discovered and recorded.

Genus *Isoperla*

Within the mostly restricted genus *Isoperla*, three species were DNA barcoded for the first time: *Isoperla bosnica* (clade No. 39, Fig. 2), *Isoperla illyrica* (clade No. 42, Fig. 2) and *Isoperla albanica* (clade No. 41, Fig. 2). *I. bosnica* is, according to morphological features, a member of the *I. oxylepis* species ~~and this species is~~ redescribed based on SEM studies of the penis and egg structure (*Murányi, 2011*), as well as the poorly known *I. illyrica* (member of *I. tripartita* species group) (*Murányi, 2011*). *I. bosnica* was hitherto reported only from type locality (SE Bosnia-

Herzegovina), NW Macedonia and Montenegro (*Murányi, 2011*) and the medial penial armature, a basic diagnostic characteristic, is like the armature of *I. oxylepis* (*Murányi, 2011*). *I. albanica* has an Eastern Alpine-Illyrian distribution and it is characterized by an undivided medial penial armature (*Murányi, 2011*). *I. illyrica,* described as an endemic species to the Postojna Cave entrance, is now common in a wide area of Dinaric karst. ~~In the~~ phylogenetic analysis ~~implemented in this study, *I. illyrica* has unresolved phylogenetic placement,~~ so ~~analysis with specimens from the whole distributional range and applying a~~ multi-gene approach is a priority (Data set 2, Table S5). ~~The same can be applied to the~~ *I. grammatica* species complex ~~whose complexity has already been observed~~ (*Murányi, 2021*).

Genus *Perla*

The taxonomy of *Perla* species is unresolved and constitutes a big challenge, and the latest revision of the genus suggests identification based on egg chorionic detail (*Sivec & Stark, 2002*) as a reliable character for species recognition. To revise such a problematic genus, the inclusion of genomic data ~~may be~~ required. For some species, it is already considered that they represent a species complex, such as *P. pallida* (distributed in the Caucasus, Anatolia, the Balkans, and the Carpathians) (*Sivec & Stark, 2002*), often erroneously identified as *P. marginata.* Furthermore, the taxonomic status for some species is ~~very~~ uncertain, such as *P. bipunctata* (*Sivec & Stark, 2002*) and specimens found within this study on Ruda Spring (marked as *Perla* sp., clade No. 29, Fig. 2), which morphologically differed from congeners but genetically represent one lineage and one BIN. *P. abdominalis* Burmeister, 1839 (reported under the name *P. burmeisteriana* Claassen, 1936 (*Kasymov 1972*)), in Croatia was recorded for the first time in 1908 and a few larvae were found at the northern foot of the Papuk Mountain (*Popijač & Sivec, 2009a*) and in 2014 on the Drava River (*I Sivec, 2014, unpublished data*). *Perla illiesi* was found in several localities in Croatia (Slovenia, Istria Peninsula and in the Gorski kotar region). Within present study, this species was recorded at two localities in Lika and DNA barcoded for the first time (clade No. 26, Fig. 2), as well as *Perla carantana* (clade No. 24, Fig. 2), which was reported at several localities in Slovenia and Austria (*Sivec & Graf, 2002*). One ~~of the last~~ recent record~~s~~ is Kupa River (*D Hlebec & I Sivec, 2021, unpublished data*).

Genus *Besdolus*

Interspecific *p*-distances between newly obtained sequences of *B. imhoffi* and *B. illyricus* retrieved from GenBank (from 0.106 to 0.124), do not support the synonymy of *B. illyricus* and *B. imhoffi*, as stated before (*Fochetti et al., 2011)* (Data set 6, Table S5).

Extinction and conservation

High sensitivity of stoneflies to changes in abiotic factors may lead to the local or global extinction of taxa (*Fochetti & Tierno de Figueroa, 2006; Graf et al., 2018*). Extensive field research that began in Europe about twenty years ago, however, found the presence of taxa that were considered extinct, but with much ~~less abundancy~~ (*I Sivec, 2021, unpublished data*). The

local and regional extinction rate of stoneflies would be the highest across the Animal Kingdom, according to IUCN criteria (*Sánchez-Bayo & Wyckhuys, 2019*). In neighboring countries, such as Italy, several species can be considered as already extinct: *Brachyptera trifasciata* (Pictet 1832), *Isogenus nubecula* Newman 1833, *Taeniopteryx nebulosa* (Linneo 1758) and *Perla abdominalis* Burmeister 1839 (*Fochetti et al., 1998; Fochetti, 2020*), while even more species can be considered threatened with extinction. The situation is, as usual, also critical for endemic species (known only from their type locality or few populations) and all microendemic taxa (*Fochetti 2020*).

Despite the relatively large effort invested in researching the stonefly fauna in Croatia (*Popijač, 2008; Popijač & Sivec, 2009a, 2009b*) several species are preserved only in the museum collections: *Perla bipunctata, Perla grandis, Isoperla obscura* and *Isogenus nubecula* (*Popijač & Sivec, 2009a*). It is questionable whether the species have gone locally extinct, or their populations have decreased so much that it is difficult to record them. In future systematic research of stonefly fauna, these species and their distribution will be one of the priorities. But in spite of that, many species that have been declared extinct in other European countries have been recorded with the implementation of present study. *Brachyptera monilicornis*, emerging throughout January, has long been considered extinct because it does not occur in Central Europe (*Zwick, 1992)*. In the border rivers of Croatia and Slovenia (Kupa and Čabranka), as well as in the streams and rivers of Papuk Mountain (largest mountain and protected area in eastern Croatia), *B. monilicornis* is very common. Furthermore, the rare and endangered lowland species, *Taeniopteryx nebulosa* as well as *Rhabdiopteryx acuminata* have been recorded at several localities at the foothills of the Papuk Mountain. *Besdolus imhoffi* (Pictet, 1841) was re-discovered in Croatia in 2005 (Plitvice Lakes National Park) (*Kovács & Murányi, 2008*) after a one-hundred-year-old record (*Popijač & Sivec, 2009).* This finding was confirmed with molecular analysis within this study at Una and Cetina Rivers. Species of the genus, and thus the species *B. imhofii* have a relictual distribution (*Zwick & Weinzierl, 1995*) and are sensitive to environmental perturbations (*Fochetti et al., 2011*). *Marthamea vitripennis* Burmeister, 1839, a species ~~largely extinct in~~ most of Europe (*Zwick, 2004*) due to the destruction of river potamon, was found in the Rába River in Hungary (*Kovács & Ambrus, 2000*), but also in Croatia in 2011 (*Popijač & Sivec, 2011*), and during field research in this study in 2021, on the rapids of the Una River. Another rare plecopteran, found in the Rába River in Hungary (*Kovács & Ambrus, 2000*) is *Agnetina elegantula*, which was recorded during this study at the Papuk Mountain and DNA barcoded for the first time.

Conclusions

The current study generated a validated national reference DNA barcode library for stoneflies in Croatia, which can support the implementation of cost-efficient DNA-based identifications and assessments to ecological status. DNA barcoding proved to be an effective tool for the identification and delimitation of some closely related species. Furthermore, this study provides several findings of ~~taxa that were considered extinct,~~ as well as the first molecular characterization of species with restricted distributions. For some genera (e.g., *Isoperla, Taeniopteryx* and *Perla*) and some collected individuals, a larger, integrative revisionary examination based on more comprehensive geographic sampling with all known variants, and

application of a multi-gene approach, especially on type specimens, is necessary for resolving taxonomic relationships. Highlighting the localities and areas with emphasized biodiversity, based on morphological variability and genetic diversity, will create preconditions for further protection of stoneflies species and their habitats.

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Figure 1

Box plot of uncorrected pairwise genetic distances (*p*-distances) (A) and results of "Barcode Gap Analysis" (B-D).

(A) Sorted by distance category: intraspecific (specimens that belong to the same species), congeneric (specimens belonging to different species, but to the same genus) and comfamiliar (specimens that belong to the same family). Boxes indicate interquartile range (IQR: between upper [Q3] and lower [Q1] quartile). Black bars designate medians, whiskers indicate values within 1.5 × IQR beneath Q1 or 1.5 × above Q3. Circles depict outliers (above or below 1.5 × IQR). (B) The barcode gap for 74 species of Croatian stoneflies shown by plotting maximum intraspecific distance against interspecific (nearest-neighbour) distance. Dots above the diagonal indicate species with a barcode gap. (C) Scatterplot plots the maximum intraspecific distances against the minimum interspecific distances. (D) Scatterplot plots the number of individuals in each species against their maximum intraspecific distances.

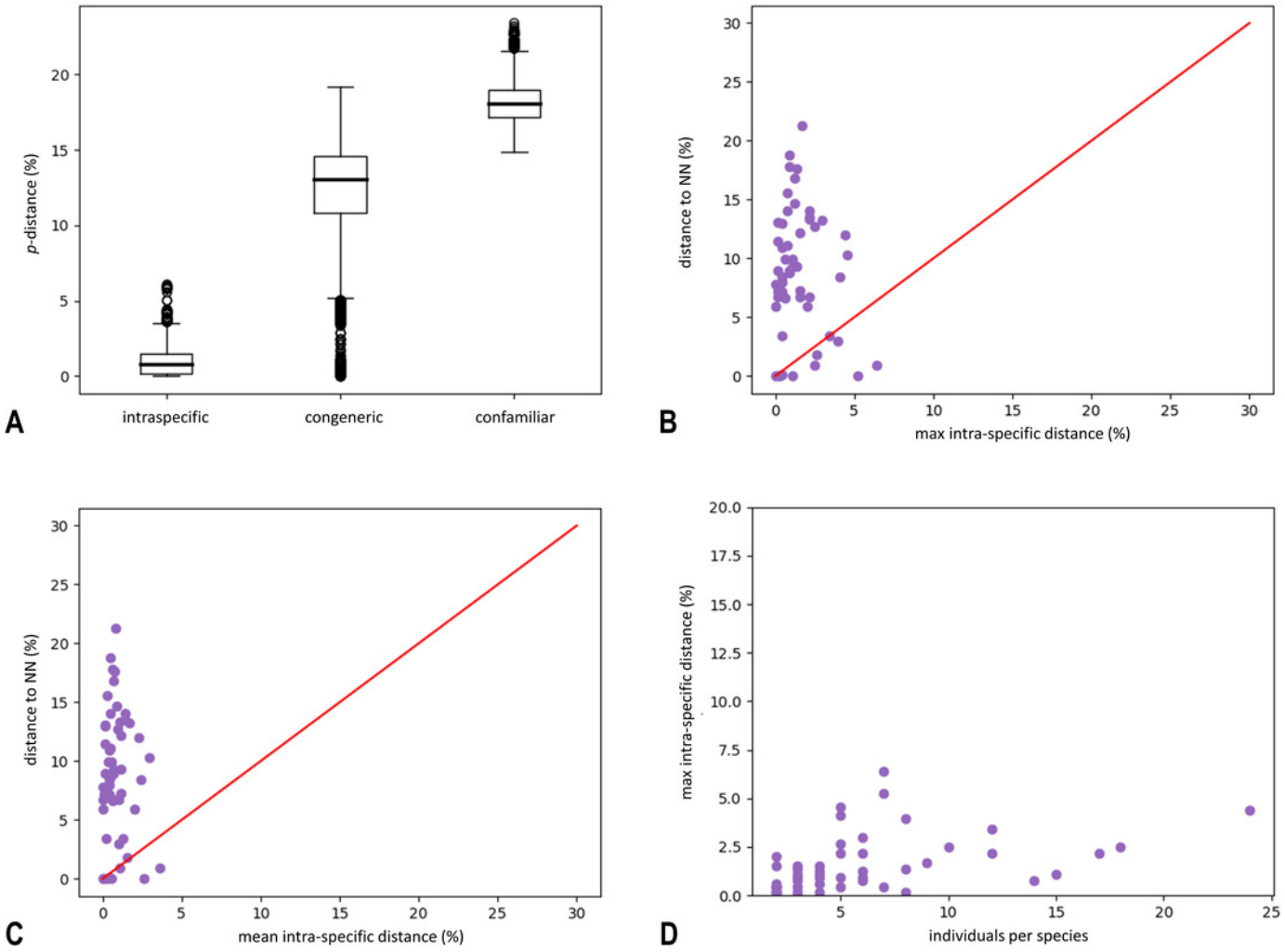
p-distance (%)
intraspecific
congeneric
confamiliar
A
distance to NN (%)
max intra-specific distance (%)
B
distance to NN (%)
mean intra-specific distance (%)
C
max intra-specific distance (%)
individuals per species
D

Figure 2

Circular maximum-likelihood (ML) phylogram from analysis of the released data set and species delineation.

Maximum likelihood phylogeny based on the DNA barcoding region (5’ fragment of the mitochondrial *COI* gene). Species are color-coded by family. Dots on nodes represent ultrafast bootstrap support values (BS) categories. The results of species delineations are represented with the bars in different colors and indicate the OTUs inferred by ABGD, ASAP and mPTP methods. Terminal codes present BOLD IDs, as in Supplemental Table S1 . An asterisk indicates two tentative species within *Isoperla inermis* specimens inferred by ASAP method. The tree was annotated in FigTree v.1.4.3. (Rambaut 2009) and iTOL v.5 (Letunic and Bork, 2021) and finished in Adobe Illustrator.

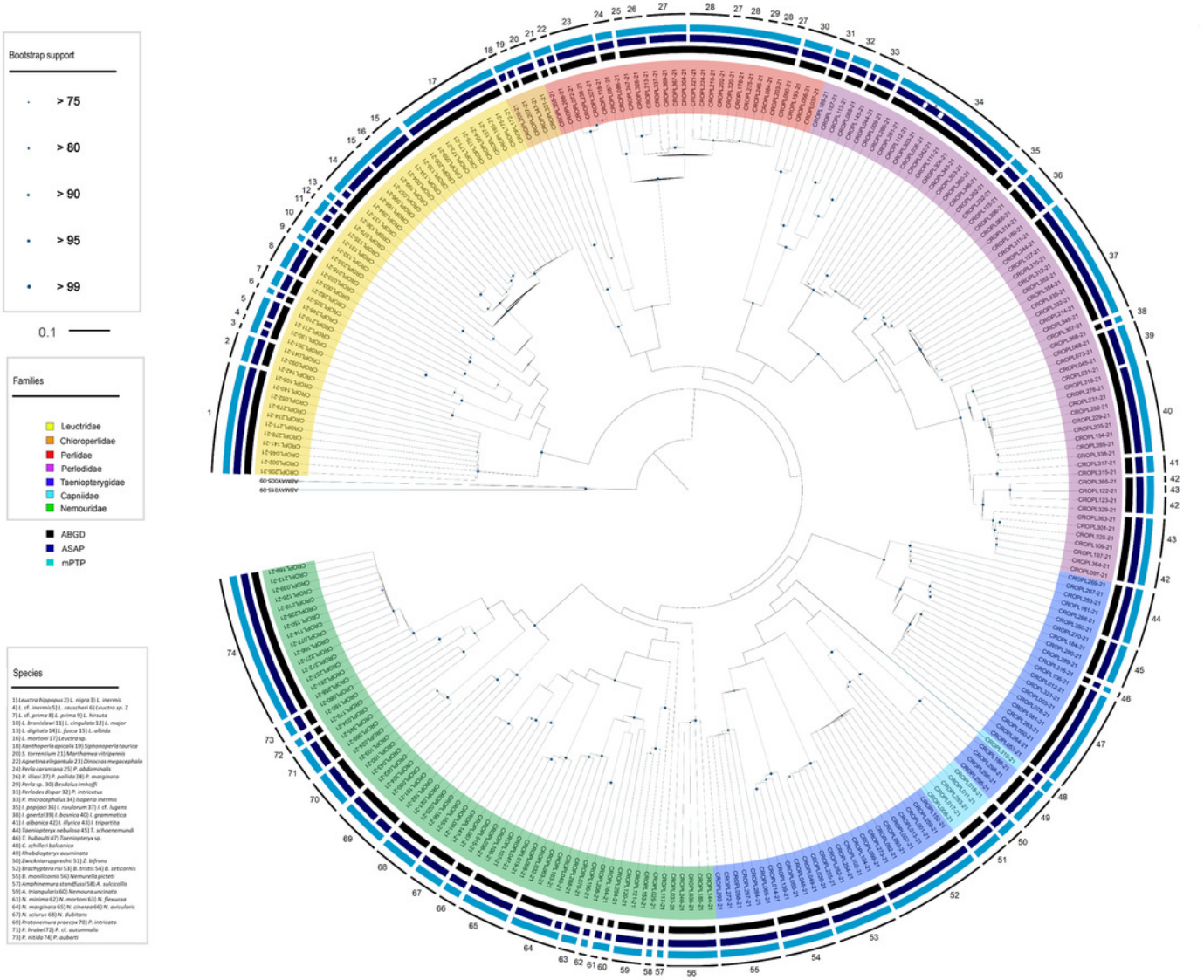
Bootstrap support
> 75
> 80
> 90
> 95
> 99
0.1
Families
Leuctridae
Chloroperlidae
Perlidae
Perlodidae
Taeniopterygidae
Capniidae
Nemouridae
ABGD
ASAP
mPTP
Species

Figure 3

Sampling map and median-joining network of *COI* sequences (658 bp long) obtained from *Isoperla* cf. *lugens*.

(A) Map of Croatia and neighboring countries with sampling localities (color coding matches insert in 3B). (B) MJ network of *COI* sequences. Colors indicate different sampling localities. Numbers of mutational steps are given as hatch marks. The black dots indicate the extinct ancestral or unsampled haplotypes. Frequencies of the haplotypes are proportional to the size of the circles. Haplotypes are labeled with BOLD IDs, as in Supplemental Table S1. Map is produced with Cartopy package 0.19 in Python.

Figure 4

Sampling sites within this study.

Colors of dots represent the number of species in each locality. Main map (B) is an enlarged framed area in the bottom left corner (A).