DNA barcoding for biodiversity assessment: Croatian stoneflies (Insecta, Plecoptera)

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Abstract

Background. The hemi-metabolous aquatic order Plecoptera (stoneflies) constitutes an indispensable part of terrestrial and aquatic food webs due to their specific life cycle and habitat requirements. Stoneflies are considered one of the most sensitive groups to environmental changes in freshwater ecosystems and anthropogenic changes have caused range contraction of many species. Given the critical threat to stoneflies, the study of their distribution, morphological

45 variability and genetic diversity should be one of the priorities in conservation biology.
46 However, some aspects about stoneflies, especially a fully resolved phylogeny and their patterns
47 of distribution are not well known. A study that includes comprehensive field research and
48 combines morphological and molecular identification of stoneflies has not been conducted in
49 Croatia so far. Thus, the major aim of this study was to regenerate a comprehensive and
50 taxonomically well-curated DNA barcode database for Croatian stoneflies, to highlight the
51 morphological variability obtained for several species and to elucidate results in light of recent
52 taxonomy.
53 **Methods.** Morphological examination of adult specimens was made using basic characteristics
54 for distinguishing species: terminalia in males and females, head and pronotum patterns, penial
55 morphology, and egg structures. DNA barcoding was applied to many specimens to help
56 circumscribe known species, identify cryptic or yet undescribed species, and to construct a
57 preliminary phylogeny for Croatian stoneflies.
58 **Results.** Sequences (658 bp in length) of 74 morphospecies from all families in Croatia were
59 recovered from 87% of the analysed specimens (355 of 410), with one partial sequence of 605 bp
60 in length for *Capnopsis schilleri balcanica* Zwick, 1984. 84% morphological species could be
61 unambiguously identified using *COI* sequences. Species delineation methods confirmed the
62 existence of five deeply divergent genetic lineages, with monophyletic origin, which also differ
63 morphologically from their congeners and represent distinct entities. BIN (Barcode Index
64 Number) assignment and species delineation methods clustered *COI* sequences into different
65 numbers of operational taxonomic units (OTUs). ASAP delimited 76 putative species and
66 achieved a maximum match score with morphology (97%). ABGD resulted in 62 and mPTP in
67 61 OTUs, indicating a more conservative approach. Most BINs were congruent with traditionally
68 recognized species. Deep intraspecific genetic divergences in some clades highlighted the need
69 for taxonomic revision in several species-complexes and species-groups. Research has yielded
70 the first molecular characterization of 9 species, with most having restricted distributions and
71 confirmed the existence of several species which had been declared extinct regionally.

Introduction

73 With 3800 described species across 17 families (*Fochetti & Tierno de Figueroa, 2008*; *DeWalt
74 et al., 2021; South et al., 2021*), the ancient (*Béthoux et al., 2011*) hemi-metabolous insect order
75 Plecoptera, commonly known as stoneflies, represents an important component of freshwater
76 ecological systems and terrestrial and aquatic food webs (*Fochetti & Tierno de Figueroa, 2008;
77 South et al., 2020*). Stoneflies are widely distributed on all continents except Antarctica (*Zwick,
78 2000*) and their range and abundance have declined rapidly in the last 30 years in Central Europe
79 (*Fochetti & Tierno de Figueroa, 2006; Bojková et al., 2012*), mainly due to anthropogenic
80 influences (i.e., habitat destruction and pollution) (*Fochetti & Tierno de Figueroa, 2008; Bálint
81 et al., 2011*). Typical stonefly habitat is a lotic system characterized by cold, fast flowing and
82 well-oxygenated water (*Sivec & Yule, 2004*) in the mountains of temperate regions. Typically,
83 stoneflies are macropterous or brachypterous, but several species, especially males at higher
84 elevations are apterous or micropterous (*Illies, 1966*). Due to their poor dispersal capacity (*Lee et
85 al., 2022*), stoneflies are ideal organisms for biogeographical studies (*Murányi, 2011; Graf et al.,
86 2012; Pessino et al., 2014; Stevens, Bishop & Picker, 2018; Gamboa et al., 2019*).

87   Since Plecoptera taxa exhibit high degrees of endemism (*Fochetti & Tierno de Figueroa, 2006;*
88   *Murányi, 2011; Murányi, Kovács & Orci, 2016*) and morphological diversity, regional field
89   research has both local and global value. Prior to 2009 when concerted efforts in field research
90   began, only 28 species of stoneflies were recorded in Croatia (*Sivec, 1980; Sivec, 1985*), a
91   surprisingly low number considering the many suitable habitats (*Illies, 1978*) and compared to
92   checklists for neighbouring countries: Slovenia with more than 100 species (*Sivec, 2001*), Bosnia
93   and Herzegovina with 73 species and subspecies (*Kaćanski, 1976*), Montenegro with 57 species
94   (*Murányi*, 2008), Hungary with 61 species (*Andrikovics & Murányi, 2001*) and Serbia with 90
95   documented species (*Petrović et al., 2014*). Greater efforts in Croatia resulted in 50 species
96   (*Popijač, 2008; Popijač & Sivec, 2009a*), but still studies were limited to narrow areas such as
97   Plitvice Lakes National Park (*Popijač & Sivec, 2009b; Ridl et al., 2018*), Cetina River (*Popijač*
98   *& Sivec, 2009b*), Čabranka and Gerovčica Rivers (*Popijač & Sivec, 2009c*) and lower reaches of
99   the Una River and its tributaries (*Popijač & Sivec, 2011*). In Plitvice Lakes National Park during
100 the above-mentioned studies, some specimens were recorded that could not be assigned with
101 certainty to known species: *Perlodes* cf. *intricatus, Isoperla* cf. *lugens*, *Leuctra* cf. *pusilla*,
102 *Leuctra* sp., *Nemoura* sp., *Protonemura* sp., *Isoperla* sp. and *Perlodes* sp. Also, during these
103 studies, several remarkable species were documented: *Marthamea vitripennis* Burmeister, 1839,
104 which was re-discovered again after one century (*Sivec, 1985; Popijač & Sivec, 2011*), *Perla*
105 *burmeisteriana* Claassen, 1936 (*Popijač & Sivec, 2009a*), *Besdolus imhoffi* Pictet, 1841 (*Popijač*
106 *& Sivec, 2010*) and *Protonemura julia* Nicolai, 1983 (*Popijač & Sivec, 2009c*).
107 Currently, the most widely accepted system of stonefly classification is by Zwick (2000), with
108 two recognized suborders: Arctoperlaria and Antarctoperlaria. To resolve deeper phylogenetic
109 relationships, research has highlighted the need for molecular data, which in the last years, at
110 least in part, has helped overcome morphology-based identification limitations. South et al.
111 (2020) produced the most complete molecular phylogenetic study of stoneflies based on the
112 North American fauna, following several studies with limited taxon sampling (*Thomas et al.,*
113 *2000; Chen et al., 2018; Wang et al., 2018; Ding et al., 2019*). Allopatric diversification, driven
114 mostly by glaciation and orogenesis, has been the main contributor to current diversity patterns
115 in Plecoptera (*Zwick, 2000; Weiss, Stradner & Graf, 2011; Theissinger et al., 2013*).

116 DNA barcoding, which uses sequence diversity in the standardized 658-bp region of the
117 mitochondrial cytochrome c oxidase subunit I (*COI*) gene to aid in species identification and
118 circumscription, is a useful tool for associating immature life stages and to identify cryptic
119 species. Such an approach is extremely important for biodiversity assessment (*Hebert et al.,*
120 *2003a*; *Hebert, Ratnasingham & deWaard, 2003b*; *Hebert et al., 2004; Valentini, Pompanon &*
121 *Taberlet, 2009; Morinière et al., 2017*). The application of DNA barcoding has aided species
122 delimitation in various groups of organisms and has pointed to divergent haplotypes and
123 hybridization (*Van Velzen et al., 2012; Szivák et al., 2017; Zangl et al., 2019; Zangl et al., 2021*).
124 Once a set of barcodes for a group of organisms is established, examination of previously
125 unidentified specimens is greatly facilitated (*DeSalle, 2006; Ratnasingham & Hebert, 2007*).
126 However, it has been observed that the results of DNA barcoding could be confounded
127 (*Havemann et al., 2018*) due to *Wolbachia* infections (*Werren, Zhang & Guo, 1995*), incomplete
128 lineage sorting (*Petit & Excoffier, 2009*), pseudogenes (*Ribeiro Leite, 2012*), introgressive
129 hybridization and recent speciation (*Raupach et al., 2014*). DNA barcoding has proved to be a

130 great tool for identification of species from the EPT (Ephemeroptera, Plecoptera and
131 Trichoptera) group (*Gill et al., 2014; Ball et al., 2005; Webb et al., 2012; Morinière et al., 2017;*
132 *Kučinić et al., 2020*). Furthermore, the analysis of DNA barcoding results is often difficult due to
133 deposited barcode sequences without scientific species names, known as "dark taxa", which
134 represent groups of organisms characterized with a lack of taxonomic expertise or undescribed
135 species (*Page, 2016; Ryberg & Nilsson, 2018*). Additionally, results from DNA barcoding and
136 the traditional taxonomic approach can be incongruent and this is not surprising given the
137 process of evolution itself (*Hendrich et al., 2010; Hendrich et al., 2014*). Therefore, for efficient
138 species delimitation, it is necessary to analyze multiple character systems and use integrative
139 taxonomy (*Vitecek et al., 2017; Zhang et al., 2013*).
140 Conducted in the framework of the project *DNA barcoding of Croatian faunal biodiversity*, the
141 present study aims at: (i) developing a DNA reference barcode library for the Croatian stonefly
142 fauna with macrophotographs of 26 species, (ii) getting first insight into inter- and intraspecific
143 genetic diversity, (iii) assessing morphological variability of stoneflies in Croatia, (iv)
144 highlighting localities with high biodiversity, especially in isolated habitats of the Dinaric Karst
145 area to assist conservation planning and strategies for protecting the genetic diversity of
146 stoneflies, and (v) filling the gaps in the Barcode of Life Data System database (BOLD).
147 Furthermore, the study will contribute to knowledge about species distribution, species
148 complexes and systematic and phylogenetic relationships.

Materials & Methods

Taxon sampling
151 Specimen collection was conducted with approval from the Ministry of Economy and
152 Sustainable Development of the Republic of Croatia (UP/I-612-07/21-48/73). A total of 337
153 stonefly specimens (Data S1, Table S1) from 95 different localities in Croatia and eighteen
154 specimens from seventeen localities in Slovenia (Fig. S1) were used in phylogenetic analysis.
155 Information regarding species determinations and details about sampling sites can be found in
156 Table S1. Adult specimens were collected using sweep nets and beating sheets, while larval
157 specimens were collected by handpicking. The aedeagus of males was everted in the field and
158 specimens were fixed and stored in 96% ethanol for morphological and molecular analysis.
159 Morphological characteristics of male terminalia were examined after KOH treatment (*Stark &*
160 *Gaufin, 1978*).
161 Comparative study on morphology was made using specimens kept in the Collection of
162 stoneflies in the Slovenian Museum of Natural History, Ljubljana, Slovenia (PMSL). Newly
163 collected specimens are deposited in the Croatian Natural History Museum, Zagreb, Croatia
164 (CNHM), under the Collection of Plecoptera Sivec & Hlebec (CPSH). Voucher information for
165 individuals used in phylogenetic analysis are publicly accessible in BOLD
166 (dx.doi.org/10.5883/DS-CROPL) and GenBank under the accession numbers as listed in Table
167 S1.

168 Individuals were sorted and identified using a Leica Wild M3Z stereomicroscope.
169 Macrophotographs were taken using a Canon EOS 5D Mark II. Morphological examination was
170 made using descriptions and identification keys: *Illies, 1955*; *Kaćanski & Zwick, 1970*; *Raušer,*

171 *1980*; *Ravizza, 2002*; *Sivec & Stark, 2002*, *Graf & Schmidt-Kloiber, 2003*; *Zwick, 2004* and
172 *Murányi, 2011*. The most reliable diagnostic characters for species determination were terminalia
173 in males and females, head and pronotum patterns, penial armatures and egg structures for
174 species within the genus *Perla*.

175 **DNA extraction, gene amplification and sequencing**
176 The number of specimens per species for *COI* marker amplification ranged from one to 24
177 (*Isoperla inermis* Kaćanski & Zwick, 1970*; see Table S1). Tissue was dissected from a single
178 leg for each specimen and genomic DNA was extracted from recently collected samples using
179 the Sigma GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, US) and from
180 specimens older than 10 years using the QIAamp DNA Micro Kit (Qiagen, Germany) and
181 eluting in 50 µl of elution buffer to increase DNA yield. A partial region of the mitochondrial
182 cytochrome c oxidase subunit I gene (*COI,* the DNA barcode region, *Hebert et al., 2003a*) was
183 amplified using two sets of primers: (a) LCO-1490/HCO-2198 (*Folmer et al., 1994*) or (b)
184 C_LepFolF/C_LepFolR (*Folmer et al., 1994; Hebert, Ratnasingham & deWaard, 2003b*). For
185 samples older than 10 years, the DNA barcode region was amplified as shorter overlapping
186 fragments with primer set: (c) MLepF1/LepR1 and MLepR1/LepF1 (*Hajibabaei et al., 2006*).
187 All polymerase chain reactions (PCRs) were carried out in a total volume of 20 µl with the PCR
188 mixture prepared following Hlebec et al. (2021). Thermocycling conditions are given in Table
189 S2. PCR products were purified using Exonuclease I (0.05 U/µL) and FastAP (0.025 U/µL)
190 enzymatic system (Thermo Fisher Scientific, Inc., US). The reaction was carried out using the
191 following conditions: 37 °C for 1 hr followed by 80 °C for 20 min. Bi-directional sequencing
192 was done by Macrogen Inc. (Amsterdam, The Netherlands), using amplification primers.

193 **Sequence editing and phylogenetic analyses**
194 In total, 355 obtained sequences were checked and inspected manually for base pair ambiguities,
195 indels and stop codons in Geneious Prime 2021.2 (Biomatters, Auckland, New Zealand) to
196 confirm overall sequences quality. Sequences were aligned using MAFFT ver. 7 (*Katoh &*
197 *Standley, 2013*). The final alignment for the *COI* gene fragment was 658 bp in length (Data S2).
198 Sequences were collapsed into 268 *COI* haplotypes using the online tool FaBox ver. 1.5
199 (*Villesen, 2007*). Evolutionary divergence was estimated using uncorrected pairwise genetic
200 distances (*p*-distances) in MEGA-X ver. 10.2.6 (*Kumar et al., 2018*) (Fig. 1A–D, mean values
201 are shown in Table S3). Phylogenetic analyses were performed using Maximum likelihood (ML)
202 optimality criteria in IQ-TREE2 (*Minh et al., 2020*) under a GTR+I+G optimal model of
203 nucleotide evolution (as determined by jModelTest ver. 2.1.10 (*Darriba et al., 2012*) under the
204 Bayesian information criterion (BIC)) and bootstrapping with 2000 ultrafast bootstrap replicates
205 (*Hoang et al., 2018*) (Fig. 2). ABMAY005-09 (Heptageniidae) and ABMAY015-09 (*Stenacron*
206 *interpunctatum* Say, 1839) were selected as outgroups. Additionally, phylogenetic relationships
207 between haplotypes were inferred using the Neighbour joining (NJ) method in MEGA-X ver.
208 10.2.6 (*Kumar et al., 2018*) (Fig. S2) and Bayesian inference (BI) in MrBayes ver. 3.2.7
209 (*Ronquist et al., 2012*) via the online CIPRES Science Gateway ver. 3.3 (*Miller, Pfeiffer &*
210 *Schwartz, 2010*) (Fig. S3). The NJ analysis was performed using the Kimura-2-parameter (K2P)
211 model with the pairwise deletion option. Bootstrap support was inferred using the fast bootstrap
212 algorithm, based on 5000 replicates. For the BI, two separate runs with four Markov chain Monte

213 Carlo (MCMC) simulations were performed for 10 million generations, sampling every 1000
214 generations, using ABMAY005-09 (Heptageniidae) as the root and discarding the initial 25% of
215 the trees as burn-in. Remaining trees were used to create a 50% majority rule consensus tree,
216 with nodal values representing the posterior probabilities. TRACER ver. 1.7.1 (*Rambaut et al.,*
217 *2018*) was used to check the convergence between the two runs. Phylogenetic trees were
218 visualized using FigTree ver. 1.4.3 (*Rambaut, 2009*) and iTOL ver. 5 (*Letunic & Bork, 2021*).
219 Existence of a barcoding gap (distance between the mean intraspecific sequence variability and
220 interspecific variability for congeneric *COI* sequences) was ascertained using the "Barcode Gap
221 Analysis" tool, provided in BOLD, using the Kimura-2-Parameter (K2P) distance metric
222 (*Puillandre et al., 2012*) (Fig. 1A–D, Table S4). To visualize phylogeographic relationships
223 among specimens from the largest observed lineage, a median joining (MJ) network among 12
224 haplotypes (*Bandelt, Forster & Röhl, 1999*) (Fig. 3, Data S3) was generated using the program
225 PopART ver. 1.7 (*Leigh & Bryant, 2015*) with default settings. Six different alignments were
226 created for additional phylogenetic (ML in IQ-TREE2 with settings as above, under the optimal
227 model of nucleotide evolution as is listed in Table S5) and species delineation analysis in order
228 to interpret the results and to check the plausibility. We applied these analyses to: (a) species
229 with high levels of intraspecific morphological variability (*Perlodes intricatus* (Pictet, 1841) and
230 *Isoperla inermis*), (b) specimens that differed from the described morphospecies (*Isoperla* cf.
231 *lugens*, *Protonemura hrabei* (Raušer, 1956) and *Taeniopteryx* n.sp. CRO-1) and (c) species,
232 which were taxonomically interesting (*Besdolus imhofii* and *B. illyricus* Kovács & Zwick, 2008).
233 Analyses were performed with sequences of these species and their closely related congeners
234 from BOLD (*Ratnasingham & Hebert, 2007,* http://www.boldsystems.org) and GenBank
235 (https://www.ncbi.nlm.nih.gov/) databases (accessed 20 August 2021). Accession numbers of all
236 sequences used in additional analysis are listed in Datasets 1–6, Table S5. Molecular species
237 delineation was achieved through four different methods: the BIN (Barcode Index Number)
238 assignment tool on the BOLD server using the refined single linkage (RESL) algorithm
239 (*Ratnasingham & Hebert, 2013*), ABGD (Automatic Barcode Gap Discovery) (*Puillandre et al.,*
240 *2012*), ASAP (Assemble Species by Automatic Partitioning) (*Puillandre, Brouillet & Achaz,*
241 *2021*) and mPTP (Multi-rate Poisson Tree Processes) (*Kapli et al., 2017*). All methods clustered
242 *COI* sequences into Operational Taxonomic Units (OTUs) based on sequence similarity.

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

251 Automatic Barcode Gap Discovery (ABGD) was carried out on the web server
252 (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html), applying the K2P model and using
253 default parameters, except for the relative gap width, which was set to X = 1.0. ASAP was also
254 carried out on the web server (https://bioinfo.mnhn.fr/abi/publi c/asap/asapw eb.html) using *p-*

255   distances with default settings. The mPTP method, run on the web server (http://mptp.h-its.org/),
256   was implemented using default settings and inputting the ML tree from IQ-TREE2 (*Minh et al.,*
257   *2020*), as the starting tree.
258   Validity and reliability of the generated DNA barcode library was evaluated by comparing
259   classical taxonomy with the counts of OTUs from the various species delineation methods.

Results

261   Field sampling and morphological identification produced 74 species of stoneflies in seven
262   families and nineteen genera. Four species (*Capnopsis schilleri balcanica, Zwicknia rupprechti*
263   Murányi, Orci & Gamboa, 2014, *Zwicknia bifrons* (Newman, 1838) and *Nemoura sciurus*
264   Aubert, 1949) were identified by comparing with available sequences in the BOLD and
265   GenBank databases.

266   Barcode sequencing was successful for 355 of 410 (87%) individuals. All *COI* sequences were
267   658 bp in length, except for one sequence of *Capnopsis schilleri balcanica*, which was 605 bp in
268   length.

269   Molecular results were largely congruent with morphological assessment such that 62 of the 74
270   morphological species (84%) could be unambiguously identified using *COI* sequences alone.
271   The median number of barcodes per species was four, and seventeen predominantly rare species
272   were known only from single specimens.
273   The average confamilial *p*-distance was 20.8% (ranging from 18.2–23.4%) while the average
274   congeneric distance was 15.6% (ranging from 12.1–19.2%). The mean intraspecific p-distance
275   was 1.3% (ranging from 0–6.8%) (Fig. 1A). The maximum intraspecific distance of 6.8% was
276   obtained for *Isoperla illyrica* Tabacaru, 1971. Most individuals were above the barcoding gap,
277   meaning that for each individual the difference between the distance to the NN (Nearest
278   Neighbour) and the distance to the furthest conspecific is above zero (Figs. 1B–C).

279   **Figure 1**
280
281   The mean intraspecific *p*-distance distribution is partially overlapping with distance to the
282   nearest neighbour distribution (Fig. 1C), but for most species, nearest-neighbour distances were
283   on average several times higher than maximum intraspecific distances (Fig. 1B). Maximum
284   intraspecific *p*-distance was positively correlated with the number of individuals per species (Fig.
285   1D) (the correlation coefficient (ρ) = 0.447). For several neighbour species pairs, maximum
286   intraspecific *p*-distance values were higher than their nearest-neighbour distance: *Leuctra albida*
287   Kempny, 1899/*Leuctra mortoni* Kempny, 1899; *Leuctra fusca* (Linnaeus, 1758)/*Leuctra albida*;
288   *Perla pallida* Guérin-Méneville, 1838/*Perla marginata* (Panzer, 1799) and *Isoperla illyrica*
289   /*Isoperla tripartita* Illies, 1954. For the following neighbour species pairs, *p*-distance to the
290   nearest neighbour was below 2%: *Leuctra albida*/*Leuctra mortoni, Nemoura* cf.
291   *rivorum*/*Nemoura flexuosa* Aubert, 1949, *Perla illiesi* Braasch & Joost, 1973/*Perla*
292   *burmeisteriana, Perla* sp./*Perla marginata, Perla pallida*/*Perla marginata* and *Isoperla*
293   *illyrica*/*Isoperla tripartita* (Illies, 1954).
294   This study resulted in five entities, which morphologically differ from their congeners and

295 genetically appeared as separate lineages. These species therefore represent candidates for new,
296 previously undescribed species: *Leuctra* cf. *prima* (clade No. 7 in Fig. 2, distance to NN =
297 9.3%), *Leuctra* cf. *inermis* (clade No. 4 in Fig. 2, distance to NN = 3.6%), *Protonemura* cf.
298 *autumnalis* (clade No. 72 in Fig. 2, distance to NN = 8.7%), *Isoperla* cf. *lugens* (clade No. 37 in
299 Fig. 2, distance to NN = 6.7%) and *Taeniopteryx* n.sp. CRO-1 (clade No. 47 in Fig. 2, distance to
300 NN = 7.1%) (Fig. 2, Table S4). Another separate lineage was recently described *Isoperla*
301 *popijaci* Hlebec & Sivec, 2021 (clade No. 35 in Fig. 2, distance to NN = 6.7%) (*Hlebec et al.,*
302 *2021*).
303 All methods used for phylogenetic reconstruction (ML (Fig. 2), NJ (Fig. S2) and BA (Fig. S3))
304 recovered the same, well-supported topology. All methods grouped phenotypically defined
305 species in distinct, highly supported monophyletic species clusters (ultrafast bootstrap support >
306 99). Phylogenetic relationships above the species level are in concordance with morphology-
307 based hypotheses. All genera were monophyletic, while analyses resulted in unresolved
308 phylogenetic relationships between families Capniidae and Taeniopterygidae.

309 **Figure 2**

310
311 Obtained sequences were allocated to 85 BINs (of which 29 were unique to BOLD), and
312 delimited OTUs were mostly consistent with the clustering pattern observed in the ML tree,
313 which was also concordant with morphological identification. Twenty-six BINs were represented
314 by a single individual (singletons).
315 One BIN often belongs to a single species delineated by traditional taxonomy (*Hausmann et al.,*
316 *2013*), and every case can be an incentive for re-evaluation of morphological and molecular data
317 (*Hendrich et al., 2014*). Specimens of several species showed deep *COI* divergence resulting in
318 multiple BINs within a species: *Protonemura praecox* (Morton, 1894) (BOLD:AEH4111 and
319 BOLD:AEH7722), *Perlodes microcephalus* (Pictet, 1833) (BOLD:AAL2343 and
320 BOLD:AEH5507), *Nemoura marginata* (BOLD:AAN1631, BOLD:AEH3564 and
321 BOLD:AEK9273), *Isoperla illyrica* (BOLD:AEH3875 and BOLD:AEH7030), *Leuctra fusca*
322 (BOLD:AAE6442 and BOLD:ACY3863), *Leuctra albida* (BOLD:AAM4011 and
323 BOLD:AEH5504), *Leuctra hippopus* Kempny, 1899 (BOLD:ACL7184 and BOLD:AEH4770),
324 *Isoperla tripartita* (BOLD:AEH3875, BOLD:AEH3876, BOLD:AEG6510 and
325 BOLD:AEH7030), *Isoperla grammatica* (Poda, 1761) (BOLD:AEH6396 and BOLD: AEG4373)
326 and *Isoperla inermis* (BOLD:ACS6073, BOLD:AAZ7905 and BOLD:AEH8653). Intraspecific
327 *p*-distances were as follows for the following species: *P. praecox* (0–3.2%), *P. microcephalus*
328 (0–4.2%), *N. marginata* (0–4.8%), *Isoperla illyrica* (0.002–6.8%), *Leuctra fusca* (0–5.8%),
329 *Isoperla tripartita* (0–6.6%), *Isoperla grammatica* (0–5.0%) and *Isoperla inermis* (0–4%).
330 A shared BIN assignment was obtained within three genera: *Isoperla, Perla* and *Nemoura,* and
331 species *Isoperla illyrica/Isoperla tripartita, Nemoura flexuosa/Nemoura* cf. *rivorum* and *Perla*
332 *pallida/Perla* sp./*Perla marginata.*
333 Overall, use of different species delineation algorithms resulted in a different number of putative
334 species. ASAP (with the best ASAP-score (6.00) which was achieved at a distance threshold of
335 2.5% (*p*-distance) delimited 76 putative species and achieved a maximum match score with
336 morphology (97%). The partition with the second-best ASAP-score (8.50, distance threshold

337 3.6%) delimits 70 species and the third partition (9.00, distance threshold 5.0%) delimits 64
338 species. The ABGD method delineated 62 putative species, while mPTP delineated 61 putative
339 species. ABGD and mPTP represent conservative estimates of DNA species. Results of all
340 species delineation methods are shown in Fig. 2.
341 Median joining (MJ) network depicted relatedness and distribution within the newly obtained
342 divergent lineage (also unique to BOLD) named as *Isoperla* cf. *lugens*. The MJ network among
343 12 unique haplotypes which were separated by a different number of mutational steps is shown
344 in Fig. 3A–B. The MJ network also revealed low haplotype sharing among sampling sites.
345 Haplotypes CROPL311-21, CROPL344-21, CROPL127-21, CROPL310-21, CROPL312-21 and
346 CROPL352-21 grouped together in a well-supported subclade (Fig. 2, Fig. 3B). Haplotype
347 CROPL352-21 (discovered in Vitunjčica River) was separated by five mutational steps from the
348 closest haplotype that was recorded in Drakulić rijeka, Crna rijeka i Čabranka River. The
349 remaining 7 haplotypes also grouped together in a well-supported subclade (Fig. 2, Fig. 3B).

350 **Figure 3**

351 # Discussion
352 The present study represents the first comprehensive research combining morphological and
353 molecular identification of stoneflies species in Croatia and establishes DNA barcoding as an
354 effective tool for reliable species identification. Such an approach enhances taxonomic resolution
355 and assists in the quality of faunal research and can be used in discovering cryptic diversity and
356 species complexes (*Previšić et al., 2014a; Zangl et al., 2021*). A taxonomically, well-curated,
357 DNA barcode library can democratize species level work for non-experts with application in
358 ecological research and in conservation biology across taxa, communities, and ecosystems. Other
359 studies with specimens from Croatia that focus on mollusks (*Buršić et al., 2021*), mosquitos
360 (*Bušić et al., 2021*) and Trichoptera (*Kučinić et al., 2013; Valladolid et al., 2020*) have obtained
361 similar results, which support the efficacy of DNA barcoding for species determination.
362 So far, sequencing of the *COI* gene fragment has been used to elucidate the systematics and
363 phylogeography of Plecoptera (*Fochetti et al., 2009; Fochetti et al., 2011; Weiss et al., 2011*),
364 and identify new species (*Boumans & Murányi, 2014; Graf, Pauls & Vitecek, 2018; Pelingen &*
365 *Freitag, 2020; South et al., 2019*) as part of DNA barcoding initiatives (*Morinière et al., 2017;*
366 *Gattolliat et al., 2016; Ferreira et al., 2020*) and revisionary systematics (*Fochetti et al., 2011*).
367 Distinct DNA lineages obtained within morphospecies indicate the need for re-examination of
368 morphological characters (*Muster & Michalik, 2020; Wachter et al., 2015*).
369 Within this study, all methods for phylogenetic reconstructions show that most species can be
370 distinguished using *COI* barcodes. Exceptions are the following pairs: *Isoperla illyrica*/*Isoperla*
371 *tripartita, Perla pallida*/*Perla sp.*, *Perla pallida*/*Perla marginata, Perla burmeisteriana*/*Perla*
372 *illiesi, Leuctra albida*/*Leuctra mortoni, Leuctra albida*/*Leuctra fusca, Nemoura* cf. *rivorum*/*N.*
373 *flexuosa*, which possessed identical or overlapping *COI* sequences. For the above-mentioned
374 species, identification is sometimes difficult due to high levels of intraspecific morphological
375 variability between closely related species (*Ravizza & Ravizza Dematteis, 1995; Sivec & Stark,*
376 *2002; Murányi, 2011*) and often both sexes and eggs are necessary for identification. BIN
377 sharing reported within the species of the four genera: *Isoperla, Perla, Leuctra* and *Nemoura*
378 potentially indicates introgression or hybridization.

379 High numbers of intraspecific BINs (obtained for ten species) can indicate discrete geographical
380 populations of a species or overlooked cryptic species (*Hawlitschek et al., 2017; Morinière et al.,*
381 *2017*), e.g., *Dinocras cephalotes* (Curtis, 1827) (*Elbrecht et al., 2014*), so the number of 85 BINs
382 cannot be a proxy for the total number of stonefly species in Croatia. Nevertheless, the
383 underlying RESL algorithm is based on a distance threshold of 2.2%, so it is expected to have a
384 larger number of BINs in the dataset. Meier et al. (2021) made a claim that the cytochrome
385 oxidase I (*COI*) barcode region cannot be used as the only/main data source for describing or
386 delimitating species (*Sharkey et al., 2021*) and stressed the importance of additional species
387 delimitation methods as well as examination of morphological characters for justifying the
388 validity of a given species. *COI* barcode clusters ("BINs") as a basis for species descriptions,
389 given the assumption that a BIN equals a species, is often not consistent with the results of other
390 species delineation methods (*Meier et al., 2021*) due to theoretical and empirical reasons
391 (*Puillandre, Brouillet & Achaz, 2021; Zhang et al., 2013*).
392 Intra- and interspecific distances overlapped for some species (Fig. 1). Existence of a barcoding
393 gap allows the use of DNA barcoding to identify species (*Meyer & Paulay, 2005*). Overlapping
394 intra- and interspecific *p*-distances can be represented against a universal cut-off value (*Collins*
395 *& Cruickshank, 2013*). Also, they can be a consequence of inaccurate taxonomy, indicating
396 oversplit or cryptic species, especially for species within the genera *Isoperla* and *Perla* (Fig. 1).
397 In the present study, the distance to the NN is predominantly higher than the maximum
398 intraspecific distance, confirming the clear local barcoding gap, enabling successful use of DNA
399 barcoding for Croatian stonefly identification (Table S4). Furthermore, the congeneric average
400 distance among stoneflies species was five times higher than the average distance within species.
401 Nevertheless, the observed overlap between intra- and interspecific *p*-distances may be related to
402 the presence of cryptic species or species complexes, which would not be surprising given the
403 results of the morphological study.

404 **Geographic morphological variation**

405

406 Genus *Perlodes*
407 Previous research of the stonefly fauna in Croatia, based exclusively on morphological analysis,
408 have established the presence of specimens, which could not be identified with certainty to
409 known species (*Popijač & Sivec, 2009b*). *Perlodes intricatus* (clade No. 32, Fig. 2) from Plitvice
410 Lakes, in which morphological differences were observed with respect to the typical *Perlodes*
411 *intricatus*, was also found during this study. Molecular methods confirmed identification,
412 whereas DNA barcoded specimens were grouped with sequences of *P. intricatus* retrieved from
413 BOLD database, into a highly supported monophyletic clade (Dataset 1, Table S5), but with high
414 intraspecific *p*-distances (5.2–5.6%), indicating the need for further field research across the
415 entire range of the species. For another species *P. microcephalus,* from the same genus, we
416 obtained high intraspecific *p*-distance*,* and it is accompanied by increasing morphological
417 variability from type specimens (Dataset 1, Table S5).

418 Genus *Isoperla*
419 The genus *Isoperla* is characterized by several poorly circumscribed West Palearctic species
420 (*Zwick, 2004*) and Murányi (2011) argued for the need of taxonomic revision. Within *Isoperla*

421 *inermis* (clade No. 34, Fig. 2), we found great morphological variability as observed in previous
422 research (*Popijač & Sivec, 2009b*). Individuals from Plitvice Lakes are almost double in size
423 compared to specimens from Cetina River (*Popijač & Sivec, 2009b*), which may be a result of
424 different climates and a longitudinal gradient. Color variation is found in the abdomen, head, and
425 pronotum, which vary from brown to black. Phylogenetic analysis of sequences from all *Isoperla*
426 species and from *I. difformis* from central Europe, resulted in a highly supported monophyletic
427 clade *I. inermis-I. difformis*, which could ultimately result in the synonymy of these species
428 (Dataset 2, Table S5).
429 *Isoperla* cf. *lugens* (clade No. 37, Fig. 2) was recorded in the area of the Plitvice Lakes (*Popijač*
430 *& Sivec, 2009b*), and was determined based on similarity of the penial armature. During
431 comprehensive field research in this study, specimens were found associated with several
432 headwaters of karst rivers. The species differs morphologically from the alpine species, *I. lugens*,
433 by having a lighter colored head and pronotum and different penial armatures. In addition to
434 these morphological characteristics, the species is also characterized by exceptional genetic
435 distinctiveness. The lowest interspecific *p*-distance value from *I.* cf. *lugens* compared to other
436 congeners from the *I. tripartita* and *I. rivulorum* species-groups is 6.7% and represents a separate
437 genetic lineage within the clade consisting of typical *I. lugens* and *I. popijaci* (clade No. 37, Fig.
438 2) (Dataset 3, Table S5). Due to the above-mentioned characteristics, *Isoperla* cf. *lugens* most
439 probably represents a new species. The MJ network (Fig. 3B) for *Isoperla* cf. *lugens* revealed
440 low haplotype sharing among sampling sites, which may be due to the small number of
441 specimens per sampling site. Additional specimens would be useful to test this hypothesis.

442 Genus *Protonemura*
443 *Protonemura hrabei* (clade No. 71, Fig. 2) from the Cetina and Zrmanja Rivers emerges at the
444 beginning of summer, mostly due to climatic conditions, in contrast populations from Central
445 Europe, which emerge in autumn (*Popijač & Sivec, 2009b*). Molecular analysis of *P. hrabei*
446 sequences and sequences of closely related *Protonemura* species, confirmed morphological
447 identification and individuals from the Cetina River form a highly supported, monophyletic clade
448 with *Protonemura hrabei* from Central Europe with an intraspecific *p*-distance of 2.5% (Dataset
449 4, Table S5).

450 Genus *Taeniopteryx*

451 In a comprehensive study of the genus *Taeniopteryx* in the framework of this study,
452 morphological differences were identified among newly collected individuals of *Taeniopteryx*
453 n.sp. CRO-1 (clade No. 47, Fig. 2), *Taeniopteryx hubaulti* (Aubert, 1946) (clade No. 46, Fig. 2)
454 and *Taeniopteryx auberti*, as well as museum specimens in Croatia, Slovenia, Bosnia and
455 Herzegovina, Montenegro and Germany. The morphological differences between *Taeniopteryx*
456 n.sp. CRO-1 and other species are accompanied by genetic distinctiveness, making *Taeniopteryx*
457 n.sp. CRO-1 a candidate for a new species (interspecific *p*-distances ranged from 7.8–9.5%) as
458 suggested by Popijač & Sivec (2009b).
459 Morphological analysis of *T. hubaulti* confirmed the variability of the femoral thorn on the hind
460 legs, present in some individuals but varying in size, even though this character was originally
461 described as absent (*Aubert, 1946*). This morphological character should be clearly visible in *T.*

462   *auberti* (*Kis & Sowa, 1964*). Genetic analyses of our sequences and those retrieved from BOLD
463   and GenBank (Dataset 5, Table S5) resulted in the unclear taxonomic status of *T. hubaulti* and *T.*
464   *auberti*, so there is the suspicion that *T. hubaulti* may be a junior synonym of *T. auberti*, and
465   morphological variability is a consequence of geographical distribution. This is also similar for
466   the species, *T. stankovitchi* and *T. schoenemundi* (Mertens, 1923), for which it has already been
467   pointed out that additional research is needed to clarify their distinction (*Fochetti & Nicolai,*
468   *1996*). As has been noted, the genus *Taeniopteryx* is, from the taxonomic point of view,
469   complicated, and oftentimes only females show reliable characters, so the whole genus needs
470   revision.

471   Genus *Leuctra*
472   Within the *Leuctra inermis* species-group, congruence of morphospecies concepts and
473   phylogenetic relationships among taxa was not studied until 2017 (*Vitecek et al., 2017*). Vitecek
474   et al. (2017) found that relationships among species remained unresolved, suggesting sister taxon
475   relationships between morphologically similar species and potential subspecies-level diversity.
476   Morphological variability was observed within geographically isolated populations and the same
477   was confirmed by the present study. As many species from the *L. inermis* species-group have
478   overlapping geographical ranges, appearance of morphological variability within several species
479   is expected (*Fochetti et al., 2011*) and mitochondrial introgression has already been confirmed
480   within the *Leuctra* species pairs, *L. fusca* and *L. digitata* (*Boumans & Tierno de Figueroa, 2016*).
481   Thus, it can be assumed that assessment of drumming call variations could be helpful in
482   resolving taxonomic relationships within this species-group (*Vitecek et al., 2017*).
483   Few individuals collected in this study appeared as a distinct lineage (Fig. 2), which possessed
484   morphological features resembling already known species: *Leuctra* cf. *inermis (*CROPL130-21)
485   collected in the Plitvice Lakes National Park, *Leuctra* sp. collected at Cetina River (clade No. 17,
486   Fig. 2), *Leuctra* sp. Z (CROPL248-21) collected at Žumberak Hills and *L.* cf. *prima*
487   (CROPL282-21, CROPL325-21 and CROPL326-21) collected at Papuk Mountain and near
488   Plitvice Lakes National Park. Due to the unavailability of specimens of *L. carphatica*, only found
489   in the Carphatian Mountains, Slovenia and Austria (*Andrikovics & Murányi, 2001*), the
490   comparison of collected individuals with this species was omitted. Morphological differences
491   were also observed among individuals of *L. mortoni* and *L. fusca*, which requires further research
492   with a more comprehensive sampling, which would contribute to a more precise taxonomy of
493   these species.

494

Dinaric Karst as a biodiversity hotspot
495
496   The Dinaric Karst system of the Dinaric Mountains, represents one of the most diverse European
497   freshwater habitats in terms of biological, geological and hydrological interplay, including many
498   available microhabitats, which has resulted in speciation and endemism (*Bonacci, 2009*).
499   Considering the results of earlier studies, high genetic diversity could be a result of the specific
500   habitat requirements and biological characteristics of individual taxa, which promoted speciation
501   and played an important role in the genetic differentiation of freshwater taxa (*Previšić et al.,*
502   *2009, 2014a, 2014b; Klobučar et al., 2013; Jelić et al., 2016; Szivák et al., 2017; Lovrenčić et*

503   *al., 2020*). The same goes for taxa restricted to particular microhabitats (caves, pits, underground
504   and intermittent rivers and streams) within the Dinaric Karst (*Bilandžija et al., 2013; Bedek et*
505   *al., 2019; Pavlek & Mammola, 2020*), which in general, can be considered as refugia from which
506   taxa re-colonise Europe following glacial periods (*Hewitt, 2000*), often showing a pattern
507   "refugia within refugia" (*Kryštufek et al., 2007; Ursenbacher et al., 2008; Previšić et al., 2009;*
508   *Jug-Dujaković et al., 2020*).

509   The diversity of the stonefly fauna in Croatia is a probable consequence of the different climatic
510   conditions in a variety of regions (Continental, Alpine and Mediterranean), a substantial
511   altitudinal gradient, and an immense number of protected habitats. Within this study, we
512   identified hotspots of elevated stonefly species. Highest levels of species richness were primarily
513   located in the northwest Dinarides (Fig. 4): the border rivers, Kupa and Čabranka; Plitvice Lakes
514   National Park; the Cetina and Una Rivers; and Mt. Papuk where several localities have five or
515   more species. Hotspots of species richness are coincident with protected areas (e.g., Plitvice
516   Lakes National Park, Mt. Papuk, Mt. Medvednica), which is not surprising, given that these areas
517   have suitable conditions and mostly include fast streams with high oxygen saturation. Current
518   research shows a significant decrease in the number of species from the northern part of the
519   Dinaric Karst (Gorski kotar and Lika) to the Cetina River and the headwater of the Una River,
520   which is consistent with the biological features of stoneflies. Great species richness is also
521   confirmed by Ridl et al. (2018), who recorded 7–18 species across different study sites (in total
522   31 species) in the Plitvice Lakes National Park. Furthermore, Popijač & Sivec (2010) found
523   significant species richness (14) within the Cetina River. The Croatian fauna shows great species
524   richness, not only for stoneflies but also for other aquatic insects (*Ivković & Plant, 2015;*
525   *Vilenica et al., 2015; Vilenica et al., 2016; Kučinić et al., 2017; Vilenica, Ternjej & Mihaljević,*
526   *2021*). Still, many parts of the Dinaric Karst are not well studied, due to the size of area and
527   inaccessibility of the habitats. Species richness patterns are likely incomplete, especially from the
528   aspect of the stonefly fauna.
529
530   **Figure 4**
531
532   **Database's enrichment and systematic implications**
533   This study provides the first molecular characterization of 9 species: *Brachyptera tristis,*
534   *Perlodes dispar, Leuctra bronislawi* Sowa, 1970*, Isoperla bosnica* (Aubert, 1964)*, Isoperla*
535   *illyrica, Isoperla albanica* (Aubert, 1964)*, Perla carantana* Sivec & Graf, 2002*, Perla illiesi* and
536   *Agnetina elegantula* (Klapálek, 1905).
537   Genus *Protonemura*
538   Our molecular characterization of *Protonemura auberti* (Illies, 1954), *P. hrabei*, *P. intricata*
539   (Ris, 1902), *P. nitida* (Pictet, 1836) and *P. praecox* should be considered in future revision of the
540   genus, a need that has already been emphasized (*Wagner et al., 2011; Vinçon, Reding & Ravizza,*
541   *2021*). The discovery of specimens from the *P. auberti* species subgroup (determined as *P*. cf.
542   *autumnalis*, clade No. 72, Fig. 2) at the Plitvice Lakes National Park with morphological

543 characteristics similar to *P. aestiva*, which emerges throughout the spring and seems to be
544 restricted to the Carpathian Mountains (*Kis, 1974; Graf et al., 2009*), suggests a hybrid of these
545 two species, with range expansion of *P. aestiva* (*Vinçon, Reding & Ravizza, 2021*). Nevertheless,
546 the production of hybrids with intermediate morphological characters has already been observed
547 in the newly described *P. bispina* (*Vinçon, Reding & Ravizza, 2021*), which is often vicariant
548 with specimens of *P. auberti*. Therefore, it is necessary to pay additional attention to the genus
549 also from that point of view. DNA barcoded specimens of *P. auberti* (CROPL257-21,
550 CROPL258-21, CROPL281-21) collected during the spring at the Una River appeared as a
551 separate clade among other specimens of *P. auberti* (clade No. 74, Fig. 2). Therefore,
552 establishing phylogenetic relationships with a multi-gene approach is necessary to unravel the
553 taxonomy of this group.
554
555 Genus *Dinocras*
556 DNA barcoding of the species *Dinocras megacephala* (Klapálek, 1907) (clade No. 23, Fig. 2),
557 the only *Dinocras* species in Croatia, which is widely distributed in its northern and central part,
558 emphasizes the need for revision of the BOLD database due to the noticeable number of
559 erroneous determinations of this species. It often appears to be misidentified as *D. cephalotes*.
560 Males of *D. cephalotes* differ from *D. megacephala* by having patches of stronger sensilla
561 basiconica on the ventral side of the abdomen. Furthermore, brachypterous males of *D.*
562 *megacephala* also occur at higher elevations (*Illies, 1966*), so brachyptery cannot be a
563 characteristic to distinguish this species from *D. cephalotes* (usually at higher elevations).
564
565 Genus *Zwicknia*
566 Morphological examination of individuals from the *Capnia bifrons* species-group, following
567 Murányi et al. (2014) determined the presence of two species in Croatia: *Zwicknia bifrons* (clade
568 No. 51, Fig. 2) and *Zwicknia rupprechti* (clade No. 50, Fig. 2). Within the family Capniidae, we
569 also discovered one of the smallest European species, *Capnopsis schilleri balcanica*
570 (CROPL319-21) from only a single locality, a surprising find given that this was 17 years since
571 the first finding (*Murányi, 2004*).
572
573 Genus *Nemoura*
574 Within the genus *Nemoura*, ten species were DNA barcoded (clades Nos. 60-68, Fig. 2): *N.*
575 *avicularis* Morton, 1894, *N. cinerea* (Retzius, 1783), *N. dubitans* Morton, 1894, *N. marginata*, *N.*
576 *minima* Aubert, 1946, *N. sciurus*, *N. flexuosa*, *N.* cf. *rivorum*, *N. mortoni* (Ris, 1902) and *N.*
577 *uncinata* Despax, 1934. The *Nemoura flexuosa-marginata* complex is one of the most enigmatic
578 assemblages of species within European stoneflies and requires revision (*Ravizza & Ravizza*
579 *Dematteis, 1995*).
580 The *flexuosa-marginata* species-group is composed of widely distributed European species (*N.*
581 *flexuosa* Aubert, 1949, *N. marginata* Pictet, 1836 and *N. uncinata* 1934) and six Italian and
582 French Alps endemic species: *N. hesperiae* Consiglio, 1960, *N. lucana* Nicolai & Fochetti, 1991,

583   *N. oropensis* Ravizza & Ravizza Dematteis, 1980, *N. pesarinii* Ravizza & Ravizza Dematteis,
584   1979 (all occurring in Italy), *N. palliventris* Aubert, 1953 and *N. rivorum* Ravizza & Ravizza
585   Dematteis, 1995 (occurring in Italy and further north in the French Alps) (*Fochetti & Vinçon,*
586   *2009*). Descriptions of two species within that complex: *Nemoura rivorum* C. Ravizza & Ravizza
587   Dematteis, 1995 and *Nemoura sabina* Fochetti & Vinçon, 2009 helped in understanding
588   morphological variation among individuals within this species complex. *Nemoura rivorum*,
589   which is endemic to the northern section of the Apennines and exhibits a variable apical and
590   arched sclerite of the epiproct, is often erroneously identified as *N. flexuosa*, especially when
591   only females are available for morphological analysis. Pregenital plate shape is similar in all
592   species belonging to the *N. flexuosa-N. marginata* species-group and separating nymphs among
593   species is almost impossible. Specimens collected as part of this study (CROPL075-21,
594   CROPL076-21 and CROPL162-21) have morphological characteristics similar to *N. rivorum*
595   (determined as *N.* cf. *rivorum*). However, based on the similarity of the sequences, these
596   specimens clustered with *N. flexuosa* (CROPL070-21, CROPL095-21, CROPL190-21), which
597   further emphasizes the importance of species-group revision, and revision of the sequences in
598   BOLD and GenBank databases, respectively.
599   Furthermore, high morphological variability has been observed even among specimens of
600   *Nemoura marginata* Pictet, 1836 (clade No. 64, Fig. 2) (*Popijač & Sivec, 2009b*). The mean
601   intraspecific *p*-distance was 3.1%. Future research should assess whether *Nemoura marginata*
602   represents a single species with large intraspecific distances and high morphological variability
603   or a species-complex, as stated earlier (*Ravizza & Ravizza Dematteis, 1995*).
604
605   *Leuctra bronislawi*
606   This study also provides the first molecular characterization of *Leuctra bronislawi* (CROPL131-
607   21 and CROPL132-21). This autumnal species, which is relatively rare and a relict species with
608   disjunct distribution in the Balkan and the Carpathians, was recently found in the Czech
609   Republic (*Kroča, 2010*) and the first early spring records were reported from the Republic of
610   Macedonia (*Murányi, Kovács & Orci, 2014*). Considering the limited knowledge of the stonefly
611   fauna in countries which can include potential distribution areas of *L. bronislawi*, it is anticipated
612   that many more populations remain to be discovered and recorded.
613
614   Genus *Isoperla*
615   Within the mostly endemic genus *Isoperla*, three species were DNA barcoded for the first time:
616   *Isoperla bosnica* (clade No. 39, Fig. 2)*, Isoperla illyrica* (clade No. 42, Fig. 2) and *Isoperla*
617   *albanica* (clade No. 41, Fig. 2)*. Isoperla bosnica* is, based on morphology, a member of the
618   *Isoperla oxylepis* species-group, which was redescribed based on SEM studies of the penis and
619   egg structure (*Murányi, 2011*). *I. bosnica* was hitherto reported only from type locality (SE
620   Bosnia-Herzegovina), NW Macedonia and Montenegro (*Murányi, 2011*) and the medial penial
621   armature, a basic diagnostic characteristic, is like the armature of *Isoperla oxylepis* (*Murányi,*
622   *2011*). *Isoperla albanica* has an Eastern Alpine-Illyrian distribution and is characterized by an

623   undivided medial penial armature (*Murányi, 2011*). *I. illyrica,* described as an endemic species to
624   the Postojna Cave entrance, is now common across a wide area of the Dinaric Karst. Our
625   phylogenetic analysis does not resolve the placement of this species. A multi-gene approach
626   across the entire range is a priority (Dataset 2, Table S5). A similar approach is warranted for the
627   *I. grammatica* species-complex (*Murányi, 2021*).
628
629   Genus *Perla*
630   The taxonomy of *Perla* species is unresolved and constitutes a big challenge, and the most recent
631   revision of the genus suggests using characters in the egg chorionic (*Sivec & Stark, 2002*) as
632   reliable for species recognition. To revise such a problematic genus, the inclusion of genomic
633   data is required. For some species, it is already considered that they represent a species-complex,
634   such as *P. pallida* (distributed in the Caucasus, Anatolia, the Balkans, and the Carpathians)
635   (*Sivec & Stark, 2002*), often erroneously identified as *P. marginata.* Furthermore, the taxonomic
636   status of some species is uncertain, such as *P. bipunctata* (*Sivec & Stark, 2002*) and specimens
637   found within this study on Ruda River (marked as *Perla* sp., clade No. 29, Fig. 2), which
638   morphologically differed from congeners but genetically represent one lineage and one BIN.
639   *Perla burmeisteriana* was recorded for the first time in 1908 and a few larvae were found at the
640   northern foot of Papuk Mountain (*Popijač & Sivec, 2009a*). *Perla illiesi* was found in several
641   localities in Croatia (Kupa and Čabranka Rivers). Within the present study, this species was
642   recorded at two localities in Lika and DNA barcoded for the first time (clade No. 26, Fig. 2), as
643   well as *Perla carantana* (clade No. 24, Fig. 2), which was reported at several localities in
644   Slovenia and Austria (*Sivec & Graf, 2002*).
645
646   Genus *Besdolus*
647   Interspecific *p*-distances between newly obtained sequences of *B. imhoffi* and *B. illyricus*
648   retrieved from GenBank (10.6–12.4) do not support the synonymy of *B. illyricus* and *B. imhoffi*,
649   as stated before (*Fochetti et al., 2011)* (Dataset 6, Table S5).
650
651
652   **Extirpation and conservation**
653   High sensitivity of stoneflies to abiotic changes may lead to local or global extirpation of taxa
654   (*Fochetti & Tierno de Figueroa, 2006; Graf et al., 2018*). Extensive field research that began in
655   Europe about twenty years ago, however, found the presence of taxa that were considered
656   extinct, but with range reduction. The local and regional extinction rate of stoneflies would be
657   the highest across the Animal Kingdom, according to IUCN criteria (*Sánchez-Bayo & Wyckhuys,*
658   *2019*). In neighbouring countries, such as Italy, several species can be considered already
659   extirpated: *Brachyptera trifasciata* (Pictet, 1832), *Isogenus nubecula* Newman, 1833,
660   *Taeniopteryx nebulosa* (Linnaeus, 1758) and *Perla abdominalis* Burmeister, 1839 (*Fochetti et*
661   *al., 1998; Fochetti, 2020*), while even more species can be considered threatened with extinction.
662   The situation is, as usual, also critical for endemic species (known only from their type locality
663   or a few populations) and all microendemic taxa (*Fochetti, 2020*).

664   Despite the relatively large effort invested in researching the stonefly fauna in Croatia (*Popijač,*
665   *2008; Popijač & Sivec, 2009a, 2009b*), several species are preserved only in museum
666   collections: *Perla bipunctata, Perla grandis, Isoperla obscura* and *Isogenus nubecula* (*Popijač*
667   *& Sivec, 2009a*). It is questionable whether these species have gone locally extinct, or their
668   populations have decreased so much that it is difficult to detect them. In future systematic
669   research of the stonefly fauna, discovering these species and their distributions will be one of the
670   priorities.
671   But in spite of that, we recorded many species that have been declared extirpated in other
672   European countries. *Brachyptera monilicornis* (Pictet, 1841) has long been considered extinct
673   because it does not occur in Central Europe (*Zwick, 1992)*. In the border rivers of Croatia and
674   Slovenia (Kupa and Čabranka), as well as in the streams and rivers of Papuk Mountain, *B.*
675   *monilicornis* is very common. Furthermore, the rare and endangered lowland species,
676   *Taeniopteryx nebulosa* and *Rhabdiopteryx acuminata* (Klapálek, 1905), have been recorded at
677   several localities in the foothills of Papuk Mountain. *Besdolus imhoffi* was re-discovered in
678   Croatia in 2005 (Plitvice Lakes National Park) (*Kovács & Murányi, 2008*) after a one-hundred-
679   year-old record (*Popijač & Sivec, 2009)*. This finding was confirmed with molecular analysis in
680   the present study at the Una and Cetina Rivers. Species of the genus, including *B. imhofii*, have
681   relictual distributions (*Zwick & Weinzierl, 1995*) and are sensitive to environmental perturbations
682   (*Fochetti et al., 2011*). *Marthamea vitripennis*, a species lost from most of Europe (*Zwick, 2004*)
683   due to destruction of river potamon, was found in the Rába River in Hungary (*Kovács & Ambrus,*
684   *2000*), but also in Croatia in 2011 (*Popijač & Sivec, 2011*), and during field research in this study
685   in 2021, on the rapids of the Una River. Another rare plecopteran, found in the Rába River in
686   Hungary (*Kovács & Ambrus, 2000*) is *Agnetina elegantula*, which we recorded during this study
687   at Papuk Mountain.

Conclusions

689   The current study generated a validated national reference DNA barcode library for stoneflies in
690   Croatia, which can support the implementation of cost-efficient DNA-based identifications and
691   assessments to ecological status. DNA barcoding proved to be an effective tool for the
692   identification and delimitation of some closely related species. Furthermore, this study provides
693   several findings of species thought to be extirpated from Croatia and neighbouring regions, as
694   well as the first molecular characterization of species with restricted distributions. For some
695   genera (e.g., *Isoperla, Taeniopteryx* and *Perla*) an integrative revisionary examination based on
696   more comprehensive geographic sampling and application of a multi-gene approach, especially
697   on type specimens, is necessary for resolving taxonomic relationships. Identifying areas with
698   high biodiversity, including both morphological variability and genetic diversity, will allow
699   further protection of stoneflies 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 confamiliar (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.
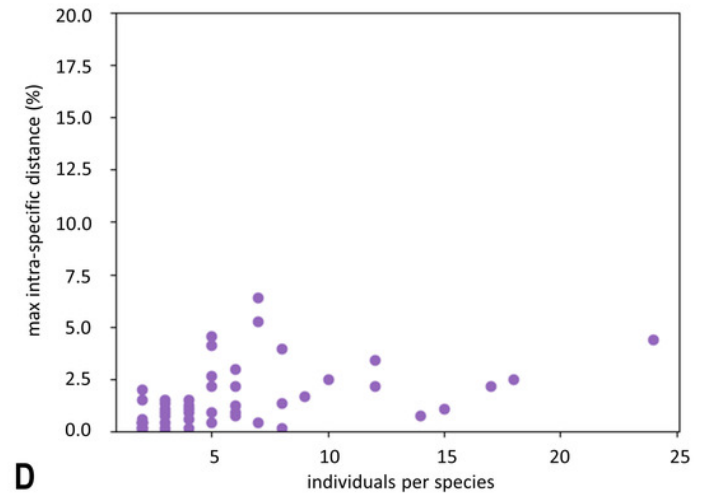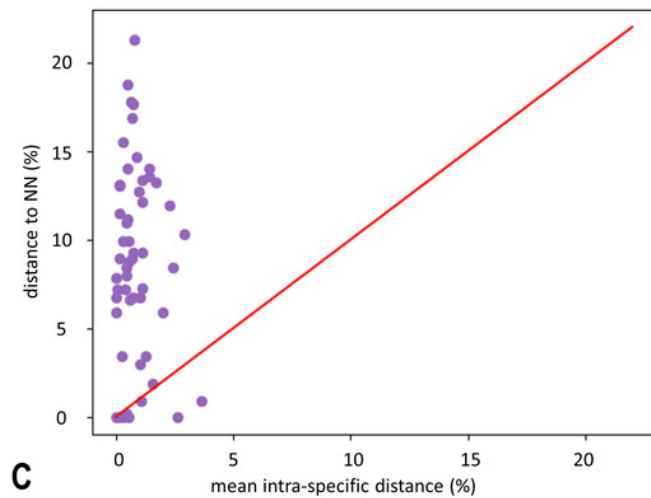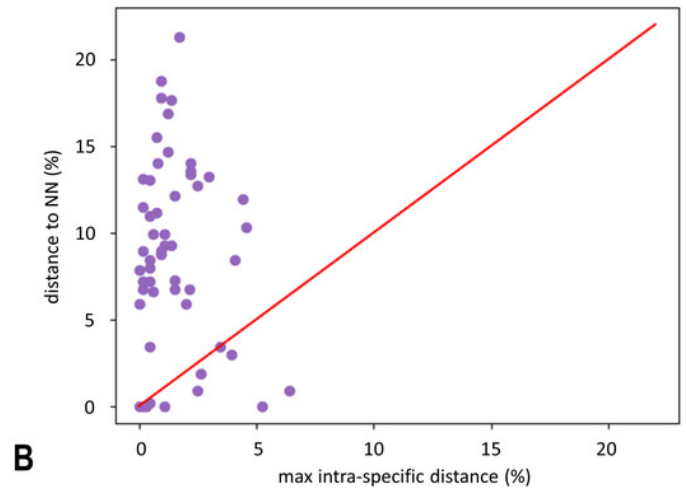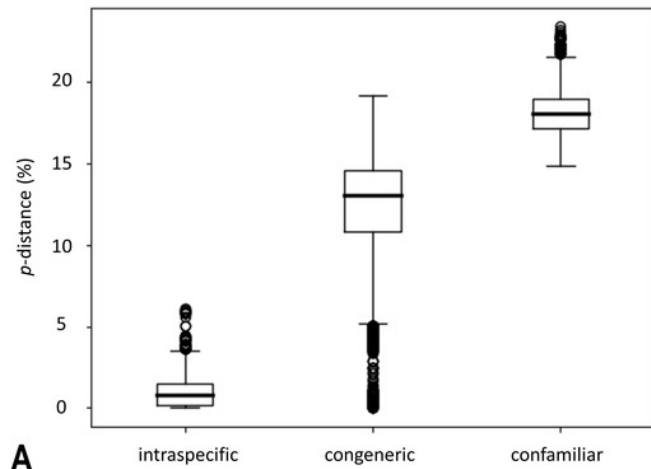
Figure 2

Circular maximum-likelihood (ML) phylogram from analysis of the released dataset and results of species delineation methods.

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 and the size of dots is proportional with the value. The results of species delineations are represented with the bars in different colours 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 ver. 1.4.3 (*Rambaut, 2009*) and iTOL ver. 5 (*Letunic & Bork, 2021*) and finished in Adobe Illustrator.

Boostrap support

- · >75
- · >80
- · >90
- · >95
- · >99

0.1

Species delimitation

■ ABGD
■ ASAP
■ mPTP

Families

☐ Leuctridae
☐ Chloroperlidae
☐ Perlidae
☐ Perlodidae
☐ Taeniopterygidae
☐ Capniidae
☐ Nemouridae

Species

1) *Leuctra hippopus* 2) *L. nigra* 3) *L. inermis* 4) *L. cf. inermis* 5) *L. rauscheri* 6) *Leuctra sp. Z* 7) *L. cf. prima* 8) *L. prima*
9) *L. hirsuta* 10) *L. bronislawi* 11) *L. cingulata* 12) *L. major* 13) *L. digitata* 14) *L. fusca* 15) *L. albida* 16) *L. mortoni*
17) *Leuctra sp.* 18) *Xanthoperla apicalis* 19) *Siphonoperla taurica* 20) *S. torrentium* 21) *Marthamea vitripennis*
22) *Agnetina elegantula* 23) *Dinocras megacephala* 24) *Perla carantana* 25) *P. abdominalis* 26) *P. illiesi* 27) *P. pallida*
28) *P. marginata* 29) *Perla sp.* 30) *Besdolus imhoffi* 31) *Perlodes dispar* 32) *P. intricatus* 33) *P. microcephalus*
34) *Isoperla inermis* 35) *I. popijaci* 36) *I. rivulorum* 37) *I. cf. lugens* 38) *I. goertzi* 39) *I. bosnica* 40) *I. grammatica*
41) *I. albanica* 42) *I. illyrica* 43) *I. tripartita* 44) *Taeniopteryx nebulosa* 45) *T. schoenemundi* 46) *T. hubaulti*
47) *Taeniopteryx n.sp. CRO-1* 48) *C. schilleri balcanica* 49) *Rhabdiopteryx acuminata* 50) *Zwicknia rupprechti*
51) *Z. bifrons* 52) *Brachyptera risi* 53) *B. tristis* 54) *B. seticornis* 55) *B. monilicornis* 56) *Nemurella picteti*
57) *Amphinemura standfussi* 58) *A. sulcicollis* 59) *A. triangularis* 60) *Nemoura uncinata* 61) *N. minima* 62) *N. mortoni*
63) *N. flexuosa/N. rivorum* 64) *N. marginata* 65) *N. cinerea* 66) *N. avicularis* 67) *N. sciurus* 68) *N. dubitans*
69) *Protonemura praecox* 70) *P. intricata* 71) *P. hrabei* 72) *P. cf. autumnalis* 73) *P. nitida* 74) *P. auberti*

Figure 3

Sampling sites and median-joining network of 658 bp long *Isoperla* cf. *lugens COI* sequences.

(A) Map of Croatia and neighbouring countries with sampling localities (colour coding matches insert in 3B). (B) MJ network of *COI* sequences. Colours and numbers 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 labelled with BOLD IDs, as in Supplemental Table S1. Map is produced with Cartopy package 0.19 in Python. In order to distinguish similar colours we included additional number coding.
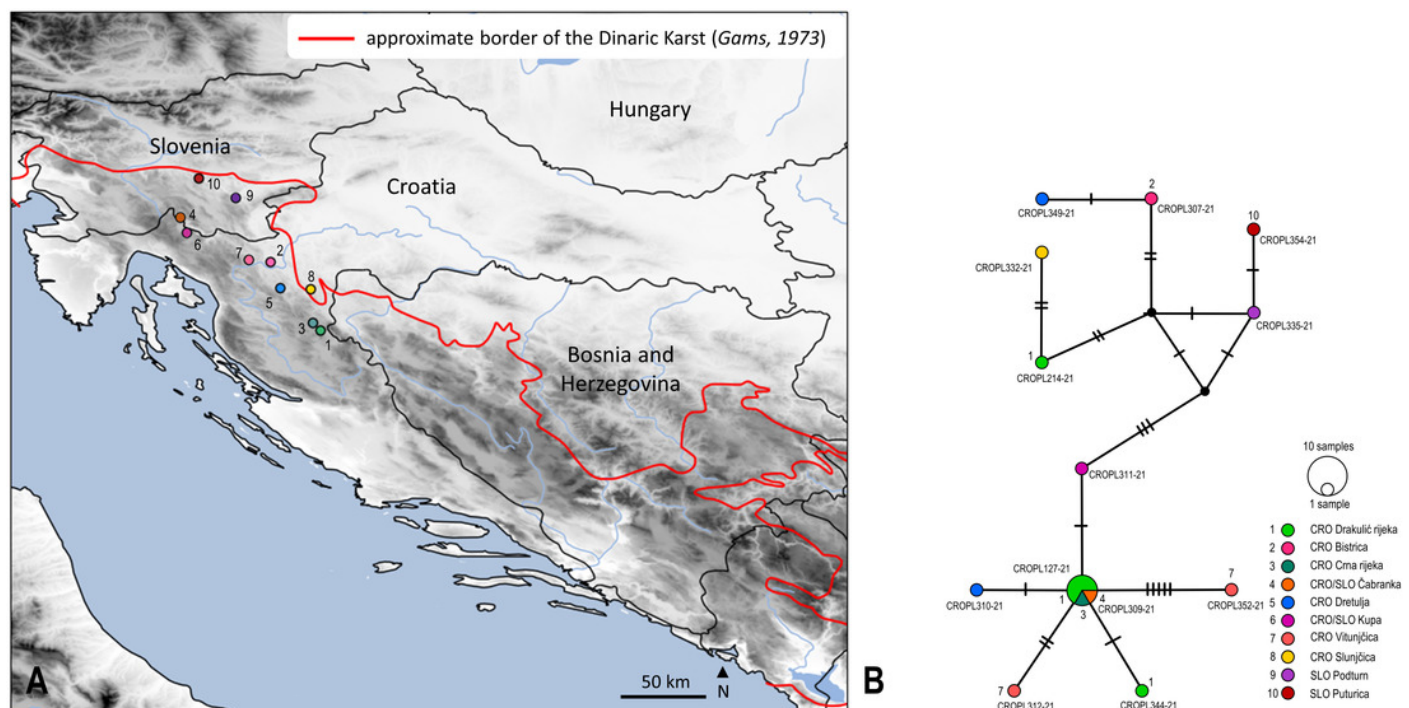
Figure 4

Geographical location of the studied stoneflies species.

Colours of dots represent the species richness in each locality and size of dots is proportional with the number. Main map (B) is an enlarge framed area in the bottom left corner (A). Map is produced with Cartopy package 0.19 in Python with use of elevation data from https://land.copernicus. eu/imagery-in-situ/eu-dem/eu-dem-v1.1 .