1 **Comparison of markers for the monitoring of freshwater benthic biodiversity through**
2 **DNA metabarcoding**

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16 Running title:

17 Markers for freshwater biomonitoring

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19 Abstract

20

21 Metabarcoding of bulk or environmental DNA has great potential for biomonitoring
22 freshwater environments. However, successful application of metabarcoding to biodiversity
23 monitoring requires universal primers with high taxonomic coverage that amplify highly
24 variable, short metabarcodes with high taxonomic resolution. Moreover, reliable and
25 extensive reference databases are essential to match the outcome of metabarcoding analyses
26 with the available taxonomy and biomonitoring indices. Benthic invertebrates, particularly
27 insects, are key taxa for freshwater bioassessment. Nevertheless, few studies have so far
28 assessed markers for metabarcoding of freshwater macrobenthos. Here we combined *in silico*
29 and laboratory analyses to test the performance of different markers amplifying regions in the
30 18S rDNA (Euka02), 16S rDNA (Inse01), and COI (BF1_BR2-COI) genes, and developed an
31 extensive database of benthic macroinvertebrates of France and Europe, with a special focus
32 on key insect orders (Ephemeroptera, Plecoptera and Trichoptera). Analyses on 1514
33 individuals representing different taxa of benthic macroinvertebrates showed very different
34 amplification success across primer combinations. The Euka02 marker showed the highest
35 universality, while the Inse01 marker showed excellent performance for the amplification of
36 insects. BF1_BR2-COI showed the highest resolution, while the resolution of Euka02 was
37 often limited. By combining our data with GenBank information, we developed a curated
38 database including sequences representing 822 genera. The heterogeneous performance of the
39 different primers highlights the complexity of the identification of the best markers, and
40 advocates for the integration of multiple metabarcodes for a more comprehensive and
41 accurate understanding of ecological impacts on freshwater biodiversity.

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43 Keywords: amplification rate; cytochrome c oxidase I; biomonitoring; biotic indices;
44 environmental DNA; freshwater biodiversity; macroinvertebrates; primer bias; taxonomic
45 resolution; universality.

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1 INTRODUCTION

Freshwater environments are essential providers of clean water and other services for human society. They also host a substantial biodiversity, still they are globally subjected to the joint impact of multiple stressors such as pollution, eutrophication, climate change and hydrological and hydromorphological modifications (Noges *et al.* 2016; Iversen *et al.* 2019). As a consequence, numerous regulations have been adopted at both the national and international level for the protection of water resources, such as the European Water Framework Directive (Directive 2000/60/EC) and the Clean Water Act of the US Environmental Protection Agency (33 U.S.C. §§1251-1387 1972; Pawlowski *et al.* 2018). These regulations generally require the monitoring of freshwater environments through a combination of physical, chemical hydrological, and biotic parameters to obtain prompt measurements of water quality and of the ecological status of ecosystems.

Multiple approaches exist to assess freshwater quality using aquatic organisms. Benthic macroinvertebrates are perhaps the most frequently used biological group in aquatic bioassessment (Birk *et al.* 2012), because (i) they are taxonomically, biologically and functionally diverse (Usseglio-Polatera *et al.* 2000; Usseglio-Polatera *et al.* 2001), (ii) they are rather easy to identify at the genus or family levels (Tachet *et al.* 2010), (iii) they are often sedentary and react rapidly to anthropogenic pressures in all types of freshwater bodies (Hering *et al.* 2006b; Archaimbault *et al.* 2010; Hering *et al.* 2013), and (iv) their occurrence integrates the effects of environmental changes over several months (Floury *et al.* 2013). Macroinvertebrate assemblages are thus a tool of choice to assess the ecological status of water bodies *(e.g.* Marzin *et al.* 2012; Hering *et al.* 2013; Mondy & Usseglio-Polatera 2013) and to demonstrate environmental degradation (Miler *et al.* 2013; Mondy & Usseglio-Polatera

72 2013; Theodoropoulos *et al.* 2020) or restoration (Arce *et al.* 2014; Kupilas *et al.* 2016;
73 Camargo 2017; Carlson *et al.* 2018).

74 Generally, bioassessment indices relying on benthic communities are based on the
75 standardized collection of macroinvertebrate assemblages from monitored sites, followed by
76 organism sorting and taxonomic identification using morphological criteria. Then, quality
77 scores can be attributed on the basis of the presence and/or abundance of certain taxa (Friberg
78 *et al.* 2006; Birk *et al.* 2012). As morphological identification is often challenging, in many
79 case protocols do not require species-level identification, and identification at the genus or
80 family level (and, in some cases, even at coarser levels) can be enough for the calculation of
81 many biotic indices evaluating the ecological status of rivers (Bailey *et al.* 2001; Chessman *et*
82 *al.* 2007; Birk *et al.* 2012). Nevertheless, the morphological identification of hundreds
83 collected individuals, including young, small-sized, larval stages and organisms damaged
84 during sampling, remains time-consuming and requires a substantial taxonomic expertise,
85 increasing the cost and time required for in-depth assessment of water quality (Haase *et al.*
86 2004; Hering *et al.* 2018).

87 DNA metabarcoding and environmental DNA (eDNA) are revolutionizing the
88 monitoring of biodiversity at all levels, because they circumvent the challenge of
89 morphological identification and allow the efficient detection of many taxa that are difficult to
90 capture and detect using traditional methods (Taberlet *et al.* 2018). Environmental DNA and
91 metabarcoding are therefore extremely promising for the assessment of freshwater
92 communities (Hering *et al.* 2018; Li *et al.* 2018; Kuntke *et al.* 2020). For metabarcoding,
93 DNA can be extracted from the tissue of pooled macroinvertebrate communities, amplified
94 using universal primers, sequenced, and identified on the basis of reference databases (Baird
95 & Hajibabaei 2012; Yu *et al.* 2012; Andújar *et al.* 2018). This approach uses the same starting
96 material than traditional biomonitoring, but allows skipping the complexity of morphology-

97 based taxonomy (Baird & Hajibabaei 2012). Alternatively, DNA can be obtained directly
98 from the water (Ficetola *et al.* 2008). Environmental DNA extracted from freshwaters allows
99 the detection of many taxa that are difficult to capture and detect using traditional methods,
100 but also poses new challenges compared to metabarcoding performed on the tissues of
101 captured individuals. In aquatic environments, DNA undergoes rapid degradation (Eichmiller
102 *et al.* 2016; Buxton *et al.* 2017); therefore eDNA is generally characterized by short fragment
103 sizes (Jo *et al.* 2017; Bylemans *et al.* 2018), but see also (Sigsgaard *et al.* 2017). Therefore,
104 primers amplifying short regions can provide a better detection compared to "standard"
105 barcode primers, which often amplify long DNA fragments (e.g. >300 bp in the most
106 frequently used COI markers; Andújar *et al.* 2018). Furthermore, highly degenerated primers
107 increase the risk of non-specific amplification, thus this kind of primers is not really suitable
108 for the amplification of the complex mix of DNA extracted from the environment. As a
109 consequence, the monitoring of benthic macroinvertebrates using eDNA requires the
110 development and assessment of primers with appropriate features.

111 Besides the length of the amplified region, three main characteristics are essential for
112 satisfactory eDNA metabarcodes (Ficetola *et al.* 2010; Wilcox *et al.* 2013). First, the eDNA
113 amplification success generally decreases with the number of mismatches between target
114 fragments and primers. Primers must therefore be designed in order to have a consistently low
115 number of mismatches within sequences of the target group (high universality or taxonomic
116 coverage; Ficetola *et al.* 2010; Piñol *et al.* 2015; Marquina *et al.* 2019). Taxonomic coverage
117 can be assessed through both *in silico* and laboratory analyses. *In silico* analyses can allow the
118 rapid assessment of all the taxa for which information is publicly available in databases, but
119 laboratory tests (hereafter, *in vitro* tests) are still needed to confirm the actual performance of
120 primers. Second, the amplified region must be highly variable, to ensure the identification of
121 amplified organisms at the desired taxonomic level (high resolution; Ficetola *et al.* 2010;

122 Tang *et al.* 2012; Marquina *et al.* 2019). Finally, extensive databases are essential if we want
123 to assign the amplified sequences to known taxa. Even though attempts have been made for
124 the assessment of environmental quality without a taxonomic assignment of DNA fragments
125 (Ji *et al.* 2013; Apothéloz-Perret-Gentil *et al.* 2017; Cordier *et al.* 2018; Cordier *et al.* 2019),
126 taxonomic assignment is essential if we want to produce data comparable with traditional
127 indices of water quality, or if we want to combine eDNA data with information obtained
128 through traditional methods (*e.g.* to analyze long-term series of water body surveys). Despite
129 several attempts to assess freshwater quality using eDNA (Hering *et al.* 2018; Li *et al.* 2018;
130 Serrana *et al.* 2019; Czechowski *et al.* 2020; Pont *et al.* 2020; Yang & Zhang 2020), so far
131 limited formal comparisons have been performed among short primers suitable for eDNA
132 metabarcoding of freshwater macrobenthos (but see Elbrecht *et al.* 2016; Elbrecht & Leese
133 2017). In addition, there is a pressing need for exhaustive reference databases for taxonomic
134 assignment (see Moriniere *et al.* 2017; Morinière *et al.* 2019; Weigand *et al.* 2019 for
135 remarkable examples).

136 In this study we combined *in vitro* and *in silico* analyses to compare the performance
137 of three primer pairs potentially suitable for the metabarcoding of bulk or environmental DNA
138 from freshwater macroinvertebrates (macrobenthos), and we developed an extensive reference
139 database for benthic macroinvertebrates living in European freshwaters. We mostly focused
140 on three insect orders (Ephemeroptera, Plecoptera and Trichoptera), which are among the
141 most frequently used macroinvertebrates for the bioassessment of streams (e.g. Brabec *et al.*
142 2004; Hering *et al.* 2006a; Gabriels *et al.* 2010; Arman *et al.* 2019; but see also Cox *et al.*
143 2019). We also considered a broad range of organisms belonging to other orders of insects
144 and other classes. We first used high-throughput DNA barcoding (Vivien *et al.* 2020) of
145 reference individuals identified by experts to obtain sequences on the broadest available
146 number of taxa from France, and then combined the obtained sequences with those available

147 in public database, to obtain extensive and reliable measures of the performance of markers,
148 and to produce an extensive reference database for the monitoring of freshwaters through
149 eDNA.

150

151 2 MATERIAL AND METHODS

152

153 We used the standardized database of European freshwater organisms (Schmidt-Kloiber &
154 Hering 2015; download on 01 March 2018) as taxonomic reference for our analyses,
155 considering all the benthic macroinvertebrates. Although in some cases this database
156 considers non-monophyletic groups (*e.g.* Crustacea), it provides an exhaustive checklist of
157 benthic macroinvertebrates that serve as an essential basis for bioassessment and monitoring.

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159 2.1 Analyses of reference individuals

160

161 Most of the reference individuals were provided by OPIE-Benthos which is a working group
162 of OPIE (Office Pour les Insectes et leur Environnement) especially dedicated to aquatic
163 insect studies and aquatic ecosystem protection in France. OPIE-Benthos has developed a
164 national inventory and reference collection of aquatic insects (http://www.opie-
165 benthos.fr/opie/insecte.php), including Ephemeroptera, Plecoptera, Trichoptera, and more
166 recently aquatic Coleoptera, aquatic and semi-aquatic Heteroptera, aquatic larval stages of
167 Megaloptera, Neuroptera and Diptera (Ptychopteridae). Corresponding organisms, identified
168 at the highest possible level (species, if possible) by experienced taxonomists, were provided
169 in triplicates (*i.e.* three individuals per taxon, if possible). The collection was completed by

170 additional taxa (*e.g.* non-insect taxa) specifically sampled by the authors for this reference
171 database.

172 Individuals were stored in 99% ethanol before DNA extraction. Total DNA was
173 extracted from the entire organism. Samples (constituted of one individual) were initially
174 incubated overnight at 56 °C in 0.5 ml of lysis buffer (Tris-HCl 0.1 M, EDTA 0.1 M, NaCl
175 0.01 M and N-lauroyl sarcosine 1%, pH 7.5–8.0). Extractions were then completed using the
176 DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany), according to the
177 manufacturer's instructions. DNA extracts were recovered in a total volume of 300 µl of
178 elution buffer. Negative extractions without individuals were systematically performed to
179 monitor possible contaminations. Three DNA amplifications were carried out for each sample
180 using the following primer pairs: Inse01, amplifying a ~155 bp region of the 16S
181 mitochondrial rDNA (Taberlet *et al.* 2018); Euka02, amplifying a ~123 bp region of the 18S
182 rDNA (Guardiola *et al.* 2015; Taberlet *et al.* 2018); and the BF1 and BR2 primers, which
183 amplify a ~316 bp region of the cytochrome c oxidase I (Elbrecht & Leese 2017). Inse01 has
184 been developed mostly to amplify insects, Euka02 to amplify all eukaryotes, while BF1 and
185 BR2 were designed to amplify freshwater macroinvertebrates (Elbrecht & Leese 2017;
186 Taberlet *et al.* 2018). The DNA of each individual was amplified in one PCR replicate per
187 each marker considered. DNA amplifications were performed in a final volume of 20 µL,
188 using 2 µL of DNA extract as template. The amplification mixture contained 10 µL of
189 Applied Biosystems™ Master Mix AmpliTaq Gold™ 360, 0.2 µg/µL of bovine serum
190 albumin (BSA, Roche Diagnostic, Basel, Switzerland) and 0.5 µM of each primer for COI
191 and Inse01, or 0.2 µM of each primer for Euka2. Forward and reverse primers were 5'-labeled
192 with eight-nucleotide tags with at least three differences between any pair of tags, so that each
193 PCR reaction was identified by a unique combination of tags. This allowed the assignment of
194 each sequence to the corresponding sample during sequence analysis (Coissac 2012; Taberlet

195 *et al.* 2018). The PCR mixture was denatured at 95°C for 10 min, followed by 35 cycles of 30
196 s at 95°C, 30 s at 52°C for COI and Inse01 or 45°C for Euka2, and 1 min at 72°C (1m 30 s for
197 COI), and followed by a final elongation at 72°C for 7 min. Ten negative DNA extraction and
198 18 PCR controls (ultrapure water) were analyzed in parallel with the samples to monitor
199 possible contaminations during the experimental process.

200 Library preparation was performed using the MetaFast protocol by Fasteris (Geneva,
201 Switzerland; https://www.fasteris.com/dna/?q=content/metafast-protocol-amplicon-
202 metagenomic-analysis), which significantly limits the tag-jump problem (Taberlet *et al.*
203 2018). For Euka02 and Inse01, sequencing was performed by 2 × 125-bp paired-end
204 sequencing on the Illumina HiSeq 2500 platform, while for BF1_BR2-COI sequencing was
205 performed by 2 × 250-bp paired-end sequencing on the Illumina MiSeq platform using default
206 settings at Fasteris (Geneva, Switzerland).

207 Sequencing data were processed using the OBITools (Boyer *et al.* 2016). All libraries
208 were processed using the same pipeline whatever the primer pair used for amplification. Raw
209 sequences were first aligned (program *illuminapairedend*) to recover the amplicon sequence.
210 Based on the alignment score, sequences were filtered for badly aligned paired-end, i.e. all
211 alignment with a score of less than 40 were discarded (a score of 40 corresponds to 10bp of
212 high sequencing quality perfectly aligned). Then sequences were demultiplexed (program
213 *ngsfilter*) to assign them to the samples, primers with up to two errors were allowed whereas
214 the tags used for demultiplexing had to be strictly conserved. The last step was dereplication
215 (program *obiuniq*) keeping track for each unique sequence of its count in the different
216 samples. For each sample, the most abundant sequence was kept as the most likely barcode.
217 Sequences having a count lesser than 1000 or an abundance ratio with the second most
218 abundant sequence above 1/10 were tagged as belonging to badly amplified samples or

samples where several products amplified. These sequences were given particular attention in the further manual selection of barcodes.

As a further validation step, all the retrieved metabarcodes were matched against NCBI using BLAST, to identify eventual cases in which the obtained metabarcode is a spurious amplification of a non-target organism (*e.g.* fungi or algae). For each taxon, the amplification success was measured as the proportion of individuals for which we obtained valid metabarcodes with a given marker. Such measure of amplification success includes the proportion of individuals that cannot be amplified for a given marker (e.g. because of mismatches in the primer region), and also possible lab failures that can reduce amplification even if the primers have a good match with the primer region (e.g. low DNA quality or other issues occurring during laboratory procedures), thus it provides a value comparable to what can occur in real-world biodiversity analyses performed using metabarcoding.

2.2 Setting up the composite reference databases

For each species within the database of European freshwater organisms (Schmidt-Kloiber & Hering 2015), we matched the binomial name with the NCBI taxonomy database to retrieve their NCBI taxonomic code (taxid). All the available metabarcodes for the three regions of interest, together with their associated taxid, were extracted from the EMBL sequence data repository (release 136) using the ecoPCR program (Ficetola *et al.* 2010) by matching the primer sequences with up to three errors and restricting the metabarcodes to relevant lengths (lengths >30 bp for Euka02, lengths 70-270 bp for Inse01, lengths 100-500 for BF1_BR2-COI). The three composite reference databases (one for each metabarcoding region) were then built by aggregating metabarcodes for each genus with those obtained from analysed specimens. In order to obtain the most complete coverage of genera found in France, we

244 obtained the taxid of all metabarcodes produced through *in vitro* analyses as well as
245 metabarcodes extracted from EMBL and associated to the taxid of a species found in France.
246 For genera for which no such metabarcode existed, we included the metabarcodes extracted
247 from EMBL and associated to the taxid of a species of the same genus found in Europe. If no
248 such metabarcode existed, we included all the metabarcodes extracted from EMBL, and
249 associated to a taxid belonging to this genus, also considering species that are not native in
250 Europe.

251

252 ## 2.3 Assessing the resolution of metabarcodes

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254 We assessed the resolution of each metabarcoding region with the same procedure. First, the
255 metabarcodes obtained as described above were compared against the ones of each specimen
256 to find identical metabarcodes; this allowed producing a list of unique metabarcodes. For each
257 unique metabarcode, we obtained the list of all the associated taxids. We tested taxonomic
258 resolution at four levels: order, family, genus, and species. More specifically, we tested if, at a
259 given taxonomic level, the list of associated taxids would collapse to a unique taxid or not (*i.e.*
260 all taxids have the same ancestor taxid at that level). If a list collapsed to more than one taxid
261 for the tested taxonomic level, it meant that this metabarcode was not discriminant for this
262 taxonomic level. Consider for instance a given metabarcode associated to multiple species
263 within multiple genera within one single family. This particular metabarcode showed a
264 family-level resolution, but not a species- or a genus-level resolution. It should be noted that
265 these measures of taxonomic resolution heavily depend on the available database (Weigand *et*
266 *al.* 2019). For example, if the database includes the metabarcode of only one species within a

267 genus, this analysis could return a species-level resolution, even though it is possible that
268 unanalyzed species within the same genus share the same metabarcode.

269

270 2.4 Statistical analyses

271

272 We used generalized linear mixed models (GLMMs) to test the significance of differences in
273 amplification success and resolution among markers. GLMMs allowed taking into account
274 non-independence, i.e. the fact that same individual and the same taxon were tested with
275 multiple markers (Pinheiro & Bates 2000). For the comparison of amplification success, we
276 used binomial GLMMs; the amplification of each individual with each marker was the
277 dependent variable, while marker and class identity were the independent factors. To take into
278 account the possibility that markers can have a different performance across taxa, we also
279 tested the interaction between marker and taxonomic class. We also considered the identity of
280 the individual, and the finest taxonomic information available for each individual (e.g.
281 species, or genus identity if species information was not available) as random effects. The
282 main GLMM detected significant differences among markers and among taxa, therefore we
283 repeated the GLMM for each of the 12 major taxonomic groups considered here (six classes
284 and six insect orders; see Table S1). Significant GLMMs were followed by Tukey's post-hoc
285 tests, to assess differences in performance of the three markers within each taxon (Hothorn *et*
286 *al.* 2008).

287 For the comparison of taxonomic resolutions, we used GLMMs with Gaussian error.
288 The taxonomic resolution of each marker within each taxonomic group was the dependent
289 variable, marker identity was the independent factor, and taxonomic group was included as a
290 random effect. Tukey's post-hoc tests were also used to compare the performance of the three

291 markers (Hothorn *et al.* 2008). GLMMs were built using the *lme4* package in R (Bates *et al.*
292 2015). Significance of GLMMs was assessed using likelihood ratio tests (binomial GLMMs)
293 or using a F test with approximated degrees of freedom (lmerTest Package in R; Kuznetsova
294 *et al.* 2017).

295

296 ## 3 RESULTS

297

298 ### 3.1 Analyses of reference individuals

299

300 We extracted and amplified DNA from 1514 individuals, belonging to 578 different taxa
301 (species, genera or families, depending on the identification level; Table 1). The majority of
302 individuals were insects, and three insect orders with macrobenthic larvae (Ephemeroptera,
303 Plecoptera and Trichoptera) altogether accounted for 80% of all the analyzed individuals. Out
304 of these individuals, 99% were morphologically identified at the family level or higher, 95%
305 at the genus level or higher, and 62% at the species level. The average number of sampled
306 individuals was 2.6 individuals per taxon (range: 1-12; median: 3). For Ephemeroptera,
307 Plecoptera, Trichoptera and Megaloptera, the analyzed specimens covered well the diversity
308 of French and European benthic fauna (100%, 74%, 78% and 100% of genera recorded in
309 France for these orders, respectively; 70%, 52%, 65% and 100% of all the genera recorded in
310 Europe; Table 2). Representation was relatively good for Coleoptera, Hemiptera and
311 Neuroptera, whereas coverage was weaker for the remaining orders of insects and for non-
312 insects.

Sequencing returned a total of 15 328 548 reads for Euka02, 14 872 950 reads for Inse01 and 6 023 376 reads for BF1_BR2-COI. After filtering, the average number of reads per individual was high for all the markers (Euka02: 6800; Inse01: 5951; BF1_BR2-COI: 1801 reads).

The amplification success was significantly different between the three markers and among classes (GLMM: differences between markers: $\chi^2_2 = 736.1$, $P < 0.0001$; differences among classes: $\chi^2_6 = 15.1$, $P = 0.020$). Furthermore, a significant interaction between class and metabarcodes indicated that the performance of markers was heterogeneous across taxonomic classes ($\chi^2_{12} = 164.5$, $P < 0.0001$). Differences in amplification success between markers were significant for all the classes except Crustacea (Fig. 1, Supplementary Table S1). Euka02 showed the highest average amplification success (88%), with consistently high amplification success in all the taxa except Malacostraca (Fig. 1). According to Tukey's post-hoc tests, Euka02 showed a particularly high amplification success for Gastropoda and Clitellata (Table S1). Within insects, Euka02 showed excellent amplification success in most of orders, with particularly good performance for Ephemeroptera and Trichoptera, while its amplification success was significantly lower than the other markers for Diptera (Fig. 1b, Table S1).

As expected, Inse01 showed good amplification success for insects (82%), while it showed a limited amplification for the remaining taxa (Fig. 1a). Within insects, Inse01 showed a particularly good performance for Coleoptera and Diptera, and an amplification success similar to Euka02 for Hemiptera and Plecoptera (Fig. 1b, Table S1). Within insects, the lowest amplification success for this marker was observed for Trichoptera (71%; Fig. 1b).

Finally, BF1_BR2-COI showed an average amplification success of 48%, with highly variable results among taxa (Fig. 1a; Table S1). BF1_BR2-COI showed a relatively good amplification success for Gastropoda, Clitellata and Malacostraca, while the rate was lower

338 for some orders of insects. Within insects, BF1_BR2-COI showed good performance for
339 Coleoptera and Diptera (amplification success ≥ 74%, significantly better than Euka02; Table
340 S1), while it amplified less than 50% of individuals from Ephemeroptera, Plecoptera and
341 Trichoptera (Fig. 1b; Table S1).

342

343 3.2 Combined database

344

345 When we combined sequences of reference individuals with those obtained from GenBank,
346 we obtained a total of 18 834 gene sequences (3 441 for Euka02, 9 715 for Inse01 and 5 678
347 for BF1_BR2-COI). Insects accounted for the majority of sequences, followed by Crustacea
348 and Clitellata (Table 3). The combined database showed a good coverage of the diversity of
349 European benthic fauna. For the Euka02 primer pair, the completeness of the database was
350 particularly good (>80%) for free-living Platyhelminthes, Coleoptera and Odonata. For
351 instance, our database included Euka02 sequences for 35 out of 43 dragonfly genera living in
352 Europe, i.e. 81% of the European fauna. For Inse01, the completeness was particularly good
353 for Coleoptera, Ephemeroptera and Odonata, while BF1_BR2-COI showed a relatively
354 homogeneous completeness across taxa, with values between 50 and 70% for most taxa (Fig.
355 2).

356

357 3.3 Taxonomic resolution of the different markers

358

359 The taxonomic resolution was strongly different among markers. At the species level, the best
360 resolution was observed for BF1_BR2-COI, with just 3% of sequences associated with more

than one species, while Inse01 showed an intermediate resolution (10% of sequences associated with more than one species; Fig. 3a). For Euka02, 21% of sequences were associated with more than one species in the database (Fig. 3a). Differences in resolution between markers were strongly significant (linear mixed models: $F_{2,12} = 271.8$, $P < 0.001$). The resolution of BF1_BR2-COI was significantly better than the one of both Euka02 and Inse01, and the resolution of Inse01 was significantly better than the one of Euka02 (Tukey's post-hoc: both $P < 0.001$).

The taxonomic resolutions of these markers were clearly better if we consider the identification at the genus level (Fig. 3b). Euka02 showed the weakest performance, with around 6% of sequences associated with more than one genus, while BF1_BR2-COI showed the best performance, with less than 1% of sequences associated with more than one genus. Inse01 showed a generally good performance, with less than 1% of sequences associated with more than one genus for most taxa. The performance was slightly poorer for Plecoptera and Trichoptera, with around 4% of sequences associated with more than one genus. Also in this case, differences in resolution among markers were significant ($F_{2,8} = 32.3$, $P < 0.001$). At the genus level, BF1_BR2-COI and Inse01 showed a similar resolution (Tukey's post hoc: $P = 0.20$), and both outperformed Euka02 (both $P < 0.001$). Family level identification was very good for all the metabarcodes, still Euka02 showed a slightly weaker performance than both BF1_BR2-COI and Inse01 (both $P \leq 0.05$). BF1_BR2-COI and Inse01 showed a comparable family-level resolution ($P = 0.24$; Fig. 3c). It should be noted that these values of resolution are calculated on an incomplete set of data, since our database did not include the sequences of many species and genera. For instance, our database only included sequences for ~60% of genera of European Trichoptera (Table 3), and all resolution estimates would probably be poorer if calculated on a complete database.

386 **4 DISCUSSION**

387

388 Metabarcoding-based biomonitoring requires the availability of primers with high
389 performance, as they must amplify all the relevant target taxa, have sufficient resolution to
390 identify them at the desired taxonomic level, and amplify short sequences usable with eDNA
391 (Ficetola *et al.* 2010; Taberlet *et al.* 2018). Finding primers with all these features is
392 challenging, and the identification of "perfect" regions for barcoding and metabarcoding has
393 often been labelled as a "search for the Holy Grail" (Rubinoff *et al.* 2006). By combining an
394 extensive high-throughput DNA barcoding analysis with the assessment of publicly available
395 sequences, our study highlights the complexity of finding all these desired features in one
396 single metabarcode. It also provides a comparison of performances, allowing the
397 identification of most appropriate markers for different aims and taxonomic groups, and it
398 produced a reference database for the taxonomic identification of a large number of benthic
399 insects.

400

401 **4.1 The importance of good reference databases**

402 Metabarcoding enables biodiversity monitoring either with or without the taxonomic
403 identification of the retrieved taxa. Taxonomic identification clearly requires appropriate
404 reference databases that can be obtained *ad hoc* (*e.g.* by amplifying sequences from all the
405 taxa from the target group; (Moriniere *et al.* 2017; Cilleros *et al.* 2019; Morinière *et al.* 2019)
406 or by searching public databases such as GenBank or BOLD. Public databases offer an ever-
407 growing resource, given that they combine the outcome of thousands of studies and produce a
408 sheer amount of data that would be unreachable by *ad hoc* studies. Public databases are not
409 error-free, still analyses showed that for animals, the error rate of GenBank for genus-level

410 identification is generally low (~0.7 / 3.5%), suggesting that it can be a formidable data
411 source for applications relying on molecular data to understand the impact of environmental
412 changes on biodiversity (Leray *et al.* 2019). However, public databases are opportunistic
413 collections of the material from multiple studies, thus they do not have the ambition of
414 taxonomic completeness. *Ad-hoc* databases (see also Ratnasingham & Hebert 2007) are thus
415 essential resources to obtain the taxonomic coverage required if we want to identify most of
416 benthic macroinvertebrates.

417 Several researchers advocated that COI-based markers should be favored for
418 metabarcoding because they are standard barcodes for animals, and thus we can expect a very
419 large availability of sequences in reference databases (Andújar *et al.* 2018; Leray *et al.* 2019).
420 For benthic macroinvertebrates, a very large number of COI sequences is available in
421 GenBank (Table 3). For instance, BF1_BR2-COI is largely the marker with the highest
422 number of sequences of benthic Diptera, with nearly 3,000 sequences of BF1_BR2-COI
423 available against just 1000 sequences of Inse01 (16S rDNA), still the number of available
424 sequences is surprisingly variable across taxa. Nevertheless, a very large number of sequences
425 does not necessarily allow a better taxonomic coverage. In fact, most of genera of benthic
426 Diptera do not have GenBank sequences for COI, and Inse01 sequences represent slightly
427 more genera than BF1_BR2-COI (25% for Inse01 against just 15% for BF1_BR2-COI; Fig.
428 3). The mismatch between number of sequences and database completeness could be related
429 to the different scopes of studies employing the different markers. COI is the most used
430 marker by standard barcoding studies, which often aim at unveiling diversity among closely
431 related, cryptic taxa, thus studies often consider many individuals from closely related,
432 morphologically similar species within genera (Hebert *et al.* 2004). Conversely, the 16S and
433 18S rDNA genes are often used to build phylogenies (*e.g.* Alvarez-Presas *et al.* 2008;
434 Criscione & Ponder 2013), and many phylogenetic studies aim at representing the largest

435 number of genera and families. Such process could also explain the strong differences among
436 taxa (*e.g.* a very high completeness for Euka02 with free-living Platyhelminthes, and the
437 better coverage for Inse01 with Gastropoda; Fig. 2). If the aim is the species-level
438 identification, databases should include all the species and markers should have a species-
439 level resolution. Luckily, for freshwater biomonitoring a genus-level identification is often
440 enough (Bailey *et al.* 2001; Mistri & Rossi 2001; Chessman *et al.* 2007; Birk *et al.* 2012),
441 thus our database provides a good completeness that can allow the identification of most of
442 genera, particularly with the markers Euka02 and Inse01. Here we tested our markers against
443 the GenBank database only, because it includes sequences from our three genomic regions.
444 However, an impressive number of additional COI sequences is available at the BOLD
445 database. Integrating GenBank and BOLD data can certainly improve the resolution of COI-
446 based metabarcoding studies.

447

448 **4.2 Metabarcoding without taxonomic identification**

449

450 Metabarcoding can provide ecological information even if reference databases are not
451 available, as molecular taxonomic units can allow the comparison of communities among
452 sites with environmental differences (Ji *et al.* 2013; Apothéloz-Perret-Gentil *et al.* 2017;
453 Cordier *et al.* 2019). The taxonomy-free approach allows overcoming the fact that, despite
454 intensive efforts, databases remain incomplete for many taxa (Fig. 3). Primers with high
455 taxonomic coverage and resolution are essential also in this case. High taxonomic coverage is
456 needed to avoid under-representation of some taxa, while resolution allows teasing apart
457 related taxa. Related taxa can have very different ecological properties, and some widespread
458 taxa, tolerant to human disturbance, can be closely related to highly sensitive specialists (Caro

459 *et al.* 2005). Therefore, ecological responses of communities can remain obscured if
460 metabarcodes are not able to resolve related taxa with different ecology. Our study focused on
461 European taxa, where taxonomic knowledge is particularly good (Moustakas & Karakassis
462 2005; Rodrigues *et al.* 2010; Brewer *et al.* 2012) and, with targeted studies, we could envisage
463 an improvement of database completeness in the next years. However, our results on primer
464 performance can be also useful in megadiverse, tropical areas, where taxonomy-free
465 biomonitoring is a viable option (Andersen *et al.* 2019).

466

467 **4.3 Universality and resolution of primers**

468

469 Our analysis did not identify one single outperforming metabarcode. The universality of
470 primers was variable among taxa, with Euka02 showing the highest performance for some
471 phyla (platyhelminthes, molluscs, annelids and even some arthropods), and Inse01 showing a
472 generally good performance for insects. However, each of these metabarcodes has some
473 drawbacks. For instance, Euka02 amplifies very long sequences for some taxa of crustaceans
474 (Isopoda and Amphipoda; Guardiola *et al.* 2015; Taberlet *et al.* 2018) thus their
475 metabarcoding with this marker is problematic. Conversely, Inse01 is a marker developed
476 specifically for insects, and fails to amplify key freshwater taxa such as free-living
477 Platyhelminthes and molluscs (Fig. 1). In our analysis, BF1_BR2-COI showed a moderate
478 amplification success, still for insects a relevant proportion of individuals was not amplified
479 (Fig. 1). This is in contrast with previous analyses that successfully amplified 100% of tested
480 insects using BF1_BR2-COI (Elbrecht & Leese 2017). Differences might be due to DNA
481 quality, as this primer amplifies relatively long metabarcodes (>300 bp). Some of our >1500
482 specimens were old, and this can cause DNA degradation, while the starting material of

Elbrecht and Leese (2017) was probably of better quality. In fact, a few species (*Ephemerella mucronata*, *Torleya major* and *Odontocerum albicorne*) were successfully amplified by Elbrecht & Leese (2017), while failed amplification here. Furthermore, in several cases BF1_BR2-COI did not amplify the DNA of our target organisms, but amplified the DNA of contaminants, i.e. other organisms for which small body fragments were probably present in the tube, and that perhaps showed excellent match with the primers. Unfortunately, these conditions (degraded DNA, and simultaneous presence of many organisms) are typical of eDNA metabarcoding studies, stressing the complexity of finding appropriate primers.

Differences in performance were also strong when considering the resolution of the markers. BF1_BR2-COI clearly showed the best resolution while Euka02 showed a very poor performance, as in many cases it failed even at the family level (Fig. 3; see also Tang *et al.* 2012). COI is a highly variable region, and this has promoted its use as standard barcode for animals (Hebert *et al.* 2003; Hebert *et al.* 2004; Andújar *et al.* 2018). The excellent performance of BF1_BR2-COI can also be explained by the relatively long amplified region. Inse01 showed an intermediate performance, as its resolution was insufficient for species-level identification, while genus level identification was good for most of taxa (Fig. 3). It must be remarked that these are optimistic values of resolution, given that our database was far from complete, particularly at the genus-level and for some taxa, therefore a more complete database could yield poorer resolution values.

4.4 No Holy Grail for macrobenthos metabarcodes?

The heterogeneous performance of the different markers highlights the complexity of the identification of the best metabarcodes. No primer showed the best performance for all the

507 considered metrics, as the most "universal" marker (Euka02) showed a generally poor
508 resolution, while the marker with the highest resolution (BF1_BR2-COI) did not successfully
509 amplify several taxa. The selection of metabarcodes for biomonitoring is therefore a trade-off,
510 depending on the aims of studies. Euka02 can allow a good assessment of overall
511 biodiversity, but it is unable to tease apart closely related taxa, thus it might be not enough to
512 define the ecological status of environments. Furthermore, the poor resolution would hamper
513 the comparison with historical data for most of taxa. Conversely, the excellent resolution of
514 BF1_BR2-COI could allow species-level identification, and might have more power to
515 distinguish different communities. However, this comes at a cost. Several taxa did not amplify
516 either because the level of DNA degradation compromises the amplification of a relatively
517 long metabarcode, or because the poor match of the primer(s) with their target. In fact, the
518 relatively long amplified region could limit its usefulness for application with environmental
519 DNA extracted from water. Finally, Inse01 showed a generally good performance, but it is not
520 appropriate for many non-insect taxa.

521 Given these limitations, it is unlikely that one single metabarcode will be able to fully
522 replace the traditional biomonitoring using macrobenthic macroinvertebrates. Nevertheless,
523 the data obtained through multiple metabarcodes can be integrated for a more comprehensive
524 and accurate understanding of ecological impacts on freshwater biodiversity. For instance,
525 highly universal markers, providing a complete but coarse picture of animal biodiversity (*e.g.*
526 Euka02) can be combined with markers providing a specific focus on key taxa (*e.g.* Inse01) or
527 a high-resolution level (*e.g.* BF1_BR2-COI). The integration of multiple metabarcodes
528 certainly increases the cost and complexity of studies, still it has the potential to provide an
529 unprecedented amount of data, thus opening unexplored avenues to biodiversity assessment.

530

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532

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539

540 **Competing interest**

541 A.V. and T.D. are research scientists at a private company specializing in the use of eDNA for
542 species detection.

543

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771

772

Data Accessibility:
The complete database is available at fighshare:

https://doi.org/10.6084/m9.figshare.12046242.v1

The filtered sequences generated by the project with length >100 bp are also available on GenBank (Euka02: accession numbers LR824819 to LR826155; Inse01: LR826156 to LR827357, BF1-BR2 COI: LR827358 to LR828086).

Author Contributions

Designed the research: Pierre Taberlet, Tony Dejean, Gentile Francesco Ficetola, Frédéric Boyer, Alice Valentini, Aurélie Bonin, Albin Meyer, Philippe Usseglio-Polatera

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Analyzed data: Gentile Francesco Ficetola, Frédéric Boyer, Aurélie Bonin

Wrote the paper: Gentile Francesco Ficetola wrote the first draft of the paper, with subsequent contribution of all the authors

791 Figure legends

792

793 Figure 1. Amplification success of the three markers across benthic macroinvertebrate taxa.
794 A): all taxa; B): insects only. Error bars are standard errors of the mean; * indicate significant
795 differences between markers. See Table S2 for the raw values used to build the plot.

796

797 Figure 2. Completeness of the combined database, combining the sequences produced in this
798 study with sequences retrieved from public databases. For each taxon, the plots report the
799 proportion of European genera of macroinvertebrates with at least one sequence in the
800 database.

801

802 Figure 3. Resolution of the three markers at the species, genus and family levels. The
803 resolution is measured as the proportion of metabarcodes that are associated with a) at least
804 two species; b) at least two genera; c) at least two families (non-identification), therefore low
805 values of non-identification indicate a better performance of the markers.

806

807 Table 1. Inventory of macrobenthos individuals from which we extracted and amplified DNA.

808

Class / subphylum / phylum		*N* individuals	*%* identified at the genus level by taxonomists	*N* genera
Platyhelminthes		7	100%	2
Bivalvia		12	100%	5
Gastropoda		29	97%	11
Clitellata		35	69%	8
Arachnida		11	9%	1
Crustacea		9	100%	4
	Coleoptera	117	97%	40
	Diptera	54	20%	6
	Ephemeroptera	338	100%	35
	Hemiptera	24	100%	14
Insecta	Lepidoptera	2	100%	2
	Megaloptera	4	100%	1
	Neuroptera	2	100%	1
	Odonata	9	78%	2
	Plecoptera	210	100%	20
	Trichoptera	651	100%	84

809

810

811 Table 2. Representativeness of reference individuals used for analyses, relative to European

812 and French genera of benthic macroinvertebrates.

813

Class / subphylum / phylum	Order (insects only)	*N* genera Europe	*N* Genera France	*N* genera for *in vitro* analyses
Platyhelminthes		29	23	2
Bivalvia		18	11	5
Gastropoda		65	34	11
Clitellata		102	64	8
Arachnida*		1	1	1
Hydracnidia		56	52	0
Crustacea		72	34	4
	Coleoptera	127	95	40
	Diptera	522	323	6
	Ephemeroptera	50	35	35
	Hemiptera	30	28	14
Insecta	Lepidoptera	8	5	2
	Megaloptera	1	1	1
	Neuroptera	3	3	1
	Odonata	43	36	2
	Plecoptera	38	27	20
	Trichoptera	130	108	84

814 * except Hydracnidia

815

816

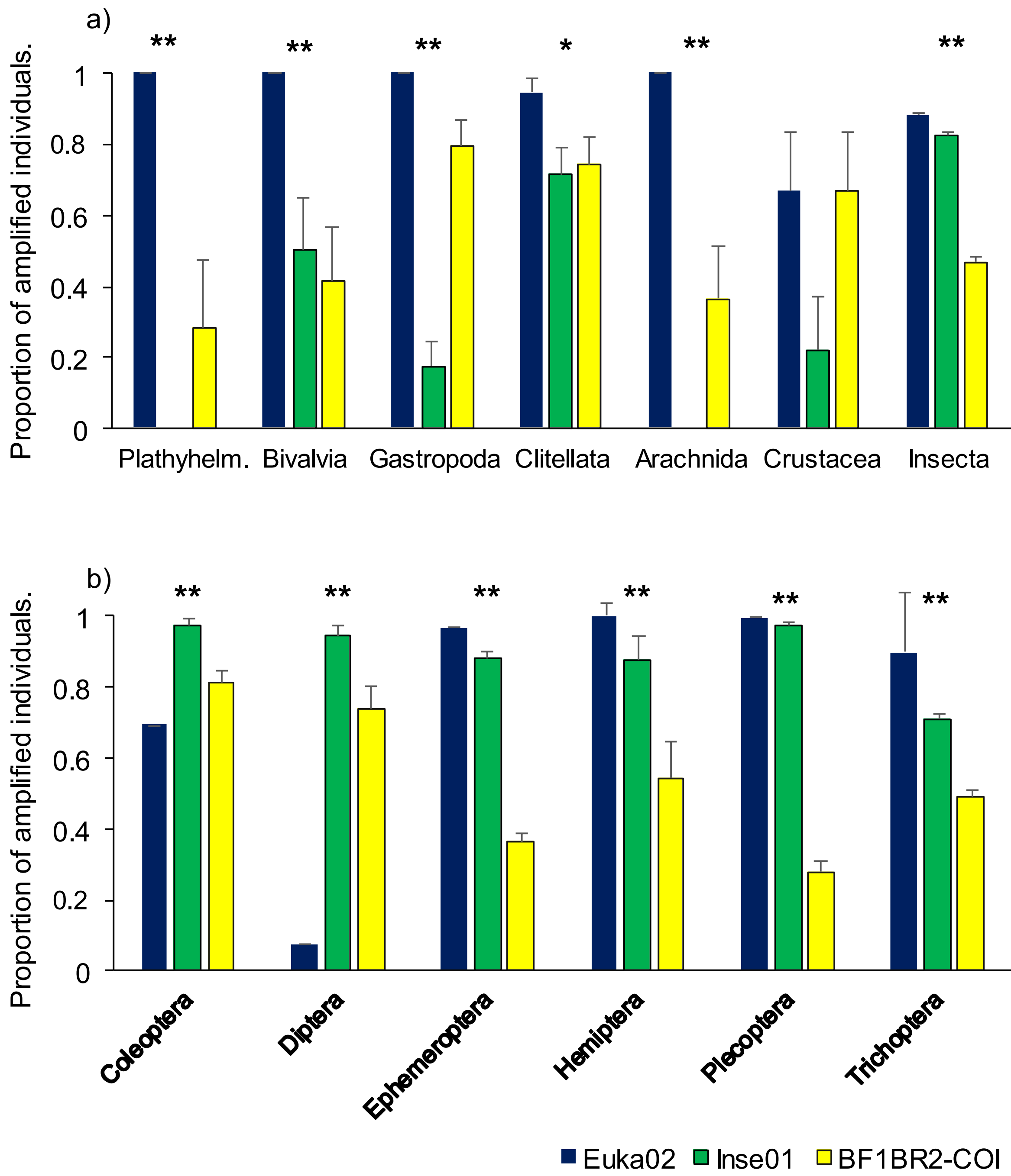
Table 3. Number of sequences and genera represented in the combined database, across taxa. Taxa for which >70% of European genera are represented in the database are highlighted in bold.

Class / subphylum / phylum	Order (insects only)	*N* sequences in the database			*N* genera Europe	*N* genera in the database			
		Euka02	Inse01	COI		Euka02	Inse01	COI	Total
Hydrozoa		31	134	21	6	**5**	**5**	3	**5**
Enopla (Nemertini)		4	1	-	1	**1**	**1**	0	**1**
Platyhelminthes		217	25	316	29	**25**	9	8	**26**
Gordioida (Nematomorpha)		9	-	-	1	**1**	-	-	**1**
Bivalvia		66	453	125	18	**14**	9	11	**16**
Gastropoda		81	1147	102	65	32	**46**	31	**51**
Clitellata		414	838	170	102	**79**	70	50	**86**
Polychaeta		33	74	64	11	**8**	4	2	**8**
Gymnolaemata (Bryozoa)		23	44	-	3	2	2	-	2
Phylactolaemata (Bryozoa)		17	36	1	6	**6**	**6**	1	**6**
Arachnida*		2	2	4	1	**1**	**1**	**1**	**1**
Hydrachnidia		70	-	15	56	24	0	8	24
Crustacea		325	1980	303	72	**61**	47	34	**65**
Insecta		2149	4981	4557	981	385	456	327	530
	Coleoptera	450	1809	333	127	**107**	**107**	68	**115**
	Diptera	228	1078	2839	522	64	131	77	170
	Ephemeroptera	396	507	397	50	**44**	**43**	29	**44**
	Hemiptera	46	206	70	30	19	**22**	16	**24**
	Hymenoptera	4	21	33	29	3	4	5	9
	Lepidoptera	2	4	5	8	2	3	3	3
	Megaloptera	9	10	6	1	**1**	**1**	**1**	**1**
	Neuroptera	6	12	5	3	**3**	**3**	2	**3**
	Odonata	164	537	294	43	**35**	**39**	27	**41**
	Plecoptera	243	233	118	38	24	24	21	26
	Trichoptera	601	564	457	130	83	79	78	**94**

* except Hydracnidia

825 Figure 1

826



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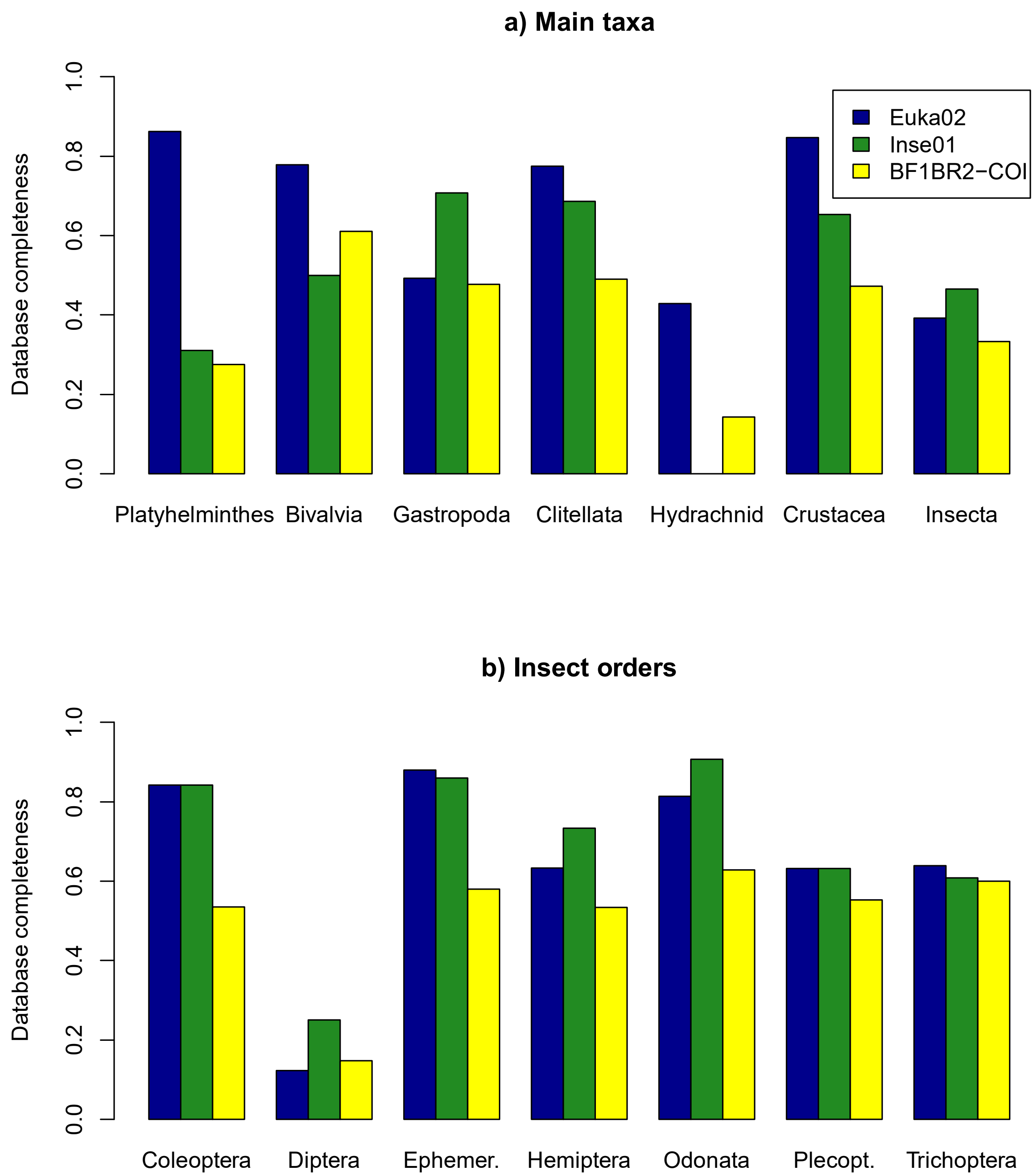
828

829 Figure 2



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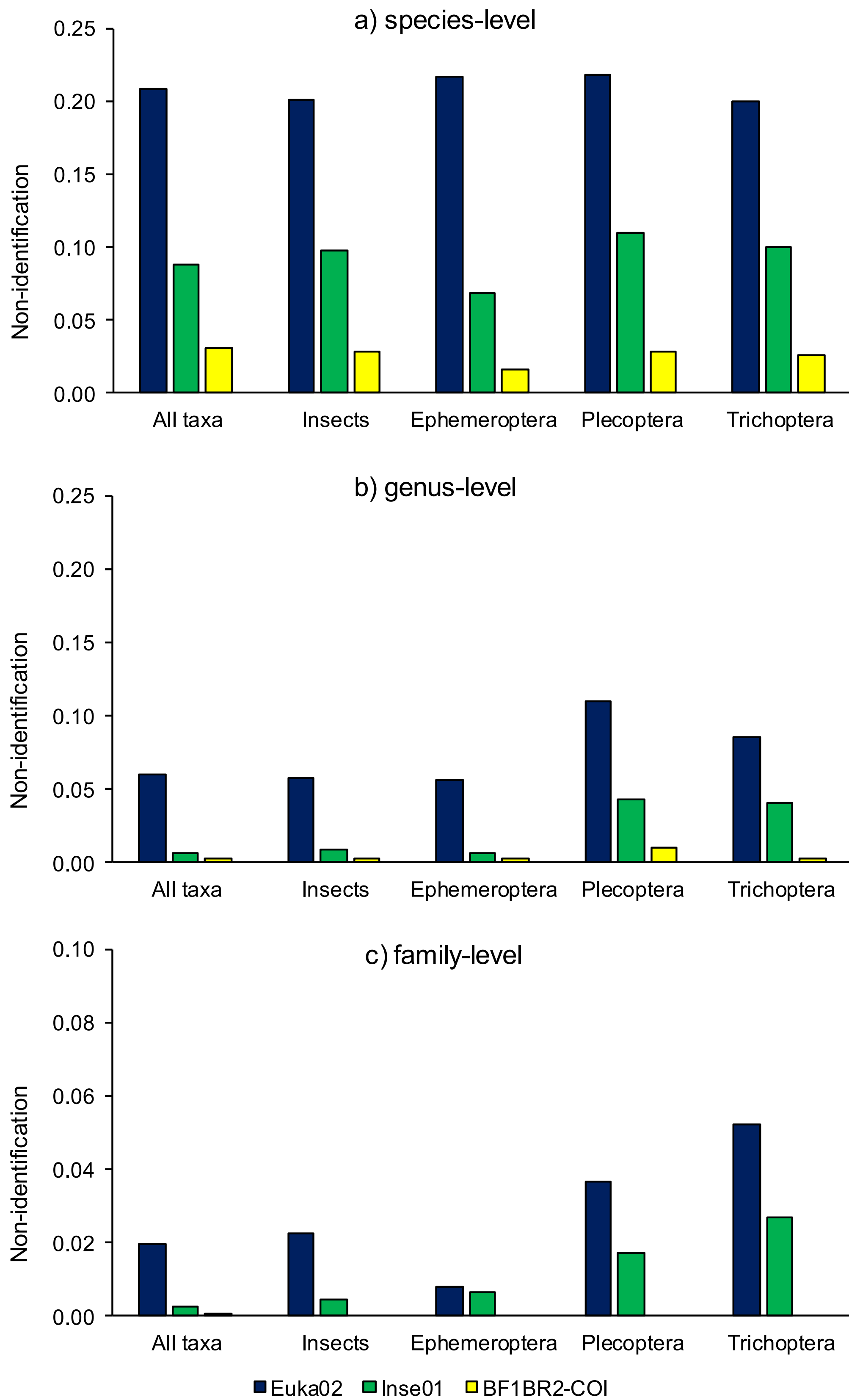
831

832 Figure 3

833



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