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

Gentile Francesco Ficetola[1,2] | Frédéric Boyer[1] | Alice Valentini[3] | Aurélie Bonin[1,2] | Albin Meyer[4] | Tony Dejean[3] | Coline Gaboriaud[3] | Philippe Usseglio-Polatera[4] | Pierre Taberlet[1,5]

[1]LECA, Laboratoire d'Ecologie Alpine, Univ. Grenoble Alpes, CNRS, Univ. Savoie Mont Blanc, Grenoble, France

[2]Department of Environmental Sciences and Policy, University of Milano, Milano, Italy

[3]SPYGEN, Le Bourget du Lac, France

[4]Université de Lorraine, CNRS, LIEC, Metz, France

[5]UiT – The Arctic University of Norway, Tromsø Museum, Tromsø, Norway

Correspondence
Gentile Francesco Ficetola, Univ. Grenoble Alpes, CNRS, Univ. Savoie Mont Blanc, LECA, Laboratoire d'Ecologie Alpine, Grenoble, France.
Email: francesco.ficetola@gmail.com

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Abstract

Metabarcoding of bulk or environmental DNA has great potential for biomonitoring of freshwater environments. However, successful application of metabarcoding to biodiversity monitoring requires universal primers with high taxonomic coverage that amplify highly variable, short metabarcodes with high taxonomic resolution. Moreover, reliable and extensive reference databases are essential to match the outcome of metabarcoding analyses with available taxonomy and biomonitoring indices. Benthic invertebrates, particularly insects, are key taxa for freshwater bioassessment. Nevertheless, few studies have so far assessed markers for metabarcoding of freshwater macrobenthos. Here we combined in silico and laboratory analyses to test the performance of different markers amplifying regions in the 18S rDNA (Euka02), 16S rDNA (Inse01) and COI (BF1_BR2-COI) genes, and developed an extensive database of benthic macroinvertebrates of France and Europe, with a particular focus on key insect orders (Ephemeroptera, Plecoptera and Trichoptera). Analyses on 1,514 individuals representing different taxa of benthic macroinvertebrates showed very different amplification success across primer combinations. The Euka02 marker showed the highest universality, while the Inse01 marker showed excellent performance for the amplification of insects. BF1_BR2-COI showed the highest resolution, while the resolution of Euka02 was often limited. By combining our data with GenBank information, we developed a curated database including sequences representing 822 genera. The heterogeneous performance of the different primers highlights the complexity in identifying the best markers, and advocates for the integration of multiple metabarcodes for a more comprehensive and accurate understanding of ecological impacts on freshwater biodiversity.

KEYWORDS

amplification rate, biomonitoring, biotic indices, cytochrome c oxidase I, environmental DNA, freshwater biodiversity, macroinvertebrates, primer bias, taxonomic resolution, universality

1 | INTRODUCTION

Freshwater environments are essential providers of clean water and other services for human society. They also host a substantial biodiversity, yet are globally subjected to the joint impact of multiple stressors such as pollution, eutrophication, climate change, and hydrological and hydromorphological modifications (Iversen et al., 2019; Noges et al., 2016). As a consequence, numerous regulations have been adopted at both the national and the 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 (a) they are taxonomically, biologically and functionally diverse (Usseglio-Polatera et al., 2000, 2001), (b) they are quite easy to identify at the genus or family levels (Tachet et al., 2010), (c) they are often sedentary and react rapidly to anthropogenic pressures in all types of freshwater bodies (Archaimbault et al., 2010; Hering et al., 2006, 2013) and (d) 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, 2013; Theodoropoulos et al., 2020) or restoration (Arce et al., 2014; Camargo, 2017; Carlson et al., 2018; Kupilas et al., 2016).

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

DNA metabarcoding and environmental DNA (eDNA) are revolutionizing the monitoring of biodiversity at all levels, because they circumvent the challenge of morphological identification and allow the efficient detection of many taxa that are difficult to capture and detect using traditional methods (Taberlet et al., 2018). eDNA and metabarcoding are therefore extremely promising for the assessment of freshwater communities (Hering et al., 2018; Kuntke et al., 2020; Li et al., 2018). For metabarcoding, DNA can be extracted from the tissue of pooled macroinvertebrate communities, amplified using universal primers, sequenced and identified on the basis of reference databases (Andújar et al., 2018; Baird & Hajibabaei, 2012; Yu et al., 2012). This approach uses the same starting material as traditional biomonitoring, but avoids the complexity of morphology-based taxonomy (Baird & Hajibabaei, 2012). Alternatively, DNA can be obtained directly from the water (Ficetola et al., 2008). eDNA extracted from freshwater allows the detection of many taxa that are difficult to capture and detect using traditional methods, but also poses new challenges compared to metabarcoding performed on the tissues of captured individuals. In aquatic environments, DNA undergoes rapid degradation (Buxton et al., 2017; Eichmiller et al., 2016); therefore, eDNA is generally characterized by short fragment sizes (Jo et al., 2017; Bylemans et al., 2018; but see also Sigsgaard et al., 2017). Therefore, primers amplifying short regions can provide a better detection compared to "standard" barcode primers, which often amplify long DNA fragments (e.g., >300 bp in the most frequently used COI markers; Andújar et al., 2018). Furthermore, highly degenerated primers increase the risk of nonspecific amplification, and thus this kind of primer is not really suitable for amplification of the complex mix of DNA extracted from the environment. As a consequence, the monitoring of benthic macroinvertebrates using eDNA requires the development and assessment of primers with appropriate features.

Besides the length of the amplified region, three main characteristics are essential for satisfactory eDNA metabarcodes (Ficetola et al., 2010; Wilcox et al., 2013). First, eDNA amplification success generally decreases with the number of mismatches between target fragments and primers. Primers must therefore be designed to have a consistently low number of mismatches within sequences of the target group (high universality or taxonomic coverage; Ficetola et al., 2010; Marquina et al., 2019; Piñol et al., 2015). Taxonomic coverage can be assessed through both in silico and laboratory analyses. In silico analyses can allow the rapid assessment of all the taxa for which information is publicly available in databases, but laboratory tests (hereafter, in vitro tests) are still needed to confirm the actual performance of primers. Second, the amplified region must be highly variable, to ensure the identification of amplified organisms at the desired taxonomic level (high resolution; Ficetola et al., 2010; Marquina et al., 2019; Tang et al., 2012). Finally, extensive databases are essential if we want to assign the amplified sequences to known taxa. Although attempts have been made to assess environmental quality without a taxonomic assignment of DNA fragments (Apothéloz-Perret-Gentil et al., 2017; Cordier et al., 2018, 2019; Ji et al., 2013), taxonomic assignment is essential if we want to produce data comparable with traditional indices of water quality, or if we want to combine eDNA data with information obtained through

traditional methods (e.g., to analyse long-term series of water body surveys). Despite several attempts to assess freshwater quality using eDNA (Czechowski et al., 2020; Hering et al., 2018; Li et al., 2018; Pont et al., 2020; Serrana et al., 2019; Yang & Zhang, 2020), so far limited formal comparisons have been performed among short primers suitable for eDNA metabarcoding of freshwater macrobenthos (but see Elbrecht & Leese, 2017; Elbrecht et al., 2016). In addition, there is a pressing need for exhaustive reference databases for taxonomic assignment (for remarkable examples, see Morinière et al., 2019; Moriniere et al., 2017; Weigand et al., 2019).

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

2 | MATERIAL AND METHODS

We used the standardized database of European freshwater organisms (Schmidt-Kloiber & Hering, 2015; download March 1, 2018) as a taxonomic reference for our analyses, considering all the benthic macroinvertebrates. Although in some cases this database considers nonmonophyletic groups (e.g., Crustacea), it provides an exhaustive checklist of benthic macroinvertebrates that serve as an essential basis for bioassessment and monitoring.

2.1 | Analyses of reference individuals

Most of the reference individuals were provided by OPIE-Benthos, which is a working group of OPIE (Office Pour les Insectes et leur Environnement) dedicated to aquatic insect studies and aquatic ecosystem protection in France. OPIE-Benthos has developed a national inventory and reference collection of aquatic insects (http://www.opie-benthos.fr/opie/insecte.php), including Ephemeroptera, Plecoptera and Trichoptera, and more recently aquatic Coleoptera, aquatic and semi-aquatic Heteroptera, and aquatic larval stages of Megaloptera, Neuroptera and Diptera (Ptychopteridae). Corresponding organisms, identified at the highest possible level (species, if possible) by experienced taxonomists, were provided in triplicate (i.e., three individuals per taxon, if possible). The collection was completed by additional taxa (e.g., noninsect taxa) specifically sampled by the authors for this reference database.

Individuals were stored in 99% ethanol before DNA extraction. Total DNA was extracted from the entire organism. Samples (consisting of one individual) were initially incubated overnight at 56°C in 0.5 ml of lysis buffer (Tris-HCl 0.1 m, EDTA 0.1 m, NaCl 0.01 m and *N*-lauroyl sarcosine 1%, pH 7.5–8.0). Extractions were then completed using the DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions. DNA extracts were recovered in a total volume of 300 μl of elution buffer. Negative extractions without individuals were systematically performed to monitor possible contaminations. Three DNA amplifications were carried out for each sample using the following primer pairs: Inse01, amplifying an ~ 155-bp region of the 16S mitochondrial rDNA (Taberlet et al., 2018); Euka02, amplifying an ~ 123-bp region of the 18S rDNA (Guardiola et al., 2015; Taberlet et al., 2018); and BF1 and BR2, which amplify an ~316-bp region of the cytochrome *c* oxidase I (COI; Elbrecht & Leese, 2017). Inse01 has been developed mostly to amplify insects, Euka02 to amplify all eukaryotes, while BF1 and BR2 were designed to amplify freshwater macroinvertebrates (Elbrecht & Leese, 2017; Taberlet et al., 2018). The DNA of each individual was amplified in one PCR (polymerase chain reaction) replicate per each marker considered. DNA amplifications were performed in a final volume of 20 μl, using 2 μl of DNA extract as template. The amplification mixture contained 10 μl of Applied Biosystems Master Mix AmpliTaq Gold 360, 0.2 μg μL^{-1} of bovine serum albumin (BSA, Roche Diagnostic) and 0.5 μm of each primer for COI and Inse01, or 0.2 μm of each primer for Euka2. Forward and reverse primers were 5′-labelled with eight-nucleotide tags with at least three differences between any pair of tags, so that each PCR was identified by a unique combination of tags. This allowed the assignment of each sequence to the corresponding sample during sequence analysis (Coissac, 2012; Taberlet et al., 2018). The PCR mixture was denatured at 95°C for 10 min, followed by 35 cycles of 30 s at 95°C, 30 s at 52°C for COI and Inse01 or 45°C for Euka2, and 1 min at 72°C (1 min 30 s for COI), and followed by a final elongation at 72°C for 7 min. Ten negative DNA extraction and 18 PCR controls (ultrapure water) were analysed in parallel with the samples to monitor possible contamination during the experimental process.

Library preparation was performed using the MetaFast protocol by Fasteris (https://www.fasteris.com/dna/?q=content/metafast-protocol-amplicon-metagenomic-analysis), which significantly limits the tag-jump problem (Taberlet et al., 2018). For Euka02 and Inse01, sequencing was performed by 2 × 125-bp paired-end sequencing on the Illumina HiSeq 2,500 platform, while for BF1_BR2-COI sequencing was performed by 2 × 250-bp paired-end sequencing on the Illumina MiSeq platform using default settings at Fasteris.

Sequencing data were processed using OBITOOLS (Boyer et al., 2016). All libraries were processed using the same pipeline whatever the primer pair used for amplification. Raw sequences were first aligned (program ILLUMINAPAIREDEND) to recover the amplicon

sequence. Based on the alignment score, sequences were filtered for poorly aligned paired-ends (i.e., all alignments with a score of less than 40 were discarded [a score of 40 corresponds to 10 bp of high sequencing quality perfectly aligned]). Then sequences were demultiplexed (program NGSFILTER) to assign them to the samples, primers with up to two errors were allowed whereas the tags used for demultiplexing had to be strictly conserved. The last step was dereplication (program OBIUNIQ) keeping track for each unique sequence of its count in the different samples. For each sample, the most abundant sequence was kept as the most likely barcode. Sequences having a count lower than 1,000 or an abundance ratio with the second most abundant sequence above 1/10 were tagged as belonging to poorly amplified samples or samples where several products amplified. These sequences were of particular note 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 nontarget organism (e.g., fungi or algae). For each taxon, amplification success was measured as the proportion of individuals for which we obtained valid metabarcodes with a given marker. Such a 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 laboratory 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), and thus it provides a value comparable to that 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 (>30 bp for Euka02, 70–270 bp for Inse01, 100–500 bp 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. To obtain the most complete coverage of genera found in France, we obtained the taxid of all metabarcodes produced through in vitro analyses as well as metabarcodes extracted from EMBL and associated with the taxid of a species found in France. For genera for which no such metabarcode existed, we included the metabarcodes extracted from EMBL and associated with the taxid of a species of the same genus found in Europe. If no such metabarcode existed, we included all the metabarcodes extracted from EMBL, and associated with a taxid belonging to this genus, also considering species that are not native to Europe.

2.3 | Assessing the resolution of metabarcodes

We assessed the resolution of each metabarcoding region with the same procedure. First, the metabarcodes obtained as described above were compared against those of each specimen to find identical metabarcodes; this allowed us to produce a list of unique metabarcodes. For each unique metabarcode, we obtained the list of all associated taxids. We tested taxonomic resolution at four levels: order, family, genus and species. More specifically, we tested if, at a given taxonomic level, the list of associated taxids would collapse to a unique taxid or not (i.e., all taxids have the same ancestor taxid at that level). If a list collapsed to more than one taxid for the tested taxonomic level, it meant that this metabarcode was not discriminant for this taxonomic level. Consider for instance a given metabarcode associated with multiple species within multiple genera within one single family. This particular metabarcode showed a family-level resolution, but not a species- or a genus-level resolution. Note that these measures of taxonomic resolution depend heavily on the available database (Weigand et al., 2019). For example, if the database includes the metabarcode of only one species within a genus, this analysis could return a species-level resolution, even though it is possible that unanalysed species within the same genus share the same metabarcode.

2.4 | Statistical analyses

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

For the comparison of taxonomic resolution, we used GLMMs with Gaussian error. The taxonomic resolution of each marker within

each taxonomic group was the dependent variable, marker identity was the independent factor, and taxonomic group was included as a random effect. Tukey's post-hoc tests were also used to compare the performance of the three markers (Hothorn et al., 2008). GLMMs were built using the LME4 package in R (Bates et al., 2015). Significance of GLMMs was assessed using likelihood ratio tests (binomial GLMMs) or using an *F* test with approximated degrees of freedom (LMERTEST Package in R; Kuznetsova et al., 2017).

3 | RESULTS

3.1 | Analyses of reference individuals

We extracted and amplified DNA from 1,514 individuals, belonging to 578 different taxa (species, genera or families, depending on the identification level; Table 1). The majority of individuals were insects, and three insect orders with macrobenthic larvae (Ephemeroptera, Plecoptera and Trichoptera) altogether accounted for 80% of all individuals analysed. Of these individuals, 99% were morphologically identified at the family level or higher, 95% at the genus level or higher, and 62% at the species level. The average number of sampled individuals was 2.6 per taxon (range: 1–12; median: 3). For Ephemeroptera, Plecoptera, Trichoptera and Megaloptera, the analysed specimens covered well the diversity of French and European benthic fauna (100%, 74%, 78% and 100% of genera recorded in France for these orders, respectively; 70%, 52%, 65% and 100% of all the genera recorded in Europe; Table 2). Representation was relatively good for Coleoptera, Hemiptera and Neuroptera, whereas coverage was weaker for the remaining orders of insects and for noninsects.

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: 6,800; Inse01: 5,951; BF1_BR2-COI: 1,801 reads).

Amplification success was significantly different between the three markers and among classes (GLMM: differences between markers: $\chi^2_2 = 736.1$, $p < .0001$; differences among classes: $\chi^2_6 = 15.1$, $p = .020$). Furthermore, a significant interaction between class and metabarcodes indicated that the performance of markers was heterogeneous across taxonomic classes ($\chi^2_{12} = 164.5$, $p < .0001$). Differences in amplification success between markers were significant for all the classes except Crustacea (Figure 1; Table S1). Euka02 showed the highest average amplification success (88%), with consistently high amplification success in all the taxa except Malacostraca (Figure 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 orders, with particularly good performance for Ephemeroptera and Trichoptera, while its amplification success was significantly lower than the other markers for Diptera (Figure 1b; Table S1).

As expected, Inse01 showed good amplification success for insects (82%), while it showed limited performance for the remaining taxa (Figure 1a). Within insects, Inse01 showed a particularly good performance for Coleoptera and Diptera, and an amplification success similar to Euka02 for Hemiptera and Plecoptera (Figure 1b;

TABLE 1 Inventory of macrobenthos individuals from which we extracted and amplified DNA

Class/subphylum/phylum		Number of individuals	Percentage identified at the genus level by taxonomists	Number of 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
Insecta				
	Coleoptera	117	97	40
	Diptera	54	20	6
	Ephemeroptera	338	100	35
	Hemiptera	24	100	14
	Lepidoptera	2	100	2
	Megaloptera	4	100	1
	Neuroptera	2	100	1
	Odonata	9	78	2
	Plecoptera	210	100	20
	Trichoptera	651	100	84

TABLE 2 Representativeness of reference individuals used for analyses, relative to European and French genera of benthic macroinvertebrates

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

[a]Except Hydracnidia.

Table S1). Within insects, the lowest amplification success for this marker was observed for Trichoptera (71%; Figure 1b).

Finally, BF1_BR2-COI showed an average amplification success of 48%, with highly variable results among taxa (Figure 1a; Table S1). BF1_BR2-COI showed a relatively good amplification success for Gastropoda, Clitellata and Malacostraca, while the rate was lower for some orders of insects. Within insects, BF1_BR2-COI showed good performance for Coleoptera and Diptera (amplification success ≥ 74%, significantly better than Euka02; Table S1), while it amplified less than 50% of individuals from Ephemeroptera, Plecoptera and Trichoptera (Figure 1b; Table S1).

3.2 | Combined database

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

3.3 | Taxonomic resolution of the different markers

Taxonomic resolution differed strongly among markers. At the species level, the best 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; Figure 3a). For Euka02, 21% of sequences were associated with more than one species in the database (Figure 3a). Differences in resolution between markers were strongly significant (linear mixed models: $F_{2,12} = 271.8$, $p < .001$). The resolution of BF1_BR2-COI was significantly better than that of both Euka02 and Inse01, and the resolution of Inse01 was significantly better than that of Euka02 (Tukey's post-hoc test: all $p < .001$).

The taxonomic resolutions of these markers were clearly better if we consider the identification at the genus level (Figure 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

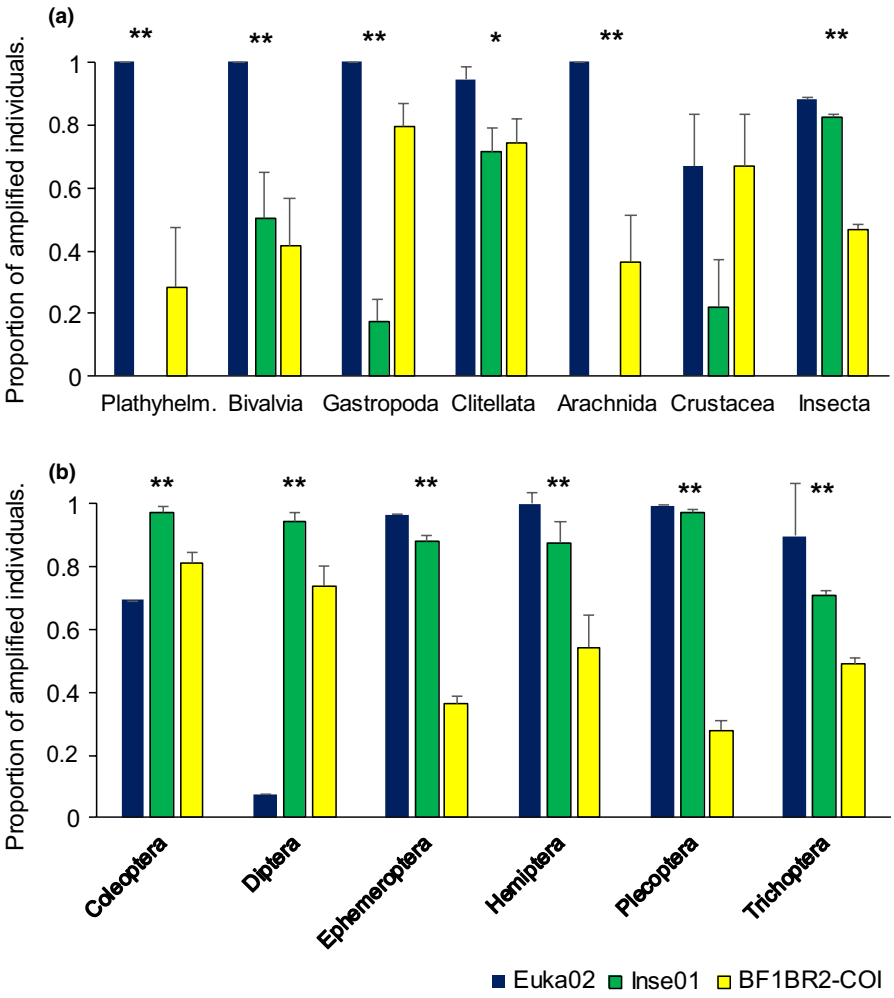

FIGURE 1 Amplification success of the three markers across benthic macroinvertebrate taxa. (a) All taxa; (b) insects only. Error bars are *SEM*; *significant differences between markers. See Table S2 for the raw values used to build the plot

than one genus for most taxa. 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 < .001). At the genus level, BF1_BR2-COI and Inse01 showed a similar resolution (Tukey's post hoc test: p = .20), and both outperformed Euka02 (both p < .001). Family-level identification was very good for all the metabarcodes, but Euka02 showed a slightly weaker performance than both BF1_BR2-COI and Inse01 (both $p \leq .05$). BF1_BR2-COI and Inse01 showed a comparable family-level resolution (p = .24; Figure 3c). Note that these values of resolution are calculated on an incomplete set of data, because 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.

4 | DISCUSSION

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

4.1 | The importance of good reference databases

Metabarcoding enables biodiversity monitoring either with or without the taxonomic identification of the retrieved taxa. Taxonomic identification clearly requires appropriate reference databases that can be obtained ad hoc (e.g., by amplifying sequences from all the taxa from the target group; Cilleros et al., 2019; Morinière et al., 2019; Moriniere et al., 2017) or by searching public databases such as GenBank or BOLD. Public databases offer an ever-growing

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)	Number of sequences in the database			Number of genera Europe	Number of 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	1,147	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[a]		2	2	4	1	**1**	**1**	**1**	**1**
Hydrachnidia		70	—	15	56	24	0	8	24
Crustacea		325	1,980	303	72	**61**	47	34	**65**
Insecta		2,149	4,981	4,557	981	385	456	327	530
	Coleoptera	450	1,809	333	127	**107**	**107**	68	**115**
	Diptera	228	1,078	2,839	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**

[a]Except Hydracnidia.

resource, given that they combine the outcome of thousands of studies and produce a sheer amount of data that would be unreachable by ad hoc studies. Public databases are not error-free, but analyses showed that for animals the error rate of GenBank for genus-level identification is generally low (~0.7/3.5%), suggesting that it can be a formidable data source for applications relying on molecular data to understand the impact of environmental changes on biodiversity (Leray et al., 2019). However, public databases are opportunistic collections of the material from multiple studies, and thus they do not have the ambition of taxonomic completeness. Ad-hoc databases (see also Ratnasingham & Hebert, 2007) are thus essential resources to obtain the taxonomic coverage required if we want to identify most benthic macroinvertebrates.

Several researchers advocated that COI-based markers should be favoured for metabarcoding because they are standard barcodes for animals, and thus we can expect a very large availability of sequences in reference databases (Andújar et al., 2018; Leray et al., 2019). For benthic macroinvertebrates, a very large number of COI sequences is available in GenBank (Table 3). For instance, BF1_BR2-COI is generally the marker with the highest number of sequences of benthic Diptera, with nearly 3,000 sequences of BF1_BR2-COI available against just 1,000 sequences of Inse01 (16S rDNA), although the number of available sequences is surprisingly variable across taxa. Nevertheless, a very large number of sequences does not necessarily allow better taxonomic coverage. In fact, most genera of benthic Diptera do not have GenBank sequences for COI, and slightly more genera have Inse01 sequences compared with BF1_BR2-COI (25% for Inse01 versus. just 15% for BF1_BR2-COI; Figure 3). The mismatch between number of sequences and database completeness could be related to the different scopes of studies employing the different markers. COI is the most widely used marker by standard barcoding studies, which often aim to unveil diversity among closely related, cryptic taxa, and thus studies often consider many individuals from closely related, morphologically similar species within genera (Hebert et al., 2004). Conversely, the 16S

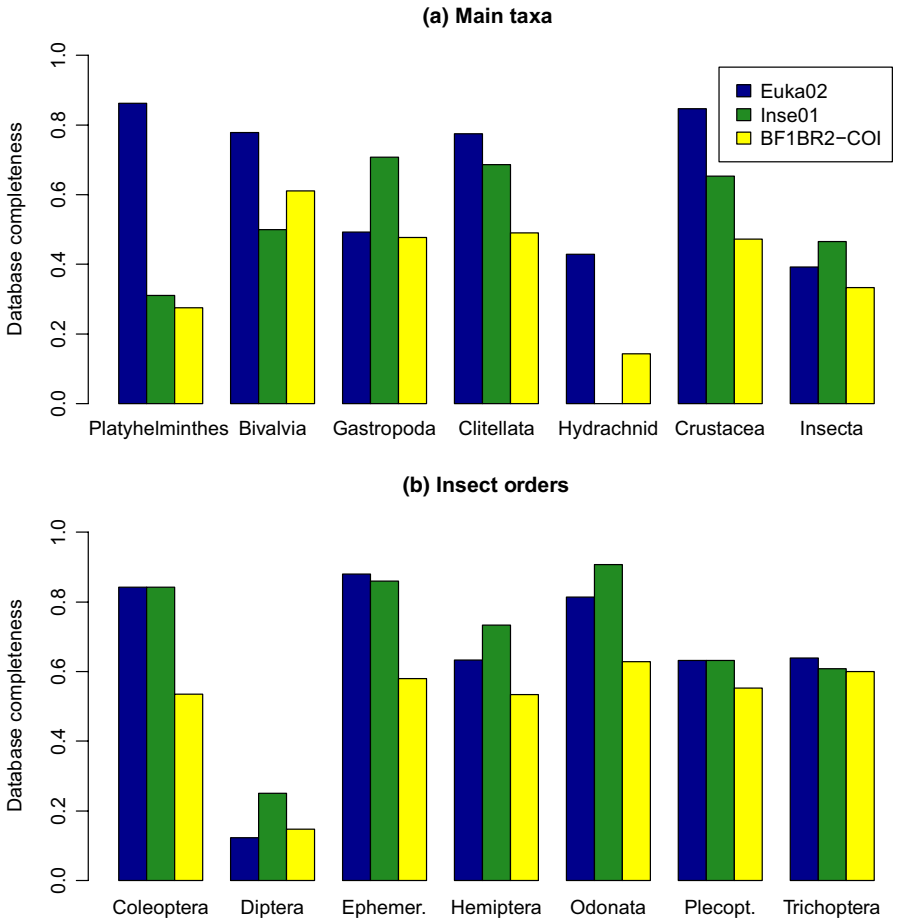

FIGURE 2 Completeness of the combined database, combining the sequences produced in this study with sequences retrieved from public databases. For each taxon, the plots report the proportion of European genera of macroinvertebrates with at least one sequence in the database

and 18S rDNA genes are often used to build phylogenies (e.g., Alvarez-Presas et al., 2008; Criscione & Ponder, 2013), and many phylogenetic studies aim to represent the largest number of genera and families. This could also explain the strong differences among taxa (e.g., a very high completeness for Euka02 with free-living Platyhelminthes, and the better coverage for Inse01 with Gastropoda; Figure 2). If the aim is species-level identification, databases should include all the species and markers should have a species-level resolution. Luckily, for freshwater biomonitoring a genus-level identification is often enough (Bailey et al., 2001; Birk et al., 2012; Chessman et al., 2007; Mistri & Rossi, 2001), and thus our database provides a good completeness that can allow the identification of most genera, particularly with the markers Euka02 and Inse01. Here we tested our markers against the GenBank database only, because it includes sequences from our three genomic regions. However, an impressive number of additional COI sequences is available in the BOLD database. Integrating GenBank and BOLD data can certainly improve the resolution of COI-based metabarcoding studies.

4.2 | Metabarcoding without taxonomic identification

Metabarcoding can provide ecological information even if reference databases are not available, as molecular taxonomic units can allow the comparison of communities among sites with environmental differences (Apothéloz-Perret-Gentil et al., 2017; Cordier et al., 2019; Ji et al., 2013). The taxonomy-free approach allows us to overcome the fact that, despite intensive efforts, databases remain incomplete for many taxa (Figure 3). Primers with high taxonomic coverage and resolution are essential also in this case. High taxonomic coverage is needed to avoid under-representation of some taxa, while resolution allows related taxa to be teased apart. Related taxa can have very different ecological properties, and some widespread taxa, tolerant to human disturbance, can be closely related to highly sensitive specialists (Caro et al., 2005). Therefore, ecological responses of communities can remain obscured if metabarcodes are not able to resolve related taxa with different ecology. Our study focused on European taxa, where taxonomic knowledge is particularly good (Brewer et al., 2012; Moustakas & Karakassis, 2005; Rodrigues et al., 2010) and, with targeted studies, we could envisage an improvement of database completeness in the next few years. However, our results on primer performance can be also useful in megadiverse, tropical areas, where taxonomy-free biomonitoring is a viable option (Andersen et al., 2019).

4.3 | Universality and resolution of primers

Our analysis did not identify a single outperforming metabarcode. The universality of primers was variable among taxa, with Euka02

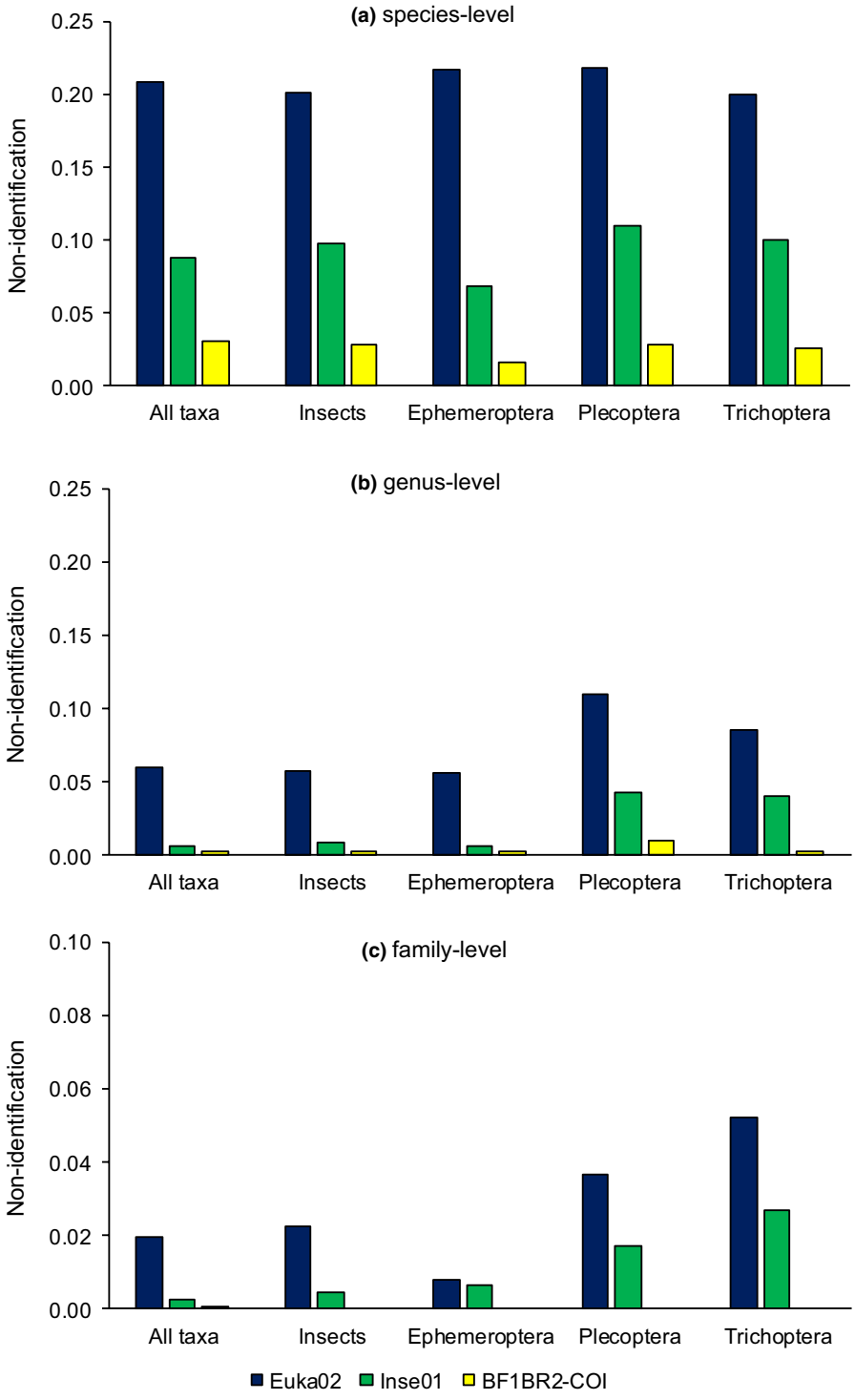

FIGURE 3 Resolution of the three markers at the species, genus and family levels. Resolution is measured as the proportion of metabarcodes that are associated with (a) at least two species, (b) at least two genera or (c) at least two families (non-identification), and therefore low values of non-identification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

showing the highest performance for some phyla (platyhelminthes, molluscs, annelids and even some arthropods), and Inse01 showing a generally good performance for insects. However, each of these metabarcodes has some drawbacks. For instance, Euka02 amplifies very long sequences for some taxa of crustaceans (Isopoda and Amphipoda; Guardiola et al., 2015; Taberlet et al., 2018), and thus their metabarcoding with this marker is problematic. Conversely, Inse01 is a marker developed specifically for insects, and fails to amplify key freshwater taxa such as free-living Platyhelminthes and molluscs (Figure 1). In our analysis, BF1_BR2-COI showed moderate amplification success, but for insects a relevant proportion of individuals was not amplified (Figure 1). This is in contrast to previous analyses that successfully amplified 100% of tested insects using BF1_BR2-COI (Elbrecht & Leese, 2017). Differences might be due to DNA quality, as this primer amplifies relatively long metabarcodes (>300 bp). Some of our >1,500 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 and Leese (2017), but failed to amplify here. Furthermore, in several cases BF1_BR2-COI did not amplify the DNA of our target organisms, but amplified the DNA of contaminants, that is 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 poor performance, as in many cases it failed even at the family level (Figure 3; see also Tang et al., 2012). COI is a highly variable region, and this has promoted its use as a standard barcode for animals (Andújar et al., 2018; Hebert et al., 2003, 2004). 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 sometimes insufficient for species-level identification, while genus-level identification was good for most of the taxa (Figure 3). It must be noted that these are optimistic values of resolution, given that our database was far from complete, particularly at the genus level and for some taxa, and 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 in identifying the best metabarcodes. No primer showed the best performance for all the considered metrics, as the most "universal" marker (Euka02) showed a generally poor resolution, while the marker with the highest resolution (BF1_BR2-COI) did not successfully amplify several taxa. The selection of metabarcodes for biomonitoring is therefore a trade-off, depending on the aims of the study. Euka02 gives good assessment of overall biodiversity, but it is unable to tease apart closely related taxa, and thus it might be not sufficient to define the ecological status of environments. Furthermore, the poor resolution would hamper the comparison with historical data for most of the taxa. Conversely, the excellent resolution of BF1_BR2-COI could allow species-level identification, and might have more power to distinguish different communities. However, this comes at a cost. Several taxa did not amplify either because the level of DNA degradation compromises the amplification of a relatively long metabarcode, or because there was poor match of the primer(s) with their target. In fact, the relatively long amplified region could limit its usefulness for application with eDNA extracted from water. Finally, Inse01 showed a generally good performance, but it is not appropriate for many noninsect taxa.

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

5 | COMPETING INTEREST

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

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AUTHOR CONTRIBUTIONS

Designed the research: P.T., T.D., G.F.F., F.B., A.V., A.B., A.M., P.U.P. Performed research: A.V., A.B., A.M., C.G., P.T., T.D., G.F.F., F.B., P.U.P. Analysed data: G.F.F., F.B., A.B. Wrote the paper: G.F.F. wrote the first draft of the paper, with subsequent contribution from all authors.

DATA AVAILABILITY STATEMENT

The complete database is available at figshare: 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 nos. LR824819 to LR826155; Inse01: LR826156 to LR827357, BF1-BR2 COI: LR827358 to LR828086).

ORCID

Gentile Francesco Ficetola https://orcid.org/0000-0003-3414-5155
Aurélie Bonin https://orcid.org/0000-0001-7800-8609
Albin Meyer https://orcid.org/0000-0002-0419-205X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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