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## Why the COI barcode should be the community DNA metabarcode for the Metazoa

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1 OPINION ARTICLE

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3 **Why the COI barcode should be the community DNA metabarcode for the**  
4 **Metazoa**

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23 Throughput Sequencing (HTS)

24 Running Title: COI barcode for metazoan metabarcoding

## 25 **Abstract**

26 **Metabarcoding** of complex metazoan communities is increasingly being used to measure  
27 biodiversity in terrestrial, freshwater, and marine ecosystems, revolutionizing our ability to  
28 observe patterns and infer processes regarding the origin and conservation of biodiversity. A  
29 fundamentally important question is which genetic marker to amplify, and although the  
30 mitochondrial cytochrome oxidase subunit I (COI) gene is one of the more widely used  
31 markers in metabarcoding **for the Metazoa**, doubts have recently been raised about its  
32 suitability. We argue that (i) the extensive coverage of reference-sequence databases for COI,  
33 (ii) the variation it presents, (iii) the comparative advantages for denoising protein coding  
34 genes, and (iv) recent advances in DNA sequencing protocols argue in favour of standardising  
35 for the use of COI for metazoan community samples. We also highlight where research  
36 efforts should focus to maximise the utility of metabarcoding.

## 38 **Introduction**

39 Metabarcoding (Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012; Yu et al.,  
40 2012), i.e. the bulk DNA amplification and high-throughput sequencing (HTS) of biological  
41 samples, is now a well-established tool for the study of biodiversity, as reflected by the rapid  
42 growth in the number of published studies since the early applications to bacteria and fungi  
43 (e.g., Buée et al., 2009; Hamady, Walker, Harris, Gold, & Knight, 2008) (Fig.1).  
44 Metabarcoding has been applied to DNA from diverse biological sources using a wide range  
45 of laboratory procedures and addressing manifold questions about spatial and temporal  
46 biodiversity patterns (e.g., Deiner et al., 2017; Taberlet, Bonin, Zinger, & Coissac, 2018). The  
47 most straightforward application of metabarcoding is the acquisition of DNA data from bulk

48 specimen samples. These are mixed species assemblages that have been extracted from their  
49 habitat matrix and combined for a single DNA extraction, followed by PCR amplification  
50 with ‘universal’ primers. This approach, referred to as community DNA metabarcoding  
51 (cMBC) (Deiner et al., 2017) is increasingly being applied to biodiversity inventories and  
52 biomonitoring in marine (e.g., Fonseca et al., 2010; Leray & Knowlton, 2015), terrestrial  
53 (e.g., Arribas, Andújar, Hopkins, Shepherd, & Vogler, 2016; Ji et al., 2013) and freshwater  
54 environments (e.g., Andújar et al., 2018; Elbrecht & Leese, 2017) (See Fig. 1). Although there  
55 are technical differences, metabarcoding of metazoan communities can also be conducted on  
56 DNA extractions directly from the external medium, such as soil or water, to gather  
57 ‘environmental DNA’ (eDNA; see glossary) (Taberlet, Coissac, Hajibabaei, & Rieseberg,  
58 2012; Deiner et al., 2017 for a comparison between community and environmental DNA  
59 metabarcoding)

60 A key design consideration for metazoan metabarcoding is the selection of the DNA  
61 marker to be amplified, a choice that may greatly affect the number of species and taxonomic  
62 groups detected and the accuracy of species identifications against marker-specific reference  
63 databases. Taxonomic bias associated with PCR primer choice has been the main reason to  
64 question the utility of several markers for DNA metabarcoding (Deagle et al., 2014; Taberlet,  
65 Coissac, Pompanon, et al., 2012), including the mitochondrial cytochrome oxidase 1 gene  
66 (COI or *cox1*) where is located the standard barcode region (COI-bcr) for metazoan DNA  
67 taxonomy (Hebert, Cywinska, Ball, & DeWaard, 2003; also see the Consortium for the  
68 Barcode of Life, CBOL; [http:// www.barcodeoflife.org/](http://www.barcodeoflife.org/)). Additional considerations for  
69 fragment choice in metazoan metabarcoding are the state of preservation of the DNA template  
70 (eDNA is often fragmented; e.g., Deagle, Eveson, & Jarman, 2006), read-length limitations of  
71 widely-used parallel-sequencing methods (e.g, a maximum read length of 300 bp of the

72 Illumina technology, limiting paired-sequencing to amplicons of maximally  $\approx 450$  bp; e.g.,  
73 Fadrosch et al., 2014), and potential co-amplification of concomitant microbial DNA (e.g., Stat  
74 et al., 2017). Due to these concerns, marker choice for metazoan metabarcoding lacks a  
75 universally agreed approach, which has resulted in a proliferation of primers with different  
76 taxon specificities and degree of universality.

77         The above-mentioned concerns are well-founded in the case of eDNA metabarcoding  
78 (Deagle et al., 2014), where DNA is often poorly preserved and frequently includes high  
79 proportions of microbial DNA (e.g., Stat et al., 2017; Yang et al., 2014). However, concerns  
80 regarding DNA integrity and co-amplification of microbial DNA are largely inconsequential  
81 for cMBC. It is largely for reasons of presumed taxonomic bias for PCR amplification of the  
82 COI-bcr that many studies have abandoned this locus, in favour of primers matching highly  
83 conserved binding sites with a presumed more even coverage of all taxa present. The most  
84 widely used alternatives are the nuclear ribosomal genes coding for the small subunit (SSU or  
85 18S rRNA) (Capra et al., 2016; Creer et al., 2010), the large subunit (LSU or 28S rRNA)  
86 (Hirai, Kuriyama, Ichikawa, Hidaka, & Tsuda, 2014), the internal transcribed spacer 2 (ITS2)  
87 (Anslan & Tedersoo, 2015; Avramenko et al., 2017), and the mitochondrial small [*rrnS* or  
88 12S rRNA] (Machida, Kweskin, & Knowlton, 2012) and large subunit rRNA [*rrnL* or 16S  
89 rRNA] (Elbrecht et al., 2016; Saitoh et al., 2016). The lack of consensus over the choice of  
90 metabarcode markers, even within the same target community, carries the risk of poor  
91 standardisation and low comparability among studies, which ultimately hampers the  
92 development of an efficient, universal system for biodiversity discovery and monitoring using  
93 cMBC.

94         Here we argue in favour of the COI-bcr as a standard for bulk-sampled metazoan  
95 cMBC and support our position with four sets of arguments. We revisit two points that have

96 made the COI-bcr the fragment of choice for barcoding in metazoans and equally apply to  
97 cMBC: the availability of large COI-bcr reference databases, and the level of nucleotide  
98 variation of COI-bcr that is appropriate for the taxonomic assignment of amplicons at the  
99 species level. Our third point is that sequencing errors and spurious sequence assemblies can  
100 be robustly identified by bioinformatic processing based on the predicted variation in protein  
101 coding regions and the limited length variation in COI-bcr. Finally, recent evidence regarding  
102 potential taxonomic amplification bias associated with the COI-bcr, a key reason for  
103 questions about its utility, can be overcome by improved design of primers. We conclude by  
104 focussing on the benefits and synergies that can emerge from standardisation, and provide  
105 recommendations for future research and applications.

106

## 107 **1. Large COI-bcr reference databases provide a powerful link to taxonomic** 108 **identity**

109 The utility of a reference sequence database for metabarcoding is a function of: (i) the  
110 inherent power of the marker for taxonomic assignment; (ii) the taxonomic coverage (number  
111 of species and phylogenetic diversity represented in the database) and depth (number of  
112 individuals sequenced per species) of reference sequences, and (iii) the adequate formatting  
113 and curation of the database and its accessibility to taxonomic-assignment software packages.

114 The taxonomic coverage and depth of COI-bcr is unparalleled. Public records at the BOLD  
115 online database (Ratnasingham & Hebert, 2007) include 1,240,301 sequences of >500 bp in  
116 length, representing 102,254 species (accessed 26 May 2018). Taking into account sequences  
117 on BOLD that are yet to be made publically available, there are 5,542,839 sequences of which  
118 3,150,643 are identified to species representing 191,568 animal species.

119 COI-bcr resources clearly exceed those available for any other DNA marker for  
120 animals. For example, *rrnL* and *rrnS* include 256,372 and 137,603 sequences on GenBank  
121 (Benson et al., 2014), while SSU include 149,119 sequences (searches on 26 May 2018 at  
122 GenBank for sequences of >500 bp within Metazoa). There were 135,416 and 127,065  
123 metazoan sequences for LSU and SSU, respectively, on the SILVA database (Quast et al.,  
124 2013) (searches on 26 May 2018). Additionally, Machida et al. (2017) have recently  
125 constructed the *Midori* database, which includes all mitochondrial genes of the Metazoa,  
126 including GenBank records available prior to September 2015. *Midori* also provides a  
127 quantitative measure of the available taxonomic coverage of different mtDNA gene regions,  
128 demonstrating the dominant representation of COI-bcr (583,043 sequences) which greatly  
129 exceeds the next-most represented regions of cytochrome oxidase b (*cob*; 223,247 sequences)  
130 and *rrnL* (146,164 sequences), and is represented for more species in almost all animal phyla  
131 (Machida et al., 2017).

132 As a reference database increases in size, the probability of false taxonomic  
133 assignment is reduced and placement to lower taxonomic ranks is improved (Somervuo et al.,  
134 2016). In this context, it is worth noting the expected future growth of the COI-bcr reference  
135 dataset due to ongoing geographically and taxonomically focused campaigns. When such  
136 campaigns incorporate historic type specimens into barcode projects (e.g., Hausmann et al.,  
137 2016), stronger linkage is forged between traditional taxonomic systems and reference  
138 sequences. Barcode campaigns that employ rigorous taxonomic identification of voucher  
139 specimens also provide a necessary step forward to identify database sequences that have  
140 been incorrectly assigned taxonomically, as it has been shown to occur in the Genbank  
141 database (Mioduchowska, Jan, Gołdyn, Kur, & Sell, 2018).

142 In addition to the availability of reference sequences, tools are needed to manage such  
143 large databases and facilitate taxonomic classification of the unprecedented volume of  
144 sequences obtained by metabarcoding (Somervuo et al., 2016). The BOLD website itself was  
145 not designed for the large-volume searches needed by metabarcoding, although an application  
146 programming interface ([v4.boldsystems.org/index.php/api\\_home](http://v4.boldsystems.org/index.php/api_home), accessed 8 Mar 2018)  
147 allows automated queries via the R *bold* package ([github.com/ropensci/bold](https://github.com/ropensci/bold), accessed 8 Mar  
148 2018), and a new BOLD database interface, suitable for large-volume queries, has recently  
149 been made publically available ([mbrave.net](http://mbrave.net), accessed 8 Mar 2018). Additionally, the *Midori*  
150 web server ([www.reference-midori.info](http://www.reference-midori.info), accessed 8 Mar 2018) provides three taxonomic-  
151 assignment methods (RDP Classifier (Wang, Garrity, Tiedje, & Cole, 2007), SPINGO  
152 (Allard, Ryan, Jeffery, & Claesson, 2015), and SINTAX (Edgar, 2016a)) for volume queries.

153

## 154 **2. Taxonomic identification and intraspecific structure – two for the price** 155 **of one**

156 Thanks to its relatively high mutation rate, COI-bcr (and other mitochondrial genes) is a  
157 powerful marker to detect intraspecific variation, which can be separated from interspecific  
158 variation using various algorithms for sequence clustering and phylogenetic rates (e.g., Hebert  
159 & Gregory, 2005; Pons et al., 2006; Puillandre, Lambert, Brouillet, & Achaz, 2012; J. Zhang,  
160 Kapli, Pavlidis, & Stamatakis, 2013) and thus improves the ability to distinguish closely  
161 related and cryptic species (Candek & Kuntner, 2015). In contrast, the *SSU* gene, widely used  
162 to characterise marine meiofauna and soil fauna (Capra et al., 2016; Creer et al., 2010; Yang  
163 et al., 2014) has a comparatively lower mutation rate, increasing the probability that related  
164 species may share the same sequence (Andújar et al., 2018; Tang et al., 2012). As well as



165 compromising species identification, such limited variation will also underestimate both alpha  
166 and beta diversity, fundamental metrics for meaningful ecological conclusions from  
167 metabarcode studies.

168         The high mutation rate of COI-bcr and resulting intraspecific variation have been  
169 widely used to investigate the structuring of genetic variation below the species level (e.g.,  
170 Bucklin, Steinke, & Blanco-Bercial, 2011; Goodall-Copestake, Tarling, & Murphy, 2012) and  
171 to inform about ecological and evolutionary processes at the community level (e.g., Baselga et  
172 al., 2013; Emerson et al., 2017). HTS data have not taken advantage of this property of the  
173 COI-bcr, largely because sequence quality has been perceived to be low, and it is effectively  
174 removed as sequence variants are clustered into OTUs. However, as read quality improves,  
175 simple clustering can be replaced by direct use of HTS reads, albeit after stringent denoising  
176 that removes spurious sequence variants (Callahan, McMurdie, & Holmes, 2017; Edgar,  
177 2016b). Denoising can be particularly efficient for COI-bcr due to the predictable pattern of  
178 nucleotide variation within protein-coding mitochondrial genes and the almost complete  
179 absence of length variation within the COI-bcr (see below). Indeed, recent work by Elbrecht,  
180 Vamos, Steinke, & Leese (2018) demonstrates the ability to recover intraspecific genetic  
181 variation from cMBC data, opening the door for the simultaneous analysis of species diversity  
182 and intraspecific variation for cMBC at the whole-community or even ecosystem level.

183

### 184 **3. The advantage of protein-coding genes to identify spurious sequences**

185 Bioinformatic steps for removing non-target sequences that can originate from PCR errors,  
186 sequencing errors, amplification of pseudogenes, and chimeric rearrangements (Edgar, 2016b;  
187 Schirmer et al., 2015) can be carried out more robustly for protein-coding genes compared to

188 ribosomal gene regions (Ramirez-Gonzalez et al., 2013; Ranwez, 2011). This is due to the  
189 pattern of variation of protein-coding mitochondrial genes, where: (i) some amino-acid  
190 residues are highly conserved; (ii) nucleotide variation is biased toward the third base  
191 positions of codons; and (iii) indels are almost completely absent (Ramirez-Gonzalez et al.,  
192 2013). Thus, COI-bcr metabarcoding reads leading to stop codons or indels are clear targets for  
193 removal, and denoising can also take advantage of known patterns of variation in protein  
194 coding sequences to detect (i) atypical ratios of synonymous/nonsynonymous mutations, (ii)  
195 atypical amino acid changes compared to representative consensus sequences, and (iii)  
196 atypical distributions of variation with respect to codon position (Ramirez-Gonzalez et al.,  
197 2013; Ranwez, 2011). These features can potentially be integrated in the denoising process to  
198 retain only well supported genetic variants from COI-bcr HTS reads.

199

#### 200 **4. Comprehensive and informative surveys with better design of primers**

201 Metabarcoding using fragments within the COI-bcr has been associated with the incomplete  
202 recovery of species from mock communities ('dropouts') (e.g., Clarke, Soubrier, Weyrich, &  
203 Cooper, 2014; Yu et al., 2012), and as a consequence the utility of the COI-bcr has been  
204 questioned (Deagle et al., 2014). A key reason for dropouts is high heterogeneity in primer  
205 binding sites and thus differential PCR efficiencies across variable templates, which results in  
206 taxonomic bias during PCR amplification. A related consequence is that differences in  
207 amplification efficiency complicate the use read frequencies as proxy measures of species  
208 abundance or biomass (Krehenwinkel et al., 2017; Piñol, Mir, Gomez-Polo, & Agustí, 2015).  
209 Proposed remedies include the use of multiple, taxon-specific primers on the same sample  
210 (Drummond et al., 2015; Stat et al., 2017).

211 Despite earlier concerns (Deagle et al., 2014), the extent to which the COI-bcr  
212 produces taxonomic bias in metazoan cMBC is unclear. Performance varies among studies,  
213 with many factors potentially explaining variation, such as target taxa, relative abundance and  
214 body size, specimen preservation, laboratory procedures, primers choice, and PCR conditions.  
215 For example, the low recovery of species documented in some studies (Brandon-Mong et al.,  
216 2015; Clarke et al., 2014; Elbrecht et al., 2016) also coincides with the use of mostly non-  
217 degenerate primers (Table 1). Yu et al. (2012) used degenerate LCO1490 and HC02198  
218 primers and inherently low-coverage 454 pyrosequencing to achieve promising results for  
219 cMBC, recovering up to 76% of the species from mock pools of known composition,  
220 including 12 different orders within the Arthropoda. Although a dropout of 24% is  
221 undesirable, Yu et al. (2012) showed that even this level of dropout did not prevent  
222 metabarcoding data from providing correct estimates of community-level metrics, namely  
223 alpha and beta diversity, and thus metabarcoding data were reliable inputs to decision-making  
224 (Ji et al., 2013).

225 Studies using redesigned, degenerate primers for various subregions of the COI-bcr  
226 have continued to reduce dropout in cMBC of Metazoa (Andújar et al., 2018; Arribas et al.,  
227 2016; Beng et al., 2016; Elbrecht & Leese, 2017; Leray et al., 2013; Prosser, Velarde-Aguilar,  
228 León-Règagnon, & Hebert, 2013; Saitoh et al., 2016) (Table 1). In a study of aquatic taxa  
229 including 52 macroinvertebrates, Elbrecht & Leese (2017) showed that the use of degenerate  
230 primers within the COI-bcr recovered almost all input taxa (42/42 insects; 9/10 other taxa)  
231 and resulted in improved estimation of relative abundances, a result that outperformed even  
232 the *rrnL* primer set (41/42 species of Insecta and 2/10 other taxa). However, it should be  
233 noted that the estimation of species abundance from metabarcode data is controversial and  
234 requires further research, probably requiring calibration studies using known amounts of

235 DNA (Bista et al., 2018; Krehenwinkel et al., 2017; Thomas, Deagle, Eveson, Harsch, &  
236 Trites, 2016). In another study of whole-community freshwater invertebrates (Andújar et al.,  
237 2018), cMBC with SSU universal primers and degenerate COI-bcr primers resulted in the  
238 detection of 2-4 times higher number of 97%-similarity OTUs (operational taxonomic units)  
239 with COI-bcr, including the main insect orders inhabiting freshwater ecosystems (Diptera,  
240 Coleoptera, Ephemeroptera, and Trichoptera), plus Crustacea, Rotifera, and Annelida  
241 (Andújar et al., 2018). However, amplification of nematodes and platyhelminthes was poor,  
242 and requires different primer sets (e.g., Prosser et al., 2013). With increasing knowledge of  
243 taxon-specific problems, primer design and combinations of primer sets can be adapted to  
244 generate increasingly complete community inventories and improved species abundance data.

245

## 246 **Concluding remarks**

247 We conclude that the much greater number of COI-bcr reference sequences, the broader  
248 taxonomic coverage and resolution of these sequences, combined with recent improvements  
249 in COI-bcr primer design, argue for the COI-bcr region as the marker of choice cMBC of bulk  
250 metazoan samples. An important caveat here is that we do not include eDNA samples in our  
251 recommendation. In the case of eDNA, the target region for the Metazoa is frequently present  
252 only at very low concentrations compared to microbial DNA (Stat et al., 2017), and it is  
253 widely found, although not generally published, that most primers within the COI-bcr amplify  
254 large proportions of microbial species (e.g., Yang et al., 2014). This fact remains the strongest  
255 reason for the use of mitochondrial rRNA markers that are much less affected by this type of  
256 cross-amplification. Ultimately, with the increasing availability of whole mitochondrial  
257 genomes, MBC studies using COI-bcr and other markers can be linked (Arribas et al. 2016).

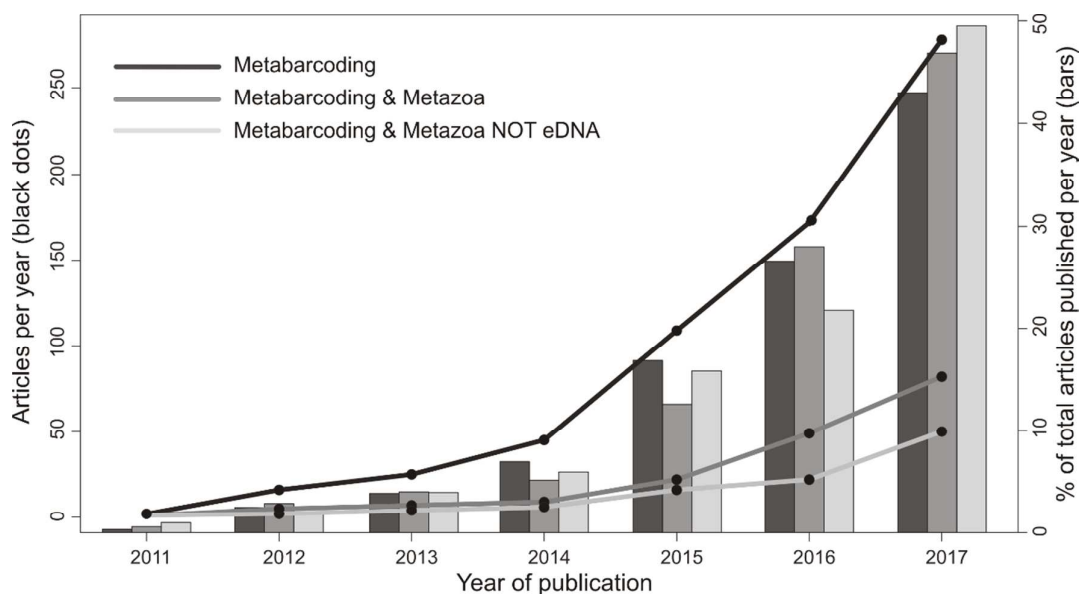
258            Looking forward, we identify the following key areas of research and development for  
259 cMBC: (1) Continued and increased funding for alpha taxonomy, DNA barcoding campaigns,  
260 and the development and maintenance of the BOLD database, increasing its functionality  
261 regarding metabarcode data. Regarding other public databases (e.g., GenBank), effort is  
262 required to identify sequences with incorrect taxonomic assignment to avoid their use as  
263 reference data (Mioduchowska et al., 2018). (2) Development and validation of detailed and  
264 standardisable methods for field work and extraction of the target specimens from their  
265 habitat matrix (water, soil, sediment etc) (e.g., Arribas et al., 2016; Fonseca et al., 2010). (3)  
266 Continued design and validation of primers for DNA fragments within the COI-bcr, with the  
267 aim of standardizing fragments of choice within the COI-bcr to maximise comparability  
268 among studies. For example, the Leray-Geller primer set (Leray et al., 2013) is now widely  
269 used because the amplicon length of 313 bp matches the read lengths of paired-end Illumina  
270 sequencing, but this primer set was largely designed for marine organisms, and thus could  
271 probably be improved upon for terrestrial taxa. Other promising primer sets include those  
272 used by Elbrecht & Leese (2017) for a fragment of 316 bp (BF1-BR2) and Shokralla et al.  
273 (2014) and Andújar et al., (2018) for a fragment around 400 bp (pair of primers Ill\_B\_F-  
274 Ill\_B\_R and Ill\_B\_F-Fol-degen-rev respectively). A related issue is that various primers  
275 target different, and frequently non-overlapping regions of the COI-bcr, which limits the  
276 direct comparisons among metabarcoding studies, in particular for those taxa without exact  
277 matches to sequences in the reference database. (4) Development and validation of denoising  
278 methods for the recovery of intraspecific genetic variation from cMBC data. This will include  
279 evolutionary models of sequence variation that go beyond the current error models based on  
280 prevalent technical artifacts of the sequencing procedure (e.g. Schirmer et al., 2015) or read  
281 abundances (Edgar, 2016b). (5) Continued development, validation, and improved availability

282 of methods for taxonomic assignment (e.g., Somervuo et al., 2016; A. Zhang, Hao, Yang, &  
283 Shi, 2016).

284           Much progress has been made in the field of cMBC in recent years, and the potential  
285 for cMBC as an integrated tool for biodiversity monitoring and management is clearly  
286 recognised (e.g. Bush et al, 2017). Standardising for the COI-bcr for cMBC and focussing on  
287 the above suggestions should increase the reliability of metabarcode data for management,  
288 policy and decision-making, while also facilitating greater comparability across independent  
289 studies.

290

For Review Only

291 **Figure 1**

292

293 **Figure 1** Temporal evolution of scientific publications on the topic *metabarcoding* (Black  
 294 line; TS = *metabarcoding*); *metabarcoding on metazoans* (Dark grey line:  
 295 TS=(*metabarcoding*) NOT TS =( \*micro\* OR \*bacteria\* OR \*myco\* OR \*archaea\* OR fungi  
 296 OR plant); and *metabarcoding on metazoans excluding eDNA studies* (Light grey line:  
 297 TS=(*metabarcoding*) NOT TS =( \*micro\* OR \*bacteria\* OR \*myco\* OR \*archaea\* OR fungi  
 298 OR plant OR eDNA OR environmental DNA). Black dots: number of publications per year  
 299 for each search. Bars: proportion of the total publications of each search per year. Searches  
 300 were performed on the Web of Science (23-04-2018), including the Science Citation Index  
 301 Expanded, Social Science Citation Index, Arts and Humanities Citation Index, and  
 302 Conference Proceedings Citation Index–Science databases for all years and restricted to  
 303 article types “article” and “review”.

304

305

**Table 1.** Overview of studies providing data on the performance of different fragments within the COI-bcr on community DNA metabarcoding (cMBC) for Metazoa.

Reference	Target taxa	Type of primers	Amplicon length(bp)	vitro/silico	Results
(Prosser et al., 2013)	Nematoda	Degenerate	650	vitro	89.5% (85/95) sequencing success on diverse parasitic nematode lineages, including members of three orders and eight families.
(Beng et al., 2016)	Arthropoda	Degenerate	ca. 400	vitro	100% in-vitro PCR efficiency on a wide range of arthropods (Chilopoda, Araneae, Hymenoptera, Blattodea, Mantodea, Coleoptera, Orthoptera, Lepidoptera, and Hemiptera)
(Beng et al., 2016)	Arthropoda	Degenerate	ca. 400	silico	100% detection success after in silico sequencing six mock communities with known arthropod composition (37 ref sequences from Genbank)
(Arribas et al., 2016)	Acari and Collembola	Degenerate	650	vitro	Detection of >100 species of Acari and Collembola from 28 families. Recovery against 79 barcoded voucher specimens in the same samples was 95% (75/79)
(Andújar et al., 2018)	Freshwater invertebrates	Degenerate	420*	vitro	COI outperformed SSU except for Nematodes and Platyhelminthes
(Saitoh et al., 2016)	Collembola	Degenerate	314	vitro	100% (7/7) recovery in mock communities. In complex soil samples, cMBC on COI outperformed morphology, and provided a similar recovery to <i>rrnL</i> (16S).
(Yu et al., 2012)	Arthropoda	Degenerate	650	vitro	Recovery rates of 76% for already barcoded species by Sanger.
(Elbrecht et al., 2016)	Freshwater invertebrates	Non-degenerate	650	vitro	Recovery of 90% (38/42) insects and 50% (5/10) other taxa in a mock community.
(Elbrecht & Leese, 2017)	Freshwater invertebrates	Degenerate	316**	vitro	Recovery of 100% (42/42) insects and 90% (9/10) other taxa in a mock community.
(Clarke et al., 2014)	Insects	Non- or low-degenerate	Several pairs	silico	For every pair of primer, recovery of <75% of insect species with complete mitochondrial genome available. <i>rrnL</i> (16S) recovered >90%.
(Clarke et al., 2014)	Insects	Non- or low-degenerate	Several pairs	vitro	Recovery of the same or less taxa than with <i>rrnL</i> (16S) on a mock community of 14 taxa.
(Brandon-Mong et al., 2015)	Arthropoda	Only forward degenerate	313	vitro	Recovery of 91% (71/78) species on a mock community with representatives for Aranea, Blattodea, Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Matodea, Odonata, Orthoptera and Collembola
(Krehenwinkel et al., 2017)	Arthropoda	Degenerate	313 and 418	vitro	Recovery of 95% (41/43) on a mock community including 19 orders in the Arachnida, Crustacea, Hexapoda & Myriapoda. Same or higher recovery than other fragments tested (Cytb, 12s, 18s, 28s, H3).

\* Refers to primers Ill\_B\_F and Fol-degen-rev. \*\* Refers to primers BF1 and BR2. \*\*\* Refers to primers mlColintF and HCO2198



## BOX 1 Glossary

*DNA barcoding.* Method for the taxonomic identification of specimens based on the sequencing of diagnostic DNA sequence regions. It was first proposed by Hebert et al (2003). Frequently used barcodes (i.e., DNA fragments used for DNA barcoding) include the COI gene for Metazoa, *rbcL* for plants, ITS for fungi and *rrnL* (16s) for bacteria.

*High-throughput sequencing (HTS).* Techniques that allow the simultaneous sequencing of millions of DNA fragments.

*DNA metabarcoding.* DNA amplification and high-throughput sequencing of a DNA extract derived from a biological sample composed of a mix of DNA from different source species, each represented by one or more individuals. After bioinformatic procedures for quality filtering, resulting DNA sequences can be subject to molecular identification using barcode reference databases.

*Environmental DNA (eDNA) metabarcoding.* DNA metabarcoding targeting DNA directly isolated from environmental samples such as soil, sediments or water, among others (Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012). DNA sources contributing to eDNA include the breakdown of body parts from organisms together with faeces, mucus, skin cells, organelles, gametes or even extracellular DNA.

*Community DNA metabarcoding (cMBC).* DNA metabarcoding targeting DNA isolated from bulk mixtures of specimens that have been extracted from their habitat matrix.

*Invertebrate ingested DNA (iDNA) metabarcoding.* DNA metabarcoding targeting vertebrate genetic material that is extracted from invertebrates (such as leeches, mosquitoes, or ticks, among others). Can be considered as an particular case of eDNA metabarcoding, as the DNA sources are of ingested material or faeces.

*Degenerate primer.* Mixture of DNA oligonucleotides that differ in base composition for one or several nucleotide positions (degenerate positions). In practice, it means that different variants of a particular oligo are synthesized and mixed to be used as primers on a PCR reaction. The higher the proportion of degenerate positions, the more degenerate a primer is.

*Universal primers.* PCR primers, degenerate or not, with the potential to amplify a particular DNA fragment within a broad taxonomic scope (e.g. all Metazoa, all Arthropoda, all Crustacea, etc). Although full universality (i.e. amplifying all species within the taxonomic scope) is unlikely, primers are often referred to as universal when they broadly function across the phylogenetic diversity within a given taxonomic scope.

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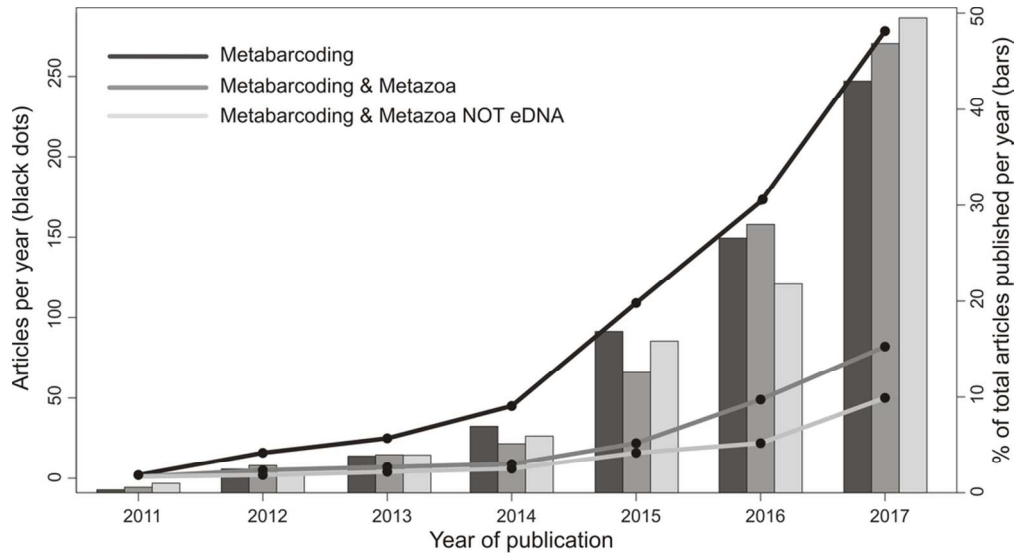


Figure 1 Temporal evolution of scientific publications on the topic metabarcoding

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