

1 **Environmental DNA Barcode Sequence Capture: Targeted, PCR-free Sequence Capture for**  
2 **Biodiversity Analysis from Bulk Environmental Samples**

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## 18 **ABSTRACT**

19 Environmental DNA analysis using PCR amplified marker genes has been a key  
20 application of high-throughput sequencing (HTS). However, PCR bias is a major drawback to  
21 gain accurate qualitative and quantitative biodiversity data. We developed a PCR-free approach  
22 using enrichment baits for species-specific mitochondrial cytochrome c oxidase 1(COI) DNA  
23 barcodes. The sequence capture was tested on species-rich bulk terrestrial and aquatic benthic  
24 samples. Hybridization capture recovered an average of 6 and 4.7 more arthropod orders than  
25 amplicon sequencing for terrestrial and benthic samples, respectively. For the terrestrial sample,  
26 the four most abundant arthropod orders comprised 94.0% of the sample biomass. These same  
27 four orders comprised 95.5% and 97.5% of the COI sequences recovered by amplification and  
28 capture, respectively. Hybridization capture recovered three arthropod orders that were detected  
29 by biomass analysis, but not by amplicon sequencing and two other insect orders that were not  
30 detected by either biomass or amplicon methods. These results indicate the advantage of using  
31 sequence capture for a more accurate analysis of biodiversity in bulk environmental samples.  
32 The protocol can be easily customized to other DNA barcode markers or gene regions of interest  
33 for a wide range of taxa or for a specific target group.

## 34 **INTRODUCTION**

35 The application of DNA sequence information for the identification of living organisms (e.g.,  
36 DNA barcoding) has revolutionized biodiversity science (1,2). Customized, public databases of DNA  
37 barcodes have been assembled through concerted efforts such as the International Barcode of Life (iBOL)  
38 project. These databases employ standardized gene regions for different groups of organisms. For  
39 example, cytochrome *c* oxidase subunit I (*COI*) has been established as the standard DNA barcode for  
40 animals (1). An increasing number of animal species have been DNA barcoded and their sequences are  
41 available publicly through the GenBank and BOLD online data portals (3,4). Similarly, other DNA

42 barcode markers have been established and public libraries built for other kingdoms of life: 16S  
43 ribosomal DNA (*16S*) for bacteria and Archaea (5); 18S ribosomal DNA (*18S*) for protists (6,7); the  
44 nuclear internal transcribed spacer (*ITS*) for fungi (8); and *rbcL* and *matK* gene regions for plants (9).

45 Initial DNA barcoding efforts were based on dideoxy chain-termination (“Sanger”) sequencing  
46 (10). While scalable, this approach is limited to producing single DNA barcode sequences for one  
47 individual at a time. High-throughput sequencing (HTS) (e.g., Illumina MiSeq) has been established as a  
48 viable means of assessing DNA barcode-based biodiversity from individuals (11-13) and mixed tissue  
49 samples (14-16). The HTS approach can produce a large number of sequences at a greatly reduced overall  
50 cost (11-13,15,17). All of these previous examples of massively parallel recovery of DNA barcode  
51 gene(s) have relied upon PCR amplification prior to HTS.

52 Protein-coding genes, such as *COI*, display more sequence variation between individuals and  
53 species as compared to ribosomal markers (18). Hence, they are generally considered more discriminating  
54 for the purposes of species identification and biodiversity analysis. However, any oligonucleotide primers  
55 designed for amplification of protein-coding gene regions may require the addition of degenerate or inert  
56 sites to improve taxonomic coverage. The use of degenerate PCR primer sets can still lead to  
57 amplification bias, especially when utilized to amplify environmental mixtures with high sequence  
58 diversity. Meanwhile, this bias can be exaggerated by the exponential nature of PCR amplification  
59 throughout the PCR cycles. This phenomenon can lead to over-amplification of some DNA templates at  
60 the expense of non- or under-amplification of other templates. Another possible effect of PCR  
61 amplification is the erroneous recovery of nuclear copies of mitochondrial DNA (i.e., NUMTs or  
62 pseudogenes). This amplification, sequencing, and reporting of paralogous gene regions can reach  
63 substantial rates in some instances (11,19).

64 It is important to note that primer amplification bias is not due solely to degeneracy and primer  
65 mismatch. Primer amplification bias exists even when highly conserved primers are used on mixed  
66 tissues. This bias is likely due to differences in GC content of amplicons, amplicon length, annealing  
67 temperature, overall genetic diversity of the initial mixture, and the binding energies of the primers

68 themselves (20-22). In order to account for PCR bias, most HTS studies design primers to amplify  
69 taxonomic groups of interest. For example, Zeale et al. (23) designed *COI* primers to amplify 12 orders of  
70 insects known to make up the diet of bats prior to amplifying and sequencing bat gut contents. In addition  
71 to taxon-specific primer design, the employment of multiple PCR reactions with different primer sets has  
72 been shown to lessen the impact of primer bias in HTS research. The multiple primer set approach has  
73 been especially effective when attempting to recover sequence data from a broad taxonomic range. In  
74 mixtures of arthropod tissue from both freshwater benthos (24) and terrestrial Malaise samples (15), the  
75 use of multiple primer sets recovers a greater proportion of taxa present than any single primer set alone.

76 In addition to the possibility of PCR primer bias producing false negative results due to selective  
77 exclusion of some species present in a mixture, the issue of organismal abundance has been raised. With  
78 the possibility of some DNA templates being selectively amplified over others during the PCR  
79 amplification process, relative abundance of DNA sequences cannot be used as a proxy for organismal  
80 abundance. Some have attempted to reconcile this discrepancy through the estimation of DNA barcode  
81 copy numbers, but this must be calculated one species at a time under controlled lab conditions (25).

82 The necessity of shorter target gene regions suitable for HTS has resulted in a move towards the  
83 use of shorter regions of *COI* contained wholly within the standard *COI* barcode region (14,15, 26,27). In  
84 each case, these shorter gene regions are tested *in silico* to ensure that the shorter region can still provide  
85 adequate species-level discrimination. In contrast, similar tests of species-level discrimination for other  
86 potential marker sequence regions (especially non-protein-coding loci) have given mixed results. For  
87 example, short segments of the *ITS* region have proven to be valuable for identification in fungi (28). On  
88 the other hand, the nuclear 16S/18S SSU rDNA region has been shown to lump species together  
89 producing artificially low reports of biodiversity (29,30).

90 Hybridization capture followed by HTS has been used in previous instances, usually focusing on  
91 individual test species. For instance, the capture method has been used to investigate vertebrate/virus  
92 genomic integration (31-33), the phylogeography of small sets of vertebrate species (34,35), and ancient  
93 plant DNA (36). When used to recover DNA regions associated with human genetic disease, the capture

94 method was able to supply accurate copy number information for multiple gene regions (37). Also using  
95 humans as test organisms, capture protocols were used to recover and reassemble complete individual  
96 mitochondrial genomes from mixed starting material (38).

97 We propose the use of hybridization capture methods, short DNA barcode regions, and HTS to  
98 eliminate the effects of PCR bias on biodiversity assessments of mixed environmental samples. We  
99 hypothesize that this approach can improve recovery of DNA barcode data from different types of bulk  
100 environmental samples as compared to PCR-based analysis. Hence, a capture-based approach should  
101 report fewer taxonomic false negatives as compared to a PCR-based method. Also, relative sequence  
102 abundance data should be more detailed and accurate than PCR-based relative sequence abundance data.  
103 This hypothesis is tested using capture baits designed from a DNA library representing 26 orders of  
104 Arthropoda and the tested tissue samples collected via two, distinct arthropod-targeting sampling  
105 methods. Malaise traps typically capture terrestrial insects, whereas benthic sampling using a pond net  
106 recovers a broad range of aquatic arthropods.

## 107 **MATERIAL AND METHODS**

### 108 **Field sampling**

109 A Malaise sample was collected at Bosque Humedo, Area de Conservación Guanacaste, northwestern  
110 Costa Rica (latitude 10.85145°N; longitude -85.60801°W; altitude 290 m; date January 24–31, 2011). The  
111 sample was collected directly into 95% ethanol, and frozen at -20°C until thawed and processed. In  
112 addition, three benthic samples (A, B, and C) were collected from Wood Buffalo National Park in  
113 Alberta, Canada (58.60273°N; 111.52612°W) in June, 2011. The samples were collected 50 meters apart  
114 from each other and were taken from the edge of the emergent vegetation zone into the submerged  
115 vegetation zone at each site. Samples were collected using a standard pond net with a sterile 400µm mesh  
116 net and attached collecting cup attached to a pole. Effort was standardized at 2 minutes per sample.  
117 Sampling was conducted by moving the net up and down through the vegetation in a sinusoidal pattern  
118 while maintaining constant forward motion. Samples were preserved in 95% ethanol in the field, and kept  
119 cold until processed. All DNA-friendly best practices—including wearing clean gloves to collect and

120 handle samples in the field and laboratory and decontaminating nets between samples—were followed to  
121 minimize the risk of DNA contamination between sites.

## 122 **Biomass calculation**

123 For the Malaise sample, all 1,066 morphologically identifiable individuals were isolated, identified to  
124 order morphologically, photographed, measured for total body length (excluding antennae) and maximum  
125 thoracic width, and tissue subsampled for individual DNA extraction. Dry biomass of each individual was  
126 estimated using order-specific length-width formulae (39).

## 127 **DNA extraction**

128 The Malaise sample and each of the benthic samples were individually blended in 95% ethanol and the  
129 resultant slurry was transferred to multiple sterile 50 mL Falcon tubes. After ethanol evaporation of the  
130 slurry at 56°C, the dried mixture was divided into three lysing matrix tubes “A” (about 100 mg each) and  
131 homogenized using the MP FastPrep-24 Instrument (MP Biomedicals Inc.) at speed 6 for 40 sec. Total  
132 DNA of this homogenized slurry was extracted using the Nucleospin tissue kit (Macherey-Nagel Inc.)  
133 following the manufacturer’s instructions and eluted in 50 µL of molecular biology grade water.

## 134 **Amplification-based approach**

135 For all samples, two fragments within the standard *COI* DNA barcode region were amplified with two  
136 primer sets (F230R and BR5 for the Malaise sample; AD and BE for the three benthic samples), in a two-  
137 step PCR amplification regime (14). The primer sequences are as follows, F:

138 5'.GGTCAACAAATCATAAAGATATTGG .3' (40), 230\_R: 5'.

139 CTTATRTTRTTTATICGIGGRAAIGC.3' (this paper), A\_F: 5'.

140 GGIGGITTTGGIAATTGAYTIGTICC.3', B\_F:5'.CCIGAYATRGCI-TTYCCICG.3', D\_R:

141 5'.CCTARIATIGAIGARAYICCIGC.3' (24); and R5: 5'.GTRATIGCICCIG-CIARIAC.3' (15). The first

142 PCR used *COI*-specific primers and the second PCR involved Illumina-tailed primers. The PCR reactions

143 were assembled in 25 µL volumes. Each reaction contained 2 µL DNA template, 17.5 µL molecular

144 biology grade water, 2.5 µL 10× reaction buffer (200 mM Tris–HCl, 500 mM KCl, pH 8.4), 1 µL MgCl<sub>2</sub>

145 (50 mM), 0.5  $\mu$ L dNTPs mix (10 mM), 0.5  $\mu$ L forward primer (10 mM), 0.5  $\mu$ L reverse primer (10 mM),  
146 and 0.5  $\mu$ L Invitrogen's Platinum *Taq* polymerase (5 U/ $\mu$ L). The PCR conditions were initiated with  
147 heated lid at 95°C for 5 min., followed by a total of 30 cycles of 94°C for 40 s., 46°C (for both primer  
148 sets) for 1 min., and 72°C for 30 s., and a final extension at 72°C for 5 min., and hold at 4°C. Amplicons  
149 from each sample were purified using Qiagen's MiniElute PCR purification columns and eluted in 30  $\mu$ L  
150 molecular biology grade water. The purified amplicons from the first PCR were used as templates in the  
151 second PCR with the same amplification condition used in the first PCR with the exception of using  
152 Illumina-tailed primers in a 15-cycle amplification regime. All PCRs were done using Eppendorf  
153 Mastercycler ep gradient S thermal cyclers and negative control reactions (no DNA template) were  
154 included in all experiments. PCR products were visualized on a 1.5% agarose gel to check the  
155 amplification success.

#### 156 **Selecting capture targets and probes design**

157 The most important factor in designing a successful Sequence Capture Developer experiment is the  
158 quality of the input sequence used to select the target and capture probes. Conservative, hypervariable  
159 regions and copy number variation can introduce problems at the design stage (31,35). These problems  
160 increase significantly when creating designs for multiple species where some of the taxa are less  
161 represented in the database. In this study, a total of 79,215 *COI* DNA barcodes (47,631,471 bp)  
162 representing 26 arthropod orders, 559 families, and 4,035 genera were downloaded from both GenBank  
163 and BOLD in October, 2012. The 98% similarity clustering of the downloaded sequences resulted in  
164 46,762 unique clusters (28,365,304 bp) (Supplementary Table S1). Sequence Capture Developer probes  
165 were designed with the help of the NimbleGen probes design team. A total of 2.1 million 50-105 mer  
166 probes (baits) were designed to cover all target clusters. Uniformity of probe abundance was considered,  
167 especially for highly conservative regions. The baits design covers 26,918,673 target bases (94.9%) from  
168 46,762 (100%) of the clusters. Four Sequence Capture Developer probes reactions were ordered from  
169 NimbleGen, catalog number 06740278001.

#### 170 **Library preparation for Illumina MiSeq sequencing**

171 For each of the Malaise and benthic samples, a total of 3 µg total DNA of each sample was sheared using  
172 a Covaris S220 Focused ultra-sonicator, resulting in a range of 300-800 bp fragments of each sample. The  
173 fragmentation efficiency was tested by running the fragmented DNA on an Agilent Bioanalyzer and DNA  
174 1000 chip. Duplicate libraries with unique indexes were prepared with KAPA LTP library preparation kit  
175 (Catalog no. KK8230) according to the manufacturer's protocol.

#### 176 **Pre-capture pooling and hybridization**

177 For the three benthic samples, the indexed libraries were quantified and equimolar concentrations of each  
178 were pooled before hybridization. According to the NimbleGen SeqCap EZ SR user guide v4.2, the  
179 pooled and un-pooled indexed libraries were blocked with a blend of COT DNA (Sigma Aldrich, catalog  
180 number: 05480647001), MB-grade fish sperm DNA (Roche Diagnostics, catalog number: 11467140001),  
181 and plant capture enhancer as a part of Sequence Capture Developer Reagent (Roche Diagnostics, catalog  
182 number: 06684335001) in addition to the universal sequencing adaptors and the used indexes. The  
183 blocked indexed libraries were hybridized to aliquots of SeqCap EZ library at 47°C for 72 hours with the  
184 thermocycler's head lid maintained at 57°C. The captured libraries were recovered using Streptavidin  
185 Dynabeads and washed twice with 1x stringent wash buffer preheated to 47°C followed by subsequent  
186 washes with wash buffer I, II, and III and finally recovered with 50 µL of molecular biology grade water.  
187 The captured libraries were amplified, purified, and quantified to be ready for sequencing.

#### 188 **Illumina MiSeq sequencing**

189 The generated amplicons and captured libraries were sequenced in two MiSeq flow cells (one for  
190 amplicons and one for captured libraries) using a V3 MiSeq sequencing kit (300 × 2)(FC-131-1002 and  
191 MS-102-3001).

#### 192 **Bioinformatic processing**

193 For the three benthic samples, a total of 24.4 million sequences were generated from PCR amplification  
194 and capture libraries, while a total of 11.0 million sequences were generated from PCR amplification and  
195 capture libraries of the Malaise sample. For each sample, the forward and reverse raw sequences were  
196 kept un-merged as the size of the capture libraries could be variable. All sequences were filtered for



197 quality using PRINSEQ software (41) with a minimum Phred score of 20, window of 10, step of 5, and a  
198 minimum length of 150 bp. A total of 17.6 million sequences for benthic samples (mean: 2.0 million  
199 reads/sample) and a total of 9.3 million reads for the Malaise sample (mean: 3.1 million reads/sample)  
200 were retained for further processing. USEARCH v6.0.307 (42), with the UCLUST algorithm, was used to  
201 de-replicate and cluster the remaining sequences using a 99% sequence similarity cutoff. This was done to  
202 de-noise any potential sequencing errors prior to further processing. Chimera filtering was performed  
203 using USEARCH with the ‘de novo UCHIME’ algorithm (43). At each step, cluster sizes were retained,  
204 singletons were retained, and only putatively non-chimeric reads were retained for further processing. All  
205 good quality, non-chimera clusters were identified using the MEGABLAST algorithm (44) against a  
206 reference library. This reference library contained all verified *COI* sequences downloaded from the  
207 GenBank database January 15, 2015 (N = 883,612 sequences). All MEGABLAST searches were  
208 conducted with a minimum alignment length of 100 bp and a minimum similarity of 90% for *COI*  
209 taxonomic identification recovery based on unambiguous top matches, and 98% for order-, family-,  
210 genus-, and species-level identification recovery. All sequencing data generated has been submitted to  
211 Dryad and can be accessed at (link will be added once accepted)

## 212 **RESULTS**

### 213 **PCR-based sequencing**

214 PCR amplification followed by sequencing produced between 285,680 and 2,559,402 total  
215 sequences for each sample (Table 1). Following quality filtering between 144,356 (50.5%) and 2,342,372  
216 (91.5%) high-quality sequences remained for analysis. Of these high-quality sequences, between 135,560  
217 (93.9%) and 2,075,555 (88.6%) sequences were identified as *COI* sequences (i.e., had at a minimum of  
218 90% similarity to a reference *COI* sequence in GenBank).

### 219 **Hybridization capture sequencing**

220 Hybridization capture followed by sequencing produced between 3,010,292 and 4,444,412 total  
221 sequences for each sample (Table 1). Following quality filtering, between 1,700,512 and 3,713,290 (up to

222 85.73%) high-quality sequences per sample remained for analysis. Of these sequences, between  
223 1,001,123 and 2,278,390 (up to 68.2%) sequences per sample were identified as *COI* sequences as  
224 mentioned before.

### 225 **Taxonomic data recovery**

226 For the benthic samples, hybridization capture followed by HTS recovered an average of 18 more  
227 orders than PCR amplification followed by HTS. For the Malaise sample, the average increase in order  
228 richness was 7. For the benthic samples, hybridization capture followed by HTS recovered an average of  
229 31.8 more families than PCR amplification followed by HTS. For the Malaise sample, the average  
230 increase in family richness was 23. For the benthic samples, hybridization capture followed by HTS  
231 recovered an average of 37.2 more genera than PCR amplification followed by HTS. For the Malaise  
232 sample, the average increase in genus richness was 19.5.

233 Differences in taxonomic recovery considering only arthropod orders—for which the sampling  
234 and capture baits were designed—were also calculated. For the benthic samples, hybridization capture  
235 followed by HTS recovered an average of 4.7 more arthropod orders than PCR amplification followed by  
236 HTS (Figure 1). For the Malaise sample, the average increase in order richness was 6. For the benthic  
237 samples, hybridization capture followed by HTS recovered an average of 15 more arthropod families than  
238 PCR amplification followed by HTS. For the Malaise sample, the average increase in arthropod family  
239 richness was 24. For the benthic samples, hybridization capture followed by HTS recovered an average of  
240 18.2 more arthropod genera than PCR amplification followed by HTS. For the Malaise sample, the  
241 average increase in arthropod genus richness was 21.5.

### 242 **Relative sequence abundance**

243 For the Malaise sample, the four most abundant orders (Diptera, Lepidoptera, Coleoptera, and  
244 Hymenoptera) were all arthropods and comprised 94.0% of the biomass of the sample (Supplementary  
245 Table S2). These same four orders comprised 95.5%, 97.5%, and 97.4% of the *COI* sequences recovered  
246 by PCR amplification, Capture 1, and Capture 2, respectively. Three arthropod orders (Blattodea,

247 Trombidiformes, and Psocoptera) were detected by morphological identification and biomass analysis,  
248 but not by any molecular method. Also, Coleoptera was present as a much higher proportion of biomass  
249 (35.6%), than as sequence proportion by any of the three methods (amplification 0.08%; capture 0.23%  
250 and 0.29%). Hybridization capture recovered three arthropod orders (Collembola, Neuroptera,  
251 Trichoptera) that were detected by biomass analysis, but not by PCR amplification. Furthermore,  
252 hybridization capture detected two insect orders (Mantodea, Plecoptera), in very low sequence numbers,  
253 which were not detected by either biomass analysis or PCR amplification (Supplementary Table S2).

254 For the Benthic A sample, at the order level, the three most abundant arthropod orders (Diptera,  
255 Hemiptera, Podocopida) combined represented 56.0%, 56.4%, and 59.3% of the *COI* sequences recovered  
256 for PCR amplification, Capture 1, and Capture 2, respectively. For the Benthic B sample, at the order  
257 level, the mentioned three most abundant arthropod orders combined represented 60.8%, 56.2%, and  
258 55.0% of the *COI* sequences recovered for PCR amplification, Capture 1, and Capture 2, respectively. For  
259 the Benthic C sample, at the order level, the three most abundant arthropod orders combined composed  
260 93.3%, 92.7%, and 92.9% of the *COI* sequences recovered for PCR amplification, Capture 1, and Capture  
261 2, respectively (Figure 2).

262 A comparison between methods of the proportion of sequences assigned to each arthropod order,  
263 family, and genus was used to generate scatter plots and correlation values (Figure 3). Regardless of  
264 sample or taxonomic level, the two rounds of hybridization capture produced highly correlated (0.99 to  
265 1.00) proportions assigned to each taxon. The degree of correlation between PCR amplification and  
266 hybridization capture varied (0.43 to 0.93), but was consistently below that of hybridization capture round  
267 one versus round two. Similarly, Bray-Curtis dissimilarity values calculated from the proportion by taxon  
268 matrices at the arthropod order, family, and genus level were calculated (Figure 4). Likewise,  
269 dissimilarity values were much lower for hybridization capture round one versus round two comparisons  
270 (0.011 to 0.077), than those for PCR amplification versus hybridization capture comparisons (0.166 to  
271 0.615).

272 **DISCUSSION**

273           This study is the first targeted attempt at PCR-free DNA barcode recovery from mixed  
274 environmental samples. Previous work employed mitochondrial enrichment followed by sequencing of  
275 total enriched mtDNA (45). This method required the exclusion of 99.5% of sequence data in order to  
276 retain only the 0.5% that comprises informative *COI* DNA barcode data. Liu et al. (46) were able to  
277 increase the efficiency of whole mitochondrial genome sequencing to about 42.5% by using a capture  
278 microarray followed by HTS in a mock community sample containing equal aliquots of genomic DNA  
279 extracts of 49 species. The present method retains 52.4 to 68.2% of the passed filter sequences produced  
280 through Illumina MiSeq sequencing for final analysis (Table 1). Whether pooled as three lower diversity  
281 samples or retained as one single, high diversity, high sequencing coverage sample, this produces one to  
282 two million *COI* sequences per sample for further analysis. This rate of analyzable sequences does not  
283 differ between benthic- and Malaise-derived tissue samples.

284           The recovery of non-target DNA regions has been noted in previous use of capture enrichment in  
285 resequencing studies where multiple regions of the genome need to be enriched and analyzed (33,34,37).  
286 This occurrence is likely due to the capture of adjacent (“flanking”) DNA regions during the  
287 hybridization process. The proportion of recovered sequences not matching targets ranges between 18 and  
288 42% in these previous studies. In the present study, 31.8 to 47.6% of high-quality sequences produced  
289 through hybridization capture recovered were not positively identified as *COI*. This rate in target  
290 recovery, while similar to previous resequencing studies, differs fundamentally from past mitochondrial  
291 enrichment experiments. Previously, hybridization baits were designed and implemented to target a  
292 number of different gene regions for a narrow range of organisms (e.g., the entire mitochondrial genome  
293 of one species). The present use of hybridization capture targets many versions of one small gene region  
294 in order to retain sequence data from a taxonomically wide range of organisms. As such, the rate of non-  
295 target sequence generation can be interpreted as either capture of non-*COI* sequences with some affinity  
296 for the oligonucleotide baits used, or else sequencing error within the Illumina MiSeq resulting in  
297 uninformative sequences. Any truly “flanking” sequences adjacent to the *COI* region targeted could still  
298 be retained and identified as *COI* sequences.

299           Hybridization capture followed by HTS recovered approximately twice as many order, family,  
300 and genus names as compared to PCR amplification for the three benthic samples (Table 1, Figure 1).  
301 Likewise, hybridization capture followed by HTS recovered an increase in order, family, and genus  
302 richness for the Malaise sample. These increases in taxonomic recovery are more pronounced when only  
303 the Arthropoda are considered. These increases in biodiversity detection reflect a reduced false negative  
304 rate due to the absence of primer amplification bias. The difference in increased taxonomic recovery at  
305 each level reveals an important difference between the benthic and Malaise samples. For both Malaise  
306 and benthic samples, the majority of the orders recovered only through hybridization capture were non-  
307 insects, including other arthropods, vertebrates, diatoms, fungi, and plants. Benthic samples, being aquatic  
308 in nature, will include a number of orders not found in a terrestrial sample, especially zooplankton and  
309 phytoplankton. To rule out false positives, we considered only the sequencing clusters containing more  
310 than ten sequences per cluster. The PCR amplification primers employed were designed for insects and  
311 are unlikely to have amplified non-insect orders. The hybridization capture, despite also being designed  
312 from insect DNA sequences, employs multiple longer oligos complementary to multiple fragments of the  
313 *COI* gene region and was better able to recover non-insect taxa.

314           It has been stated that PCR primer bias is likely to make conventional barcode copy abundance  
315 calculations from HTS environmental DNA studies impossible (e.g., 47). The removal of this bias  
316 facilitates a shift towards more meaningful interpretations of sequence numbers recovered from  
317 environmental samples and decreases chances of false negatives for taxa with a small biomass or rare taxa  
318 (15,17). For all benthic and Malaise samples, there was consistency in relative sequence frequencies  
319 between hybridization capture attempts at the arthropod genus, family, and order levels (Figure 2, Table  
320 2). In all samples, PCR amplification recovered a different community profile from hybridization capture  
321 (Figures 2, 3; Table 1).

322           In the Malaise sample, when compared to biomass calculations, the difference in proportion of  
323 sequences assigned to each taxon is noticed as an overabundance of Lepidoptera sequences at the expense  
324 of Coleoptera. In addition, three orders (Blattodea, Trombidiformes, and Psocoptera) are not detected by

325 HTS methods at all. This result is likely a product of the taxonomic composition of GenBank *COI*  
326 libraries. An abundance of tropical Lepidoptera are represented as GenBank records whereas few Costa  
327 Rican Blattodea, Psocoptera, and Trombidiformes are included. This lack of likely matches, coupled with  
328 our strict 98% similarity cut-off, resulted in a failure to recover these orders. As a further analysis, when  
329 similarity cut-offs were loosened to 90% similarity, with the same GenBank library, Blattodea,  
330 Trombidiformes, and Psocoptera were recovered with HTS methods, and Coleoptera was recovered as a  
331 much greater proportion of sequences (results not shown). However, a relaxed cut-off for sequence  
332 analyses such as BLAST could also result in reduced confidence in taxonomic identification of sequences  
333 or may result in false positives. As reference DNA barcode libraries are populated with diverse taxa and  
334 include better representation of local populations (haplotypes), sequence capture studies can benefit from  
335 these additional sequences both for the design of capture probes and in subsequent analyses of sequences.  
336 In fact, the use of standard DNA markers (e.g., barcodes) and provisioning reference databases with  
337 voucher specimens could facilitate large-scale analyses of environmental samples.

338         The use of HTS to allow parallel sequencing of multiple genetic markers in both library building  
339 and environmental mixture research has been previously proposed and demonstrated (11,12,15). While  
340 the present study focused on *COI* region as the most widely used DNA barcodes, the protocol could be  
341 easily adapted to include other DNA barcode regions, phylogenetic markers, or functional genes. The  
342 presented approach offers the flexibility to be customized to specific target groups and multiple barcoding  
343 markers. It also overcomes the challenges in PCR-based methods including primer design in  
344 hypervariable markers and the associated bias. The ability to multiplex many samples and recover  
345 individual sample data at sufficient sequencing depth is shown here to have no negative effect on  
346 biodiversity data recovery. The push to include as many samples and genetic markers as desired in future  
347 HTS-based biodiversity studies would be readily accommodated by the current protocol.

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485 **Figure legends**

486 **Figure 1.** Taxonomic richness recovery at three levels for four different subsamples using PCR  
487 amplification and hybridization capture.

488 **Figure 2.** Proportion of DNA sequences identified to order for four different subsamples using PCR  
489 amplification and hybridization capture. Amp – sequences recovered via PCR amplification. Cap1, Cap2 -  
490 sequences recovered via hybridization capture in the first and second attempts.

491 **Figure 3.** Scatter plots and correlation values of proportion of sequences assigned to each arthropod  
492 order, family, and genus for four different subsamples using PCR amplification and hybridization capture.

493 **Figure 4.** Bray-Curtis dissimilarity values based on proportion of sequences assigned to each arthropod  
494 order, family, and genus for four different subsamples using PCR amplification and hybridization capture.

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513 **Tables**

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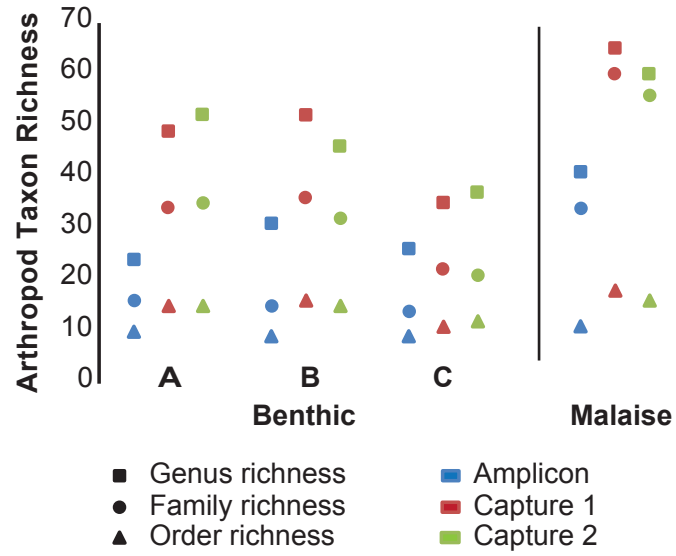
515 Table 1. Summary of number of DNA sequences produced, filtered, and used via four different samples

516 and two different methods.

<b>Sample</b>	<b>Process</b>	<b># raw DNA sequences</b>	<b># sequences passing quality filter</b>	<b># <i>COI</i> sequences</b>	<b>% high quality sequences used in <i>COI</i> analysis</b>
	<b>Amplicon</b>	285,680	144,356	135,560	93.9
<b>Benthic A</b>	<b>Capture 1</b>	4,331,460	3,713,290	1,954,225	52.4
	<b>Capture 2</b>	4,023,194	2,993,002	1,939,291	64.8
<b>Benthic B</b>	<b>Amplicon</b>	582,590	341,528	304,548	89.2
	<b>Capture 1</b>	4,042,778	2,703,271	1,695,684	62.7
	<b>Capture 2</b>	3,010,292	1,700,512	1,001,123	58.9
<b>Benthic C</b>	<b>Amplicon</b>	510,396	411,362	373,668	90.8
	<b>Capture 1</b>	3,438,490	2,558,027	1,744,542	68.2
	<b>Capture 2</b>	4,177,500	3,027,892	2,046,278	67.6
<b>Malaise</b>	<b>Amplicon</b>	2,559,402	2,342,372	2,075,555	88.6
	<b>Capture 1</b>	4,444,412	3,499,795	2,278,390	65.1
	<b>Capture 2</b>	4,042,698	3,436,537	2,041,300	59.4

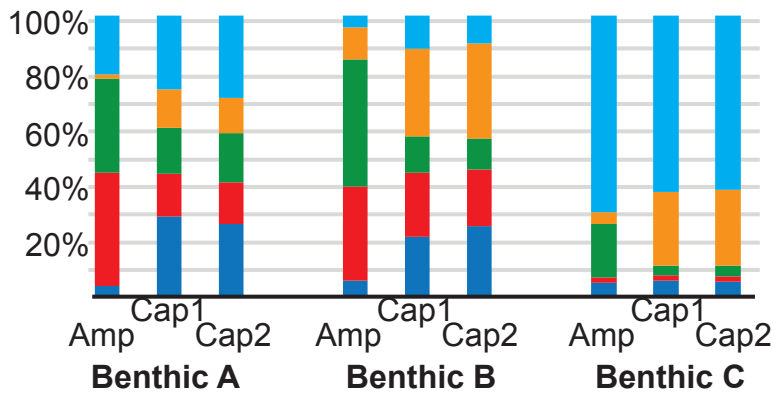
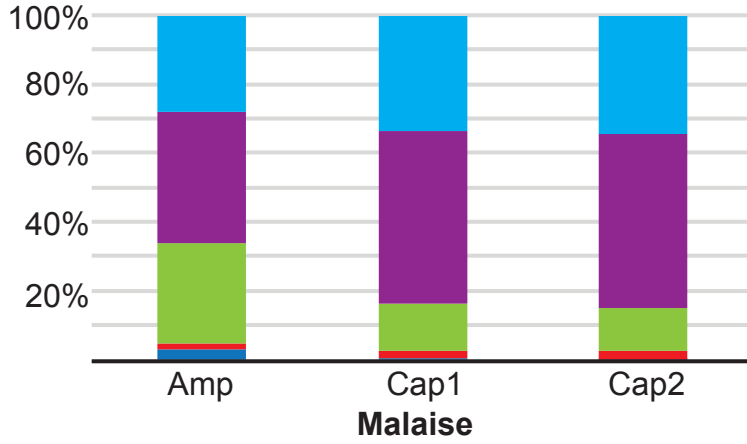
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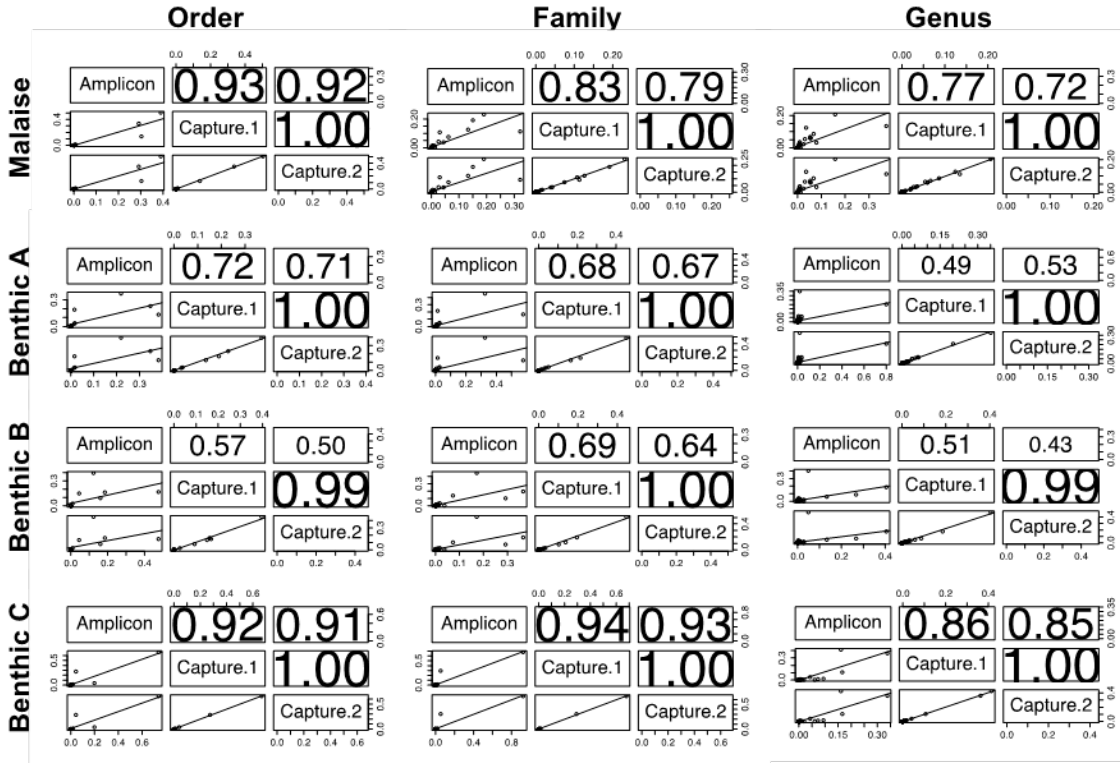
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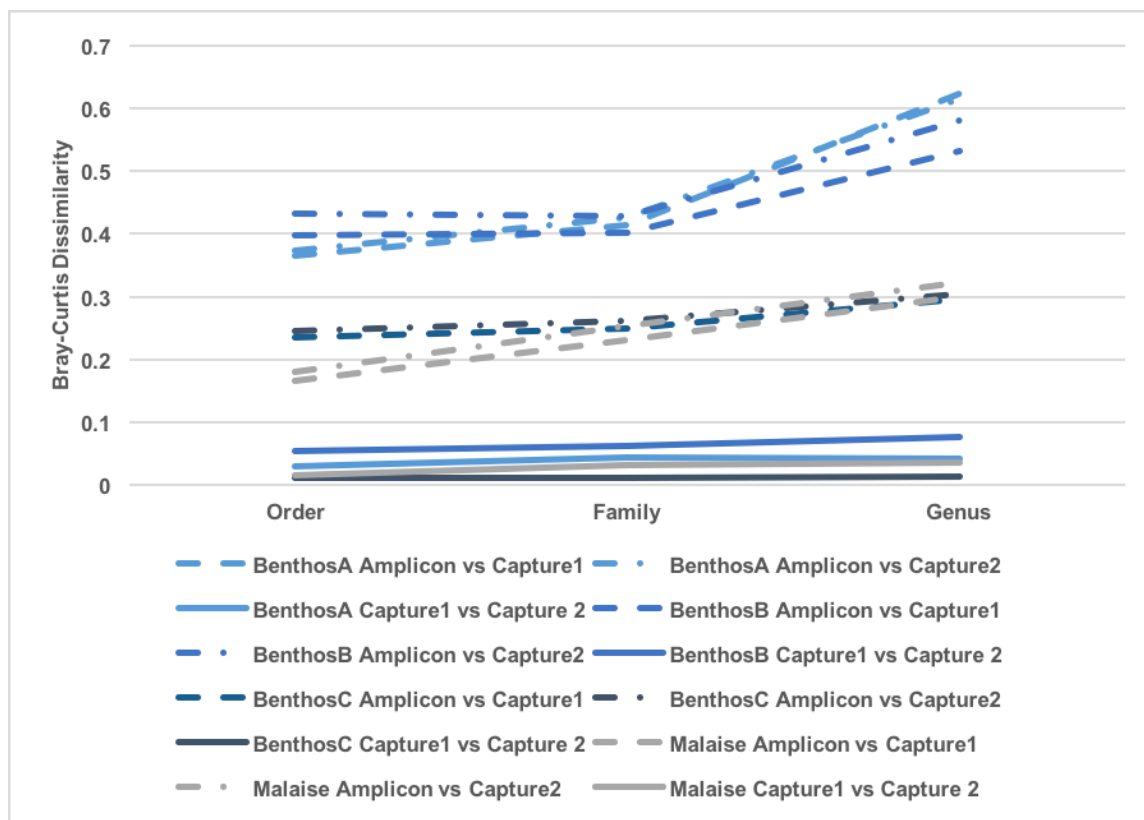
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527 **Supplementary material**

528 **Table S1.** Summary of sequences included in oligo development.

<b>Order</b>	<b># Families</b>	<b># Genera</b>	<b>#of sequences</b>	<b># of bases</b>
<b>COLEOPTERA</b>	80	764	6,531	3,756,110
<b>DIPTERA</b>	62	640	11,056	6,885,416
<b>HEMIPTERA</b>	60	407	4,010	2,390,925
<b>HYMENOPTERA</b>	36	257	14,358	8,745,477
<b>ARACHNIDA</b>	68	498	8,587	5,389,853
<b>ORTHOPTERA</b>	14	189	1,586	875,654
<b>PSOCOPTERA</b>	6	25	497	276,365
<b>STREPSIPTERA</b>				
<b>THYSANOPTERA</b>				
<b>DERMAPTERA</b>	1	1	34	21,751
<b>ISOPODA</b>	28	92	1193	702,654
<b>EPHEMEROPTERA</b>	15	47	2895	1,786,921
<b>Blattaria</b>	49	172	1,964	1,196,335
<b>Dermaptera</b>				
<b>Isoptera</b>				
<b>Mecoptera</b>				
<b>Megaloptera</b>				
<b>Neuroptera</b>				
<b>Odonata</b>				
<b>PLECOPTERA</b>	11	42	1,624	1,005,905
<b>TRICHOPTERA</b>	43	196	6,986	4,421,067

529	<b>MANTODEA</b>	12	90	135	79,097
530	<b>TROMBIDIFORMES</b>	17	22	11,453	6,095,705
	<b>COLLEMBOLA</b>	16	38	3,265	2,056,296
531	<b>LEPIDOPTERA</b>	39	552	2,997	1,918,309
532	<b>PHASMATODEA</b>	2	3	44	27631

533 **Table S2.** Proportion of calculated total biomass and proportion of *COI* DNA sequences identified to insect,  
534 arthropod, and non-arthropod orders via two different methods for a single Malaise sample.

	<b>Order</b>	<b>Biomass</b>	<b>Amplicon</b>	<b>Capture 1</b>	<b>Capture 2</b>
<b>Insects</b>	<b>Blattodea</b>	0.710%			
	<b>Coleoptera</b>	35.600%	0.080%	0.235%	0.291%
	<b>Diptera</b>	27.500%	28.288%	33.595%	34.687%
	<b>Ephemeroptera</b>		0.018%	0.031%	0.024%
	<b>Hemiptera</b>	1.390%	0.580%	0.953%	0.992%
	<b>Hymenoptera</b>	26.310%	29.461%	13.775%	12.243%
	<b>Lepidoptera</b>	4.620%	37.835%	50.324%	50.635%
	<b>Mantodea</b>			0.005%	0.002%
	<b>Neuroptera</b>	0.030%		0.008%	0.005%
	<b>Orthoptera</b>	2.470%	0.817%	0.886%	0.933%
	<b>Plecoptera</b>			0.002%	0.002%
	<b>Psocoptera</b>	1.050%			
	<b>Thysanoptera</b>	0.080%	0.013%	0.016%	0.025%
	<b>Trichoptera</b>	0.020%		0.008%	0.023%
	<b>Non-Insect</b>	<b>Amphipoda</b>			0.002%
<b>Arthropods</b>	<b>Araneae</b>	0.090%	0.031%	0.002%	
	<b>Collembola</b>	0.020%		0.013%	0.008%

<b>Decapoda</b>			0.002%	
<b>Diplostraca</b>		0.012%	0.026%	0.027%
<b>Podocopida</b>		0.023%	0.037%	0.031%
<b>Trombidiformes</b>	0.080%			
<b>Haplotaxida</b>		0.026%	0.035%	0.025%
<b>Rickettsiales</b>		2.805%	0.028%	0.027%
<b>Boraginales</b>		0.003%		
<b>Phyllodocida</b>			0.002%	0.002%
<b>Naviculales</b>			0.002%	0.002%
<b>Lumbriculida</b>		0.005%	0.005%	0.006%
<b>Basommatophora</b>		0.003%	0.008%	0.006%
<b>Scale</b>		<b>0.005%</b>	<b>5.000%</b>	<b>50.000%</b>

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