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Lessons from genome skimming of arthropod-preserving ethanol

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24 **Running title**

25 Metagenome skimming of preservative ethanol

26

27 **Abstract**

28 Field-collected specimens of invertebrates are regularly killed and preserved in ethanol, prior
29 to DNA extraction from the specimens, while the ethanol fraction is usually discarded.
30 However, DNA may be released from the specimens into the ethanol, which can potentially
31 be exploited to study species diversity in the sample without the need for DNA extraction
32 from tissue. We used shallow shotgun sequencing of the total DNA to characterize the
33 preservative ethanol from two pools of insects (from a freshwater and terrestrial habitat) to
34 evaluate the efficiency of DNA transfer from the specimens to the ethanol. In parallel, the
35 specimens themselves were subjected to bulk DNA extraction and shotgun sequencing,
36 followed by assembly of mitochondrial genomes for 39 of 40 species in the two pools.
37 Shotgun sequencing from the ethanol fraction and read-matching to the mitogenomes detected
38 ~40% of the arthropod species in the ethanol, confirming the transfer of DNA whose quantity
39 was correlated to the biomass of specimens. The comparison of diversity profiles of
40 microbiota in specimen and ethanol samples showed that ‘closed association’ (internal tissue)
41 bacterial species tend to be more abundant in DNA extracted from the specimens, while ‘open
42 association’ symbionts were enriched in the preservative fluid. The vomiting reflex of many
43 insects also ensures that gut content is released into the ethanol, which provides easy access to
44 DNA from prey items. Shotgun sequencing of DNA from preservative ethanol provides novel
45 opportunities for characterising the functional or ecological components of an ecosystem and
46 their trophic interactions.

47

48 **Introduction**

49 The exploration of biodiversity using high-throughput sequencing (HTS) opens a path to new
50 questions and novel empirical approaches. Although initially focusing on microbial diversity
51 (Sogin *et al.* 2011), more recent HTS studies have tackled the characterisation of complex
52 communities of macroscopic organisms (e.g. Fonseca *et al.* 2010; Ji *et al.* 2013; Andújar *et al.*
53 2015). The high sensitivity of these methods also permits the study of DNA isolated directly
54 from the environment (eDNA), such as soil (e.g. Andersen *et al.* 2012) and water (e.g. Jerde
55 *et al.* 2011; Thomsen *et al.* 2012), or ingested DNA from the gut of predators (Paula *et al.*
56 2014) or blood-sucking invertebrates (iDNA) (e.g. Schnell *et al.* 2012). Most studies have
57 used PCR amplification for targeting particular gene regions and taxonomic groups
58 (metabarcoding), and result in a set of sequences used for profiling the species mixture (Ji *et*
59 *al.* 2013). As an alternative to metabarcoding, the DNA of such mixtures can also be
60 characterised by metagenomic shotgun sequencing, in a procedure commonly referred to as
61 ‘genome skimming’ (GS) (Straub *et al.* 2012) and its extension to metagenomes
62 (‘metagenome skimming’, MGS) (Linard *et al.* 2015). Shallow sequencing of the total DNA
63 and subsequent assembly of reads with genome assemblers preferentially extracts the high-
64 copy number fraction of a sample including the mitochondrial genomes (Gillett *et al.* 2014;
65 Andújar *et al.* 2015; Crampton-Platt *et al.* 2015; Tang *et al.* 2015). In addition, MGS can
66 provide useful information about the species’ nuclear genomes and concomitant biodiversity
67 such as bacterial symbionts or gut content (e.g. Paula *et al.* 2014; Linard *et al.* 2015).

68 Assemblages of invertebrates, which may be a primary target of such HTS efforts, are
69 frequently collected into ethanol as preservative in the field until DNA extraction is
70 performed at some later point. Frequently, multiple conspecific or heterospecific individuals

71 and even complete communities are stored together in a single container, under the
72 assumption that cross-contamination is too low to be detectable in the Sanger sequencing of
73 the individual specimens. However, reports of PCR amplification of arthropod genes from
74 ethanol and even from alcoholic beverages indicate that traces of DNA are transferred from
75 the specimen to the preservative (e.g. Shokralla *et al.* 2010; Hajibabaei *et al.* 2012), and with
76 the much greater sensitivity of single-molecule sequencing, the question about the magnitude
77 of cross-contamination takes on a new significance. In addition, detecting low concentration
78 DNAs in the preservative opens exciting new opportunities for the study of bulk biodiversity
79 samples, as extractions directly from the ethanol may avoid the need for tissue preparations
80 and the resulting damage to specimens caused by standard methods. This would be
81 particularly useful for the sequencing of spirit-preserved collections in the world's natural
82 history museums.

83 In a recent metabarcoding study of benthic arthropods, the set of species obtained directly
84 from the specimen mixture were reported to be detectable also in the ethanol in which these
85 specimens had been stored (Hajibabaei *et al.*, 2012). However, these PCR-based studies did
86 not provide a quantitative measure of the amount of transferred DNA. The great sequencing
87 depth achievable with Illumina sequencing now permits a more direct approach to address the
88 question about DNA transfer to the ethanol with PCR-free methods by shotgun sequencing of
89 DNA from the preservative ethanol. This approach could be a straightforward, non-
90 destructive way to study bulk-collected arthropods. In addition, the non-targeted sequencing
91 of total DNA could also be used to explore specific fractions of the associated biodiversity
92 that are released into the preservative, e.g. from the gut or attached to the exoskeleton, which
93 may be different in composition from the directly sequenced specimen. Therefore, shallow

94 metagenomic sequencing of preservative ethanol could be used as an alternative tool to study
95 species diversity and biotic associations.

96 Here, we conducted shotgun sequencing on DNA extracted from ethanol used as a killing
97 agent and preservative in field collecting of mixed arthropods (one freshwater and one
98 terrestrial pool). We also extracted DNA from the ethanol-preserved specimens and
99 assembled complete mitochondrial genome sequences from shotgun sequencing thereof.
100 These assemblies served as reference sequences to map the reads from the ethanol fraction, as
101 a measure of the magnitude of DNA transfer from the specimens to the preservative medium.
102 In addition, we extensively explored the concomitant biodiversity detectable in the
103 preservative fluid, with special attention to potential gut content released from the live
104 specimens when placed in the ethanol. The collection fluid therefore may be enriched for food
105 items and gut bacteria, but may be impoverished for internal parasites and bacterial
106 endosymbionts if compared with specimen DNA extractions. Considering that field collection
107 of bulk arthropod communities into preservative ethanol remains the primary step in most
108 biodiversity surveys, sequencing of ethanol-derived DNA may be a powerful approach for the
109 study of species diversity and ecology.

110

111 **Materials and Methods**

112 **Specimen collection**

113 Two arthropod pools were generated with specimens collected from terrestrial and aquatic
114 environments in Richmond Park, Surrey, UK (coordinates: 51.456083, -0.264840). Aquatic
115 arthropods were collected along the edge of a pond using a 5 mm mesh. Live specimens were

116 transferred to a 100 ml sterile vial containing 80 ml of 100% (pure) ethanol to generate a
117 pooled 'aquatic' sample (Figure 1A). A 'terrestrial' sample was obtained by hand collection of
118 beetles under stones and logs in the area surrounding the pond. Both were conserved for less
119 than a day at ambient temperature and maintained at -18°C for two weeks before DNA
120 extraction was performed. The specimens occupied up to half of the volume of the collecting
121 vial, reducing the final concentration of the ethanol to an unknown degree.

122 **Mitochondrial metagenomics of voucher specimens**

123 Specimens from each pool (*vouchers*) were individually removed from the ethanol using
124 sterilised forceps, identified to genus level, grouped by morphospecies, and their body length
125 measured (Figure 1B). Individual non-destructive DNA extraction was performed on up to
126 four specimens of each morphospecies using the DNeasy Blood & Tissue Spin-Column Kit
127 (Qiagen). The 5' half of the *cox1* gene (barcode fragment) was PCR amplified using the *FoldF*
128 and *FoldR* primers (see Suppl. File S1 for details) and the PCR products were Sanger
129 sequenced with ABI technology. Morphological identifications were validated by BLAST
130 searches against the NCBI and BOLD databases (accessed on 29-04-2015). DNA
131 concentrations of specimen extractions were estimated using the Qubit dsDNA HS Assay Kit
132 (Invitrogen) and equimolar pooled aliquots were used to prepare two specimen pools:
133 *Terrestrial Vouchers* (TV) and *Aquatic Vouchers* (AV). Two Illumina TruSeq DNA PCR-free
134 libraries were prepared and sequenced on an Illumina MiSeq sequencer (2 x 250 bp paired-
135 end reads).

136 Raw paired reads were trimmed to remove residual library adaptors with Trimmomatic v0.32
137 (Bolger *et al.* 2014), and Prinseq v0.20.4 (Schmieder & Edwards 2011) was used for filtering
138 low-quality reads. Filtered reads from each pool were then assembled using four different

139 assemblers; Celera Assembler v7.0 (Myers 2000), IDBA-UD v1.1.1 (Peng *et al.* 2012),
140 Newbler v2.7 (Miller *et al.* 2010) and Ray-meta v1.6.5 (Boisvert *et al.* 2012). Contigs with
141 regions of high similarity produced by the different assemblers were merged with the '*De*
142 *Novo Assembly*' function of Geneious v7.1.8 (minimum overlap = 500 bp; minimum overlap
143 identity = 99%). The resulting mitogenomes were first annotated with the MITOS server
144 (Bernt *et al.* 2013), then manually curated to validate all protein-coding, rRNA and tRNA
145 genes. Finally, mitogenomes were matched with the corresponding Sanger *coxI* sequences for
146 species assignment. For further details on the mitochondrial metagenomics pipeline see
147 Crampton-Platt *et al.* (2015) and Suppl. File S1.

148 **Metagenomics of voucher specimens and preservative ethanol**

149 The preservative ethanol from the terrestrial and aquatic pools was decanted and centrifuged
150 (Figure 1C) at 14000 *g* for 30 min at 6°C to allow for sedimentation of precipitated DNA
151 (Tréguier *et al.* 2014). The supernatant was discarded, the precipitate was dried, and DNA
152 was extracted using the DNeasy Blood & Tissue Spin-Column Kit (Qiagen). Concentrations
153 of total DNA extracts were estimated using the Qubit dsDNA HS Assay Kit (Invitrogen) and
154 the two pools representing the terrestrial and aquatic specimens, respectively, in equal
155 concentrations were used to prepare TruSeq DNA PCR-free libraries, referred to as
156 *Terrestrial Ethanol* (TE) and *Aquatic Ethanol* (AE), and Illumina sequenced (2 x 250 bp
157 paired-end reads for AE; 2 x 300 bp paired-end reads for TE) using 5 and 4% of a flow cell on
158 the MiSeq. Adapter removal and quality control followed the same protocol as described
159 above for the *vouchers* (TV and AV; also see Suppl. File S1).

160 *Voucher species recovery from the preservative ethanol*

161 Species recovery from the preservative ethanol was assessed by matching the filtered TE and
162 AE reads against the voucher sequences using BLAST ($\geq 97\%$ similarity over ≥ 150 bp).
163 Sanger sequences, full-length assembled mitogenomes, and the protein-coding genes only (i.e.
164 excluding the less variable rRNA genes) were used as references to check for differences in
165 species recovery depending on the voucher information used. The biomass of each species in
166 the pools was estimated using specimen length as a proxy for body size, multiplied by number
167 of specimens, and was subsequently correlated with the number of matching reads from the
168 *ethanol* libraries.

169 *Phylogenetic profile of the vouchers and the preservative ethanol*

170 The diversity of concomitant DNA (reads presumed not to be derived from the genomes of
171 voucher specimens) was estimated for each library (Figure 1C) by (i) a general taxonomic
172 characterisation of the paired reads and (ii) a more precise assignment of the reads to
173 mitochondria, plastids, nuclear rRNAs and putative bacterial symbionts. The general
174 taxonomic characterisation is based on a custom database combining the whole content of the
175 preformatted NCBI *nt* (nucleotides) database and all coleopteran assemblies currently
176 available in the NCBI *wgs* database (Suppl. File S1 for the reason motivating this choice).
177 Each library was aligned to this custom database with megaBLAST from the BLAST+
178 package (Camacho *et al.* 2009), retaining only hits with a maximum E-value of $1e-15$.
179 BLAST outputs were then analysed with MEGAN 5.10.3 (Huson *et al.* 2007). The MEGAN
180 LCA (Lowest Common Ancestor) clustering was set to consider paired reads as belonging to
181 the same entity and only the top 20% of BLAST hits were considered for taxonomic
182 assignments, with all other MEGAN clustering parameters kept at default values. Pie charts

183 describing the taxonomic content of the *voucher* and *ethanol* libraries were also generated
184 with MEGAN.

185 Assignment of reads to four specific categories of DNA markers was based on read matches
186 to four custom reference databases, including (i) “Mitochondria” containing all complete and
187 partial mitochondrial genomes (minimum 10 kb) from the NCBI *nt* database (downloaded on
188 05-05-2015); (ii) “Plastids” obtained by retrieving all complete and fragmented plastid
189 genomes (minimum 10 kb) from the NCBI Nucleotide database (downloaded on 04-05-2015);
190 (iii) “Symbionts” based on all complete genomes available from NCBI for a panel of bacterial
191 genera known for their symbiotic interactions in different arthropod lineages, including 27
192 bacterial genera reported in Russel et al. (2012) (retrieved from the NCBI Genome database
193 on 08-07-2014; details in Suppl. File S1); (iv) “Nuclear rRNAs” corresponding to the whole
194 content of the SILVA database (Quast *et al.* 2013) (release 119, containing manually curated
195 18S and 28S rRNAs for 2,100,000 bacteria, 49,000 archaea, 95,000 eukaryotes and 44,000
196 unclassified cultured organisms). Reads of all libraries were aligned to these databases with
197 megaBLAST and the taxonomic classification of the BLAST best hit was assigned based on
198 stringent similarity thresholds (Suppl. File S1). Mitochondrial and plastid reads were then
199 grouped according to high taxonomic levels (Arthropods, Plants, Fungi, etc.), while bacterial
200 symbionts and rRNA reads were assigned to genera when more than 99% similar to a
201 reference for >90% of the read. Only taxa supported by more than 5 matching reads in one of
202 the libraries were considered for further analyses.

203 The proportion of reads assigned to the above four classes of DNA markers in different taxa
204 were compared between the *vouchers* (AV, TV) and the *ethanol* (AE, TE) libraries. For a
205 single library, a marker proportion is reported as the ratio of base pairs assigned to a particular

206 taxon over the total number of base pairs sequenced in the library. The percentage difference
 207 (increase or decrease) of this proportion in the ethanol compared to the voucher libraries was
 208 calculated. Formally, in a library L of size S (bp) we define a pair $\{C, M\}$ representing a clade
 209 C and a DNA marker M . In L , the number of bp n associated to M and identified as belonging
 210 to C is noted $n_{\{C,M\}}^L$ and is then converted to a library proportion $P_{\{C,M\}}^L$ with the formula:

$$P_{\{C,M\}}^L = \frac{n_{\{C,M\}}^L}{S^L}$$

211
 212 The percentage change (% change) observed for a pair $\{C,M\}$ in a library L_2 compared to a
 213 library L_1 , as well as the magnitude of change corresponding to this increase (when positive)
 214 or decrease (when negative) is then defined as:

$$\% \text{ change}_{L_2/L_1} = \frac{P_{\{C,M\}}^{L_2} - P_{\{C,M\}}^{L_1}}{P_{\{C,M\}}^{L_1}} \times 100$$

215
 216 Typically, L_2 will correspond to an *ethanol* library (E) that is compared to L_1 constituting a
 217 *voucher* library (V) and a pair of clade and marker could be for instance {Bacterial symbiont,
 218 rRNAs}. Then, the differential recovery obtained from the ethanol is reported as the order of
 219 magnitude (\log_{10}) of the difference ΔF_{EV} in nucleotide counts between both libraries, i.e.

$$\Delta F_{L_2/L_1} = \log_{10}(|\% \text{ change}_{L_2/L_1}|)$$

220
 221 For instance, for the pair {Bacterial symbiont, rRNAs} a $\Delta F_{EV} = 2$ indicates a recovery of
 222 symbionts rRNA base pairs 100 times higher in the *ethanol* (preservative) compared to the
 223 *voucher* (the specimen itself).

224

225 **Results**

226 **Assembly of mitogenomes from voucher specimens**

227 A total of 126 and 49 specimens were collected respectively in the aquatic and terrestrial
228 habitats, which in total represented 38 morphospecies from the order Coleoptera and one
229 morphospecies each of Trichoptera and Megaloptera encountered as larval stages in the
230 freshwater pool. Representatives of all morphospecies were selected as vouchers, and
231 depending on body size and where possible, up to four specimens were subjected to DNA
232 extractions (to standardize the amount of DNA for improved assembly), for a total of 72
233 specimens (see Table 1). Sanger sequencing generated successful *coxI* barcodes for 37 of the
234 40 morphospecies (Table 1). BLAST matches of these voucher *coxI* sequences against the
235 NCBI and BOLD databases showed good agreement with the morphospecies identifications
236 (Table 1). The voucher DNA extracts were pooled in equal concentrations to generate two
237 mixtures, one terrestrial (TV) and one aquatic (AV). Illumina MiSeq sequencing on these
238 pools produced, respectively, 10,782,446 and 26,867,180 paired reads after quality control
239 and resulted in successful assembly of complete or nearly complete mitochondrial genomes
240 for 39 of the 40 morphospecies (Table 1).

241 **Metagenomics of voucher specimens and preservative ethanol**

242 *Voucher species recovery from the preservative ethanol*

243 The TE and AE libraries built from the preservative ethanol produced a total of 1,960,740 and
244 1,772,094 paired reads, respectively. Matching these reads against the voucher *coxI*
245 sequences recovered only 4 species, while using the full-length and protein-coding genes of
246 the assembled mitogenomes recovered 15 and 13 species. The species with highest recovery

247 were those with high biomass in the samples, including the larval specimens of *Sialis sp.*
248 (Neuroptera) and *Dorcus sp.* (Coleoptera:Lucanidae) (see Table 1), and a strong correlation
249 was found between the log transformed number of reads in the preservative ethanol and the
250 estimated biomass of each species (Pearson R = 0.88, p-value = 0.0001; Figure 2).

251 *Phylogenetic profile of the vouchers and the preservative ethanol*

252 The general taxonomic characterisation of the paired reads showed that in all libraries a large
253 proportion of reads has no BLAST hits to our custom reference databases, with 95.3, 95.5,
254 93.0 and 95.2% of reads unmatched in AV, TV, AE and TE, respectively. The inclusion of
255 coleopteran genome assemblies (from NCBI *wgs* data) in the reference database contributed
256 significantly to the MEGAN identification of arthropod nuclear DNA (compared to using
257 NCBI Nucleotide reference set alone; see Suppl. File S2). This was particularly striking for
258 the aquatic pool, for which the number of identified coleopteran reads increased by a factor
259 4.4 in AV and 14.1 in AE, while this factor was 1.8 and 1.3 in the terrestrial TV and TE pools.

260 Identified reads showed different profiles in the voucher and ethanol libraries, but also
261 between the two habitats (Figure 3). In the *voucher* libraries the great majority of these reads
262 were apparently derived from the target specimens, with 78.6 and 77.4% identified as
263 arthropod reads in AV and TV. This proportion was reduced in the *ethanol* libraries to 17.2
264 and 7.1% in AE and TE. Other DNAs were present in low proportions in the vouchers but
265 dominant in the preservative ethanol. In both *voucher* libraries, Proteobacteria were the 2nd
266 most dominant clade. In AV, Proteobacteria are followed by Nematoda, Platyhelminthes and
267 Chordata reads in decreasing proportions, with more than half of the Chordata reads identified
268 as sequences of *Cyprinus carpio* (common Eurasian carp). Within Platyhelminthes, 10,158
269 reads were assigned at the species level to the tapeworm *Hymenolepsis diminuta*. No species-

270 level identifications were obtained for Nematoda, which produced scattered matches to
271 numerous sub-taxa. TV showed a similar profile with a dominance of Proteobacteria,
272 followed by a more diverse pattern of various bacterial phyla.

273 The *ethanol* libraries were characterized by a high diversity of bacterial taxa. Again,
274 Proteobacteria were prevalent but the TE sample clearly differed from all others by showing a
275 large proportion of reads matching Firmicutes (36.5%). In addition, a high diversity of
276 eukaryotic clades was recovered. Ascomycota (fungi) were observed in both habitats with a
277 greater prevalence in TE (6.2%). Chordata and Streptophyta (land plants and green algae)
278 were identified in AE.

279 Further analyses allowed the assignment of the reads to three main groups, including (i)
280 arthropods, (ii) taxa potentially associated to the gut or the environment, and (iii) bacterial
281 endosymbionts. Their relative proportion was compared in the *voucher* and *ethanol* libraries
282 (Figure 4, Suppl. Table S3). Generally, DNA reads were recovered, in decreasing order of
283 abundance, from plastids, mitochondria and rRNA genes in eukaryotes, and from complete
284 genomes and rRNAs in bacterial symbionts, reflecting that longer markers produced more
285 read matches. In agreement with Figure 3, the proportion of Arthropoda reads in the *ethanol*
286 was much lower than in the *vouchers* for both habitats. On average, a two-orders of
287 magnitude ($F=2.0$) loss was observed for both the mitochondrial and the rRNA sequences
288 (Figure 4A). In contrast, read numbers for some taxa potentially associated with the
289 environment and gut content (Figure 4B) were increased in the *ethanol* by between 2.2 (Fungi
290 rRNA) to 4.6 (Annelida rRNA) orders of magnitude. Following Douglas et al. (2015), the
291 symbiont species were divided into those with “closed associations” representing strict
292 bacterial symbionts confined to bacteriocytes or specific host tissues, and those in “open

293 associations” representing bacterial infections, loose symbiotic interactions or commensals of
294 the gut. All genera in closed associations (*Wolbachia*, *Rickettsia*, *Regiella*) showed a lower
295 recovery from the *ethanol* compared to the *vouchers*, and *Wolbachia* and *Rickettsia*,
296 respectively, were absent altogether in TE and AE, despite their strong signal in the *vouchers*
297 (Figure 4C). On the other hand, symbiont genera with open associations showed more
298 complex patterns, but in general recovery was higher or at least at similar levels in the *ethanol*
299 than in the *vouchers*. Interestingly, in both TV and TE we noticed the presence of rRNA
300 genes from endosymbionts typically associated with Collembola, possibly providing indirect
301 evidence for predation on arthropod microfauna in some of the voucher specimens of the
302 terrestrial pool (Figure 4C).

303

304 **Discussion**

305 **Species recovery and shotgun metagenomic sequencing from preservative ethanol**

306 Earlier PCR-based studies have demonstrated that specimen DNA can be obtained from the
307 preservative ethanol (e.g. Shokralla *et al.* 2010; Hajibabaei *et al.* 2012), while here we
308 established the power of direct shotgun sequencing, for a broader characterisation of the
309 sampled specimens. PCR-based approaches are effective for detection of low DNA
310 concentration templates, and thus have been successful for generating fairly complete species
311 inventories from the ethanol fraction (Hajibabaei *et al.* 2012). We show that the number of
312 DNA reads pertaining to the specimens themselves is rather low and, at the selected
313 sequencing depth, less than half of species present in the samples could be identified from the
314 reads, despite the availability of complete reference mitogenomes. If it is the aim of a study to

315 detect all species in the sample, PCR amplification may be the more efficient approach, but
316 with the proviso that the specific primers used in the assay limit the outcome of the detected
317 taxa (only *coxI* was used in previous studies). Alternatively, a combination of primer sets
318 (Hajibabaei *et al.* 2012) can be used but holds the risk of cross-sample contamination, in
319 particular if samples differ greatly in the concentration of DNA. In addition, the PCR
320 approach may not be universally successful. In our attempts to replicate the *coxI* results on
321 the ethanol samples generated here, we experienced a complete failure of amplification
322 despite the use of various primers and PCR protocols (data not shown). The DNA
323 concentration and level of preservation were sufficient for metagenomic libraries, which
324 generally requires much more DNA template than the PCR, ruling out issues affecting the
325 quality or quantity of the template for PCR failure. Instead, PCR inhibitors from the
326 environment or the gut may be enriched in the ethanol fraction, which apparently affects the
327 PCR, but less so the library construction and direct sequencing of the DNA.

328 In addition, the shotgun approach provides a better quantitative measure of the DNA
329 concentrations for each species, as it is not affected by uneven amplification of templates in
330 the mixture. We find that the DNA pool was dominated by two large-bodied species present
331 in multiple individuals (*Dorcus sp.* in TE and *Sialis sp.* in AE) that accounted for >23% of all
332 mitochondrial reads. Both species were encountered in the larval stages, whose soft cuticle
333 may have facilitated the release of DNA into the ethanol. Some species with low biomass
334 (body size x specimen number) or hard cuticle remain below the detection limit but should
335 be recovered with deeper sequencing of ethanol libraries beyond the ~5% of a MiSeq flow
336 cell used here. Similarly, recovery of low-biomass species could be improved if great
337 differences in DNA concentration are avoided by sorting according to body size or life stage
338 during field collecting.

339 The availability of reference sequences was a key requirement for the shotgun approach. We
340 generated an almost-complete reference set of mitogenomes following an established protocol
341 (Crampton-Platt *et al.* 2015, 2016). At the read depth used here (approximately 1% of a
342 MiSeq flow cell per species) this procedure was highly efficient and even exceeded the
343 species identification rate of *cox1* PCR-based Sanger sequencing of the same specimens. In
344 addition, the *ethanol* libraries produced many matches to arthropod nuclear DNA, including
345 rRNA genes that could be identified against external databases (Figure 4A). Although
346 complementing mitochondrial references with rRNA markers would greatly increase the
347 sensitivity of species recovery, the assembly of rRNA genes remains challenging. In our tests,
348 no unequivocal contigs were produced in both TV and AV, despite the use of four different
349 assemblers (Suppl. Table S4). While present in high copy number in metazoan genomes,
350 alternating highly conserved and rapidly evolving expansion segments in the primary
351 sequence of rRNA genes (Stage & Eickbush 2007) currently prevent the assembly from short
352 sequence reads.

353 **Exploration of concomitant biodiversity from the preservative ethanol**

354 The *ethanol* libraries may be considered as complex ‘environmental DNA’ (eDNA) mixtures
355 that include the DNA released from the focal specimens, together with organisms associated
356 with these specimens and potentially unconnected organisms carried over from the wider
357 ecosystem (Bohmann *et al.* 2014). Bacteria are expected to have a high chance of recovery in
358 the DNA reads, as they are present in high copy numbers and they are detected by read
359 matching against full genomes. Some bacterial genera detected in the ethanol are known to be
360 associated to specific habitats (e.g. *Acinetobacter*, *Hydrogenophaga*; Figure 4B). These were
361 present in small proportions (Figure 3), as would be expected in specimens collected

362 manually from the environment, which limits these contaminants. A larger proportion of the
363 ethanol-enriched clades seems to be associated with gut content such as *Proteobacteria* or
364 *Firmicutes*, which are generally dominant microbiota of insect guts, followed by
365 *Bacteroidetes*, *Actinobacteria* and *Tenericutes*. The libraries recovered very similar profiles to
366 those obtained in a recent study of insect gut microbiomes (see Figure S2; Yun *et al.* 2014).
367 Bacterial clades known to be gut-specific are part of this profile in both habitats, i.e. high
368 proportions of Enterobacteriales (*Proteobacteria*) and “open associations” symbionts
369 (*Serratia*, *Rickettsiella*, etc.). Hence, the vomiting of many arthropods at the moment of being
370 immersed in the ethanol (which is seen in many insects but particularly in predatory beetles)
371 appears to be an effective mechanism for the release of gut content to the preservative
372 medium. These DNA profiles from specimen mixtures reflect compound microbiota that are
373 determined by the species composition and relative abundance of the insect communities and
374 their habitat, diet and developmental stage. A case in point are the *Firmicutes* that include the
375 obligatory anaerobic Clostridiales known to be present primarily during larval stages (Yun *et*
376 *al.* 2014). This group dominated in particular the terrestrial sample with 55% of all reads
377 compared to 34% in the aquatic sample (Table 1, Figure 3), which is consistent with the
378 higher biomass of larvae in the former.

379 Other “closed association” bacterial endosymbionts show the reverse pattern, i.e. a higher
380 DNA proportion in the vouchers than in the preservative ethanol. These species reside in the
381 bacteriocytes, specialized intracellular compartments that are not expected to be released into
382 the preservative medium. Specifically, *Wolbachia*, *Regiella* and *Rickettsia* are present in most
383 arthropod communities (Werren *et al.* 2008) and in our samples are easily detectable in the
384 voucher libraries but are poorly, if at all, recovered from the ethanol (Figure 4C). By contrast,
385 several bacterial genera implicated in “open” symbiotic associations as commensals outside

386 of the bacteriocytes (Moran *et al.* 2005) show more mixed patterns. This category of bacteria
387 appears to be the main candidate if one intends to use the preservative ethanol for the study of
388 insect symbiont communities. Finally, some eukaryotic species relevant to insect biology
389 were also detected (Figure 4). The Viridiplantae and Stramenophiles were greatly enriched in
390 the ethanol (Figure 4) and may represent ingested food items. Potential infectious agents, such
391 as the entomopathogenic fungus *Metharizium* (Jackson & Jaronski 2009) represented as much
392 as 75% of fungal reads in TE. In contrast, the fungal genus *Hymenolepis* known to have
393 parasitic life cycles using insects as intermediary hosts (Shostak 2014) is strongly detected in
394 AV (10,160 reads identified to genus level) and its absence in AE suggests an association
395 with internal tissues but not the gut content.

396 **The value of the preservative ethanol**

397 The increasing depth of modern sequencing technology is changing the analysis of field-
398 collected preserved samples. Each specimen can be seen as an ecosystem in its own right
399 harbouring microbiota, parasites and ingested food. Deep sequencing therefore shifts the
400 focus of metagenomic studies of bulk specimen samples, which were initially geared towards
401 the analysis of species and phylogenetic diversity of a local insect community (e.g. Gómez-
402 Rodríguez *et al.* 2015; Andújar *et al.* 2015; Crampton-Platt *et al.* 2015; Tang *et al.* 2015), but
403 now can take a holistic view that provides new opportunities for research.

404 For bulk samples the interactions cannot be ascribed to any particular species in the mixture,
405 but the information is still highly valuable to characterise the functional or ecological
406 components of an ecosystem *in toto*, for example through the parallel study of macro- and
407 microbiomes of bulk samples. For higher precision, the methodology can be modified to
408 include only members of a single species or possibly individually preserved specimens,

409 allowing comparisons among co-distributed species for analyses of resource segregation or
410 the turnover in feeding source for a given species or assemblage among different sites.
411 Additionally, the regurgitation of gut content into the ethanol provides a procedure for non-
412 invasive DNA isolation for identification of food items, and it overcomes the problem that the
413 degraded DNA of the gut content makes up only a small proportion of sequence reads
414 compared to the well-preserved gut tissue that cannot be removed even with careful
415 dissections (e.g. Paula *et al.* 2014). The greatest value of these techniques lies in the
416 possibility for making comparison of numerous samples, each of them surveyed for multiple
417 types of trophic interactions, given a different ecological context in which the target taxa are
418 found. The high cost of shotgun sequencing relative to PCR-based metabarcoding may be a
419 deterrent for such studies, but due to the emergence of cheaper methods for library
420 construction (e.g. Baym *et al.* 2015) and the limited amount of sequencing required (e.g. 5%
421 of MiSeq per sample in the current study), these costs are not prohibitive. Thus, the use of the
422 preservative ethanol extends the metasytematic approach to biodiversity assessment and
423 environmental monitoring, for more effective analysis and management of complex
424 ecosystems (Gibson *et al.* 2014). The biomass-dependence of shotgun sequencing is another
425 strength of this approach, to provide abundance estimates for ecological studies, while also
426 recovering rare components without PCR biases. Increased sequencing depth and/or biomass
427 pre-processing of the samples could be useful strategies when recovering low biomass entities
428 is required. At the same time, the extension of reference databases, including complete
429 mitochondrial genomes or nuclear genomes, will also increase the reliability of these
430 approaches, reducing their dependency on the completeness of existing public databases.
431 Beyond the study of freshly collected samples, the significance of bulk sampling and
432 preservative sequencing may arise from the molecular analysis of historical spirit collections.

433 Museum collections provide enormous resources as a base-line against which modern
434 observations can be compared, helping us to build predictive models in a world increasingly
435 influenced by human activities (Suarez & Tsutsui 2004). A holistic approach to the study of
436 preservative ethanol (specimen + eDNA) should reconsider specimen collection and storage
437 practices. A widespread practice to obtain 'cleaner' samples from field collections is the
438 replacement of the original ethanol fraction, which is usually discarded, but this procedure
439 loses valuable information and efforts should be made to store this initial preservative (as
440 volume can easily be reduced through evaporation). Ethanol should also be carefully
441 considered in the management and maintenance of these collections, such as following
442 protocols based on a "topping-up" of the ethanol (e.g. Notton 2010) instead of replacement.

443 Long-term microbiota characterisation appears to be a potential outcome from insect spirit
444 collections. The ability to quantify the microbiotas in insect specimen vs. ethanol fractions
445 can establish their relationships with the 'host' specimens, while the co-existence of similar
446 organisms within samples from different ecosystems may uncover the pathogenic or
447 ecological role played by the insect microbiome (Mira *et al.* 2010). Similarly, organisms
448 attached to the surface of specimens, such as pollen in the leg baskets of bees or fungi
449 contained in the mycangia of wood-boring beetles, may be present in the preservative
450 medium. Such molecular information can complement the information associated to
451 collection records making the ethanol metagenome itself a record from which more
452 associations may be identified in the future when more DNA reads will be identified against
453 the growing genome reference set. Further studies on the dynamics of DNA transfer from
454 specimens to ethanol under different conditions and how this DNA degrades through time are
455 needed to uncover the full potential of the preserving ethanol into which specimens are
456 collected. But it appears that preservative ethanol is an unexpected source of molecular

457 knowledge: it will contain both the specimen and concomitant biodiversity and can provide
458 valuable biological information when subjected to shallow metagenomic sequencing.

459

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470 **Data Accessibility**

471 GenBank Accessions numbers for voucher specimens are KT876876-KT876902; KT876904-
472 KT876915; original datasets have been uploaded as fastq files in Dryad doi:
473 doi:10.5061/dryad.jr6r5; all supplementary details, tables and figures cited in the main text
474 have been uploaded as online Supporting Information.

475 **Author Contributions**

476 B.L., P.A. and C.A. conceived the study; B.L., P.A., C.A. and A.C.P. conducted the specimen
477 collection; P.A. obtained the molecular data; B.L., P.A., C.A. and A.C.P. analysed the data;
478 B.L., P.A., A.P.V. wrote the manuscript and all the authors contributed to the final version.

479

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623

Table 1. Dataset description and voucher species recovery from the preservative ethanol. Ethanol reads correspond to the number of quality filtered reads from the ethanol libraries matching vouchers sequences.

Species	Community	Stage	Total specimens	Specimens used as vouchers	Total estimated biomass	cox1_Sanger	mitogenome	ethanol reads matching cox1	ethanol reads matching complete mitogenomes	ethanol reads matching protein-coding mito-genes
<i>Acilius sulcatus</i> BMNH1425211	Aquatic	adult	2	1	36	X	X	0	0	0
<i>Berosus affinis</i> BMNH1425169	Aquatic	adult	3	2	13.5	X	X	0	0	0
<i>Colymbetes fuscus</i> BMNH1425212	Aquatic	adult	5	2	90	X	X	0	15	15
<i>Dryops luridus</i> BMNH1425163	Aquatic	adult	4	3	20	X	X	0	2	1
<i>Haliphus immaculatus</i> BMNH1425121	Aquatic	adult	3	2	9	X	X	0	0	0
<i>Haliphus lineatocollis</i> BMNH1425118	Aquatic	adult	5	3	15	X	X	0	2	0
<i>Helochaeres</i> sp. BMNH1425100	Aquatic	adult	10	4	60	X	X	0	0	0
<i>Hydrochus</i> sp. BMNH1425167	Aquatic	adult	2	2	6	X	X	0	0	0
<i>Hydroporus planus</i> BMNH1425115	Aquatic	adult	1	2	4.5	X	X	0	0	0
<i>Hydroporus discretus</i> BMNH1425116	Aquatic	adult	2	2	8	X	X	0	0	0
<i>Hydroporus gyllenhalii</i> BMNH1425127	Aquatic	adult	2	2	7	X	X	0	2	0
<i>Hydroporus obscurus</i> BMNH1425129	Aquatic	adult	1	2	3.5	X	X	0	0	0
<i>Hydroporus erythrocephalus</i> BMNH1425131	Aquatic	adult	27	3	81	X	X	0	2	2
<i>Hydropsyche pellucidulla</i> BMNH1425186	Aquatic	larva	4	2	56	X	X	2	55	25
<i>Hygrobia hermanni</i> BMNH1425190	Aquatic	adult	3	1	30	X	X	0	0	0
<i>Hygrotus inaequalis</i> BMNH1425126	Aquatic	adult	1	1	3	X	X	0	1	1
<i>Hygrotus impressopunctatus</i> BMNH1425158	Aquatic	adult	5	3	25	X	X	0	0	0
<i>Hygrotus confluens</i> BMNH1425172	Aquatic	adult	1	1	3.5	X	X	0	0	0
<i>Liopterus haemorrhoidalis</i> BMNH1425193	Aquatic	adult	6	2	42	X	X	0	0	0
<i>Noterus clavicornis</i> BMNH1425090	Aquatic	adult	22	3	99	X	X	0	9	5
<i>Sialis lutaria</i> BMNH1425199	Aquatic	larva	11	2	154	NO	X	24	476	432
<i>Abax parallelepipedus</i> BMNH1425236	Terrestrial	adult	2	1	40	X	X	0	0	0
<i>Agriotes obscurus</i> BMNH1425233	Terrestrial	larva	2	1	30	X	X	0	0	0
<i>Anisosticta novemdecimpunctata</i> BMNH1425231	Terrestrial	adult	1	1	3.5	NO	X	0	0	0
<i>Athous haemorrhoidalis</i> BMNH1425235	Terrestrial	larva	1	1	9	X	X	0	1	1
<i>Atrecus affinis</i> sp. BMNH1425232	Terrestrial	adult	1	1	7	X	X	0	2	2
<i>Calathus melanocephalus</i> BMNH1425227	Terrestrial	adult	1	1	7	NO	X	0	0	0
<i>Cyphon variabilis</i> BMNH1425225	Terrestrial	adult	2	2	9	X	X	0	0	0
<i>Dorcus parallelepipedus</i> BMNH1425260	Terrestrial	larva	7	1	175	X	X	17	478	360
<i>Melanotus villosus</i> BMNH1425245	Terrestrial	larva	8	4	45	X	X	0	6	4
<i>Nalassus laevioctostriatus</i> BMNH1425217	Terrestrial	adult	5	2	42.5	X	X	0	0	0
<i>Nebria brevicollis</i> BMNH1425256	Terrestrial	adult	1	1	14	X	X	0	0	0
<i>Ocyopus olens</i> BMNH1425259	Terrestrial	larva	1	1	16	X	X	0	0	0
<i>Pterostichus niger</i> BMNH1425241	Terrestrial	adult	4	1	84	X	X	0	12	5
<i>Pterostichus madidus</i> BMNH1425238	Terrestrial	adult	4	2	64	X	X	0	2	2
<i>Stenus clavicornis</i> BMNH1425222	Terrestrial	adult	3	2	18	X	X	0	0	0
<i>Stenus boops</i> BMNH1425230	Terrestrial	larva	1	1	5	X	X	0	0	0
<i>Stomis pumicatus</i> BMNH1425229	Terrestrial	adult	1	1	6.5	X	X	0	0	0
<i>Tasgius</i> sp. BMNH1425251	Terrestrial	adult	2	1	34	X	NO	7	0	0
<i>Uloma</i> sp. BMNH1425257	Terrestrial	larva	2	2	26	X	X	0	0	0

Figure 1

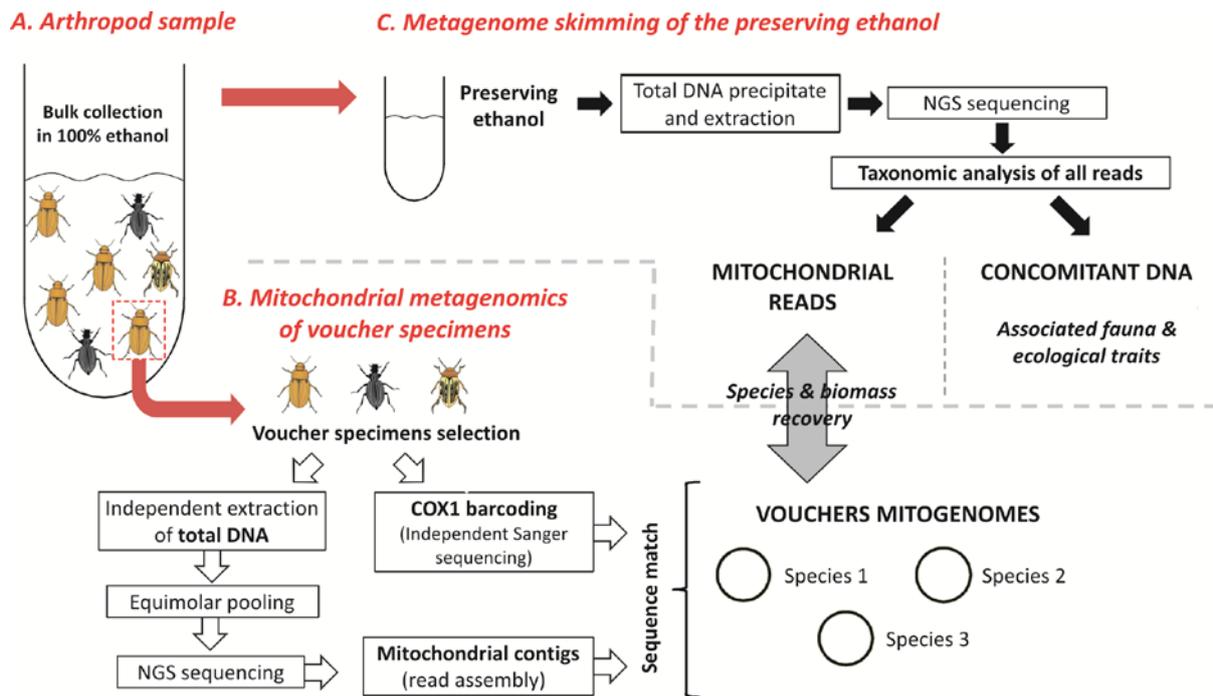


Figure 1 Schematic representation of the experimental design and bioinformatics pipeline followed in this study.

Figure 2

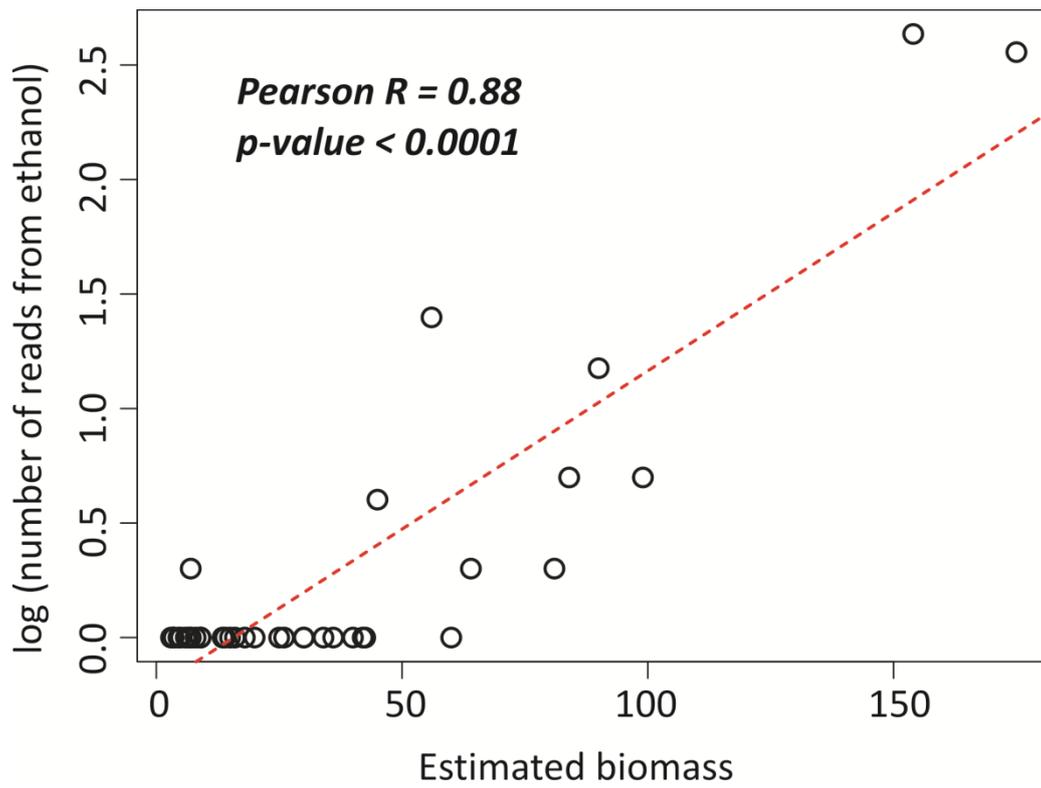


Figure 2 Relationship between numbers of metagenomic reads from the preservative ethanol for each species and its estimated biomass in the samples.

Figure 3

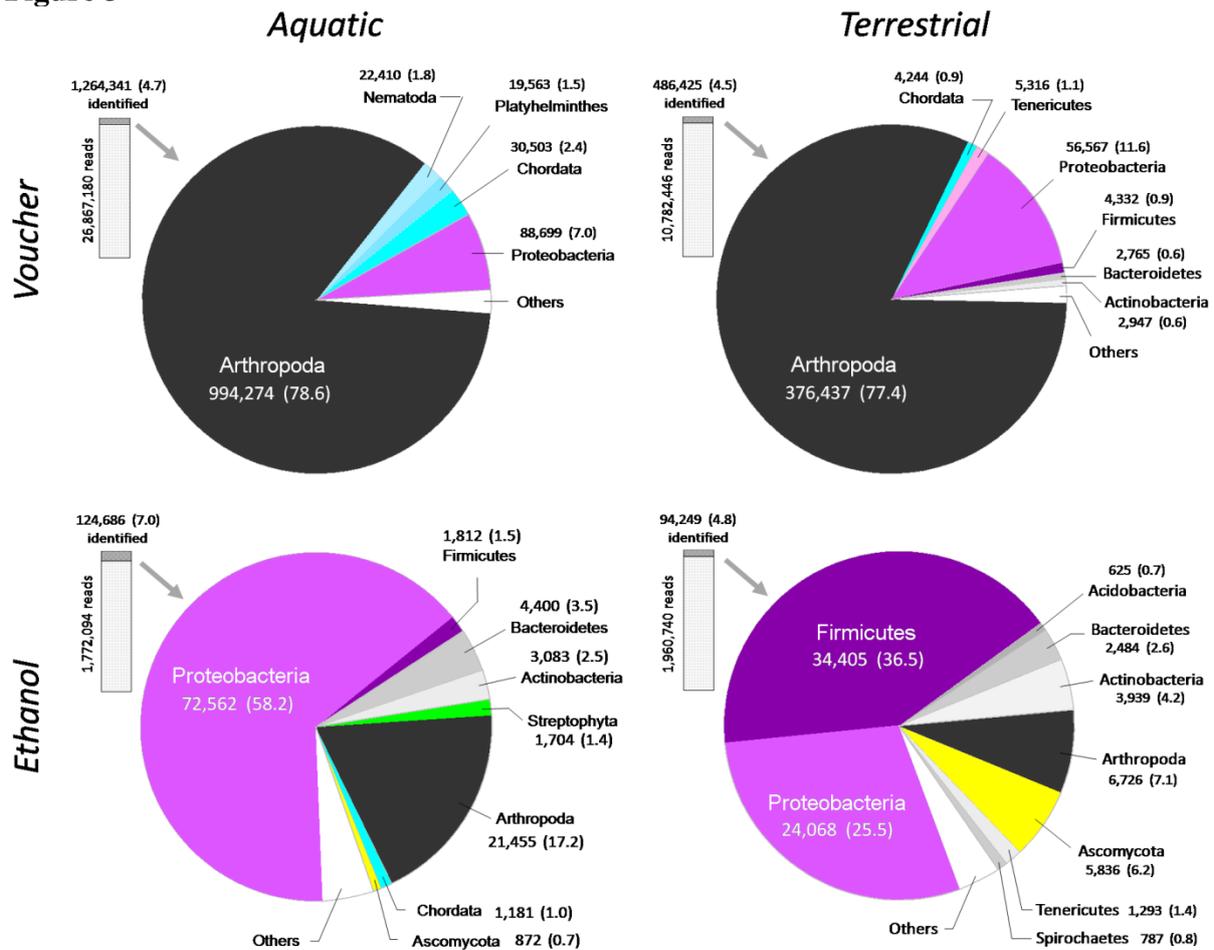


Figure 3. Taxonomic composition of the identified DNA reads. MEGAN-based identifications are reported for the four libraries. The names of the most abundant taxa are reported while all minor taxa are grouped in the “other” fraction. The pie charts represent the DNA reads identified as the given taxonomic group and their percentage of the total number of identified reads is given in parentheses. The bars next to each pie chart indicate the number of reads in the library identified to a taxonomic group and their proportion of total reads in parentheses.

Figure 4

	Clade	Marker	Aquatic		$\Delta F_{E/V}$ (log)	Terrestrial		$\Delta F_{E/V}$ (log)	Comments	
			V	E		V	E			
A.	Arthropoda	Mito			1.9			2.0		
		rRNA			2.0			2.0		
B. Environment and gut content	Eukaryota	Annelides			4.6				>99% similar to Enchytraeidae and Naididae, found in benthic and wet soil habitats ^{a,b}	
		Fungi			---			1.8	In TE, 75% of mito. reads are >99% similar to Metarhizium, an entomopathogen genera ^c	
		Viridiplantae	Plastid			3.7			3.2	
			Mito			3.5			3.0	
		rRNA			4.6			---		
	Stramenophiles	Plastid			3.3			3.2		
		Mito			2.3			2.9		
		Blastocystis			4.1			3.2	Insect gastrointestinal tracts habitat ^d	
	Bacteria	Acinetobacter	rRNA			2.7			1.8	Soil mineralization and found in beetle guts ^{e,f}
		Hydrogenophaga	rRNA			2.7			Oxygenates-rich water habitats ^g	
Variovorax		rRNA			2.8			Soil and water habitats ^{h,i}		
C. Bacterial symbionts	Closed association	Wolbachia	Genomes			1.9		>2.0	Intracellular facultative endosymbiont, Widespread in arthropods ^j	
		rRNA			>2.0			>2.0		
		Regiella	Genomes			1.8			1.9	Facultative symb. associated to bacteriocytes ^k
	Rickettsia	Genomes			>2.0				"Scattered" association to bacteriocytes ^l	
	Open association	Collembola endosym.	rRNA						1.7	Coxiellaceae symbiont (unpublished, gi:13507245)
		Rickettsiella	Genomes			3.6			1.9	Intracellular pathogens of arthropods ^m interacting with coexisting endosymbionts ⁿ
			rRNA						2.0	
Serratia		Genomes			2.5			1.1	Genera found ubiquitously in water, soil and insect guts habitats ^o Some species are facultative symbionts playing a role in bacteriocyte/embryo transmission ^p	
rRNA							2.4			
Spiraplasma	Genomes						1.5	Found in plants/insect guts ^q , heritable symbiont in some insect species ^r		

Fig. 4 Ethanol recovery for concomitant DNA. The number of base pairs identified for four types of markers (plastids, mitochondria, rRNAs and symbiont genomic DNA) in different taxa was quantified in the *vouchers* and *ethanol* metagenomes and normalized by library size. Taxa (1st column) are grouped in Arthropoda (A), Environment and Gut (B) and Bacterial symbionts categories (C) based on literature information about the identified taxa ('Comment'). Circle areas represent the square root of the relative proportion of each taxon/marker combination detected in the *vouchers* library (V columns) and the *ethanol* libraries (E columns) in both habitats and their colours are matching taxa in Figure 3. The increased or reduced recovery in the *ethanol* relative to the *vouchers* libraries is indicated by green or red arrows, and the magnitude of change is given as the log₁₀ of the factor change ($\Delta F_{E/V}$, see Methods). For instance, a F=2.0 lower recovery for a selected taxon/marker indicates that 100 times fewer base pairs were recovered in *ethanol* compared to *vouchers*. References in the last column are: a. Caspers (1986) b. Envall *et al.* (2006) c. Jackson & Jaronski (2009) d. Yoshikawa *et al.* (2007) e. Morales-Jiménez *et al.* (2009) f. L. Dijkshoorn (2008) g. Willems (2014) h. Carbajal-Rodríguez *et al.* (2011) i. Carrino-Kyker & Swanson (2008) j. Sicard *et al.* (2014) k. Moran *et al.* (2005) l. Caspi-Fluger *et al.* (2011) m. Cordaux *et al.* (2007) n. Tsuchida *et al.* (2014) o. Grimont & Grimont (2006) p. Koga *et al.* (2012) q. Gasparich *et al.* (2004) r. Haselkorn *et al.* (2009).