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1 **From environmental DNA sequences to ecological conclusions: How strong is the influence of**
2 **methodological choices?**

3
4 **Running title:** Sensitivity of eDNA-based ecological results

5
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19 alpes.fr)).

20 **ABSTRACT**

21 **Aim**

22 Environmental DNA (eDNA) is increasingly used for analysing and modelling all-inclusive
23 biodiversity patterns. However, the reliability of eDNA-based diversity estimates is commonly
24 compromised by arbitrary decisions for curating the data from molecular artefacts. Here, we test the
25 sensitivity of common ecological analyses to these curation steps, and identify the crucial ones to
26 draw sound ecological conclusions.

27 **Location**

28 Valloire, French Alps.

29 **Taxon**

30 Vascular plants and Fungi.

31 **Methods**

32 Using soil eDNA metabarcoding data for plants and fungi from twenty plots sampled along a 1000-m
33 elevation gradient, we tested how the conclusions from three types of ecological analyses: (i) the
34 spatial partitioning of diversity, (ii) the diversity-environment relationship, and (iii) the distance-decay
35 relationship, are robust to data curation steps. Since eDNA metabarcoding data also comprise
36 erroneous sequences with low frequencies, diversity estimates were further calculated using
37 abundance-based Hill numbers, which penalize rare sequences through a scaling parameter, namely
38 the order of diversity q (Richness with $q=0$, Shannon diversity with $q=1$, Simpson diversity with $q=2$).

39 **Results**

40 We showed that results from different ecological analyses had varying degrees of sensitivity to data
41 curation strategies and that the use of Shannon and Simpson diversities led to more reliable results.
42 We demonstrated that MOTU clustering, removal of PCR errors and of cross-sample contaminations
43 had major impacts on ecological analyses.

44 **Main conclusions**

45 In the Era of Big Data, eDNA metabarcoding is going to be one of the major tools to describe, model
46 and predict biodiversity in space and time. However, ignoring crucial data curation steps will impede
47 the robustness of several ecological conclusions. Here, we propose a roadmap of crucial curation steps
48 for different types of ecological analyses.

49

50 **KEYWORDS:** Data curation strategies, distance-decay, environmental DNA, Hill numbers,
51 metabarcoding, sensitivity analysis, spatial partitioning of diversity.

52 **1 INTRODUCTION**

53 Understanding the structure and distribution of biodiversity across space and time is a critical
54 goal in ecology. The development of environmental DNA (eDNA) metabarcoding approaches now
55 facilitates the monitoring of species at biogeographical scales and across the whole tree of life
56 (Drummond et al., 2015; Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). It is now
57 possible to tackle unresolved questions that could not be addressed with traditional biodiversity
58 surveys so far. For example, eDNA-based biodiversity studies have enabled the spatial partitioning of
59 diversity (i.e. gamma, alpha and beta diversity) of so far elusive taxa in both terrestrial and marine
60 environments (e.g. marine viruses and protists, soil fungi and bacteria), thereby improving our
61 understanding of their community assembly processes and of their role in structuring communities
62 and networks at global scales (e.g. Lima-Mendez et al., 2015; Tedersoo et al., 2014). However, while
63 the eDNA metabarcoding approach promises substantial advances in macroecology and multi-taxa
64 studies, it requires an appropriate and careful processing of the tremendous amount of sequences
65 generated to draw robust and ecologically meaningful conclusions.

66
67 Indeed, the analyses of diversity patterns (e.g. alpha- and beta-diversity; Whittaker, 1960) across
68 space and of the processes generating these patterns are traditionally based on community matrices
69 representing the presence/abundance of species across samples. In eDNA metabarcoding surveys, the
70 data consist of hundreds to millions of DNA sequencing reads from the hundreds to thousands of
71 species co-occurring within samples. Using bioinformatics, these data are then transformed in
72 community matrices, but with species replaced by DNA sequences, and species abundance replaced
73 by a number of sequencing reads. While, in an ideal world, one sequence should correspond to a
74 single species, in practice, it can correspond to several species if the DNA region has a low taxonomic
75 resolution, and more critically, one species can be represented by tens to thousands of variant
76 sequences. Amongst those variants, a few are biologically meaningful (e.g. intraspecific variability),
77 but the large majority of them are technical errors produced at the different stages of the lab
78 treatments, from DNA extraction to sequencing (see Table 1 and Appendix S1, Bálint et al., 2016;
79 Taberlet, Bonin, Zinger, & Coissac, 2018). These errors can represent more than 70% of the
80 sequences in raw metabarcoding datasets, and have usually low frequencies (e.g. singletons; S. P.
81 Brown et al., 2015). If interpreted as genuine, these sequences can therefore inflate diversity by
82 several orders of magnitude and lead to flawed ecological interpretations (Kunin, Engelbrekton,
83 Ochman, & Hugenholtz, 2010). Molecular protocols are thus applied to reduce and/or control specific
84 technical errors accumulated during the data production. For example, replicated PCR amplification
85 and use of negative controls allow identifying artefactual sequences resulting from random errors
86 introduced by DNA polymerases or sequencers, as well as reagent contaminants (de Barba et al.,
87 2014). However, error rates remain high even with the most stringent molecular protocols (Bálint et
88 al., 2016; Taberlet et al., 2018), which has led to the development of bioinformatics algorithms aiming

89 at detecting errors known to occur during data generation (e.g. PCR errors or chimeric sequences).
90 Also, most of these tools require specifying thresholds and parameter values, which are usually based
91 on arbitrary decisions and visual assessments. An example is the classification of sequence variants
92 into MOTUs (Molecular Operational Taxonomic Units) based on the similarity of sequences. While
93 this step is critical because MOTUs are used as a proxy for species in the majority of DNA
94 metabarcoding studies (Appendix S1), MOTUs are commonly defined using a 97% sequence
95 similarity threshold, a value historically defined as the similarity level of full-length 16S rRNA
96 barcodes below which bacterial strains necessarily belong to different species (Stackebrandt &
97 Goebel, 1994). However, the optimal threshold value to define MOTUs depends on the focal taxa and
98 polymorphism/length of the DNA marker used (e.g. E. A. Brown, Chain, Crease, MacIsaac, &
99 Cristescu, 2015; Kunin et al., 2010). It also depends on the PCR/sequencing error rate, which varies
100 across molecular protocols, and depends on the amount of target DNA: when it is low, each genuine
101 DNA fragment has a higher probability of being amplified at each PCR cycle (Taberlet et al., 2018).

102

103 Hence, using DNA metabarcoding requires taking several methodological choices. Beyond those
104 related to molecular protocols and bioinformatics software, one of the most critical choice is to decide
105 which data curation steps to include in the curation procedure. Indeed, each step directly affects the
106 community matrix obtained, by influencing the final list of MOTUs and/or their frequencies within
107 samples. Previous methodological studies have thus underlined the importance of data curation steps
108 on the reliability of ecological analyses and provided guidelines for bioinformatics decision-making
109 (e.g. Alberdi, Aizpurua, Gilbert, & Bohmann, 2018; Schloss, 2010). However, most of these studies
110 tested the influence of data curation procedures on a single metric or ecological question. However,
111 questions related to local community richness can be very sensitive to errors (Flynn, Brown, Chain,
112 MacIsaac, & Cristescu, 2015), while comparisons of communities' composition might be less affected
113 (Leray & Knowlton, 2015; Taberlet et al., 2018). In addition, most studies have focused on microbial
114 communities (bacteria or fungi), and few have addressed such questions to macro-organisms. Finally,
115 most published tests have so far relied on mock communities (i.e. positive controls) usually made of
116 DNA extracts for few known species. While mock communities are useful to identify errors and
117 estimate error rates, the conclusions cannot easily be translated to realistic environments with rich and
118 complex communities (Alberdi et al., 2018).

119

120 Here, we address how methodological choices related to the DNA metabarcoding data curation
121 strategy influence the results for different types of ecological analyses and their related diversity
122 metrics. We used soil eDNA data from an elevation gradient in the French Alps, and focused on
123 plants and soil fungi to represent both macro- and microorganisms, as well as DNA markers with
124 different length (Table 2). Patterns of plant diversity have been extensively studied in this area (e.g.
125 Chalmandrier, Münkemüller, Lavergne, & Thuiller, 2015) and serve as a good reference to evaluate

126 the results estimated from eDNA metabarcoding data. We subjected these data to 256 different data
127 curation strategies, which correspond to all possible combinations of seven critical data curation steps.
128 We then tested how the curation strategies influence the inferences drawn from three different
129 ecological analyses: 1) a spatial partitioning of diversity (i.e. gamma, alpha and beta diversities) to
130 estimate the regional and local diversity of the gradient, 2) a diversity-environment relationship, to
131 analyse the influence of environment on the local community diversity (alpha), and 3) a distance-
132 decay analysis, to evaluate if similarities between communities (beta) decrease with increasing
133 geographic distances. To this end, we first checked the accuracy of eDNA metabarcoding data in
134 detecting ecological patterns by comparing the eDNA-based diversity patterns with the expected
135 values based on mock communities and traditional botanical surveys (only available for plants).
136 Second, we did an overall sensitivity analysis to test the sensitivity of ecological results to the data
137 curation strategy. Finally, with a variance partitioning analysis we identified the crucial curation steps
138 (i.e. those that introduced more variance to the results) to include or consider in the curation
139 procedure.

140

141 To achieve these objectives, we built on Hill numbers (Hill, 1973) to estimate diversity, which
142 unifies mathematically the best known diversity measures in ecology through a unique parameter q
143 (i.e. Richness at $q=0$, the exponential of Shannon entropy at $q=1$ and the inverse of Simpson at $q=2$).
144 In this framework, the weight of rare species decreases when increasing the value of the parameter q .
145 This feature is particularly relevant for DNA metabarcoding data, since artefactual sequences are
146 usually rare compared to the genuine ones (Bálint et al., 2016; Taberlet et al., 2018). Hill numbers
147 can thus penalize these rare sequences at different degrees: $q=1$ is the order of diversity that levels the
148 MOTUs exactly according to their relative abundances, while $q<1$ overweigh rare MOTUs and $q>1$
149 overweight abundant MOTUs. As a result, we could expect that diversity measures that give less
150 importance to rare sequences (i.e. $q>0$) are less sensitive to the data curation strategy, because they
151 penalize the artefactual sequences targeted by the curation steps.

152

153 **2 MATERIAL AND METHODS**

154 **2.1 Sample data**

155 Soil cores were sampled at 10 different elevations equally distributed across an elevation gradient in
156 the northern French Alps (from 1748 m to 2725 m a.s.l.) in 2012. At each elevation, two 10m × 10m
157 plots were selected (20 plots in total). In each plot, 21 soil cores distributed along the two diagonals
158 were sampled. Soil corers were cleaned and sterilized between each sample collection. Extracellular
159 DNA was then extracted twice, from 15 g as described in Taberlet, Prud'Homme, et al., (2012).
160 Aboveground plant community information (hereafter observed plant diversity) was obtained in each
161 plot with a botanical survey conducted during the annual productivity peak (mid-July) using the
162 Braun-Blanquet cover-abundance scale (Braun-Blanquet, 1946).

163

164 **2.2 Molecular analyses**

165 eDNA-based plant diversity was estimated by targeting a vascular plant specific marker (P6 loop of
166 chloroplast trnL, Table 2). It targets highly conserved priming sites across vascular plants and
167 amplifies a short region, which is desired when working with degraded DNA. eDNA-based fungal
168 diversity was assessed using the nuclear ribosomal Internal Transcribed Spacer 1 (ITS1, Table 2). For
169 each DNA extract, PCRs were run in duplicate leading to four technical replicates per core sample
170 and DNA marker. PCR thermocycling conditions and mixes composition and purification can be
171 found in Table S2.1 in Appendix S2. To control for potential contaminants, extraction and PCR blank
172 controls were included in the experiment. To control for false positives caused by tag-switching
173 events, we also defined “sequencing blank controls”, i.e. tag combinations not used in our
174 experimental design, but that could be formed at the library preparation or sequencing stage (See
175 Appendix S1). We also included positive controls in this experiment, which consisted of a mix of
176 DNA extracted from 16 plant species. For this, genomic DNA was extracted from leaf tissue using the
177 DNeasy Plant Kit (Qiagen GmbH, Hilden, Germany), quantified, diluted at different concentrations
178 for each species and mixed to form a mock community (species composition provided in Table S2.2,
179 Appendix S2). Positive controls allow for quantification of technical biases introduced by PCR and
180 sequencing. Illumina sequencing was performed on a HiSeq platform (2x100bp paired-end reads) for
181 plant amplicons and on a MiSeq (2x250bp paired-end reads) for fungi amplicons, both using the
182 paired-end technology.

183

184 **2.3 Bioinformatics analyses**

185 The Illumina sequencing paired-end reads (Table S2.3) were pre-processed for each marker with three
186 procedures: (i) assembling forward and reverse paired-end reads based on their overlapping 3'-end
187 sequences, (ii) assigning each read to its respective sample (demultiplexing) and (iii) combining
188 strictly identical sequences into unique DNA sequences while keeping information on their abundance
189 (number of sequencing reads) in each sample (dereplication). Then we systematically processed the
190 dereplicated sequences following common data curation procedures that included removal of
191 sequences with low paired-end alignment scores, removal of singletons, removal of short sequences
192 and removal of sequences containing ambiguous bases (not to be confounded with a phred-quality
193 filtering; Fig.1a, Table 1 and Table S2.4). Singletons are sequences that occur only once in the whole
194 dataset and many studies agree that their removal is necessary to reduce data
195 complexity/computational time and because they mostly correspond to molecular artefacts that may
196 inflate disproportionately diversity indices (S. P. Brown et al., 2015; Kunin et al., 2010). In our data,
197 they represented 70-80% of the total number of sequences but only 1-15% of the total number of
198 sequencing reads for plants and fungi respectively (Table S2.3 in Appendix S2). We finally assigned
199 each remaining sequence to a taxonomic clade with the *ecotag* command from the OBITOOLS

200 software package (Boyer et al., 2016) that uses a lowest common ancestor algorithm for the
201 assignment, and the EMBL database version 133 as a reference.

202

203 Next, data from each marker were processed following a range of different data curation
204 strategies to test the sensitivity of ecological analyses to different methodological choices (Fig. 1b).
205 To do so, we selected seven important steps: (i) removal of PCR errors, (ii) filtering of highly
206 spurious sequences, (iii) removal of chimeras, (iv) sequence classification into MOTUs (MOTU
207 clustering), (v) removal of reagent contaminants, (vi) cross-sample contamination cleaning and (vii)
208 dysfunctional PCRs filtering (see Table 1, Appendix S1 and Table S2.4 in Appendix S2 for target
209 errors and step descriptions). Curation steps were either kept or excluded, and were always performed
210 in the same order in each data curation strategy. For the MOTU clustering step, when kept, three
211 clustering thresholds were tested (1, 2 or 3 mismatches allowed between pairwise aligned sequences).
212 We used here raw mismatches rather than percentages of dissimilarities because the DNA markers
213 used are short (<100 bp) and/or highly polymorphic in length. Using the percentages of dissimilarity
214 in this case would penalize more little differences when alignments are short than when they are long.

215

216 All different possible combinations of these curation strategies were implemented (Fig.1b). Most
217 of the curation steps were done using the software OBITOOLS (Boyer et al., 2016). Chimera detection
218 was performed with UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011) and we used
219 SUMACLUSt (Mercier, Boyer, Bonin, & Coissac, 2013) for MOTU clustering due to its ability in
220 handling large datasets and its flexibility for defining the clustering threshold (see Table S2.4 for
221 more details on the algorithm). After data curation, PCR replicates were summed and standardized by
222 the total number of reads in each core sample. We then pooled the samples for each of the 20 plots to
223 obtain a single community per plot. For this, MOTUs abundance (already standardized by the number
224 of reads) were summed and standardized by the number of samples in each plot. For each of the data
225 curation strategies, we obtained a community matrix with rows representing plots and columns
226 representing all the MOTUs obtained after curation, which we used here as a proxy for species.
227 Therefore, our sensitivity analysis was conducted on a total of 256 matrices for each DNA marker
228 (Fig.1c).

229

230 **2.4 Ecological questions**

231 We tested the sensitivity of the results for three common ecological analyses to the above-
232 mentioned data curation strategies using MOTUs as equivalent of species:

233

234 *Spatial partitioning of diversity* - We used the multiplicative diversity partitioning approach
235 (Whittaker, 1960) to analyse gamma (here the diversity across the entire gradient), alpha (diversity of
236 local communities) and beta diversity (diversity between communities). In the Hill numbers

237 framework, gamma diversity is the effective number of species in the pooled meta-community (i.e.
238 across all plots), alpha diversity is the effective number of species per community (i.e. plot), and beta
239 diversity is the effective number of communities, calculated as the ratio of gamma diversity to alpha
240 diversity. We followed Chao, Chiu, & Jost, (2014)'s definition where beta diversity is independent of
241 alpha and ranges from 1 (all communities are identical) to the total number of communities N (when
242 N=20 all communities are different). We limited our study to taxonomic diversity, because the DNA
243 markers we used here are rather short (Table 2) and are highly variable in length, which make them
244 not suitable for inferring accurate phylogenetic relationships at the scale of the community.

245

246 *Diversity-environment relationship (Alpha~SOM content)* – Diversity is often linked to abiotic
247 drivers, and a common ecological research question is how alpha diversity changes along an
248 environmental gradient. Here, we fitted a linear model to determine changes in alpha diversity along a
249 gradient of soil organic matter content (SOM content), known to be a strong predictor of diversity
250 changes in the study site (Ohlmann et al., 2018).

251

252 *Distance-decay relationship (Similarity~geographic distance)* – Species' distributions and resulting
253 diversity patterns are controlled by both species dispersal abilities and spatial turnover of
254 environmental conditions (Tuomisto, 2003). One hypothesis is thus that spatially distant communities
255 are more different than close communities ("distance-decay", Green et al., 2004; Tuomisto, 2003).
256 We used the Jaccard-type overlap (U_{qN}) as a measure of similarity (Chao et al., 2014) and we fitted a
257 linear model using the log transformation of similarity against the geographic distance to evaluate the
258 distance-decay. The geographic distance between plots was calculated with Euclidean distances using
259 the elevation values of the plots.

260

261 For each DNA marker (plant and fungi), we calculated the gamma, alpha and beta diversities
262 (spatial partitioning of diversity) for each of the 256 community matrices obtained from the different
263 metabarcoding data curation strategies using Hill numbers with values of $q=\{0,0.5,1,2\}$. For the
264 diversity-environment and the distance-decay relationships, we fitted our models to each community
265 matrix and extracted the slopes and the R-squares of the models. Alpha diversity and community
266 similarity were calculated using Hill numbers with values of $q=\{0,1,2\}$.

267

268 **2.5 Sensitivity analyses**

269 *Detectability of ecological patterns* - To test the ability of eDNA metabarcoding data and of the
270 different data curation strategies to detect ecological patterns we (1) evaluated the completeness of the
271 sampling unit (plot), and (2) used the observed plant diversity and positive controls as references to
272 evaluate the accuracy of the ecological results. We acknowledge that eDNA-based diversity is
273 expected to slightly diverge from observed diversity (see discussion) but they should follow similar

274 trends (Hiiesalu et al., 2012; Träger, Öpik, Vasar, & Wilson, 2019; Yoccoz et al., 2012). The
275 sampling completeness of each plot was evaluated with rarefaction curves for the different orders of
276 diversity $q=\{0,1,2\}$ and for three data curation strategies with varying filtering stringency: a “no data
277 curation” strategy with no curation step at all; a “basic curation” strategy including only the chimera
278 removal and a traditional clustering threshold allowing three mismatches between clustered sequences
279 and, a “rigorous curation” strategy, including all the curation steps considered here and a clustering
280 threshold allowing two mismatches.

281

282 *Overall sensitivity analyses* - To test the sensitivity of the results for the different ecological analyses
283 and their related diversity metrics to the data curation strategy, we used the variance of each diversity
284 estimate, obtained across the 256 community matrices and for each marker (Fig. 1c). For the
285 diversity-environment and the distance-decay relationships, we looked at the variance in the slope and
286 the R-square of the linear regression across the 256 models for each marker. In addition, we used ‘the
287 rigorous’ and ‘the basic’ curation strategies explained above, that correspond to commonly used
288 pipelines, to exemplify how results can differ between studies.

289

290 *Identifying the crucial steps of the curation procedure* - To identify the crucial steps we did a variance
291 partitioning analysis for each diversity metric. In respect to spatial partitioning of diversity, the
292 diversity metrics (gamma, alpha and beta diversities) were used as the response variable in function of
293 the curation steps. For the diversity-environment and the distance-decay relationships we used the
294 slope and the R-square of the models as the response variable in function of the curation steps.
295 Variance partitioning analyses were done with the R package RELAIMPO (Grömping, 2006).

296

297

298 **3 RESULTS**

299 **3.1 Detectability of ecological patterns with eDNA metabarcoding data**

300 *Sampling completeness of the plots* – For both markers/taxa, the total diversity was well represented
301 by the number of reads sequenced, when considering the diversity at $q=\{1,2\}$ (Fig.S2.1 and Fig.S2.2
302 in Appendix S2). At $q=\{0\}$, the rarefaction curve rarely saturated, but we obtained more asymptotic
303 curves when increasing the stringency of the data curation strategy.

304

305 *Spatial partitioning of diversity* – Overall, we found that alpha diversity estimates at $q=\{1,2\}$ were
306 closer to the observed plant diversity (Fig.2b) and to the positive controls composition (Fig. 3) than at
307 $q=\{0,0.5\}$. However, diversity at $q=\{1\}$ slightly underestimated gamma (Fig.2a) and beta (Fig.2c)
308 while all diversity components were underestimated for most curation strategies at $q=\{2\}$ (Fig.2a-c).

309 Richness ($q=0$) was always overestimated. While we obtained very accurate results for diversity at
310 $q=\{0.5\}$ when using a rigorous pipeline, a basic pipeline led to a substantial overestimation.

311

312 *Diversity-environment relationship* – While the expected positive slope was in most cases detected
313 (Fig.2g) and its value was on average very similar to the one observed for plant diversity, especially
314 when using a rigorous pipeline, it was highly overestimated for some data curation strategies at
315 $q=\{0,1\}$.

316

317 *Distance-decay relationship* – The expected negative slope of the distance-decay curve was always
318 detected (Fig.2k). However, independently of the data curation strategy, the slope was always
319 underestimated compared to the curve calculated with observed plant diversity. Also, the R-square of
320 the distance-decay relationship was reduced at $q=\{2\}$ (Fig.2l).

321

322 **3.2 Overall sensitivity of ecological questions and diversity metrics**

323 The results of different ecological questions had varying degrees of sensitivity to the data curation
324 strategies. While the estimates in all ecological questions were highly sensitive (width of the boxplots
325 in Fig. 2), the main signal of the diversity-environment and the distance-decay relationships was
326 consistent across most curation strategies.

327

328 *Spatial partitioning of diversity* - Sensitivity of gamma, alpha and beta diversity decreased for higher
329 values of q , i.e. weighing down rare MOTUs (Fig.2a-f). Diversity estimates at $q=\{0\}$ were the most
330 sensitive, with more than two orders of magnitude for both gamma and alpha (Fig.2a & b) diversities
331 of plants. Likewise, the rigorous and basic curation strategies (circles and triangles in Fig.2) exhibited
332 a steep difference at $q=\{0\}$, which decreased when using higher values of q in the majority of cases.

333

334 *Diversity-environment relationship* - The interpretation of the alpha-SOM content relationship could
335 change depending on the data curation strategy used. However, the alpha-SOM content relationship
336 was more robust when using $q=\{1,2\}$, i.e. a positive relation between alpha diversity and SOM
337 content was detected independently of the data curation strategy used (Fig.2g,h). Patterns in fungi
338 diversity were more robust, i.e. no relation between fungi diversity and SOM content was detected
339 across the different pipelines. A very weak positive relation between fungi diversity and SOM content
340 was observed for $q=\{1,2\}$. The rigorous and the basic strategies led to very similar results for both
341 DNA markers/taxa.

342

343 *Distance-decay relationship* – In contrast, a significant distance-decay relationship was always
344 detected from eDNA metabarcoding data independently of the data curation strategy, but the rate at
345 which similarity decays with increasing distance between plots (i.e. slope) slightly changed across

346 strategies. While very similar results were found between the rigorous and the basic strategies for the
347 distance-decay curve of plants, the slope of the distance-decay curve for fungi was very low when
348 using a basic instead of a rigorous strategy.

349

350 **3.3 Crucial steps of the curation procedure**

351 Overall, we found that two curation steps, the removal of PCR error and the clustering to define
352 MOTUs, explained most of the variation in diversity estimates across data curation strategies (more
353 than 15% each and usually more than 40% in total) for most of the diversity metrics in the ecological
354 analyses and for both markers/taxa (Fig.4 and Fig.S2.3 in Appendix S2). Also, cross-sample
355 contamination removal explained large parts of the variance of beta diversity in the spatial
356 partitioning of diversity analyses (Fig.4a,b) and of R-squares and slopes in the diversity-environment
357 (Fig.4c,d) and distance-decay (Fig.4e,f) relationships analyses.

358

359

360 **4 DISCUSSION**

361 Ecologists do now increasingly rely on DNA metabarcoding to measure biodiversity as this approach
362 holds the promise of allowing testing long-standing hypotheses at spatial, temporal and taxonomic
363 scales that were hitherto inaccessible with traditional approaches. However, the technique is still
364 hampered by a substantial amount of technical errors (Table 1, Appendix S1; Bálint et al., 2016;
365 Taberlet et al., 2018). Here, we sought at testing the sensitivity of the conclusions drawn from
366 different ecological analyses and diversity metrics to the steps commonly used to curate DNA
367 metabarcoding data from such errors. We show that ecological conclusions had varying degrees of
368 sensitivity to the data curation strategies and that the use of metrics that are less sensitive to rare
369 species/MOTUs (i.e. Shannon and Simpson diversity) leads to more robust diversity estimates. Also,
370 we demonstrated that MOTU clustering, removal of PCR errors and removal of cross-sample
371 contaminations have a major influence on ecological results, and must always be carefully included
372 when curating DNA metabarcoding data.

373

374 The breadth of our study makes our findings generalizable to other systems. Indeed, we found
375 similar trends in the sensitivity of gamma and alpha diversity estimates for both our observed plant
376 diversity and the mock community (Figure 2 vs Figure 3). Second, our study focus on both plants and
377 fungi, that widely differ in their ecological properties and the length of their markers (on average 50
378 bp for plants vs. 225 bp for fungi). Still, while they do not share the same diversity patterns, their
379 sensitivity to data curation strategies were comparable. Further, we expect that our study and the
380 experimental testing design we developed will stimulate further methodological studies (e.g. for

381 tropical or aquatic systems and other markers/taxa) and that they will serve as a guide to prioritize
382 some curation steps when deciding for a curation strategy.

383

384 **4.1 Linking methodological choices with ecological questions**

385 The ecological question(s) underlying a study should lead the prioritization of the curation steps to be
386 included in the data curation procedure, as well as the selection of appropriate diversity metrics (Fig.
387 5). If the aim of the study is to estimate the spatial partitioning of diversity (Fig.5a), it is important to
388 keep in mind that all diversity components are biased by the data curation steps. Richness is highly
389 sensitive to error accumulation, and was hence the metric responding the strongest to the data curation
390 strategy. Consequently, if measuring richness is crucial for the study, and, thus, rare species are
391 important, the reliability of the results must be confirmed with additional analyses. For example, a
392 more conservative strategy (i.e. keeping only MOTUs present in more than a certain number of PCR
393 replicates) can improve the reliability of final results, but with the risk of missing species represented
394 by few sequences in only a few samples due to the sampling process occurring when preparing
395 aliquots of one DNA extract (Alberdi et al., 2018). Verifying the pertinence of species detected by
396 looking in detail into the taxonomic assignments can also improve the reliability of results, even
397 though this could be problematic for poorly known taxa with incomplete reference databases
398 (Cristescu, 2014). Also, positive controls (with mock communities) and numerous negative controls
399 (extraction, PCR) must be included in all the phases of sequence generations to ensure the accuracy of
400 richness estimates (Bálint et al., 2016). In any cases, a certain degree of uncertainty will always
401 remain because of the complexity of deciding objectively which sequences are genuine and which are
402 artefactual.

403

404 We corroborated that richness is a very sensitive metric and is always overestimated (Fig.2a-
405 c). The intrinsic properties of eDNA can inflate the diversity compared to traditional surveys because
406 eDNA can persist in the environment or be transported through space depending on the abiotic
407 conditions (e.g. water transport, temperature, UV, or microbial activity; Barnes & Turner, 2016). This
408 means that the diversity eDNA estimates not only encompass local and current species, but also
409 species that are dormant (Hiiesalu et al., 2012), that were present in the recent past (Yoccoz, 2012) or
410 that are present in the vicinity of the studied area (Taberlet et al., 2018). In other words, the
411 spatiotemporal window captured by local eDNA diversity estimates may be larger than that captured
412 by traditional approaches, a property that can be desirable or not depending on the question addressed.
413 Distinguishing this feature from methodological bias remains at this stage difficult, as it may look like
414 cross-contamination, and also because the cycle of eDNA in the environment remains poorly
415 understood (Barnes & Turner 2016). However, it is crucial to account for eDNA properties when
416 interpreting richness-based studies to avoid meaningless conclusions.

417

418 When the detection of rare species is not of importance, Hill numbers are a promising
419 solution to increase the robustness of results and to avoid the inflation of diversity estimates. The Hill
420 numbers approach has been already proposed to better estimate microbial diversity (e.g. Bálint et al.,
421 2016; Chiu & Chao, 2016), and we corroborate its efficiency for estimating plant diversity and
422 potentially other macro-organisms from metabarcoding data. Both, Shannon and Simpson diversity
423 measures led to a satisfying representativeness of the sampling unit diversity and were robust to the
424 different data curation strategies tested here, but Shannon diversity was less biased. In the same way
425 that richness overestimated diversity, Simpson diversity tended to underestimate diversity. Diversity
426 measures, other than richness (i.e. $q > 0$), account for species/MOTUs abundance structure. The factors
427 determining species' abundances in a community are not the only factors determining the MOTUs'
428 abundances. These correspond to a pool of DNA fragments from current, dormant, or past populations
429 (e.g. microbes) down to one (or part of one) single multicellular individual that are besides amplified
430 by PCR. Consequently, a highly abundant MOTU does not necessarily imply that more individuals of
431 the corresponding taxon were present, it could also be due to e.g. higher body mass, larger root
432 systems, or slower DNA decomposition. Besides, given the exponential nature of the PCR
433 amplification, abundant taxa become even more abundant in this step and this could lead to an
434 underestimation of Simpson diversity. Hence, interpreting MOTUs frequency directly as species
435 abundance can be highly misleading, and estimating species abundance in terms of number of
436 individuals or biomass from eDNA is still a major challenge in the field (Deiner et al., 2017).
437 However, MOTUs frequency correlates to a certain extent to species relative abundance, and more
438 importantly, errors are usually rarer than genuine sequences (reviewed in Taberlet et al., 2018).
439 Accordingly, Shannon diversity from eDNA samples appears here as a balanced diversity measure,
440 robust to the data curation strategy, and hence, to rare errors. This can be generalized to all ecological
441 analyses tested in this study. Given these results, we argue that using a complete diversity profile (for
442 example, with q values between 0 and 2) may allow improving confidence in diversity estimates from
443 eDNA data while getting information about MOTUs structure of abundances.

444

445 Another important outcome of our assessment is that despite the above-mentioned limits,
446 robust conclusions can be obtained from eDNA metabarcoding data if the aim is to link local diversity
447 (alpha) or community similarity (beta) to environmental or geographic gradients (Fig.5b). Changes in
448 local diversity across an environmental gradient were more sensitive to the data curation strategies
449 than the distance-decay relationship. Our results thus corroborate other studies that demonstrated the
450 robustness of beta diversity to bioinformatics analyses (Botnen, Davey, Halvorsen, & Kauserud,
451 2018; Deiner et al., 2017). However, the slope of the distance-decay was always underestimated
452 compared to that obtained from observed plant diversity. On one hand, this could result from a lack of
453 phylogenetic resolution of the genetic marker used here, which is relatively short. In alpine
454 ecosystems, it is common to see abundant species replaced by closely related species across an

455 elevation gradient (Chalmandrier et al., 2015). A genetic marker with a low phylogenetic resolution
456 would not detect these changes and as a consequence, gamma and beta diversities would be
457 underestimated. However, the underestimation of gamma diversity relative to alpha diversity is not
458 strong enough, suggesting that other reasons may also explain the lower slope of the distance-decay
459 curve for eDNA-based plant diversity. Botanical surveys used in this study represent just a local
460 snapshot of the visible plant diversity at the sampling time, and, unlike the eDNA approach, may miss
461 species with an offset phenology or present only in the vicinity of the sampling area (Hiiesalu et al.,
462 2012). We can expect that the larger spatiotemporal window captured by the eDNA metabarcoding
463 approach would thus result in higher similarity among the sites, which could be tested by increasing
464 the botanical sampling effort across seasons and years to reduce botanical surveys biases related to the
465 differentiated phenology of the species.

466

467 **4.2 Crucial steps for designing a careful curation protocol**

468 While we included here curation steps that are common to most bioinformatic tools (e.g. QIIME,
469 USEARCH), we acknowledge that algorithms within OBITOOLS have their own particularities, as
470 each of the other packages, and that the results obtained here may not be directly transferable.
471 However, we expect that the differences from a specific software are minor compared to the
472 differences caused by the choice of specific curation steps (Bonder, Abeln, Zaura, & Brandt, 2012).
473 In general, we corroborate past studies concluding that the clustering threshold used for defining
474 MOTUs leads to significant changes in diversity estimates and that this is especially important for
475 alpha and gamma diversities, but less so for beta diversity (Botnen et al., 2018; E. A. Brown et al.,
476 2015; Kunin et al., 2010). Additionally, we found that PCR errors and cross-sample contaminations
477 are critical steps and that including them leads to more realistic spatial diversity patterns and estimates
478 of diversity components. These two steps correct the diversity at local levels (i.e. sample level) and
479 are especially important when comparing communities. To our knowledge, this is the first study
480 testing in a systematic way the effect of these curation steps on results across different types of
481 ecological analyses. We recommend carefully choosing the MOTU clustering threshold, e.g.
482 empirical means can be estimated for each marker or targeted taxa using *in silico* methods with
483 reference databases (Taberlet et al., 2018) or experimentally, using mock communities (E. A. Brown
484 et al., 2015), and considering removing PCR errors and cross-sample contaminations when designing
485 a curation protocol to study biodiversity patterns. Furthermore, a rigorous data curation strategy
486 including all the curation steps of the present study allowed obtaining accurate diversity estimates and
487 diversity-environment and distance-decay relationships. This demonstrates that the other curation
488 steps should not be neglected.

489

490 **5 DATA AVAILABILITY STATEMENT**

491 Pre-filtered sequencing data as well as associated metadata are available on the Dryad Digital
492 Repository ([doi:10.5061/dryad.0t39970](https://doi.org/10.5061/dryad.0t39970)).

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615

616

617 **BIOSKETCH:** Irene Calderon-Sanou is a PhD student aiming at a better understanding of multi-
618 trophic assemblages through the use of environmental DNA. Author contributions: WT initiated the
619 overall idea, and together with ICS, LZ and TM conceived the overall analyses. ICS, LZ and FB
620 conceptualized the data curation strategies, ICS run the curation procedures and analysed all the
621 results, and led the writing with the significant contribution of all co-authors.

622

623

624 **TABLE CAPTION**

625

626 **TABLE 1** Brief description of classical technical errors occurring in DNA metabarcoding data, the
627 associated data curation steps tested in the present study and the curation methodology. Target errors
628 make reference to the errors described further in Appendix S1. See also Table S2.4 for more details on
629 the curation steps used in this study.

630

631 **TABLE 2** Characteristics of the DNA markers used to estimate eDNA-based diversity in this study.

632

633

634
635
636

TABLES
TABLE 1

| Target error | Definition | Curation step (Abbreviation) and methodology |
|---|---|---|
| Mixed | Common obvious molecular/sequencing errors such as mis-paired reads, sequences with ambiguous bases, that are too short or singletons. | Common basic filtering : Removal of sequences meeting these criteria. This step is not tested here and has been applied systematically. |
| PCR error | Base mis-incorporation by the DNA polymerase during the PCR amplification. | PCR errors removal (PCR error): Identification of PCR errors using a model-based classification of sequences based on their similarities and abundances. The model reflects the accumulation of base mis-incorporation across PCR cycles, where genuine sequences remain more abundant than their respective errors. |
| Highly spurious sequences | Chimeras from multiple parents, primers dimers, etc. or sequences from highly degraded DNA fragments that largely differ from any known sequence. | Highly spurious sequences removal (Spurious): Removal of sequences of whose similarity with their closest match in public reference databases is below 70% (plants) or 50% (fungi). |
| Chimeras | Sequences obtained from the recombination of two or more parent sequences | Chimera detection and removal (Chimeras): Removal of sequences that have a high probability to be a subsequence from other, more abundant sequences in the dataset. |
| Remaining PCR errors/ biological variation | Sequences from the same species either resulting from a PCR error that could not be filtered above, or from intraspecific variability | MOTU clustering (Clustering): Clustering of sequences into MOTUs on the basis of their pairwise similarity. Here done at different sequence similarity thresholds. |
| External contaminants | DNA coming from an external source other than the biological sample | Reagent contaminants cleaning (Reagent): Removal of sequences that are more abundant in negative controls relative to biological samples because of the absence of other competing DNA fragments during the amplification process. |
| Cross-contaminations or Tag-jumps | Genuine sequences present in a sample where actually absent, either due to cross-contaminations at the bench, or due to tag-jumps occurring during the library preparation or the sequencing , i.e. switches of nucleotidic labels used to assign the sequencing reads to their samples. These contaminants are usually of much lower abundance than in their sample of origin. | Cross-sample contamination curation (Cross): If the abundance of a given MOTU in a given sample is below 0.03% of the total MOTU abundance in the entire dataset, it is considered as absent in this sample. |
| Dysfunctional PCRs | PCRs that are too different in comparison with their technical replicates. | Dysfunctional PCR removal (DysPCR): Removal of PCR replicates from a single biological sample that are more dissimilar to each other in MOTUs composition and structure than are the PCR obtained from other biological sample. |

637 **TABLE 2**
 638
 639

| DNA Marker | Target taxa | Forward primer (5'-3') | Reverse primer (5'-3') | Length [range] (bp) | References |
|--|--------------------|---------------------------------|-------------------------------------|----------------------------|---|
| P6 loop of the chloroplast <i>trnL</i> intron | Vascular plants | g: GGGCAATCCTGAGCCAA | h: CCATTGAGTCTCTGCACCTATC | 48 [10-220] | Taberlet et al., 2007 |
| Nuclear ribosomal DNA Internal Transcribed Spacer 1 (ITS1) | Fungi | ITS5: GGAAGTAAAAGTCGTAACAAGG | Fung02: CCAAGAGATCCGTTGYTGAAAGTK | 226 [68-919] | White, Bruns, Lee, & Taylor, 1990; Taberlet et al., 2018 |

640 **FIGURE CAPTION**

641

642 **FIGURE 1** Workflow of the sensitivity analysis. (a) Raw data are curated with basic filtering steps for
643 each DNA marker (plants: trnL-P6 loop, fungi: ITS1). (b) Filtered data are processed using seven
644 curation steps that were varied or removed in each data curation strategy making a total of 256
645 possible combinations. As a result, 256 community matrices are obtained per DNA marker and used
646 to (c) conduct three types of ecological analyses. The range of values obtained for each ecological
647 analysis and diversity metric represents the variance due to the data curation strategy.

648

649 **FIGURE 2** Estimated values of the spatial partitioning of diversity components (a-f), of the regression
650 parameters from the diversity-environment (g-j), and of distance-decay (k-n) relationships across the
651 256 curation strategies for different diversity metrics (Hill numbers, $q=\{0,0.5,1,2\}$). The top row (a-c,
652 g-j, k-l) corresponds to the plant DNA marker (trnL-P6 loop) and bottom row (d-f, i-j, m-n) to the
653 fungi DNA marker (ITS1). Size of each box (including whiskers) represents the sensitivity of the
654 diversity metrics or the model parameters to the data curation strategy. The circle and the triangle
655 symbols indicate the values obtained from a rigorous and a basic curation strategy, respectively. The
656 star symbol indicates the values calculated from botanical survey (only represented for plants, top
657 row).

658

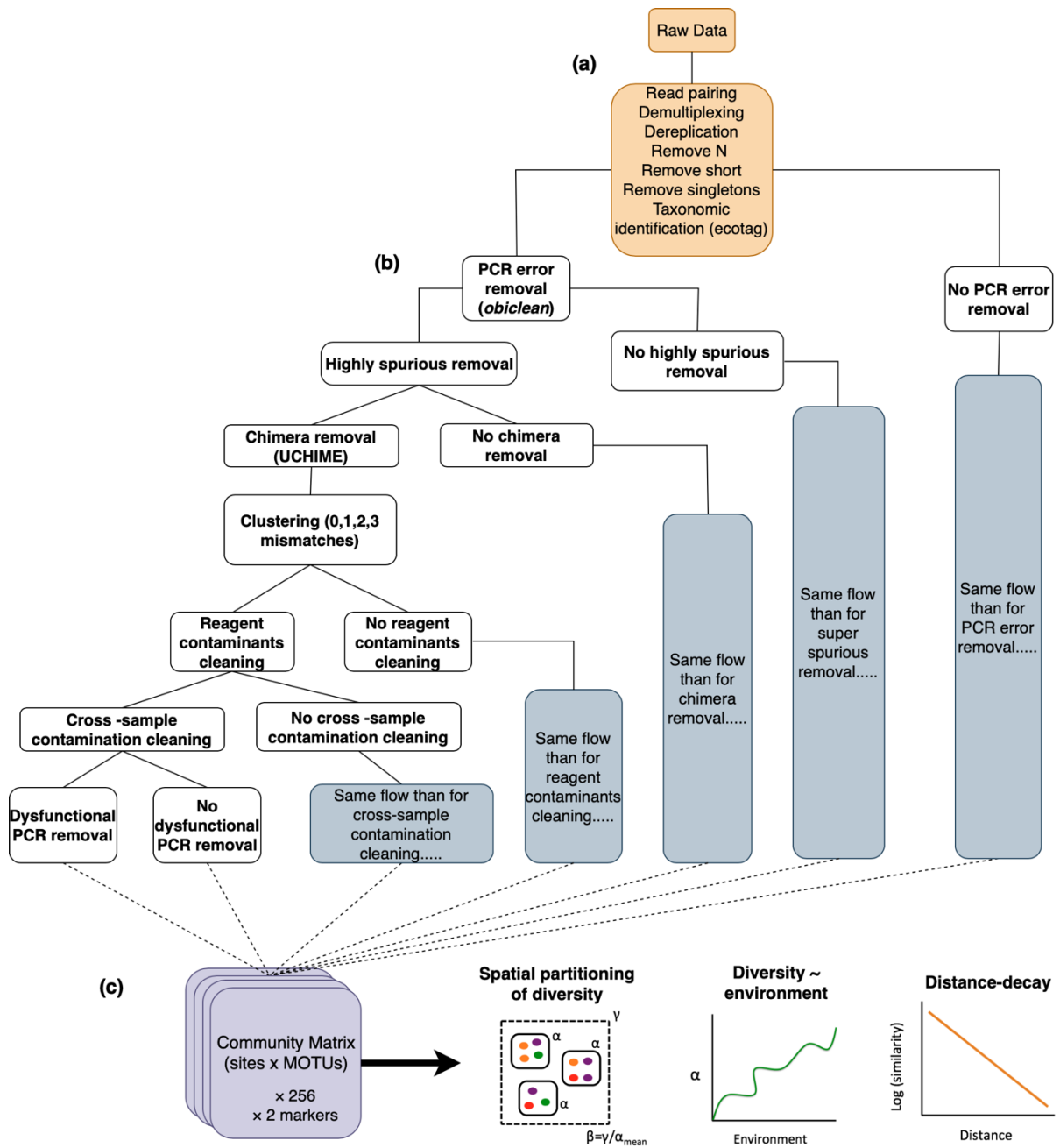
659 **FIGURE 3** Mean diversity estimated in positive controls across the 256 data curation strategies for
660 different diversity metrics (Hill numbers, $q=\{0,0.5,1,2\}$). Size of each box (including whiskers)
661 represents the sensitivity of the diversity metrics to the data curation strategy. The star symbol
662 indicates the values calculated from the known species composition in positive controls, the other
663 symbols are as in Fig. 2.

664

665 **FIGURE 4** Relative importance (% of variance explained) of the data curation steps on the variability
666 of estimated values of the spatial partitioning of diversity components (a,b) and of the parameters
667 from the diversity-environment (c,d) and distance-decay (e,f) relationships, using Hill numbers at
668 $q=\{1\}$ (see Fig.S2.3 for the other q values). The top row (a,c,e) corresponds to the plant DNA marker
669 (trnL-P6 loop) and bottom row (b,d,f) to the fungi DNA marker (ITS1). A model was fitted
670 independently for each diversity component (a,b) or model parameter (c-f) as response variable, with
671 curation steps as main effects.

672

673 **FIGURE 5** Guidelines to improve the reliability of ecological results when analysing eDNA
674 metabarcoding data.

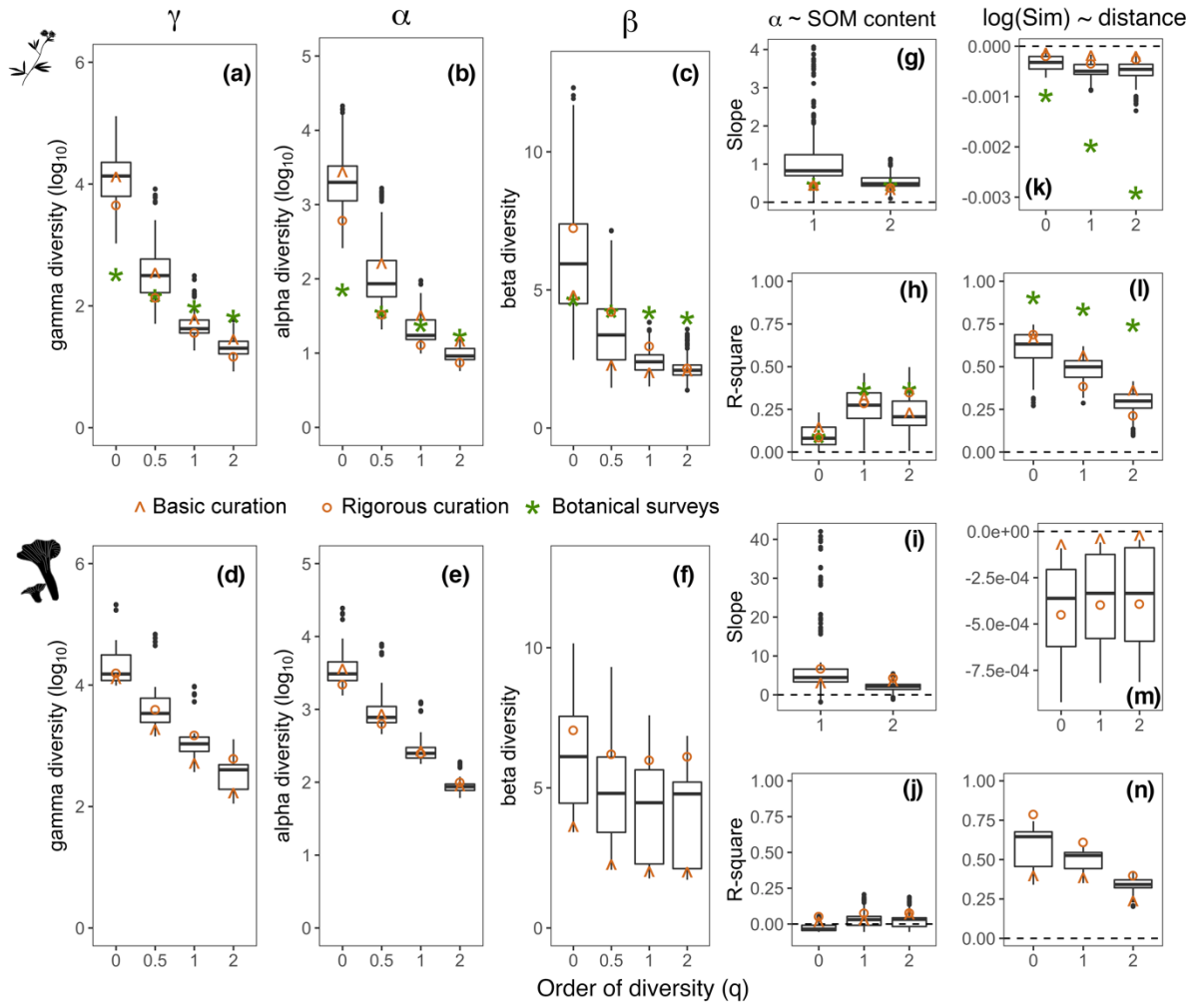


675
676
677

FIGURE 1

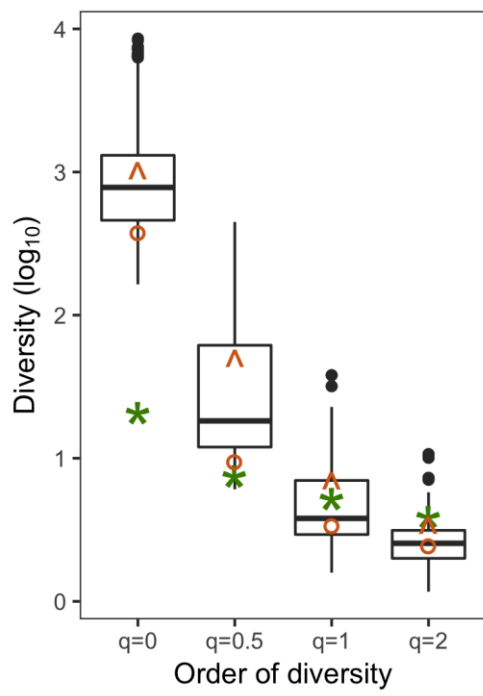
Spatial partitioning of diversity

Diversity ~ environment Distance-decay



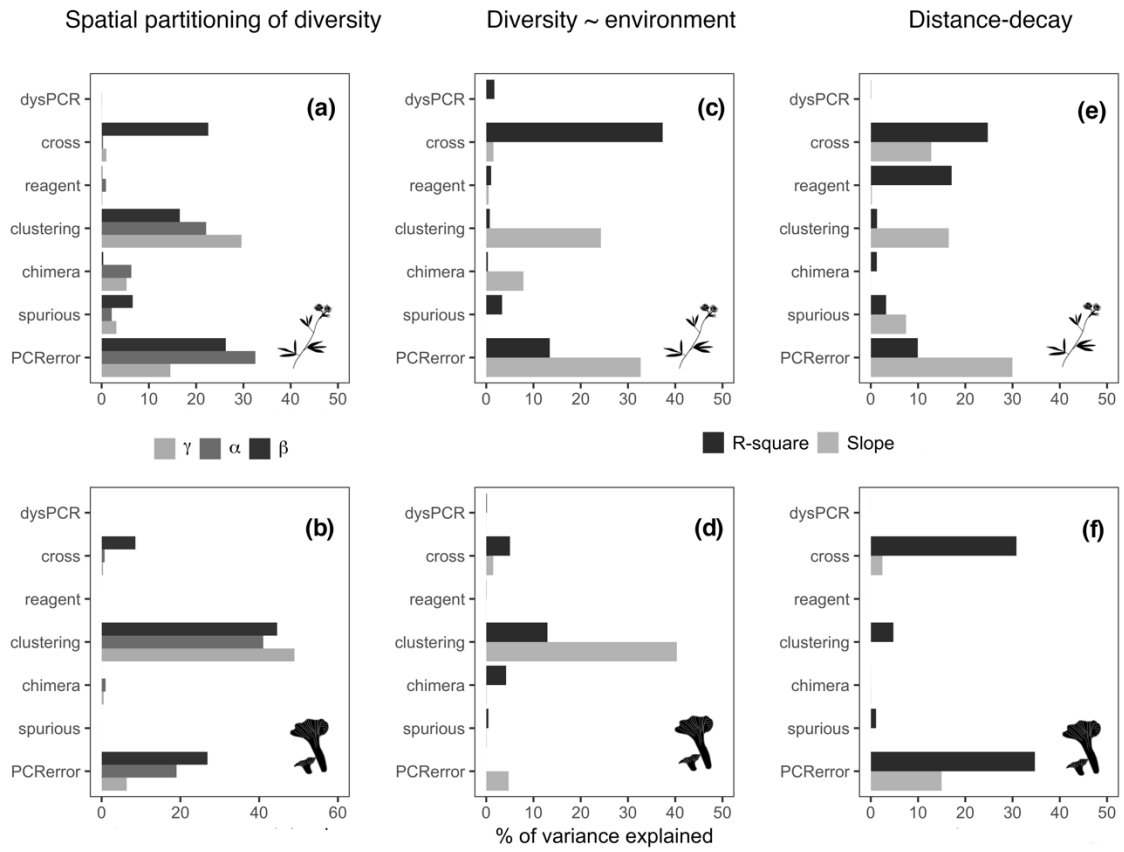
678
679
680

FIGURE 2



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683

FIGURE 3



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686

FIGURE 4

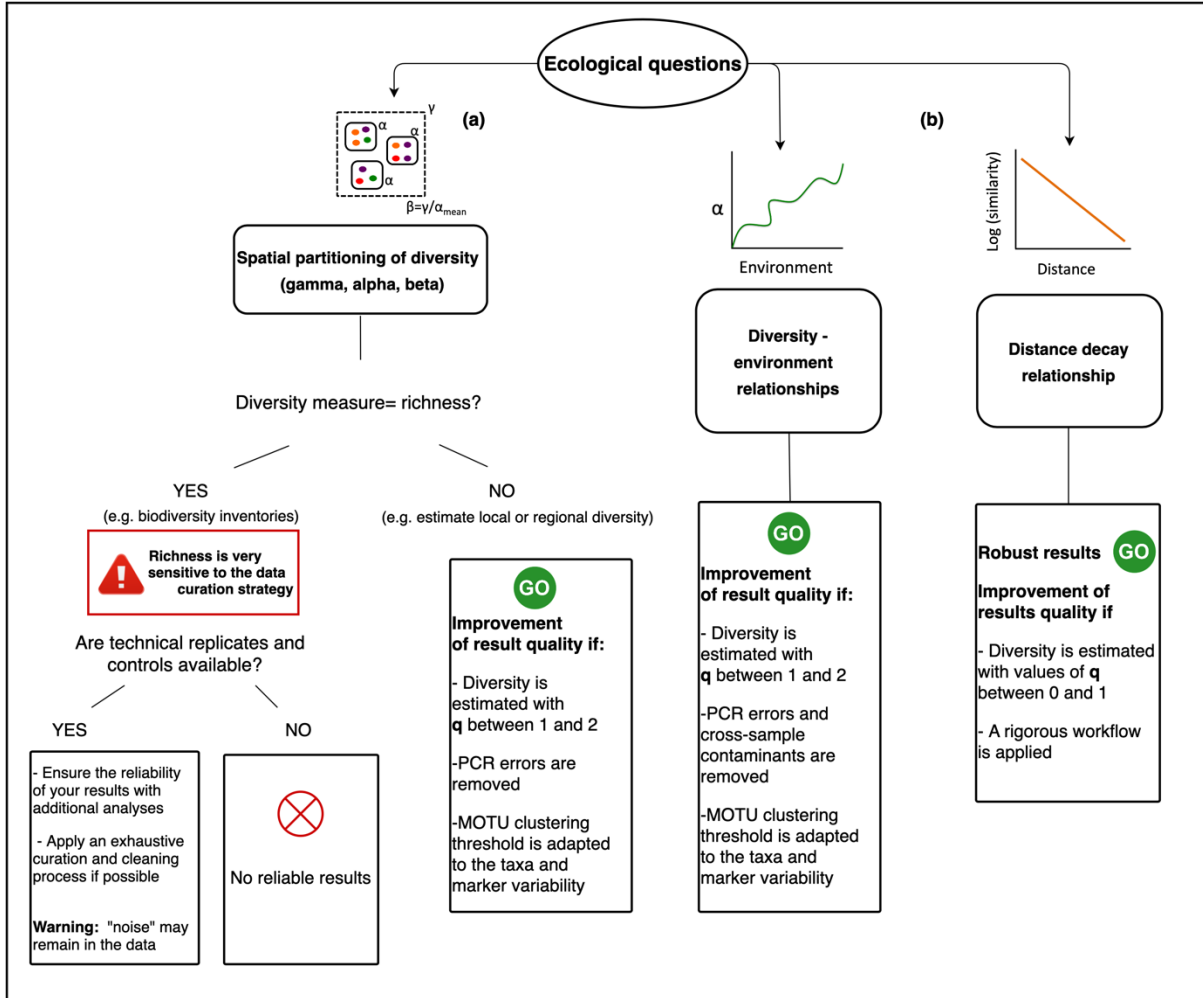


FIGURE 5