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# **Influence of homogenization methods on lichen species detection from environmental DNA metabarcoding**

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3 **Influence of homogenization methods on lichen species detection from**  
4 **environmental DNA metabarcoding**

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13 **Abstract**

14 Environmental DNA (eDNA) techniques are increasingly employed in biodiversity monitoring of  
15 terrestrial animals, plants, and fungi, holding great potential to revolutionize biodiversity assessments  
16 on land. However, sampling and basic laboratory protocols still require refinement to optimize DNA  
17 metabarcoding performance. Homogenization as a pretreatment for eDNA extraction is known to  
18 enhance the concentration and quality of extracted eDNA for some groups of organisms. We  
19 developed a simple and efficient method for capturing arboreal biodiversity using stemflow as a  
20 source of eDNA; however, its performance with or without homogenization had not yet been  
21 compared. In this study, we evaluated the performance of two different homogenization methods  
22 using eDNA metabarcoding and qPCR assays. Metabarcoding analyses revealed that the method  
23 without homogenization detected the fewest species, while nearly identical and higher numbers of  
24 species were detected in samples subjected to bead-beating and frozen bead-beating homogenization.  
25 Similarly, qPCR analyses revealed that the method without homogenization yielded the lowest DNA  
26 concentration, while nearly identical and higher DNA yields were observed for bead-beating and  
27 frozen bead-beating homogenization. These findings suggest that, considering cost and effort, the  
28 bead-beating method without freezing is the most advantageous.

29

30 **Key Words**

31 eDNA, lichen, stemflow, metabarcoding, homogenization, laboratory protocols

32

## 33 **Introduction**

34 Over the last decade, environmental DNA (eDNA) metabarcoding has rapidly proliferated as a  
35 method for monitoring biodiversity in aquatic environments. This technique has proven particularly  
36 effective for fish biodiversity monitoring, often outperforming or complementing traditional survey  
37 methods (Jerde et al. 2011, Hinlo et al. 2017, Pont et al. 2018, Cantera et al. 2019, Fujii et al. 2019).  
38 The eDNA Society published a comprehensive manual for aquatic biodiversity monitoring using  
39 eDNA metabarcoding (Miya and Sado 2019), which has significantly facilitated the widespread  
40 adoption of this method. The manual provides detailed instructions for sampling, filtration methods,  
41 fish eDNA metabarcoding, and species-specific detection using real-time PCR, enabling researchers  
42 to conduct biodiversity monitoring with greater ease and accuracy.

43 It is now recognized that terrestrial animal, plant, and fungus communities can be surveyed using  
44 eDNA techniques (Johnson et al. 2023). To advance the use of eDNA metabarcoding for terrestrial  
45 biodiversity monitoring, simple, effective, and appropriate methods need to be established. However,  
46 several challenges remain in monitoring terrestrial organisms, such as efficient DNA extraction,  
47 effective removal of PCR inhibitors, development of optimized primers, and the need for  
48 comprehensive reference sequence libraries. These challenges can significantly affect the accuracy  
49 and reliability of eDNA metabarcoding results (Schrader et al. 2012, Guo and Zhang 2013, Wilcox et  
50 al. 2018, Johnson et al. 2019, 2023, Prieto et al. 2021).

51 Homogenization before eDNA extraction is known to improve the concentration and quality of  
52 extracted eDNA for bacteria and prokaryotes (Guo and Zhang 2013, Albertsen et al. 2015, Ushio  
53 2019). Based on these findings, bead-beating homogenization was used as a pretreatment in the DNA  
54 extraction process for stemflow samples (Sakata et al. 2023). However, the effectiveness of this  
55 method compared to non-homogenization methods has not been thoroughly evaluated.

56 The objective of this study was to establish an efficient method for extracting eDNA from stemflow.  
57 To achieve this, we compared three extraction methods for detecting lichen species. The first method  
58 did not involve homogenization, while the second and third methods involved homogenization of  
59 lichen fragments, with or without freezing as a pretreatment. We assessed the performance of these  
60 methods using eDNA metabarcoding and qPCR analyses.

## 61 **Methods**

### 62 **Ethics statement**

63 The field experiments conducted in Aoba-no-Mori Park were carried out with the permission of the  
64 park administrator.

## 65 **Collection of eDNA samples from stemflow**

66 Stemflow was collected from a Japanese apricot (*Prunus mume*) in Aoba-no-Mori Park, Chiba City,  
 67 Japan, on a rainy day on July 1, 2023, from 08:30 to 17:00. Stemflow sampling was conducted in  
 68 accordance with Sakata et al. (2023), with a modification to the gauze collection method. In this  
 69 modified method, the gauze was retrieved from the tree trunk and placed in the stemflow collected in  
 70 a plastic bag for filtration, rather than being soaked in Milli-Q water in a separate 50 mL syringe. The  
 71 collected stemflow (approximately 1 liter in a plastic bag) was subjected to gravity filtration following  
 72 Oka et al. (2022). The stemflow was successively filtered into 15 Sterivex filter cartridges (pore size  
 73 0.45 µm; Merck Millipore, MA, USA), with each cartridge receiving approximately 50 mL of filtrate,  
 74 as measured using a plastic measuring cup. After filtration, 1–2 mL of RNAlater (Thermo Fisher  
 75 Scientific, DE, USA) was added to each cartridge through the inlet using a disposable pipette (Nihon  
 76 Medical Science, Osaka, Japan). The Sterivex filter cartridges were stored in a freezer at –20°C until  
 77 DNA extraction.

## 78 **DNA extraction and bead-beating**

79 The 15 frozen filter cartridges were left at room temperature, and the liquid remaining in the cartridges  
 80 was removed in accordance with Minamoto et al. (2021). The trapped lichen fragments on the filters  
 81 were either processed without homogenization or homogenized before eDNA extraction.

82 **Without bead-beating homogenization:** This method was applied to five cartridges, which  
 83 proceeded directly to DNA extraction without homogenization.

84 **Bead-beating homogenization:** One gram of zirconia beads (φ 0.5 mm; AsOne, Tokyo, Japan)  
 85 was added to 10 filter cartridges from the inlet using folded weighing paper. Then, 100 µL of AP1  
 86 buffer and 1 µL of RNase A stock solution were added to each filter cartridge containing zirconia  
 87 beads. Five of these cartridges were attached to the Vortex Adapter 24 (Qiagen, Hilden, Germany)  
 88 and vortexed for 3 minute.

89 **Frozen bead-beating homogenization:** The remaining five cartridges with zirconia beads were  
 90 inserted into a customized tube block for an Automill (Tokken, Chiba, Japan) and soaked in liquid  
 91 nitrogen for approximately 5 minutes. The frozen filter cartridges in the tube block were then  
 92 homogenized at 2,000 rpm for 3 minute using the Automill.

93 DNA extraction from all 15 cartridges was performed using the DNeasy Plant Mini Kit (Qiagen)  
 94 following the kit instructions and the methods described by Miya et al. (2016).

## 95 **PCR and parallel sequencing**

96 The targeted lichen eDNA fragments were amplified using a two-step PCR process to prepare paired-

97 end libraries for metabarcoding analysis on the MiSeq platform (Illumina, CA, USA) (Miya et al.  
98 2015).

99 The first round of PCR (1st PCR) was conducted with 35 cycles in a reaction volume of 12  $\mu\text{L}$ ,  
100 containing 6  $\mu\text{L}$  of 2 $\times$  PCR buffer, 2.4  $\mu\text{L}$  of 2 mM dNTPs, 1.4  $\mu\text{L}$  of multiplexed primers (5  $\mu\text{M}$ ;  
101 ITS-PHLichenF, ITS-PHLichenR, ITS-PALichenF, and ITS-PALichenR; Sakata et al., 2023), 0.24  
102  $\mu\text{L}$  of KOD FX NEO DNA polymerase (Toyobo, Osaka, Japan), 1  $\mu\text{L}$  of Milli-Q water, and 1  $\mu\text{L}$  of  
103 template, with two technical replicates. The thermal cycling profile, after an initial 2-minute  
104 denaturation at 94°C, was as follows: denaturation at 98°C for 10 seconds, annealing at 55°C for 30  
105 seconds, extension at 68°C for 1 minute, with a final elongation at 72°C for 7 minutes. Duplicate 1st  
106 PCR products were pooled in a 1.5 mL tube and purified using SPRIselect (Beckman Coulter, Brea,  
107 CA). The concentration and product size distribution of purified PCR products were measured using  
108 a TapeStation 4200 (Agilent, Tokyo, Japan). The concentration of the 1st PCR products was adjusted  
109 to 0.1 ng/ $\mu\text{L}$  using Milli-Q water. Samples with concentrations less than 0.1 ng/ $\mu\text{L}$  were used as  
110 templates without dilution.

111 The second round of PCR (2nd PCR) was conducted with 10 cycles in a 15  $\mu\text{L}$  reaction volume  
112 containing 7.5  $\mu\text{L}$  of 2 $\times$  KAPA HiFi HotStart ReadyMix, 0.88  $\mu\text{L}$  each of 2nd-PCR forward and  
113 reverse primers (Miya and Sado, 2019) with each different combination of indices (5  $\mu\text{M}$ ), 3.88  $\mu\text{L}$   
114 of Milli-Q water, and 1.88  $\mu\text{L}$  of template. The thermal cycling profile, after an initial 1-minute  
115 denaturation at 95°C, was as follows: denaturation at 98°C for 20 seconds, and annealing and  
116 extension combined at 72°C for 30 seconds, with a final elongation at 72°C for 5 minutes. Indexed  
117 2nd PCR products were pooled, and the target-sized DNA was excised using E-Gel SizeSelect 2%  
118 (Thermo Fisher Scientific, MA, USA) with the E-Gel Pre-cast Agarose Electrophoresis System  
119 (Thermo Fisher Scientific). The concentration of the library was quantified using a Qubit dsDNA HS  
120 Assay Kit and a Qubit fluorometer (Thermo Fisher Scientific). The concentration of the library was  
121 adjusted to 4 nM using Milli-Q water. The library was sequenced using a MiSeq Reagent Kit v2 with  
122 150 bp  $\times$  2 paired-end sequencing.

123 All raw DNA sequence data and associated information were deposited in the  
124 DDBJ/EMBL/GenBank database and are available under accession number DRAxxxxxx.

## 125 **Sequence analysis**

126 Raw MiSeq reads were preprocessed and analyzed using PMiFish ver. 2.4  
127 (<https://github.com/rogotoh/PMiFish>; Miya et al. 2020). Data preprocessing followed several steps  
128 implemented in USEARCH version 10.0.240 (Edgar 2010). Forward and reverse paired-end reads  
129 were merged using the *fastq\_mergepairs* command. During this process, low-quality tail reads were

130 discarded based on a cut-off threshold set at a quality (Phred) score of 2. Additionally, reads that  
 131 were too short (<64 bp) after tail trimming and paired reads with too many differences (>5 positions)  
 132 in the aligned region (approximately 70 bp) were also removed. Primer sequences were removed from  
 133 the merged reads using the *fastx\_truncate* command. Reads that lacked primer sequences underwent  
 134 further quality filtering using the *fastq\_filter*. Low-quality reads with an expected error rate > 1% and  
 135 shorter than 50 bp were removed using the *fastq\_filter*. The filtered reads were dereplicated using the  
 136 *fastx\_uniques* command, and singletons, doubletons and tripletons were excluded from further  
 137 analysis to avoid false positives, as suggested by Edgar (2010). The dereplicated reads were denoised  
 138 using the *unoise3* command, which removed putative chimeric and erroneous sequences prior OTU  
 139 assignment. Processed reads were assigned to OTUs with > 98% sequence identity (query coverage  
 140  $\geq 90\%$ , allowing two or three nucleotide mismatches) using the *usearch\_global* command. Reads with  
 141 a sequence identities between 80–98% were designated as “U98 OTU” and clustered at 98 %  
 142 similarity using *cluster\_smallmem* command. After rarefaction, all minor molecular taxonomic units  
 143 (MOTUs) with read counts less than 0.01% of the total reads (<2 reads) were excluded from the  
 144 taxonomic table to ensure conservative estimates of MOTU diversity (Sakata et al. 2023).

145 For reference sequences, the nuclear ribosomal internal transcribed spacer (ITS) sequences of  
 146 lichens were independently determined and registered to the database. In addition to those sequences,  
 147 sequence data, primarily from mainly Japanese and Korean species of sequence data that were  
 148 visually confirmed to grow in the vicinity of the survey site, were downloaded the top ca. 10 search  
 149 results from NCBI on March 10, 2023, and saved them in FASTA format. To select sequences, the  
 150 combined FASTA-formatted sequences were initially aligned using MAFFT (Rozewicki et al. 2019)  
 151 on the web server (<https://mafft.cbrc.jp/alignment/software/>) with default parameters. A Maximum  
 152 Likelihood (ML) tree was then constructed from the aligned sequences using IQ-TREE  
 153 (Trifinopoulos et al. 2016) with auto substitution model. Node support was estimated using bootstrap  
 154 method (Felsenstein 1985) with 1000 pseudoreplicates. Visually inspected the phylogenetic tree and  
 155 selected one to two sequences that formed groups with many other sequences, and used as a reference  
 156 database for taxonomic assignment (Table. 1).

## 157 **qPCR**

158 Quantitative real-time PCR (qPCR) was performed using the ITS-PHLichenF and ITS-PHLichenR  
 159 primer pair and eDNA samples with a StepOnePlus real-time PCR system (Thermo Fisher Scientific).  
 160 The alternative primer pair (PALichenF and ITS-PALichenR) was excluded from the qPCR assay due  
 161 to low amplification efficiency observed in preliminary experiments. Each 20  $\mu$ l reaction contained  
 162 2  $\mu$ l of eDNA template, 900 nM of each primer in PowerUp SYBR Green Master Mix (Thermo Fisher

163 Scientific). The thermal cycling profile consisted of an initial 10-minute denaturation at 95°C,  
164 followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Three replicates were amplified for  
165 each eDNA sample, as well as for 100, 10, and 1 pg of standard DNA (a mixture of tissue-derived  
166 DNA from *Kashiwadia orientalis*, *Hyperphyscia crocata*, and *Dirinaria applanata*), and a PCR  
167 negative control.

## 168 **Statistical analysis**

169 To examine whether there were statistically significant differences in the number of detected species  
170 and the concentration of detected DNA among the three DNA extraction methods, Dunn's multiple  
171 comparison test was performed. The test was conducted in R v4.2.2 (R-Core-Team 2022) using the  
172 `dunn.test` package (Dinno and Dinno 2017).

## 173 **Results and discussion**

### 174 **Library preparation and parallel sequencing**

175 The pooled 102 libraries (including 87 libraries from other projects) were sequenced, and the MiSeq  
176 run yielded a total of 2,747,887 reads, with an average of 94.2% base calls having Phred quality  
177 scores of  $\geq 30.0$  (Q30; error rate = 0.1% or base call accuracy = 99.9%). This run was highly successful,  
178 considering that the manufacturer's guidelines (Illumina Publication no. 7702011-001 as of May 27,  
179 2014) recommend  $>80\%$  bases  $\geq Q30$  at  $2 \times 150$  bp.

180 A total of 452,297 reads were assigned to the 15 libraries, and the number of raw reads for each  
181 library ranged from 16,486 to 45,390 with an average of 30,153 reads. Merging the two overlapping  
182 paired-end fastq files yielded 444,249 reads (98.2%). The sequences from which the primer sequences  
183 were removed were subjected to quality filtering to eliminate low-quality reads, resulting in 438,472  
184 reads (96.9%). The remaining reads were dereplicated for subsequent analysis, and singletons to  
185 tripletons were removed from the unique sequences (Miya et al. 2022). Thereafter, the reads were  
186 denoised to remove putatively erroneous and chimeric sequences. The remaining 404,170 reads  
187 (92.2%) were subjected to taxon assignments, with 403,891 reads (99.9% of the denoised reads)  
188 putatively considered lichen sequences.

189 We analyzed these 403,891 reads (average 26,926 reads per sample) from the 15 samples, with five  
190 replicates for each of the three eDNA extraction methods. The average read counts for the three  
191 methods—without bead-beating, with bead-beating, and with frozen bead-beating—were similar,  
192 being 29,535, 28,608, and 22,635, respectively. Negative controls yielded no denoised reads across  
193 all methods.

194 **Number of detected species**

195 Results from the automatic taxon assignments, with manual adjustments based on the genus-level NJ  
 196 trees, are summarized in Table 2. In total, 11 species from four families and eight genera were detected  
 197 across the three methods, with a total detection frequency of 100.

198 The number of detected species per sample was lowest in the eDNA extraction method without  
 199 bead-beating (NB; Fig. 1), ranging from 2 to 6 species across the five samples, with a total of 7 species  
 200 detected (Table 2). In contrast, the number was higher in the two methods with bead-beating (BB and  
 201 FB), ranging from 7 to 9 species across the 10 samples, with a total of 9 species detected for each  
 202 method (Fig. 1, Table 2). Statistically significant differences were found between the methods with  
 203 and without bead-beating (NB vs. BB and NB vs. FB;  $p < 0.05$ , Dunn's multiple comparison test),  
 204 while no significant difference was found between the two bead-beating methods (BB vs. FB;  $p =$   
 205 1.000, Dunn's multiple comparison test) (Fig. 1).

206 **DNA concentration**

207 As with the number of detected species (Fig. 1), DNA concentration was lowest in the eDNA  
 208 extraction method without bead-beating (NB), with a mean of  $7.2 \pm 4.2$  pg/2 $\mu$ L (mean  $\pm$  1SD; Fig.  
 209 2). In the two methods with bead-beating (BB and FB), it was significantly higher, with means of  
 210  $53.5 \pm 15.3$  and  $53.1 \pm 11.0$  pg/2 $\mu$ L, respectively (Fig. 2). Statistically significant differences were  
 211 found between the methods with and without bead-beating (NB vs. BB and NB vs. FB;  $p < 0.01$ ,  
 212 Dunn's multiple comparison test), while no significant difference was found between the two bead-  
 213 beating methods (BB vs. FB;  $p = 1.000$ , Dunn's multiple comparison test) (Fig. 2).

214 As expected from the similar patterns of variation in the number of detected species (Fig. 1) and  
 215 DNA concentration (Fig. 2), a significant positive correlation was found between the two variables,  
 216 with a coefficient of determination ( $R^2$ ) of 0.707 ( $p < 0.01$ ) (Fig. 3). These observations indicate that  
 217 bead-beating residues from stemflow not only increase the amount of extracted DNA but also  
 218 consequently lead to an increase in the number of detected species (Ushio 2019).

219 **Detection frequency**

220 Of the 11 detected species, six were found in all 10 samples pretreated with bead-beating (BB and  
 221 FB; detection frequency = 5; Table 2). In contrast, the detection frequencies of these six species in  
 222 the five samples without bead-beating (NB) varied from 2 to 5, with a mean of 3.33 (Table 2).  
 223 Additionally, two species, *Phaeophyscia rubropulchra* and *Physciella melanctra*, which were  
 224 undetected in only a few of the 10 samples in BB and FB, were not detected at all in NB (Table 2).  
 225 Furthermore, the remaining three species were detected only once across the three methods. These

226 patterns of detection frequencies were consistent with the overlap of detected species among the three  
227 extraction methods, as shown in the Venn diagram (Fig. 4).

228 The average read number per sample for the 11 species was plotted against detection frequency  
229 (Fig. 5). The read number per sample for the two species with the highest detection frequency of 15  
230 (*Dirinaria applanata* and *Kashiwadia orientalis*) was exceptionally high, both exceeding  $10^5$ . It  
231 ranged between  $10^3$  and  $10^4$  for the four species with intermediate detection frequencies (12 and 13),  
232 while it was below  $10^3$  for the remaining five species with lower detection frequencies (1–9). These  
233 results indicate that detection frequency is reflected in the read number from MiSeq sequencing and  
234 suggest that sequencing depth should be increased for comprehensive detection of species  
235 composition in the extracted DNA (Gweon et al. 2019, Singer et al. 2019).

### 236 **Species composition**

237 Figure 6 shows the variation in pairwise dissimilarities (Jaccard distance) among the 15 samples (105  
238 pairs), partitioned into three within-pretreatment groups (NB, BB, FB) and two between-treatment  
239 groups (BB vs. FB and NB vs. BB + FB). In the two pretreatments involving bead-beating (BB and  
240 FB), which resulted in higher amounts of extracted DNA (Fig. 2), the dissimilarities were relatively  
241 low both within and between samples, with averages ranging from 0.1 to 0.2. In contrast, the average  
242 dissimilarity within samples that were not pretreated with bead-beating (NB), which had significantly  
243 lower amounts of extracted DNA, was approximately 0.5, indicating a higher level of dissimilarity.  
244 Similar levels of dissimilarity were observed between samples with and without bead-beating  
245 pretreatment (NB vs. BB and NB vs. FB).

246 When environmental DNA is present at very low concentrations (e.g., due to dilution or  
247 degradation), PCR amplification can be less efficient and may fail to consistently amplify target  
248 sequences within the extracted DNA, leading to instability in the detection of specific species (Deiner  
249 et al. 2017). This reduced detection reliability can result in variability in the number and types of  
250 species detected across samples, potentially increasing dissimilarity between them (Kelly et al. 2014).  
251 Consequently, the detected species may fluctuate randomly due to low template concentration,  
252 reducing the similarity between samples (Ficetola et al. 2016).

### 253 **Concluding remarks**

254 This study demonstrated that bead-beating as a pretreatment for eDNA extraction effectively  
255 increases both the yield of lichen DNA and the number of detected species from environmental  
256 samples. In contrast, the use of liquid nitrogen freezing before eDNA extraction, which is effective  
257 for DNA extraction from lichen tissue fragments (Cubero et al. 1999), did not show any significant

258 effect on the results of eDNA metabarcoding. Considering the time, effort, and cost associated with  
259 freezing, bead-beating alone appears to be sufficient as a pretreatment for extracting lichen eDNA.  
260 However, further research is needed to determine whether these findings are consistent across  
261 different environmental samples and other arboreal organisms. The application of this optimized  
262 method could enhance the accuracy and efficiency of biodiversity monitoring and conservation efforts  
263 involving lichens.

## 264 **Author contributions**

265 AS, TM, TS, and MM conceived and designed the study. AS selected and provided appropriate  
266 materials and performed the field survey. AS, TM, TS, and MM conducted the laboratory experiments  
267 and data analysis. AS, TM, and MM wrote and edited the first draft of the manuscript. All authors  
268 discussed the results and contributed to the development of the manuscript.

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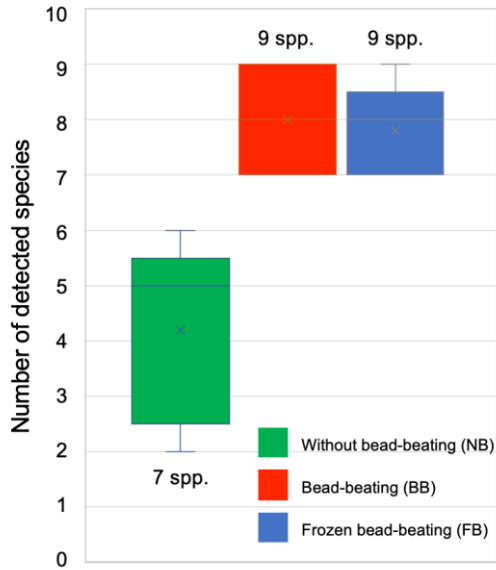
## 273 **References**

- 274 Albertsen M, Karst SM, Ziegler AS, Kirkegaard RH, Nielsen PH (2015) Back to Basics – The  
275 influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge  
276 communities. PLoS ONE 10: e0132783. <https://doi.org/10.1371/journal.pone.0132783>
- 277 Cantera I, Cilleros K, Valentini A, Cerdan A, Dejean T, Iribar A, Taberlet P, Vigouroux R, Brosse  
278 S (2019) Optimizing environmental DNA sampling effort for fish inventories in tropical streams  
279 and rivers. Scientific Reports 9: 3085. <https://doi.org/10.1038/s41598-019-39399-5>
- 280 Cubero OF, Crespo A, Fatehi J, Bridge PD (1999) DNA extraction and PCR amplification method  
281 suitable for fresh, herbarium-stored, lichenized, and other fungi. Plant Systematics and Evolution  
282 216: 243–249.
- 283 Deiner K, Bik HM, Mächler E, Seymour M, Lacoursière-Roussel A, Altermatt F, Creer S, Bista I,  
284 Lodge DM, Vere N, Pfrender ME, Bernatchez L (2017) Environmental DNA metabarcoding:  
285 Transforming how we survey animal and plant communities. Molecular Ecology 26: 5872–5895.  
286 <https://doi.org/10.1111/mec.14350>

- 287 Dinno A, Dinno MA (2017) Package ‘dunn.test.’ CRAN Repos 10: 1–7.
- 288 Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*  
289 26(19): 2460–2461. <https://doi.org/10.1093/bioinformatics/btq461>
- 290 Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*  
291 39: 783–791.
- 292 Ficetola GF, Taberlet P, Coissac E (2016) How to limit false positives in environmental DNA and  
293 metabarcoding? *Molecular Ecology Resources* 16: 604–607. [https://doi.org/10.1111/1755-](https://doi.org/10.1111/1755-0998.12508)  
294 [0998.12508](https://doi.org/10.1111/1755-0998.12508)
- 295 Fujii K, Doi H, Matsuoka S, Nagano M, Sato H, Yamanaka H (2019) Environmental DNA  
296 metabarcoding for fish community analysis in backwater lakes: A comparison of capture  
297 methods. *PLoS ONE* 14: e0210357. <https://doi.org/10.1371/journal.pone.0210357>
- 298 Guo F, Zhang T (2013) Biases during DNA extraction of activated sludge samples revealed by high  
299 throughput sequencing. *Appl Microbiol Biotechnol* 97: 4607–4616.  
300 <https://doi.org/10.1007/s00253-012-4244-4>
- 301 Gweon HS, Shaw LP, Swann J, Maio ND, AbuOun M, Niehus R, Hubbard ATM, Bowes MJ,  
302 Bailey MJ, Peto TEA, Hoosdally SJ, Walker AS, Sebra RP, Crook DW, Anjum MF, Read DS,  
303 Stoesser N, Abuoun M, Anjum M, Bailey MJ, Barker L, Brett H, Bowes MJ, Chau K, Crook  
304 DW, Maio ND, Gilson D, Gweon HS, Hubbard ATM, Hoosdally S, Kavanagh J, Jones H, Peto  
305 TEA, Read DS, Sebra R, Shaw LP, Sheppard AE, Smith R, Stubberfield E, Swann J, Walker  
306 AS, Woodford N (2019) The impact of sequencing depth on the inferred taxonomic composition  
307 and AMR gene content of metagenomic samples. *Environmental Microbiome* 14: 7.  
308 <https://doi.org/10.1186/s40793-019-0347-1>
- 309 Hinlo R, Furlan E, Sutor L, Gleeson D (2017) Environmental DNA monitoring and management of  
310 invasive fish: comparison of eDNA and fyke netting. *Management of Biological Invasions* 8:  
311 89–100. <https://doi.org/10.3391/mbi.2017.8.1.09>
- 312 Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) “Sight-unseen” detection of rare aquatic  
313 species using environmental DNA. *Conserv Letter* 4: 150–157. [https://doi.org/10.1111/j.1755-](https://doi.org/10.1111/j.1755-263x.2010.00158.x)  
314 [263x.2010.00158.x](https://doi.org/10.1111/j.1755-263x.2010.00158.x)
- 315 Johnson MD, Cox RD, Barnes MA (2019) Analyzing airborne environmental DNA: A comparison  
316 of extraction methods, primer type, and trap type on the ability to detect airborne eDNA from  
317 terrestrial plant communities. *Environmental DNA* 1: 176–185. <https://doi.org/10.1002/edn3.19>

- 318 Johnson MD, Freeland JR, Parducci L, Evans DM, Meyer RS, Molano-Flores B, Davis MA (2023)  
319 Environmental DNA as an emerging tool in botanical research. *American Journal of Botany* 110:  
320 e16120. <https://doi.org/10.1002/ajb2.16120>
- 321 Kelly RP, Port JA, Yamahara KM, Crowder LB (2014) Using Environmental DNA to census  
322 marine fishes in a large mesocosm. *PLoS ONE* 9: e86175.  
323 <https://doi.org/10.1371/journal.pone.0086175>
- 324 Minamoto T, Miya M, Sado T, Seino S, Doi H, Kondoh M, Nakamura K, Takahara T, Yamamoto  
325 S, Yamanaka H, Araki H, Iwasaki W, Kasai A, Masuda R, Uchii K (2021) An illustrated manual  
326 for environmental DNA research: Water sampling guidelines and experimental protocols.  
327 *Environmental DNA* 3: 8–13. <https://doi.org/10.1002/edn3.121>
- 328 Miya M, Gotoh RO, Sado T (2020) MiFish metabarcoding: a high-throughput approach for  
329 simultaneous detection of multiple fish species from environmental DNA and other samples.  
330 *Fisheries Science* 86: 939–970. <https://doi.org/10.1007/s12562-020-01461-x>
- 331 Miya M, Minamoto T, Yamanaka H, Oka S, Sato K, Yamamoto S, Sado T, Doi H (2016) Use of a  
332 filter cartridge for filtration of water samples and extraction of Environmental DNA. *Journal of*  
333 *Visualized Experiments* (117): 54741. <https://doi.org/10.3791/54741>
- 334 Miya M, Sado T (2019) Multiple species detection using MiFish primers. In: Committee eDNA MS  
335 (Ed.), *Environmental DNA Sampling and Experiment Manual Version 2. 1*. The eDNA Society,  
336 Otu, Japan, 55–92.
- 337 Miya M, Sado T, Oka S, Fukuchi T (2022) The use of citizen science in fish eDNA metabarcoding  
338 for evaluating regional biodiversity in a coastal marine region: A pilot study. *Metabarcod*  
339 *Metagenom* 6: 133–144. <https://doi.org/10.3897/mbmg.6.80444>
- 340 Miya M, Sato Y, Fukunaga T, Sado T, Poulsen JY, Sato K, Minamoto T, Yamamoto S, Yamanaka  
341 H, Araki H, Kondoh M, Iwasaki W (2015) MiFish, a set of universal PCR primers for  
342 metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine  
343 species. *Royal Society Open Science* 2: 150088. <https://doi.org/10.1098/rsos.150088>
- 344 Oka S, Miya M, Sado T (2022) Gravity filtration of environmental DNA: A simple, fast, and  
345 power-free method. *MethodsX* 9: 101838. <https://doi.org/10.1016/j.mex.2022.101838>
- 346 Pont D, Rocle M, Valentini A, Civade R, Jean P, Maire A, Roset N, Schabuss M, Zornig H, Dejean  
347 T (2018) Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers  
348 despite its downstream transportation. *Scientific Reports* 8: 10361.  
349 <https://doi.org/10.1038/s41598-018-28424-8>

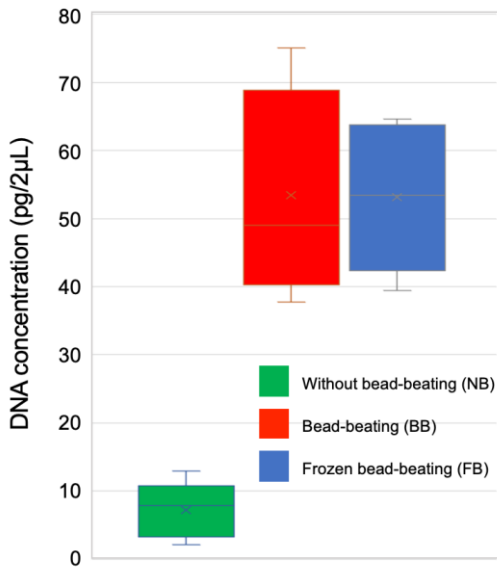
- 350 Prieto AE, Hardion L, Beisel J-N (2021) Designing robust DNA barcode libraries for  
351 metabarcoding of freshwater plants by integrating herbarium collections and contemporary  
352 floristic inventories. ARPHA Conference Abstracts 4: e64713.  
353 <https://doi.org/10.3897/aca.4.e64713>
- 354 R-Core-Team (2022) R: A language and environment for statistical computing. Available from:  
355 <https://www.r-project.org/index.html>.
- 356 Rozewicki J, Li S, Mar Amada K, Standly D M, Katoh K (2019) MAFFT-DASH: integrated protein  
357 sequence and structural alignment. Nucleic acids Research 47(W1): W5-W10.
- 358 Sakata A, Sado T, Oka S, Ushio M, Miya M (2023) Collection of environmental DNA from  
359 stemflow for monitoring arboreal biodiversity: Preliminary validation using lichens. MethodsX  
360 11: 102448. <https://doi.org/10.1016/j.mex.2023.102448>
- 361 Schrader C, Schielke A, Ellerbroek L, Johne R (2012) PCR inhibitors – occurrence, properties and  
362 removal. Journal of Applied Microbiology 113: 1014–1026. [https://doi.org/10.1111/j.1365-  
363 2672.2012.05384.x](https://doi.org/10.1111/j.1365-2672.2012.05384.x)
- 364 Singer GAC, Fahner NA, Barnes JG, McCarthy A, Hajibabaei M (2019) Comprehensive  
365 biodiversity analysis via ultra-deep patterned flow cell technology: a case study of eDNA  
366 metabarcoding seawater. Scientific Reports 9: 5991. [https://doi.org/10.1038/s41598-019-42455-  
367 9](https://doi.org/10.1038/s41598-019-42455-9)
- 368 Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ (2016) W-IQ-TREE: a fast online  
369 phylogenetic tool for maximum likelihood analysis W-IQ-TREE: a fast online phylogenetic tool  
370 for maximum likelihood analysis. Nucleic acids research 44(W1): W232-W235.
- 371 Ushio M (2019) Use of a filter cartridge combined with intra-cartridge bead beating improves  
372 detection of microbial DNA from water samples. Methods in Ecology and Evolution: 435305.  
373 <https://doi.org/10.1111/2041-210x.13204>
- 374 Wilcox TM, Zarn KE, Piggott MP, Young MK, McKelvey KS, Schwartz MK (2018) Capture  
375 enrichment of aquatic environmental DNA: A first proof of concept. Molecular Ecology  
376 Resources 18: 1392–1401. <https://doi.org/10.1111/1755-0998.12928>
- 377



378

379 **Figure 1.** Box plots showing the variation in the number of detected species per sample among the  
 380 three eDNA extraction methods. Numerals beside the boxes indicate the total number of detected  
 381 species across the five samples for each method.

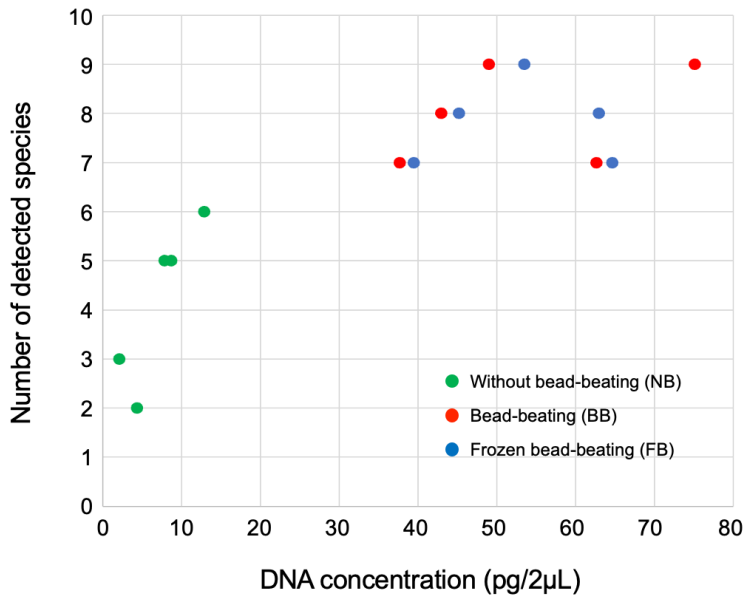
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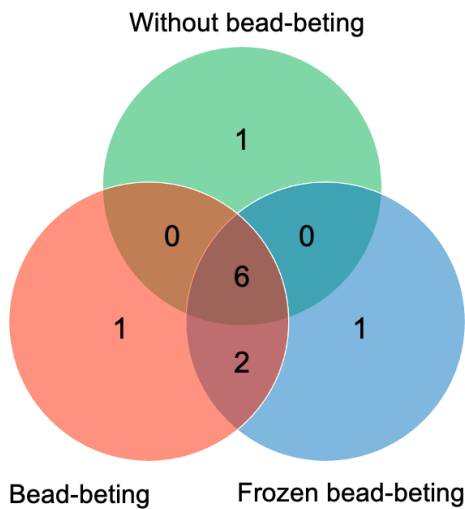
383

384 **Figure 2.** Box plots showing the variation in DNA concentration, as determined by qPCR, among the  
 385 three eDNA extraction methods.

386

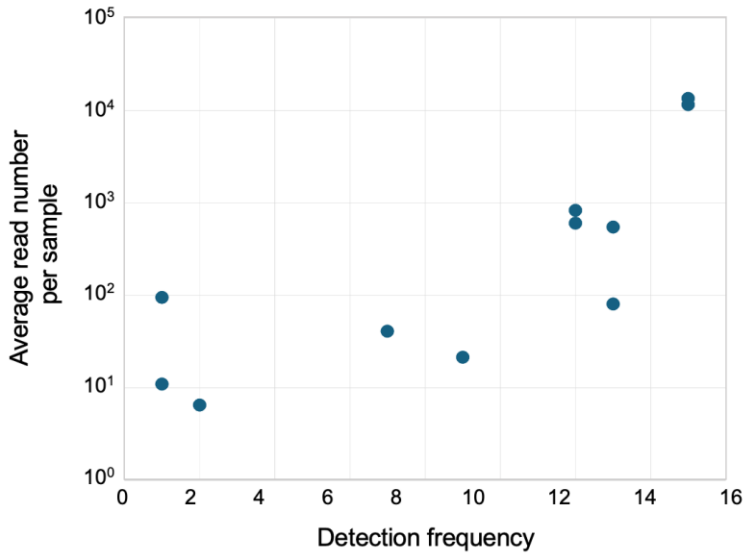


387  
 388 **Figure 3.** Scatter plot showing the relationship between DNA concentration and the number of  
 389 detected species.

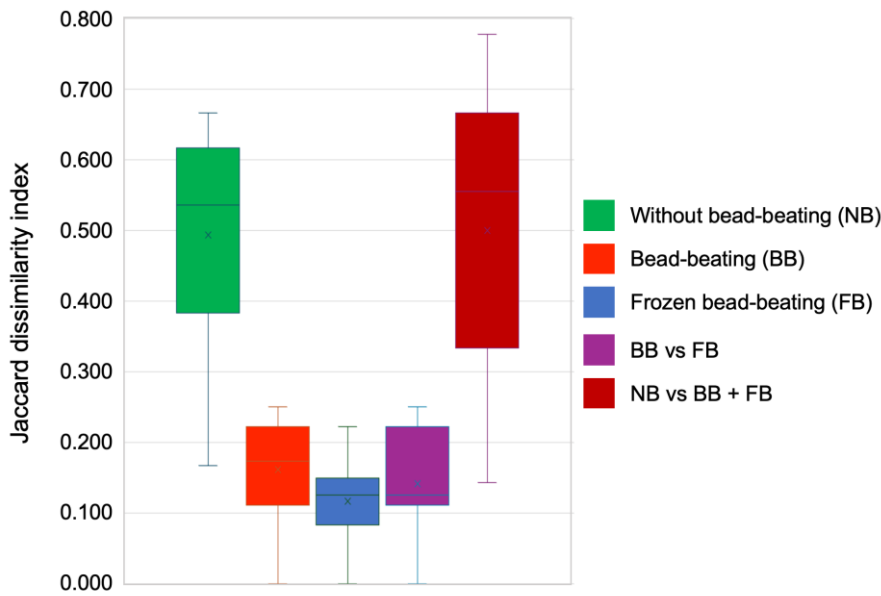


391  
 392 **Figure 4.** Venn diagram showing the overlap of detected species among the three eDNA extraction  
 393 methods.

394



395  
 396 **Figure 5.** Relationship between the detection frequencies of 11 species and the average read number  
 397 per sample from MiSeq sequencing.



399  
 400 **Figure 6.** Box plots showing the variation in pairwise dissimilarities of species composition among  
 401 the three eDNA extraction methods. The dissimilarities are partitioned into three within-pretreatment  
 402 groups (NB, BB, FB) and two between-pretreatment groups (BB vs. FB and NB vs. BB + FB).

403

404 **Table 1.** A list of the nuclear ribosomal internal transcribed spacer (ITS) regions of lichens species  
 405 used as a reference database during taxonomic assignment.

Family	Species	Locality	specimen voucher	collector	Acc. No.
Caliciaceae	<i>Amandinea punctata</i>	South Korea	163344 (KoLRI 041589)	Kondratyuk S. Y.	MF398994
Caliciaceae	<i>Dirinaria applanata</i>	Japan: Chiba	CBM:Sakata 6044	Ayumi Sakata	LC771175
Candelariaceae	<i>Candelaria concolor</i>	Canada	personal:hb. Haughland:UoA-CC- 2019-96	Sydney Toni & Alessandra Hood	ON116022
Chrysothricaceae	<i>Chrysothrix xanthina</i>	South Africa	Curtis (B 60 0202469)	-	MH714516
Graphidaceae	<i>Graphis scripta</i>	-	45918	James Lendemer	MK092086
Lecanoraceae	<i>Lecanora argentata</i>	-	-	-	MN654584
Lecanoraceae	<i>Lecanora fulvastra</i>	Japan	CBM:Sakata 3591	Ayumi Sakata	LC269720
Lecanoraceae	<i>Lecanora imshaugii</i>	-	-	-	JQ782717
Lecanoraceae	<i>Lecanora leprosa</i>	Thailand	-	-	JQ782721
Lecanoraceae	<i>Lecanora pulverulenta</i>	Japan	TNS:YO7700	Yoshihito Ohmura	LC669640
Lecanoraceae	<i>Lecanora pulverulenta</i>	Japan	TNS:YO7700	Yoshihito Ohmura	LC669640
Lecanoraceae	<i>Lecidella euphorea</i>	-	-	-	HQ650596
Parmeliaceae	<i>Canoparmelia aptata</i>	South Korea	KoLRI013328	-	KM250224
Parmeliaceae	<i>Flavoparmelia caperata</i>	Japan	TNS:YO6863	Yoshihito Ohmura	LC669627
Parmeliaceae	<i>Parmelinopsis minarum</i>	South Korea	KoLRI001017	-	KM250245
Parmeliaceae	<i>Parmotrema austrosinense</i>	Japan: Chiba	CBM:Sakata 5664	Ayumi Sakata	LC773250
Parmeliaceae	<i>Parmotrema clavuliferum</i>	South Korea	K.H. Moon 13397	-	KU354438
Parmeliaceae	<i>Parmotrema clavuliferum</i>	South Korea	K.H. Moon 13920	-	KU354444
Parmeliaceae	<i>Parmotrema reticulatum</i>	Portugal	MAF-Lich 20577	-	KX457730
Parmeliaceae	<i>Parmotrema tinctorum</i>	Japan: Chiba	Sakata 5823	Ayumi Sakata	LC773248
Parmeliaceae	<i>Parmotrema tinctorum</i>	Japan: Chiba	Sakata 3545	Ayumi Sakata	LC461188
Parmeliaceae	<i>Punctelia borneri</i>	Japan	TNS:YO6831	Yoshihito Ohmura	LC669679
Physciaceae	<i>Hyperphyscia adglutinata</i>	-	BCN-Lich 15516	-	GU247153
Physciaceae	<i>Hyperphyscia crocata</i>	Japan	TNS:YO7701	Yoshihito Ohmura	LC669636
Physciaceae	<i>Kashiwadia orientalis</i>	Japan: Chiba	Sakata 5890	Ayumi Sakata	LC773249
Physciaceae	<i>Phaeophyscia limbata</i>	Japan	TNS:YO6847	Yoshihito Ohmura	LC669666
Physciaceae	<i>Phaeophyscia limbata</i>	Japan: Hokkaido	-	-	LC700480
Physciaceae	<i>Phaeophyscia rubropulchra</i>	Japan	TNS:YO7690	Yoshihito Ohmura	LC669671
Physciaceae	<i>Phaeophyscia spinellosa</i>	Japan: Akita	CBM:FL-41438	Harada Hiroshi et al.	LC547498
Physciaceae	<i>Physciella melanchra</i>	Japan	TNS:YO6841	Yoshihito Ohmura	LC669677
Stereocaulaceae	<i>Lepraria cupressicola</i>	Japan	TNS:YO7702	Yoshihito Ohmura	LC669648
Stereocaulaceae	<i>Lepraria cupressicola</i>	Japan	TNS:YO7702	Yoshihito Ohmura	LC669648

406

407

408 **Table 2.** Summary of taxon assignment results for the 15 libraries from the three different eDNA  
 409 extraction methods

Family	Species	Ave. Identity	Total reads	Without bead-beating	Bead-beating	Frozen bead-beating	Total frequency
Caliciaceae	<i>Amandinea punctata</i>	92.8	1,043	3	5	5	13
Caliciaceae	<i>Dirinaria applanata</i>	100.0	174,672	5	5	5	15
Lecanoraceae	<i>Lecanora fulvastra</i>	83.8	11	1	0	0	1
Parmeliaceae	<i>Parmotrema austrosinense</i>	100.0	10,012	2	5	5	12
Parmeliaceae	<i>Parmotrema clavuliferum</i>	100.0	95	0	0	1	1
Parmeliaceae	<i>Parmotrema tinctorum</i>	100.0	7,227	2	5	5	12
Physciaceae	<i>Hyperphyscia adglutinata</i>	96.7	7,165	3	5	5	13
Physciaceae	<i>Hyperphyscia crocata</i>	99.5	13	0	2	0	2
Physciaceae	<i>Kashiwadia orientalis</i>	97.8	203,176	5	5	5	15
Physciaceae	<i>Phaeophyscia rubropulchra</i>	97.6	192	0	4	5	9
Physciaceae	<i>Physciella melanchra</i>	98.4	285	0	4	3	7
Total frequency				21	40	39	100
Number of detected species				7	9	9	11