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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 1 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 1 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 μL ,
100 containing 6 μL of 2 \times PCR buffer, 2.4 μL of 2 mM dNTPs, 1.4 μL of multiplexed primers (5 μM ;
101 ITS-PHLichenF, ITS-PHLichenR, ITS-PALichenF, and ITS-PALichenR; Sakata et al., 2023), 0.24
102 μL of KOD FX NEO DNA polymerase (Toyobo, Osaka, Japan), 1 μL of Milli-Q water, and 1 μL of
103 template, with two technical replicates. The thermal cycling profile, after an initial 2-minute
104 denaturation at 94 $^{\circ}\text{C}$, was as follows: denaturation at 98 $^{\circ}\text{C}$ for 10 seconds, annealing at 55 $^{\circ}\text{C}$ for 30
105 seconds, extension at 68 $^{\circ}\text{C}$ for 1 minute, with a final elongation at 72 $^{\circ}\text{C}$ 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/ μL using Milli-Q water. Samples with concentrations less than 0.1 ng/ μL were used as
110 templates without dilution.

111 The second round of PCR (2nd PCR) was conducted with 10 cycles in a 15 μL reaction volume
112 containing 7.5 μL of 2 \times KAPA HiFi HotStart ReadyMix, 0.88 μL each of 2nd-PCR forward and
113 reverse primers (Miya and Sado, 2019) with each different combination of indices (5 μM), 3.88 μL
114 of Milli-Q water, and 1.88 μL of template. The thermal cycling profile, after an initial 1-minute
115 denaturation at 95 $^{\circ}\text{C}$, was as follows: denaturation at 98 $^{\circ}\text{C}$ for 20 seconds, and annealing and
116 extension combined at 72 $^{\circ}\text{C}$ for 30 seconds, with a final elongation at 72 $^{\circ}\text{C}$ 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 (<https://github.com/rogotoh/PMiFish.git>; Miya et al. 2020) to perform OTU clustering and taxonomic assignment. After
127 rarefaction, all minor molecular taxonomic units (MOTUs) with read counts of less than 0.01% of
128 the total reads (<2 reads) were excluded from the taxonomic table to ensure conservative estimates
129

130 of MOTU diversity (Sakata et al. 2023). To refine the taxonomic assignments, genus-level
131 phylogenies were reconstructed from MOTU sequences and reference sequences belonging to the
132 respective genera. Representative MOTU sequences and reference sequences were saved in a FASTA
133 format for each genus. Sequences were initially aligned using MAFFT (Rozewicki et al. 2019) with
134 default parameters. A neighbor-joining (NJ) tree was subsequently constructed using the aligned
135 sequences in MEGA X (Stecher et al. 2020).

136 **qPCR**

137 Quantitative real-time PCR (qPCR) was performed using the ITS-PHLichenF and ITS-PHLichenR
138 primer pair and eDNA samples with a StepOnePlus real-time PCR system (Thermo Fisher Scientific).
139 The alternative primer pair (PALichenF and ITS-PALichenR) was excluded from the qPCR assay due
140 to low amplification efficiency observed in preliminary experiments. Each 20 μ l reaction contained
141 2 μ l of eDNA template, 900 nM of each primer in PowerUp SYBR Green Master Mix (Thermo Fisher
142 Scientific). The thermal cycling profile consisted of an initial 10-minute denaturation at 95°C,
143 followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Three replicates were amplified for
144 each eDNA sample, as well as for 100, 10, and 1 pg of standard DNA (a mixture of tissue-derived
145 DNA from *Kashiwadia orientalis*, *Hyperphyscia crocata*, and *Dirinaria applanata*), and a PCR
146 negative control.

147 **Statistical analysis**

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

152 **Results and discussion**

153 **Library preparation and parallel sequencing**

154 The pooled 102 libraries (including 87 libraries from other projects) were sequenced, and the MiSeq
155 run yielded a total of 2,747,887 reads, with an average of 94.2% base calls having Phred quality
156 scores of ≥ 30.0 (Q30; error rate = 0.1% or base call accuracy = 99.9%). This run was highly successful,
157 considering that the manufacturer's guidelines (Illumina Publication no. 7702011-001 as of May 27,
158 2014) recommend >80% bases \geq Q30 at 2×150 bp.

159 A total of 452,297 reads were assigned to the 15 libraries, and the number of raw reads for each
160 library ranged from 16,486 to 45,390 with an average of 30,153 reads. Merging the two overlapping

161 paired-end fastq files yielded 444,249 reads (98.2%). The sequences from which the primer sequences
162 were removed were subjected to quality filtering to eliminate low-quality reads, resulting in 438,472
163 reads (96.9%). The remaining reads were dereplicated for subsequent analysis, and singletons to
164 tripletons were removed from the unique sequences (Miya et al. 2022). Thereafter, the reads were
165 denoised to remove putatively erroneous and chimeric sequences. The remaining 404,170 reads
166 (92.2%) were subjected to taxon assignments, with 403,891 reads (99.9% of the denoised reads)
167 putatively considered lichen sequences.

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

173 **Number of detected species**

174 Results from the automatic taxon assignments, with manual adjustments based on the genus-level NJ
175 trees, are summarized in Table 1. In total, 11 species from four families and eight genera were detected
176 across the three methods, with a total detection frequency of 100.

177 The number of detected species per sample was lowest in the eDNA extraction method without
178 bead-beating (NB; Fig. 1), ranging from 2 to 6 species across the five samples, with a total of 7 species
179 detected (Table 1). In contrast, the number was higher in the two methods with bead-beating (BB and
180 FB), ranging from 7 to 9 species across the 10 samples, with a total of 9 species detected for each
181 method (Fig. 1, Table 1). Statistically significant differences were found between the methods with
182 and without bead-beating (NB vs. BB and NB vs. FB; $p < 0.05$, Dunn's multiple comparison test),
183 while no significant difference was found between the two bead-beating methods (BB vs. FB; $p =$
184 1.000, Dunn's multiple comparison test) (Fig. 1).

185 **DNA concentration**

186 As with the number of detected species (Fig. 1), DNA concentration was lowest in the eDNA
187 extraction method without bead-beating (NB), with a mean of 7.2 ± 4.2 pg/2 μ L (mean \pm 1SD; Fig.
188 2). In the two methods with bead-beating (BB and FB), it was significantly higher, with means of
189 53.5 ± 15.3 and 53.1 ± 11.0 pg/2 μ L, respectively (Fig. 2). Statistically significant differences were
190 found between the methods with and without bead-beating (NB vs. BB and NB vs. FB; $p < 0.01$,
191 Dunn's multiple comparison test), while no significant difference was found between the two bead-
192 beating methods (BB vs. FB; $p = 1.000$, Dunn's multiple comparison test) (Fig. 2).

193 As expected from the similar patterns of variation in the number of detected species (Fig. 1) and

194 DNA concentration (Fig. 2), a significant positive correlation was found between the two variables,
195 with a coefficient of determination (R^2) of 0.707 ($p < 0.01$) (Fig. 3). These observations indicate that
196 bead-beating residues from stemflow not only increase the amount of extracted DNA but also
197 consequently lead to an increase in the number of detected species (Ushio 2019).

198 **Detection frequency**

199 Of the 11 detected species, six were found in all 10 samples pretreated with bead-beating (BB and
200 FB; detection frequency = 5; Table 1). In contrast, the detection frequencies of these six species in
201 the five samples without bead-beating (NB) varied from 2 to 5, with a mean of 3.33 (Table 1).
202 Additionally, two species, *Phaeophyscia rubropulchra* and *Physciella melanctra*, which were
203 undetected in only a few of the 10 samples in BB and FB, were not detected at all in NB (Table 1).
204 Furthermore, the remaining three species were detected only once across the three methods. These
205 patterns of detection frequencies were consistent with the overlap of detected species among the three
206 extraction methods, as shown in the Venn diagram (Fig. 4).

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

215 **Species composition**

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

225 When environmental DNA is present at very low concentrations (e.g., due to dilution or
226 degradation), PCR amplification can be less efficient and may fail to consistently amplify target

227 sequences within the extracted DNA, leading to instability in the detection of specific species (Deiner
228 et al. 2017). This reduced detection reliability can result in variability in the number and types of
229 species detected across samples, potentially increasing dissimilarity between them (Kelly et al. 2014).
230 Consequently, the detected species may fluctuate randomly due to low template concentration,
231 reducing the similarity between samples (Ficetola et al. 2016).

232 **Concluding remarks**

233 This study demonstrated that bead-beating as a pretreatment for eDNA extraction effectively
234 increases both the yield of lichen DNA and the number of detected species from environmental
235 samples. In contrast, the use of liquid nitrogen freezing before eDNA extraction, which is effective
236 for DNA extraction from lichen tissue fragments (Cubero et al. 1999), did not show any significant
237 effect on the results of eDNA metabarcoding. Considering the time, effort, and cost associated with
238 freezing, bead-beating alone appears to be sufficient as a pretreatment for extracting lichen eDNA.
239 However, further research is needed to determine whether these findings are consistent across
240 different environmental samples and other arboreal organisms. The application of this optimized
241 method could enhance the accuracy and efficiency of biodiversity monitoring and conservation efforts
242 involving lichens.

243 **Author contributions**

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

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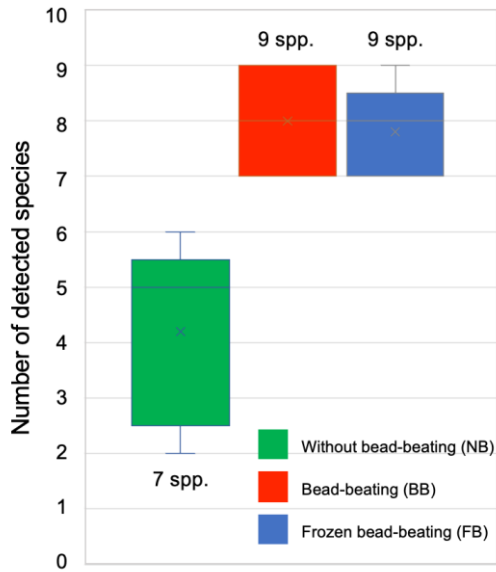
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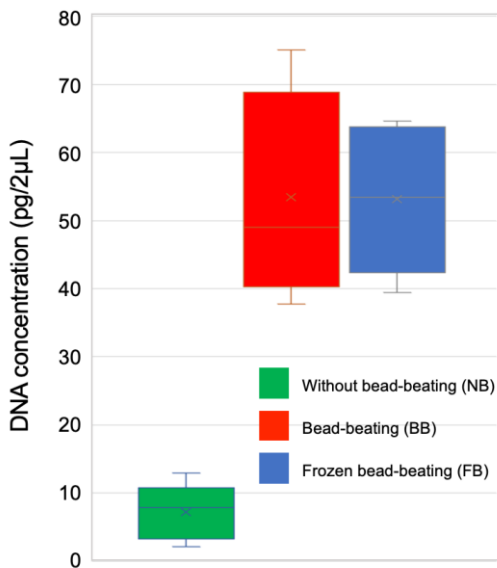
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348

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

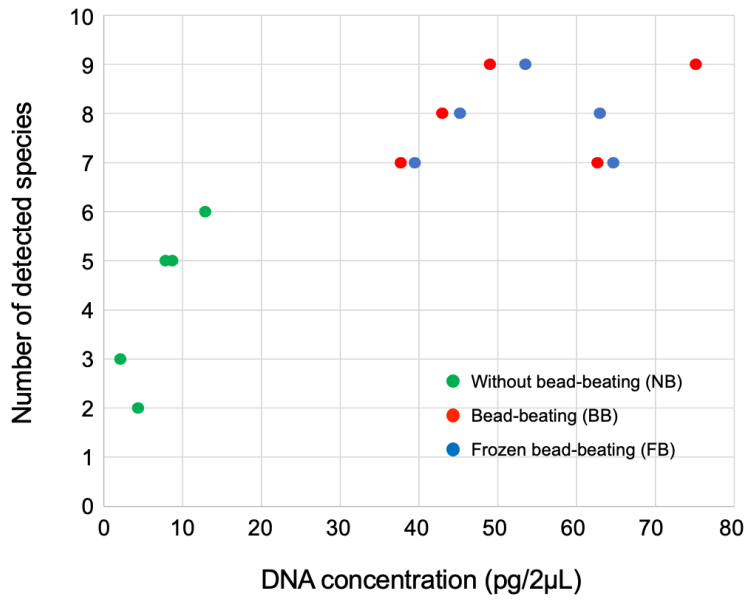
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353

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

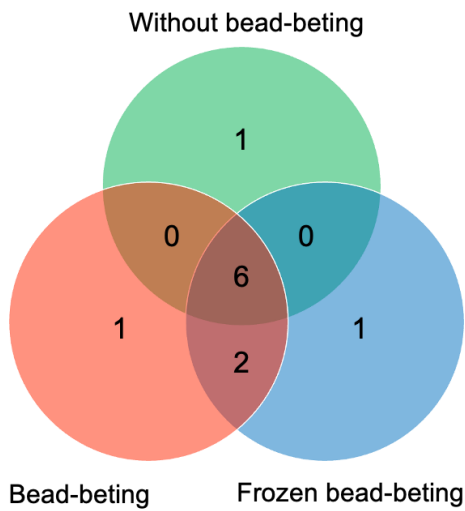
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357

358 **Figure 3.** Scatter plot showing the relationship between DNA concentration and the number of
 359 detected species.

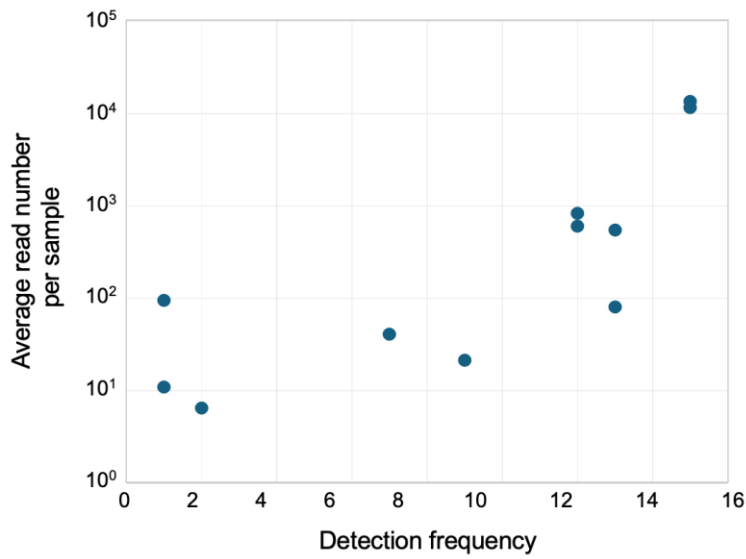
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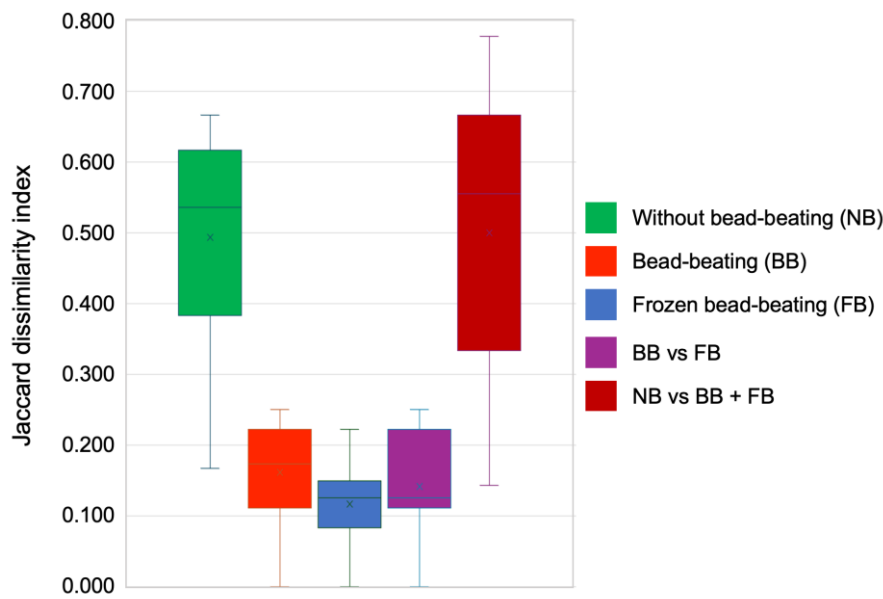
361

362 **Figure 4.** Venn diagram showing the overlap of detected species among the three eDNA extraction
 363 methods.

364



365
 366 **Figure 5.** Relationship between the detection frequencies of 11 species and the average read number
 367 per sample from MiSeq sequencing.



369
 370 **Figure 6.** Box plots showing the variation in pairwise dissimilarities of species composition among
 371 the three eDNA extraction methods. The dissimilarities are partitioned into three within-pretreatment
 372 groups (NB, BB, FB) and two between-pretreatment groups (BB vs. FB and NB vs. BB + FB).

373

374 **Table 1.** Summary of taxon assignment results for the 15 libraries from the three different eDNA
 375 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</i> sp.	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

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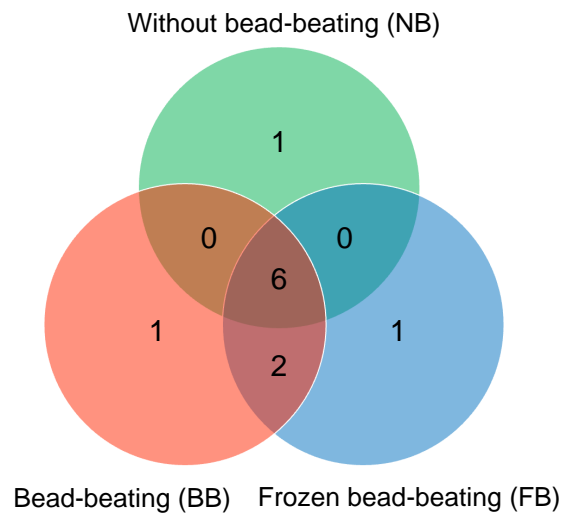


Figure 4

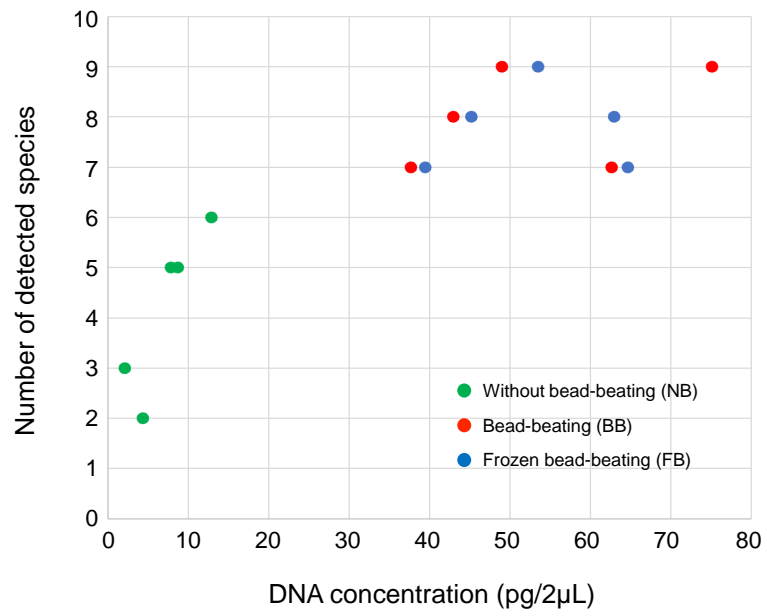


Figure 3

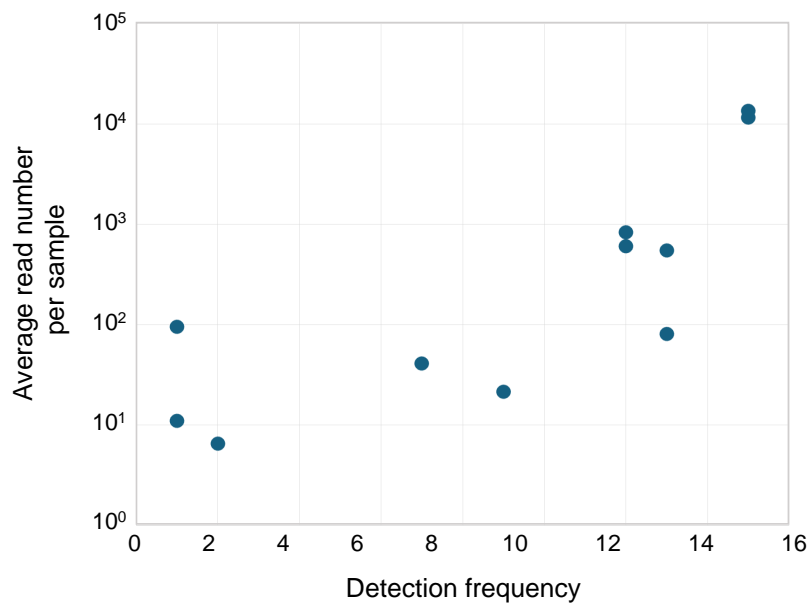


Figure 5

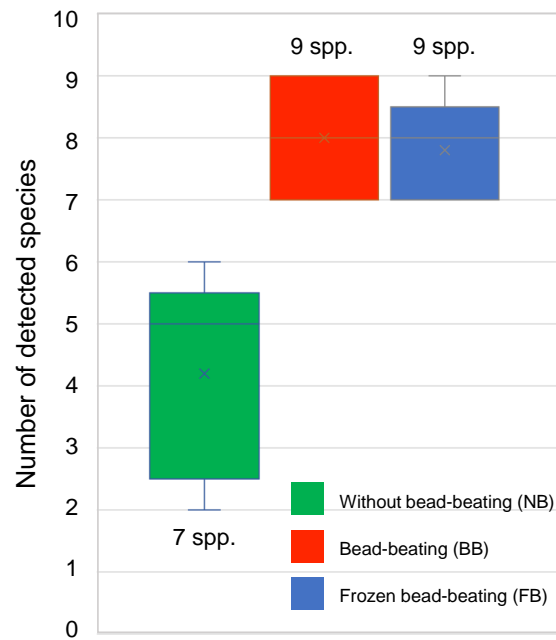


Figure 1

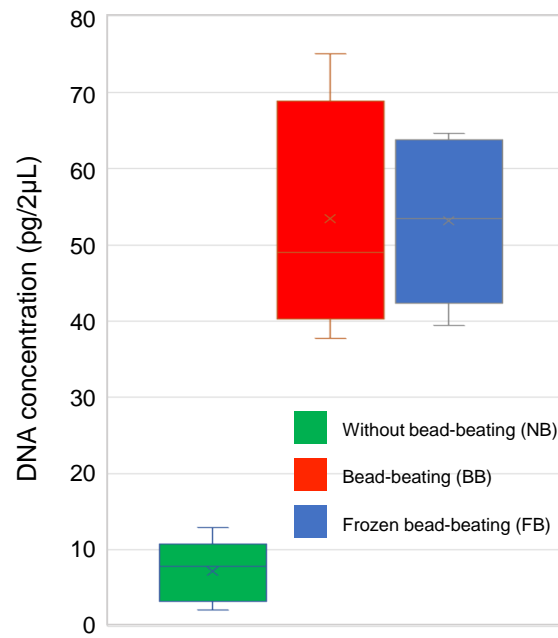


Figure 2

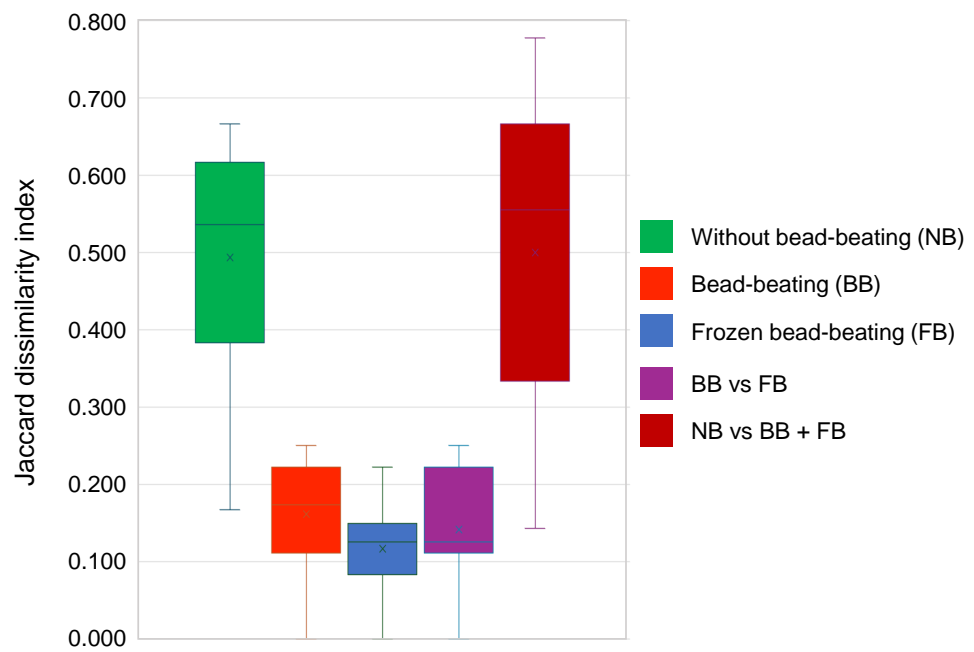


Figure 6

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