Effects of species traits and ecosystem characteristics on species detection by eDNA metabarcoding in lake fish communities

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Abstract

Although environmental DNA (eDNA) metabarcoding is acknowledged to be an exceptionally useful and powerful tool for monitoring surveys, it has limited applicability, particularly for nationwide surveys. To evaluate the performance of eDNA metabarcoding in broad-scale monitoring, we examined the effects of species ecological/biological traits and ecosystem characteristics on species detection rates and the consequences for community analysis. We conducted eDNA metabarcoding on fish communities in 18 Japanese lakes on a country-wide scale. By comparing species records, we found that certain species traits, including body size, body shape, saltwater tolerance, and habitat preferences, influenced eDNA detection. We also found that the proportion of species detected decreased significantly with an increase in lake surface area, owing to an ecosystem-size effect on species detection. We conclude that species traits, including habitat preferences and body size, and ecosystem size should be taken into consideration when assessing the performance of eDNA metabarcoding in broad-scale monitoring.

Keywords: environmental DNA, community, high-throughput parallel DNA sequencing (HTS), MiFish primers

Introduction

An ongoing global decline in biodiversity is beyond doubt and is particularly pronounced in freshwater ecosystems[1-3]. Nevertheless, despite concerns regarding the loss of local/global freshwater fish diversity[1,2,4], there has been a recent tendency to scale back on investments in monitoring and museum collections[5,6]. An understanding of the prevailing status and trends of biodiversity provides a bedrock for ecological research and conservation biology[1,2], and in this regard, Matsuzaki et al.[7] highlighted that long-term trends in local-scale fish diversity have not been comprehensively monitored at broader scales (i.e., national or continental scales) and that performing broad-scale surveys of fish communities is essential for planning fish management and conservation measures[8]. However, the number of lake fish surveys conducted by national and local governments have recently declined[5,6,9]. Given this undesirable trend, it would be prudent to develop broad-scale survey methods for fish monitoring, which could be employed in conducting national-level surveys and thereby provide a basis for formulating conservation policies.

Recent advances in molecular ecology have seen the emergence of environmental DNA (eDNA) analysis as a useful approach for investigating the distribution and richness of aquatic and terrestrial organisms[10-16], and high-throughput parallel DNA sequencing has recently been applied to eDNA methods for simultaneous detection of multiple taxa, known as eDNA metabarcoding[16-21]. For example, Miya et al.[22] designed and applied universal PCR primers (MiFish primers) for fish eDNA metabarcoding, and MiFish primers recently developed for different fish taxa[15,16,23] have shown higher performance compared with other primers types[24] and PCR conditions[25].

74 eDNA metabarcoding is acknowledged to be an exceptionally useful and
75 powerful tool for community surveys[16-20,26,27], and consequently, in recent years, this
76 technique has been widely applied in aquatic community surveys worldwide[28].
77 However, despite the growing number of eDNA metabarcoding studies being
78 conducted, the performance of eDNA metabarcoding applied in broad-scale (e.g.,
79 national-wide) surveys has, with a few exceptions[18,26,29], yet to be sufficiently evaluated
80 quantitatively and statistically[28].
81 From the perspective of evaluating the performance of eDNA metabarcoding
82 surveys, we hypothesized that the ecological/biological traits of targeted species and
83 ecosystem characteristics would influence the detection of species when using eDNA
84 metabarcoding; based on the fact that fish ecological/biological traits (e.g., body size
85 and habitat preference) have been considered to be factors that affect the detectability of
86 eDNA[30,31]. In this regard, Pont et al.[31] revealed differences in the traits of fish detected
87 in a large river using eDNA metabarcoding and traditional survey methods. Differences
88 in the relative abundance of fish related to habitat preference (benthic or pelagic),
89 dietary type, and level of tolerance to water pollution have also been detected in
90 comparisons between eDNA metabarcoding and traditional surveys[31]. The findings of
91 these studies indicate that the ecological/biological traits of fish species, such as body
92 size and shape, habitat preference, and environmental tolerance, may influence specific
93 detection rates when using eDNA metabarcoding. However, despite the increasing
94 evidence of the effects of species traits on the detectability of eDNA, assessments of
95 these effects across spatially broad areas, for example, entire countries with diverse
96 species, are still minimal. Given the same sampling effort, the detection of species
97 eDNA may also depend on certain ecosystem characteristics, such as ecosystem size

(area and volume) and habitat types and variation. For example, eDNA metabarcoding may achieve a lower rate of species detection in larger lakes where greater sampling effort is required to characterize fish communities.

In an effort to gain a better understanding of the factors that could potentially influence to the efficacy of eDNA metabarcoding, we conducted surveys of fish communities in 18 lakes distributed throughout Japan using universal MiFish primers[22] and compared the results obtained with the records of over 200 fish species (including non-native species) inhabiting these lakes[7]. Based on this comparison, we were able to assess the validity of the aforementioned hypotheses regarding the use of eDNA metabarcoding surveys. Moreover, to evaluate the utility of different sampling methods for broad-scale eDNA surveys, we also conducted a survey using individual and mixed water sampling techniques and different cooling and freezing methods to evaluate the effects of sampling and transportation on the findings of eDNA surveys. Finally, we discuss the applicability of eDNA metabarcoding for broad-scale monitoring in lake fish, taking into consideration species traits and ecosystem characteristics.

Results

Sequencing and eDNA metabarcoding overview

Given that we obtained a sufficient proportion of sequences from the raw data and that, with the exception of Lake Shikotsu, there were very few non-fish sequences (Table S2), we assumed MiSeq sequencing and pipeline analysis of the sequence data to have been successful. MiSeq paired-end sequencing for library construction (N = 178: 144 samples, 18 field and filter blanks, and 16 PCR negative controls) yielded a total of

121 5,208,062 sequences [29,258 ± 23,237 (mean ± SD) sequences for each sample, Table
122 S2].
123
124 *Number of detected fish taxa*

125 In total, 119 fish taxa were detected based on eDNA metabarcoding, and all detected
126 taxa were identified to the species or genus level (Table S4, Figs. 1 and S3). We found
127 significant differences among the different sampling methods with respect to the
128 number of fish taxa detected using eDNA metabarcoding (nested ANOVA with LMM,
129 $P < 0.001$, Table S5 and Fig. S4). However, we also detected six species in 23 of the
130 negative and field controls. We obtained sequence reads from six species (*Carassius*
131 *auratus, Chelon haematocheilus, Micropterus salmoides*, *Pagrus major*, *Rhinogobius*
132 spp., and *Tribolodon hakonensis*, Table S4). Furthermore, five species found in the fish
133 records had lower sequence reads (sequences < 276) in the controls than in the samples.
134 In contrast, *Pagrus major* (Red seabream) was not found in the records and does not
135 inhabit the surveyed lakes. The number of sequence reads obtained for *P. major*
136 (sequences = 29 and 452) were found to be higher than those species in the samples
137 (sequences= 20 and 21). Thus, we assumed that the species DNA was contaminated and
138 excluded the species from further analyses.
139 We found differences in the number of fish taxa in the different lakes (Figs. 1
140 and S5), and the number of fish taxa detected using the Mix_cool and Mix_freeze
141 methods was significantly higher than those detected based on individual sampling (Fig.
142 2a, nested ANOVA with LMM, $P < 0.001$ and Tukey's multiple comparison, Table S5).
143 Nevertheless, we found that some species detected in the individual samples were not
144 detected in the Mix_cool and Mix_freeze samples (Table S4).

145

146 *Percentage record covered*

147 To evaluate the species detection performance of eDNA metabarcoding, we used
148 "percentage record covered (%)" as a measure to indicate the proportion of eDNA-
149 detected species (number of species) matching those in the species records. We
150 accordingly found that the percentage record covered did not differ significantly among
151 the three sampling methods (Fig. 2b, nested ANOVA with LMM, $P = 0.249$, Table S5),
152 although values were found to differ among the surveyed lakes (Fig. S6).

153 We also compared the ecological/biological traits of species groups frequently
154 detected using eDNA metabarcoding or in the species records, which was evaluated
155 based indicator taxa analysis (Fig. 3). All the nested ANOVAs with linear mixed
156 models (LMMs) for the fish traits revealed significant differences between eDNA
157 metabarcoding and the species records, whereas no significant differences were detected
158 among the different sampling methods (Table S6). We found that species detected at a
159 higher frequency using eDNA metabarcoding tended to have shorter body lengths (Fig.
160 3a), mainly inhabit the benthopelagic zone (Fig. 3b), and are saltwater tolerant (Fig. 3c).
161 With respect to lateral body shape types, smaller proportions of eel-like species, either
162 short, deep bodied, or both, were identified among those species detected with a higher
163 frequency using eDNA metabarcoding than in the species records (Fig. 3d).

164 To evaluate the effect of lake limnological features on the percentage record
165 covered, we used generalized linear models (GLMs), which revealed a significant
166 negative effect of surface area on the percentage record covered ($P < 0.018$, Table S7,
167 Fig. 4), whereas for all sampling methods used, there were no significant differences
168 with respect to latitude, mean water depth, trophic state, or water type ($P > 0.05$, Table

S7). The relationship between the percentage record covered and the non-included factors to the final GLMs are shown in Fig. S7.

Community analysis

Using indicator taxa analysis, we established that certain species were detected at a significantly higher frequency using eDNA metabarcoding than indicated in the species records for the 18 surveyed lakes (please refer to Table 1 for species with significantly higher frequencies determined by eDNA metabarcoding and all results in Table S8). For example, *C. auratus* was significantly more frequently detected using all three sampling methods, whereas *Zacco platypus* was significantly more frequently detected in the individual and Mix_freeze samples, *Hemibarbus labeo* and *Hypophthalmichthys nobilis* were more frequently detected in individual samples, and *Rhinogobius* spp. were significantly more frequently detected in Mix_freeze samples.

Non-metric multidimensional scaling (NMDS) ordination analysis revealed dissimilarities in fish communities among the surveyed lakes and between eDNA metabarcoding and the species records (Fig. 5 for Mix_cool and Figs. S8 and S9 for individual and Mix_freeze, respectively). Similarly, PERMANOVA analysis revealed significant differences among the lakes and between eDNA metabarcoding and the species records ($P < 0.003$, Table S9).

Discussion

In this study, we observed a difference in the ecological/biological traits of fish species and lake limnological features relating to the eDNA-evaluated community structure and species detection based on broad-scale eDNA and direct surveys.

We initially compared the efficacy of three different sampling methods (individual, Mix_cool, and Mix_freeze sampling) for water collection, and accordingly found that the number of fish taxa detected using Mix_cool and Mix_freeze methods was significantly higher than that detected using individual sampling. In contrast, we detected no significant differences among these sampling methods based on the percentage of record covered, NMDS, or indicator taxa analyses. In this regard, Sato et al.[20] suggested that mixed samples can be used for fish community comparisons despite a somewhat lower detection rate for certain rare species, and this indeed appeared to hold true with respect to the lakes we sampled in the present study.

Our findings tended to indicate that the water transportation method (cool vs. frozen) had no significant influence on the efficiency of eDNA detection, which is consistent with the findings of Deiner et al.[32], who reported a non-significant difference between cool and frozen water transportation on eDNA detection by quantitative PCR (qPCR), which is in contrast to the findings of Takahara et al.[33], who found a reduction in the fish species detected using frozen preservation of water samples based on qPCR analyses. We thus believe that both methods may be useful for transporting water samples for eDNA analysis. In this regard, as a consequence of a field survey we conducted in 2015, Yamanaka et al.[15] developed a water preservation method for eDNA analysis that included the addition of the quaternary ammonium compound benzalkonium chloride (BAC), and we recommend using this BAC method for water transportation.

In order to evaluate the performance of eDNA metabarcoding, we examined the percentage record covered for all three sampling methods. We accordingly found that the values obtained for species records were approximately 30%, thereby indicating

that many of the species inhabiting the surveyed lakes, particularly pelagic species and those with lower occurrence, would not be detected using eDNA metabarcoding. Below, we consider the factors (limnological features and species traits) that might contribute to the lower detection rates obtained using eDNA metabarcoding.

As factors that could potentially influence the efficacy of eDNA metabarcoding, we examined the selected ecological/biological traits of fish species, and accordingly found that species with shorter body length and normal body shape, those inhabiting benthopelagic habitats, and those showing tolerance to saltwater were frequently detected using eDNA metabarcoding. In previous eDNA studies, certain biological traits of fish, notably body size, have been considered to influence eDNA detection[30,31]. However, whereas the findings of several experimental studies have indicated that fish of larger body size release larger quantities of eDNA[30], we found that species with shorter body length, as an index of body size, were detected more frequently using eDNA metabarcoding than their occurrence in the species records. We suspect that these differences are probably attributable to the size–abundance distribution of fish communities, which indicates that species with smaller body sizes are more abundant in lakes than are those of larger body size[34]. Lakes, particularly those characterized by brackish waters, harbor numerous marine-migratory fish species that show tolerance to saltwater, and eDNA metabarcoding surveys of lakes have detected many such migratory species[16,27]. Given that the timing of occurrence of species influences the detection of their eDNA[35], the timing of migration would influence the detection of migratory species in lakes using eDNA metabarcoding, and consequently, may contribute to the infrequent detection of saltwater-tolerant species by eDNA metabarcoding in eDNA sampling surveys. Spatial heterogeneity in fish eDNA

242 concentrations/detection in aquatic ecosystems has previously been observed[10,15,25,26,]
243 and Pont et al.[31] found that benthic species were detected at a higher rate using eDNA
244 metabarcoding than by using traditional methods. This phenomenon would appear to
245 suggest that the habitat preferences of fish might influence their detectability using
246 eDNA metabarcoding. In this regard, the lakeshore water sampling conducted in the
247 present study revealed the presence of numerous benthopelagic species based on eDNA
248 metabarcoding, but relatively few pelagic species. Accordingly, in order to detect a
249 larger number of pelagic species, we would need to perform offshore sampling, for
250 example, using drone-assisted water collection for eDNA surveys[36]. The lateral body
251 shape traits of species may also be associated with their micro-habitat use; for example,
252 species with eel-like and short and/or deep body shapes appear to prefer sheltered
253 micro-habitats (i.e., beneath stones) and pelagic zones. Consequently, the preference for
254 macrohabitats, such as the benthic and pelagic zones, and their microhabitat use with
255 respect to body shape types could serve as useful indices for evaluating eDNA
256 detectability.

257 Evaluation of the effects of lake characteristics on the percentage record
258 covered based on our modeling results revealed significant negative effects of lake
259 surface area. Lakes with a larger ecosystem size are characterized by a broad diversity
260 of habitats types, and correspondingly tend to support a higher diversity of fish
261 species[37,38] and an increased proportion of pelagic zones[39]. Consequently, the size of
262 lake ecosystems will tend to influence the rate of detection using eDNA metabarcoding.
263 With respect to rivers, Bylemans et al.[17] found that river morphology influences the
264 optimal sampling strategy for eDNA metabarcoding in these habitats, and in the present
265 study, we identified a similar effect of ecosystem morphology on eDNA sampling

strategy in lakes. Taking into consideration the factors of sampling effort and expenditure, broad-scale surveys, such as nationwide surveys, tend to be constrained with respect to limited sample sizes, and given these circumstances, it may thus be important to assess the performance of eDNA metabarcoding-based limited sampling effort. In this regard, our GLM analysis predicted that the percentage record covered would decline to almost half (for example, from 30% to 15%) with an increase in lake surface area from 0.1 to 100 km^2.

The utility of eDNA metabarcoding in evaluating fish community structure in lakes has been examined in previous studies that have compared eDNA metabarcoding and traditional sampling methods[16,26]. Similar to the previous results, we observed significant differences in the fish communities detected using eDNA metabarcoding and those in the species records. As discussed above, lake ecosystem size and the traits of fish species can influence the results obtained using eDNA metabarcoding, and consequently, we would expect these effects to be reflected in differences between fish community structures determined based on eDNA metabarcoding and the species record. We indeed found that similar to that shown in the species records, the fish community structure determined by eDNA metabarcoding showed significant differences among the surveyed lakes, thereby indicating that community analysis using this technique could compare the communities of different lakes. Although eDNA metabarcoding would be useful for broad-scale community surveys at low survey costs, we should carefully consider the influence of ecosystem characteristics and the traits of species within fish communities in such surveys.

We acknowledge that the present study does have certain limitations, and thus the conclusions we draw should be viewed as provisional and in need of further

verification. Notably, as we performed only single samplings for the eDNA survey, we were unable to evaluate the false negative/positive detection rates for fish species, which is a vital consideration in applying eDNA metabarcoding to biomonitoring[40]. eDNA sampling strategies are also prone to false-positive errors, owing to contamination and/or errors in PCR or sequencing, which may result in the spurious detection of species[41,42]. Accordingly, further studies are needed to confirm false negative/positive detection rates in lake eDNA surveys.

In conclusion, on the basis of a comparison with existing fish records, we were able to establish that certain traits of fish species and characteristics of ecosystems, particularly body size/shape, species habitat preferences, and ecosystem size, would be useful indices for optimizing eDNA metabarcoding sampling strategies, as well as for assessing the rates of species detection using eDNA methods. From the perspective of designing eDNA metabarcoding-based surveys to monitor aquatic communities across wide geographical areas, we should consider the ecological/biological traits of species and the characteristics of an ecosystem that can potentially influence species detectability.

Methods

Study lakes and sampling sites

For the survey described herein, we selected 18 lakes distributed throughout Japan (Fig. 1), the locations of which are listed in Table S1. The lakes differed with respect to the surface area, water depth, volume, trophic state, and water type (brackish and freshwater), as shown in Table S1. Along the shores of each lake, we established six sampling sites separated by approximately equal distances (Fig. S1), none of which were located in the vicinity of river inflows/outflows.

As species-area accumulation curves in community surveys[43,44], increasing the sample size would increase the detectability of species by eDNA metabarcoding[20,45]. Given that one of our aims in this study was to evaluate the effect of lake size on the detectability of species using eDNA metabarcoding, we conducted surveys with an equal number of sampling sites regardless of lake size.

Collection water samples for eDNA survey

Between 14 July and 4 November 2015, we collected samples of water at each of the six sampling sites located along the shores of the surveyed lakes, using three sampling methods, namely, "Individual," "Mix_cool," and "Mix_freeze" (Fig. S1) (Table S1). For convenience, to evaluate the monitoring methods, we collected water samples from the lakeshore and thereby avoided contamination via floating gears[14]. Initially, for the individual samples, we collected 1 L of surface water in a bottle. To avoid DNA contamination, we sterilized the bottles and all other equipment used in sampling, including filtering apparatus, using 10% commercial bleach (ca. 0.6% hypochlorous acid) followed by washing with DNA-free distilled water. For the Mix_cool and Mix_freeze samples, we collected two 150-mL samples of surface water at each of the six sampling sites, which were mixed in two sterilized bottles to give two composite water samples with final volumes of 900 mL. The "field blank" sample contained 1 L of DNA-free water, which we brought to the field and treated identically to the other water samples, with the exception that it was not exposed to the external environment at the field sites. The individual, Mix_cool, and field blank water samples were stored on-site in a cooler with ice packs and transported to the laboratory in a 4°C refrigerator within 2

338 days. The Mix_freeze samples were stored on-site in a cooler with ice packs, transferred
339 to a -18°C freezer within 12 h, and transported frozen at -18°C within 2 days.
340
341 *Water filtering and eDNA extraction from filter samples*
342 Bottled water samples were vacuum-filtered through 47-mm GF/F glass fiber filters
343 (nominal pore size: 0.7 µm; GE Healthcare, Little Chalfont, UK) in the laboratory.
344 Following filtration, all filters were stored at -20°C prior to eDNA extraction. The field
345 blank samples were processed in a similar manner. As an equipment control, we used 1
346 L of Milli-Q water to monitor contamination during the filtering of the samples from
347 each site and during the subsequent DNA extraction.
348 eDNA was extracted from filters using the method developed by Uchii et
349 al.[46]. We incubated filters by submerging in a mixed buffer comprising 400 µL of
350 Buffer AL (Qiagen, Hilden, Germany) and 40 µL of Proteinase K (Qiagen, Hilden,
351 Germany) using a Salivette tube (Sarstedt, Nümbrecht, Germany) at 56°C for 30 min.
352 The Salivette tube containing filters was centrifuged at 5000 × *g* for 5 min, after which
353 we added 220 µL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH = 8.0) onto the
354 filter, which was then centrifuged at 5000 × *g* for 5 min. The DNA in the eluted solution
355 was purified using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), which
356 extracted the DNA in 200 µL of Buffer AE. Samples were stored at 20°C until used for
357 the 1st PCR assay.
358
359 *Library preparation and MiSeq sequencing*
360 Details of the two-step PCR procedure used for Illumina MiSeq sequencing have been
361 described previously by Fujii et al.[16]. We performed the 1st PCR using MiFish-U-F and

MiFish-U-R primers[22], which were designed to contain Illumina sequencing primer regions and 6-mer random bases, as follows:

Forward: 5′-*ACACTCTTTCCCTACACGACGCTCTTCCGATCT* NNNNNN GTCGGTAAAACTCGTGCCAGC-3′;

Reverse: 5′-*GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT* NNNNNN CATAGTGGGGTATCTAATCCCAGTTTG-3′,

in which the italicized and non-italicized base sequences represent the MiSeq sequencing primers and MiFish primers, respectively, and the six random bases (N) were used to enhance cluster separation on flow cells during the initial base call calibrations on MiSeq.

We performed the 1st PCR using a 12-μL reaction volume containing 1× PCR Buffer for KOD FX Neo polymerase (Toyobo, Osaka, Japan), 0.4 mM dNTP mix, 0.24 U KOD FX Neo polymerase, 0.3 μM of each primer, and 2 μL of template DNA. The thermocycling conditions for this step were as follows: initial denaturation at 94°C for 2 min; followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 65°C for 30 s, and extension at 68°C for 30 s; and a final extension at 68°C for 5 min. The 1st PCRs were performed using eight replicates[25], and the products of individual 1st PCR replicates were pooled and purified using AMPure XP (Beckman Coulter, Brea CA, USA) as templates for the 2nd PCR. The Illumina sequencing adapters and 8-bp identifier indices (the X sequence in the following primers) were added to the subsequent PCR process using the following forward and reverse fusion primers:

Forward: 5′-*AATGATACGGCGACCACCGAGATCTACA* XXXXXXXX ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′;

385 Reverse: 5′-*CAAGCAGAAGACGGCATACGAGAT* XXXXXXXX
386 GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3′,
387 in which the italicized and non-italicized base sequences represent MiSeq P5/P7 adapter
388 and sequencing primers, respectively. The eight X bases represent dual-index sequences
389 inserted to identify different samples[47]. We performed the 2nd PCR with 12 cycles
390 using a 12-μL reaction volume containing 1× KAPA HiFi HotStart ReadyMix, 0.3 μM
391 of each primer, and 1.0 μL of the 1st PCR product. The thermocycling conditions after
392 an initial 3-min denaturation at 95°C were as follows: denaturation at 98°C for 20 s,
393 followed by combined annealing and extension at 72°C (shuttle PCR) for 15 s; and a
394 final extension at the same temperature for 5 min. The 2nd PCR products were pooled
395 in equal volumes and purified using AMPure XP.
396 The purified PCR products were loaded onto a 2% E-Gel SizeSelect agarose
397 gel (Thermo Fisher Scientific, Waltham, MA, USA), and those of the target size
398 (approximately 370 bp) were collected. The collected samples were quantified using a
399 Qubit dsDNA HS assay kit and a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). The
400 amplicon libraries were sequenced using 2 × 250 bp paired-end sequencing on the
401 MiSeq platform using an MiSeq v2 Reagent Kit. Note that sequencing runs were
402 performed for a total of 415 libraries, comprising 178 libraries constructed in the
403 present study (144 samples, 18 field and filter blanks, and 16 PCR negative controls)
404 and 237 libraries constructed in previous research projects. MiSeq sequencing was
405 conducted at the Department of Environmental Solution Technology, Faculty of Science
406 and Technology, Ryukoku University. All sequence data generated have been deposited
407 in the DNA Data Bank of Japan [DRA, Accession number: (submitted)].
408

Bioinformatic analysis for MiSeq sequencing

The detailed procedures used for bioinformatics analyses have been described previously by Fujii et al.[16]. Initially, low-quality tails were trimmed from each read, and paired-end reads were then assembled. For the 5,225,947 reads thus obtained, primer sequences were removed, and identical sequences (i.e., 100% sequence similarity) were merged using UCLUST (usearch 7.0.1001)[48]. Sequences with ten or more identical reads were subjected to downstream processing. For taxonomic annotation, we conducted a local BLASTN search using BLAST 2.2.29 based on a previously established reference database of fish species for processed reads[22]. For each assessed sequence, the top BLAST hit with a sequence identity ≥98.6% was used for species detection. Note that a majority of the species were identified with a match of ≥99%. Based on the BLAST results, we identified the species and genus using previously described methods[16,20]. The sequence reads in the pipeline processes are listed in Table S2.

Fish fauna record data

We used fish fauna data from Japanese lakes published previously by Matsuzaki et al.[7,49]. These studies assembled information on the distribution (i.e., presence or absence) of strictly freshwater fish that are intolerant of saltwater and inhabit freshwater environments for their entire life cycle, based on extensive surveys of scientific papers, monographs and books, online databases (National Survey on the Natural Environment by the Ministry of the Environment and National Censuses on River Environments by the Ministry of Land, Infrastructure, Transport and Tourism), museum specimen databases collected by the National Museum of Nature and Science, local museum

specimens, prefectural and municipal reports, and gray literature, including reports of non-governmental organizations (NGOs) and universities. In the present study, using the same sources, we further added the distributions of secondary freshwater fish, which have a certain degree of salt tolerance and are occasionally able to cross narrow sea barriers, and peripheral freshwater fish, which are derived from marine ancestors and include diadromous fish that migrate between fresh and marine environments. Having compiled the distribution data, we generated a comprehensive dataset of fish fauna consisting of native and exotic fishes after extirpations and introductions. Finally, we obtained records for a total of 242 fish taxa (Table S3). To evaluate the performance of our eDNA metabarcoding survey, we assumed that the records obtained provided a reliable indication of the fish communities inhabiting the selected lakes.

We acknowledge the potential limitations associated with analyzing such compiled data, given that these data were not systematically collected. Moreover, fish were collected using a variety of apparatus and techniques (such as electrofishing, bag seines, trap nets, minnow traps, gill nets, hand nets, and fyke nets), by different entities, and within different studies with differing objectives. However, the utilization of integrated multiple sources can serve to minimize bias and summarize fish fauna in terms of temporal and spatial replication[7,48].

,

Fish trait data

We obtained details on the ecological and biological traits of the detected and recorded fish species from FishBase[50], which was searched using species names, and obtained the related data using the “species” and “ecology” functions of the “rfishbase” package (ver. 3.1.0) on March 4, 2020. We were able to obtain sufficient data on body length

(cm, including both total and standard lengths, but mainly standard length), recorded longevity in the wild (years), lateral body shape types, habitat types, and saltwater tolerance, but were unable to obtain sufficient data (for less than 12% of taxa) for certain traits, such as trophic position, and body weight, for further analysis.

Lake morphology and characteristic data

We obtained data on lake morphology [surface area (km^2), maximum water depth (m), mean water depth (m), and volume (km^3)], and lake types [trophic state and water types (brackish or freshwater)] from Tanaka[51] (Table S1).

Statistical analyses

To evaluate the performance of eDNA metabarcoding with respect to detecting species, we used "percentage record covered (%)" as a measure of the proportion of eDNA-detected species matching species recorded in the target lakes, which was calculated from the number of eDNA-detected species matched to the fish records per total number of recorded species in the dataset.

All statistical analyses and graphic preparations were performed using R ver. 3.6.0[52], and statistical values were evaluated at a significance level of $\alpha = 0.05$.

To compare eDNA metabarcoding and species record data, we compared taxonomic levels in the species list compiled based on visual surveys with those in the lists compiled using eDNA metabarcoding data (Table S1, S2) with reference to previous studies that have used MiFish primers[16,20]. To confirm that the sequencing depth was sufficient to detect fish diversity in the samples, we used the "rarefy" and

480 “rarecurve” functions of the “vegan” package (ver. 2.5.6) (Fig. S2). Thus, we used raw
481 data for further analyses without rarefying the data.
482 We examined differences in the number of detected fish taxa and percentage
483 records covered evaluated by eDNA metabarcoding among the sampling methods
484 (individual, Mix_cool, Mix_freeze, and field and negative controls) using nested
485 analysis of variance (nested ANOVA) based on LMMs with “lake” as the nested
486 (random) factor, using the “anova.lme” and “lme” functions of the “nlme” package (ver.
487 3.1.139), respectively. Tukey’s post hoc test was used for multiple comparisons for
488 least-squares means using the “lsmeans” function of the “lsmeans” package (ver.
489 2.30.0).
490 Differences in fish traits, body length (numeric data), habitats, lateral body
491 shape types, and saltwater tolerance (categorical data) between high frequency of
492 species detection (eDNA metabarcoding vs. species record) among the sampling
493 methods (individual vs. Mix_cool vs. Mix_freeze) were examined using nested
494 ANOVA and an LMM as described above. Prior to the GLM analyses, we calculated a
495 variance inflation factor (VIF) to check the collinearity of the explanatory factors of the
496 GLMs. We found that the maximum VIF value was 1.23 and that all VIF values were
497 less than 5.
498 We also performed GLM analysis to evaluate the relationships between the
499 percentage record covered of the species record and the explanatory factors of lake
500 ecosystems, including lake latitude and altitude, morphology (surface area, maximum
501 water depth, mean water depth, and volume), trophic state (eutrophic vs. meso- and
502 oligotrophic), and water types (freshwater vs. brackish) using the "glm" function. We
503 set "binomial" as the error distribution of the GLMs and added the total number of

504 species records as the offset factor. In this case, we found that the maximum VIF value
505 was 49.7, indicating that collinearity among the factors could potentially influence the
506 parameter estimations in the GLMs. After removing the explanatory factors with a VIF
507 value >5 to reduce the collinearity effect on the GLMs, we finally performed the GLMs
508 using latitude, surface area, mean water depth, trophic state, and water types for all
509 sampling methods.

510 We performed indicator taxa analysis[53] to determine those taxa showing
511 significantly different frequencies between the eDNA metabarcoding and species record
512 species lists. The analysis was performed using the "signassoc" function of the
513 "indicspecies" package (ver. 1.7.6) based on presence/absence data[53]. We used mode =
514 1 (group-based) and calculated the P values with 999 permutations after applying
515 Sidak's correction for multiple testing.

516 Differences in community compositions were visualized using non-metric
517 multidimensional scaling (NMDS) with 500 separate runs of real data. The community
518 dissimilarity for NMDS was calculated using incidence-based Jaccard indices. We also
519 calculated NMDS stress to confirm the representation of the NDMS ordination and
520 evaluated differences in community structures between sampling methods and sites
521 using PERMANOVA, for which we used an incidence-based Jaccard similarity matrix
522 and calculated the statistical values with 999 permutations. For NMDS and
523 PERMANOVA analyses, we used the "metaMDS" and "Adonis" functions of the
524 "vegan" package (ver. 2.5.6), respectively.

525

526 **Acknowledgments**

This study was supported by the Environment Research and Technology Development Fund (4-1602, 4-1705, 4-2004) of the Environmental Restoration and Conservation Agency, Japan, and JST-CREST (JPMJCR13A2).

Data availability

All data obtained from MiSeq sequencing are available in DRA [Accession number: DRA (submitted)], and all data used, including all species detected based on MiSeq sequencing, the recorded species data, and lake data used for analysis, are shown in the Supplemental tables.

Author contributions

HD designed the study, and HA, KI, AK, KK, NM, T Mitsuzuka, TT, KT, NU, TW, and KY contributed to field sampling. SSM contributed to preparing the dataset of the fish presence records. S. Matsuoka, MN, HD, HS, HY, S Matsuhashi, SY, T Minamoto, and MM contributed to laboratory and molecular experiments. HD, S Matsuoka, and SSM analyzed the data. HD, S Matsuoka, SSM, and MM wrote the initial draft of the manuscript. All other authors critically reviewed the manuscript.

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735
736 **Table**

737 Table 1. Significantly higher frequencies of species detected using eDNA
738 metabarcoding compared with species records, as determined based on indicator taxa
739 analysis ($P < 0.05$). "Best" indicates the method facilitating the most frequent detection.
740 P values were calculated based on 999 permutations subsequent to Sidak's correction
741 for multiple testing. Species detected at non-significant, and significantly lower
742 frequencies in the species records are shown in Table S7.

Method	Species name	P value for eDNA	P value for species records	Best method	P value for multiple testing
Individual	*Carassius auratus*	0.001	1	eDNA	0.001999
Individual	*Hemibarbus labeo*	0.001	1	eDNA	0.001999
Individual	*Hypophthalmichthys nobilis*	0.001	1	eDNA	0.001999
Individual	*Zacco platypus*	0.001	1	eDNA	0.001999
Mix_cool	*Carassius auratus*	0.001	1	eDNA	0.001999
Mix_cool	*Zacco platypus*	0.023	0.997	eDNA	0.045471
Mix_freeze	*Carassius auratus*	0.001	1	eDNA	0.001999
Mix_freeze	*Rhinogobius* spp.	0.023	0.997	eDNA	0.045471

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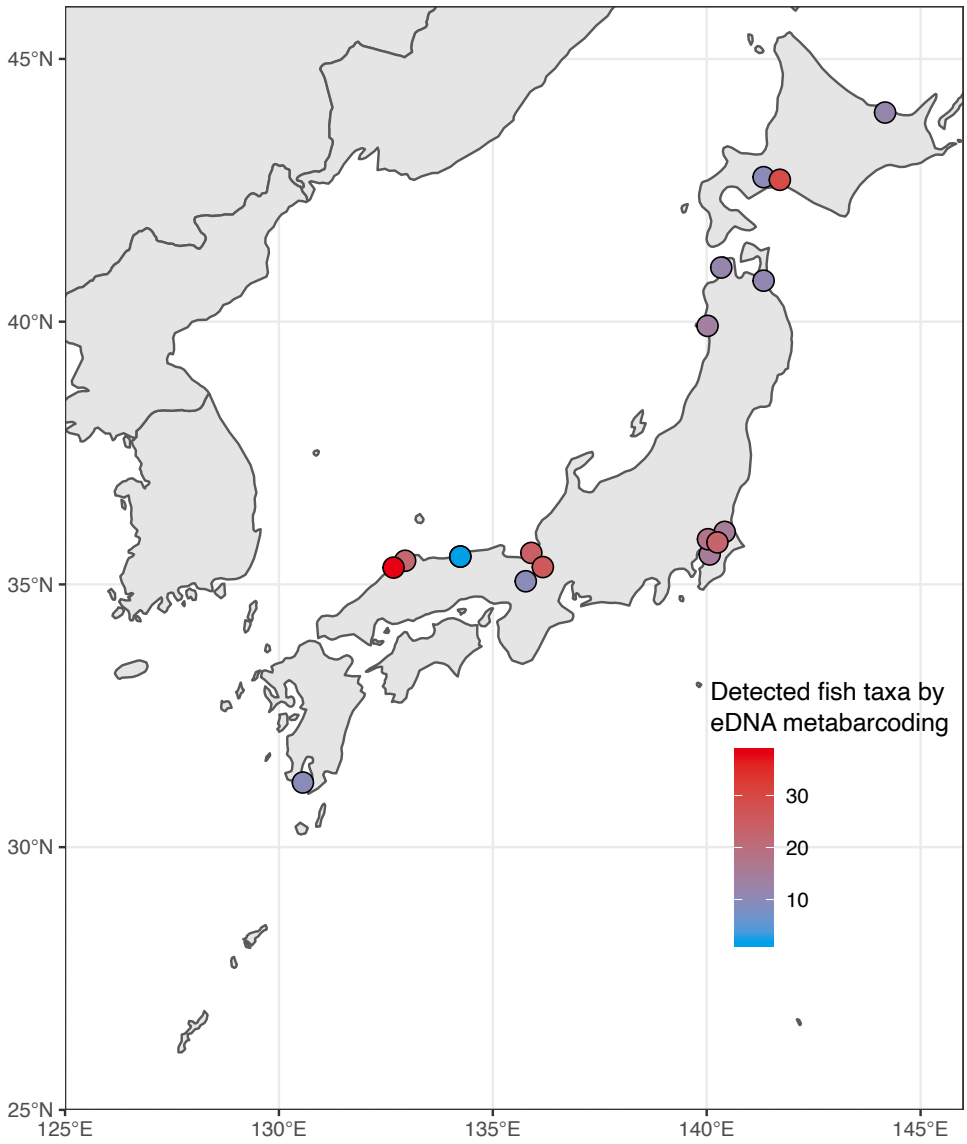
746 **Figures**



747

748 Figure 1. A map of Japan showing the locations of the 18 study lakes. The color of
749 circles indicates the approximate numbers of fish taxa detected using eDNA
750 metabarcoding based on the Mix_cool sampling method. Total numbers of fish taxa
751 detected using other sampling methods (Individual and Mix_freeze) are shown in Fig.
752 S3.
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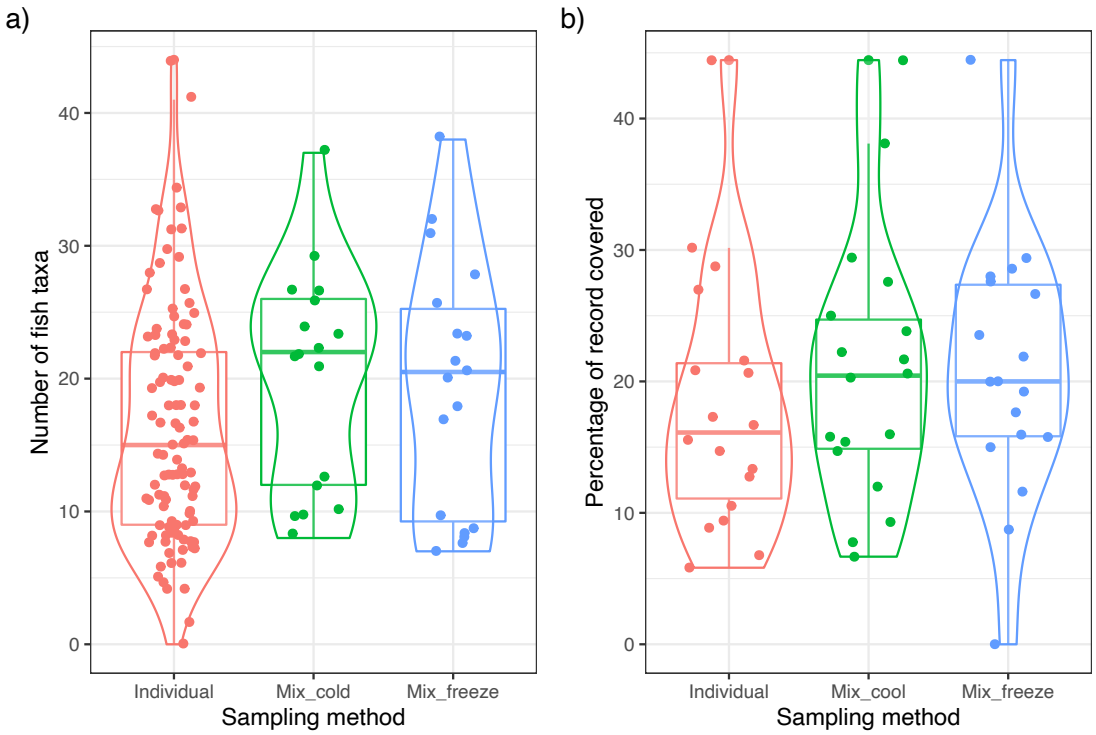

Figure 2. Box and violin plots showing (a) the fish taxa detected and (b) the percentage of record covered (%) with eDNA metabarcoding using different sampling methods. The boxes and bars in the box plots indicate median ± inter-quartiles and ±1.5 × inter-quartiles, respectively. The points represent individual data values. The smooth lines indicate the distribution of the data using violin plots. The violin plot outlines illustrate kernel probability density, i.e., the width of the enclosed area represents the proportion of the data located.

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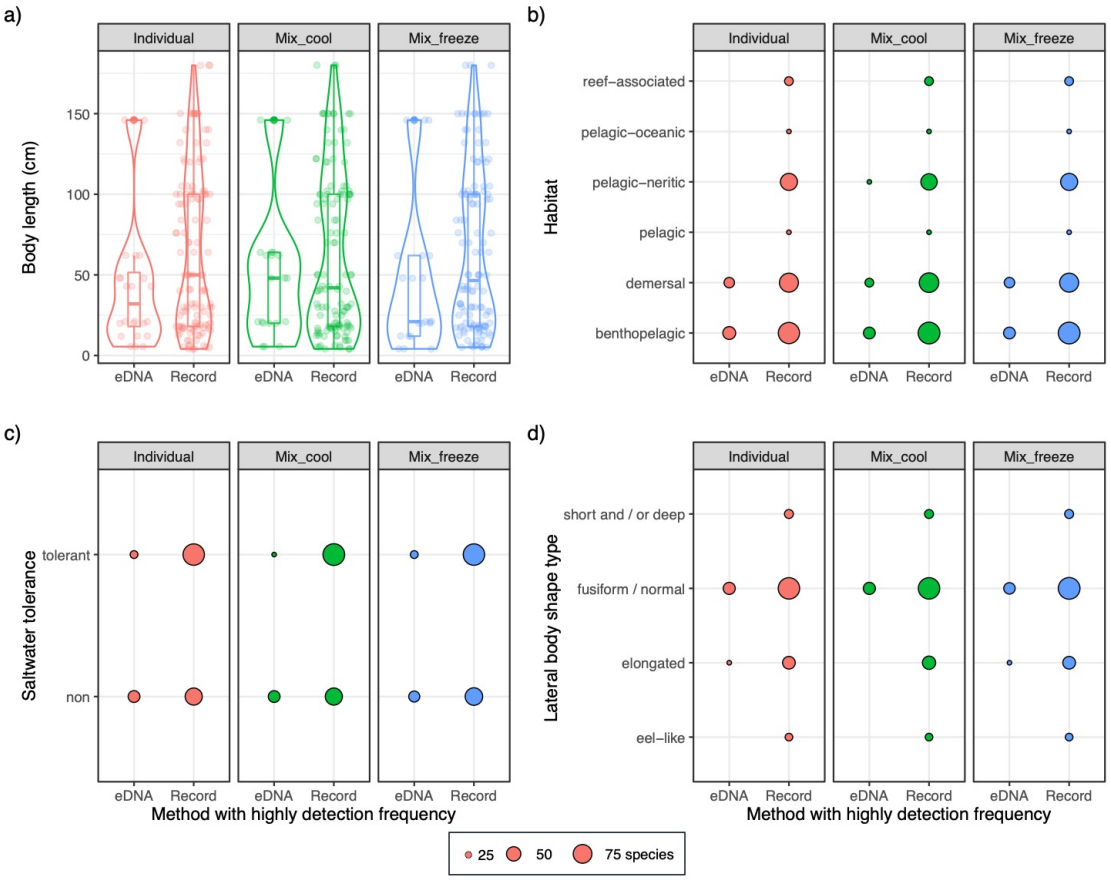

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770 Figure 3. The ecological/biological traits of fish species in species group most
771 frequently detected based on eDNA metabarcoding and in the species records using the
772 different sampling methods, as evaluated based on indicator taxa analysis. (a) Box and
773 violin plots for body length. The boxes and bars in the box plots indicate median ±
774 inter-quartiles and ±1.5 × inter-quartiles, respectively. The smooth lines indicate the
775 distribution of the data using violin plots. The violin plot outlines illustrate kernel
776 probability density, i.e., the width of the enclosed area represents the proportion of the
777 data located. The points represent individual data values. (b-d) Balloon plots indicating
778 the habitat preference (b), saltwater tolerance (c), and lateral body shape type (d) of fish
779 species. The sizes of balloons in (b), (c), and (d) indicate the number of species in each
780 category, as shown in the bottom legend.

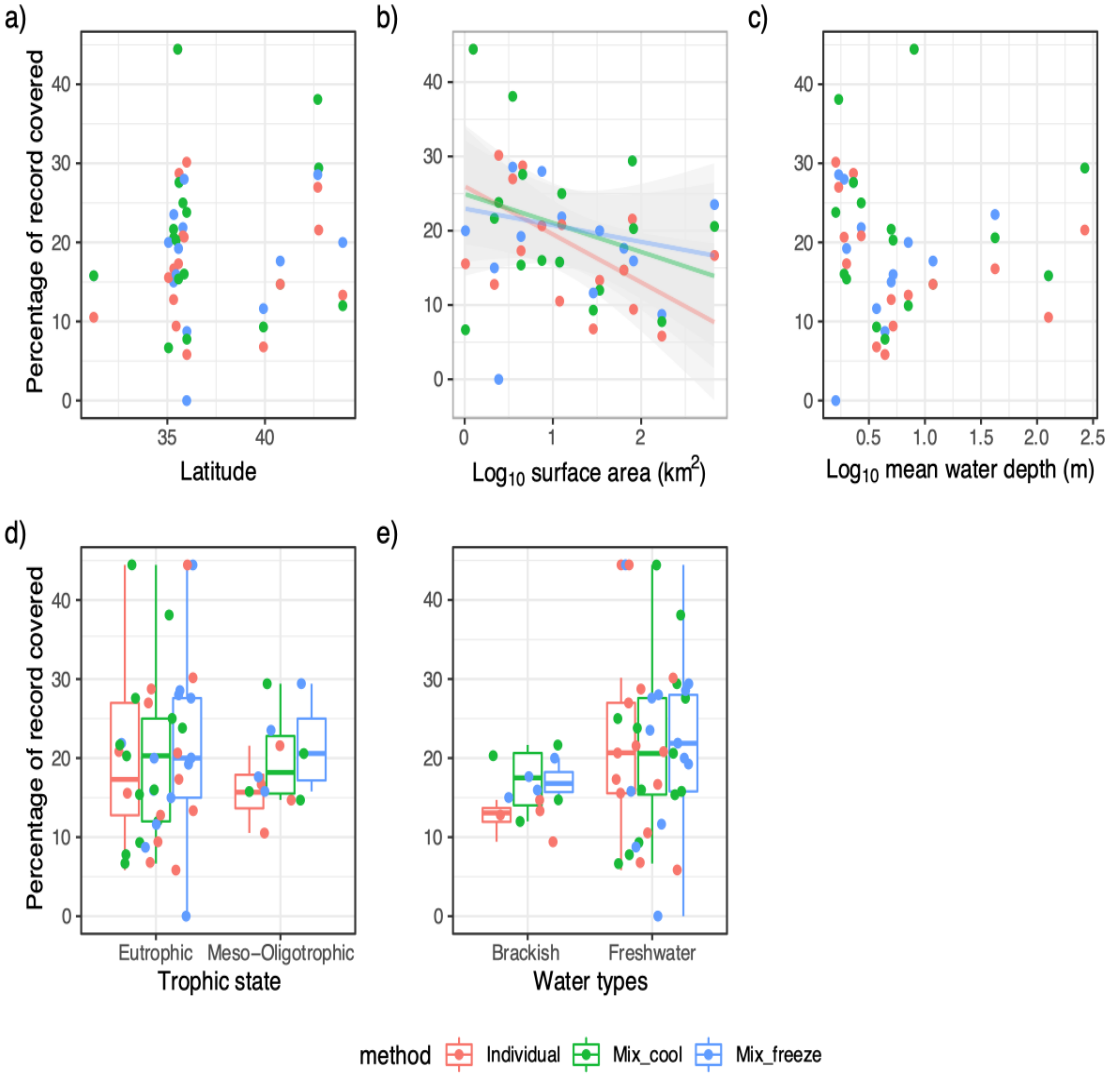
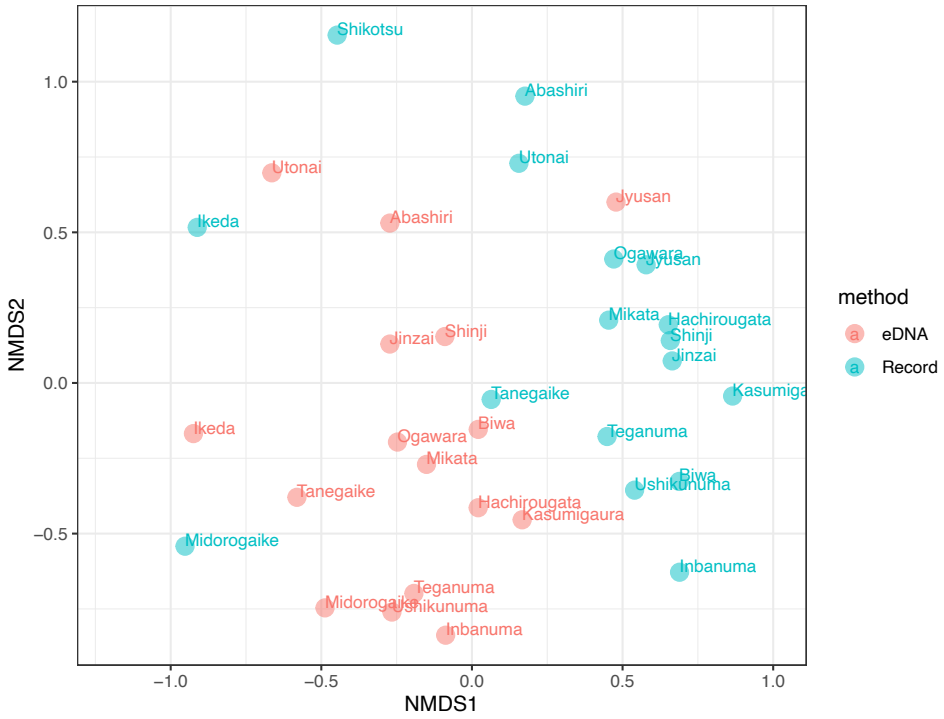


Figure 4. The relationships between the percentage of record covered by eDNA metabarcoding (%) against the fish records and (a) lake latitude, (b) lake surface area, (c) lake mean depth, (d) trophic state, and (e) water type, including the final GLMs, using the different sampling methods (red = Individual, green = Mix_cool, and blue = Mix_freeze). Plots show the relationships with other factors are shown in Fig. S8. The solid and gray areas indicate the regression line from the significant GLM results and the 95% CI, respectively. The boxes and bars in the box plot indicate median ± inter-quartiles and ±1.5 × inter-quartiles, respectively. The points represent individual data values.



793
794 Figure 5. A non-metric multidimensional scaling (NMDS) ordination plot for fish
795 communities based on species records and eDNA metabarcoding using Mix_cool
796 samples. The NMDS stress was 0.174. NDMS plots for Individual and Mix_freeze data
797 are shown in Figs. S8 and S9, respectively.