Evaluation of biodiversity metrics through environmental DNA metabarcoding
outperforms visual and capturing surveys

Hideyuki Doi1,*,†, Ryutei Inui2,*,†, Shunsuke Matsuoka1,†, Yoshihisa Akamatsu2, Masuji Goto2, and Takanori Kono2

1Graduate School of Simulation Studies, University of Hyogo, 7-1-28 Minatojima Minami-machi, Chuo-ku, Kobe, 650-0047, Japan
2Graduate School of Science and Engineering, Yamaguchi University, 2-16-1 Tokiwadai, Ube, Yamaguchi, 755-8611, Japan
†These authors equally contributed to this study.
*Corresponding authors:
Hideyuki Doi (hideyuki.doi@icloud.com)
Ryutei Inui (inuiryutei@gmail.com)
Abstract

Information on alpha (local), beta (between habitats), and gamma (regional) diversity is fundamental to conserving biodiversity and the functions and stability of ecosystem processes. Robust methods like environmental DNA (eDNA) metabarcoding are currently considered useful to investigate biodiversity. However, the performance of eDNA methods in evaluating diversity has not been tested quantitatively. We compared the performance of eDNA metabarcoding and visual and capturing surveys in estimating alpha, beta, and gamma diversity in river fish communities, particularly considering community nestedness and turnover. In five rivers across west Japan, when compared with visual and capturing surveys, eDNA metabarcoding detected higher alpha and gamma diversity in local habitats, and indicated differences in beta diversity more clearly; this suggests the superiority of eDNA metabarcoding over visual/capturing surveys in estimating diversity. The statistical frameworks, particularly nestedness and turnover, can provide quantitative evidences needed to assess diversity component estimation by new survey methods.
The maintenance of biodiversity underpins the stability of ecosystem processes in constantly changing environments\(^1,2,3\). Moreover, biodiversity loss affects ecosystem functions and services and consequently human society\(^1,2,3\). Ecologists have made efforts to conserve biodiversity based on essential biodiversity data, e.g., species richness and distribution\(^1,2,3,4\). Biodiversity can be evaluated in different ways: viz., by estimating alpha (local), beta (between habitats), and gamma (regional), diversity. To conserve local communities, ecologists incorporated these diversity measurements into management decision-making\(^1,2,5\). For example, beta diversity can quantify biodiversity loss and inform the placement of protected areas and the management of biological invasions and landscapes\(^5\). Thus, robust methods for monitoring biodiversity are fundamental for biodiversity and environmental management.

Since recent advances in molecular techniques, environmental DNA (eDNA) analysis has been considered a useful tool to investigate the distribution and richness of aquatic and terrestrial organisms\(^6,7,8,9,10,11,12,13,14\). High-throughput sequencing (HTS) derived from eDNA, called “eDNA metabarcoding”, became a useful and powerful tool for biodiversity surveys\(^13,16,17,18,19,20\). eDNA metabarcoding has recently been applied in fish community surveys, e.g., Miya et al.\(^11\) (2015) developed the universal PCR primers for fish community (MiFish primers). To confirm the usefulness of eDNA metabarcoding for community assessment, many studies conducted eDNA metabarcoding comparing the observed species list with traditional direct surveys including visual and capturing surveys\(^13,16,20,21,22\). However, evaluating the performance of eDNA metabarcoding estimating alpha and beta diversity was still limited quantitively and statistically, especially when evaluating beta diversity and comparing to traditional surveys.
Beta diversity is a fundamental aspect for communities, and it is important for evaluating community responses to environmental gradients and the spatial locations across the different habitats and ecosystems\textsuperscript{5,23,24,25}. Beta diversity itself and the trends along environmental gradients such as productivity and disturbance can be affected by alpha diversity\textsuperscript{24}. Furthermore, beta diversity can decline even if alpha and gamma diversity remain unchanged or even increase in the area, mainly due to biological homogenization\textsuperscript{26,27,28}. Therefore, beta diversity evaluation of communities would be considerably different among the survey methods when alpha and gamma diversity evaluations were different between the survey methods, i.e., eDNA metabarcoding and traditional surveys.

Beta diversity is considered to reflect two different components: nestedness and spatial turnover\textsuperscript{29,30,31}. Nestedness of species assemblages in the communities occurs when the community at the sites with less species are subsets of the community at the sites with higher species richness\textsuperscript{32,33}. Nestedness generally reflects a non-random process of species loss as a consequence of any factor that promotes the orderly disaggregation of assemblages\textsuperscript{34}. Contrastingly, spatial turnover implies the replacement of some species by others because of environmental sorting or spatial/historical constraints\textsuperscript{31}. Baselga\textsuperscript{31} (2010) developed statistical separation methods to evaluate beta diversity considering nestedness and spatial turnover and applied them to beta-diversity evaluation in various systems\textsuperscript{31,35}. However, the method has never been applied to evaluate the performance of eDNA metabarcoding estimating beta diversity. Moreover, Baselga's framework\textsuperscript{31} (2010) can be applied to comparing the performance among methods when evaluating alpha diversity via nestedness and species turnover.

Using statistical methods, we can quantitatively and statistically compare the performance of eDNA metabarcoding and traditional surveys in community evaluation.
using alpha, beta, and gamma diversity. Here, we tested the performance of eDNA metabarcoding in five river systems in different regions with various fish species. We conducted eDNA metabarcoding using the MiFish universal primer for fish and also identified the fish by visual snorkeling and hand-net capturing surveys. We evaluated the performance of eDNA metabarcoding by comparing the obtained fish community structure to that evaluated by visual/capturing survey with special regard to nestedness and spatial turnover. We finally validated the ability of eDNA metabarcoding to estimate biodiversity.

Results

Overview. We detected 53 fish taxa, almost all identified to species or genus level, by eDNA metabarcoding in five rivers (Table S1, and S2) and visually observed 38 fish taxa in total. A MiSeq paired-end sequencing for the 40 libraries (30 samples plus 10 negative controls) yielded a total of 1,601,816 reads (53,351 ± 17,639; mean ± S. D. for each sample, Table S2). We confirmed very low reads from negative controls (Table S2).

Alpha, beta, and gamma diversity between the methods. We found significant differences in fish richness between eDNA metabarcoding and visual/capturing surveys (Fig. 1, GLMM, t = −5.45, P = 0.000018). Richness was not significantly different among river segments (t = −5.85, P = 0.000004) but not among rivers (t = 1.737, P = 0.0942), indicating higher alpha and gamma diversity estimated by eDNA metabarcoding than by visual/capturing surveys.
We found differences in community structures between the two methods by NMDS ordination (Fig. 2). PERMANOVA results for the ordination suggested there were differences in community composition evaluated by each method, eDNA metabarcoding and visual/capturing survey (P = 0.003, Table S3 for full statistical results). Moreover, communities from the combined results of eDNA metabarcoding and visual/capturing survey were significantly different among rivers and segments (P = 0.001, Table S3). We found different patterns in ordinated river sites for each method (Fig. 2). The PERMANOVA results determined that communities were significantly different among the rivers by eDNA metabarcoding (P = 0.011) but not by visual/capturing survey (P = 0.12, Table S4 for full statistical results). Conversely, the communities were significantly different among river segments by visual/capturing survey (P = 0.011) but marginally not significantly different by eDNA metabarcoding (P = 0.061, Table S4). The differences in PERMANOVA results suggested that differences in beta-diversity among rivers across regions can be detected by eDNA metabarcoding but not by visual/capturing survey.

Indicator taxa analysis comparing the communities estimated by both methods, eDNA metabarcoding and visual/capturing survey, detected significantly different method preferences of several taxa, including Japanese eel (Anguilla japonica), salmon (e.g., Oncorhynchus keta), and Amur catfish (Silurus asotus), between both methods (p < 0.05, Table 1 for statistically significant taxa, Table S5 for all taxa).

**Nestedness and turnover.** We compared the pairwise standardized effect size (pSES) between eDNA metabarcoding and visual/capturing survey in nestedness and species turnover (Fig. 3). Nestedness pSES was significantly positive without overlapping the
zero-pSES 95% confidence interval, indicating that the visual/capturing survey community was nested in that detected by eDNA metabarcoding. The significantly negative pSES in species turnover indicated that the taxa did not significantly turnover in the community evaluated by eDNA metabarcoding and visual/capturing survey comparing with random communities.

We compared the longitudinal nested structure (upstream to downstream) of the fish community in the study rivers using nested pSES (Fig. 4a). The longitudinal nested structure was not significantly different between both methods (GLMM, \( P = 0.302 \)). The positive pSES indicated that downstream community significantly nested the upstream ones. The species per segment nested as downstream > mid-stream > upstream in both methods (Fig. S4, 5, NOFD, \( P < 0.001 \)). We also compared the longitudinal spatial turnover of fish community in the study rivers using turnover pSES (Fig. 4b). The longitudinal spatial turnover was not significantly different between both methods (GLMM, \( P = 0.280 \)).

**Discussion**

We found that river fish communities estimated by eDNA metabarcoding significantly nested the communities estimated by visual/capturing survey. Furthermore, the species turnover in the communities was very weak between methods. Nestedness of species assemblages occurs when the communities obtained by the method estimating lower number of species are subsets of the communities estimated by other methods with higher species richness. Although it is difficult to assume the effort of sampling was enough to evaluate the species compositions, we assumed the efforts for the visual and capturing survey are enough to confirm the species composition in the study sites.
because we combined two high-detection surveys, i.e. visual observation and capturing surveys\textsuperscript{12,13}. Therefore, the performance of eDNA metabarcoding in estimating alpha and gamma diversity outperformed that of visual/capturing survey for river fish community. In fact, the fish local and regional richness (alpha and gamma diversity) evaluated by eDNA metabarcoding was significantly higher, including almost all taxa evaluated by visual/capturing survey.

eDNA metabarcoding has been reported to perform better than traditional methods in evaluating species richness\textsuperscript{13,16,19,20,21,22}. Nakagawa et al.\textsuperscript{21} (2018) investigated freshwater fish community in 100 rivers and confirmed that the community detected by eDNA metabarcoding was similar to the species list observed in governmental-authorized monitoring. Furthermore, several eDNA metabarcoding studies on fish communities have been performed in other river systems\textsuperscript{20}, marine habitats\textsuperscript{22}, and freshwater lakes\textsuperscript{13,19}. Deiner et al.\textsuperscript{16} (2016) showed that river eDNA metabarcoding can reflect the community in a watershed, indicating that eDNA metabarcoding has high-performance gamma diversity evaluation. These studies indicated the great potential of eDNA metabarcoding as a useful tool for alpha and gamma diversity assessment by simply comparing the community data obtained from eDNA metabarcoding and traditional surveys. However, previous studies did not evaluate the performance by nestedness and species turnover between eDNA metabarcoding and other community data. Here, we supported previous literature by showing nestedness, species turnover, and species preferences between the communities detected by eDNA metabarcoding and traditional methods, and we found the nestedness structure eDNA metabarcoding > visual/capturing survey, with scarce species turnover in the community.
We especially focused on beta-diversity for assessing the performance of eDNA metabarcoding compared to the visual/capturing survey. Higher beta diversity of the rivers were statistically detected by eDNA metabarcoding than by visual/capturing surveys. Furthermore, the spatial nestedness and turnover were not significantly different between eDNA metabarcoding and traditional methods. These results suggested that eDNA metabarcoding also outperformed visual/capturing survey in evaluating river fish community beta diversity, probably due to the lower alpha diversity detected by visual/capturing survey, especially in the upstream segments. Moreover, beta diversity between segments could be detected by visual/capturing surveys but not by eDNA metabarcoding. These differences in beta diversity evaluation may lead us to interpret beta diversity using the results from both survey methods, eDNA and traditional survey. Deiner et al.\(^\text{16}\) (2016) suggested that eDNA sampling allows an estimate of catchment-level diversity and integrates this information across space due to downstream transport of eDNA. We agree with the suggestion for estimate of catchment-level diversity and further suggest that the catchment-level diversity metrics including alpha, beta, and gamma diversity, can be evaluated similarly to the direct survey.

We further compared the indicator taxa for the communities obtained from both eDNA metabarcoding and visual/capturing survey and concluded that several taxa, including eel, salmon, and catfish, were significantly better detected by eDNA metabarcoding, whereas non-indicator taxa were detected by visual/capturing surveys. These results indicated that eDNA metabarcoding outperformed visual/capturing surveys in fish taxa detection. The community structures estimated by eDNA metabarcoding and visual/capturing survey were slightly different, as reported in previous studies\(^\text{13,19}\), probably because of the differences in taxa-detection
performances. Discriminated taxa in this analysis included eel, salmon, and catfish, which mostly had larger body size and lower abundances in these rivers\textsuperscript{36,37}. In fact, the Japanese eel \textit{Anguilla japonica}, was difficult to find by visual observation, probably due to its hiding behavior\textsuperscript{38}. Such endangered species would be important as top-predator\textsuperscript{37}. eDNA metabarcoding can evaluate the distribution of such rare and important taxa in fish communities better than traditional surveys.

To evaluate the comparing among the local sites and the rivers, we examined the performances of eDNA metabarcoding at three sites of five rivers with eDNA sampling from only two habitats. Thus, our understanding of details in the fish community spatial structure in the rivers and the performance of community evaluation in local habitats, such as backwater, was still limited. In fact, Bylemans et al.\textsuperscript{20} (2018) found that river morphology in the habitats influenced the optimal sampling strategy for eDNA metabarcoding. Moreover, in backwater lakes, the performance of eDNA metabarcoding varied with different lake morphology\textsuperscript{13}. However, testing the usefulness of eDNA metabarcoding for assessing river fish community biodiversity has been limited. Further research is needed to evaluate fish community spatial structure in rivers.

In conclusion, eDNA metabarcoding outperformed the visual/capturing survey in evaluating alpha, beta, and gamma diversity of fish communities in five rivers across the west Japan region. Biodiversity testing using statistical frameworks, especially community nestedness and turnover, provided the quantitative evidence needed to support the advantages of eDNA metabarcoding against traditional surveys. eDNA methods for biodiversity assessment may provide more information to us, as shown here, but we should also pay attention to the unknown characteristics of eDNA, such as the origins, degradation, and transport of eDNA in the water which are still
unknown in various habitats\textsuperscript{39,40}. To routinely use eDNA methods to assess biodiversity, we encourage testing the effect of eDNA degradation and transport on the performance of eDNA methods for biodiversity evaluation.

**Methods**

**Study sites.** In 2016, we conducted field surveys in five river systems across Japan (river map in Fig. S1): the Kyuragi River on 10\textsuperscript{th} October, the Koishiwara River on 21\textsuperscript{st} October, the Yato River on 25\textsuperscript{th} October, the Hazuki River on 2\textsuperscript{nd} November, and the Oze River on 6\textsuperscript{th} November. The survey sites were set at three sites in each of three segments (upstream, mid-stream, and downstream, the internal distances ranged from 4.5 to 25.8 km, Fig. S1) for each river. Each site was set so that the length in the up-down direction is approximately 100 m and with a riffle at the downstream end (e.g., Fig. S2).

**Water collection for eDNA survey.** In each site, we collected 1 L of surface water in bleached bottles at two points, the stream near the downstream end and the shore with static or semi-static water (Fig. S2). The bottles and filtering equipment removed the DNA using 10\% commercial bleach (approximately 0.6\% hypochlorous acid) and washed it using DNA-free distilled water. One milliliter of benzalkonium chloride (BAC, 10\% w/v) was added per liter of water sample to avoid a decrease in eDNA concentration in the samples\textsuperscript{41}. During transport, samples were stored in a cooler with ice packs. The ‘cooler blank’ contained 1 L DNA-free water, which we brought to the field and was treated identically to the other water samples, except that it was not opened at the field sites.
Visual observation and capturing methods. After water sampling, the fish fauna survey was conducted by visual observation with snorkel and collection with hand net. For visual observation, we observed and recorded fish species by snorkeling in 100-m area (1 person × 1 h, Fig. S2). We observed at various micro habitats including riffle, pool, and shore bank from downstream end to upstream end. We also conducted the hand-net capturing survey (1 person × 1 h) using a D-flame net (net opening: 0.16 m²) in the various habitats in the river including riffle, pool, and shore bank. In this study, Because of the impossibility of sampling some habitats such as behind stones, we did not use electrofishing to observe species. Fishes were identified according to Nakabo et al. (2013) at the survey site. We used the combined taxa list from both surveys to compare with that from eDNA metabarcoding. In order to prevent contamination of DNA, the investigator who collected and identified the fish and the investigator who sampled water were different.

Water filtering and eDNA extraction from filter samples. Bottled water samples were vacuum-filtered in the laboratory into 47 mm GF/F glass filters (pore size: 0.7 µm, GE Healthcare, Little Chalfont, UK) within 24 h after sampling. After filtration, all filters were stored at -20 °C before eDNA extraction. The cooler blank was also processed in the same manner. A liter of Milli-Q water was used as the equipment control to monitor contamination during filtering in each site and during subsequent DNA extraction.

To extract the DNA from the filters, we followed the methods described in Uchii, Doi & Minamoto (2016). We incubated the filter by submerging the mixed buffer of 400 µL of Buffer AL (Qiagen, Hilden, Germany) and 40 µL of Proteinase K
(Qiagen, Hilden, Germany), using a Salivette tube (Sarstedt, Nümbrecht, Germany) at 56 °C for 30 min. The Salivette tube with filters was centrifuged at 5000 × g for 5 min and then we added 220 µL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH: 8.0) onto the filter and again centrifuged at 5000 × g for 5 min. The DNA in the eluted solution was purified using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. The final volume of the extracted sample was 200 µL with Buffer AE of DNeasy Blood & Tissue Kit. Samples were stored at -20 °C until qPCR assay.

**Library preparation and MiSeq sequencing.** The procedures used for molecular methods are described in Fujii et al.\(^\text{13}\) (2019). A two-step PCR-procedure was used for library preparation of Illumina MiSeq sequencing. As the first step, a fragment of the mitochondrial 12S rRNA gene was amplified using the MiFish-U-F and MiFish-U-R primers\(^\text{11}\) (Miya et al. 2015) which were designed to contain Illumina sequencing primer regions and 6-mer Ns;

Forward: 5´-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNN

GTCGGTAAACTCGTGCCAGC-3´,

Reverse: 5´-GTGACTGGAGTTCAACGCTTGCTCTTCCGATCTNNNNNN

CATAGTGAGGTATCTAACTCCAGTTTG-3´

The italicized and normal letters represent MiSeq sequencing primers and MiFish primers, respectively, and the six random bases (N) were used to enhance cluster separation on the flow cells during initial base call calibrations on MiSeq. We used a KOD FX Neo polymerase (Toyobo, Osaka, Japan) for the first PCR to facilitate amplifications of DNA from crude extracts. The first PCR was performed with a 12 µL reaction volume containing 1× PCR Buffer for KOD FX Neo, 0.4 mM dNTP mix, 0.24
U KOD FX Neo polymerase, 0.3 μM of each primer, and 2 μL template (Sato et al. 2017). The thermal cycles of 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 elongation at 68 °C for 30 s, followed by final elongation at 68 °C for 5 min. The first PCRs were performed using eight replicates to mitigate false negatives (PCR dropouts). Thereafter, individual first PCR replicates were pooled. Each PCR product (30 μL) was purified using AMPure XP (Beckman Coulter, Brea CA, USA) and eluted with 30 μL of sterilized water. The purified first PCR products were used as templates for the second PCR. The Illumina sequencing adaptors and the eight bp identifier indices (XXXXXXXX) were added to the subsequent PCR process using a forward and reverse fusion primer:

Forward: 5´-AATGATACGGCGACCACCGAGATCTAC... XXXXXXXX
ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3´
Reverse: 5´-CAAGCAGAAGACGGCATACGAGAT... XXXXXXXX
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3´

The italicized and normal letters represent MiSeq P5/P7 adapter and sequencing primers, respectively. The 8X bases represent dual-index sequences inserted to identify different samples (Hamady 2008). The second PCR was conducted with 12 cycles of a 12 μL reaction volume containing 1× KAPA HiFi HotStart ReadyMix, 0.3 μM of each primer, and 1.0 μL template from the first PCR production. The thermal cycle profile after an initial 3 min denaturation at 95 °C was as follows: denaturation at 98 °C for 20 s, annealing, and extension combined at 72 °C (shuttle PCR) for 15 s, with the final extension at the same temperature for 5 min. The second PCR products were pooled in equal volumes and purified using AMPure XP as in the first PCR.
The purified PCR products were loaded on a 2% E-Gel SizeSelect (Thermo Fisher Scientific, Waltham, MA, USA) and the target size of the libraries (approximately 370 bp) was collected. The library concentration and quality were estimated by a Qubit dsDNA HS assay kit and a Qubit 2.0 (Thermo Fisher Scientific). The amplicon libraries were sequenced by 2 × 250 bp paired-end sequencing on the MiSeq platform using the MiSeq v2 Reagent Kit according to the manufacturer’s instructions. Note that the sequencing run contained a total of 339 libraries including 40 of our libraries (30 samples plus ten negative controls) and 299 libraries from other research projects. The MiSeq sequencing was conducted in the Department of Environmental Solution Technology, Faculty of Science and Technology, Ryukoku University. Sequence data were deposited in the Sequence Read Archive of DNA Data Bank of Japan (DRA, Accession number: DRA008090).

**Bioinformatic analysis for MiSeq sequencing.** The procedures used for bioinformatics analysis are described in Fujii et al.13 (2019). The processing formality of the MiSeq reads was evaluated by the FASTQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). After confirming a lack of technical errors in the MiSeq sequencing, low-quality tails were trimmed from each read using “DynamicTrim.pl” in the SOLEXAQA software package44 (Cox et al. 2010) with a cut-off threshold set at a Phred score of 10. The trimmed paired-end reads (reads 1 and 2) were then merged. The assembled 1,823,446 reads were further filtered by custom Perl scripts to remove reads with either ambiguous sites (Ns) or those exhibiting unusual lengths with reference to the expected size of the PCR amplicons (297 ± 25 bp). The software TagCleaner45 (Schmieder et al. 2010) was used to remove primer sequences with a maximum of three-base mismatches and transform the FASTQ format.
into FASTA. The pre-processed reads with an identical sequence (i.e., 100% sequence similarity) were assembled using UCLUST (Edgar 2010) and the number of identical reads was added to the header line of the FASTA formatted data file. The sequences represented by 10 or more identical reads were subjected to the downstream analyses. Processed reads were subjected to local BLASTN searches on the previously established comprehensive reference database of fish species (Miya et al. 2015). The top BLAST hit with a sequence identity $\geq 97\%$ and the E-value threshold of $10^{-5}$ was applied to species detection of each sequence but the species were mostly identified with $\geq 99\%$ match. From the BLAST results, we identified the species using methods previously described (Sato et al. 2017).

**Statistical analyses.** All statistical analyses and graphics were conducted in R ver. 3.4.4 (R Core Team, 2018). All statistics were set at the significance level $\alpha = 0.05$. To compare between eDNA metabarcoding and visual survey data, the taxonomic levels in the species list from visual survey were adjusted to the lists from eDNA metabarcoding (Table S1, S2) in reference to previous studies using MiFish primer (Sato et al. 2017, Fujii et al. 2019). Before statistical analysis, we confirmed that the sequencing depth was sufficient to detect alpha diversity in the samples by “iNEXT” and “ggiNEXT” functions in “iNEXT” package (Fig. S3). We merged the community data from two points, the stream near the downstream end and the shore, to compare with visual/capturing surveys.

We tested the differences in fish richness of sites, segments, and rivers between both methods by general linear mixed model (GLMM) with “lmer” function in “lme4” package. In the GLMM models, the method was treated as a fixed effect, and the rivers and segments were treated as the random effects.
The differences in community compositions were visualized using nonmetric multidimensional scaling (NMDS) with 500 separate runs of real data. For NMDS, the community dissimilarity was calculated based on incidence-based Jaccard indices. We evaluated the differences in community structures between methods and sites using Permutational multivariate analysis of variance (PERMANOVA). For PERMANOVA, we used Jaccard similarity matrix and calculated the statistical values with 999 permutations. We used “metaMDS” and “adonis” functions in vegan package for NMDS ordination and PERMANOVA, respectively.

For the communities evaluated by both methods, indicator taxa analysis was performed to determine which taxa had significantly different frequencies between both methods. The analysis was performed using the “signassoc” function in the “indicspecies” package on the present/absence data. We used mode = 1 (group-based) and calculated the p-values with 999 permutations after Sidak’s correction of the multiple testing.

To compare community composition (i.e., beta diversity) focusing on nested and turnover structures between eDNA metabarcoding and visual/capturing survey, we calculated the pairwise indices for nestedness and turnover (Baselga 2010; Baselga 2012) using the “beta.pair” function in the “betapart” package. Standardized effective size (SES) of nestedness and turnover indices were calculated to show the degree of nestedness and turnover structure. The significance was defined by deviation from zero, the expectation of random assembly (a null model) estimated with 999 random sampling replicates. The SES was defined as: 
\[ \frac{\beta_{\text{obs}} - \beta_{\text{null}}}{\beta_{\text{sd}}} \]

where \( \beta_{\text{obs}} \) is the observed beta diversity, \( \beta_{\text{null}} \) is the mean of the null distribution of beta diversity, and \( \beta_{\text{sd}} \) is the standard deviation of the null distribution. SES values greater than zero indicate statistically stronger nestedness or turnover structure than expected under a random
model of community assembly, while negative values indicate weaker nestedness or
turnover than expected. The randomized community data were generated with
independent swap algorithm\(^{50}\) (Gotelli 2000) using “randomizeMatrix” function in
“picante” package. Then, to evaluate the fish community longitudinal nestedness and
turnover structure along with river flow (i.e., upstream to downstream) by each method,
a nestedness metric\(^{51}\) (NODF, Almeida-Neto et al., 2008) and pairwise indices were
calculated. First, to test whether the communities show significant longitudinal
nestedness, SES value of NODF and their SES were calculated with 999 randomizations
using “oecosimu” function in “vegan” package. Then, the SES of pairwise nestedness
and turnover were calculated for each sample pair within the river. Finally, we tested
the differences in SES of pairwise indices between the survey methods (eDNA
metabarcoding vs. visual/capturing survey) by general linear mixed model (GLMM)
with “lmer” function in “lme4” package. In the GLMM models, the SES of pairwise
indices was treated as a fixed effect, and the rivers-pairs and segment-pairs (i.e., three
pairs in each river) were treated as the random effects.

For the map graphics in Supplemental Materials, we used the stream and coast line data
from the National Land Information Division, Ministry of Land, Infrastructure,
Transport and Tourism of Japan (http://nlftp.mlit.go.jp/ksj/).

**Data availability**

All data of the MiSeq sequencing was shared in DRA (Accession number:
DRA008090), and the observed species data was shared in Table S2 in Supplemental
Materials.

**References**


   Taxonomic homogenization of woodland plant communities over 70 years. Proc.
31. Baselga, A. Multiplicative partition of true diversity yields independent alpha and
32. Wright, D. H. & Reeves, J. H. On the meaning and measurement of nestedness of
35. Baselga, A., Gómez-Rodríguez, C. & Lobo, J. M. Historical legacies in world
   amphibian diversity revealed by the turnover and nestedness components of beta
36. Kawanabe, H., Mizuno, N. & K. Hosoya (eds.) Freshwater Fishes of Japan Yama-
37. Nakabo, T. Fishes of Japan with pictorial keys to the species, 3rd ed. Tokai
38. Itakura H, et al. Environmental DNA analysis reveals the spatial distribution,
   abundance, and biomass of Japanese eels at the river-basin scale. Aquat.


https://www.R-project.org/.


**Acknowledgement**

MiSeq sequencing was conducted in the Department of Environmental Solution Technology, Faculty of Science and Technology, Ryukoku University, and we thank to H. Yamanaka and H. Sato for supporting the experiments involving MiSeq sequencing.

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

**Author contributions.** HD, RI, and YA designed the study, RI, MG, TK, and YA contributed to field survey and sampling. SM, HD, RI, and MG contributed to molecular experiments. SM and HD analyzed the data and interpreted the results. HD, SM, and RI wrote the initial draft of the manuscript. All other authors critically reviewed the manuscript.
Competing financial interests. The authors declare no competing financial or non-financial interests.

Materials & Correspondence. H. D. (hideyuki.doi@icloud.com) and R. I. (inuiryutei@gmail.com)
Figure legends

Figure 1. Venn diagrams for the number of detected taxa. eDNA metabarcoding (eDNA, blue) and visual/capturing methods (V/C, red) in each site of the five study river systems. The numbers mean the number of taxa.

Figure 2. Nonmetric multidimensional scaling (NMDS) ordination. Fish communities evaluated by eDNA metabarcoding (blue) and visual/capturing methods (red) in each site of the river. MDS stress was 0.158.

Figure 3. Pairwise effect size (SES, standard effect size). Comparison between community nestedness and spatial turnover between the communities detected by eDNA metabarcoding and visual/capturing methods. Error bars indicate 95% confidence interval. The horizontal dotted line represents SES = 0, indicating non-significant effect.

Figure 4. Effect size (SES, standard effect size). a) community nestedness and b) spatial turnover SES among the segment communities evaluated by eDNA metabarcoding and visual/capturing methods. The error bars indicate 95% confidence interval. The horizontal dotted line represents SES = 0, indicating non-significant effect.
Figure 1
Figure 2
Figure 3

- Nestedness
- Turnover

Pairwise standardized effect size (pSES)
Figure 4

(a) community nestedness

(b) spatial turnover