Vol. 688: 99–112, 2022
https://doi.org/10.3354/meps13994

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published April 28

Small-scale heterogeneity of fish diversity evaluated by environmental DNA analysis in eelgrass beds

Kyosuke Momota[1,2,*], Shinya Hosokawa[2], Takashi Komuro[2]

[1]Central Laboratory, Marine Ecology Research Institute, Iwawada, Onjuku, Isumi, Chiba 299-5105, Japan
[2]Port and Airport Research Institute, Nagase, Yokosuka, Kanagawa 239-0826, Japan

ABSTRACT: Seagrass beds are a target for conservation worldwide and are frequently the focus of bioassessment and biomonitoring surveys. However, these surveys often employ destructive methods and involve considerable effort and cost. Recently, environmental DNA (eDNA) techniques have been attracting attention as a low-impact alternative for evaluating species diversity. However, the relationship between the detection ability of eDNA metabarcoding and the ecology of the organisms from which eDNA is derived has not been explicitly investigated. In this study, we examined this relationship for fishes in 2 eelgrass *Zostera marina* beds in temperate Japan, with a focus on 2 ecological characteristics (swimming position and appearance frequency in seagrass beds). We used eDNA metabarcoding to identify fish DNA collected from seawater samples and compared species inventories between 2 sampling positions (within vs. above eelgrass meadows) and 2 survey methods (eDNA vs. sledge-net sampling). Our results show that eDNA metabarcoding is much more effective than sledge-net sampling in detecting fish species, and that the detection ability of eDNA metabarcoding differs with water-sampling position. In particular, the relationship between fish ecology and survey detection ability appears to differ between the 2 eelgrass beds. Our results indicate that prior consideration of the spatial structure and fish communities of eelgrass beds is necessary for a reliable estimation of fish diversity in vegetated marine habitats.

KEY WORDS: eDNA · Metabarcoding · Biodiversity · Seagrass bed · Fish communities · Bioassessment · Biomonitoring · Water sampling

1. INTRODUCTION

Marine plants (e.g. seagrasses, algae, and mangroves) form communities that provide habitats for a variety of organisms in coastal marine zones. Seagrass beds are a typical example of a vegetated marine habitat that features high biodiversity and productivity (Duffy 2006, Short et al. 2007). However, seagrass bed habitats are declining globally due to climate and human-induced changes (Waycott et al. 2009, Duffy et al. 2019). Assessment and monitoring surveys are being used to help manage the remaining habitat. Generally, direct survey methods (e.g. diving, underwater cameras, autonomous underwater vehicles, sledge nets, and trawls) are used to evaluate aquatic biological communities. However, these survey methods are not always environmentally friendly and often entail considerable effort and cost (Wheeler et al. 2004, Thomsen & Willerslev 2015). Therefore, cheaper and less destructive survey methods are needed.

Environmental DNA (eDNA; Taberlet et al. 2012) — DNA collected from the environment (e.g. water, sediment, and soil) — has recently become a popular alternative tool for identifying species present and evaluating species diversity in aquatic ecosystems

*Corresponding author: kyo.momota@gmail.com

Publisher: Inter-Research · www.int-res.com

(Miya et al. 2020). Survey methods using eDNA may involve less effort, cost, and have a lower impact on the environment than traditional direct survey methods. In particular, species diversity is evaluated by using eDNA metabarcoding, a method that can be used to detect multiple species by amplifying an appropriately variable DNA barcoding region from a bulk environmental sample (Valentini et al. 2016, Stat et al. 2017). eDNA-derived data can be used to identify species and capture changes in species composition over time with higher resolution than direct survey methods; thus, eDNA metabarcoding is potentially a powerful tool in the management and conservation of marine vegetated habitats, including seagrass beds (Foster et al. 2020, Oka et al. 2021). In terms of spatial resolution, eDNA is accurate enough to distinguish among species compositions in coastal marine habitats separated by distances of several 10s of meters to several kilometers (Port et al. 2016, Jeunen et al. 2019, Nguyen et al. 2020). Although previous studies in seagrass beds have demonstrated the effectiveness of this technique for species detection (e.g. Cowart et al. 2015, Lobo et al. 2017), further examination of the unknown dynamics of eDNA and variation in the detection ability of eDNA metabarcoding is still needed to expand its applicability.

In seagrass beds, seagrass shoots (i.e. leaf blades, sheaths, and stems) may affect how eDNA is distributed through modification of various physical and chemical properties of the water column (Gambi et al. 1990, Robbins & Bell 1994, Abdelrhman 2003). The spatial and temporal distribution of eDNA in water can be influenced by its dynamics (e.g. advection, diffusion and degradation) (Kelly et al. 2018, Pont et al. 2018, Andruszkiewicz et al. 2019, Harrison et al. 2019). Specifically, distribution of eDNA within a seagrass meadow (i.e. at the same level as the seagrass shoots) could differ from distribution in the water above it (i.e. in the open water column). Additionally, the relationship between habitat structure (e.g. vegetation density and shape) and the ecology of target species (e.g. fish and invertebrates) may also be important. Specifically, the results of eDNA metabarcoding could be affected by the relationship between the sampling location and the location of target species. Many seagrass-associated species prefer specific micro-habitats within seagrass beds (Connolly 1994, Horinouchi 2007, Momota & Nakaoka 2017), each of which could have distinct effects on the dynamics of eDNA distribution. At the inter-habitat scale, eDNA detection patterns may differ among habitats, depending on the habitat preferences of target species or taxa (Jeunen et al. 2019), suggesting that results can be affected by species ecology even at small spatial scales. However, the magnitude and nature of any such effects have not been adequately investigated in vegetated marine habitats.

In this paper, we term the eDNA obtained from within a meadow as '$eDNA_{in}$' and from above a meadow as '$eDNA_{out}$'. We conducted eDNA metabarcoding of both $eDNA_{in}$ and $eDNA_{out}$ and sledge-net sampling in eelgrass *Zostera marina* beds in Kasado and Kurihama Bays in temperate Japan. We then compared the species inventories obtained with each method to examine any differences in species detectability (i.e. the detection ability of the method for a given species) among methods and to identify the relationship between detectability and the ecology (i.e. swimming position and appearance frequency in seagrass beds) of detected fish species. We tested 2 predictions: (1) fish inventories will differ between eDNA and sledge-net sampling regardless of sampling position (i.e. we compared sledge-net sampling to both $eDNA_{in}$ and $eDNA_{out}$ analysis), and (2) different sampling positions will result in different fish inventories even within the same sampling method (i.e. we compared $eDNA_{in}$ analysis to $eDNA_{out}$ analysis). For Prediction 1, we expected that the fish species detected with sledge-net sampling would be most similar to those detected using $eDNA_{in}$ analysis because of the similar sampling depths of the 2 methods. For Prediction 2, we expected that analysis of $eDNA_{in}$ would be more likely to detect frequently occurring fish species in eelgrass beds and those with swimming positions near the seabed than $eDNA_{out}$. Additionally, we discuss whether any associations identified are location-specific by comparing results from the 2 bays.

2. MATERIALS AND METHODS

2.1. Study site

We conducted seawater sampling (for eDNA metabarcoding) and fish sampling (for sledge-net surveys) in eelgrass beds located in Kasado Bay and Kurihama Bay in temperate Japan (Fig. 1). We sampled from one eelgrass bed from each bay to determine whether our results are location-specific. Surveys were conducted during the summer, when eelgrass ecosystems are stable and most productive. Sampling was carried out when the tide was high enough (water depth ca. 2 m) to allow for comparison of fish eDNA inventories within and above eelgrass meadows. Suitable sampling areas (i.e. without

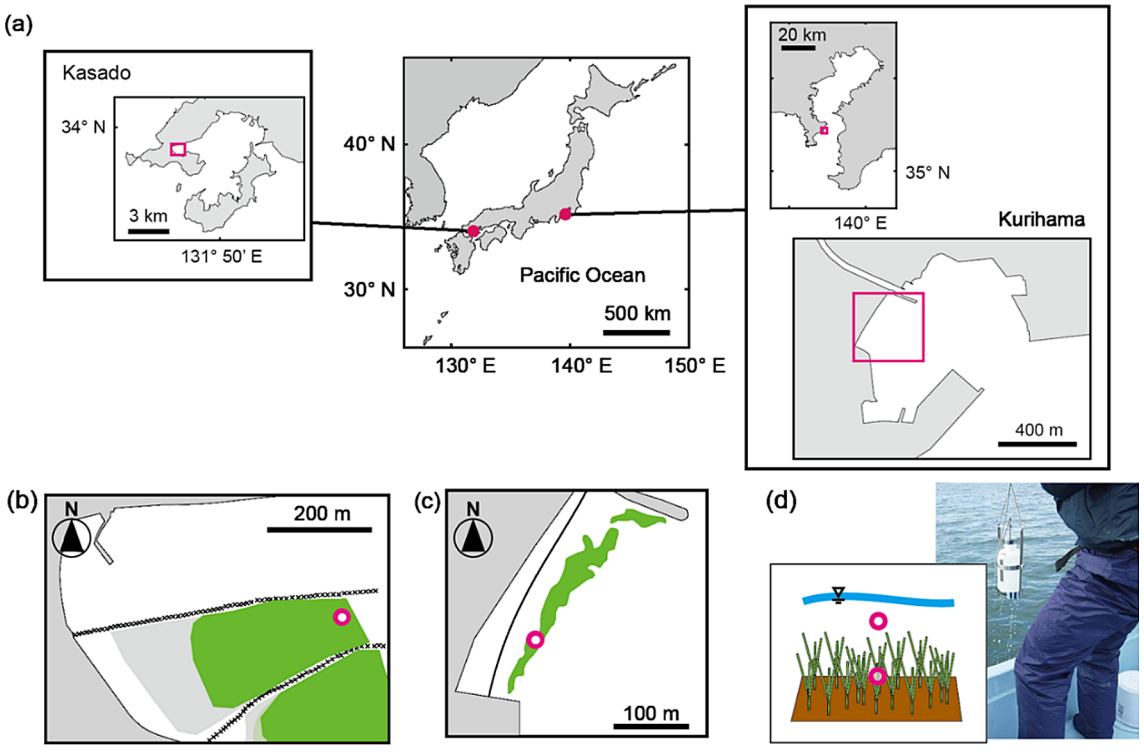

Fig. 1. (a) Locations of study sites (red rectangles) in Kasado Bay and Kurihama Bay, Japan. Detailed maps of the study sites are shown in (b) for Kasado Bay and (c) for Kurihama Bay. Green shaded regions indicate eelgrass beds. Circles indicate sampling zones. (d) Schematic of seawater sampling design (circles indicate sampling positions)

patches, gaps, or seafloor structures) were identified by SCUBA divers several days before sampling started.

Kasado Bay is located in the Seto Inland Sea (Fig. 1a,b). We established our sampling area in an eelgrass bed off the Oshima artificial tidal flats (Table 1). These artificial tidal flats were constructed with dredged soil (approx. 1.2 million m^3) to create a habitat for the manila clam *Ruditapes philippinarum* and are separated by submerged masonry breakwaters. We collected all samples in an area with a mean depth of 2.5 m. The bottom sediment in the target area is primarily sandy, because a sea-sand layer was layered over the dredged-soil layer during the construction of the tidal flats.

Table 1. Environmental conditions in eelgrass beds

Factor	Kasado Bay	Kurihama Bay
Mean water depth (m)	2.5[a]	1.5[a]
Water temperature range (°C)	23.5–23.7[a]	21–25[b,c]
Salinity range	30.3–30.7[a]	29–33[d]
Eelgrass density range (shoots m^{-2})	84–140[a]	144–160[a]

[a]This study; [b]Hosokawa et al. (2015); [c]Hosokawa et al. (2016); [d]Tokoro et al. (2014)

Kurihama Bay is located near the mouth of Tokyo Bay (Fig. 1a,c). The mouth of Kurihama Bay is partially obstructed by a thermal power plant and breakwaters. Perennial eelgrass has been colonizing the bay since 2000 and currently forms a banded community in the 0.4–1.6 m depth range along the shoreline (Hosokawa et al. 2015, S. Hosokawa & K. Momota pers. obs. 2019). Patchy (i.e. low shoot density) beds are more prevalent near the mouth of the Hirasaku River, which enters the bay. We collected all of our samples in a high shoot-density area opposite the river mouth at a depth of approximately 1.5 m (Table 1).

2.2. Sledge-net sampling

We sampled fish at intervals of several days within each eelgrass bed by using a sledge net (mouth size: 60 cm width × 40 cm height) to minimize stress on the sampled ecosystems. Sampling was conducted on 22, 26, and 30 July 2019 in Kasado Bay and

on 27 June and 2 and 9 July 2019 in Kurihama Bay, for a total of 3 samplings per bay. Net transects (50 m) were placed parallel to the shoreline within each eelgrass bed, such that the lines did not overlap with the seawater sampling zone or other sampling lines, to minimize the effects of disturbance across samplings. All fish samples were fixed in 10% seawater-formalin solution in the field. We then counted and identified the fish in the laboratory.

2.3. Seawater sampling

If sledge-net and seawater sampling occurred on the same day, we conducted the seawater sampling (which caused less environmental disturbance) prior to net sampling. We collected seawater samples for eDNA by using a 2l Heyroht water sampler (Miyamoto Riken) with an unused sterilized plastic bottle. A total of 24 samples were collected per site. Sampling was conducted in Kasado Bay on 26 July, 30 July, and 3 August and in Kurihama Bay on 2 July, 9 July, and 12 July. We collected 4 replicates within (0.5–0.7 m above the seafloor) and above (approx. 0.3 m below the water surface) the eelgrass meadow, for each sample (Fig. 1d). Sampling locations were at least 5 m apart to reduce the effects of our approach by boat.

We conducted seawater sampling and eDNA analysis based on the water-sampling protocol described in (Minamoto et al. 2021) and Miya et al. (2015). To avoid cross-contamination, we used new sterilized latex gloves for each sample collection. After sample collection, we immediately added 1 ml of 10% benzalkonium chloride solution per 1 l of sample volume to delay eDNA decay. We also transported the sample bottles in cooler boxes to prevent eDNA decay due to direct sunlight and high temperature. All sampling equipment was washed with a 10% household bleach solution before sample collection to prevent contamination by residual DNA. We transported sample bottles filled with Milli-Q water (field blanks) together with seawater samples to monitor for contamination during transport to the laboratory. Two field blanks were prepared for each sampling date (i.e. 12 blanks in total).

2.3.1. DNA extraction and PCR amplification

We concentrated biometabolites by filtering 2 l seawater samples through a 0.45 µm Sterivex cartridge filter unit (Millipore SVHV010RS, Merck). We then added 400 µl of Buffer AL (Qiagen) and 40 µl of proteinase-K to the Sterivex filter cartridge, which was then rotated on a mini-rotator in a fan oven at 10 rpm and heated at 56°C for 30 min for proteolysis. We collected the extracted DNA in a 2.0 ml tube by inserting the inlet port of the Sterivex filter cartridge into the tube (contained in a 50 ml conical tube) and centrifuging at 4000 × *g* for 3 min. After adding 400 µl of 99.5% ethanol to each tube, the sample DNA was purified by using an automated DNA extraction system (QIAcube, Qiagen). DNA extraction was performed by using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. DNA was eluted with the elution buffer (100 µl) provided in the kit. The DNA products were frozen at −25°C until we were ready to conduct a 2-step tailed PCR consisting of first- and second-round PCR for paired-end library preparation (Miya et al. 2015). We prepared negative controls using Milli-Q water (filtration blanks) for each filtering day to monitor contamination during the filtration process.

For first-round PCR, we used a SimpliAmp thermal cycler (Thermo Fisher Scientific) to amplify regions of interest. The 12 µl reaction solution consisted of 6.0 µl of KAPA HiFi HotStart ready mix (KAPA Biosystems), 2.8 µl of primer mix, 1.2 µl of Milli-Q water, and 2.0 µl of template (extracted DNA). We used Milli-Q water as the template (non-template PCR blank) in place of extracted DNA to monitor contamination. The thermal cycle profile was as follows: 95°C for 3 min (initial DNA denaturation); 40 cycles of 98°C for 20 s, 65°C for 15 s, and 72°C for 15 s; and 72°C for 5 min (final extension). For all blanks, the number of cycles was shortened to 35 cycles to avoid false positives. The primer mix was made by mixing multiple MiFish primers (Table S1 in the Supplement at www.int-res.com/articles/suppl/m688p099_supp.pdf; Minamoto et al. 2021). MiFish primers were chosen because of their superior detection ability, reproducibility, and estimation efficiency (Collins et al. 2019, Miya et al. 2020). The mixing ratio was MiFish U-F/R:MiFish Ev2-F/R:MiFish U2-F/R = 2:1:1. We analyzed 8 replicates for each template to reduce PCR dropouts. Finally, the replicates were combined and sequenced together. After first-round PCR, target PCR products were purified by using Agencourt AMPure XP magnetic beads (Beckman Coulter) with a ratio of products to magnetic beads of 1:0.8. We then quantified the purified products (i.e. double-stranded DNA) by using a Quantus Fluorometer and a QuantiFluor ONE dsDNA System (Promega).

To prepare the templates for the second-round PCR, we diluted each sample down to 0.1 ng of product by using a portion of the purified product.

Samples with <0.1 ng of purified product DNA were used as templates for the second-round PCR without dilution. In the second-round PCR, we used the Nextera XT Index Kit (Illumina) to append dual-index sequences. The 12 µl reaction solution consisted of 6.0 µl of KAPA HiFi HotStart ready mix, 0.83 µl of XT index primer F/R (Illumina), 2.34 µl of Milli-Q water, and 2.0 µl of template. As in the first-round PCR, we used a SimpliAmp thermal cycler with the following thermal cycle profile: 95°C for 3 min (initial DNA denaturation); 12 cycles of 98°C for 20 s and 72°C for 15 s; and 72°C for 5 min (final extension).

2.3.2. DNA sequencing and bioinformatics

The indexed second-round PCR products were pooled in equal volumes, and 22.5 µl of the pooled libraries mixed with 2.5 µl of 10× buffer was subjected to agarose gel electrophoresis using E-Gel SizeSelect II 2% (Thermo Fisher Scientific) to excise the target size of the libraries (ca. 370 bp). We measured the concentration of the size-selected libraries using a Quantus Fluorometer and a QuantiFluor ONE dsDNA System. The double-stranded DNA concentration of the pooled library was adjusted to 1 nM (assuming 1 bp equals 660 g mol^{-1}) using Milli-Q water. To improve the data quality of low-diversity samples such as single-PCR amplicons, we prepared 10 µl of the 1 nM library by adding 0.5 µl of PhiX DNA spike-in control (1 nM) to 9.5 µl of the 1 nM library. Double-stranded DNA in the 1 nM library was denatured with 10 µl of fresh 0.2 N NaOH. After 5 min, we neutralized it with 10 µl of 200 nM Tris-HCL. Finally, we prepared an 11 pM final library by adding 870 µl of HT1 buffer to the denatured library (10 µl; 2 nM). We sequenced 600 µl of the final library on the Miseq platform (Illumina) by inputting it into the MiSeq Reagent Kit v2 (2 × 150 bp PE; Illumina).

We performed all data preprocessing and analysis of the MiSeq raw reads by using USEARCH v10.0.240 (Edgar 2010) as follows (Takeuchi et al. 2019). (1) Forward and reverse reads were merged using the *fastq_mergepairs* command. Low-quality reads below a *Phred* score threshold of 20 and paired reads with >5 base differences in the aligned region (ca. 70 bp) were discarded in this process. (2) Primer sequences were removed from the merged reads by using the *fastx_truncate* command. (3) Reads with the primer sequences removed underwent quality filtering using the *fastq_filter* command to remove low-quality reads with an expected error rate of >1% and reads of <50 bp. (4) The preprocessed reads were dereplicated using the *fastx_uniques* command, and singletons, doubletons, and tripletons were removed from subsequent analysis as recommended by the developer. (5) Noise sequences, chimeric sequences, and sequences presumed to be errors were removed from the depleted reads using the *unoise3* command to generate amplicon sequence variants. These sequences were also separated from subsequent operational taxonomic unit assignments. (6) Species names were assigned to operational taxonomic unit clusters with sequences having >98.5% homology (2 nucleotide differences allowed) by using the *usearch_global* command. For species name assignment, we used MitoFish (http://mitofish.aori.u-tokyo.ac.jp/; Sato et al. 2018) and 12s rRNA regions of fishes extracted from the NCBI non-redundant nucleotide database.

2.4. Data analysis

We categorized the fish species inventories into 3 sampling types: $eDNA_{in}$, $eDNA_{out}$ and sledge-net sampling. Although eDNA metabarcoding (based on 2 l seawater samples) and sledge-net sampling (used in 50 m net transects) have different targets and spatiotemporal scales, we compared inventories obtained using these 2 methods under the assumption that the methods are based on equivalent sampling effort for a single sample in the field. All statistical and graphical analyses were performed using R v.4.0.2 (R Development Core Team 2020). In our analysis, we assumed that false positives arising from cross-contamination or tag-jumping (Schnell et al. 2015) could have a critical impact on fish species inventories. Additionally, we removed all unexpected species (e.g. freshwater and deep-sea fish) prior to analysis.

2.4.1. Univariate analyses of fish species detectability

To compare detection ability among the 3 sampling types, we compared the number of detected fish species by fitting generalized linear models (GLMs) with Poisson distributions. First, we used sampling type as an explanatory factor in the GLMs. To determine if the explanatory factor was significant, we compared the model to a null model by using likelihood-ratio chi-square tests (Type II χ^2 tests) implemented in the *Anova* function in the *car* package (Fox et al. 2020). Type II χ^2 tests can eliminate artifacts stemming from the order of explanatory factors in models with un-

balanced designs. In cases where the effect of sampling type was significant, we used the *emmeans* function in the *emmeans* package (Lenth et al. 2020) to perform pairwise (post-hoc) tests to identify which pairs of sampling types had significantly different detection ability. We used the false discovery rate method (Benjamini & Hochberg 1995) to control for Type I error in post-hoc tests.

Moreover, we examined differences in detection ability among sampling dates for $eDNA_{in}$ and $eDNA_{out}$ in terms of data reproducibility (variability) during the study period. Following the same analytical procedure used to compare sampling types (described above), we compared the number of species detected among sampling dates by using the GLMs with sampling date as an explanatory factor.

2.4.2. Multivariate analyses of fish species inventory

Fish species inventories were compared by multivariate analysis based on Sørensen similarity. The Sørensen similarity index takes values between 0 and 1, with a value closer to 1 indicating a higher degree of similarity between the 2 inventories. We analyzed the factors underlying any differences among species inventories by performing a permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) by using the *adonis* function in the *vegan* package (Oksanen et al. 2020). We used sampling type as an explanatory factor. We also examined differences in detection ability among sampling dates for both $eDNA_{in}$ and $eDNA_{out}$ during the study period by using sampling date as an explanatory factor. If a significant effect of sampling type was detected in the PERMANOVAs, we performed pairwise (post-hoc) tests (pairwise PERMANOVA) to identify which pairs of inventories were significantly different by using the *pairwise.perm.manova* function in the *RVAideMemoire* package (Hervé 2020). To confirm the homogeneity of the multivariate dispersions between all pairs of the groups (sampling type or date), we performed permutation tests for multivariate homogeneity of dispersions (PERMDISP) using the *betadisper* function with the group centroid in the *vegan* package (Oksanen et al. 2020). Additionally, we performed nonmetric multidimensional scaling (nMDS; Clarke 1993) by using the *metaMDS* function in the *vegan* package to visually compare inventories (Oksanen et al. 2020). The goodness-of-fit of nMDS ordination plots was evaluated by using stress values; ordination plots were accepted if stress values were <0.2 (Clarke 1993). We used 9999 permutations for PERMANOVA, pairwise PERMANOVA, nMDS ordination, and permutational dispersion tests.

Furthermore, we explored how the detectability of fish DNA is affected by interactions between seawater sampling position and the ecology of detected fish by performing a fourth-corner analysis test on data for seawater sampling position (R matrix), species presence/absence (L matrix), and ecological characteristics (Q matrix) (Brown et al. 2014). We used the *anova.traitglm* function in the *mvabund* package (Wang et al. 2020) to perform a log-likelihood ratio statistic test for the R–Q interaction by fitting multivariate GLMs (multi-GLMs) with a binomial distribution using 9999 iterations via PIT-trap block resampling (Warton et al. 2017). We then fitted the multi-GLMs with a least absolute shrinkage and selection operator (LASSO) penalty to estimate standardized coefficients for the relationships between fish ecological characteristics and sampling positions by using the *traitglm* function in the *mvabund* package. The LASSO penalty is a method that sets model terms to zero if they do not reduce the Bayesian information criterion, thereby selecting multi-GLMs with relatively high prediction accuracy and interpretability (Wang et al. 2012). The ecological characteristics we examined were swimming position (bottom or surface swimmer) and appearance frequency in seagrass beds (year-round, seasonal, transient, or casual) (Tables 2 & S2). We defined bottom swimmers (including midwater swimmers and bottom-dwellers) as fish species whose primary swimming position is within eelgrass meadows or on the seafloor. We predicted that swimming position and seawater sampling position would be positively related, and that fish species with high appearance frequency were more likely to be detected. To identify ecological characteristics, we used online databases (Fish Base: www.fishbase.org; World Register of Marine Species: www.marinespecies.org) and the relevant literature (Table S2).

3. RESULTS

3.1. Kasado Bay

A total of 72 fish species were detected (Table S2) in Kasado Bay. A total of 70 species were detected by eDNA metabarcoding, with 52 detected in the 24 samples obtained from $eDNA_{in}$ and 52 in 24 samples from $eDNA_{out}$. No DNA contamination was observed as a result of transport or during analysis. A total of 8 species were collected during 3 separate dates of

Table 2. Fish species ecological categories and description

Ecological characteristic	Description
Swimming position	
Bottom swimmer	Primary swimming position is within eelgrass meadows or on the seafloor
Surface swimmer	Primary swimming position is near the surface
Appearance frequency	
Year-round resident	Inhabits seagrass beds perennially
Seasonal resident	Inhabits seagrass beds during specific times or growth stages, but not perennially
Transient species	Frequently inhabits seagrass beds. Includes species that do not fit the descriptions of the above 2 categories but which have been captured more than once in seagrass beds
Casual species	Found incidentally in seagrass beds. Includes species with few capture records and different preferred habitats

sledge-net sampling (Fig. 2a). A total of 34 species were shared among the $eDNA_{in}$ and $eDNA_{out}$ inventories. Two fish species collected by sledge-net sampling (rockfish *Sebastes inermis*, crowned seahorse *Hippocampus coronatus*) were not detected by eDNA metabarcoding. Only 4 fish species (striped sandgoby *Acentrogobius pflaumii*, perch sculpin *Pseudoblennius cottoides*, white-spotted pygmy filefish *Rudarius ercodes*, rockfish *Sebastes cheni*) were shared among the $eDNA_{in}$, $eDNA_{out}$, and sledge-net inventories (Fig. 2a).

Throughout the study period, we detected a mean of 22.1 fish species from each $eDNA_{in}$ sample, 17.9 fish species from each $eDNA_{out}$ sample, and 5.0 fish species from each sledge-net sample (Fig. 3a). Sampling type had a significant effect on the number of species detected among the 3 sampling types (Type II χ^2 tests: $\chi^2 = 49.770$, df = 2, $p < 0.0001$). Pairwise tests showed significant differences between all pairs of the 3 sampling types (Fig. 3a).

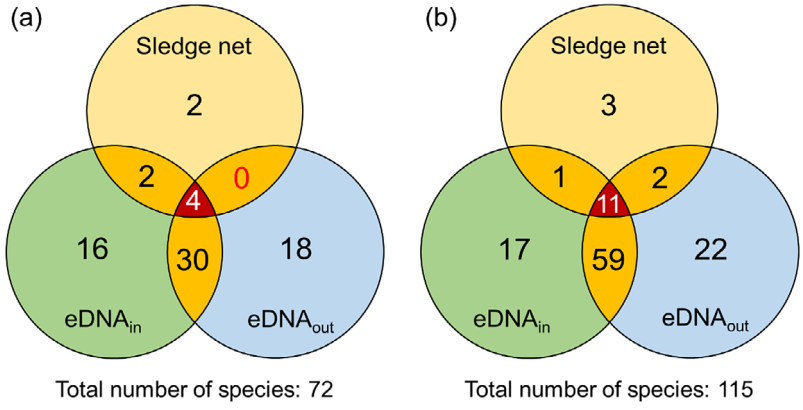

Fig. 2. Number of species detected by and shared among sampling types at (a) Kasado Bay and (b) Kurihama Bay. $eDNA_{in}$ indicates eDNA metabarcoding of water samples collected from within eelgrass beds (i.e. at the level of the shoots) and $eDNA_{out}$ indicates eDNA metabarcoding of water samples collected from above eelgrass beds (i.e. in the open water column)

Significant differences in the number of species among sampling dates were not detected for $eDNA_{in}$ ($\chi^2 = 4.945$, df = 2, p = 0.084), but were detected for $eDNA_{out}$ ($\chi^2 = 10.756$, df = 2, p = 0.005) (Fig. S1). However, pairwise tests for $eDNA_{out}$ only revealed a significant difference between the first and third sampling dates (p = 0.005).

Fish species inventories obtained with eDNA metabarcoding were clearly different from those obtained with sledge-net sampling (Fig. 4a), and the effect of sampling type was significant (PERMANOVA: $F_{2,24} = 14.776$, $R^2 = 0.552$, $p < 0.0001$). Pairwise PERMANOVAs for sampling type showed significant differences between all pairs of the 3 sampling types ($eDNA_{in}$–$eDNA_{out}$: p = 0.0003; $eDNA_{in}$–sledge-net sampling: p = 0.002; $eDNA_{out}$–sledge-net sampling: p = 0.002). More specifically, mean (±SD) Sørensen similarities among the sampling types were 0.482 (0.101) between $eDNA_{in}$ and $eDNA_{out}$, 0.168 (0.055) between $eDNA_{in}$ and sledge-net sampling, and 0.085 (0.075) between $eDNA_{out}$ and sledge-net sampling. On the other hand, significant differences in the multivariate homogeneity of dispersions were detected between $eDNA_{in}$ and $eDNA_{out}$ ($F_{1,22} = 14.768$, p = 0.001), but not between $eDNA_{in}$ and sledge-net sampling ($F_{1,13} = 1.391$, p = 0.260) or $eDNA_{out}$ and sledge-net sampling ($F_{1,13} = 0.572$, p = 0.449). This indicates that the inventories differ between eDNA metabarcoding and sledge-net sampling, but do not differ between $eDNA_{in}$ and $eDNA_{out}$ because of the different dispersions. Across the sampling dates, mean (±SD) similarities were 0.668 (0.077) for $eDNA_{in}$ and 0.558 (0.096) for $eDNA_{out}$.

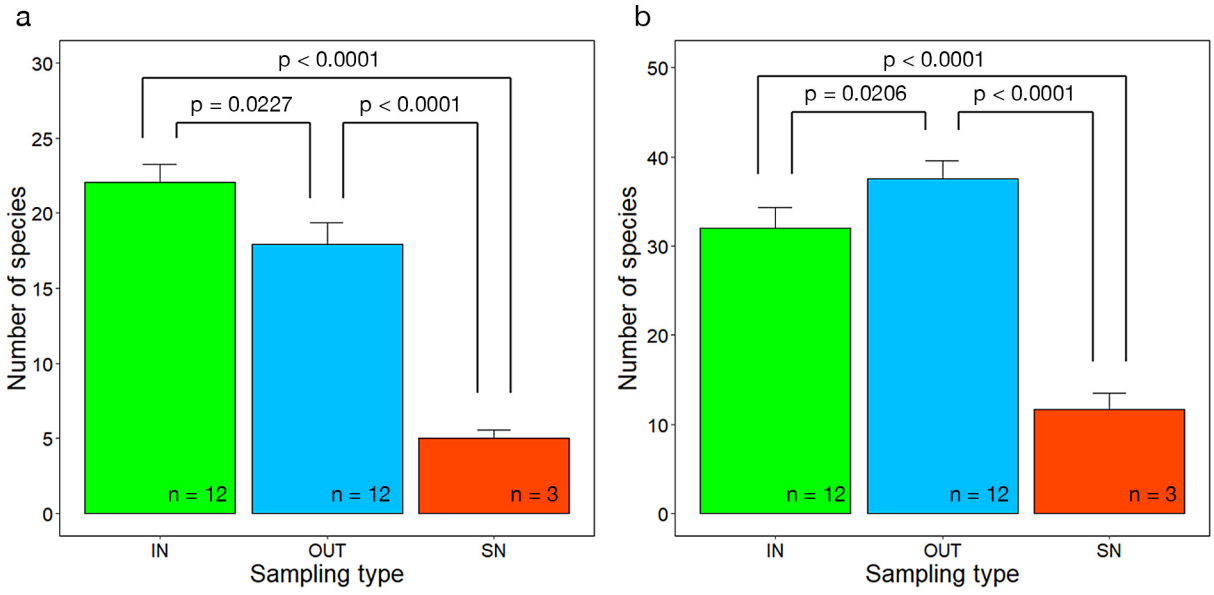

Fig. 3. Mean number of detected fish species for each sampling type across sampling dates at (a) Kasado Bay and (b) Kurihama Bay. Error bars are SE; p-values indicate the results of pairwise (post-hoc) tests. IN: $eDNA_{in}$; OUT: $eDNA_{out}$; SN: sledge net

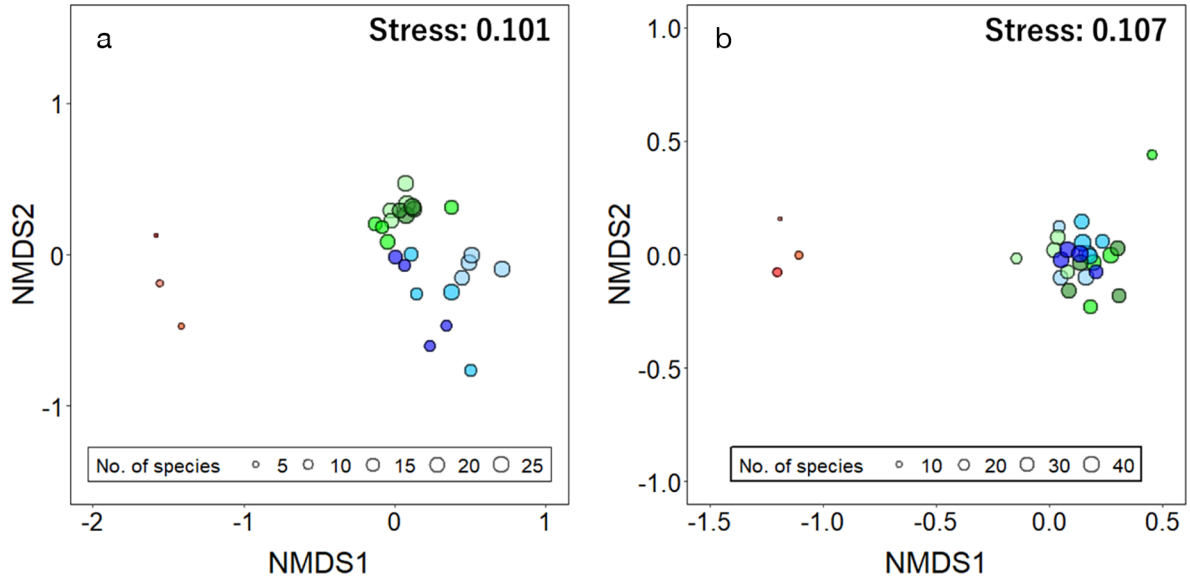

Fig. 4. Non-metric multidimensional scaling ordination plots based on Sørensen dissimilarity matrices of fish species inventories obtained from sledge-net sampling and eDNA metabarcoding at (a) Kasado Bay and (b) Kurihama Bay. Red: sledge-net sampling; blue: eDNA sampled $eDNA_{out}$; green: $eDNA_{in}$. Paler colors indicate samples collected earlier in the study

Significant differences in the eDNA metabarcoding inventories across sampling dates were detected for both $eDNA_{in}$ ($F_{2,9}$ = 1.884, R^2 = 0.295, p = 0.013) and $eDNA_{out}$ ($F_{2,9}$ = 2.424, R^2 = 0.350, p = 0.011). Significant differences in multivariate homogeneity of variance were not detected for $eDNA_{in}$ ($F_{2,9}$ = 3.483, p = 0.076), but were detected for $eDNA_{out}$ ($F_{2,9}$ = 7.263, p = 0.013). Thus, the differences in species inventory across sampling dates detected for $eDNA_{out}$ likely reflect in heterogeneity of variance rather than actual differences in inventories. Although there appeared to be statistically significant differences in $eDNA_{in}$ inventories among the sampling dates, pairwise comparisons of the sampling dates revealed no significant differences, probably because the contribution of sampling date to the overall variation was low (R^2 = 0.295). For $eDNA_{in}$, the mean (±SD) similarity between pairs of sampling dates was 0.662 (0.069) for the first and second sampling dates, 0.628 (0.058) for the second and third sampling dates, and 0.724

(0.039) for the first and third sampling dates. For $eDNA_{out}$, the values were 0.528 (0.057) for the first and second sampling dates, 0.588 (0.113) for the second and third sampling dates, and 0.502 (0.102) for the first and third sampling dates.

The interaction of water sampling position and fish ecological characteristics had a significant effect on detection ability (deviance = 84.48, $p < 0.0001$). For $eDNA_{in}$, bottom swimmers and seasonal species were more easily detected than infrequently occurring species (i.e. transient and casual species) (Fig. 5a). Conversely, for $eDNA_{out}$, infrequently occurring species were more easily detected. There were no significant differences in detection ability between $eDNA_{in}$ and $eDNA_{out}$ for surface swimmers and year-round species.

3.2. Kurihama Bay

A total of 115 fish species were detected in Kurihama Bay (Table S2). A total of 112 species were detected by eDNA metabarcoding, with 88 detected in the 24 samples obtained from $eDNA_{in}$ and 94 in the 24 samples from $eDNA_{out}$. Although the field

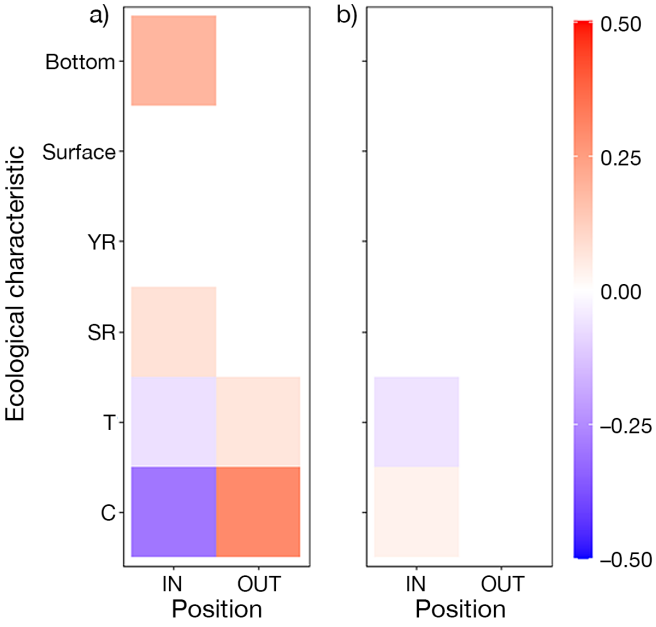

Fig. 5. Fourth-corner modelling results showing standardized coefficients for the relationships between fish ecological characteristics and water sampling positions in (a) Kasado Bay and (b) Kurihama Bay. Positive associations are shown in red and negative associations in blue. Brighter colors indicate stronger associations. Bottom: bottom swimmer; surface: surface swimmer; YR: year-round resident; SR: seasonal resident; T: transient species; C: casual species (see Table 2 for details)

blanks were contaminated by DNA from mullet *Mugil cephalus* and freshwater loach *Barbatula barbatula* during DNA analysis, we considered our inventory creation to be successful because we only detected an infinitesimal amount of *M. cephalus* DNA (4 reads), and because *B. barbatula* could be easily discarded, as its distribution does not overlap with our study area. A total of 17 species were collected during the 3 days of sledge-net sampling (Fig. 2b). A total of 70 species were shared between the $eDNA_{in}$ and $eDNA_{out}$ inventories. Three of the species collected by sledge-net sampling (black cardinalfish *Apogonichthyoides niger*, crowned seahorse *Hippocampus coronatus*, perch sculpin *Pseudoblennius cottoides*) were not detected by eDNA metabarcoding. Eleven fish species were shared among the $eDNA_{in}$, $eDNA_{out}$, and sledge-net inventories (Fig. 2b).

We detected a mean of 32.0 fish species from each $eDNA_{in}$ sample, 37.6 fish species from each $eDNA_{out}$ sample, and 11.7 fish species from each sledge-net sample throughout the study period (Fig. 3b). Sampling type had a significant effect on the number of species detected (Type II χ^2 tests: $\chi^2 = 62.392$, df = 2, $p < 0.0001$). Pairwise tests showed significant differences between all pairs of the 3 sampling types (Fig. 3b).

No significant differences among the sampling dates for the number of species detected were found for $eDNA_{in}$ ($\chi^2 = 0.975$, df = 2, $p = 0.614$) or $eDNA_{out}$ ($\chi^2 = 0.554$, df = 2, $p = 0.758$) (see Fig. S1b).

As in Kasado Bay, there were clear differences between fish species inventories obtained from eDNA metabarcoding and sledge-net sampling (Fig. 4b). The effect of sampling type was significant (PERMANOVA: $F_{2,24} = 7.712$, $R^2 = 0.391$, $p < 0.0001$). Pairwise tests for sampling type only showed significant differences between pairs that compared eDNA metabarcoding against sledge-net sampling ($eDNA_{in}$–$eDNA_{out}$: $p = 0.558$; $eDNA_{in}$–sledge-net sampling: $p = 0.032$; $eDNA_{out}$–sledge-net sampling: $p = 0.032$). Mean (±SD) similarities among the sampling dates were 0.589 (0.079) between $eDNA_{in}$ and $eDNA_{out}$, 0.232 (0.069) between $eDNA_{in}$ and sledge-net sampling, and 0.239 (0.035) between $eDNA_{out}$ and sledge-net sampling. Significant differences in multivariate homogeneity of dispersions were detected between $eDNA_{in}$ and $eDNA_{out}$ ($F_{1,22} = 5.545$, $p = 0.024$), but not detected between $eDNA_{in}$ and sledge-net sampling ($F_{1,13} = 3.377$, $p = 0.088$) and $eDNA_{out}$ and sledge-net sampling ($F_{1,13} = 1.038$, $p = 0.326$). These results indicate that the inventories obtained from $eDNA_{in}$ are not significantly different from those obtained from $eDNA_{out}$, but those obtained

from both types of eDNA are different from those obtained from sledge-net sampling. Across the sampling dates, mean similarities were 0.550 (0.091) for $eDNA_{in}$ and 0.629 (0.057) for $eDNA_{out}$.

Significant differences in the eDNA-based species inventories between sampling dates were detected for both $eDNA_{in}$ ($F_{2,9}$ = 1.937, R^2 = 0.301, p = 0.006) and $eDNA_{out}$ ($F_{2,9}$ = 1.659, R^2 = 0.269, p = 0.009). No significant differences in multivariate homogeneity of variance were detected for either $eDNA_{in}$ ($F_{2,9}$ = 1.758, p = 0.232) or $eDNA_{out}$ ($F_{2,9}$ = 0.904, p = 0.433). For both $eDNA_{in}$ and $eDNA_{out}$, pairwise tests showed significant differences between the first and second samplings ($eDNA_{in}$: p = 0.044, $eDNA_{out}$: p = 0.043) and between the first and third samplings ($eDNA_{in}$: p = 0.044, $eDNA_{out}$: p = 0.043), but not between the second and third samplings ($eDNA_{in}$: p = 0.506, $eDNA_{out}$: p = 0.255). For $eDNA_{in}$, the mean (±SD) similarity between pairs of sampling dates was 0.505 (0.097) for the first and second sampling dates, 0.568 (0.102) for the second and third sampling dates, and 0.540 (0.069) for the first and third sampling dates. For $eDNA_{out}$, the values were 0.607 (0.053) for the first and second sampling dates, 0.647 (0.067) for the second and third sampling dates, and 0.610 (0.049) for the first and third sampling dates.

The interaction between seawater sampling type (i.e. sampling position) and ecological characteristics did not have a significant effect on detection ability (deviance = 10.23, p = 0.111). In pairwise comparisons of sampling positions and ecological characteristics, the only associations detected were weak associations for $eDNA_{in}$ (Fig. 5b).

4. DISCUSSION

eDNA metabarcoding (both $eDNA_{in}$ and $eDNA_{out}$) had, on average, a 3-fold higher detection ability than sledge-net sampling for the 2 eelgrass beds, resulting in significantly different fish inventories, as expected in Prediction 1. Our results also show that the detection ability of eDNA metabarcoding depends on water sampling position, as expected in Prediction 2. The difference in the detected inventories between $eDNA_{in}$ and $eDNA_{out}$ was much smaller than the difference between eDNA metabarcoding and sledge-net sampling. Additionally, the relationship between the detection ability of eDNA metabarcoding and the ecology of the detected fish species differed between the 2 eelgrass beds (Fig. 5). These results indicate the importance of considering fish community structures (i.e. functional compositions) and the spatial structuring of eelgrass beds prior to eDNA sampling to enhance use efficiency.

4.1. Sledge-net sampling vs. eDNA metabarcoding

We confirmed, in both eelgrass beds, that eDNA metabarcoding was more effective than sledge-net sampling in detecting fish species, which is consistent with previous reports (Thomsen et al. 2012, Valentini et al. 2016, Yamamoto et al. 2017, Nguyen et al. 2020, Zou et al. 2020, Afzali et al. 2021). The species inventories obtained also differed according to method. Comparisons of other traditional sampling methods with eDNA metabarcoding have also demonstrated the superior detection ability of eDNA metabarcoding (Thomsen et al. 2012, Fujii et al. 2019). However, standard eDNA-metabarcoding-based methods for quantifying abundance, biomass, or life-history characteristics (e.g. size distributions) are still under development. Therefore, eDNA metabarcoding remains best suited for use as a complement to traditional methods, as has been previously proposed (Stat et al. 2019, Zou et al. 2020, Afzali et al. 2021, Antich et al. 2021). Nonetheless, the use of traditional methods should in future be considerably reduced to avoid unnecessary damage to seagrass beds during long-term monitoring. An alternative may be to replace some traditional surveys with eDNA analysis, e.g. by conducting a thorough initial survey with both traditional and eDNA methods and then switching to eDNA once sufficient data has been accumulated.

Although we expected to find more overlap between the species inventories obtained by sledge-net sampling and $eDNA_{in}$ than between those obtained by sledge-net sampling and $eDNA_{out}$, our results are not consistent with this expectation. Additionally, it is possible that abundant species in eelgrass beds are particularly easily detected by sledge-net sampling (Table S2). It is of concern that sledge-net sampling successfully detected fish species such as striped sandgoby *Acentrogobius pflaumii*, perch sculpins *Pseudoblennius* spp., white-spotted pygmy filefish *Rudarius ercodes*, and rockfish *Sebastes cheni* in the 2 seagrass beds, which are commonly found in temperate seagrass beds in Japan, whereas eDNA metabarcoding did not detect all these common species. Differences in species distributions in each area may explain differences in the detection of related

species with similar ecological characteristics (i.e. the perch sculpins *P. cottoides* vs. *P. percoides*, or the rockfishes *S. cheni* vs. *S. inermis*), yet the fact that the seagrass-associated seahorse *Hippocampus coronatus* was only detected from sledge-net samples could have broader implications for the use of eDNA metabarcoding in seagrass beds. In contrast to previous reports that have emphasized the high detection ability of eDNA metabarcoding, this suggests that certain species may be difficult to detect through eDNA metabarcoding. It is essential to identify more fish species that are similarly prone to being missed by eDNA methods in order to determine the best combination of sampling methods for each region.

4.2. $eDNA_{in}$ vs. $eDNA_{out}$

Fish inventories obtained with $eDNA_{in}$ were more similar to those obtained with $eDNA_{out}$ in Kurihama Bay than in Kasado Bay. The mean Sørensen similarity index between $eDNA_{in}$ and $eDNA_{out}$ was 0.482 in Kasado Bay, against 0.589 in Kurihama Bay. This indicates that eDNA distributions within the eelgrass meadow differed from those above the eelgrass meadow in Kasado Bay, and that the spatial gradient in eDNA distribution was small in Kurihama Bay.

The relationship between eDNA detection ability and the ecology of the detected fish species was unclear in Kurihama Bay because the water masses are sometimes indistinguishable. This is because the bay is so shallow that the upper parts of the eelgrass shoots emerge from the water during certain low tides in this bay. This likely reduces any spatial gradient in eDNA distribution and obscures the relationship between the detectability of eDNA and the ecological characteristics of the detected fish species.

In Kasado Bay, the water depth was always sufficient to keep the eelgrass submerged, and the relationship between eDNA detection ability and fish ecology was more apparent. This may indicate that the depth range used by each ecological group was distinct or that eDNA distributions were to some extent separated by depth zones. Notably, the results for bottom swimmers and transient and casual species (Fig. 5a) highlight that different fish behavioral characteristics can affect the detection ability of eDNA (Jeunen et al. 2019). For the transient and casual species, it is most likely that their DNA was carried into the eelgrass bed by tidal flow from other habitats (i.e. their primary habitats), as most of the detected species were bottom swimmers (Fig. S2, Table S2). The large difference in the detectability of these species between $eDNA_{in}$ and $eDNA_{out}$ is presumably attributable to differences in the amount of DNA present in each depth zone. Also, in Kasado Bay, any DNA carried in from other habitats may be less susceptible to physical processes that promote spatial homogenization such as sedimentation, trapping within eelgrass meadows, and vertical mixing. Such processes could be less active in this eelgrass bed with sufficient water depth, since flow and material transport were reduced within the meadows and enhanced in the water above them (Abdelrhman 2003).

In both Kasado Bay and Kurihama Bay, there was no significant difference between the sensitivity of $eDNA_{in}$ and $eDNA_{out}$ for seagrass-dependent species (i.e. year-round and seasonal residents) or surface swimmers. For species dependent on seagrass meadows, their DNA detectability is likely to be high because they are always widely distributed within eelgrass beds. Most surface swimmers were school-forming and migratory, and their numbers of reads were relatively high when they were detected (Table S2). Although the surface swimmers are typically less dependent on seagrass meadows (i.e. transient and casual species) because of their large numbers when schooling, large amounts of their DNA are likely released into eelgrass beds on any occasions when they are present. Therefore, their DNA may also be spatially widespread in eelgrass beds, although this will ultimately depend on their biomass and frequency of occurrence.

In both eelgrass beds, there was a difference of 5 species between the mean number of species detected with $eDNA_{in}$ and $eDNA_{out}$. However, the type of eDNA sampling that detected more species was different in the 2 eelgrass beds (Fig. 3). The fact that water samples were taken at high tide could help explain the higher detection ability of $eDNA_{out}$ in Kurihama Bay; water inflow and mixing from other habitats at high tide could have caused a temporary increase in the diversity of $eDNA_{out}$ in this habitat. More studies on the effects of water depth and tidal range on detection ability are needed in the future.

Both $eDNA_{in}$ and $eDNA_{out}$ provided highly reproducible results across both space and time in both eelgrass beds. The MiFish (fish-specific) primer used in this study is highly reliable for species identification and is known to provide high estimation efficiency and reproducibility (Collins et al. 2019). Nevertheless, it is important to optimize the number of replicates and the spatial distribution of sampling points to maximize detection ability and repro-

ducibility. Although the fish inventories obtained in this study were quite similar, a certain number of replicates was still needed to increase species coverage (Fig. S3), and the differences between Kasado Bay and Kurihama Bay should be considered when determining how to best target a given species, especially when using eDNA in intertidal and shallow subtidal seagrass beds. Differences in reproducibility over time (i.e. across different intervals between water sampling) should be investigated in future studies to examine the effects of factors such as weather and tides.

5. CONCLUSIONS

Our results demonstrate that adequate consideration of the environmental conditions surrounding seagrass vegetation and the ecology of target fish species can improve the detection ability of eDNA-based diversity estimates in seagrass beds. Our study also shows that eDNA metabarcoding can provide high spatial resolution even at very small spatial scales (i.e. the microhabitat scale); this finding agrees with previous reports that eDNA methods can distinguish among biological communities in different habitats (Port et al. 2016, Stat et al. 2019, Lafferty et al. 2021, Oka et al. 2021). Additionally, our results suggest that tidal changes affect eDNA signals at intra-habitat scales even within a spatially heterogeneous habitat, unlike what has been previously reported at inter-habitat scales (Kelly et al. 2018, Lafferty et al. 2021). Our findings can provide useful data for survey planning in other marine vegetated habitats with high microscale spatial heterogeneity (e.g. kelp forests, sargassum beds, and mangrove forests). In these habitats, eDNA methods often have a significant advantage over traditional survey methods, which can have problems with accessing the sites (Afzali et al. 2021). Although eDNA methods still face many technical issues, more empirical evaluations of their performance will support their application in various environments.

Acknowledgements. We are grateful to the Yokohama Research and Engineering Office for Port and Airport of the Kanto Regional Development Bureau, Ministry of Land, Infrastructure, Transport and Tourism, and the Hiroshima Research and Engineering Office for Port and Airport of the Chugoku Regional Development Bureau, Ministry of Land, Infrastructure, Transport, and Tourism for providing opportunities to conduct the field surveys. We thank S. Okura and the other members of the Marine Environmental Information Group of the Port and Airport Research Institute, Japan; Y. Mito at Fukken Co., Ltd., Japan; and T. Iwashita at Tokyo Kyuei Co., Ltd., Japan, for assistance in field surveys, and N. Shibata at Environmental Research & Solutions Co., Ltd. for assistance and advice in DNA analysis.

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Editorial responsibility: Rob Toonen, Kāneʻohe, Hawaiʻi
Reviewed by: 3 anonymous referees

Submitted: May 11, 2021
Accepted: January 6, 2022
Proofs received from author(s): April 6, 2022