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Short Communication

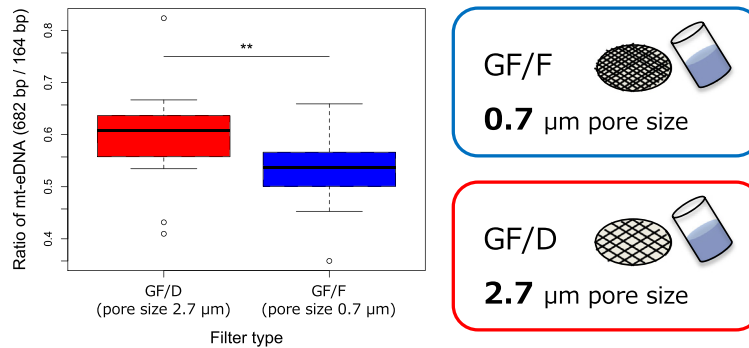
Selective collection of long fragments of environmental DNA using larger pore size filter

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HIGHLIGHTS

- Selective collection of eDNA at larger size fractions increased the ratio of long to short DNA fragments in water.
- The ratio of nuclear to mitochondrial eDNA concentrations differed depending on filter pore size.
- We show the potential to select ecological information from eDNA by focusing on eDNA of specific size and state.

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental DNA (eDNA) can exist in water with various sizes and states. Among them, relative to extra-cellular DNA, intra-cellular DNA such as cell and tissue fragments can mainly be detected at larger size fractions, and may be protected from enzymatic DNA degradation processes. Here, we verified the hypothesis that the selective collection of such large-sized eDNA enhances the efficiency of capturing less-degraded eDNA, based on a tank experiment using Japanese Jack Mackerel (*Trachurus japonicus*) as a model species. We concentrated different volumes of rearing water using the filters with different pore sizes (0.7 μm and 2.7 μm), and quantified the copy number of short and long mitochondrial and short nuclear DNA fragments of target species in water samples. As a result, the ratio of long to short eDNA concentrations was higher in the larger pore size filter, which would support our stated hypothesis. In addition, the ratio of nuclear to mitochondrial eDNA was lower in the larger pore size filter. These results imply a difference in the persistence of nuclear and mitochondrial DNA between intra- and extra-cellular environments. Moreover, larger filter pore size did not necessarily decrease the yields of eDNA, and there was little difference in yields in smaller filtration volumes. The findings of this study indicate the potential to select information from eDNA signals by focusing on eDNA of specific size and state, which may contribute to efficient utilization of the information on species taxonomy and physiology in water samples.

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1. Introduction

For the rapid and extensive detection of threatened rare and invasive species in the aquatic environment, environmental DNA (eDNA) analysis has recently been developed (Bohmann et al., 2014; Deiner et al., 2017a; Takahara et al., 2012). Environmental DNA is defined as the genetic materials in environment derived from mucus, feces, skin, scale, and gametes of organisms (Barnes and Turner, 2016). The detection of eDNA infers the presence of target species without capturing or observing them, and thus analyzing eDNA is less-invasive and more cost-effective than traditional methods (Darling and Mahon, 2011; Thomsen and Willerslev, 2015). Ever since the first inception of eDNA analysis (Ficetola et al., 2008), its applicability has been demonstrated for various taxa and environments (Bista et al., 2017; Carraro et al., 2018; Minamoto et al., 2012; Nichols and Marko, 2019; Thomsen et al., 2016; Ushio et al., 2017).

Some studies have previously reported that aqueous eDNA can be detected at various size fractions (<0.2 to >180 μm) (Jo et al., 2019a; Turner et al., 2014; Wilcox et al., 2015). This implies that eDNA can exist with various physical and physiological states in water (Barnes and Turner, 2016). With regards to macro-organisms' eDNA, not only cell and tissue fragments but also nuclei and mitochondria, and even extra-membrane nuclear and mitochondrial DNA can potentially be detected. Among them, cell and tissue fragments are likely to be detected as intra-cellular DNA and at larger size fractions than extra-cellular DNA, which in contrast would be detected at smaller size fractions (Jo et al., 2019a). In addition, owing to the presence of a cellular membrane, such large-sized and intra-cellular eDNA may be protected from various DNA degradation processes (e.g., enzymatic and mechanical fragmentation by microbes) compared with small-sized and extra-cellular eDNA (Nielsen et al., 2007; Torti et al., 2015). Deiner et al. (2017b) reported the successful sequencing of fish mitochondrial genomes (>16 kbp)

from water samples, which might have attributed to the presence of large-sized eDNA that was covered with the cellular membrane. Thus, we hypothesized that selective collection of large-sized eDNA can result in the effective collection of the less-degraded eDNA.

Here, we verified the aforementioned hypothesis by the filtration using filters with different pore sizes (described below). That is, water filtration with a larger pore size filter leads to the selective collection of the eDNA at larger size fractions. We collected water samples from a tank in which Japanese Jack Mackerels (*Trachurus japonicus*) were kept, and quantified the copy numbers of short and long mitochondrial DNA fragments in water samples. We expected that the filtration with a larger pore size filter would increase the relative yield of long DNA fragments (i.e., the ratio of long to short DNA fragments) from water samples. Moreover, we quantified the copy number of short nuclear DNA fragment in water. The findings of some previous studies have indicated that the persistence of nuclear eDNA in water could be lower than that of mitochondrial eDNA (Jo et al., 2019a, 2019b), and that the persistence of nuclear and mitochondrial DNA could differ between intra- and extra-cellular environments using tissue samples (Foran, 2006). Accordingly, it is conceivable that the yields of nuclear and mitochondrial eDNA and their ratios may differ depending on size fractions and filter pore sizes.

2. Materials and methods

2.1. Water sampling

We sampled seawater from a 500-L tank, in which around 30 individuals of Japanese Jack Mackerels were kept, at the Maizuru Fisheries Research Station (MFRS) of Kyoto University, Japan, in September 2019 (Fig. 1). We used this species because the primers/probe sets targeting its mitochondrial and nuclear DNA with different fragment

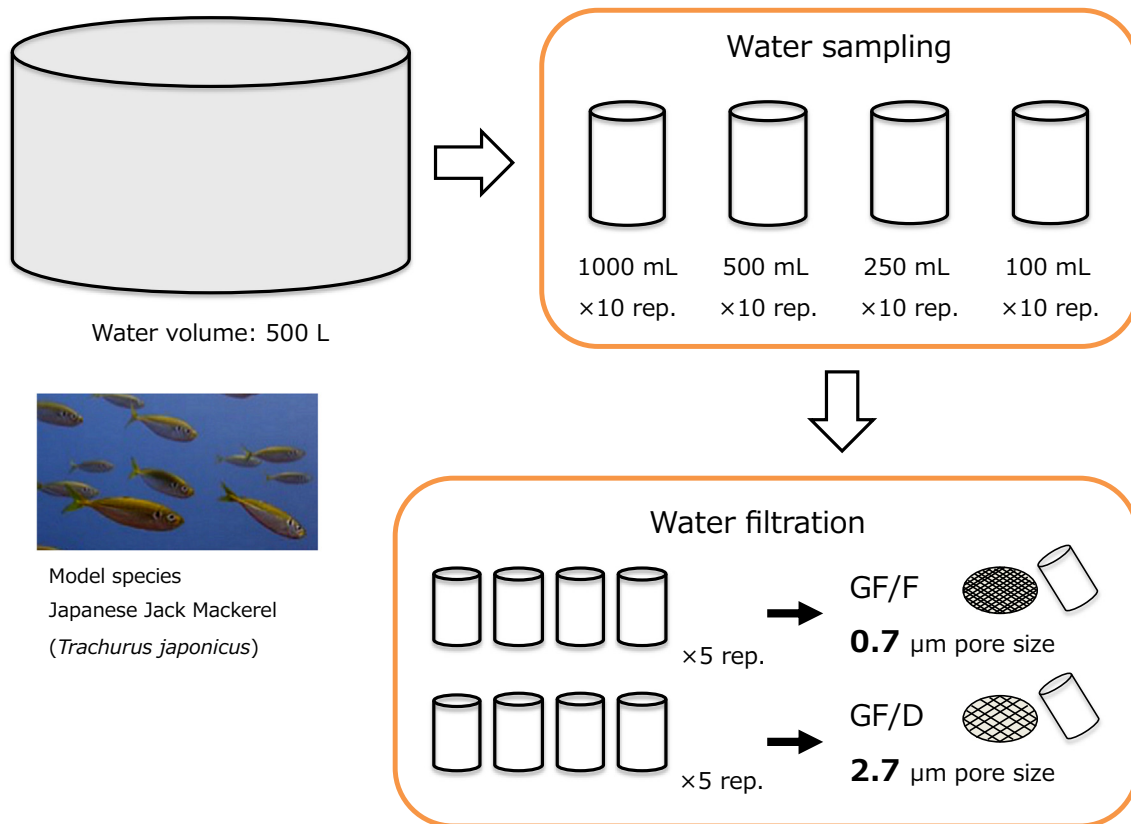


Fig. 1. Overall flowchart of the tank experiment. We collected 1000, 500, 250, and 100 mL of rearing water samples from a 500-L tank, in which Japanese Jack Mackerels were kept, and then randomly assigned them into two groups: one filtered by GF/F (nominal pore size of 0.7 μm) and the other filtered by GF/D (nominal pore size of 2.7 μm). After the filtration, we quantified the copy number of short mitochondrial, long mitochondrial, and short nuclear eDNA in filter samples.

sizes were available (Jo et al., 2017, 2019b; Yamamoto et al., 2016). The tank was aerated by a pump, and filtered seawater, which was pumped from 6 m depth off the coast of the station, was used as the inlet water into the tank. We collected 10 replicates of 100, 250, 500, and 1000 mL of tank water samples using 1.3 L plastic bottles, and randomly filtered the five replicates of each volume of water samples with a 47 mm-diameter glass microfiber filter GF/F (nominal pore size 0.7 μm ; GE Healthcare Life Science, U.K.), and the other five replicates with a 47 mm-diameter glass microfiber filter GF/D (nominal pore size 2.7 μm ; GE Healthcare Life Science). The water temperature was 25.5 $^{\circ}\text{C}$ when collecting water samples. We simultaneously filtered 500 mL of distilled water as filtration negative controls and 500 mL of inlet water into the tank to evaluate the background concentrations of target eDNA using both filters. Throughout the sampling, disposable gloves were worn, and the filtering devices (i.e., filter funnels [Magnetic Filter Funnel, 500 mL capacity; Pall Corporation, Westborough, MA, USA], 1 L beakers, tweezers, and sampling bottles) were bleached before every use in 0.1% sodium hypochlorite solution for at least 5 min (Yamanaka et al., 2017). We kept all the filter samples at -20°C until DNA extraction.

2.2. DNA extraction and quantitative real-time PCR

Total eDNA on the filter was extracted by DNeasy Blood and Tissue Kit (Qiagen, Germany) following Jo et al. (2017) (Appendix S1). We estimated the Japanese Jack Mackerel eDNA concentration in water samples by quantifying the copy number of mitochondrial cytochrome *b* (CytB) genes and nuclear internal transcribed spacer-1 (ITS1) regions of ribosomal RNA genes using the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, U.S.). We used three primers/probe sets that specifically amplified the 164-bp fragment of CytB gene (mtS), the 682-bp fragment of CytB gene (mtL), and the 164-bp fragment of ITS1 region (nuS) from the target species (Table 1). The species-specificity of each primers/probe set had already been in vitro checked (Jo et al., 2017, 2019b; Yamamoto et al., 2016). Each 20 μL of TaqMan reaction contained a 2 μL template DNA, a final 900 nM concentration of each forward and reverse primer, and 125 nM of TaqMan probe in 1 \times TaqPath™ qPCR Master Mix, CG (Thermo Fisher Scientific). We simultaneously analyzed 2 μL of pure water as PCR negative controls. We performed qPCRs using a dilution series of standards containing 3×10^1 to 3×10^4 copies of a linearized plasmid containing synthesized artificial DNA fragments from a full CytB gene (1141 bp) or partial ITS1 region (237 bp) of the target species (Jo et al., 2019b). We performed all qPCRs for eDNA samples, standards, and negative controls in triplicate. Thermal conditions of quantitative real-time PCR were as follows: 2 min at 50 $^{\circ}\text{C}$, 10 min at 95 $^{\circ}\text{C}$, 55 cycles of 15 s at 95 $^{\circ}\text{C}$, and 1.5 min at 60 $^{\circ}\text{C}$ for mtS and nuS (2-step PCR), and 2 min at 50 $^{\circ}\text{C}$, 10 min at 95 $^{\circ}\text{C}$, 55 cycles of 15 s at 95 $^{\circ}\text{C}$, 30 s at 60 $^{\circ}\text{C}$, and 1 min at 72 $^{\circ}\text{C}$ for mtL (3-step PCR). We calculated eDNA concentrations by averaging the triplicate, and each PCR-negative replicate (indicating non-detection) was regarded as containing zero copies (Ellison et al., 2006).

2.3. Statistical analyses

For each type of eDNA (mtS, mtL, and nuS), we characterized linear relationships between eDNA concentrations (log-transformed) and the volume of water filtration (log-transformed). The interactions between covariates (filtration water volume) and factors (filter types) were significant or marginal (see Results), which meant that the effect of covariates on eDNA concentrations was different between factors. Thus, analyses of covariance (ANCOVA) could not be applied to our dataset and, instead, we compared log-transformed eDNA concentrations between filter pore sizes for each filtration water volume using Student's *t*-test. In addition, we calculated the ratios of long to short mitochondrial eDNA (i.e., mtL: mtS) and short nuclear to short mitochondrial eDNA (i.e., nuS: mtS) concentrations, and compared them between the filters using Mann-Whitney's *U* test. Moreover, we calculated the coefficients of variations (CVs; standard deviations divided by mean values) for each type of filter and eDNA. For the calculation of the ratios and CVs, we used the raw eDNA concentrations and pooled the eDNA data from different filtration volumes to increase the sample size. All the statistical analyses were performed by R version 3.6.1 (R Core Team, 2019).

3. Results and discussion

The eDNA concentrations in inlet water samples and filtration negative controls were at most 28.8 and 1.0 copies per PCR reaction respectively, which was negligible relative to those in tank water samples (Table S1). No amplification was observed in any of the PCR negative controls. DNA concentrations in all tank water samples were larger than 30 copies/reactions, which is the lowest value of a dilution series of standards (Table S1). PCR information for each type of eDNA is shown in Table S2.

3.1. The ratio of long to short mitochondrial eDNA

The ratio of long to short mitochondrial eDNA concentrations (mtL: mtS) was significantly higher for GF/D (2.7 μm pore size) than GF/F filters (0.7 μm pore size) ($P = 0.0020$; Fig. 2). As expected, the use of a larger pore size filter increased the relative yield of long DNA fragments, which is the most important finding in the study. After released by the organisms, aqueous eDNA is degraded by mainly microbes and extra-cellular enzymes (Barnes et al., 2014; Collins et al., 2018). Its persistence in water can be lower for longer DNA fragments (Bista et al., 2017; Jo et al., 2017), while it is considered that, due to its cellular membrane, DNA fragmentation can be suppressed for intra-cellular DNA relative to extra-cellular DNA in natural environment (Nielsen et al., 2007; Torti et al., 2015). Thus, the result would indicate the increase of the relative yield of less-degraded eDNA by the selective collection of large-sized and intra-cellular DNA. Such long DNA fragments in water may have the potential to improve the identification of closely related species and the evaluation of intra-specific genetic diversity based on

Table 1
Primers/probe sets used in this study.

ID	Target region	Sequences (5' → 3')	Amplicon size with the forward primer (bp)	T _m (°C)	Reference
Tja_CytB_F	Mitochondrial cytochrome <i>b</i> (CytB)	CAG-ATA-TCG-CAA-CCG-CCT-TT		58.7	Yamamoto et al. (2016)
Tja_CytB_R164		TTC-TTT-GTA-GAG-GTA-CGA-GCC-G	164	59.8	Jo et al. (2019a)
Tja_CytB_R682		ATT-GAT-CGG-AGA-ATG-GCG-TAT	682	57.3	Jo et al. (2017)
Tja_CytB_P		[FAM]-TAT-GCA-CGC-CAA-CGG-CGC-CT-[TAMRA]		67.9	Yamamoto et al. (2016)
Tja_ITS1_F	Nuclear internal transcribed spacer-1 (ITS1)	GCG-GGT-ACC-CAA-CTC-TCT-TC		60.1	Jo et al. (2019a)
Tja_ITS1_R		CCT-GAG-CGG-CAC-ATG-AGA-G	164	63.2	
Tja_ITS1_P		[FAM]-CTC-TCG-CTT-CTC-CGA-CCC-CGG-TCG-[TAMRA]		70.8	

Note that we shared the forward primer and TaqMan probe for the primers/probe sets targeting mtS and mtL, and changed only reverse primers to adjust the length of PCR amplicon.

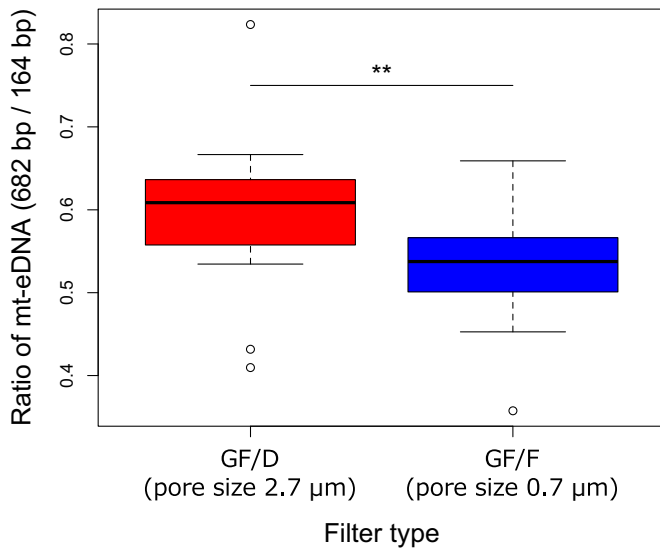


Fig. 2. The comparison of the ratio of long (682-bp) to short (164-bp) mitochondrial eDNA concentrations between GF/F and GF/D. The boxplots were drawn based on the raw eDNA concentrations. The double star represents the significant difference ($P < 0.01$) between filter types by Mann-Whitney's U test.

eDNA analysis (Sigsgaard et al., 2017; Uchii et al., 2016; Williams et al., 2019). Also, the detection of long DNA fragments may provide a more precise temporal inference of an organism's biomass/abundance contrary to short DNA fragments (Jo et al., 2017). As only two kinds of filter pore sizes were tested in this study, future studies using various pore sizes of filters will strengthen our findings.

3.2. The ratio of nuclear to mitochondrial eDNA

The ratio of nuclear to mitochondrial eDNA concentrations (nuS: mtS) was significantly lower for GF/D than GF/F filters ($P < 0.0001$; Fig. 3). There was little difference in yields between nuclear and mitochondrial eDNA using GF/F, whereas the yield of nuclear eDNA tended to be lower than that of mitochondrial eDNA using GF/D (Table S1). Considering that a GF/D filter (2.7 µm pore size) could mainly capture the intra-cellular eDNA while a GF/F filter (0.7 µm pore size) could capture both intra- and extra-cellular eDNA, it is likely that extra-cellular nuclear eDNA from target species is more abundant in rearing water than extra-cellular mitochondrial eDNA. So far as being covered with its non-porous membranes, mitochondrial DNA could be more persistent to enzymatic activities than nuclear one, which is covered with porous membrane (Ellenberg et al., 1997; Ernster and Schatz, 1981). However, once having lost their membranes, extra-cellular mitochondrial DNA might be degraded faster than extra-cellular nuclear DNA in water. Foran (2006) reported that the degradation of tissue-derived DNA was faster for nuclear than mitochondrial DNA without homogenization (assuming intra-cellular DNA), whereas the result was reversed in homogenized tissues (assuming extra-cellular DNA) and the degradation of mitochondrial DNA was faster than that of nuclear DNA. Therefore, the result of our study might be partly attributed to the reversal in the degradative vulnerability of mitochondrial and nuclear DNA between intra- and extra-cellular environments (Foran, 2006).

Alternatively, the fusion of mitochondria in cells to form dynamic inter-connecting networks for the maintenance of their integrities (Koshiba et al., 2004; Suen et al., 2008) would possibly have brought the result; there could be some mitochondria larger than the nuclei due to the fusion, which might decrease the omission of mitochondrial eDNA with an increase in filter pore size relative to nuclear one. Further studies would be needed to investigate how the cellular environment and DNA structure, could physically, chemically, and biologically influence the persistence of mitochondrial and nuclear DNA. It would help

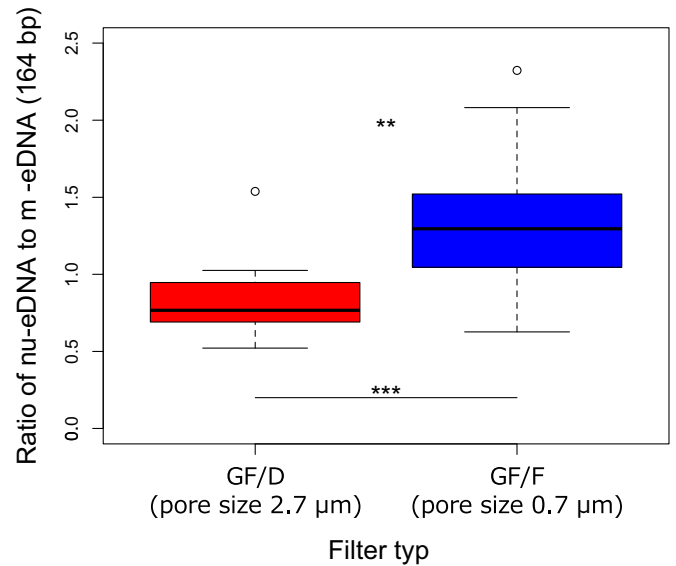


Fig. 3. The comparison of the ratio of nuclear to mitochondrial (both 164-bp) eDNA concentrations between GF/F and GF/D. The boxplots were drawn based on the raw eDNA concentrations. The triple star represents the significant difference ($P < 0.001$) between filter types by Mann-Whitney's U test.

understand the characteristics and dynamics of nuclear and mitochondrial eDNA in water.

3.3. The difference of eDNA capture efficiencies between filters

According to results of Student's *t*-tests, all types of eDNA concentrations were significantly higher for GF/F (pore size: 0.7 µm) than GF/D filters (pore size: 2.7 µm) in 1000 mL of filtration water volume (all $P < 0.01$; Table 2). In addition, short nuclear eDNA concentrations were significantly higher for GF/F than GF/D filters in any of the filtration volumes (all $P < 0.05$), while short and long mitochondrial eDNA concentrations did not significantly differ between filter pore sizes in <500 mL of filtration volume ($P > 0.1$). All types of eDNA concentrations were generally higher for the smaller pore size filter, whereas the differences of eDNA concentrations tended to be unclear when smaller volume of water samples was filtered (Fig. 4). In a natural environment especially with high turbidity, larger filter pore size enables to prevent a filter clogging and to increase the filtration efficiencies (Robson et al., 2016; Wilson et al., 2014), and thus the lower capture efficiency of GF/D may not be the major problem. For example, the yield of eDNA by GF/F filtrations of 100 mL water samples can be recovered by GF/D filtrations of at most 250 mL water samples (Fig. 4). It would

Table 2

Results of Student's *t*-tests for comparisons of eDNA yields between filter pore sizes (GF/F and GF/D).

eDNA type	Filtration volume	<i>P</i> value	
Short mt-eDNA	100 mL	0.1359	
	250 mL	0.8455	
	500 mL	0.1130	
	1000 mL	0.0016	**
Long mt-eDNA	100 mL	0.2467	
	250 mL	0.6721	
	500 mL	0.1474	
	1000 mL	0.0051	**
Short nu-eDNA	100 mL	0.0108	*
	250 mL	0.0312	*
	500 mL	0.0063	**
	1000 mL	0.0007	***

Note: Asterisks represent the statistical significances of the parameters (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). All eDNA concentrations were log-transformed.

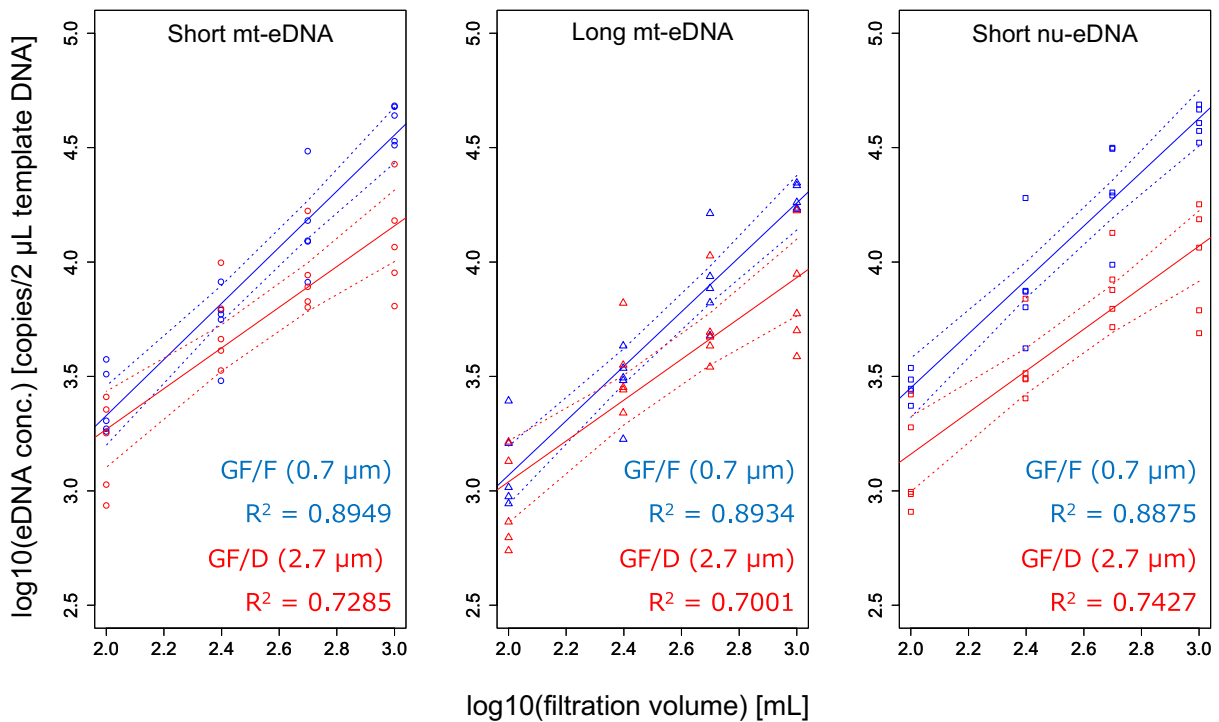


Fig. 4. Relationships between eDNA concentrations for each type (mts, mtL, and nuS) and filtration water volume (both log-transformed) using different filters, GF/F and GF/D. Blue and red plots are derived from GF/F and GF/D, respectively. Regression lines and their 95% confidence intervals (CIs) for each plot are shown by solid and dotted lines, respectively. R² values represent the fitness of each regression line.

also result in the increase of the relative yield of long DNA fragments from water samples.

In contrast, higher CVs for GF/D relative to GF/F filters were observed in all types of eDNA (Table 3). A similar result was reported previously (Minamoto et al., 2016), and it may decrease the precision of biomass estimation based on eDNA analysis (Mauvisseau et al., 2019). The heterogeneous distribution of aqueous eDNA is likely attributed to the large-sized eDNA such as aggregation of cells and tissues (Turner et al., 2014; Furlan et al., 2016; Song et al., 2017). Therefore, selective collection of such large-sized eDNA might possibly disperse the eDNA concentrations; however, we were unable to determine the statistical significance of differences, as our findings were based on samples collected from a single experimental tank. For the improvement of eDNA-based biomass estimation, the effect of filter pore size and materials on the precision of eDNA quantification would be needed, which will be a focus of future studies.

4. Conclusions

So far, the filter with larger pore size has been used in eDNA studies to prevent the filter clogging and to increase the filtration volume (Robson et al., 2016; Wilson et al., 2014) except for Fremier et al. (2019), which focused on the transport of only intra-cellular DNA using a larger pore size filter. However, we showed that the use of larger pore size filter could improve the relative capture efficiency of long DNA

fragments from water samples. Our study suggests that the selective collection of the specific size, and possibly the state, of aqueous eDNA may allow to improve the eDNA-based taxonomy and biomass/abundance estimation. In addition, the ratio of nuclear to mitochondrial eDNA concentrations varied depending on filter pore sizes. Although further study would be needed from physiological and cytological aspects, our findings may reflect the potential difference of nuclear and mitochondrial DNA persistence between cellular and the aquatic environment.

There remain some issues to be verified in this study. First, all the eDNA data was based on a single experimental tank, and thus our results might be less statistically robust, even though we assessed a large number of water filtration and PCR replicates. Second, it would be necessary to verify whether the approach used in the present study is practically applicable to natural environments. As mentioned above, in the environment difficult for water filtration (e.g., high turbidity), the use of larger pore size filter to improve the relative capture efficiency of long DNA fragments might be more efficient. Third, as mentioned above, the findings of the present study were based on the analysis using filters of only two pore sizes, and thus future analyses using filters of a larger range of pore sizes would conceivably contribute to the robustness of our results.

Nevertheless, as far as we know, this is the first report to show the possibility to control the ecological, and possibly physiological, information from eDNA by utilizing the knowledge of its size and state in water. Some studies suggested that the combination of nuclear and mitochondrial eDNA could imply the spawning activity and the age structure of fish (Jo et al., 2019b; Bylemans et al., 2017), and that the combination of long and short DNA fragments in water allowed to remove the effect of eDNA from carcasses and to obtain fresher ecological information (Jo et al., 2017). By targeting the large-sized and less-degraded eDNA, the results shown in these studies might be more obvious not only in mesocosm but also in a natural environment. The selective collection of eDNA based on its size and state would be able to extend the eDNA applications for ecological monitoring in the future.

Table 3

The results of the coefficients of variations (CVs) for each type of eDNA and filter.

Type of eDNA	Type of filters	CVs [%]
Short mitochondrial	GF/F	40.0
	GF/D	46.5
Long mitochondrial	GF/F	38.2
	GF/D	52.0
Short nuclear	GF/F	39.8
	GF/D	44.5

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2020.139462>.

Data accessibility

The raw data for the qPCR experiments is included in the Supporting Information.

CRediT authorship contribution statement

Toshiaki Jo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Hiroaki Murakami:** Resources, Writing - original draft, Writing - review & editing. **Reiji Masuda:** Resources, Writing - original draft, Writing - review & editing, Supervision. **Toshifumi Minamoto:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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