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Estimating shedding and decay rates of environmental nuclear DNA with relation to water temperature and biomass

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Abstract

Background: Environmental DNA (eDNA) analysis has been recently applied to the surveillance of species distribution and composition in aquatic ecosystems. However, most eDNA studies have used mitochondrial DNA markers, and those using nuclear DNA markers are quite scarce. Moreover, although some studies reported the availability of nuclear DNA markers for eDNA analyses, the characteristics and dynamics of nuclear environmental DNA (nu-eDNA) of macro-organisms remain unknown. Herein, we re-analyzed eDNA samples described in a previously published paper to investigate the shedding and decay rates of nu-eDNA from Japanese Jack Mackerel (*Trachurus japonicus*) and compared them to those of mt-eDNA (mitochondrial environmental DNA).

Materials & Methods: Tank experiments consisting of 12 combinations of four temperatures and three fish biomass levels were performed, and four tank replicates were prepared for each treatment level. Before and after removing the fish from the tanks, we sampled rearing water over time to quantify nu-eDNA copy numbers.

Results & Discussion: Model fitting to eDNA decay curves demonstrated that nu-eDNA decay rates increased in higher water temperature and with larger fish biomass. The estimated shedding rates of nu-eDNA also increased with higher temperature and larger biomass. These results were generally consistent with those of mt-eDNA. Moreover, the ratio of mt-eDNA to nu-eDNA shedding and concentration decreased with larger fish biomass levels, which implied that these values may be among the potential indices for estimating the age and body size of organisms from environmental samples. Our findings contribute to the understanding of eDNA characteristics and dynamics between different DNA markers and may help us to interpret future results of eDNA surveillance.

KEYWORDS

environmental DNA, internal transcribed spacer-1, Japanese Jack Mackerel (*Trachurus japonicus*), mitochondrial DNA, nuclear DNA, quantitative real-time PCR

1 | INTRODUCTION

During the last decade, environmental DNA (eDNA) analysis has been developed as a novel tool for the assessment and management of aquatic ecosystems (Bohmann et al., 2014; Ficetola, Miaud, Pompanon, and Taberlet, 2008; Minamoto, Yamanaka, Takahara, Honjo, and Kawabata, 2012; Taberlet, Coissac, Hajibabaei, and Rieseberg, 2012; Thomsen and Willerslev, 2015). Organisms release DNA into the environment in the form of mucus, feces, scales, and gametes (Bylemans et al., 2017; Martellini, Payment, and Villemur, 2005; Merkes, McCalla, Jensen, Gaikowski, and Amberg, 2014; Sassoubre, Yamahara, Gardner, Block, and Boehm, 2016), and this genetic material is called eDNA. The analysis of eDNA has enabled us to obtain information on species distribution and composition quickly, extensively, and noninvasively (Balasingham et al., 2017; Biggs et al., 2015; Fukumoto, Ushimaru, and Minamoto, 2015; Yamamoto et al., 2017).

To date, most eDNA analyses relating to macro-organisms have targeted mitochondrial DNA (mtDNA) as a genetic marker (Dougherty et al., 2016; Ficetola et al., 2008; Goldberg et al., 2013; Takahara et al., 2012; Ushio et al., 2018). This is mainly because a single cell has multiple mitochondrial genomes (tens to thousands of mtDNA copies), contrary to the nuclear genome (Foran, 2006; Robin and Wong, 1988). However, some studies have suggested the use of nuclear DNA (nuDNA) markers, particularly the markers targeting multiple copies of ribosomal RNA genes such as internal transcribed spacer (ITS) regions, and reported that the regions could be sensitive genetic markers for eDNA analyses (Dysthe, Franklin, McKelvey, Young, and Schwartz, 2018; Gantz, Renshaw, Erickson, Lodge, and Egan, 2018; Minamoto, Uchii, et al., 2017). The mtDNA copy numbers per cell may vary depending on individual body condition and cell type, whereas those of nuDNA do not depend on such factors (Long and Dawid, 1980). In addition, some regions of nuDNA have high interspecific variation (Booton et al., 1999), which could be useful for distinguishing closely related species via eDNA analysis. Therefore, testing the availability of nuDNA marker is important for the expansion of eDNA applicability in the field.

Given the possibility and prospect of using nuDNA marker in eDNA analyses, it is important to understand the characteristics and dynamics of nuclear and mitochondrial eDNA (nu-eDNA and mt-eDNA, respectively). For example, some studies have examined how various environmental factors may influence the shedding and degradation of eDNA (Barnes and Turner, 2016; Hansen, Bekkevold, Clausen, and Nielsen, 2018; Strickler, Fremier, and Goldberg, 2015). For mt-eDNA, previous studies reported that its shedding is mainly affected by the biomass/abundance of organisms and temperature (Klymus, Richter, Chapman, and Paukert, 2015; Maruyama, Nakamura, Yamanaka, Kondoh, and Minamoto, 2014; Jo, Murakami, Yamamoto, Masuda, and Minamoto, 2019; Takahara et al., 2012), whereas eDNA degradation is affected by different water chemistries, temperature, and microbial activity (Barnes et al., 2014; Eichmiller, Best, and Sorensen, 2016; Jo, Murakami, et al., 2019; Seymour et al., 2018; Strickler et al., 2015). Although some studies have examined the detectability, amount, and persistence of eDNA among different DNA markers (Bylemans et al., 2017; Bylemans, Furlan, Gleeson, Hardy, and Duncan, 2018; Gantz et al., 2018; Minamoto, Uchii, et al., 2017), the influence of environmental factors on the shedding and degradation of nu-eDNA has not been formally evaluated, and such information is needed to evaluate the feasibility of using nu-eDNA in future studies.

In this study, using Japanese Jack Mackerel (*Trachurus japonicus*) as a model species, we estimated the shedding and decay rates of nu-eDNA and investigated the effects of water temperature, biomass of organisms, and type of DNA marker (nuclear or mitochondrial) on eDNA shedding and degradation. For this, we first developed a novel

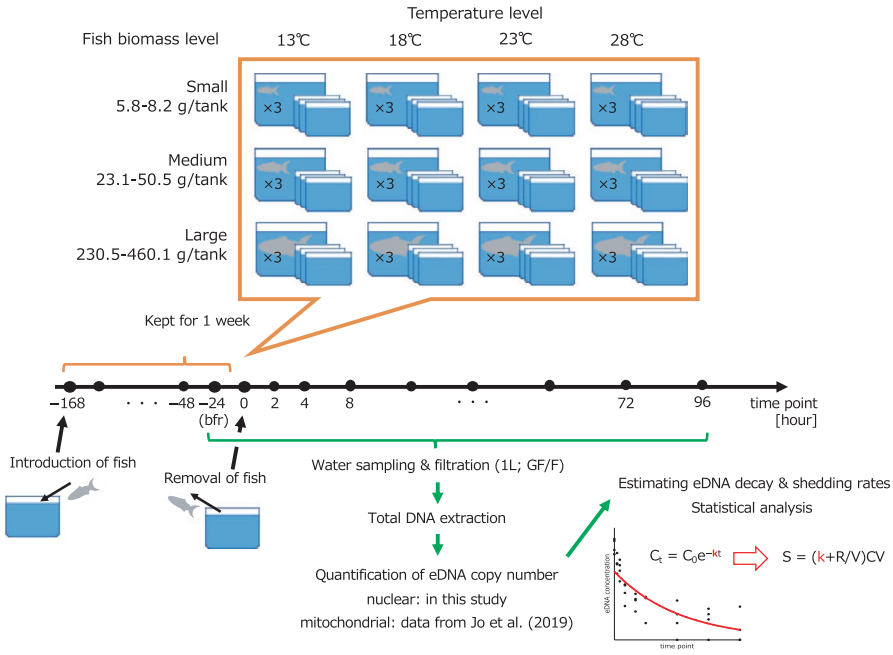

FIGURE 1 The overall flowchart of our tank experiment. Three Japanese Jack Mackerels were introduced into each 200-L tank with 12 different combinations of four temperature and three fish biomass levels. After the 1-week acclimation, the fish were removed from each tank. Time-series water sampling was performed on the day before and after the fish removal. By model fitting to eDNA decay curves from times 0–96, the eDNA decay rates were estimated for each tank. Using the decay rate constants and eDNA concentrations at time bfr, eDNA shedding rates were calculated for each treatment

primer/probe set that specifically amplified a nuDNA fragment of Japanese Jack Mackerel, an economically important marine fish in East Asia (Sassa and Konishi, 2006; Zhang and Lee, 2001). Considering that a large proportion of eDNA exists as intracellular DNA, such as cell and tissue fragments in water (Jo, Murakami, et al., 2019; Turner et al., 2014), it was expected that the tendencies of eDNA shedding and degradation would be similar between nu- and mt-eDNA.

2 | MATERIALS AND METHODS

2.1 | Experimental design

All extracted eDNA samples used were from Jo, Murakami, et al., (2019 Briefly, we assigned 200-L acrylic tanks to four water temperatures (13, 18, 23, and 28°C) and three fish biomass levels (Small, Medium, and Large), resulting in 12 treatment levels (Figure 1). Four tank replicates were prepared per treatment. Temperature levels were set based on previous studies that reported the range of water temperature when the species was recorded at the sampling site (Masuda, 2008) and the preferred temperature of our model species (i.e., around 20°C; Nakamura and Hamano, 2009; Tsuchida, 2002). Fish biomass levels were determined by the difference in fish body size. All tanks housed the same number of fish individuals. Water temperature was kept constant for each tank throughout the experiment. All tanks were individually aerated using a pump and flow-through until the fish were removed from the tank. Filtered seawater was pumped from a 6 m depth at the Research Station and used as the inlet water for each tank (flow velocity: 600 ml/min).

Three Japanese Jack Mackerels were added to each tank and kept there for 1 week. A small amount of krill was used to feed the fish every morning until the day before water sampling. The bottom of each tank was cleaned an hour after feeding to remove the effect of the feces from the analyses. The fish were starved on the sampling day. Total lengths (TLs) and wet weights of the fish were 6.2 ± 0.4 cm and 2.3 ± 0.5 g (Small), 11.7 ± 1.2 cm and 13.4 ± 4.2 g (Medium), and 21.4 ± 3.1 cm and 106.5 ± 48.4 g (Large) (mean ± 1 *SD*). In addition, the age of each fish was estimated by the growth model for Japanese Jack Mackerel (Mitani and Ida, 1964). Ages were 0.16 ± 0.02 year (Small), 0.37 ± 0.06 year (Medium), and 1.04 ± 0.26 year (Large) (mean ± 1 *SD*).

2.2 | eDNA sampling and extraction

After a 1-week acclimation period, we quickly and carefully removed the fish from each tank using a net. Flow-through was switched off after removing the fish. The time point immediately after removing the fish from each tank was defined as time 0. Water samples were collected with plastic bottles from the tanks 0, 2, 4, 8, 16, 24, 48, 72, and 96 hr after time 0; these time points are referred as times 0–96. At each time point, 1 L of rearing water was collected from each tank and filtered using a 47-mm-diameter glass microfiber filter GF/*F* (nominal pore size 0.7 μm; GE Healthcare Life Science, Little Chalfont, UK). Water samples were also collected the day before time 0, which was defined as the time before fish removal (time bfr), to measure eDNA concentrations at a steady state (i.e., the time at which eDNA shedding was in equilibrium with total eDNA degradation; Jo, Murakami, et al., 2019; Sassoubre et al., 2016). Besides, 1 L of distilled water was filtered at each time point as a filtration negative control, and 1 L of inlet water put into each tank was filtered at time 24, when flow-through had already been switched off, to evaluate the background Japanese Jack Mackerel eDNA concentration in it.

Disposable gloves were used during water samplings, and the outer part of sampling bottles was washed with tap water after water samplings. Filtering devices (i.e., filter funnels [Magnetic Filter Funnel, 500 ml capacity; Pall Corporation, Westborough, MA, USA], 1 L beakers, tweezers, and plastic bottles) used for water sampling were bleached after every use in 0.1% sodium hypochlorite solution for at least 5 min (Yamanaka et al., 2017). All filter samples were kept at −20°C until DNA extraction. Total DNA from each filter was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) (Jo, Murakami, et al., 2019). All eDNA samples were kept at −20°C until quantitative PCR analysis.

2.3 | Primers and probe development

We designed a novel primer/probe set that specifically amplified the DNA fragment of the nuclear internal transcribed spacer-1 (ITS1) region of Japanese Jack Mackerel. This region was targeted because from tens to tens of thousands of copies of ribosomal RNA genes, including ITS1 regions, are present in the nuclear genome (Prokopowich, Gregory, and Crease, 2003), which is fixed regardless of the individual's body condition or cell type (Hillis and Dixon, 1991; Long and Dawid, 1980). Because of the paucity of publicly available sequence data for this region, we sequenced the ITS1 region of the model species and related species from Maizuru Bay (Amberfish [*Decapterus maruadsi*], Amberjack [*Seriola quinqueradiata*], and Greater Amberjack [*Seriola dumerili*]) to use as reference sequences for primers and probe development. We newly captured eight Japanese Jack Mackerels in the west Maizuru Bay (Nagahama, Maizuru, Kyoto, Japan; 35°29′N and 135°22′E) in June 2018, and tissue samples were collected. Tissue samples of the related fishes were obtained from the fish collection of Kyoto University (FAKU). Total DNA was extracted from the tissues using the DNeasy Blood and Tissue Kit following manufacturers' guidelines. These DNA extracts were amplified in a Veriti Thermal Cycler (Applied Biosystems) using the universal ITS1 primer pair (forward primer: 5′-TCC GTA GGT GAA CCT GCG G-3′; reverse primer: 5′-CGC TGC GTT CTT CAT CG-3′), which was designed to amplify the ITS1 region of a wide variety of marine animals (Chow, Ueno, Toyokawa, Oohara, & Takeyama, 2009). Each 25 μl PCR reaction contained 2 μl of DNA extract, 0.4 μM of each primer, 0.1 mM of dNTPs, and 1 U of ExTaq™ DNA polymerase (Takara Bio, Tokyo, Japan) in 1× ExTaq Buffer (Takara Bio, Tokyo, Japan). PCR was performed with the following conditions: 2 min at 94°C, 55 cycles of 30 s at 96°C, 30 s at 50°C, and 1.5 min at 72°C, and

7 min at 72°C. PCR products were visualized using electrophoresis on 1.5% agarose gels stained with Midori Green (NIPPON Genetics Co, Ltd., Japan). The agarose gels with the band of target length were then purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA). These products were commercially Sanger-sequenced using the 3130xl Genetic Analyzer (Applied Biosystems) and BigDye Terminator v3.1 (Thermo Fisher Scientific, Waltham, MA, USA) to obtain the reference sequences for the primers and probe development.

Using the produced sequences and those available in the National Center for Biotechnology Information (NCBI) database (Table S1), a species-specific primer/probe set was designed using Primer Express 3.0 (Thermo Fisher Scientific) with default settings. In vitro specificity of the assay was then checked using the StepOnePlus Real-Time PCR system (Applied Biosystems). Each 20 μl TaqMan reaction contained 100 and 10 pg of template DNA (from one individual of Japanese Jack Mackerel or of a related species described above), a final concentration of 900 nM of forward and reverse primers, and 125 nM TaqMan probe in a 1× TaqMan Gene Expression PCR Master Mix (Thermo Fisher Scientific). PCR was performed with the following conditions: 2 min at 50°C, 10 min at 95°C, and 55 cycles of 15 s at 95°C and 1 min at 60°C. A 2 μl pure water sample was simultaneously analyzed as a PCR-negative control.

2.4 | Quantification of eDNA samples

The amount of Japanese Jack Mackerel nu-eDNA in water samples was evaluated by quantifying the ITS1 region copy number using the StepOnePlus Real-Time PCR system. Each 20 μL TaqMan reaction contained 2 μL of template DNA, a final concentration of 900 nM of forward and reverse primers, and 125 nM of TaqMan probe in a 1× TaqMan Gene Expression PCR Master Mix. Thermal conditions of the quantitative real-time PCR were the same as described above. The ITS1 region copy number in each 2 μL template DNA was quantified by simultaneously performing a qPCR with a dilution series of standards containing 3×10^1–3×10^4 copies of a linearized plasmid that contained synthesized artificial DNA fragments of the partial sequence of the ITS1 region (237 bp) of Japanese Jack Mackerel. A negative PCR control was included by simultaneously analyzing 2 μL of pure water. All quantitative mt-eDNA data used for comparisons were obtained from Jo, Murakami, et al. (2019. All qPCRs for eDNA extracts, standards, and negative controls were performed in triplicate, and the eDNA concentrations were calculated by averaging the triplicate. Each PCR-negative replicate (indicating nondetection) was regarded as containing zero copies (Ellison et al., 2006).

2.5 | Statistical analyses

R version 3.2.4 (R Core Team, 2016) was used for all statistical analyses. One of the tanks (treatment 28°C/Large fish biomass level) was excluded from all analyses because of fish mortality. Japanese Jack Mackerel eDNA decay rates were first estimated using the time-series change of their eDNA concentrations after fish removal from each tank. Previous studies estimated eDNA decay rates by fitting a first-order exponential decay model (Collins et al., 2018; Eichmiller et al., 2016; Minamoto, Fukuda et al., 2017; Sassoubre et al., 2016; Thomsen et al., 2012) as follows:

$$C_t = C_0 e^{-k*t} \quad (1)$$

where C_t is eDNA concentration at time t (copies/L), C_0 is eDNA concentration at time 0, and k is the decay rate constant (per hour). We expanded this model to include the effects of water temperature and total fish biomass in the tank (Jo, Murakami, et al., 2019) as follows:

$$C_t = C_0 e^{-(b*T+c*D+a)*t} \quad (2)$$

where T is water temperature (°C), D is total wet weight of Japanese Jack Mackerel in each 200-L tank (log-transformed, g/200 L), and a, b, and c are constants estimated using the nonlinear least-squares regression of the function *nls* in the R software. The eDNA concentrations at each time point were adjusted with those at time 0 (i.e., C_0 was regarded as one). All eDNA samples whose concentrations were below one copy per reaction (Doi et al., 2017; Katano, Harada, Doi, Souma, and Minamoto, 2017; Takahara et al., 2012) were excluded from model fitting. In addition, the eDNA samples with concentrations below the background eDNA signal, as measured from the inlet water, were excluded from model fitting. Using these parameters and constants, nu-eDNA and mt-eDNA decay rates were, respectively, calculated for each tank.

Japanese Jack Mackerel eDNA shedding rates per treatment were then estimated following Jo, Murakami, et al. (2019). The ordinary differential equation was assumed to represent the change with time of eDNA abundance in the tank (Maruyama et al., 2014; Sassoubre et al., 2016; Thomsen et al., 2012) as follows:

$$V\frac{dC}{dt} = S - \beta CV \quad (3)$$

where V is the volume of the tank (L), C is eDNA concentration from Japanese Jack Mackerel (copies/L), S is eDNA shedding rate (copies/hour), and β is total eDNA degradation rate (per hour). β included eDNA decay rates estimated above (k) and eDNA dilution rates resulting from a flow-through system (i.e., R/V; R is the flow rate of the inlet water [L/hour]). At steady state (i.e., time bfr), eDNA shedding (S) was assumed to be in equilibrium with total eDNA degradation ($\beta = k + R/V$), which resulted in $dC/dt = 0$. The equation above can therefore be expressed as follows:

$$S = \left(k + \frac{R}{V}\right) \times C_{\text{cons.}} \times V \quad (4)$$

where $C_{\text{cons.}}$ is eDNA concentrations at time bfr (copies/L). Using this equation, eDNA shedding rates were calculated for each tank. A three-way ANOVA was performed to investigate the effects of temperature (°C), fish biomass (Small, Medium, and Large), type

TABLE 1 The primer/probe set used in this study

Primer or Probe ID	Target region	Sequences (5′→3′)	Length (bp)	Tm (°C)	Reference
TjaITS1_F	Nuclear internal transcribed spacer−1 (ITS1)	GCG-GGT-ACC-CAA-CTC-TCT-TC	164	60.1	This study
TjaITS1_R		CCT-GAG-CGG-CAC-ATG-AGA-G		63.2	
TjaITS1_P		[FAM]-CTC-TCG-CTT-CTC-CGA-CCC-CGG-TCG-[TAMRA]		70.8	
Tja_CytB_F2	Mitochondrial cytochrome b (CytB)	CAG-ATA-TCG-CAA-CCG-CCT-TT	127	58.7	Yamamoto et al. (2016)
Tja_CytB_R2		CCG-ATG-TGA-AGG-TAA-ATG-CAA-A		57.6	
Tja_CytB_P2		[FAM]-TAT-GCA-CGC-CAA-CGG-CGC-CT-[TAMRA]		67.9	

of DNA markers, and their interaction on eDNA shedding rates, where eDNA shedding rates were log-transformed to reduce skewness.

Furthermore, we calculated Ratio_s and Ratio_c for each tank as follows:

$$\text{Ratio}_S = \frac{\log_{10}(S\text{of mt} - \text{eDNA})}{\log_{10}(S\text{of nu} - \text{eDNA})} \quad (5)$$

$$\text{Ratio}_C = \frac{\log_{10}(C_{\text{cons.}}\text{of mt} - \text{eDNA})}{\log_{10}(C_{\text{cons.}}\text{of nu} - \text{eDNA})} \quad (6)$$

where Ratio_S was the ratio between the nu- and mt-eDNA shedding rates, and Ratio_c was the ratio between nu- and mt-eDNA concentrations at time bfr. Although a single-copy nuclear gene would be more suitable, it would be difficult to detect and quantify the single-copy nuDNA in water samples. Thus, by using the copy number of the ITS1 region instead, whose copy number is fixed among cells, we assumed that the indices above are a measurement of the amount of mtDNA per cell. The Kruskal–Wallis rank sum test and post hoc Wilcoxon rank sum test with Bonferroni adjustment were performed to investigate the effects of fish biomass on the ratios of eDNA shedding and concentration (Ratio_S and Ratio_C), where eDNA shedding rates and concentrations were log-transformed in the same manner as above. Four temperature levels were pooled to increase sample size per biomass level.

3 | RESULTS

We successfully developed a novel primer/probe set to specifically amplify the ITS1 region of nuDNA from the Japanese Jack Mackerel (Table 1). The in vitro specificity check showed no PCR amplification of any related species DNA and PCR-negative controls. In addition, in all qPCR runs of tank experiments for nu-eDNA, the R^2 values, slope, Y-intercept, and PCR efficiency of the standard curves were 0.992 ± 0.008, −3.779 ± 0.177, 45.034 ± 2.442, and 83.613 ± 4.998, respectively (mean ± 1 *SD*). PCR amplifications were confirmed in some of the inlet water samples and filtration negative controls. Concentrations of nu-eDNA in the inlet water samples ranged from 0.0 to 570.9 copies/reaction, which corresponded to 0.0–20.0% of the water samples at time bfr. In addition, nu-eDNA concentrations in filtration negative controls ranged from 0.0 to 418.3 copies/reaction, which corresponded to 0.0–13.1% of nuDNA concentration compared with water samples at the same sampling time point (Table S2). Thus, the Japanese Jack Mackerel eDNA in inlet water and low-level cross-contamination among samples were not likely to have affected our conclusions. No PCR amplification was observed in any PCR-negative controls.

Concentrations of nu-eDNA at time 0 drastically increased compared with those at time bfr, which resulted from the stress caused by the removal of fish from the tanks (Figure 2). After fish removal, nu-eDNA concentrations decreased exponentially in all treatment levels. Coefficients from model fitting showed that higher temperature and fish biomass significantly increased nu-eDNA decay rates (Table 2). In addition, nu-eDNA decay rates were higher than those of mt-eDNA in all treatment levels (Table 2). Moreover, three-way ANOVA showed that eDNA shedding rates were significantly different among water temperatures, fish biomass, and type of DNA markers (all $p < .0001$). The interactions between fish biomass and type of DNA markers ($p < .001$) and temperature and fish biomass ($p < .05$) also significantly influenced eDNA shedding rates. Other interactions among factors were not significant ($p > .1$) (Figure 3).

Furthermore, the ratios of mt-eDNA to nu-eDNA shedding rates and concentrations at time bfr ($Ratio_S$ and $Ratio_C$) changed depending on fish biomass levels (Figure 4). Both $Ratio_S$ and $Ratio_C$ were significantly different among fish biomass levels (both $p < .0001$), and they were significantly lower for Large fish biomass level than for Small (both $p < .0001$) and Medium ones (both $p < .01$). There were no significant differences between Small and Medium fish biomass levels for $Ratio_S$ ($p = .6420$) and $Ratio_C$ ($p = .3226$).

4 | DISCUSSION

Although some studies have focused on the characteristics and dynamics of eDNA, our understanding of them may still be limited.

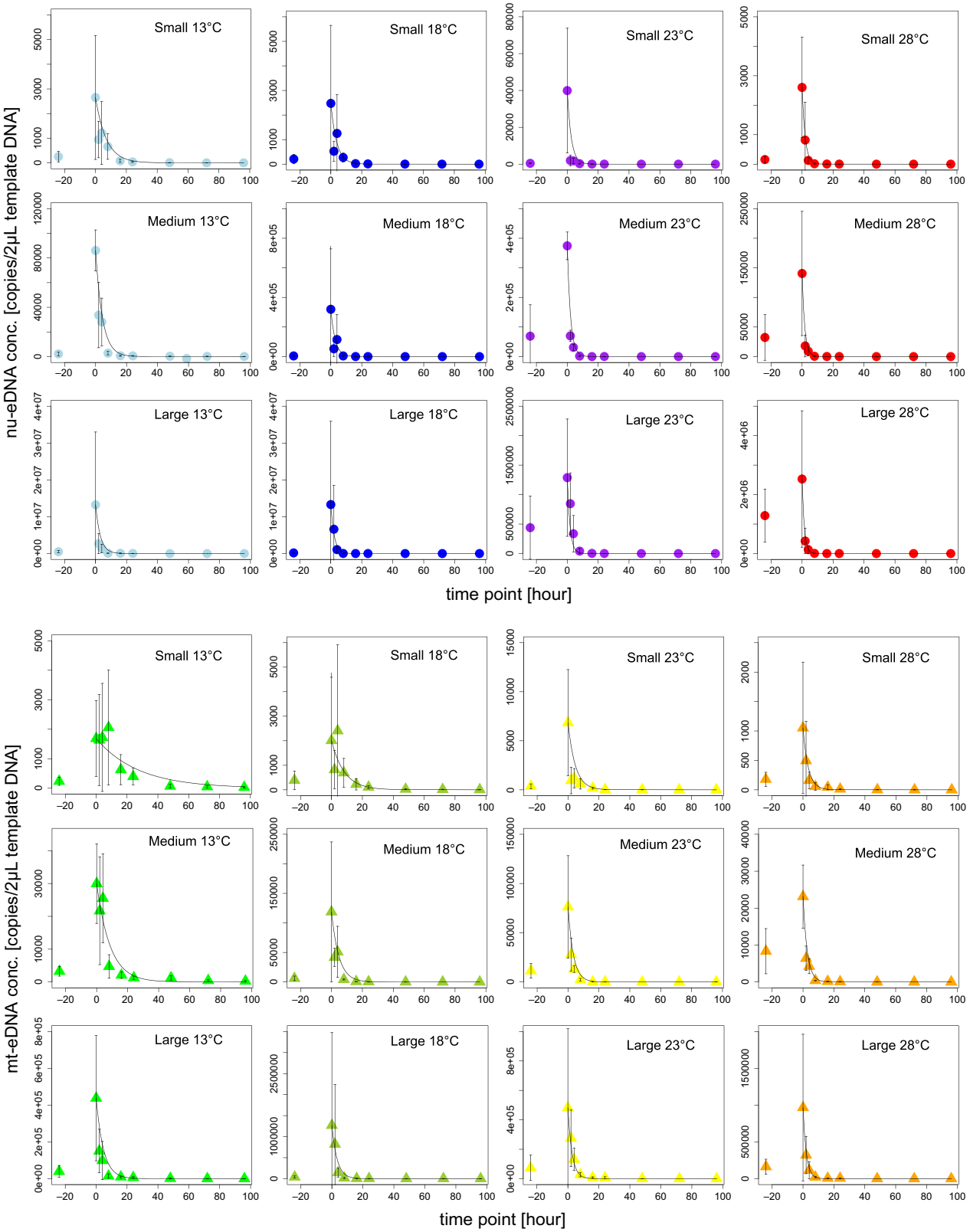

FIGURE 2 Overall nu-eDNA (upper) and mt-eDNA (lower) decay curves from the tank experiments. Dots show eDNA concentrations per PCR reaction at each time point (average of four tank replicates). Error bars show the standard deviations (*SD*) of tank replicates. The exponential curve fitted to each eDNA decay curve is based on the mean eDNA concentration at time 0 and the decay rate constant estimated by the nonlinear least-squares regression for each treatment level. All raw mt-eDNA data are from Jo, Murakami, et al. (2019)

TABLE 2 Parameters from the nonlinear least-squares regression for the eDNA decay curves (upper) and the eDNA decay rates calculated by these parameters (lower, mean ± 1 *SD*)

Target region	Parameter	Coefficient	*SE*	*p* Value
Nuclear ITS1	C_0	0.9733	0.0339	***
	b	0.0247	0.0050	***
	c	0.1092	0.0349	**
	a	−0.2690	0.0857	**
Mitochondrial CytB	C_0	1.0025	0.0316	***
	b	0.0173	0.0024	***
	c	0.1030	0.0173	***
	a	−0.2744	0.0331	***

		Fish biomass level		
Target region	Temperature level (°C)	Small	Medium	Large
Nuclear ITS1	13	0.1432 ± 0.0034	0.2264 ± 0.0094	0.3280 ± 0.0075
	18	0.2650 ± 0.0060	0.3559 ± 0.0058	0.4510 ± 0.0094
	23	0.3932 ± 0.0064	0.4757 ± 0.0060	0.5660 ± 0.0062
	28	0.5133 ± 0.0064	0.5910 ± 0.0142	0.6969 ± 0.0164
Mitochondrial CytB	13	0.0366 ± 0.0032	0.1151 ± 0.0089	0.2109 ± 0.0070
	18	0.1215 ± 0.0056	0.2073 ± 0.0055	0.2969 ± 0.0089
	23	0.2124 ± 0.0060	0.2903 ± 0.0056	0.3755 ± 0.0059
	28	0.2957 ± 0.0060	0.3690 ± 0.0134	0.4690 ± 0.0155

Note: Each parameter is derived from the equation for model fitting to eDNA decay curves. C_0 is the adjusted eDNA concentration at time 0, and b, c, and a are constants estimated by the nonlinear least-squares regression (see Materials and Methods). Asterisks show that the corresponding coefficients were statistically significant (**p < .01; ***p < .001) in the model fitting. All raw mt-eDNA data are from Jo, Murakami, et al. (2019).

Moreover, almost all the studies targeted only mt-eDNA. In the present study, we addressed this knowledge gap by estimating the shedding and decay rates of nu-eDNA from Japanese Jack Mackerel and compared them with those of mt-eDNA. We found that higher water temperature and larger fish biomass accelerated both shedding and degradation of nu-eDNA, and the observed patterns were generally similar to those of mt-eDNA (Jo, Murakami, et al., 2019). In addition, we found that the ratios of mt-eDNA to nu-eDNA shedding and concentration ($Ratio_S$ and $Ratio_C$) changed depending on total fish biomass.

The tendencies of eDNA shedding and degradation for nu-eDNA and mt-eDNA in different water temperature and biomass levels generally showed similar patterns; these were accelerated with higher water temperature and larger fish biomass (Figure 3; Table 2). The effects of temperature and biomass on mt-eDNA shedding and degradation rates have been previously investigated. Moderately high temperatures (less than 50°C) and high densities of organisms could facilitate the activity of microbes and extracellular enzymes, therefore increasing mt-eDNA degradation (Bylemans et al., 2018; Jo, Murakami, et al., 2019; Levy-Booth et al., 2007; Nielsen, Johnsen, Bensasson, and Daffonchio, 2007; Strickler et al., 2015). In addition, mt-eDNA shedding increased with an increase in biomass/abundance/size of organisms, stress introduction, and, possibly, metabolic activation at higher temperature (Maruyama et al., 2014; Mizumoto, Urabe, Kanbe, Fukushima, & Araki, 2018; Jo, Murakami, et al., 2019; Klymus et al., 2015; Sassoubre et al., 2016). The findings that the shedding and degradation of nu-eDNA showed generally similar patterns to those of mt-eDNA may help the understanding of nu-eDNA properties and the interpretation of nu-eDNA detection in natural environments. In addition, the findings could support the hypothesis that the majority of eDNA exists in the form of intracellular DNA, such as cell and tissue fragments in water (Jo, Murakami, et al., 2019; Turner et al., 2014).

On the other hand, some differences between nu-eDNA and mt-eDNA were found. Nonlinear least-squares regression revealed that nu-eDNA degraded faster than mt-eDNA in all treatments (Table 2). Although the fragment size of nu-eDNA (164 bp) was slightly larger than that of mt-eDNA (127 bp), our experiment comparing mt-eDNA concentrations in water samples between different fragments revealed no statistical differences in concentrations of 127- and 164-bp mt-eDNA (see Appendix S1). This suggests that fragment size did not affect the difference in nu- and mt-eDNA decay rates and that the difference in decay rates between nu- and mt-eDNA in our study likely depended primarily on DNA characteristics, including structure and packaging. The nuclei in eukaryotic cells have a chromatin structure (Grunstein, 1997; Kornberg, 1974), which may prevent nuDNA from attack by nucleases, whereas mtDNA has a simple cyclic structure (Lindahl, 1993; Shadel and Clayton, 1997). In contrast, the linearity of nuDNA might make it susceptible to exonucleases that do not digest circular DNA molecules such as mtDNA

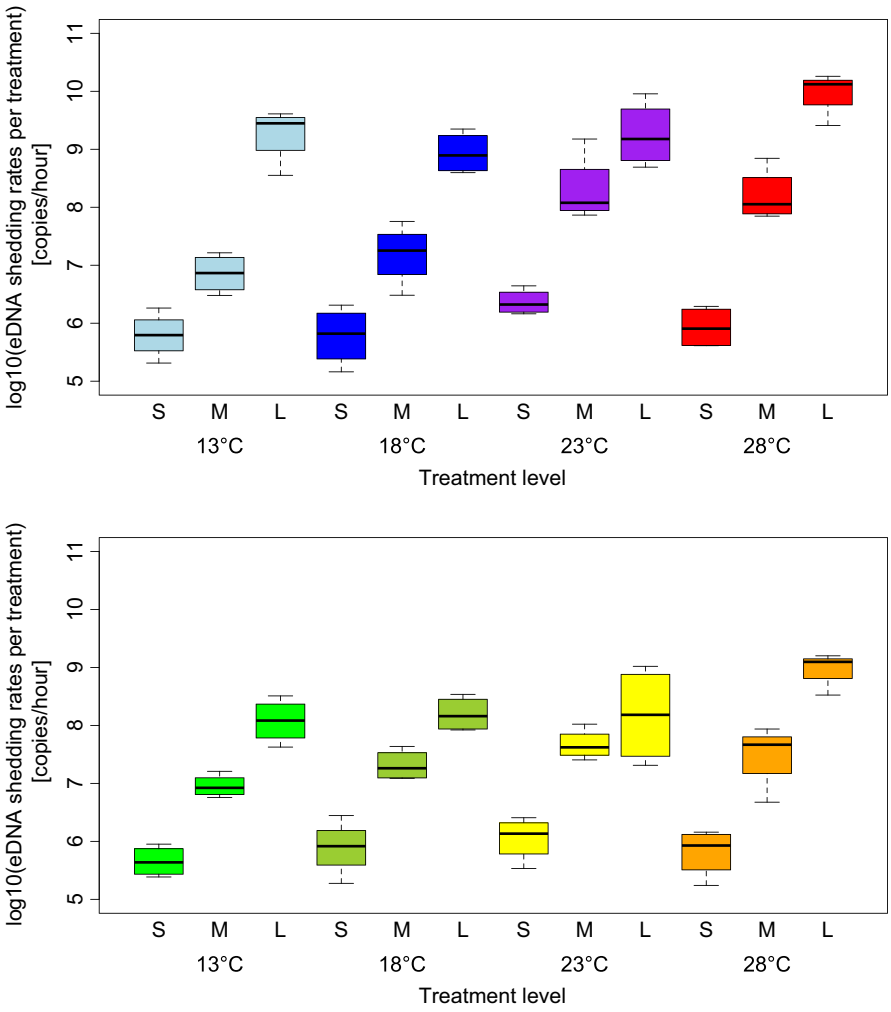

FIGURE 3 Results of nu-eDNA (upper) and mt-eDNA (lower) shedding rates per treatment. Fish biomass level: S, Small; M, Medium; L, Large. All raw mt-eDNA data are from Jo, Murakami, et al. (2019)

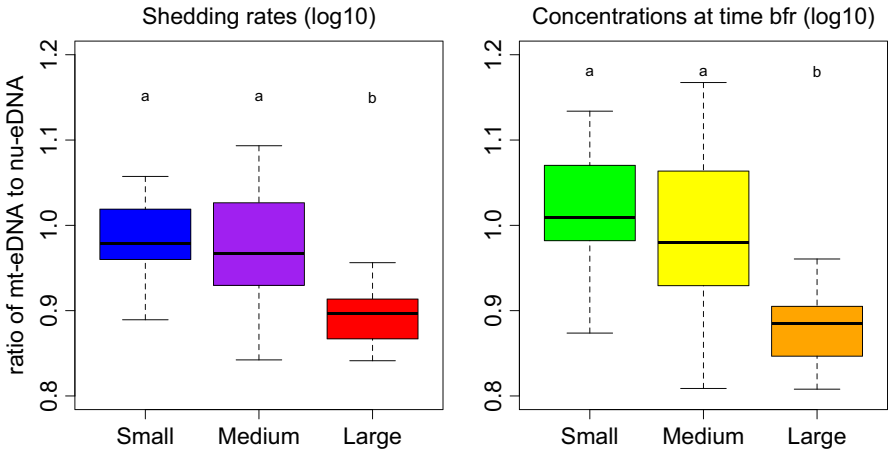

FIGURE 4 Results of the ratios of mt-eDNA to nu-eDNA shedding rates (left) and concentrations at time bfr (right); the four temperature levels were pooled to increase sample size. Factor levels with different letters are significantly different based on a post hoc Wilcoxon rank sum test with Bonferroni adjustment ($p < .05$)

(Alaeddini et al., 2010; Hosfield et al., 1998). Foran (2006) reported that degradation of mtDNA was slower than that of nuDNA in tissue samples, and some eDNA studies also implied that the nu-eDNA of macro-organisms degrades faster than mt-eDNA (Jo, Arimoto, Murakami, Masuda, & Minamoto et al., 2019; Moushomi et al., 2019). On the basis of these facts, it is likely that the persistence of mtDNA is longer than that of nuDNA in tissues as well as aquatic environments. However, it remains unknown which environmental factors the persistence of nuDNA and mtDNA depend on. Further studies are required to evaluate the structural and cellular differences between nuclei and mitochondria that may affect eDNA persistence and degradation.

The shedding rates of Japanese Jack Mackerel nu-eDNA showed similar patterns to those of mt-eDNA. On the other hand, we detected the interaction between fish biomass and type of DNA markers, where nu-eDNA shedding rates appeared to be

higher than those of mt-eDNA especially for Large fish biomass levels (Figure 3). Thus, we expected that the ratios of mt-eDNA to nu-eDNA shedding and concentrations ($Ratio_S$ and $Ratio_C$) might change depending on the fish biomass and body size and consequently confirmed that both $Ratio_S$ and $Ratio_C$ decreased with larger fish biomass levels (Figure 4). It implies that the increment of mt-eDNA shedding as increasing fish biomass and body size is smaller than that of nu-eDNA. Interestingly, the mtDNA copy number per cell or per gram of tissue is known to decrease with larger body size and/or aging for various taxa, which is caused by the accumulation of mtDNA point mutations and deletions (Hartmann et al., 2011; Hayakawa, Torii, Sugiyama, Tanaka, and Ozawa, 1991; Montier, Deng, and Bai, 2009). In our experiment, the fish in Small and Medium biomass levels were estimated to be 0+ years and those in Large biomass level to be 1+ years old (Mitani and Ida, 1964). Thus, our results may partly reflect the decrease of mtDNA in a cell, and likely free-floating DNA released from the cell, with maturity and aging. If the physiological phenomenon within organisms was reflected to environmental samples, the error of eDNA-based estimation of species biomass/abundance associated with age and developmental stage might be smaller for nu-eDNA than for mt-eDNA. Considering the higher concentrations and detectability of nu-eDNA compared with mt-eDNA (Dysthe et al., 2018; Minamoto et al., 2017), our results suggest that nuDNA marker may rather be superior to mtDNA marker for the quantification of eDNA in the field. As we did not target Japanese Jack Mackerels of older ages (to 5+ year; Mitani and Ida, 1964), further studies are needed to reveal the relationships between the eDNA ratios and body size with wider age structures.

In conclusion, we compared the shedding and degradation of nu-eDNA and mt-eDNA and showed that both processes were facilitated by high temperatures and large biomasses, which was generally similar between both markers. In addition, we found that the ratio of mt-eDNA to nu-eDNA was dependent on the fish biomass. These findings can contribute to an understanding of the characteristics and dynamics of eDNA, especially the similarity and difference between DNA markers, which would lead to the improvement of eDNA analysis (Barnes and Turner, 2016; Goldberg, Strickler, and Pilliod, 2015). On the other hand, some issues still need to be verified. Using mtDNA markers, several studies have reported the relationships between various environmental factors and eDNA production, persistence, and transport (Barnes et al., 2014; Buxton, Groombridge, and Griffiths, 2017; Eichmiller et al., 2016; Pilliod, Goldberg, Arkle, and Waits, 2014; Seymour et al., 2018). It is necessary to study more how environmental factors influence eDNA dynamics, as well as evaluate whether the influence of environmental factors is different for different DNA markers. These findings may help to interpret the eDNA detection and quantification for different DNA markers. Moreover, it would be subjects of future studies whether the ratio of mt-eDNA to nu-eDNA could depend on body size and ages for other species and in the field. If such relationships are found to be consistent in various situations, the ratio may offer a suitable index to estimate the age structure of a population from environmental samples. Alternatively, in combination with other indices (e.g., messenger RNA and protein), the ratio may help to estimate biological and physiological information (Barnes and Turner, 2016). This information would enable a more cost-effective and noninvasive conservation tactics for the management of aquatic ecosystems than those provided by traditional tools. Our study has addressed the groundwork required for the understanding of different eDNA characteristics and dynamics, and has provided valuable information to further utilize nuDNA markers for eDNA-based species conservation and management.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

T.J., R.M., and T.M. designed the experiments. T.J. and H.M. performed tank experiments. T.J. and M.A. performed molecular analysis, analyzed the data, and wrote the first draft of the manuscript. All authors edited and provided feedback for the manuscript.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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