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Utility of environmental DNA analysis for effective monitoring of invasive fish species in reservoirs

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Abstract. Dam-mediated biological invasions are a serious problem all over the world. Once established in reservoirs, the invasive species have catastrophic impacts on the river ecosystems downstream, and thus, rapid monitoring of invasive species is an urgent issue for the effective removal of them and the conservation management of native ecosystems. Here, we verified the utility of environmental DNA (eDNA) analysis as a tool to effectively monitor three invasive fish species (bluegill, largemouth bass, and smallmouth bass) in reservoirs using multiplex real-time PCR. First, to determine the optimal sampling location and season to detect eDNA from these species, we analyzed the eDNA in water samples from shore and offshore sites in three reservoirs all year around. We found that eDNA detection rates either did not differ between sampling locations or were higher for shore than offshore sites. In addition, eDNA detection rates were higher in spring (breeding season of target species) and/or summer than winter. Second, we extensively surveyed the distribution of the three species in 30 reservoirs in Japan using eDNA analysis. Consequently, a single eDNA-based surveillance in summer allowed to match approximately 90% of the presence/absence of the invasive fish species known from 27 yr of administrative capture-based surveillances. Given these results, we recommend collecting the replicated water samples from shore sites in summer or the breeding season for the effective detection of invasive fish eDNA in reservoirs. Our eDNA assays with multiplex real-time PCR enable the rapid and sensitive monitoring of invasive fish distribution in reservoirs.

Key words: biological invasions; bluegill (*Lepomis macrochirus*); environmental DNA; largemouth bass (*Micropterus salmoides*); multiplex real-time PCR; reservoirs; smallmouth bass (*M. dolomieu*).

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Introduction

A reservoir, or impoundment, is defined as the lentic environment upstream of a water storage dam, and it is used for flood control and irrigation (Tanida et al. 2014, Bellmore et al. 2017). There are more than 2700 dams in Japan (Japan Dam Foundation 2020), because the country has many steep and small riverine areas prone to flooding and drought (Umitsu 1981, Takemura 2007). By transforming rivers into semi-lentic systems, dam construction drastically alters the hydrological dynamics and has negative impact on the surrounding landscape and ecosystems, including habitat and biodiversity loss, taxonomic homogenization of fish species communities, landscape and habitat fragmentation, and the degradation of water quality due to eutrophication (De Ceballos et al. 1998, Hazell et al. 2001, Clavero and Hermoso 2011, Emer et al. 2013, Tanida et al. 2014, Benchimol and Peres 2015, Agostinho et al. 2016, Marques et al. 2018).

Moreover, biological invasions in reservoirs have recently been reported worldwide (Dudgeon et al. 2006, Usio et al. 2009, Clavero and Hermoso 2011, Ortega et al. 2015). In Japan, bluegill (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), and smallmouth bass (*M. dolomieu*) are known to be the most notorious invasive fish species, which are transported mainly by human release, passive migration from upstream, and contamination in stocking other fishes, and have seriously altered the species diversity in their colonized environments (Maezono et al. 2005, Takamura 2007, Yoshida et al. 2007). Once established in reservoirs, the fishes are dispersed downstream with dam water discharge and have catastrophic impacts on the native river ecosystems (Dudgeon et al. 2006, Tanida et al. 2014). In addition, their complete eradication and removal are considerably time- and labor-intensive. Therefore, rapid, extensive, and non-disruptive monitoring of invasive fish species in reservoirs is critical for effective management and conservation of native freshwater ecosystems. In Japan, control management of invasive fishes in reservoirs has so far been performed by capturing the fish using cast net, electrofishing, suppression of their breeding activities by collecting their broods spawned on artificial devices and drying up of their broods by the programmed water level drawdown (Azami et al. 2008, Tanida et al. 2014, Matsuzaki et al. 2019). However, these traditional methods for fish fauna surveys in reservoirs require substantial effort and cost due to their huge surface areas and water depth and are disruptive for native fauna (Darling and Mahon 2011).

Environmental DNA (eDNA) analysis can satisfy such requirements, allowing more effective and less disruptive monitoring of the distribution and composition of aquatic species (Minamoto et al. 2012, Bohmann et al. 2014, Deiner et al. 2017). Detection of eDNA, the genetic materials released by organisms into the environment, can indicate the presence of the target species without the need for direct observation nor capture (Bohmann et al. 2014). In addition, the rapid and extensive monitoring of invasive fish distribution *via* eDNA could help to eradicate them in reservoirs (Kamoroff et al. 2020). Although eDNA analysis has been applied to various taxa and natural environments (Fukumoto et al. 2015, Minamoto et al. 2017, Ushio et al. 2017, Carraro et al. 2018), the application of eDNA analysis for biodiversity assessment and ecological monitoring in reservoirs is relatively limited (Lim et al. 2016, Perez et al. 2017, Hayami et al. 2020). For example, Lim et al. (2016) concluded that eDNA could be useful for freshwater bioassessment in reservoirs by applying eDNA analysis using universal metazoan primers, and Hayami et al. (2020) investigated the seasonal change of fish communities in reservoirs using eDNA metabarcoding. Conversely, Perez et al. (2017) compared the distribution and biomass of largemouth bass and gizzard shad (*Dorosoma cepedianum*) in a reservoir between eDNA analysis and capturing and suggested that further refinement could be needed in eDNA sampling protocols.

In the present study, we verified the utility of eDNA analysis as a tool in monitoring representative invasive fish species (bluegill, largemouth bass, and smallmouth bass) in reservoirs in Japan. Here, we used the multiplex real-time PCR approach, which simultaneously detects target eDNA using multiple species-specific primers/probe sets labeled with different fluorescent dyes, which could reduce the analysis cost and detect the eDNA of several species with higher sensitivity than multiple single PCR runs (Jo et al. 2020). First, due to its huge surface area and water

depth, eDNA detection rates in reservoirs may vary depending on sampling location. In addition, given that eDNA production and persistence can be influenced by water temperature (Jo et al. 2019), eDNA detection rates in reservoirs may also vary depending on sampling season. Therefore, to optimize the efficiency of eDNA monitoring, we examined the effects of water sampling location (shore or offshore) and seasonality on eDNA detection rates. For the purpose, we collected water samples from three reservoirs in Japan all year around. Second, to evaluate the detectability of invasive fish species via eDNA analysis, we conducted the extensive surveillances of these invasive fish eDNA distribution in 30 reservoirs in Japan and compared the results with national census data. Since 1990, for the appropriate promotion of the projects and managements in Japanese dams, biological monitoring in reservoirs and their surroundings has been conducted by the M*inistry of* L*and*, I*nfrastructure, and* T*ransport*, targeting numerous taxa including fish (National Census on River and Dam Environments). The national census data are thus suitable as a comparison and reference for the results of eDNA detection.

Methods

Water sampling for the evaluation of sampling location and seasonality

We conducted seasonal water sampling in three reservoirs in Japan (Fig. 1). For these three reservoirs, all eDNA samples were the same as those described in Hayami et al. (2020). Briefly, in Miharu reservoir (Fukushima Pref., constructed in 1998), we selected ten shore and five offshore sites (15 sites in total) and monthly collected a 2 L surface water sample using a plastic bottle at each site from July 2015 to August 2016. In Okawa reservoir (Fukushima Pref., constructed in 1987), we selected six shore and three offshore sites (nine sites in total) and collected a 2 L surface water sample at each site from December 2015 to October 2016. In Sugo reservoir (Hyogo Pref., constructed in 1978), we selected two shore and three offshore sites (five sites in total) and collected a 2 L surface water sample at each site from September 2015 to September 2016. The number of sampling sites in each reservoir was determined according to its surface area (Miharu: 2.9 km^2, Okawa: 1.9 km^2, Sugo: 0.13 km^2). The maximum water depth is 65.0, 75.0, and 55.0 m in Miharu, Okawa, and Sugo reservoirs, respectively. When collecting water samples, we measured environmental parameters (water depth, water temperature, pH, and electrical conductivity [EC]; Data S1) at every sampling site. At Sugo, we prepared 2 L of distilled water as a field negative control, which was brought to the field and stored in a cooler box with the water samples.

After transporting water samples in a cooler box immediately to laboratory, we filtered them using a 47 mm diameter glass microfiber filter GF/F (nominal pore size 0.7 μm; GE Healthcare Life Science, Little Chalfont, UK). Each water sample was filtered through two GF/F filters (i.e., 500 mL × 2 filters = 1 L in total). On each sampling day, we filtered 1 L distilled water as a filtration negative control. After every use, we bleached all sampling bottles and filtering devices using 0.1% sodium hypochlorite solution for at least 5 min (Yamanaka et al. 2017). Until DNA extraction, we covered all filter samples with commercial aluminum foil and stored them at −20°C.

Capture surveys of our target fish species have been conducted by the National Census on River and Dam Environments in Miharu and Okawa reservoirs. In Miharu reservoir, bluegill and largemouth bass have been captured in every census to date, but no smallmouth bass has yet been captured in this reservoir. In Okawa reservoir, smallmouth bass has previously been captured in 2006 and 2011, but no bluegill and largemouth bass have been captured yet. Unfortunately, no such census has been conducted in Sugo reservoir, and thus, there is no reference for the presence/absence of the target species in this reservoir.

Water sampling for fish distribution survey in 30 reservoirs

We conducted water sampling in 30 reservoirs across Japan, where the presence/absence of target species was known from the National Census on River and Dam Environments (Fig. 1; Data S1). In the census, thorough capture-based surveys were conducted once every five years using cast nets, gill nets, and stationary nets. The year of construction, surface area, and water depth of the studied reservoirs ranged from 1954 to 2006, 0.42 to 8.90 km^2, and 45.0 to 156.0 m, respectively (Data

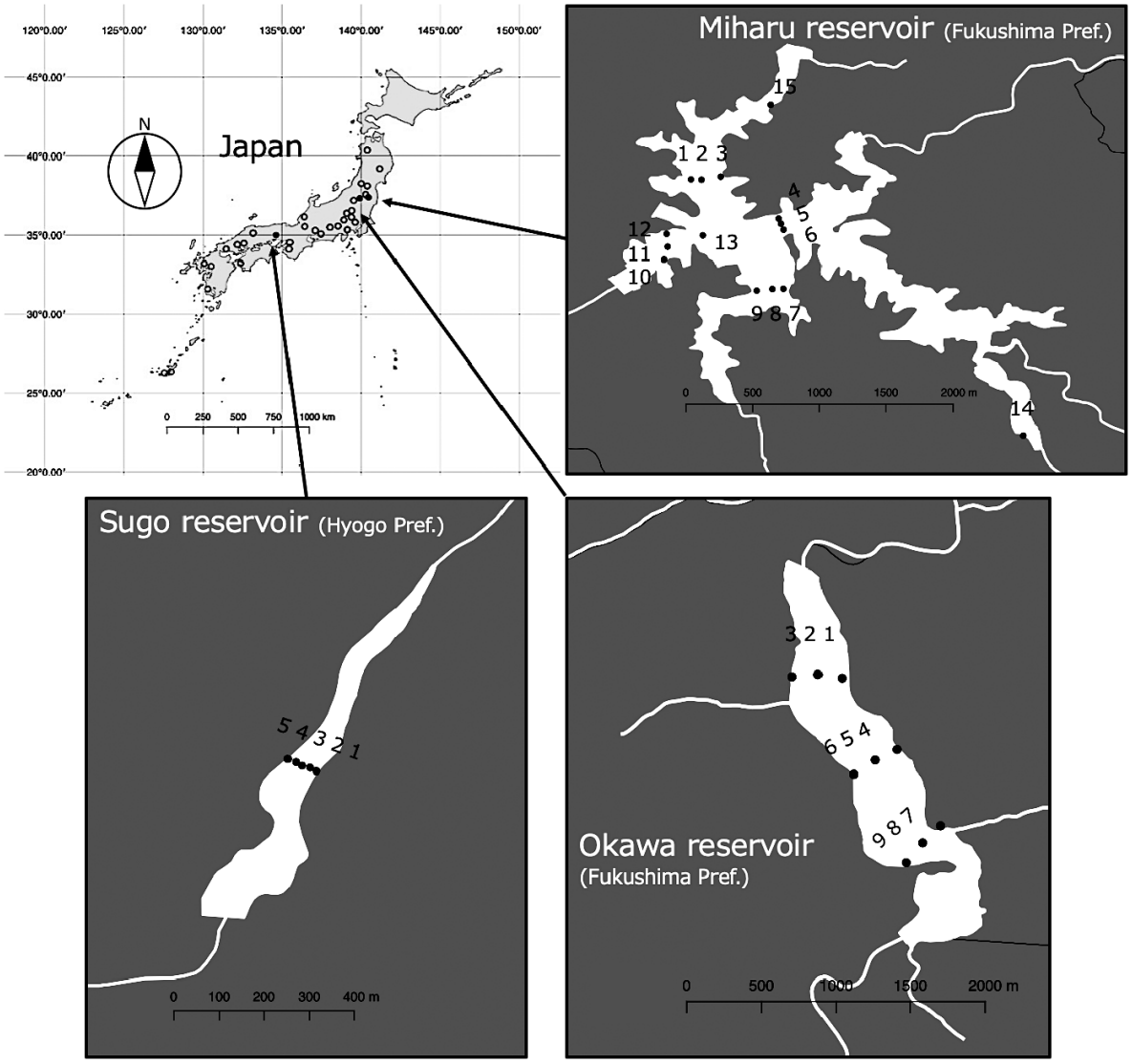

Fig. 1. Map of all reservoirs in Japan used for evaluation of the effects of sampling location and seasonality on eDNA detection rates (closed circles) and surveillances of fish eDNA distributions in 30 reservoirs (open circles). Site IDs of Miharu, Okawa, and Sugo reservoirs are shown on each reservoir.

S1). We selected two shore sites and one offshore site per reservoir (three sites in total, except for some reservoirs; Data S1) and collected a 2 L surface water sample at each site in August or September 2016. Sampling season was based on the results of the seasonality evaluation described above. We then added benzalkonium chloride (BAC) to each water sample, to a final concentration of 0.01%, to suppress DNA degradation (Yamanaka et al. 2017). After transporting the water samples to the laboratory, we filtered them on the same day using GF/F filters following the protocol described above. On each day of water filtration, we also filtered 1 L distilled water as a filtration negative control. We stored all filter samples at −20°C until DNA extraction.

DNA extraction

We extracted total DNA on GF/F filters using a DNeasy Blood and Tissue Kit (Qiagen, Hilden,

Germany) according to the method described in a previous study (Minamoto et al. 2019) with slight modifications. Briefly, we placed each filter in the suspended part of a Salivette tube (Sarstedt, Numbrecht, Germany) and then added 440 μL of solution, composed of 40 μL Proteinase K and 400 μL Buffer AL, to the filter. After incubation at 56°C for 30 min, we collected the liquid held on the filter by centrifugation. To increase the yield of eDNA, we rewashed the filter with 300 μL TE buffer for 1 min and collected the liquid again by centrifugation. We added 900 μL ethanol to the collected liquid and transferred the mixture to a spin column. Subsequently, we followed the manufacturer's instructions, and the total eDNA was eluted in 100 μL Buffer AE. All DNA extracts were stored in a freezer at −20°C until multiplex real-time PCR analysis.

Development of primers/probe sets for multiplex real-time PCR

We took the species-specific primers/probe sets that specifically amplified the mitochondrial cytochrome b (CytB) genes of bluegill, largemouth bass, and smallmouth bass from Jo et al. (2020; Table 1). We first re-checked the cross-reactivity for each primers/probe set because we used a different PCR platform from that used in Jo et al. (2020). Multiplex real-time PCR was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). Each 20 μL TaqMan reaction contained a combination of 3 μL of linearized plasmids containing PCR products (bluegill; Takahara et al. 2013) or synthesized artificial DNA fragments (largemouth bass, Yamanaka et al. 2016; smallmouth bass, this study) from the CytB gene of each target species (Data S1), a final concentration of 300 nmol/L of all forward and reverse primers, 40 nmol/L of all TaqMan probes, and 100 nmol/L of AmpErase Uracil N-Glycosylase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in 1× TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific). Thermal conditions of real-time PCR were as follows: 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. We simultaneously analyzed 3 μL pure water as a PCR negative control. All PCRs for template DNA and negative controls were performed in triplicate.

We then checked the sensitivity of the assay. Each 20 μL TaqMan reaction contained $3 \times 10^0 - 10^3$ copies of artificial CytB fragments of each target species described above, a final concentration of 300 nmol/L of all forward and reverse primers, 40 nmol/L of all TaqMan probes, and 100 nmol/L of AmpErase Uracil N-Glycosylase in 1× TaqMan Environmental Master Mix 2.0. Thermal conditions of PCR were the same as above. All PCRs for template DNA and negative controls were performed in triplicate except 3×10^0 copies of DNA with six replicates.

Multiplex real-time PCR for eDNA detection in reservoirs

The presence of each target species' eDNA was evaluated by a multiplex real-time PCR assay. Each 20 μL TaqMan reaction contained 5 μL of

Table 1. Primers/probe sets used in this study.

Target species	Name	Sequence (5′ → 3′)	Fragment size (bp)	Tm (°C)
Bluegill (*Lepomis macrochirus*)	Lma_F	GCC-TAG-CAA-CCC-AGA-TTT-TAA-CA	100	59.2
	Lma_R	ACG-TCC-CGG-CAG-ATG-TGT	100	61.3
	Lma_P	[FAM]-CGA-CAT-CGC-AAC-TGC-CTT-CTC-TTC-AGT-[BHQ1]	100	67.1
Largemouth bass (*Micropterus salmoides*)	Msa_F	GCC-CAC-ATT-TGT-CGT-GAT-GTA-A	106	59.5
	Msa_R	AGC-CCC-GGC-CGA-TAT-G	106	58.4
	Msa_P2	[Cy5]-CTA-ACG-GTG-CAT-CCT-TCT-TTT-TCA-TCT-GCA-[BHQ2]	106	65.9
Smallmouth bass (*Micropterus dolomieu*)	Mdo_F	TTG-CCT-TCC-ATT-TCT-TAT-TTC-CTT	114	57.1
	Mdo_R	TAT-CAG-CGT-CAG-AGT-TCA-ATC-CTA-A	114	59.6
	Mdo_P	[VIC]-TCG-CTG-CTG-CCA-CAG-TAA-TTC-ACC-TG-[NFQ]-[MGB]	114	67.4

Notes: Primers/probe sets were taken from Jo et al. (2020) except for Msa_P2, which was newly developed for the present study (we changed only the fluorescence dye at the end of 5′). All primers/probe sets targeted mitochondrial cytochrome b genes of the target species. Tm is the melting temperature for PCR.

template DNA, a final concentration of 300 nmol/L of each forward and reverse primer, 40 nmol/L of each TaqMan probe, and 100 nmol/L of AmpErase Uracil N-Glycosylase in 1× TaqMan Environmental Master Mix 2.0. Thermal conditions of PCR were the same as above. Five microlitre of tissue-derived DNA from each species and pure water was analyzed simultaneously as PCR positive and negative controls. All PCRs for eDNA extracts, positive controls, and negative controls were performed in triplicate. We regarded all eDNA signals with an obvious amplification curve and Ct values less than 50 as PCR positive. If any of the triplicates for each sample yielded a PCR positive, target eDNA was considered to be detected in the sample.

Statistical analyses

All statistical analyses were performed using R version 3.6.1 (R Core Team 2019). The data sets of smallmouth bass eDNA in Miharu, Okawa, and Sugo reservoirs and those of all fish eDNA in Okawa reservoir were excluded from further statistical analyses due to very low frequencies of target eDNA detection. We first evaluated the effect of sampling location on fish eDNA detection rates. Using the Wilcoxon rank sum test, we compared the rates of positive PCR replicates per month (i.e., the proportion of the number of positive PCR replicates out of all PCR replicates in each month), which were discrete and thus not parametric, between shore and offshore sites.

We then evaluated the seasonal fluctuation of fish eDNA detection rates by fitting a generalized additive mixed model (GAMM) with the Poisson distribution using the gamm4 package ver. 0.2-5 (Wood and Scheipl 2017). We included the number of PCR positives for each target eDNA in Miharu or Sugo reservoirs (i.e., 0, 1, 2, or 3) as the response variable, month as the smoothing term, and the number of sampling replication in each reservoir (i.e., $N = 15$ in Miharu and $N = 5$ in Sugo) and sampling year (2015 or 2016) as the random effects. Given the difference in eDNA detection rates between sampling locations (described below), shore and offshore water samples were individually analyzed for Miharu reservoir but were pooled for Sugo reservoir.

For the 30 reservoirs across Japan, the presence/absence of each fish species was based on the national census data and was compared with eDNA analysis by the exact McNemar test in the package exact2x2 ver. 1.3.6.1 (Fay 2010). Each fish species was considered to be present in the reservoir if its presence had been confirmed in any past National Census on River and Dam Environments surveillances (from 1990 to 2017). We compared the results of the eDNA analysis with the all-year and most recent (from 2013 to 2017) census data. In addition, the presence of largemouth bass in Kanna reservoir had been confirmed by the administrator of the dam. We compared the rate of positive PCR replicates in each reservoir between shore and offshore sites using the Wilcoxon rank sum test following the same method described above. In this analysis, we excluded the reservoir samples in which target eDNA was not detected from either shore or offshore sites.

Results

Cross-reactivity and sensitivity check for the eDNA assay

We confirmed that there was no cross-reactivity between any primers/probe sets (Data S1), which means that our multiplex real-time PCR assay could accurately detect the DNA from each target species. In the sensitivity check, all PCR replicates were positive at $3 \times 10^1 - 10^3$ copies of artificial DNA from each target species. In addition, four, one, and one out of six replicates were positive at three copies of DNA from bluegill, largemouth bass, and smallmouth bass, respectively.

Effects of sampling location and seasonality on eDNA detection rates

In Miharu reservoir, the detection rate of largemouth bass eDNA was significantly higher in shore than in offshore sites ($P < 0.001$; Fig. 2). Although the bluegill eDNA detection rate was not significantly different between sampling locations ($P = 0.1065$), the detection rate tended to be higher in shore than in offshore sites, particularly in May through August. Smallmouth bass eDNA was only detected in May and June at low detection rates, and no eDNA was detected in other months in Miharu (Data S1). In Sugo, there were no significant differences in bluegill and largemouth bass eDNA detection rates between sampling locations (both $P > 0.1$; Appendix S1:

Fig. S1). No smallmouth bass eDNA was detected in Sugo. In Okawa, largemouth bass and smallmouth bass eDNA were detected at very low detection rates in July through October, and no bluegill eDNA was detected (Data S1).

GAMM fitting generally showed statistical significance in seasonal fluctuations of invasive fish eDNA detection rates in Miharu and Sugo reservoirs (Fig. 3; Table 2). In Miharu, the detection rate of bluegill eDNA reached peaks in May and August for shore (estimated degrees of freedom [edf] = 6.4430, $P < 0.0001$) and in May and September for offshore (edf = 5.0950, $P < 0.05$) sites. Largemouth bass eDNA detection rates were the highest in May through September for shore (edf = 3.1090, $P < 0.0001$) and reached peaks in May and September for offshore (edf = 4.8530, $P < 0.05$) sites. In Sugo, there was no apparent seasonality to bluegill eDNA detection rates (edf = 1.000, $P = 0.6120$), possibly owing to its high detection rates around the year. In contrast, largemouth bass eDNA detection rates reached a peak in early June (edf = 3.8940, $P < 0.001$). There was no PCR amplification from any of the field, filtration, and PCR negative controls.

Surveillances of fish eDNA distributions in 30 reservoirs

We detected bluegill, largemouth bass, and smallmouth bass eDNA in 9, 13, and 4 reservoirs, respectively (Table 3). The results indicate that the concordances between eDNA analysis and the national census from 1990 to 2017 on the presence/absence of each fish species were 90.0%, 83.3%, and 90.0% for bluegill, largemouth bass, and smallmouth bass, respectively. No target eDNA was detected in the reservoir in which the fish had never been captured. Conversely, although the concordances of the results between methods were not very different, we detected target eDNA from the reservoirs where the corresponding fish species were regarded to be absent by the latest national census; bluegill eDNA was detected in the Ryumon reservoir, largemouth bass eDNA was detected in the Miyagase reservoir, and smallmouth bass eDNA was detected in the Watarase reservoir (Data S1 and Appendix S1: Table S1). The exact McNemar tests showed no significant differences in the detectability of each fish species between methods, regardless of the census year range (Tables 3 and Appendix S1: Table S1). There were no significant differences in bluegill and smallmouth bass eDNA detection rates between sampling locations (both $P > 0.05$; Appendix S1: Fig. S2), but largemouth bass eDNA detection rate was significantly higher in shore than in offshore sites ($P < 0.05$). There was no PCR amplification from any of the filtration and PCR negative controls.

Discussion

We evaluated the effects of sampling location and seasonality on eDNA detectability in reservoirs and optimized the strategy of eDNA sampling to effectively and sensitively monitor invasive fish species in reservoirs. These findings will lead to a decrease in false-negative detections of target eDNA caused by a very low DNA concentration in the water (Darling and Mahon 2011). In addition, the intensive eDNA monitoring in summer detected target eDNA in many reservoirs known to contain the target species. This is the first report on the utility of eDNA analysis as the effective monitoring tool of invasive fish species in reservoirs.

Sampling location

Current techniques for eDNA sampling in lentic freshwater ecosystems often involve grab sampling from the shore (Takahara et al. 2013, Dougherty et al. 2016, Bista et al. 2017, Sengupta et al. 2019). Such inshore sampling is simple, but it may not necessarily be effective due to the heterogeneous distribution of aqueous eDNA (Barnes and Turner 2016, Furlan et al. 2016), which may be especially important in large-scale ecosystems such as reservoirs. We thus compared the eDNA detection rates in reservoirs between shore and offshore sites.

Detection rates of all invasive fish eDNA did not differ between sampling locations or were higher in shore than in offshore sites. This is an important finding to simplify eDNA application in reservoirs because it is consuming to prepare bleaching solutions for a boat/ship required for offshore water sampling (Doi et al. 2017). These invasive fish species prefer the calm, shallow, and inshore habitats for foraging and breeding in warmer seasons (Iguchi et al. 2001, Nakao et al.

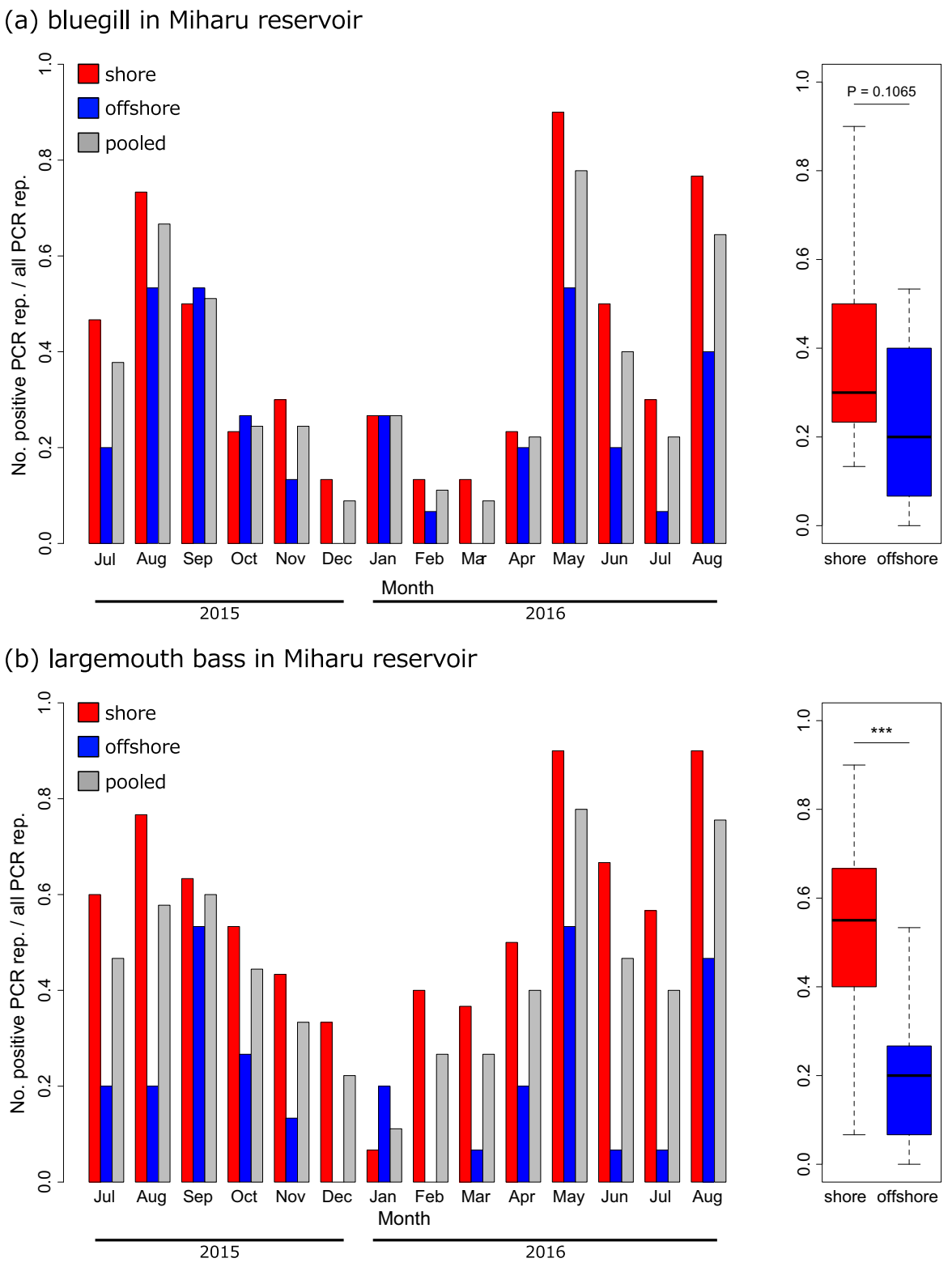

Fig. 2. Comparisons of monthly fish eDNA detection rates (i.e., the proportion of the number of positive PCR

(Fig. 2. *Continued*)

replicates out of all PCR replicates) between sampling locations in Miharu reservoir. Upper and lower graphs show the results of (a) bluegill and (b) largemouth bass. Bar graphs show detection rates of target eDNA each month, and right-side boxplots show annual averages. Asterisks *** indicate a significant difference in detection rates between sampling locations (Wilcoxon rank sum test; $P < 0.001$). Each color indicates as follows: shore sites ($N = 10$) in red, offshore sites ($N = 5$) in blue, and both sites ($N = 15$) in gray. Results for Okawa and Sugo reservoirs are shown in Appendix S1: Figs. S1, S2.

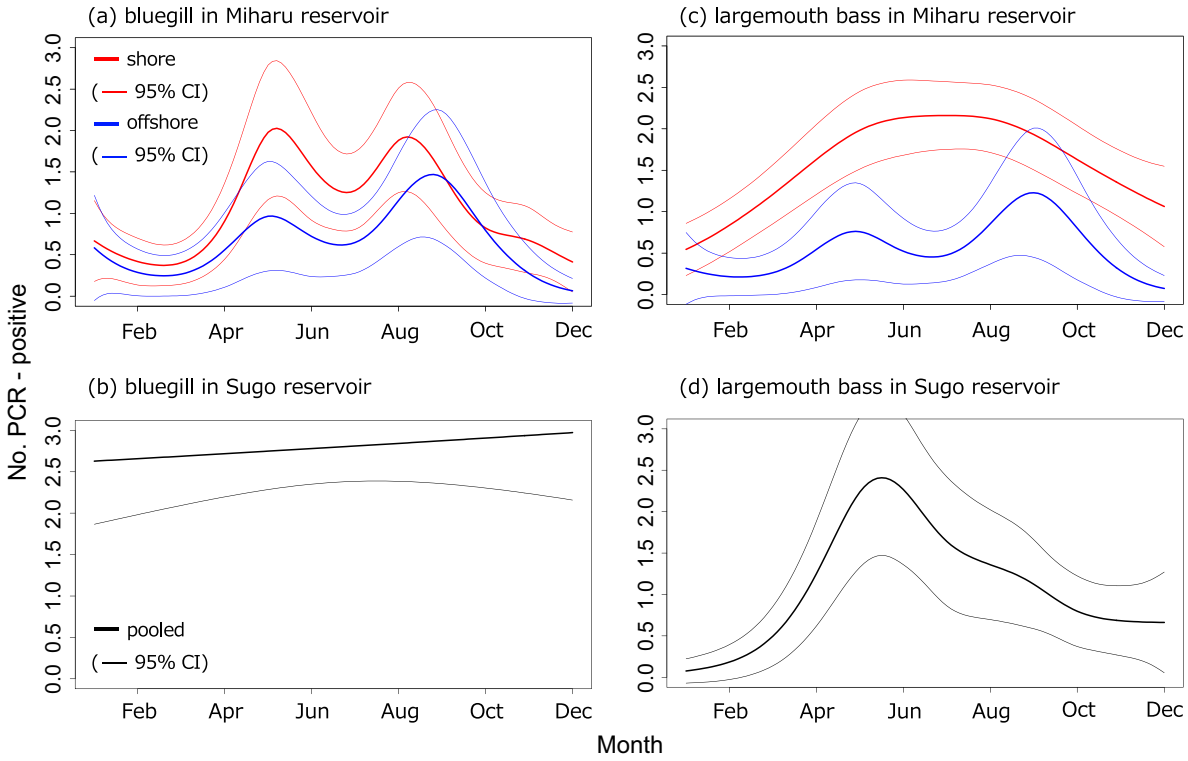

Fig. 3. Results of GAMM fitting for seasonal fluctuations in the number of PCR positives in Miharu (a, c) and Sugo (b, d) reservoirs. Model fittings for bluegill (a, b) and largemouth bass (c, d) eDNA detections are shown in left-side and right-side graphs. Bold lines show the target eDNA detection rates predicted by model fitting, and fine lines show their 95% confidence intervals (CI). Shore and offshore sites in Miharu reservoirs are shown in red and blue, while those in Sugo reservoirs were pooled and are shown in black.

2006, Takamura 2007, Tanida et al. 2014), and thus, the higher eDNA detection rates in shore sites could be associated with the spatial ecology of these fishes, which also supports shore-based eDNA sampling in freshwater ecosystems with a lower cost and requiring less effort than offshore sampling.

Alternatively, Hänfling et al. (2016) reported that the number of fish species estimated by eDNA metabarcoding was higher in shore than in offshore sites in lakes in the U.K. and suggested that fish eDNA might accumulate in the shore direction, which may increase the eDNA detection rates in shore sites. The hypothesis can also support the reasonability of eDNA sampling in shore sites, although eDNA signals would not necessarily indicate the presence of the species at or near the sampling site. However, some of

Table 2. Results of GAMM fitting to seasonal fluctuations of eDNA detection rates.

Reservoir	Target species	Location	Parametric coefficients (intercept)			Approximate significance of smooth terms (month)	
			Estimate	SE	*P*	edf	*P*
Miharu	Bluegill	Shore	−0.0341	0.1353	0.8010	**6.4430**	<0.0001
Miharu	Bluegill	Offshore	**−0.6087**	0.1950	0.0018	**5.0950**	0.0190
Miharu	Largemouth bass	Shore	**0.4194**	0.0712	<0.0001	**3.1090**	<0.0001
Miharu	Largemouth bass	Offshore	**−0.8047**	0.2323	0.0005	**4.8530**	0.0327
Sugo	Bluegill	Pooled	**1.0289**	0.0772	<0.0001	1.0000	0.6120
Sugo	Largemouth bass	Pooled	−0.2828	0.1839	0.1240	**3.8940**	0.0003

Notes: In Sugo reservoir, shore and offshore sites were pooled because there were no significant differences in eDNA detection rates between sampling locations. The abbreviation "edf" is the estimated degrees of freedom in GAMM fitting. The statistically significant estimates are shown in bold.

Table 3. Presence/absence of each fish species in 30 reservoirs in Japan according to eDNA analysis and the National Census on River and Dam Environments.

Species	Presence/absence	eDNA detection		Concordance rate (%)	*P*
		Positive	Negative		
Bluegill	Presence	9	3	90.0	0.2500
	Absence	0	18		
Largemouth bass	Presence	13	5	83.3	0.625
	Absence	0	12		
Smallmouth bass	Presence	4	3	90.0	0.2500
	Absence	0	23		

Notes: The exact McNemar tests show that there were no significant differences ($P > 0.05$) in the detectability of each fish species between methods. Shore and offshore eDNA samples were pooled in this analysis. Each table shows the concordance rate of the presence/absence of target species based on the national census compared with the detection/non-detection of target eDNA. See Table 1 for scientific names of target species.

recent studies have implied that eDNA distribution in lentic ecosystems is rather localized in space and time (Harper et al. 2019, Lawson Handley et al. 2019, Li et al. 2019, Jeunen et al. 2020). Therefore, although further refinement of water sampling locations in lentic systems is still needed, it is likely that the spatial ecology of our target fishes strongly contributed to an increase in eDNA detectability at the shore in the present study.

Seasonality

Detection rates of target eDNA were generally higher in the late spring through late summer than in autumn to winter. Remarkably, in some data sets, there were two peaks of target eDNA detection rates according to GAMM fitting. Given that all of the fish species in the present study reproduce in late spring to early summer (Iguchi et al. 2001, Nakao et al. 2006, Shindo et al. 2006), the first peak of eDNA detection could be due to spawning activity, male-to-male competition, and the aggregation of juveniles along the shore (Bylemans et al. 2017, Xu et al. 2018), while the second could be due to an increase in fish activity with warmer temperatures (Clarke and Johnston 1999, Takahara et al. 2012, Jo et al. 2019).

There was no seasonal fluctuation in the largemouth bass eDNA detection rate in Sugo, which might be attributed to the reservoir's relatively small water volume and high biomass density of the fish throughout the year. Conversely, the detection of smallmouth bass eDNA in Miharu reservoir could be a false-positive because this species has never been confirmed to be present in this reservoir (National Census on River and Dam Environments). Although smallmouth bass are known to exist in the Abukuma River, this river is just downstream of Miharu reservoir.

Besides, the national census has confirmed the presence of a number of great cormorants (*Phalacrocorax carbo*), which prey on the fish, in Miharu reservoir. Thus, the cormorants' feces could likely be the source of smallmouth bass eDNA in the reservoir.

The timing of the peaks of eDNA detection might also be associated with the seasonal movement of the fish in the reservoirs. In Miharu reservoir, after the reproduction period in late spring to early summer, 0+-yr juveniles are observed to aggregate along the shore and around artificial surface structures such as floating barriers and water intake facilities in summer to early autumn (Jiro Okitsu and Katsuki Nakai, *personal observation*). In addition, detection frequencies of the fishes by electrofishing and observation become fewer after mid-October (Jiro Okitsu, *personal observation*), which suggests that these fishes migrated from the shallows to deeper waters before overwintering. Thus, the seasonal pattern of migration of the fishes likely affected the detectability of their eDNA in the reservoirs.

Comparison of invasive fish distributions in reservoirs between methods

The presence/absence of each species was determined based on intensive capture-based surveys from 1990 to 2017 (National Census on River and Dam Environments), which served as a highly reliable reference for the eDNA monitoring in the present study. Here, we showed that a single eDNA monitoring in summer allowed to match approximately 90% of the known presence/absence of the targeted fish species in 30 reservoirs. This result indicates that eDNA analysis can rapidly and accurately detect aquatic species with low biomass/abundance, and thus outperform traditional monitoring methods such as observation and capture in reservoirs, as well as other natural environments (Darling and Mahon 2011, Takahara et al. 2013, Fukumoto et al. 2015, Dougherty et al. 2016). In addition, invasive fish eDNA was detected in some of the reservoirs where the presence of corresponding fish species was not confirmed by the latest census (from 2013 to 2017), implying that, regardless of the year-on-year fluctuation of species abundance in a reservoir, eDNA analysis can detect invasive fishes with higher sensitivity than traditional methods.

In some reservoirs, we failed to detect target eDNA despite the species being present according to the national census. These inconsistencies appeared not to correlate with hydrogeographic factors (year of construction, surface area, and water depth of reservoirs). In addition, among the reservoirs in which the target species was known to be present, target eDNA was not detected in shore sites only at Shin-Toyone reservoir. Thus, such false-negative eDNA detections might be suppressed by increasing the number of sampling replicates taken from shore sites. Some preceding studies suggested that more sampling replicates than ours could lead to the sufficient eDNA detection (i.e., >95% of detection probability; Hunter et al. 2015, Furlan et al. 2019), although it depends on the environmental conditions and may increase overall survey costs. In the future, the trade-offs among eDNA detectability, sampling replicates and volumes, and survey costs will need to be investigated to determine the most practical methods for eDNA monitoring of fish in reservoirs. Nevertheless, there is little doubt that eDNA analysis can infer the presence/absence of invasive species with much less effort, and similar or higher accuracy and sensitivity than conventional methods.

Conclusions and perspectives

We found that the replicated water sampling from shore sites in summer or the breeding season led to highly accurate estimations of the presence/absence of invasive fish species in reservoirs. In addition, eDNA monitoring in reservoirs could be made more cost-effective in the future by identifying the trade-offs among eDNA detectability, the number of sampling replicates, and overall survey costs. As far as we know, this is the first study to determine the location and season appropriate for sampling invasive fish eDNA and to maximize eDNA detectability in reservoirs. Our findings could contribute to effective monitoring and management of invasive species in reservoirs.

We used multiplex real-time PCR, which can simultaneously detect multiple target eDNA with high sensitivity in a single analysis (Jo et al. 2020). The approach is preferable to species-specific eDNA detection and eDNA metabarcoding in terms of sensitivity and cost-effectiveness, which is important for rapid monitoring of

multiple invasive fish distributions not only in reservoirs but also in other freshwater ecosystems. Therefore, our eDNA assay might also contribute to the early detection and management of introduced invasive species *via* eDNA analysis in other freshwater ecosystems such as farm ponds, where biological invasions have seriously been reported (Dudgeon et al. 2006, Takamura et al. 2007, Jo et al. 2020). Our study would provide the information valuable for effective monitoring of invasive fish species distribution in freshwater ecosystems including reservoirs.

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Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.3643/full