Vol. 609: 187–196, 2019
https://doi.org/10.3354/meps12828

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published January 17

First use of oceanic environmental DNA to study the spawning ecology of the Japanese eel *Anguilla japonica*

Aya Takeuchi[1], Shun Watanabe[2,*], Satoshi Yamamoto[3], Michael J. Miller[4], Tatsuhiro Fukuba[5], Tetsuya Miwa[5], Tatsufumi Okino[6], Toshifumi Minamoto[3], Katsumi Tsukamoto[1,4]

[1]Graduate School of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa-shi, Kanagawa 252-0880, Japan
[2]Department of Fisheries, Faculty of Agriculture, Kindai University, Nara 631-8505, Japan
[3]Graduate School of Human Development and Environment, Kobe University, Hyogo 657-8501, Japan
[4]Department of Marine Science and Resource, Nihon University, 1866 Kameino, Fujisawa-shi, Kanagawa 252-0880, Japan
[5]Marine Technology and Engineering Center, Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima-cho, Yokosuka, Kanagawa 237-0061, Japan
[6]Faculty of Environmental Earth Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan

ABSTRACT: Environmental DNA (eDNA) analysis is emerging as an innovative tool to assess the distribution and biomass of species in aquatic environments that enables quick and non-invasive surveys compared to conventional sampling methods such as fishing, hydroacoustics, and diving observations. We applied eDNA analysis to spawning ecology research of the Japanese eel *Anguilla japonica* in the western North Pacific for the first time. A preliminary laboratory experiment using tank water containing eels confirmed that our specific primers and probe could identify the Japanese eel from a small amount of eDNA (about 0.5 ng per PCR reaction). During a 19–20 May 2015 ocean survey at the southern West Mariana Ridge, seawater samples were collected at 12 water depths at 9 stations in the spawning area of *A. japonica*. Out of 108 seawater samples, DNA was successfully amplified from 3 samples, which were collected at 250 and 400 m. According to published research on Japanese eel spawning ecology and eDNA dynamics, the eDNA positive signals obtained from the oceanic survey could have been from adult Japanese eels or from larvae from the last spawning events. Thus onboard eDNA analysis proved to be a viable approach for detecting Japanese eels in their spawning area along the southern West Mariana Ridge.

KEY WORDS: eDNA · Freshwater eel · Real-time PCR · Spawning ecology · Oceanic survey

1. INTRODUCTION

Environmental DNA (eDNA) refers to genetic material in the environment from the metabolic wastes, damaged tissues, or sloughed skin cells of multicellular organisms (Kelly et al. 2014, Barnes & Turner 2016). eDNA analysis of collected water samples can detect aquatic organisms and is now emerging as a quick, inexpensive, and non-invasive tool for studying the presence or relative abundance of organisms in natural environments (Ficetola et al. 2008, Takahara et al. 2013). Recent studies have used eDNA analysis to evaluate the distribution of introduced species and endangered or rare species (Jerde et al. 2011, Fukumoto et al. 2015, Bergman et al. 2016), estimate the abundance and biomass of target

*Corresponding author: swpc@nara.kindai.ac.jp

species (Pilliod et al. 2013, Yamamoto et al. 2016), and monitor fish fauna in freshwater areas, coastal waters, and coral reefs (Thomsen et al. 2012, Miya et al. 2015). The new approach using eDNA has the potential to revolutionize ecological and biological studies, biodiversity monitoring, and conservation assessments of aquatic ecosystems.

The Japanese eel *Anguilla japonica* migrates thousands of kilometers to breed in the open ocean after its growth stage in freshwater and estuarine habitats, and like other anguillid species, its spawning area had remained a mystery because of its unique life cycle as a catadromous fish (Tsukamoto 2009). Intensive research has been conducted to discover the Japanese eel spawning area, and has included sampling with large plankton or trawl nets to collect transparent leaf-like larvae, called leptocephali (Tsukamoto 1992, Shinoda et al. 2011), or adult eels (Chow et al. 2009). Genetic species identification of eggs and newly hatched larvae (preleptocephali) collected during research cruises, which earlier was usually carried out in the laboratory after a cruise (Ishikawa et al. 2001, Yoshinaga et al. 2011), can now be done soon after specimens are collected using onboard real-time PCR (Watanabe et al. 2004, Minegishi et al. 2009). Collections of eggs, preleptocephali, and adult eels using conventional methods have revealed that the spawning area of the Japanese eel is located along the seamount chain of the southern West Mariana Ridge in the western North Pacific (Tsukamoto 2006, Chow et al. 2009, Tsukamoto et al. 2011).

Several biological and oceanographic factors appear to influence the longitude, latitude, timing, and potential depths where spawning of the Japanese eel occurs. From 2005 to 2009, more than 1000 preleptocephali were collected within a narrow area from 12° to 15° 30′ N and 140° 30′ to 143° E along the southern part of the West Mariana Ridge (Fig. 1B; Tsukamoto et al. 2011, Aoyama et al. 2014). Female adult eels and preleptocephali were also collected at the southern West Mariana Ridge (Kurogi et al. 2011). The seamount chain includes the shallow Pathfinder, Arakane, and Suruga seamounts that appeared to be possible locations for the spawning area of the Japanese eel, which led to the 'seamount hypothesis' that eels might spawn at the seamounts (Tsukamoto et al. 2003), and the seamount ridge seems to determine the longitude of its spawning area. The distribution patterns of small leptocephali led to the hypothesis that a salinity front formed by low-salinity surface water in the North Equatorial Current may influence the latitude where spawning occurs (Tsukamoto 1992), because the position of the front can change among dif-

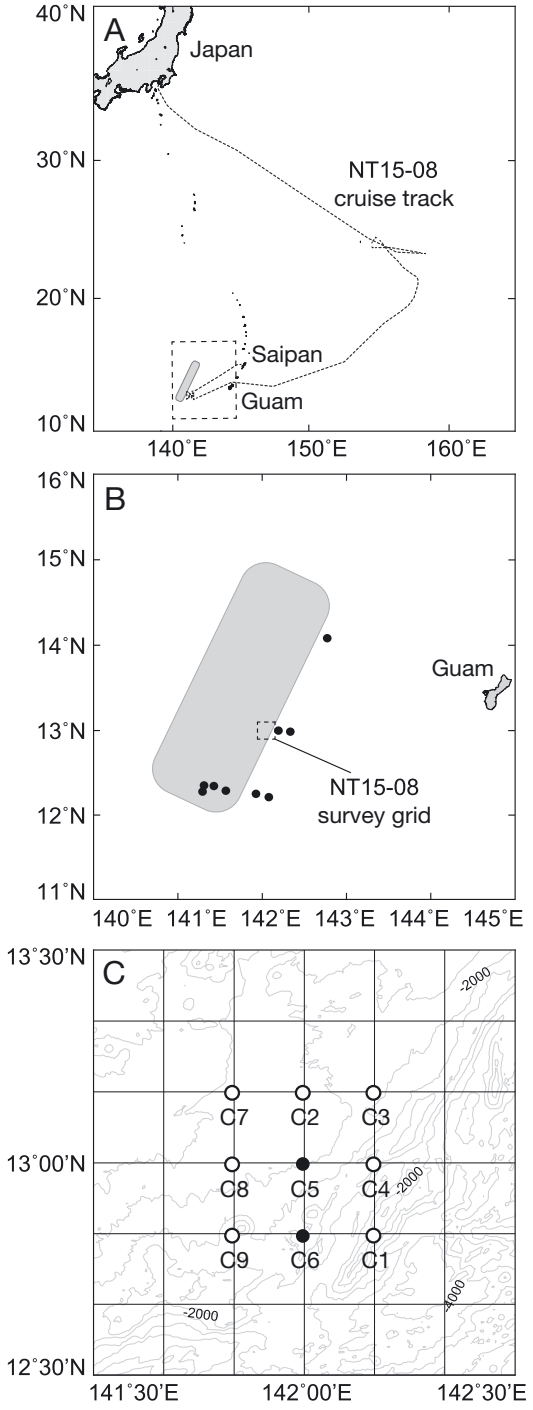

Fig. 1. Study area, showing (A) the cruise track of the NT15-08 research cruise and how the ship moved to an eastern region to avoid rough seas in the survey area caused by 2 typhoons, (B) the locations where Japanese eel adults (black circles) and preleptocephali (gray shaded area) were collected in previous cruises from 2005 to 2009 (Tsukamoto et al. 2011, Aoyama et al. 2014), and (C) the NT15-08 survey grid that consisted of 9 water sampling stations (circles) along the western side of the southern West Mariana Ridge. Black circles in (C) show the stations that were positive for eDNA

ferent months or years (Kimura & Tsukamoto 2006, Aoyama et al. 2014). Analyses of the back-calculated hatching dates determined from the otolith microstructure of Japanese eel leptocephali collected near the spawning area led to the development of the 'new moon hypothesis', which proposed that the adults spawn periodically and synchronously once a month at new moon during their spawning season (Ishikawa et al. 2001, Tsukamoto et al. 2003). In addition, the depths where adults and preleptocephali were captured suggested that Japanese eel spawning occurred in shallower layers of about 150 to 200 m, which was at the upper limit of the thermocline and not at greater depths (Tsukamoto et al. 2011, Aoyama et al. 2014). Although some information was gathered about the spawning area of Japanese eels, their precise spawning sites and ecology still remain unknown, especially regarding how they form spawning aggregations and what their natural spawning behaviors are.

Efforts were made during a previous cruise to observe natural aggregations and spawning behavior of Japanese eels using the Shinkai 6500 submersible and Deep-Tow camera system, which were able to record pelagic eels including a possible male *A. japonica* (Tsukamoto et al. 2013, Miller et al. 2014). Fukuba et al. (2015) reported on the deployment of new drifting camera systems that included live artificially matured female and male Japanese eels held in chambers as an attractant source, and pelagic eels were also observed. These camera systems have shown potential for observing the aggregations or spawning behavior of Japanese eels in the open ocean, but clear images of this species have not been recorded yet due in part to the vast scale of the possible areas to deploy the camera systems, and the need to narrow down the search areas.

To observe the spawning behavior of Japanese eels, it may be useful to estimate their real-time presence while onboard a research vessel and to help infer the spawning sites where spawning-condition female and male eels aggregate. In this study, therefore, we attempted to infer the presence of Japanese eels using eDNA analysis in their spawning area and tested the viability of this type of analysis during a research cruise (Fig. 1).

2. MATERIALS AND METHODS

2.1. Laboratory eDNA experiment

To test whether the eDNA of Japanese eels *Anguilla japonica* can be detected from water samples using real-time PCR, we conducted a laboratory experiment before the cruise to detect eDNA in a water sample from a rearing tank. This preliminary laboratory experiment was conducted in April 2015. We collected a water sample from a 100 l freshwater tank (45 × 89 × 45 cm) containing 3 Japanese eels (40–80 cm in total length) caught in rivers. The Japanese eels were held in the tank with a recirculating filtration system and constant aeration at about 20°C for about 3 yr, and were fed on an irregular basis. A water sample (500 ml) was vacuum-filtered onto a 47 mm diameter glass fiber filter (pore size 0.3 µm, Advantec), and then the eDNA was extracted from the filter using a DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol and Miya et al. (2015). The total eDNA was eluted in 50 µl of buffer supplied by the manufacturer. The concentration of eDNA extract was measured using a Nano-drop 2000 spectrophotometer (Thermo Fisher Scientific), and the extract was diluted 10-fold to experimental concentrations of 10, 1, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} ng μl^{-1}.

Real-time PCR (not qPCR) for eDNA analysis was carried out on the different eDNA concentrations using a Light Cycler® Nano (Roche) thermocycler with 65 cycles of 2-step amplification (denaturation at 95°C for 20 s, and annealing at 60°C for 40 s) after an initial 10 min denaturation at 95°C. The reaction mixture (20 µl) contained 2.8 µl PCR-grade water (Roche), 1 µl of each primer (10 µM), 0.2 µl of TaqMan probe (10 µM), 10 µl of 2× Master Mix (Roche), and 5 µl of diluted eDNA as template. Total DNA extracted from Japanese eel tissues was used as a positive control for PCR. A negative control (pure water as template) was analyzed with the same protocol to monitor contamination during filtering, eDNA extraction, and real-time PCR. We used species-specific primers (forward: 5'-AATCAGTAA TAAGAGGGCCCAAGC-3', reverse: 5'-TGTTGGGT TAACGGTTTGTGGTA-3') and a TaqMan probe (5'-CACATGTGTAAGTCAGAACGGACCGACC-3') developed by Watanabe et al. (2004), which amplified a 107 bp fragment of the 16S rRNA region of mitochondrial DNA (mtDNA), for the real-time PCR of the eDNA analysis. The mtDNA was used as the genetic marker because the copy numbers of mtDNA are greater than those of nuclear DNA per cell, and the detection rate is likely to be higher in the former even when DNA is present at low concentration and/or is degraded (Rees et al. 2014). Watanabe et al. (2004) checked the specificity of the primers and probe using DNA from tissue of *A. japonica*, *A. marmorata*, *A. bicolor pacifica*, and the 6 anguilli-

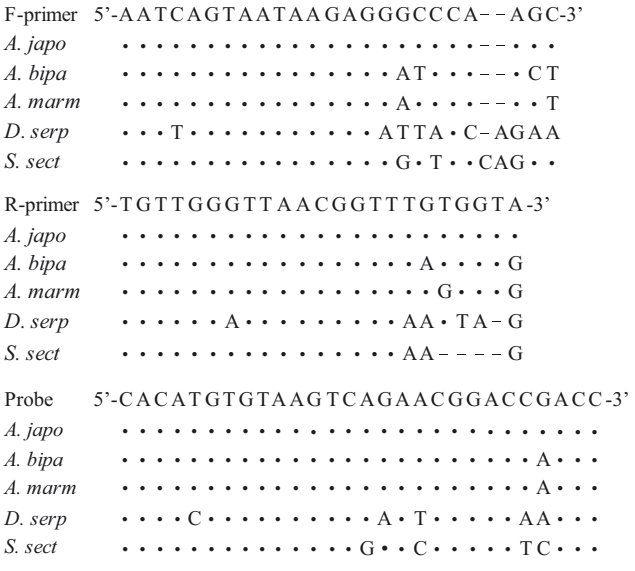

Fig. 2. Nucleotide variations in the primers and probe of the mitochondrial 16S rRNA gene between *Anguilla japonica* (*A. japo*), *A. bicolor pacifica* (*A. bipa*), *A. marmorata* (*A. marm*) (from Watanabe et al. 2004, Minegishi et al. 2009), *Derichthys serpentinus* (*D. serp*, GenBank accession number AP010851), and *Serrivomer sector* (*S. sect*, GenBank accession number AP007250). Dots indicate nucleotides identical to those of the primers and probe on the top line, and gaps show insertions/deletions

form species *Conger myriaster*, *Serrivomer sector*, *Strophidon ui*, *Rhinomuraena quaesita*, *Uropterygius* sp., and *Stemonidium hypomelas*, and only the DNA of *A. japonica* showed successful amplification, although that of *A. marmorata* also amplified at a clearly slower rate and to a much lower degree. The sensitivity of the primers and probe was also investigated using template DNA (concentrations from 2.0 pg μl^{-1} to 7.0 ng μl^{-1}) of *A. japonica*, *A. marmorata* and *A. bicolor pacifica*, and *A. japonica* was specifically distinguished from the other anguillids (Minegishi et al. 2009). These specific primers and probe could even identify the Japanese eel from a low amount of DNA (about 6.0 pg per PCR reaction) from a diluted DNA solution extracted from tissue (Minegishi et al. 2009). Therefore, the primers and probe have high specificity for the Japanese eel (Fig. 2), which can be adapted for eDNA analysis in aquatic environments that may have low amounts of eDNA of the target species.

2.2. Oceanic eDNA survey

The eDNA field survey was designed to estimate the presence of Japanese eels and their spawning sites during the RV 'Natsushima' cruise (NT15-08) from 5 to 21 May 2015 (new moon: 18 May). After a delay caused by 2 successive typhoons, intensive research was conducted in the survey area (Fig. 1) from 19 to 20 May 2015, which were the first and second days after the new moon. According to the new moon hypothesis (Ishikawa et al. 2001, Tsukamoto et al. 2003) and previous collections of eggs (Tsukamoto et al. 2011, Aoyama et al. 2014), the spawning activity of that month should have already begun and was possibly finished. The eDNA survey grid was originally planned to be set based on hydrographic features such as the location of the salinity front because spawning of Japanese eels would likely occur just at the southern side of the front (Tsukamoto et al. 2011, Aoyama et al. 2014) and possibly in a high-energy part of the internal tide field, which might be used as a signpost by the eels to detect their spawning sites (T. Higuchi et al. unpubl. data). As we had no time to implement all research plans, the grid was arranged around 142° E and 13° N (called the 'Kaiyo point') where Japanese eel eggs (Tsukamoto et al. 2011, Aoyama et al. 2014) and adults (Chow et al. 2009) had been caught previously, and this location included a patch of high internal tide energy.

The eDNA analysis was conducted using 2 l seawater samples in plastic bottles that were collected during 19 and 20 May 2015 from each of 11 water depths (100, 200, 250, 300, 400, 500, 600, 700, 800, 900, and 1000 m) at 9 stations with the Niskin water samplers of the CTD system (Fig. 1C). New gloves were worn at each water sampling station to avoid cross contamination. These CTD deployments were also used for collecting hydrographic data to depths of 1000 m for analysis of the hydrographic structure. Two liter water samples from 3.6 m depth were collected from the faucet of the ship's scientific seawater pumping system at each station as a surface water sample, and 2 l of pure water were used as a negative control for monitoring any contamination at each station. The 2 l of each water sample from the 12 water depths (11 CTD samples, 1 surface water sample) at each station and pure water were vacuum-filtered onto a 0.3 µm pore size (47 mm diameter) glass fiber filter (Advantec) onboard, and a total of 117 filters including 9 filtration blanks were obtained. All filters were preserved by freezing at −20°C until eDNA extraction onboard or mostly later in the laboratory.

To test the ability to conduct eDNA analysis onboard, eDNA was extracted within 30 min after filtration of 32 samples that consisted of 4 filtration blanks and 28 seawater samples collected at 7 of the water depths (200, 250, 300, 600, 700, 800, and 900 m) at Stations (Stns) C1, C2, C3, and C4. The negative con-

trol blank made at each of these 4 stations was processed using the same filtering and eDNA extraction procedures as the other samples that were extracted onboard the ship. Real-time PCR assays (not qPCR) were then carried out with the 32 eDNA extracts and PCR blanks onboard. The procedure of eDNA extraction, the number of amplification cycles, amplification parameters, primers and probe were the same as those used in the preliminary laboratory experiment described above (see laboratory eDNA analysis). The eDNA extracts from the 32 samples and the PCR amplicons were stored at −20°C immediately after the PCR assay. Since the ship time was limited, the remaining 85 filters were wrapped in aluminum foil with tweezers and stored at −20°C until eDNA extraction on land.

We transported 85 filters, 32 eDNA extracts, and the PCR amplicons on ice in a cooler box from the ship to the laboratory. Total eDNA was extracted from the remaining 85 filters including 5 filtration blanks. eDNA was also extracted from pure water alongside the 85 filters; thus we obtained 80 eDNA extracts, 5 filtration blanks, and 5 extraction blanks in the laboratory. All eDNA samples extracted both on the ship and in the laboratory were subjected to real-time PCR (not qPCR) with the same conditions as the 32 samples analyzed onboard. Each of the real-time PCR runs contained 3 replicates of each extracted eDNA sample solution and each filtration, extraction, and PCR blank. Tissue-derived Japanese eel DNA was used as a positive control for the real-time PCR. The real-time PCR products showing amplifications were verified by electrophoresis using a 2% agarose gel, stained with SYBR® safe DNA gel stain (Invitrogen), and visualized under ultraviolet light on a Gel Doc™ EZ imager (Bio Rad).

For 11 eDNA seawater samples, a lower amplification curve was observed than with the other positive samples (fluorescence intensities were less than 0.5), so inhibition tests were conducted to verify that these samples were not inhibited. First, we analyzed 8 PCR solutions with 100 pg of tissue-derived DNA of the Japanese eel as a template using the real-time PCR (not qPCR) in the same protocol as the preliminarily laboratory experiment (see laboratory eDNA analysis) to investigate the variation of the cycle threshold (Ct) values within a single run. The Ct values varied from 0.01 to 0.62 among the 8 PCR solutions. Then 100 pg of Japanese eel DNA were added to the 11 seawater samples and amplified along with a control sample also containing 100 pg of tissue-derived Japanese eel DNA. We considered that the 11 seawater samples that included pure tissue-derived DNA would have been inhibited if the difference between their Ct values and that of the control was more than 0.6 in the run.

Both on the research vessel and in the laboratory, all equipment (e.g. plastic bottles, filter funnels, and tweezers) were soaked in a 0.5% bleach solution for at least 5 min and rinsed with pure water before reuse to remove any residual eDNA. Work spaces were wiped using paper and a 0.5% bleach solution, filter pipet tips were used, and all eDNA extractions and PCR set-ups were conducted in separate working places, where PCR products had not been handled previously, and the real-time PCR machine was set outside of this space to minimize contamination.

3. RESULTS

3.1. Laboratory eDNA experiment

Japanese eel *Anguilla japonica* eDNA was detected from the tank water using the species-specific primers and probe in the real-time PCR system. The eDNA samples diluted to concentrations of 10, 1, and 10^{-1} ng μl^{-1} (50, 5, and 0.5 ng eDNA per PCR reaction) showed DNA amplification curves (Fig. 3A), and their Ct values (unitless) were 28.4, 31.8, and 34.8 respectively. This indicates that the higher eDNA amounts resulted in lower Ct values (amplification after fewer cycles). The eDNA extracts of 10^{-2}, 10^{-3}, and 10^{-4} ng μl^{-1} (respectively 50, 5, and 0.5 pg eDNA per PCR reaction) showed no amplification (Fig. 3A).

3.2. Oceanic eDNA survey

To test the viability of our eDNA methodology and estimate the presence of Japanese eels during the research cruise, 32 eDNA samples including 4 filtration blanks were analyzed onboard. No DNA amplification was observed when using real-time PCR. The remaining 85 filters were processed in our laboratory and not onboard due to a lack of time at the end of the cruise, and all eDNA samples (including the 32 eDNA samples analyzed onboard) were analyzed in the laboratory with exactly the same protocol as on the ship. Three out of 117 samples analyzed in the laboratory, which were obtained from 400 m at Stn C5 (142° 00′ E, 13° 00′ N), and 250 and 400 m at Stn C6 (142° 00′ E, 12° 50′ N), showed DNA amplifications by real-time PCR. The Ct values were 39.5, 37.6, and 38.3, respectively (Table 1, Fig. 3B).

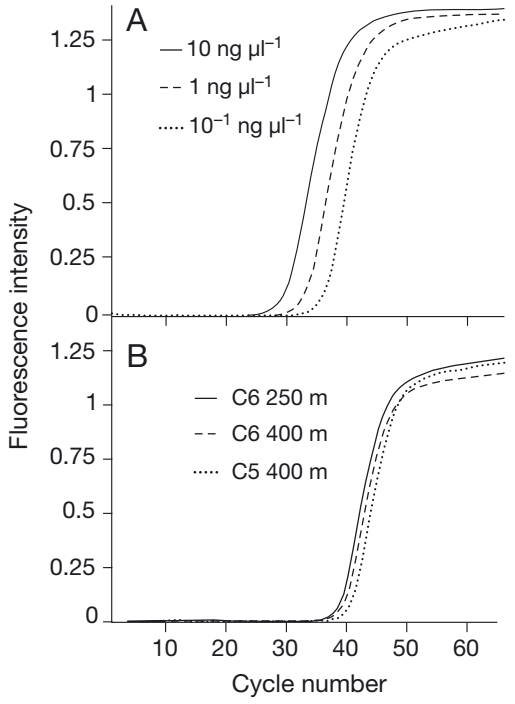

Fig. 3. eDNA amplification curves from the real-time PCR analysis for Japanese eel DNA using (A) eDNA extracts from tank water with 10, 1, 10^{-1} ng μl^{-1} concentration (50, 5, 0.5 ng eDNA per PCR reaction, respectively) and (B) eDNA extracts from seawater from Stn C5 at a depth of 400 m and Stn C6 at 250 and 400 m. All eDNA extracts in (B) were analyzed with 3 real-time PCR replicates (20 µl), but only the one successful amplification curve from each replicate is depicted

Notably, these successful DNA amplifications were obtained in only 1 of the 3 real-time PCR replicates of each sample extract (Fig. 3B), suggesting the extracts included small amounts of Japanese eel DNA. The presence of targeted amplicons in the 3 positive extracts from the oceanic survey samples was verified by gel electrophoresis in the laboratory. Since the primers and probe had specificity to the Japanese eel (Fig. 2), we considered the 3 oceanic survey samples with real-time PCR amplifications as positive results. The 3 positive samples were collected on 19 May, which was 1 d after the new moon (Table 1). The distance between Stns C5 and C6 was approximately 18.5 km (Fig. 1C).

Table 1. Summary of the sampling time, depth, hydrographic characteristics, and real-time PCR cycle threshold (Ct) value of each water sample that had a positive eDNA detection in the spawning area of the Japanese eel *Anguilla japonica*. All 3 samples were collected on 19 May 2015 (1 d after the new moon)

Stn	Sampling time (h)	Water depth (m)	Temperature (°C)	Salinity	Ct value
C5	19:20	400	7.75	34.34	39.5
C6	21:20	250	13.58	34.43	37.6
C6	21:30	400	8.16	34.31	38.3

Additional inhibition tests were performed to verify that the 11 samples with lower amplification curves were not inhibited. Ct values of all 11 seawater samples were not different from the pure Japanese eel tissue-derived DNA control by more than 0.6, and these samples showed no presence of PCR amplicons in the gel electrophoresis. We therefore regarded these 11 samples as being negative. No eDNA of *A. japonica* was detected by real-time PCR assays from all negative controls used in the study.

The hydrographic characteristics at the stations of the sampling grid were generally similar, especially at the depths where *A. japonica* eDNA was detected (Fig. 4). The positive sample from 250 m at Stn C6 was from near the bottom of the thermocline and below the layer of the high-salinity Subtropical Underwater (see Schabetsberger et al. 2016). The samples from 400 m at Stns C5 and C6 were collected in a uniform layer of 8°C water where salinity starts to gradually increase with depth. Only minor variations in temperature occurred among stations while a slightly lower surface salinity occurred at Stn C1. The chlorophyll concentration in the upper 200 m ranged from 0.2 to 0.5 µg l^{-1}, and the highest chlorophyll concentration of 0.5 µg l^{-1} was observed near Stn C6.

4. DISCUSSION

4.1. Viability and refinement of oceanic DNA analysis

During the intensive research efforts on the 2 d near the end of the cruise, we were able to successfully implement the series of procedures for eDNA analysis onboard the moving ship along the southern West Mariana Ridge. This resulted in the first use of eDNA analysis to detect the presence of Japanese eels *Anguilla japonica* in their spawning area, which suggests that this technique can be used to improve the existing methods of spawning area research on this species. Conventional methods such as plankton net sampling and trawl net towing have been useful to outline the spawning area of Japanese eels by collecting eggs, larvae, and spawning-condition eels (Tsukamoto 1992, 2006, Chow et al. 2009, Tsukamoto et al. 2011). However, these methods are insufficient to precisely locate the sites where male and female eels form aggregations to spawn, because it is not

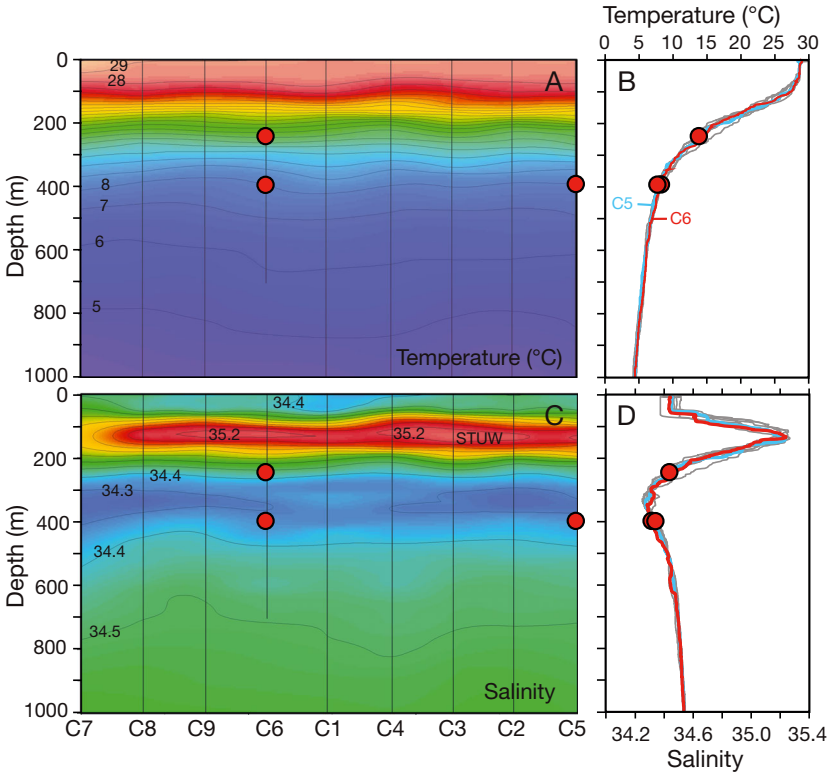

Fig. 4. Hydrographic structure and vertical distribution of (A,B) temperature and (C,D) salinity at the 9 stations sampled for Japanese eel eDNA between the surface and 1000 m. The CTD data at Stn C6 only extended down to 700 m because of a CTD problem. Red circles indicate the water depths with eDNA positive signals (400 m at Stn C5, 250 and 400 m at Stn C6). The high-salinity core of Subtropical Underwater (STUW) was present at all stations. The thicker blue and red lines show the data from Stns C5 and C6, respectively, in B and D (other stations are shown by grey lines)

known at what depth and position during a net tow they were collected. In contrast, eDNA analysis from water samples at known locations provides very specific information about the possible presence or absence of Japanese eels that can guide the deployment of underwater camera systems or submersibles to the locations and depths where spawning events might occur. Therefore, our method using eDNA may be an effective tool to help understand the spawning ecology of the Japanese eel in the open ocean by determining the possible locations of aggregations of eels before their new moon spawning periods and then also by detecting the locations and depths of spawning during or after spawning events.

Our study also suggests that several optimizations for water sampling, and efficient eDNA analysis, may improve the chances of finding and observing the spawning aggregations of Japanese eels in future cruises. An obvious improvement is that an optimal water sampling strategy needs to be developed that can effectively detect low concentrations of eDNA before spawning events occur or after the eDNA from spawning is dispersed. Only 1 of the 3 real-time PCR replicates showed successful amplification for each of the 2 l water samples (n = 3) that were positive for Japanese eel eDNA in our study, which suggests that the concentrations of eDNA were low at those locations. This also suggests that false negative results (species is not detected where it is present) could have occurred at locations where concentrations were low. For example, a previous experiment for testing the detection rate of 2 trout species showed that water samples had 100% amplification success when their eDNA concentrations were ≥2.5 target copies μl^{-1}, whereas false negatives occurred at the eDNA concentration of 0.5 copies μl^{-1} (Wilcox et al. 2013). To improve the chances of species detection, increasing the number of water samples from a site (Ficetola et al. 2008, Takahara et al. 2012, Goldberg et al. 2013) or increasing the amount of water filtered (Miya et al. 2016) are possible ways to increase eDNA detections. Our oceanic cruise used only one 2 l water sample from each of 12 water depths at 9 water sampling sites because of the limited ship time. Conducting a survey in the open ocean where there may be low densities of target species eDNA, and a high chance of occurrences of false negative samples, probably requires the analysis of multiple water samples or a larger amount of water at each location or depth (e.g. 10 or 20 l of seawater).

A second possible improvement would be to use quantitative real-time PCR (qPCR) during future research cruises. To define criteria for positive results, most researchers have examined the limit of detection (LOD, the minimum amount of target DNA sequence that can be detected in the sample) and the limit of quantification (LOQ, the lowest amount of target DNA that yields an acceptable level of precision and accuracy) using templates of known concentration (e.g. Takahara et al. 2013, Tréguier et al. 2014). We did not quantify the LOD because a qPCR system was not used in this study. The acceptable LOD probably should have been determined using qPCR to decide if a water sample is a true positive or negative. The use of a qPCR approach can identify

fish spawning activity based on the change of eDNA concentrations in freshwater (Bylemans et al. 2017, Doi et al. 2017), and thus the spawning events of the Japanese eel should also be detectable by eDNA concentrations in seawater. Japanese eels likely aggregate prior to spawning (Tsukamoto et al. 2003, Kurogi et al. 2011), and larger amounts of eDNA should be present at the spawning site because of the positive relationships between eDNA concentrations and animal abundance that have been found (Takahara et al. 2012, Pilliod et al. 2013) as well as the materials released during spawning.

A possible improvement that is unique to our type of oceanic surveys searching for spawning eels, would be to reduce the time required for obtaining eDNA filter samples by directly filtering seawater as it flows out of the Niskin sampler bottles instead of transferring it into water sample bottles. Transfer of seawater from the Niskin samplers to plastic bottles took about 1 h, whereas each of the other steps of the analysis, including the CTD cast with the Niskin water samplers, water vacuum-filtration, eDNA extraction, real-time PCR set-ups, real-time PCR run time, and decontamination of used equipment took between 0.5 and 2 h, and the total time for eDNA analysis onboard was approximately 9.5 h station^{-1}. Eliminating the step of transferring seawater samples from the Niskin bottles to other bottles would allow the survey plan to be adjusted sooner if eDNA is detected. For example, once a strong eDNA signal is detected, the deployment of a submersible or camera system as soon as possible could increase the chance of observing spawning aggregations and the behavior of Japanese eels.

4.2. Interpretation of eDNA positive signals from seawater samples

The most significant finding of our study is that we were able to detect 3 positive eDNA signals of the Japanese eel from seawater samples at 2 different stations using real-time PCR. However, in our study, as in other marine field surveys using eDNA analysis, the evaluation of the possible origins of the eDNA that is detected is influenced by the initial concentration when released, the degradation rate, and the amount of dilution of the eDNA that may have occurred before its detection. Sassoubre et al. (2016) estimated the decay rate of the eDNA of 3 marine fishes as being from 0.068 to 0.101 h^{-1} at 17.7–23°C, with the eDNA being undetectable within 3–4 d. With initial concentrations of 48 and 214 molecules in 400 ml of seawater, at 15°C, the eDNA of the three-spined stickleback *Gasterosteus aculeatus* was degraded within 0.9 d and that of the European flounder *Platichthys flesus* was degraded within 6.7 d (Thomsen et al. 2012). The decay rates along with the amount of water movement will affect how far eDNA can disperse away from its release point. For example, the eDNA of the harbor porpoise *Phocoena phocoena*, a marine mammal, was only detected at a distance of less than 10 m from a pen with 4 porpoises (Foote et al. 2012). Yamamoto et al. (2016) concluded that the spatial scale of the association between eDNA and fish biomass in a coastal bay was relatively small (10–150 m). Marine eDNA also reflected changes in vertebrate communities at a scale of ~60 m in a kelp forest ecosystem (Port et al. 2016). eDNA decay rate may vary among aquatic ecosystems though, because many other factors including water temperature, salinity, pH, oxygen, ultraviolet light, wind, currents, tides, and eddies can influence eDNA degradation and the potential distance that eDNA can be transported in the ocean (Thomsen et al. 2012, Merkes et al. 2014, Barnes & Turner 2016, Port et al. 2016, Sassoubre et al. 2016, Minamoto et al. 2017). As a consequence of continuous dilution, the probability of detecting eDNA from Japanese eels in marine waters very likely decreases rapidly with distance from its source, which suggests that most eDNA will be detected near where it was released (Thomsen et al. 2012).

For Japanese eels, eDNA of approximately 100 times higher concentrations was detected in seawater tanks that contained 3 artificially matured male eels and 1 female eel after they spawned in the tanks compared with the concentrations before spawning (A. Takeuchi et al. unpubl. data). Since the amounts of eDNA obtained at Stns C5 and C6 during the oceanic sampling of the present study were estimated to be much lower than those detected after spawning in the laboratory, if the oceanic eDNA positive signals resulted from recent spawning of Japanese eels, the eDNA must have been greatly diluted or degraded. It seems possible that some eDNA from a spawning event could have remained at those stations until the day of water sampling. Based on egg and adult collection data in the spawning area (Tsukamoto et al. 2011, Aoyama et al. 2014), spawning may occur at depths as shallow as about 150–200 m. All previous information from collections of eggs, preleptocephali, and the back-calculated hatching dates of larvae indicated that spawning occurs around the new moon (Ishikawa et al. 2001, Tsukamoto et al. 2003, 2011, Aoyama et al. 2014). However,

a more detailed spawning date analysis using egg collection dates and their stages and developmental rates (Ahn et al. 2012) during 3 previous surveys (T. Higuchi et al. unpubl. data) suggested that spawning of Japanese eels likely occurs about 2–4 d before the new moon, with a peak on the third day before new moon, which in our survey would have been 3–5 d before sampling. Thus, it is unclear if the eDNA from spawning would have lasted for 3–5 d, if the eDNA was from the larvae that were produced from spawning, or if it could have been from adult eels that may vertically migrate through the 250–400 m depths (Higuchi et al. 2018).

Although the source of the Japanese eel eDNA cannot be determined, our study established that this type of methodology can be helpful in spawning ecology research. It is clear that we need to learn more about Japanese eel eDNA production and degradation. Information such as the influence of body size and sex on eDNA concentration is lacking, so this study must rely on inference. To plausibly infer the distribution and spawning events of Japanese eels along the southern West Mariana Ridge, laboratory experiments are needed to examine eDNA decay rates in seawater and the effect of life history stages and the numbers of Japanese eels on eDNA release rates.

4.3. Prospects

Our first attempt to detect Japanese eel eDNA in their spawning area indicates that this technique has the potential to provide information about the locations and depths where they are present, which can assist efforts to observe their spawning aggregations and behavior. Although eDNA detections have uncertainties associated with the life history stages of target species (larvae, juveniles, or adults), eDNA dispersion distance, decay rate, and the source of eDNA (from sperm, eggs, other tissues), combining onboard eDNA analysis results with the information from historical spawning area research (Tsukamoto et al. 2003, 2011, Aoyama et al. 2014) has the potential to facilitate efforts to increase understanding of the spawning ecology of Japanese eels in the near future. Moreover, this non-invasive technique is likely transferrable for use in studying other anguillid species, and endangered and key commercial species such as bluefin tuna *Thunnus thynnus*, to make significant contributions to species management, population dynamics, and biodiversity conservation in the marine environment.

Acknowledgements. We sincerely thank M. Kawabe, T. Higuchi, K. Serizawa, S. Tanaka, and S. Inaba for help with the water sampling onboard. We thank the crew of the RV 'Natsushima' for their excellent effort and assistance during the cruise, and the support of the JAMSTEC cruise administration team is also appreciated. We are grateful to M. Miya who provided the procedure for the eDNA analysis and useful suggestions and to R. Yama for assistance with many aspects of the study. This study was partially supported by a Sasakawa Scientific Research Grant from The Japan Science Society to A.T., JST CREST Grant Number JPMJCR13A2 to T.M., JSPS KAKENHI Grant Number 26450268 to S.W., and KAKENHI Grant Numbers 21228005, 26252030, and 17K19300 to K.T. from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and research funds from Toyo Suisan Kabushiki Kaisha Ltd.

LITERATURE CITED

Ahn H, Yamada Y, Okamura A, Horie N, Mikawa N, Tanaka S, Tsukamoto K (2012) Effect of water temperature on embryonic development and hatching time of the Japanese eel *Anguilla japonica.* Aquaculture 330-333: 100–105

Aoyama J, Watanabe S, Miller MJ, Mochioka N, Otake T, Yoshinaga T, Tsukamoto K (2014) Spawning sites of the Japanese eel in relation to oceanographic structure and the West Mariana Ridge. PLOS ONE 9:e88759

Barnes MA, Turner CR (2016) The ecology of environmental DNA and implications for conservation genetics. Conserv Genet 17:1–17

Bergman PS, Schumer G, Blankenship S, Campbell E (2016) Detection of adult green sturgeon using environmental DNA analysis. PLOS ONE 11:e0153500

Bylemans J, Furlan EM, Hardy CM, McGuffie P, Lintermans M, Gleeson DM (2017) An environmental DNA-based method for monitoring spawning activity: a case study, using the endangered Macquarie perch (*Macquaria australasica*). Methods Ecol Evol 8:646–655

Chow S, Kurogi H, Mochioka N, Kaji S, Okazaki M, Tsukamoto K (2009) Discovery of mature freshwater eels in the open ocean. Fish Sci 75:257–259

Doi H, Inui R, Akamatsu Y, Kanno K, Yamanaka H, Takahara T, Minamoto T (2017) Environmental DNA analysis for estimating the abundance and biomass of stream fish. Freshw Biol 62:30–39

Ficetola GF, Miaud C, Pompanon F, Taberlet P (2008) Species detection using environmental DNA from water samples. Biol Lett 4:423–425

Foote AD, Thomsen PF, Sveegaard S, Wahlberg M and others (2012) Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. PLOS ONE 7:e41781

Fukuba T, Miwa T, Watanabe S, Mochioka N and others (2015) A new drifting underwater camera system for observing spawning Japanese eels in the epipelagic zone along the West Mariana Ridge. Fish Sci 81:235–246

Fukumoto S, Ushimaru A, Minamoto T (2015) A basin-scale application of environmental DNA assessment for rare endemic species and closely related exotic species in rivers: a case study of giant salamanders in Japan. J Appl Ecol 52:358–365

Goldberg CS, Sepulveda A, Ray A, Baumgardt J, Waits LP (2013) Environmental DNA as a new method for early

detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). Freshw Sci 32:792–800
Higuchi T, Watanabe S, Manabe R, Kaku T and others (2018) Tracking *Anguilla japonica* silver eels along the West Mariana Ridge using pop-up archival transmitting tags. Zool Stud 57:24
Ishikawa S, Suzuki K, Inagaki T, Watanabe S and others (2001) Spawning time and place of the Japanese eel *Anguilla japonica* in the north equatorial current of the western North Pacific Ocean. Fish Sci 67:1097–1103
Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) 'Sight-unseen' detection of rare aquatic species using environmental DNA. Conserv Lett 4:150–157
Kelly RP, Port JA, Yamahara KM, Martone RG and others (2014) Harnessing DNA to improve environmental management. Science 344:1455–1456
Kimura S, Tsukamoto K (2006) The salinity front in the north equatorial current: a landmark for the spawning migration of the Japanese eel (*Anguilla japonica*) related to the stock recruitment. Deep Sea Res II 53:315–325
Kurogi H, Okazaki M, Mochioka N, Jinbo T and others (2011) First capture of post-spawning female of the Japanese eel *Anguilla japonica* at the southern West Mariana Ridge. Fish Sci 77:199–205
Merkes CM, McCalla SG, Jensen NR, Gaikowski MP, Amberg JJ (2014) Persistence of DNA in carcasses, slime and avian feces may affect interpretation of environmental DNA data. PLOS ONE 9:e113346
Miller MJ, Koyama S, Mochioka N, Aoyama J, Watanabe S, Tsukamoto K (2014) Vertical body orientation by a snipe eel (Nemichthyidae, Anguilliformes) in the deep mesopelagic zone along the West Mariana Ridge. Mar Freshw Behav Physiol 47:265–272
Minamoto T, Fukuda M, Katsuhara KR, Fujiwara A and others (2017) Environmental DNA reflects spatial and temporal jellyfish distribution. PLOS ONE 12:e0173073
Minegishi Y, Yoshinaga T, Aoyama J, Tsukamoto K (2009) Species identification of *Anguilla japonica* by real-time PCR based on a sequence detection system: a practical application to eggs and larvae. ICES J Mar Sci 66: 1915–1918
Miya M, Sato Y, Fukunaga T, Sado T and others (2015) MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R Soc Open Sci 2:150088
Miya M, Minamoto T, Yamanaka H, Oka S and others (2016) Use of a filter cartridge for filtration of water samples and extraction of environmental DNA. J Vis Exp 117:e54741
Pilliod DS, Goldberg CS, Arkle RS, Waits LP (2013) Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Can J Fish Aquat Sci 70:1123–1130
Port JA, O'Donnell JL, Romero-Maraccini OC, Leary PR and others (2016) Assessing vertebrate biodiversity in a kelp forest ecosystem using environmental DNA. Mol Ecol 25: 527–541
Rees HC, Maddison BC, Middleditch DJ, Patmore JRM, Gough KC (2014) The detection of aquatic animal species using environmental DNA—a review of eDNA as a survey tool in ecology. J Appl Ecol 51:1450–1459
Sassoubre LM, Yamahara KM, Gardner LD, Block BA, Boehm AB (2016) Quantification of environmental DNA (eDNA) shedding and decay rates for three marine fish. Environ Sci Technol 50:10456–10464
Schabetsberger R, Miller MJ, Dall'Olmo G, Kaiser R and others (2016) Hydrographic features of anguillid spawning areas: potential signposts for migrating eels. Mar Ecol Prog Ser 554:141–155
Shinoda A, Aoyama J, Miller MJ, Otake T and others (2011) Evaluation of the larval distribution and migration of the Japanese eel in the western North Pacific. Rev Fish Biol Fish 21:591–611
Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Z (2012) Estimation of fish biomass using environmental DNA. PLOS ONE 7:e35868
Takahara T, Minamoto T, Doi H (2013) Using environmental DNA to estimate the distribution of invasive fish species in ponds. PLOS ONE 8:e56584
Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E (2012) Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLOS ONE 7:e41732
Tréguier A, Paillisson JM, Dejean T, Valentini A, Schlaepfer MA, Roussel JM (2014) Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. J Appl Ecol 51:871–879
Tsukamoto K (1992) Discovery of the spawning area for Japanese eel. Nature 356:789–791
Tsukamoto K (2006) Spawning of eels near a seamount. Nature 439:929
Tsukamoto K (2009) Oceanic migration and spawning of anguillid eels. J Fish Biol 74:1833–1852
Tsukamoto K, Otake T, Mochioka N, Lee TW and others (2003) Seamounts, new moon and eel spawning: the search for the spawning site of the Japanese eel. Environ Biol Fishes 66:221–229
Tsukamoto K, Chow S, Otake T, Kurogi H and others (2011) Oceanic spawning ecology of freshwater eels in the western North Pacific. Nat Commun 2:179
Tsukamoto K, Mochioka N, Miller MJ, Koyama S, Watanabe A, Aoyama J (2013) Video observation of an eel in the *Anguilla japonica* spawning area along the West Mariana Ridge. Fish Sci 79:407–416
Watanabe S, Minegishi Y, Yoshinaga T, Aoyama J, Tsukamoto K (2004) A quick method for species identification of Japanese eel (*Anguilla japonica*) using real-time PCR: an onboard application for use during sampling surveys. Mar Biotechnol (NY) 6:566–574
Wilcox TM, McKelvey KS, Young MK, Jane SF, Lowe WH, Whiteley AR, Schwartz MK (2013) Robust detection of rare species using environmental DNA: the importance of primer specificity. PLOS ONE 8:e59520
Yamamoto S, Minami K, Fukaya K, Takahashi K and others (2016) Environmental DNA as a 'snapshot' of fish distribution: a case study of Japanese jack mackerel in Maizuru Bay, Sea of Japan. PLOS ONE 11:e0149786
Yoshinaga T, Miller MJ, Yokouchi K, Otake T and others (2011) Genetic identification and morphology of naturally spawned eggs of the Japanese eel *Anguilla japonica* collected in the western North Pacific. Fish Sci 77: 983–992

Editorial responsibility: Philippe Borsa, Montpellier, France

Submitted: November 8, 2017; Accepted: November 19, 2018
Proofs received from author(s): December 11, 2018