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Estimations of Riverine Distribution, Abundance, and Biomass of Anguillid Eels in Japan and Taiwan Using Environmental DNA Analysis

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Although populations of anguillid eels have declined remarkably in recent decades, monitoring data on the spatial and temporal variation in their dynamics are often limited, particularly for tropical eel species. As there are often sympatries of multiple eel species in tropical rivers, identifying eel species based solely on morphological characteristics is challenging. Basin-scale surveys were conducted in rivers of southern Japan and northern Taiwan to investigate (1) whether the spatial distribution, abundance, and biomass of the tropical eel species, the giant mottled eel (*Anguilla marmorata*), can be monitored in rivers by comparing the results obtained from environmental DNA (eDNA) analysis with data from electrofishing and (2) the riverine distribution of the sympatric *A. marmorata* and the temperate eel species, the Japanese eel (*Anguilla japonica*), in this region using eDNA analysis. Although we found an much lower abundance of *A. marmorata* in the study region, we identified the eDNA of the species from all of the study sites (21 sites) where it was collected by electrofishing, in addition to 22 further study sites where it was not collected directly. This indicates that eDNA analysis has a greater sensitivity for detecting *A. marmorata*, making it a powerful tool for monitoring the spatial distribution of the species in rivers. We found a significant positive relationship between eDNA concentration and both the abundance and biomass of *A. marmorata*, and eDNA concentration seemed to better reflect the abundance of the species than did biomass. eDNA of both *A. japonica* and *A. marmorata* was identified from almost all rivers, indicating the sympatry of these species in this region, although the degree of sympatry differed between rivers. Though the eDNA concentration of *A. japonica* decreased significantly with increasing distance from the river mouth, no significant relationship was found for *A. marmorata*. This study is the first to demonstrate the potential usefulness of eDNA analysis for estimating the spatial distribution, abundance, and biomass of tropical eels in rivers and to further apply this method to investigate sympatry among anguillid species. eDNA analysis can help in obtaining data on the population dynamics of tropical eels, providing invaluable information for managing these species.

Key words: *Anguilla japonica*, *Anguilla marmorata*, Environmental DNA, Monitoring, Spatial distribution.

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BACKGROUND

The genus *Anguilla* contains 16 species and 3 subspecies, which spawn in the open ocean and grow in continental waters. Populations of anguillid eels are distributed across more than 150 countries, and their ecological, commercial, and cultural importance are common globally (Jacoby et al. 2015). However, in recent decades, there have been significant declines in the stocks of some anguillid eels, and 10 of the 16 species are now listed as "Threatened" or "Nearly Threatened" in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN 2019). This critical situation among anguillid eel populations highlights that conservation and monitoring these populations is an urgent global issue.

Ideally, to enable direct comparison of results obtained from different regions or studies, a consistent protocol should be used to monitor the dynamics of a target population quantitatively and continuously throughout its distribution range. However, a quantitative monitoring survey necessitates extensive fieldwork and considerable effort, in addition to different sampling protocols in different environments. Anguillid eels have broad geographic ranges because they migrate between saline and freshwater environments and inhabit diverse habitats, from saline bays to upland headwaters (Moriarty 2003). They also exhibit hiding behaviors in refuges (Aoyama et al. 2005). All these factors create challenges for accurate and continuous monitoring using standardized capture-based methods throughout their range. Thus, data on spatial and temporal variation in anguillid eel population dynamics are often either sparse, patchy, or imbalanced (Jacoby et al. 2015).

Still, data on population dynamics and ecological knowledge of various life stages have accumulated for eels distributed in temperate regions (i.e., temperate eels), including the American eel (*Anguilla rostrata*), the European eel (*Anguilla anguilla*), and the Japanese eel (*Anguilla japonica*), which have long been commercially important species. Conversely, though most anguillid eel species (10 species) are found in the tropical Indo-Pacific regions (*i.e.*, tropical eels) (Ege 1939; Aoyama et al. 2018), there are limited monitoring data on the population dynamics of tropical eels, and ecological studies are, as yet, incomplete (Jacoby et al. 2015), perhaps due to these eels' relatively low commercial importance. However, data collection for tropical eels is particularly important, given a recent increase in demand for tropical eels, such as the giant mottled eel (*Anguilla marmorata*) and *Anguilla bicolor*, as a replacement for the temperate eels, especially in East Asia (Gollock et al. 2018).

Continental waters in each region usually contain a single temperate eel species because, generally, the geographic distribution of temperate eels does not overlap (with the exception of *Anguilla dieffenbachii* and *Anguilla australis*; Glova et al. 1998). Conversely, there are sympatries of temperate and tropical eels in the marginal regions of their distribution range (Shiao et al. 2003; Leander et al. 2012; Hsu et al. 2019), and sympatries of multiple eel species often occur in tropical rivers (Arai and Abdul Kadir 2017; Hagihara et al. 2018). Accurate identification based solely on morphological characteristics is difficult for some eel species in the tropical regions (Watanabe et al. 2004), which often leads to eel species being misidentified. For instance, *Anguilla bengalensis bengalensis* has been misidentified as *A. marmorata* (Arai and Abdul Kadir 2017). Genetic analysis is preferred to identify eel species in tropical regions (Arai and Abdul Kadir 2017; Hagihara et al. 2018; Nguyen et al. 2018). Moreover, recent studies indicate that different eel species can have different habitat use and riverine distribution patterns (Shiao et al. 2003; Chino and Arai 2010; Arai and Abdul Kadir 2017; Hagihara et al. 2018; Nguyen et al. 2018; Hsu et al. 2019), which makes it difficult to monitor their population dynamics. Most of these studies have examined eel distribution in tropical regions using either qualitative or non-standardized sampling protocols among different environments and have used otolith microelement analysis to estimate eels' habitat utilization. Although these studies have obviously provided important new information on eels, other quantitative methods are required to understand their distributions precisely.

Environmental DNA (eDNA) analysis is increasing rapidly in popularity as a cost-efficient and non-lethal monitoring tool for studying and managing organisms in aquatic ecosystems (Lodge et al. 2012; Rees et al. 2014). It has been used effectively in determining the presence of aquatic species inhabiting lakes, rivers, and marine habitats (Dougherty et al. 2016; Yamamoto et al. 2016; Yamanaka and Minamoto 2016). Furthermore, as this approach can be more sensitive than conventional capture-based sampling methods for detecting the presence or absence of fish (Jerde et al. 2013; Wilcox et al. 2016; Sakata et al. 2017; Itakura et al. 2019), this is a particularly useful tool for monitoring rare and endangered species (Fukumoto et al. 2015; Sakata et al. 2017). eDNA analysis can also be used in both freshwater and marine habitats to estimate abundance and biomass (Pilliod et al. 2013; Dougherty et al. 2016; Yamamoto et al. 2016; Doi et al. 2017; Minamoto et al. 2017; Itakura et al. 2019). For anguillid eels, Itakura et al. (2019) conducted basin-scale surveys of Japanese eels across 10 rivers in Japan and detected the eDNA of the species at 92% (56 of 61 sites) of study sites from

which individuals were collected by electrofishing as well as at 35 additional sites where individuals were not directly collected. This indicates that eDNA analysis has greater sensitivity for detecting the presence of Japanese eels. The study also showed that eDNA analysis can be used to estimate the abundance and biomass of Japanese eels in rivers, requiring less time and effort than electrofishing. As some tropical eels cannot be identified without using genetic characteristics, eDNA analysis is considerably helpful for monitoring and allows investigators to undertake large-scale surveys throughout the eels' distribution ranges using a consistent method. However, eDNA analysis has not yet been applied to detect tropical eels that are often present sympatrically in rivers.

In this study, we used eDNA analysis to carry out basin-scale surveys of *A. marmorata* and *A. japonica* in 11 rivers in southern Japan and northern Taiwan. We chose these species and regions because (1) the two eel species are sympatric due to their overlapping geographic distributions (Shiao et al. 2003; Han et al. 2012a; Hsu et al. 2019), thereby providing suitable sites for applying eDNA analysis to sympatric anguillid eel species; (2) use of eDNA analysis for *A. marmorata* is particularly important, as misidentification of this species using only morphological characters has been reported previously (Arai and Abdul Kadir 2017); and (3) a relatively low abundance of *A. marmorata* is expected in some parts of these regions, as it is thought to be close to the northern limit of the distribution range of this species (Jacoby and Gollock 2014a), thus offering suitable sites for investigating the sensitivity of eDNA analysis for detecting the presence of *A. marmorata*. For example, the abundance of *A. marmorata* has been reported to be an order of magnitude lower than that of *A. japonica* in a river in Taiwan (Hsu et al. 2019).

We first evaluated whether eDNA analysis can be used to estimate not only the spatial distribution but also the abundance and biomass of *A. marmorata* in rivers. To this end, we compared the results of eDNA analysis with those of the electrofishing method to estimate the presence or absence of *A. marmorata* in sampling sites in three rivers in Japan and two rivers in Taiwan. Moreover, we investigated the relationship between the eDNA concentration and the abundance of *A. marmorata* in the Oganeku River on Amami-Oshima Island, a subtropical island of Japan where *A. marmorata* is known to be predominant (Wakiya et al. 2019). Lastly, we carried out further eDNA sampling in six rivers in Japan and Taiwan, and discuss the riverine distribution of the two eel species in this region based on the eDNA concentration results from all of the study rivers. Samples of *A. japonica* collected from two Japanese rivers (the Atsumari and Mawatari rivers) in the current study were also used in an eDNA study for this species (Itakura et al. 2019).

MATERIALS AND METHODS

Study species

Anguilla japonica spawn in the waters west of the Mariana Islands in the western North Pacific Ocean (Tsukamoto et al. 2011), and their larvae drift westward to growth habitats in East Asia including Taiwan, eastern China, Korea, and Japan. After metamorphosing into glass eels (early juvenile phase), they migrate into brackish and freshwater habitats, where they remain as growth-phase yellow eels. They grow in diverse habitats within rivers, ranging from brackish estuaries to upland headwaters, lakes, and saline bays (Kaifu et al. 2010; Wakiya et al. 2016; Itakura et al. 2018; Itakura et al. 2019). Once they are sexually mature, they metamorphose into reproductive-stage silver eels and then migrate to their spawning areas (Han et al. 2016; Chen et al. 2018; Higuchi et al. 2018). This species is a commercially important species in East Asia and, due to a notable decline in its abundance, is classified as Endangered on the IUCN Red List of Threatened Species (Jacoby and Gollock 2014b)

Anguilla marmorata is the most widespread anguillid species in the world, ranging from the western Indian Ocean, across the Indo-Pacific, to French Polynesia in the South Pacific Ocean (Ege 1939; Watanabe et al. 2004). The species has four genetically distinct populations (Minegishi et al. 2008), one of which spawns in the same region as *A. japonica* (Kuroki et al. 2009; Tsukamoto et al. 2011). Although *A. marmorata* tends to live in freshwater areas rather than brackish water or seawater (Shiao et al. 2003; Nguyen et al. 2018), the species occupies a broad range of habitats from brackish estuaries to upland headwaters (Arai and Abdul Kadir 2017; Arai and Chino 2018; Hagihara et al. 2018; Wakiya et al. 2019). *A. marmorata* is one of the tropical eel species for which demand is increasing, and their glass eels are being used increasingly to stock farms in East Asia (Gollock et al. 2018). This species has been classified as of Least Concern in the IUCN Red List of Threatened Species (Jacoby and Gollock 2014a).

Study area

To investigate both whether eDNA analysis can detect the presence or absence of *A. marmorata* in rivers and the sensitivity of the approach, we carried out eDNA sampling and conventional capture-based

sampling (electrofishing) at 49 study sites from the lower to the upper reaches of rivers in Kagoshima Prefecture, Japan (the Atsumari and Mawatari rivers on the southern main island of Japan and the Oganeku River on Amami-Oshima Island) and the Fengshan and Shuang rivers in Taiwan (Table 1; Figs. 1, 2). To test whether eDNA analysis can be used to estimate the abundance and biomass of *A. marmorata*, we investigated the relationship between the eDNA concentration and the abundance and biomass of *A. marmorata* in the Oganeku River (Table 1; Fig. 1), which is approximately 0.5 km in length. We collected eels over the entire area of the Oganeku River and conducted eDNA samplings at 20 m intervals from the river mouth to the uppermost reaches of the river (25 sites in total). We did not conduct surveys at study sites between 0 to 20 m from the river mouth because these were sandy beaches with very little water flow. To further study the riverine distribution of *A. marmorata* and *A. japonica* based on their eDNA concentration, we carried out eDNA sampling at 81 sites in five rivers (Hetsuka, Hirose, Kedo, Kubota, and Mae) in Kagoshima Prefecture, Japan, and three rivers (Fengshan, Nan-ao South, and Shuang) in Taiwan (Table 1; Figs. 1, 2).

We carried out eDNA sampling and eel collection from September 2016 to October 2017 (Table 1). The length and width of study sites in the Atsumari River ranged from 20.0 to 34.0 m and 3.3 to 8.1 m, respectively, and those in the Mawatari River ranged from 13.0 to 20.0 m and from 3.0 to 8.0 m, respectively (for details, see Itakura et al. 2019), whereas we did not measure those in the Taiwanese rivers. The width of the study sites in the Oganeku River ranged from 0.5 to 13.7 m, with a mean ± standard deviation of 3.4 ± 2.5 m. We measured water velocity at the center of the downstream point at each study site of the Oganeku River in August and November 2016, and we used the mean of the three measurement points for analysis (see Data analysis). Though the Mae and Kedo rivers flow through residential areas, all the other rivers flow through agricultural and forest lands. Most study sites were located in freshwater, and some sites were in brackish water.

eDNA analysis

Water sampling

Water sampling was conducted at the downstream side of each study site. Surface water (1 L) was collected at the center of the river by submerging a bottle approximately 10 cm in depth. Following Yamanaka et al. (2017), benzalkonium chloride solution (1 mL) was added immediately to each water sample to prevent eDNA degradation. After collection, each water sample was vacuum-filtered through either one or two 47 mm GF/F glass filters (pore size *c*. 0.7 μm; GE Healthcare Life Science, Whatman) within an average of 2 days (maximum 5 days). Next, the filters were wrapped immediately in commercial aluminum foil and stored at -20°C until eDNA extraction. The bottles and the filtering devices (*i.e.*, filter funnels and measuring cups used for filtration) were bleached with 0.1% sodium hypochlorite. The bottles were further washed two or more times with surface river water from each sampling site immediately before collecting the water. In study sites at which eels were also collected, water

Table 1. Characteristics of the study rivers and samplings for the environmental DNA (eDNA) analysis and electrofishing of anguillid eels (*Anguilla japonica* and *Anguilla marmorata*)

Country	River	No. study sites	Sampling date	Length (km)	eDNA copies L^{-1}	
					A. japonica	*A. marmorata*
Japan	Atsumari	10 (10)	23–25 September 2016	5.9	354 ± 394	1929 ± 1951
	Hetsuka	11 (0)	17–18 July 2017	4.2	9 ± 0	25 ± 22
	Kedo	16 (0)	27 September 2017	11.5	45 ± 33	234 ± 233
	Hirose	8 (0)	17–18 July 2017	8.7	208 ± 314	84 ± 111
	Kubota	8 (0)	17–18 July 2017	14.8	43 ± 42	17 ± 17
	Mawatari	7 (7)	23–25 September 2016	11.5	66 ± 55	3291 ± 4392
	Mae	9 (0)	17–18 July 2017	20.0	265 ± 273	38 ± 29
	Oganeku	25 (25)	26–29 July 2017	0.5	-	922 ± 900
Taiwan	Fengshan	11 (5)	2–6 October 2017	45.5	13 ± 11	-
	Nan-ao South	12 (0)	2–6 October 2017	30.6	107 ± 109	17 ± 11
	Shuang	13 (2)	2–6 October 2017	26.8pare	55 ± 0	31 ± 31

Number of study sites for electrofishing is indicated in parentheses. eDNA copies at sites where eDNA of anguillid eels was detected is indicated as mean ± standard deviation.

was sampled done before eel collection.

eDNA extraction

eDNA was extracted from the filters following the method of Yamamoto et al. (2016). Total eDNA was extracted from each filter using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), with a minor modification to adjust for eDNA extraction. In brief, the sample filter was placed in the suspended insert within a Salivette tube (Sarstedt, Nümbrecht, Germany), and 420 µL of a solution comprising 20 µL Proteinase K, 200 µL AL buffer, and 200 µL water was poured onto the filter. Then, the tube was incubated for 30 min at 56°C, after which the liquid held in the filter was collected via centrifugation. To increase the eDNA yield, 200 µL TE buffer (pH 8.0) was poured onto the filter, and the liquid was again collected via centrifugation. Next, 200 µL AL buffer and 600 µL ethanol were added to the collected liquid, the mixture was transferred to a spin column, and the final volume of eDNA was eluted in 100 µL AE buffer, according to the manufacturer's instructions. During the eDNA extraction procedures, we checked for cross-contamination by extracting eDNA simultaneously from pure water (extraction negative control) as one sample for every extraction procedure (*i.e.*, there was one negative control for every 7–23 river water samples).

Real-time qPCR

We quantified the eDNA samples by real-time TaqMan qPCR using a StepOnePlus Real-Time PCR system (Life Technologies, Foster City, USA). The mitochondrial 16S ribosomal RNA (rRNA) gene fragments were amplified and quantified using species-specific primers and probes. The primers and a probe for *A. japonica* were developed by Watanabe et al.

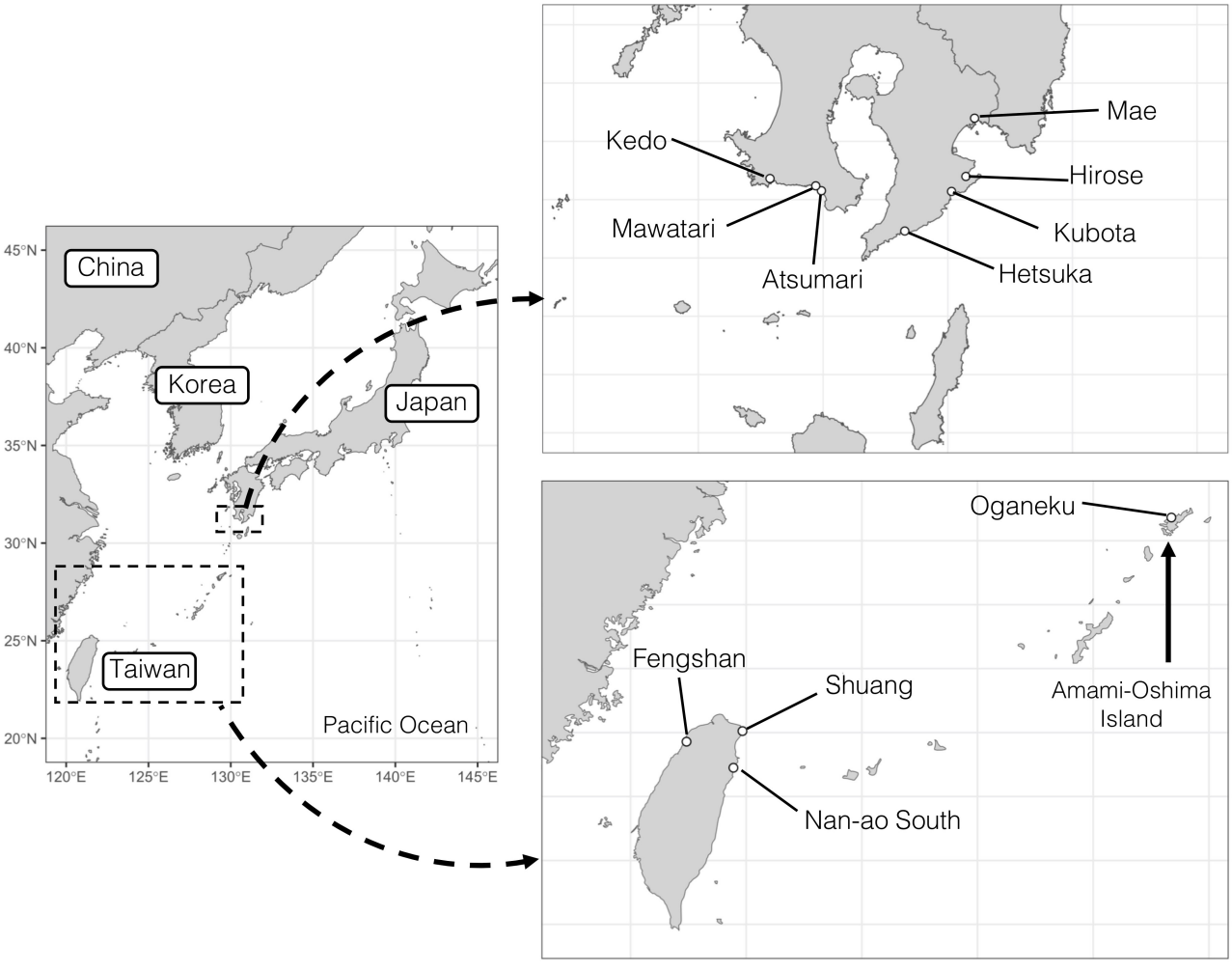

Fig. 1. Locations of the study region and study rivers in Japan and Taiwan used for environmental DNA (eDNA) analysis and/or electrofishing of anguillid eels (*Anguilla japonica* and *Anguilla marmorata*).

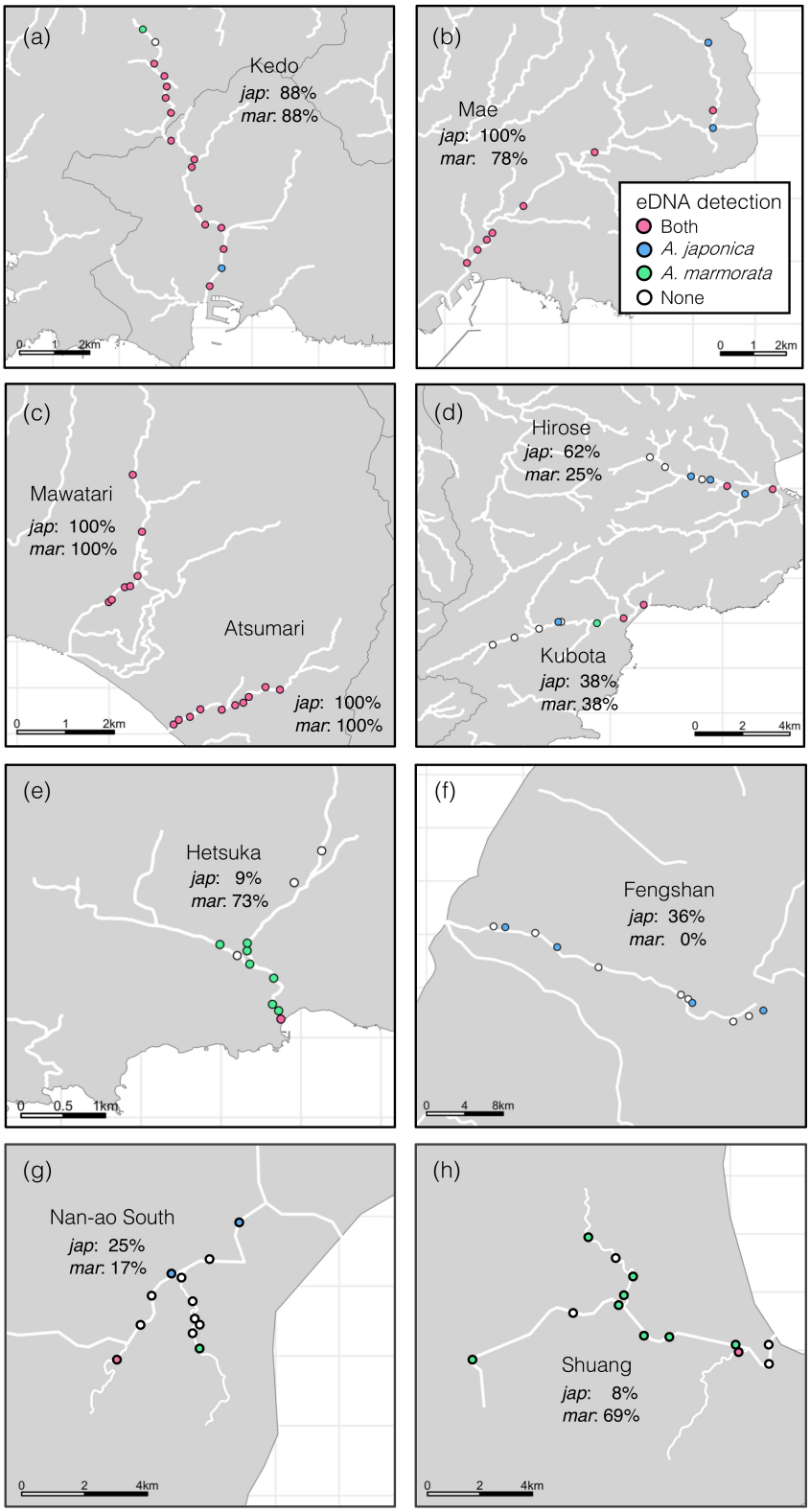

Fig. 2. Maps showing the locations of the sites in each river and the environmental DNA (eDNA) detections of anguillid eels (*Anguilla japonica* and *Anguilla marmorata*). The presence or absence of eDNA detections are shown as different colored circles. The numbers below each river name indicate the proportion of eDNA detection sites for both species (*jap*: *A. japonica*; *mar*: *A. marmorata*). (a) Kedo River, (b) Mae River, (c) Atsumari and Mawatari rivers, (d) Hirose and Kubota rivers, (e) Hetsuka River, (f) Fengshan River, (g) Nan-ao South River, and (h) Shuang River. Map scales differ among panels.

(2005), whereas those for *A. marmorata* were designed for this study (Table 2). These primers specifically amplified 153 and 171 bp fragments of 16S rRNA gene for *A. japonica* and *A. marmorata*, respectively. DNA sequences of *A. marmorata* and other related species (*i.e.*, *A. japonica*, *Anguilla bicolor pacifica*, and *Anguilla luzonensis*) potentially living in Japan and Taiwan were collected from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) nucleotide database. The primers and probe set for *A. marmorata* was designed using UGENE v1.26.1 (Unipro, Russia) and Primer Express software (Thermo Fisher Scientific, Waltham, MA, USA), checking the sequence mismatching between the target and other related species. Each 20 μL TaqMan reaction contained 2 μL extracted eDNA solution, a final concentration of 900 nM forward and reverse primers, 125 nM TaqMan probe, and 0.1 μL of AmpErase Uracil N-Glycosylase (Thermo Fisher Scientific) in 1× Environmental Master Mix 2.0 (Life Technologies). We carried out qPCR in triplicate for each eDNA sample under 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.

To quantify the number of 16S rRNA genes in a 2 μL eDNA solution sample, a dilution series of standards consisting of $3 \times 10^1 – 3 \times 10^4$ copies of a commercially synthesized artificial DNA fragment inserted into a plasmid was analyzed in triplicate simultaneously in each round of qPCR. In addition, pure water (2 μL) was analyzed in triplicate in all rounds of qPCR as a negative control. We found that the calibration curves from all rounds of qPCR had R^2 values of 0.983–0.997, slopes between -3.175 and -2.954, and intercept values of 36.253–39.712 for *A. japonica*, and R^2 values of 0.971–0.997, slopes between -3.662 and -3.303, and intercept values of 40.098–41.914 for *A. marmorata*. Based on the calibration curve of each run and the Ct value of each sample, we calculated the copy number of 16S rRNA gene fragments by averaging the three replicate values of each sample.

Watanabe et al. (2005) confirmed the specificity of the primers and probe for *A. japonica* by using tissue-derived DNA extractions from related species. The same primers and probe also enabled us to specifically detect eDNA of *A. japonica* from tanks, rivers, and the open ocean (Itakura et al. 2019; Takeuchi et al. 2019a b). We confirmed the specificity of the primers and probe for *A. marmorata* by using real-time PCR to determine the DNA sequences of some of the amplified samples. We collected these samples from rivers in which abundant *A. marmorata* had been captured by electrofishing (Wakiya et al. 2019). The DNA sequencing was performed commercially (Fasmac Co., Ltd, Kanagawa, Japan). Basic Local Alignment Search Tool searches using the NCBI nucleotide database showed that all the sequenced fragments from samples had only 16S rRNA gene sequences of *A. marmorata*.

Eel collection

Eels were collected from the downstream to upstream direction of each study site in Japanese rivers using an electroshocker (LR-20B, Smith-Root, Inc., Vancouver, WA, USA). Captured fish were euthanized with > 10% eugenol solution (FA100; DS Pharma Animal Health Co., Ltd. Japan). Though fish from the Oganeku River were released subsequently into capture sites after measurements for other studies, fish from other rivers were stored at -20°C until measurements were made. Each specimen was identified morphologically following Watanabe et al. (2004). The growth stage of each specimen was confirmed based on the color of its body and pectoral fins following previous studies (Okamura et al. 2007; Hagihara et al. 2012). The total length and body weight of each eel were measured to the nearest 1 mm and 0.1 g, respectively. In addition, we calculated the observed abundance and biomass densities of *A. marmorata* at 10 m intervals in the Oganeku River by dividing either the number or the total mass of captured eels, respectively, by the area of the study site (m^2). Eel sampling was conducted both under the guidance of and with the permission of the Fisheries Adjustment Rules of Kagoshima Prefectures.

Table 2. Specific primers and probe for the Japanese eel (*Anguilla japonica*) and the giant mottled eel (*Anguilla marmorata*) used in this study

Target species	Primer and probe	Sequences (5'–3')	Source
A. japonica	Forward primer	AATCAGTAATAAGAGGGCCCAAGC	Watanabe et al. 2005
	Reverse primer	TGTTGGGTTAACGGTTTGTGGTA	
	Probe	FAM-CACATGTGTAAGTCAGAACGGACCGACC-TAMRA	
A. marmorata	Forward primer	GGACATAAATGAGCAGTTATCCTGA	This study
	Reverse primer	TGGTTGATTTCGTATACCGACG	
	Probe	FAM-CTCTAATGCTATTCCTAATTAC-MGB-NFQ	

In the Taiwanese rivers, eels were collected from each study site using an electroshocker (Taixing Battery Store). Captured fish were released into capture sites once the species and growth stage were determined. Eel sampling was approved by the Fishery Agency, Council of Agriculture, Executive Yuan, Taiwan.

Data analysis

All statistical analyses were carried out using R 3.6.0. To assess the relationship between eDNA concentration and the abundance and biomass of *A. marmorata* in the Oganeku River, we used two different models: i) a Type I regression model and ii) a Type II regression model with the standardized major axis method (*sma* in the package *smatr*). Type II regression models can treat two variables (x and y) with an equal magnitude of random variation. These models included eDNA concentrations as a response variable and the abundance or biomass of *A. marmorata* as an explanatory variable. eDNA concentrations were log-transformed. Using the two models, we first tested the extent to which eDNA concentration reflected the abundance of eels in the river. To accomplish this, we calculated the abundances of eels between each water sampling point and over different distances by varying the distance from 10 to 150 m with 10 m increments. Furthermore, as the transport distance of eDNA from the source organisms varies with water velocity (Deiner and Altermatt 2014; Pilliod et al. 2014; Jane et al. 2015; Wilcox et al. 2016), the watershed was divided into two river basins (*i.e.*, downstream and upstream) according to water velocity characteristics. To identify major changes in water velocity throughout the river, we carried out a changepoint analysis based on a likelihood ratio test (*cpt. mean* in the package *changepoint*). The findings showed that the mean water velocity of the river changed drastically at 150 and 160 m from the river mouth in August and November 2016, respectively (Fig. S1). Therefore, we calculated the abundances of eels between each water sampling point and the various consecutive distances for the two basins separated at the point where the water velocity changed dramatically (*i.e.*, 160 m from the river mouth). Lastly, we used Akaike's information criterion (AIC) and R^2 values to evaluate 225 candidate models for Type I and II regression models.

As studies have reported that the abundance of anguillid eels declines with increasing distance from the river mouth (*e.g.*, Yokouchi et al. 2008; Kaifu et al. 2010), we also examined the spatial distribution of eDNA concentration. In the Oganeku River, we used a linear regression model (Type I regression model) with eDNA concentration of *A. marmorata* as a response variable and distance from the river mouth as an explanatory variable. In the other rivers, we used a linear mixed model (LMM: *lmer* in the package *lme4*) with eDNA concentration of *A. japonica* or *A. marmorata* as a response variable, distance from the river mouth as an explanatory variable, and the river as a random effect. We log-transformed eDNA concentrations by adding 1 to the variable. In addition, we compared the proportion of eDNA detection sites between the two eel species for each river using Fisher's Exact Test.

RESULTS

Number of collected eels

In all, 135 individuals of the two eel species were collected in this study (Table 3); 46 *Anguilla japonica* and 9 *A. marmorata* were collected from the Atsumari and Mawatari rivers on the southern main island of Japan (Fig. 3), 60 *A. marmorata* were collected from the Oganeku River on Amami-Oshima Island, Japan, and 18 *A. japonica* and 2 *A. marmorata* were collected from the two Taiwanese rivers (Fig. 3). We collected no *A. japonica* from the Oganeku and Shuang rivers and no *A. marmorata* from the Fengshan River. All of the eels captured were in their growth phase, apart from one *A. marmorata* glass eel collected from the Atsumari River. No anguillid species other than *A. japonica* and *A. marmorata* were collected in this study. The total length of the collected *A. marmorata* in the Oganeku River ranged from 78 to 600 mm, with a mean ± SD of 303 ± 156 mm.

Although both species were collected from the lower to upper reaches of the Atsumari and Mawatari rivers, higher numbers of *A. japonica* were collected from the lower reaches (Fig. 3). In the Fengshan River, *A. japonica* were collected only from the lower reaches. In the Shuang River, *A. marmorata* were collected only from the upper reaches because we did not sample the lower reaches of the river (Fig. 3).

Comparison of presence or absence of anguillid eels using eDNA and electrofishing

A. marmorata were collected from 21 of the 49 (43%) study sites via electrofishing in the Atsumari, Mawatari, Oganeku, Fengshan, and Shuang rivers, whereas eDNA of the species was detected not only at all sites where eels were collected but also at 22 sites where the species was not collected directly (Table 3). We identified eDNA of *A. marmorata* from all sites in these Japanese rivers and from nine of the 24 sites in the Taiwanese rivers (51 of 66, 77% in total). We did

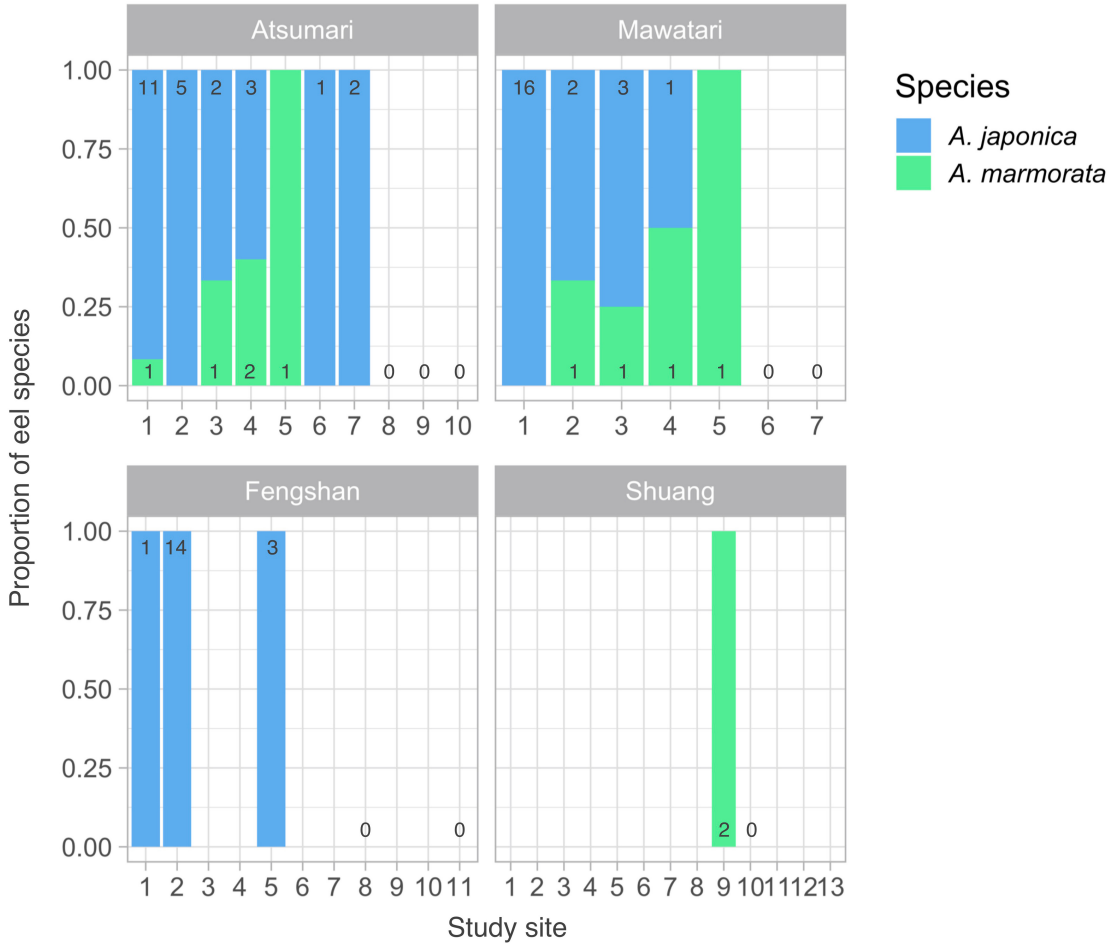

Fig. 3. Species composition of anguillid eels (*Anguilla japonica* and *Anguilla marmorata*) collected in the Atsumari and Mawatari rivers in Japan and the Fengshan and Shuang rivers in Taiwan. The numbers in each bar chart indicate the number of *A. japonica* or *A. marmorata* captured at each study site. The blank indicates that no sampling was conducted.

Table 3. Summary of the results of the environmental DNA (eDNA) analysis and the electrofishing surveys of anguillid eels (*Anguilla japonica* and *A. marmorata*)

Country	River	No. of captured eels and total length (mm)				Eel capture sites (%)	eDNA detection sites (%)	eDNA detection sites where eels were captured (%)	eDNA detection sites where no eels were captured (%)
		A. japonica		*A. marmorata*					
		No.	TL	No.	TL	*A. marmorata*			
Japan	Atsumari	24	422 ± 158 (154–780)	5	279 ± 300 (57–805)	4/10 (40)	10/10 (100)	4/4 (100)	6/6 (100)
	Mawatari	22	240 ± 93 (140–470)	4	456 ± 460 (67–1120)	4/7 (57)	7/7 (100)	4/4 (100)	3/3 (100)
	Oganeku	0	-	60	345 ± 160 (70–656)	12/25 (48)	25/25 (100)	12/12 (100)	13/13 (100)
Taiwan	Fengshan	18	-	0	-	0/5 (0)	0/11 (0)	0/0 (0)	0/11 (0)
	Shuang	0	-	2	-	1/2 (50)	9/13 (69)	1/1 (100)	0/1 (0)
Total		64	-	71	-	21/49 (43)	51/66 (77)	21/21 (100)	22/29 (76)

TL of eels is indicated as mean ± standard deviation (range).

not find *A. marmorata* in the Fengshan River, either by electrofishing or eDNA analysis.

In Taiwan, we collected *A. japonica* by electrofishing at three of the five study sites in the Fengshan River. Conversely, we identified the eDNA of the species from one of three sites (33%) where the eels were collected and from two sites where the species was not collected directly (results not shown).

Relationships between eDNA concentration and abundance and biomass of *A. marmorata*

We investigated whether eDNA concentration reflected the abundance and biomass of *A. marmorata* in the Oganeku River, Japan. We found a significant positive relationship between eDNA concentration and the abundance of *A. marmorata* in all candidate models with different abundances of eels at each sampling site (Type I or II regression model, $p < 0.05$). The AIC and R^2 values of both regression models considerably decreased or increased, respectively, when the abundance of eels was calculated more than 50 m from the water sampling point in the lower reaches of the river and more than 70 m from the water sampling point in the upper reaches (respectively) (Fig. S2). Using these distances, the models revealed that the eDNA concentration of *A. marmorata* was significantly positively related to both the abundance (Type I: coefficient ± SE = 1.416 ± 0.237, $t = 5.965$, $p < 0.001$, $R^2 = 0.590$, AIC = 76.65; Type II: , $R^2 = 0.607$, $p < 0.001$, AIC = 121.88) and biomass (Type I: coefficient ± SE = 0.859 ± 0.284, $t = 3.029$, $p = 0.006$, $R^2 = 0.254$, AIC = 91.63; Type II: , $R^2 = 0.285$, $p < 0.006$, AIC = 141.88) of eels (Fig. 4).

Spatial distribution of anguillid eels inferred by eDNA analysis

In this study, we identified eDNA of both eel species in all the rivers apart from the Fengshan River in Taiwan—we did not identify eDNA of *A. marmorata* from any study site in this river (Table 3; Fig. 2). However, the proportion of eDNA detection sites among the two eel species varied between rivers. Though the proportion of eDNA detection sites of *A. marmorata* was significantly higher in the Hetsuka and Shuang rivers than was that of *A. japonica* (Fisher's Exact Test, $p < 0.05$; Fig. 2), in other rivers, the proportions of eel species did not differ significantly (Fisher's Exact Test, $p > 0.05$; Fig. 2). In particular, eDNA of both species was detected at most study sites in the Atsumari, Kedo, Mae, and Mawatari rivers.

We found the eDNA concentration of *A. japonica* to be higher in downstream sites than in more upstream sites in each river, and it declined significantly with increasing distance from the river mouth (LMM: coefficient ± SE = -0.079 ± 0.025, $t = -3.212$, $p = 0.002$; Fig. 5). Conversely, we did not find a significant

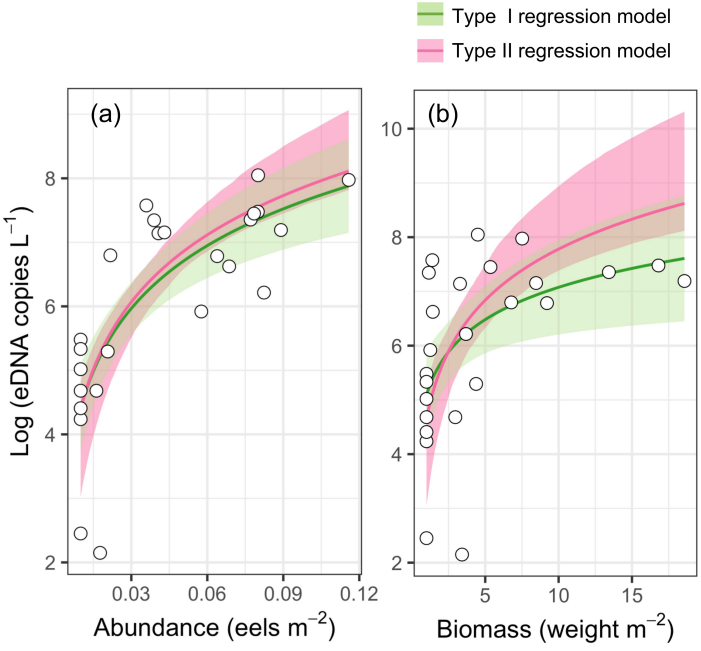

Fig. 4. Relationships between the environmental DNA (eDNA) concentrations for the giant mottled eel (*Anguilla marmorata*) in the surface waters of the Oganeku River in Amami-Oshima Island, Japan, and their (a) abundance and (b) biomass. The green and red lines and shaded areas indicate the best regression slope and 95% confidence intervals (CI) of the Types I and II regression models, respectively.

relationship between the eDNA concentration of *A. marmorata* and distance from the river mouth (LMM: coefficient ± SE = -0.043 ± 0.026, t = -1.629, p = 0.107; Fig. 5). However, the eDNA concentration of *A. marmorata* in the Oganeku River was higher in downstream sites than in more upstream sites, and it declined significantly with increasing distance from the river mouth (Type I: coefficient ± SE = -0.009 ± 0.001, t = -6.678, p < 0.001).

DISCUSSION

Effectiveness of eDNA analysis for estimating the distribution of *Anguilla marmorata*

In this study, we carried out basin-scale surveys of *Anguilla marmorata* from near the river mouths to the upstream reaches of five rivers in southern Japan and northern Taiwan, and we compared the results of eDNA analysis and direct collection of fish by electrofishing.

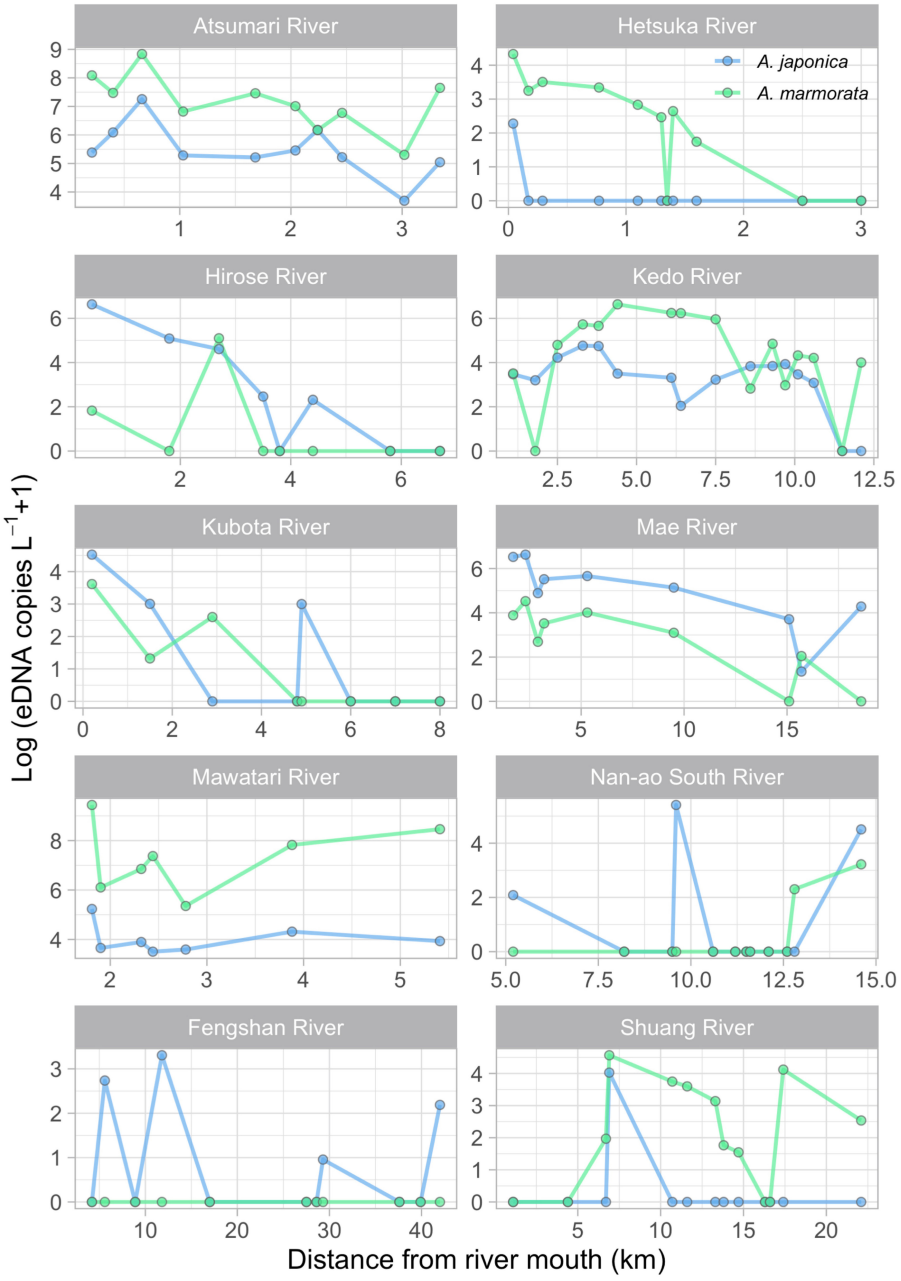

Fig. 5. Environmental DNA (eDNA) concentrations of anguillid eels (*Anguilla japonica* and *A. marmorata*) along distance from the river mouth of each study river. The species are shown as different colored circles and lines. The scales of the x and y axes differ among rivers.

As anticipated, we collected only 11 *A. marmorata* (apart from the Oganeku River on Amami-Oshima Island, from which we collected 60 individuals), suggesting that the abundance of *A. marmorata* in the study region was extremely low compared to that of *A. japonica* (64 individuals collected). Still, we identified *A. marmorata* eDNA not only from all the study sites (21 sites) from which species was collected by electrofishing but also from a further 22 study sites where the species was not collected directly. The sites were located mainly in the upper reaches of the rivers, where eel densities are generally low (Itakura et al. unpublished data). Thus, eDNA analysis seems to be more sensitive than conventional survey techniques for detecting the presence of *A. marmorata*, as reported previously for *A. japonica* (Itakura et al. 2019) and other fish (Jerde et al. 2013; Wilcox et al. 2016; Sakata et al. 2017), and it is therefore likely a powerful tool for monitoring the spatial distribution of *A. marmorata* in rivers.

In addition, irrespective of model type, we found a significant positive relationship between the eDNA concentration and the abundance and biomass of *A. marmorata* in the Oganeku River, indicating that eDNA analysis can facilitate estimation of the abundance and biomass of this species in rivers. Although we found a significant positive relationship between eDNA concentration and the abundance of eels in all candidate models with different abundances of eels at each sampling site, this relationship increased in strength when we calculated the abundance of eels more than 50 and 70 m ahead of water sampling points in the lower and upper reaches of the river, respectively. The eDNA concentration appears to reflect the abundance of eels inhabiting areas more distant from the water sampling points in the upper reaches of the river than in the lower reaches. This is likely due to the higher water velocity in the upper reaches and the distance that the eDNA travels from the source being larger at higher water velocity (Jane et al. 2015). Although the distance that the eDNA migrates in rivers ranges from several tens of meters to kilometers (Deiner and Altermatt 2014; Pilliod et al. 2014; Jane et al. 2015; Wilcox et al. 2016), our findings indicate that the eDNA concentration would reflect the abundance of this species at more than 50 and 70 m ahead of water sampling points in the lower and upper reaches of the river, respectively. Itakura et al. (2019) did not verify that the distances that eDNA travels reflects eel abundance; thus, our findings provide new information that will help in understanding the result of eDNA concentration of anguillid eels.

The AIC and R^2 values of the models indicate that the eDNA concentration of *A. marmorata* reflects their abundance better than it does their biomass. Although this has been reported for *A. japonica* in rivers (Itakura et al. 2019), this finding does not correspond with an aquarium experiment for other fish, which found that eDNA analysis reflects fish biomass better than it does fish density (Maruyama et al. 2014). This may be due to differential eDNA release at different developmental stages of eels and biased distribution of body size and individual density of eels in rivers. The rate of release of eDNA per weight decreases as the life history stages develop; thus, the release rate is higher in juvenile than in adult fish (Maruyama et al. 2014; Takeuchi et al. 2019a), possibly resulting from an ontogenetic decrease in the metabolic rate. This may lead to eDNA analysis overestimating the biomass of a population that is dominated by juveniles (Maruyama et al. 2014). In fact, we identified relatively high concentrations of eDNA in the lower reaches (sampling sites 100–200 m from the river mouth) of the Oganeku River, which included low eel biomass sites because of the dominance of small eels (< 200 mm in TL) (Itakura et al. unpublished data). Conversely, relatively moderate or low concentrations of eDNA were identified more downstream or upstream of the river, which included high or moderate eel biomass sites due to the dominance of larger eels (Itakura et al. unpublished data). This mismatch between high eDNA concentration and the low biomass of *A. marmorata* in the lower reach of the river could result in the model for biomass performing lower than that for abundance observed in this study. As the body size and individual density of anguillid eels tend to vary depending on the distance from the river mouth (Smogor et al. 1995; Glova et al. 1998; Goodwin and Angermeier 2003; Lasne and Laffaille 2008; Yokouchi et al. 2008; Kaifu et al. 2010), eDNA concentration can reflect the abundance of anguillid eels in rivers better than it can reflect their biomass.

The abundance of *A. marmorata* has been shown to decline with increasing distance from the river mouth when the species is highly dominant throughout the river (Robinet et al. 2007). Similarly, our results showed that the eDNA concentration of *A. marmorata* decreased significantly with increasing distance from the mouth of the Oganeku River. As eDNA does not accumulate in downstream reaches, being removed from there through processes such as settling or destruction (Jane et al. 2015; Laramie et al. 2015), our findings suggest that eDNA analysis can be used to identify such habitat utilization of *A. marmorata*.

We did not identify eDNA of *A. marmorata* in any study sites in the Fengshan River in Taiwan from where the species was not collected directly using electrofishing. However, *A. marmorata* has been collected previously in the upper reaches of the river using electrofishing (Hsu et al. 2019). This failure to identify eDNA of *A. marmorata* was likely due to an

exceptionally low eDNA concentration of *A. marmorata* arising from lower density of the species and the larger scale of the river compared with other study rivers from which *A. marmorata* was collected. Hsu et al. (2019) collected 240 *A. japonica* and 24 *A. marmorata* individuals in the Fengshan River over 4 years. This ten-fold difference in the relative abundances of *A. japonica* and *A. marmorata* is higher than that observed in the Atsumari and Mawatari rivers in this study (one-fifth), suggesting that the density of *A. marmorata* in the Fengshan River is relatively low. Moreover, as the Fengshan River (45.5 km) is longer than the Shuang (26.8 km), Nan-ao South (30.6 km), Atsumari (5.9 km), and Mawatari (11.5 km) rivers, there should be greater attenuation of *A. marmorata* eDNA concentration in the Fengshan River than in the other rivers. For *A. japonica* study, Itakura et al. (2019) detected the eDNA of *A. japonica* from nearly all study sites where the species was collected directly; however, the study was conducted in relatively small-scale rivers (3.0–18.0 km). Together, the low density of *A. marmorata* and the large size of the river may have resulted in an exceptionally low eDNA concentration of the species that is below the detectable concentration from the 1 L river water samples collected from each study site. Therefore, it is likely that either using more than 1 L per sample of river water or increasing the sampling numbers is required (Santas et al. 2013; Moyer et al. 2014) to identify the presence of *A. marmorata* in rivers with a very low concentration of eDNA.

Spatial distribution of anguillid eel eDNA in rivers that multiple eel species inhabit sympatrically

We identified both *A. japonica* and *A. marmorata* eDNA from nearly all rivers, suggesting that they are sympatric in the same watershed in the study region. However, the degree of sympatry of these species varied between rivers; though *A. japonica* and *A. marmorata* were identified at either an equal or similar proportion of eDNA detection sites in some rivers, either species was dominant in other rivers. This finding is consistent with previous studies that observed a difference in percentage composition of *A. marmorata* and *A. japonica* in rivers in Taiwan (Shiao et al. 2003). Although both *A. marmorata* and *A. japonica* can potentially recruit to the study region, the relative recruitments of these species can vary even in geographically close regions (Han et al. 2012a). As these species are transported passively by oceanic currents to their growth habitats (Kimura et al. 1999; Han et al. 2012b 2016), the positional relationship between the mouths of each river and the oceanic currents may be an important factor determining whether they can recruit to the growth habitats. As such, differences in recruitment between rivers might lead to differences in the relative abundance of these species between the study rivers.

Another potential reason for the difference in predominant eel species between rivers is the difference in their environmental preferences of growth habitat after recruitment (Shiao et al. 2003). Small and steep streams and gravel and rocky substrates dominate the upper reaches of rivers, whereas gradual flow and sandy and muddy substrates dominate the lower reaches. *A. japonica* tends to inhabit the lower reaches of rivers in Taiwan, whereas *A. marmorata* tends to inhabit the upper reaches (Shiao et al. 2003; Hsu et al. 2019). Conversely, in the subtropical regions, only *A. marmorata* is found in some small and steep rivers, where the environments of the lower reaches and upper reaches are quite similar to each other (Shiao et al. 2003; Wakiya et al. 2019). Thus, if glass eels of both species recruit to rivers, one of them might not be able to survive to the juvenile or growth phase because of unsuitable environmental conditions; however, it should be noted that we did not study the environments in each river. Studying the recruitment and environmental preferences of juvenile eels in growth habitats will help in elucidating their spatial distribution.

A. japonica are generally predominant in the lower reaches of any river, whereas the riverine distribution of *A. marmorata* appears to be completely different. This study found that there was a tendency for the eDNA concentration of *A. japonica* to be higher at downstream sites in each river and to decline significantly with increasing distance from the river mouth. This agrees with previous studies showing that *A. japonica* tends to inhabit lower reaches (Shiao et al. 2003; Hagihara et al. 2018; Hsu et al. 2019) and with findings from this study using electrofishing, in addition to findings showing declines in abundance of *A. japonica* with increasing distance from the river mouth in rivers where only this species is present (Yokouchi et al. 2008; Kaifu et al. 2010; Itakura et al. 2019). Conversely, there was no significant (negative) relationship between the eDNA concentration of *A. marmorata* and the distance from the river mouth, which is not consistent with previous studies that demonstrate a negative relationship between the abundance of *A. marmorata* and the distance from the river mouth in rivers where this species is predominant (Robinet et al. 2007; Itakura et al. unpublished data). *A. marmorata* tends to be dominant in the upper reaches of rivers in which *A. japonica* is dominant in the lower reaches (Shiao et al. 2003; Hsu et al. 2019), but this species is also found in varying habitat types, ranging from estuary to headstream waters (Chino and Arai 2010; Arai and

Abdul Kadir 2017; Arai and Chino 2018; Hagihara et al. 2018; Nguyen et al. 2018). Therefore, the variability of eDNA concentrations of *A. marmorata* found in the present study might reflect the plasticity of their pattern of habitat use in rivers.

We caught more *A. japonica* (n = 46) than *A. marmorata* (n = 9) in the Atsumari and Mawatari rivers (Table 3); however, we detected higher eDNA concentrations for *A. marmorata* than for *A. japonica* (Fig. 5; Table 1). This may be related to differences in eDNA release and microhabitat preferences among species. *A. marmorata* is heavier than *A. japonica* at similar lengths (Shiao et al. 2003), which indicates that, per individual, eDNA release is higher for *A. marmorata* than for *A. japonica*. Moreover, *A. marmorata* is one of the largest anguillid species, and the largest total length of *A. marmorata* (1,120 mm) collected in the rivers was considerably higher than that of *A. japonica* (780 mm) (Table 3). This larger body size of *A. marmorata* may result in an increase in eDNA release, which may contribute to the higher concentration of eDNA of *A. marmorata* compared with that of *A. japonica* observed in the rivers. In rivers where the two species are present sympatrically, *A. marmorata* appears to prefer deep pools, whereas *A. japonica* favors sandy and muddy substrates (Shiao et al. 2003). This suggests that there may be a greater abundance of *A. marmorata* residing in deeper parts of the rivers or upstream waters, none of which could be sampled by electrofishing in this study. It should be noted that there was no such deep water in the Oganeku River, where the relationship between eDNA concentration and abundance and biomass of *A. marmorata* was investigated, and only *A. marmorata* was collected in the river. Although understanding of the riverine distribution pattern of anguillid eels is still poor, it may be affected by both environmental conditions and interspecific competition related to either species composition or relative abundance (Arai and Abdul Kadir 2017; Hagihara et al. 2018), which may, in turn, be influenced by recruitment. Quantitative approaches for studying anguillid eels throughout their ranges, such as eDNA analysis, combined with measurement of detailed environmental conditions, are required to elucidate their patterns of riverine distribution and the mechanism underlying these patterns.

CONCLUSIONS

The present study is the first to demonstrate the potential usefulness of eDNA analysis for estimating the spatial distribution, abundance, and biomass of the tropical eel *A. marmorata* in rivers. As Itakura et al. (2019) demonstrated similar results for the temperate eel *A. japonica*, eDNA analysis could be applied to anguillid eels in general to monitor their riverine populations. We further applied this method to basin-scale surveys to investigate the riverine distribution of sympatric *A. japonica* and *A. marmorata*, and our findings indicated that eDNA analysis could reflect their spatial distributions. eDNA analysis involves less time and fewer human resources than does electrofishing (Itakura et al. 2019) and enables eel populations to be monitored over large spatial and temporal scales using a consistent protocol. This approach could be particularly beneficial in tropical regions, where identifying eel species based solely on morphological characteristics is difficult, as several eel species often co-inhabit rivers in these regions (Watanabe et al. 2004). Therefore, eDNA analysis can help in collecting data not only on the population dynamics of these anguillid species but also on their ecology, such as their spatial distribution, knowledge of both of which are limited considerably in tropical regions (Jacoby et al. 2015), thereby providing invaluable information for managing these eels.

List of abbreviations

AIC, Akaike's information criterion.
CI, Confidence intervals.
IUCN, International Union for Conservation of Nature.
LMM, Linear mixed model.
NCBI, National Center for Biotechnology Information.

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Authors' contributions: HI, RW conceived the study and designed the methodology; SCC, YCH, YSH, HYH, HI, RW and CSY collected the data; HI, TM, MKS and SY analyzed the eDNA samples; HI analyzed the data; HI led the writing of the article. All authors contributed

critically to the drafts and gave final approval for publication.

Competing interests: The authors declare that they have no conflict of interests.

Availability of data and materials: The data that support the findings of this study are not currently available because we are drafting another manuscript using some parts of these data.

Consent for publication: Not applicable.

Ethics approval consent to participate: Captured fish were euthanized with > 10% eugenol solution to euthanize them to satisfy both national and institutional standards.

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Supplementary Materials

Fig. S1. Water velocity plotted against distance from the river mouth in the Oganeku River in the Amami-Oshima Island, Japan. The observation values on (a) August and (b) November 2016. The red line indicates the mean values of water velocity at each section that were separated by changepoint method based on likelihood ratio test. (download)

Fig. S2. Relationships between Akaike's information criterion (AIC) or R^2 values and distances in lower and upper reaches that abundance of *A. marmorata* was calculated in the Oganeku River in the Amami-Oshima Island, Japan. AIC and R^2 values were calculated by a generalised linear model (GLM) and Type II regression model. (download)