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Abstract: The first step toward solving the problems caused by an invasive alien species is to know the distribution of the species. However, species in underwater environments are difficult to investigate. The recent development of environmental DNA (eDNA) analysis has made it possible to investigate the distribution of a target species simply by analyzing the DNA in the water. To date, few investigators have used eDNA detection of aquatic plants. We established an eDNA detection method for *Egeria densa*, an invasive aquatic plant species in Japan; used eDNA detection to survey the species in aquaria; and applied this method to water samples from 23 outdoor ponds. We also used visual observations of the ponds. The aquarium experiments revealed that the eDNA concentration in the water increased rapidly and peaked 1 or 2 d after starting the experiment, after which it decreased rapidly, reaching its lowest point on the 5th day. In the field surveys, we visually observed *E. densa* at 5 ponds, and the eDNA of *E. densa* was detected from the same 5 ponds. Thus, the eDNA results perfectly matched the observational results. Our work confirms that detection of aquatic plants by eDNA analysis is feasible.

Key words: aquatic plant, *Egeria densa*, environmental DNA (eDNA), aquarium, pond, quantitative real-time PCR (qPCR), invasive species

Loss of global biodiversity is a serious problem, and preserving biodiversity is now an important concern (Vitousek et al. 1996). One cause of biodiversity loss is the intentional or inadvertent introduction of invasive alien species from their natural range into other habitats (McGeoch et al. 2010). Many invasive aquatic plant species have increased their biomass rapidly by both sexual reproduction and vegetative propagation in their new environments. They displace native plants because they tend to grow quickly and monopolize resources and light (Cronk and Fennessy 2001). To address the problem, the distribution of invasive species must be known (Pyšek and Richardson 2010).

The usual manner of investigating the distribution of plant species in freshwater ecosystems, such as ponds, rivers, and lakes, is capturing or observing the target species directly. However, monitoring plant species across whole ponds or lakes is difficult because these methods generally require a huge amount of labor and time. Moreover, taxonomic expertise is required to identify target species on the basis of morphological characters. Environmental DNA (eDNA) analysis, which is the analysis of DNA molecules released into the surrounding environment from organisms, was invented to reduce the labor and time of fieldwork (Ficetola et al. 2008, Minamoto et al. 2012, Janosik and Johnston 2015, Thomsen and Willerslev 2015). An environmental DNA (eDNA) approach enables investigators to search for the target species by collecting and analyzing eDNA in water samples. The advantage of the eDNA method is that the distribution of the target species can be determined by analyzing the DNA in the collected water without capturing or viewing the organisms directly. In addition, this approach places far less stress on organisms and habitats than the traditional survey method.

eDNA detection has been reported for a number of aquatic species, including fish (Jerde et al. 2011, Minamoto et al. 2012, 2016, Maruyama et al. 2014, Doi et al. 2015a, b,

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Miya et al. 2015, Sigsgaard et al. 2015), amphibians (Ficetola et al. 2008, Goldberg et al. 2011, Olson et al. 2012, Pilliod et al. 2013, Biggs et al. 2015, Fukumoto et al. 2015), mammals (Foote et al. 2012, Thomsen et al. 2012), and invertebrates (Thomsen et al. 2012, Deiner and Altermatt 2014, Treguier et al. 2014, Deiner et al. 2015). However, the detection of eDNA from aquatic plants has been attempted only in aquarium experiments (Scriver et al. 2015) and has not been reported from field studies. A field eDNA survey would be a particularly useful technique for invasive submerged plants because such species are difficult to distinguish by visual observation from the shore (Haga and Ishikawa 2014). Moreover, taxonomic expertise usually is required to identify submerged plant species, and obtaining plants from the bottoms of ponds or lakes is sometimes difficult. Taking a water sample while standing on the shore is much easier than approaching the plant itself. Application of eDNA analysis for submerged plants in the field may address such difficulties associated with traditional sampling.

We used eDNA analysis with the Brazilian waterweed (*Egeria densa*) in the field. *Egeria densa* is a submerged plant that is native to South America (Cook and Urmi-König 1984) and was imported into Japan as an experimental plant in the 1910s (Ohtaki and Ishido 1980). The exotic, submerged plant soon established a population in the natural waters of Japan, where it competes with native species. The biomass of native plants declined drastically after its introduction (Ikusima 1991). *Egeria densa* is listed as an invasive alien species by the Japanese Ministry of the Environment and should be exterminated. An eDNA analysis of this species would help reveal its distribution and inform efforts to conserve native species.

To achieve our objective to develop an eDNA detection method for the species in the field, we: 1) established an eDNA detection method for *E. densa*, 2) tested eDNA detection of the species in aquarium experiments, and 3) applied this method to water samples from various ponds, including some in which the species had been observed and some in which it had not.

METHODS

Development of specific primers and probe for *E. densa*

The most closely related syntopic species to *E. densa* in Japan is *Elodea nuttallii* (Kadono 2004). The genetic marker for the analysis was chosen from the DNA region in which the sequence obviously differs between *E. densa* and *E. nuttallii* (see Results). The species-specific polymerase chain reaction (PCR) primer and TaqMan probe for *E. densa* was designed by Primer Express 3.0 (Life Technologies, Foster City, California) and based on the chloroplast DNA data of *E. densa* (GenBank Accession number JF703276). We selected the region of an intergenic spacer between *trnL* and *trnF* in chloroplast DNA as the marker (see Results).

PCR testing for extracted DNA sample

We extracted DNA from purchased *E. densa* and wild *E. nuttallii* collected at Lake Biwa, Shiga Prefecture, Japan, by means of the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). To check the specificity of the primers, we conducted quantitative real-time PCR (qPCR) on the DNA samples (50 pg, 10 pg, and 5 pg/reaction) of *E. densa* and *E. nuttallii* with the designed primer sets. The conditions for qPCR are described below.

Aquarium experiment

We used 3 tanks for experimental treatments and 1 as a blank control that did not include any plants. The tanks were 17.5 cm long × 11.3 cm wide × 11 cm high. We bleached the tanks before using them to reduce DNA contamination. We filled each tank with 990 mL of aged tap water and 10 mL of liquid fertilizer (AlgoFlash [N 30 mg/L]; COMPO, Münster, Germany). The filled tanks were maintained at 25°C in an incubator and received light from 0500 to 2100 h daily (L : D = 16:8 h).

We measured the fresh mass of each plant and adjusted it to 4 g by cutting. We kept plants in a stock aquarium for 1 wk at 25°C under the same light conditions before the experiment started. We transferred 1 plant to each experimental tank and collected a 15-mL water sample in a 50-mL sterilized tube from each tank 0, 1.17, 2, 4, 8, 24, 48, 120, and 168 h after plants were introduced. We collected eDNA by ethanol precipitation. We added 1.5 mL of 3 M sodium acetate (pH 5.2) and 30 mL of absolute ethanol to each 15-mL water sample and kept the mixture in a freezer for >1 h. Next, we centrifuged the samples for 20 min at 5350*g* and discarded the supernatants. We redissolved the pellets with 200 μL of sterilized water and used a DNeasy Blood and Tissue Kit (QIAGEN) to extract the DNA to reach a final volume of 100 μL of DNA solution (instead of the manufacturer's recommended 200 μL).

Field surveys

We surveyed 23 ponds in Hiroshima Prefecture, Japan. We conducted the field survey in ponds P6–P17 on 11 June, P1–P5 and P18–P21 on 19 June, and P22 and P23 on 16 July 2014 (Fig. 1). We visually observed each pond for 10 min from the shoreline, looking for *E. densa*. We collected 1 L of water from the shore of each pond with equipment, including the bottle and filter funnel, that had been bleached for 10 min before use to avoid contamination. We filtered the water through a glass-fiber filter (GF/F; GE Healthcare Life Sciences, Little Chalfont, UK) on the day of collection. We prepared 1 negative control for each survey day to confirm the absence of contaminating DNA in the instruments. Thus, we used 23 environmental samples and 3 negative controls for DNA extraction and measurement.

DNA trapped on the filter was extracted with a Salivette tube (Sarstedt, Nümbrecht, Germany) and DNeasy

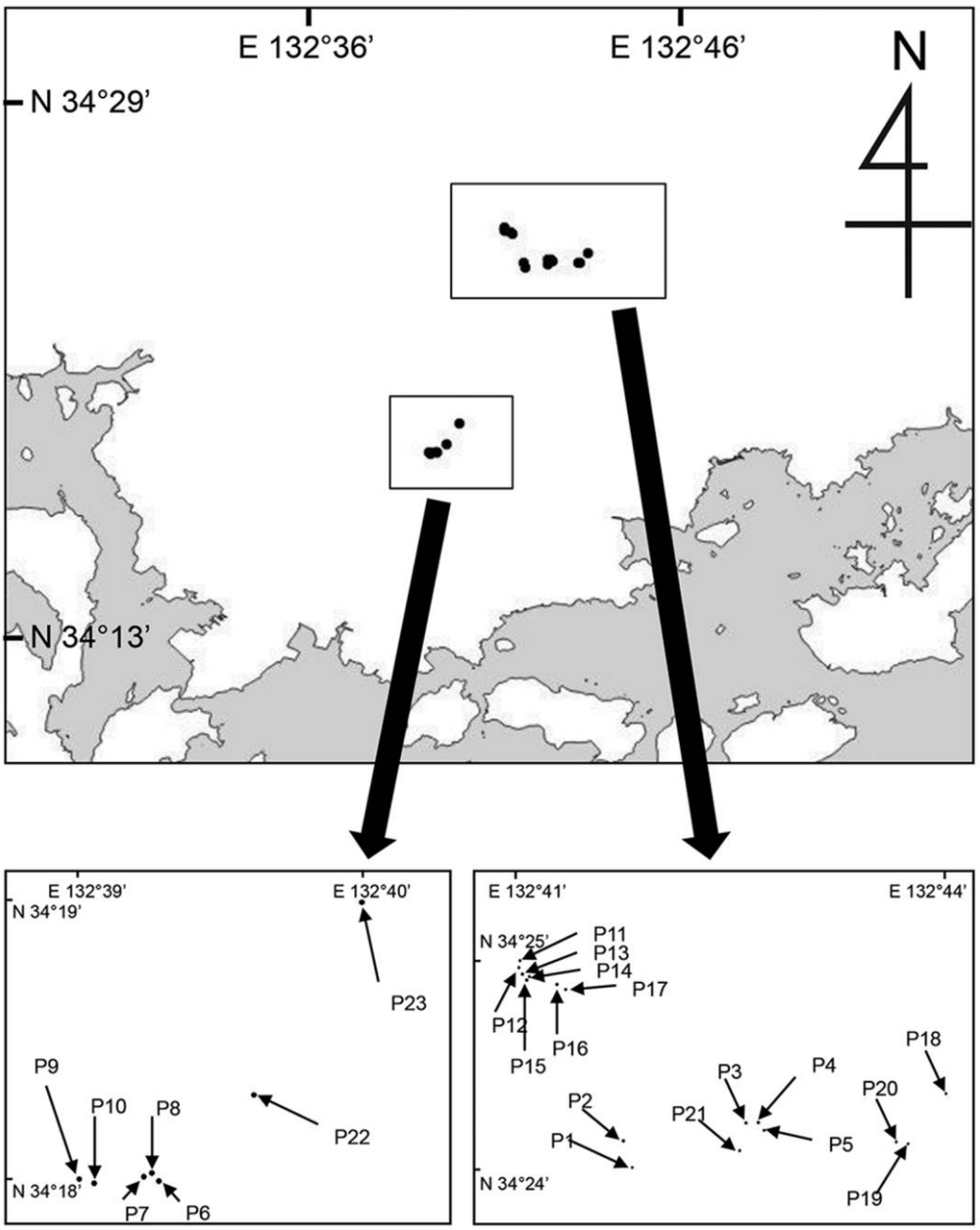

Figure 1. Sites for field surveys (Higashi–Hiroshima City, Hiroshima Prefecture, Japan). Water samples were collected from 23 ponds.

Blood and Tissue Kit (Qiagen). Briefly, the filter was placed in a Salivette tube and centrifuged for 3 min at 5000*g*. We added 200 μL of buffer AL, 200 μL of water, and 20 μL of proteinase K per sample to the filter in the Salivette tube. Tubes were maintained at 56°C for 30 min and then centrifuged for 3 min at 5000*g*. To retrieve the DNA from the filter, we added 200 μL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), allowed it to stand for 1 min, and centrifuged it for 3 min at 5000*g*. After adding 200 μL of buffer AL and 600 μL of ethanol, we transferred the sample solution into a DNeasy Mini Spin Column and centrifuged it for 1 min at 6000*g*. Next, we conducted DNA extraction according to the protocol of the DNeasy Blood and Tissue Kit, except that we used a final volume of DNA of 100 μL instead of the manufacturer's recommended 200 μL.

Analysis of aquarium and field samples using qPCR

To prepare a quantification standard, we inserted 373 base pairs (bp) of the *trnL–trnF* intergenetic spacer region, including the target region, into a pUC57 plasmid

and digested with a restriction enzyme, EcoRI. We measured the DNA concentration with a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California) and calculated the number of DNA copies. We used a standard curve with 30,000, 3000, 300, and 30 copies/PCR reaction. The 20-μL qPCR reaction mixtures contained 1×TaqMan Gene Expression Master Mix (Life Technologies), 900 nM of each primer, 125 nM of TaqMan probe, and 2 μL of DNA sample. PCR was performed for 2 min at 50°C and 10 min at 95°C, followed by 55 cycles of 95°C at 15 s and 60°C at 1 min. We conducted each qPCR with 3 and 6 replicates for aquarium and field samples, respectively. Three or 6 wells of negative control were used per PCR plate. To avoid contamination, we conducted prePCR and PCR experiments in different rooms.

Statistical analysis

For the tank experiments, we compared the eDNA concentration among sampling times and among tanks with a generalized linear model (GLM) and assumed Gaussian distribution. For the field results, we calculated Cohen's κ and compared the number of ponds in which *E. densa* were detected through visual observation and through eDNA in the sampled water. Cohen's κ should be between 0 and |1|, with |1| referring to the highest matching for the proportion. We conducted all statistical analyses in R (version 3.1.1; R Project for Statistical Computing, Vienna, Austria.)

RESULTS

Design of primers

First, we compared the sequence of *matK* between *E. densa* and *E. nuttallii*. The DNA sequences of *matK* of *E. densa* (GenBank accession number AB002567) and *E. nuttallii* (GenBank accession number AB002568) were 99% identical (13 substitutions within 1299 bp). In addition, we compared the sequences of *rbcL*, which is used for DNA barcoding in plant species, between these 2 species, and the sequences were 99% identical (12 substitutions within 1182 bp; AB004887 for *E. densa* and AB004888 for *E. nuttallii*). Last, we designed a set of PCR primers in the region of intergenic spacer between *trnL* and *trnF* (Appendix S1): a forward primer (5′-CATTTCTCCTTCATTGTATTCTTTCACA-3′), a reverse primer (5′-ATTTCTATCTGTATCGTAGCCACCAA-3′), and a TaqMan probe (5′-FAM-CGGGTCCGAACAGAAATGCTTCTCTCT-TAMRA-3′). To check the specificity of the designed primer and probe set, we conducted qPCR amplification with 50, 10, and 5 pg of *E. densa* and *E. nuttallii* DNA. We confirmed that our primers and probe set amplified *E. densa* but not *E. nuttallii*.

Aquarium experiment

We detected the eDNA of *E. densa* in all experimental tanks. The volume of DNA peaked at 48, 24, and 48 h after it was introduced to tanks 1, 2, and 3, respectively. The concentration of the plant eDNA rapidly decreased and reached its lowest value by 120 h. At 168 h, the eDNA concentration remained low in tanks 1 and 2 but increased in tank 3 (Fig. 2). The eDNA concentration was higher at 24 h (GLM, $p = 0.030$), 48 h ($p = 0.0054$), and 168 h ($p = 0.044$) than at 0 h, and that in tank 3 was higher than that in tank 1 ($p = 0.011$). No positive signal was detected in the control tank throughout experimental period or in the PCR negative controls.

Field surveys

Our visual census identified *E. densa* in P6, P7, P8, P22, and P23 but not in any of the other ponds (Table 1). The qPCR examination generated positive signals from the samples of P6, P7, P8, P22, and P23 (Table 1). Thus, the eDNA results perfectly matched our observations. Cohen's κ value for the proportion was 1, indicating that the proportions were the same ($z = 4.8$, $p < 0.001$, $n = 23$). All 6 PCR replicates were positive for samples collected at P6, P7, P22, and P23, and 2 were positive for P8 (Table 1). No amplification was found either in the equipment blanks or in the PCR negative controls.

DISCUSSION

We established a species-specific detection system for *E. densa*. We demonstrated the change in the concentration of eDNA during 1 wk in an aquarium experiment and successfully detected the eDNA of aquatic plants in the field for the first time. Our results show that plants release DNA into their environments, enabling detection of plant eDNA from field-collected water samples. The eDNA approach could reduce the labor and time required for investigations of species distribution and improve our knowledge of the distribution of aquatic plants.

We had to design primer sets that could discriminate *E. densa* from *E. nuttallii* because these species co-occur

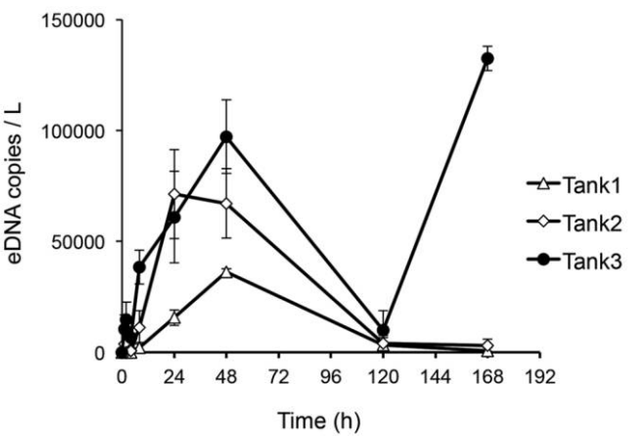

Figure 2. Mean (±1 SD) environmental DNA (eDNA) concentration over time in the aquarium experiment.

Table 1. Results of field surveys and quantitative real-time polymerase chain reaction (qPCR) with field water samples. For the visual census, + means that we visually confirmed the presence of *E. densa* in the pond and – means that we did not. The qPCR results show the number of positives in 6 qPCR replicates. The environmental DNA (eDNA) concentration at each site was estimated by qPCR.

Site	East longitude	North latitude	Visual census	qPCR results	eDNA concentration (copies/L)
P1	132°42′25.10″	34°24′18.756″	–	0/6	0
P2	132°42′22.74″	34°24′27.997″	–	0/6	0
P3	132°43′4.329″	34°24′34.115″	–	0/6	0
P4	132°43′8.500″	34°24′34.306″	–	0/6	0
P5	132°43′10.509″	34°24′31.566″	–	0/6	0
P6	132°39′47.966″	34°18′52.261″	+	6/6	512.7
P7	132°39′45.185″	34°18′53.26″	+	6/6	1402.3
P8	132°39′46.652″	34°18′53.728″	+	2/6	12.1
P9	132°39′32.902″	34°18′52.580″	–	0/6	0
P10	132°39′35.760″	34°18′51.750″	–	0/6	0
P11	132°41′46.425″	34°25′29.620″	–	0/6	0
P12	132°41′46.38″	34°25′27.326″	–	0/6	0
P13	132°41′47.236″	34°25′25.64″	–	0/6	0
P14	132°41′49.515″	34°25′24.203″	–	0/6	0
P15	132°41′48.781″	34°25′22.993″	–	0/6	0
P16	132°41′59.54″	34°25′21.527″	–	0/6	0
P17	132°42′2.106″	34°25′19.775″	–	0/6	0
P18	132°44′13.311″	34°24′44.121″	–	0/6	0
P19	132°44′0.179″	34°24′27.41″	–	0/6	0
P20	132°43′56.162″	34°24′27.678″	–	0/6	0
P21	132°43′2.12″	34°24′24.555″	–	0/6	0
P22	132°40′5.945″	34°19′8.546″	+	6/6	3788.0
P23	132°40′26.319″	34°19′45.21″	+	6/6	2999.5

in Japan. Primer sets for *E. densa* were reported by Scriver et al. (2015), but the reported primer set targeted the *matK* region, which is a conserved gene among plant species and has few variations among related species (Tanaka et al. 1997). DNA sequences of *matK* for *E. densa* and *E. nuttallii* showed 99% identity. We designed a primer set specific to the *trnL–trnF* intergenic spacers of *E. densa* that can detect *E. densa* where other plants, including *E. nuttallii*, occur.

Data from the aquarium experiments indicated that the temporal change in the eDNA concentrations of *E. densa* was similar to that of fish (Takahara et al. 2012). In all experimental tanks, the eDNA concentration increased rapidly and peaked 1 or 2 d after the introduction of the plants. Then the concentration decreased and became stable by the 5th day after starting the experiment. In the case of animals, such an early high concentration may be the result of the stress caused by changing the surrounding water environment (Takahara et al. 2012), and this explanation may also apply to plants. More plant debris was present in tank 3 than in the other tanks at the end of the experiment, and larger amounts of eDNA were found in tank 3 than in the other 2 tanks (Fig. 2). The concentration of eDNA may reflect the decomposition of plant tissue.

The ponds in which *E. densa* was detected by the eDNA method corresponded with the ponds in which the species was observed visually, suggesting that eDNA analysis is at least as sensitive as field observation. The eDNA method may allow the distribution of *E. densa* to be revealed quickly, but more empirical studies of other taxa are needed to determine the broad utility of eDNA detection for aquatic plants.

We targeted *E. densa* because the characteristics of this species (e.g., rapid growth and establishment of large colonies) (Kadono 2004) enabled us to confirm its occurrence in the ponds visually. However, many aquatic plants are difficult to observe and identify by visual census (e.g., they inhabit ponds and lakes with more depth or turbidity). The eDNA approach may allow the occurrence of such species to be confirmed without collecting or observing the plants themselves. Moreover, with eDNA analysis, we

can reduce misidentifications of plants. Thus, even people without taxonomic expertise can investigate plant distribution. In addition, in many plant species, the identification of the species is based on flowers or seeds as key characters. The period of field surveys is highly restricted because those characters may be present only during a very short period. As noted, eDNA analysis may provide more accurate identification and extended survey periods for aquatic plant species.

We developed a procedure for using eDNA to detect aquatic plants in the field. Further progress on eDNA analytical methods will enable us to estimate the biomass of plant species as in previous studies (Eichmiller et al. 2014, Doi et al. 2015a) and to investigate the biodiversity of aquatic plants by metabarcoding with plant-universal primers. These advances will enable us to cope with the challenge of preserving rare species and expelling invasive species.

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