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Seasonal change in environmental DNA concentration of a submerged aquatic plant species

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Abstract: Environmental DNA (eDNA) methods are increasingly used to detect aquatic organisms. To optimize survey efficiency in natural environments, it is necessary to understand the seasonal change in eDNA concentrations of target species and identify the season and environmental conditions in which eDNA concentrations are highest. Recently, eDNA methods have been developed to detect aquatic plant species, but the seasonal change in the eDNA concentrations remains uninvestigated. Many aquatic plants undergo considerable changes in abundance, size, and shape throughout the year. Therefore, their eDNA concentration may change along with their phenology. We investigated the seasonal change in the eDNA concentration of an aquatic submerged species, *Hydrilla verticillata*, in agricultural ponds in Japan by measuring the eDNA concentration of *H. verticillata* from 5 ponds 5× in a year. This species has a dormant period during winter and a growth period from spring to autumn. The eDNA concentrations were higher during the growth period than during the dormant period. Management and conservation surveys that use eDNA for species detection should be done when eDNA concentrations are highest to maximize detection probabilities and therefore survey efficiency.

Key words: eDNA, Hydrocharitaceae, *Hydrilla verticillata*, phenology, seasonality, biological invasions, local extinction

Environmental DNA (eDNA) methods are used to detect and quantify DNA in environmental samples and obtain information about the occurrence of target species in the environment (Ficetola et al. 2008, Jerde et al. 2011, Minamoto et al. 2012, Rees et al. 2014). eDNA methods have been applied to field research in various taxa such as fish (Jerde et al. 2011, Thomsen et al. 2012a, Takahara et al. 2013, Yamamoto et al. 2017), amphibians (Thomsen et al. 2012b, Goldberg et al. 2013, 2016 Fukumoto et al. 2015, Valentini et al. 2016), reptiles (Piaggio et al. 2014, Davy et al. 2015), mammals (Foote et al. 2012, Thomsen et al. 2012b), crustaceans (Deiner and Altermatt 2014, Ikeda et al. 2016), and plants (Fujiwara et al. 2016, Matsuhashi et al. 2016). However, eDNA concentrations and detection rates of a target species may change seasonally in natural environments, which could influence estimates of organism abundance and distribution.

eDNA concentration and detection rate change seasonally in the salamander species *Cryptobranchus alleganiensis* (Spear et al. 2015), *Triturus cristatus* (Buxton et al. 2017), *Andrias japonicus*, and *A. davidianus* (Fukumoto et al. 2015). In ayu, *Plecoglossus altivelis*, eDNA concentrations were correlated with population abundance or biomass changes from spring to autumn (Doi et al. 2017a). These results suggest that the eDNA concentration and detection rate of a target species may change with seasonal changes in its abundance, life stage events such as breeding, and behaviors such as movement. Seasonal changes in the target species, therefore, need to be considered when interpreting the results of eDNA analysis of natural populations because the distributions of target species could be underestimated without information about seasonality. Moreover, if the eDNA concentration and detection rates of a target species vary seasonally, eDNA analysis should be done when eDNA concentrations are highest to increase detection rate and survey efficiency and, therefore, optimize field surveys.

Recently, eDNA methods have been applied to aquatic plant species (Scriver et al. 2015, Fujiwara et al. 2016, Matsuhashi et al. 2016). The abundance, size, and shapes of the bodies and leaves of aquatic plants can change sea-

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sonally. Therefore, their eDNA concentrations may change with their phenology. However, the seasonal change in eDNA concentration and detection rate has not been studied in any aquatic plants.

Here, we investigate the seasonal change in eDNA detection rate and concentration in an aquatic submerged species, *Hydrilla verticillata* (Hydrocharitaceae). *H. verticillata* is a perennial plant that changes markedly in abundance and shape throughout the year. The stem and leaves of *H. verticillata* grow from tubers or turions every year, and then the leafy part of the plant dies and the plant forms tubers during winter (Haller et al. 1976). An eDNA assay is available for surveys of natural populations of this species (Matsuhashi et al. 2016). If leaves release DNA, eDNA concentrations could increase when the above-ground part of *H. verticillata* grows and its biomass increases. In contrast, if DNA is released from cells or fragments of the plants when they die, the eDNA concentration might instead increase in winter when portions of the plants die. By investigating the seasonal change in the eDNA concentration and detection rate and comparing them among seasons, we demonstrate the importance of eDNA analysis that takes into account the phenology in natural populations of aquatic plants.

METHODS

Study species

H. verticillata is a submerged aquatic plant species native to Asia and Australia (Cook and Lüönd 1982, Buckingham and Bennett 1996). In Japan, especially in the eastern region, this plant species is threatened with local extinction (see the local Red Data Books: Tochigi Prefecture 2006, Ishikawa Prefecture 2010, and Tokushima Prefecture 2014). In contrast, it is an invasive species in North America, South America, New Zealand, Africa, and Europe, where it has caused serious damage to native species, ecosystems, and the economy in invaded areas (Murphy 1988, Langeland 1996, Hershner and Havens 2008, Sousa 2011).

In Japan, this species germinates in spring (May–Jun) as an herb morph, transforms to a grass morph from Jul to Oct (Haramoto and Ikusima 1988), and then blooms from Aug to Oct (Kadono 2014). Finally, the stem and leaves of the plant die, and it overwinters as turions and tubers (Haramoto and Ikusima 1988).

Sample collection

We studied 5 ponds (Fig. 1) in which we had found *H. verticillata* eDNA in a previous study (Matsuhashi et al. 2016). To monitor the eDNA concentrations throughout the year, we sampled the water from each pond 5× over 9 mo. (Apr 25, Jul 10, Aug 21, Oct 5, and Dec 11, 2015). At each sampling time we collected a single sample of 1 L of surface water in a plastic sampling bottle from the accessible shore of each pond. The plastic sampling bottles, including a negative control, were bleached with a sodium hypochlorite solution before sampling to avoid DNA contamination. The bottles were filled with a 0.06% sodium hypochlorite solution for more than 5 min, and then rinsed with water more than 10×. The samples were immediately placed in a cooler until filtration. We also recorded the temperature (YSI ProODO; YSI Inc. Yellow Springs, Ohio) and pH (Twin pH; Horiba Co., Tokyo, Japan) of the water because these variables can affect eDNA degradation (Strickler et al. 2015). We also noted whether *H. verticillata* was visible in each pond at each sampling time by visually examining the pond from the shore for 2 to 5 min, depending on the size of the pond. To determine whether the sample bottles were contaminated in the cooler during sampling and transportation, we placed a 1-L bottle of DNA-free distilled water in the cooler on each sampling day as a field control.

DNA extraction and quantitation

Each water sample, including the field control, was filtered onto 2 GF/F glass filters (mesh size: ~0.7 μm; GE Healthcare Japan, Tokyo, Japan) within 6 h of sampling. For most samples we filtered the entire 1 L (0.5 L onto each filter). However, we stopped filtering whenever a filter became clogged, so for some samples we filtered as little as 0.45 L onto each filter. All filtration equipment was bleached in the same manner as the sample bottles and rinsed with DNA-free pure water between rounds of filtration to prevent cross-contamination. To determine whether contaminating DNA could have persisted on filtering equipment during filtration, 1 L of DNA-free distilled water was filtered through apparatuses as an equipment control after each sample was filtered and the equipment was decontaminated. We stored all filters at −20°C until DNA extraction. We collected the DNA from each filter by centrifuging a Salivette tube (Sarstedt, Nümbrecht, Germany), and extracted the DNA with a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the method of Uchii et al. (2016). DNA was eluted in 100 μL of Buffer AE and stored at −20°C until polymerase chain reaction (PCR) was conducted.

Quantitative PCR was done with the PikoReal Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts) with a primer/probe set developed for *H. verticillata* (Matsuhashi et al. 2016). Each TaqMan reaction contained 900 nM of each primer, 125 nM TaqMan probe, 5 μL of PCR master mix (2 × TaqMan Environmental Master Mix 2.0; Thermo Scientific), and 3 μL of the DNA solution, for a final volume of 10 μL. The PCR conditions were as follows: 10 min at 95°C, 55 cycles of 15 s at 95°C, and 1 min at 58°C. PCR was done in triplicate, and the mean concentration of the 3 replicates was treated as the concentration of each sample. If the target DNA was not detected in a replicate, we assumed the concentration of target DNA was 0 (Takahara et al. 2012). To prepare the DNA standard, the target sequence of the amplification

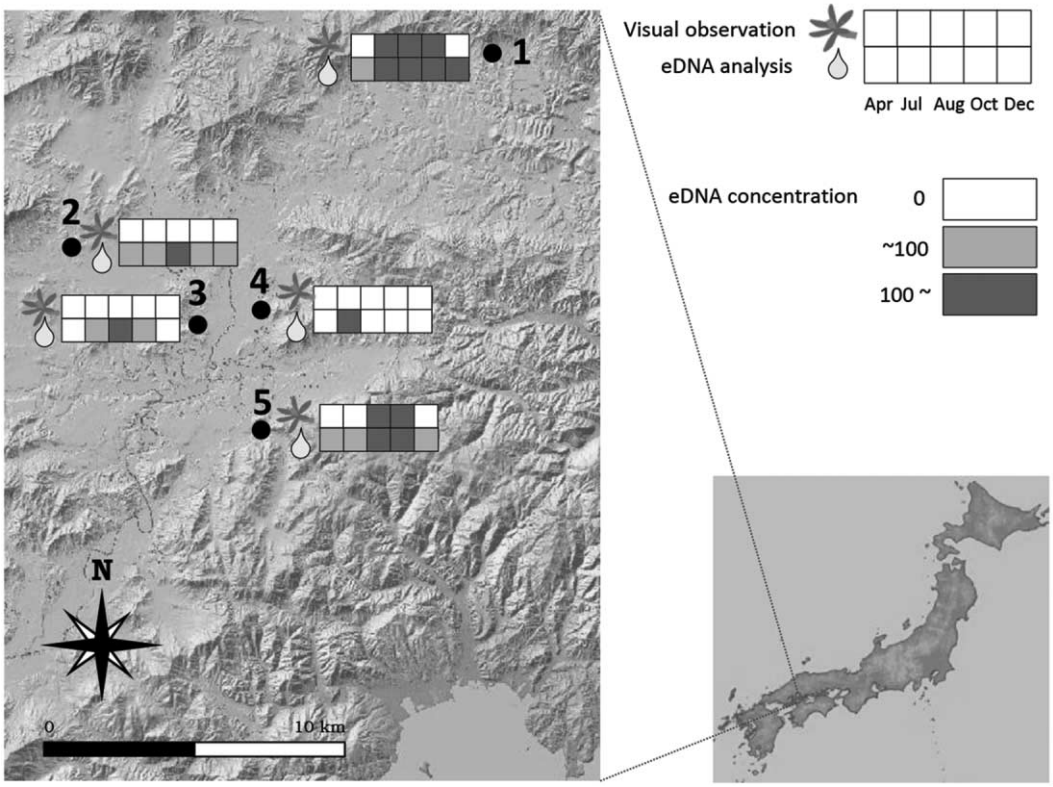

Figure 1. Location of the 5 study ponds. The numbers correspond to the pond identifications (IDs) in Table 1. For each site, sampling month (Apr, Jul, Aug Oct, and Dec 2015), the results of the 5 visual observations and eDNA analyses are shown. Closed boxes in the upper and lower portions indicate the detection of *Hydrilla verticillata* by visual observation and eDNA analysis, respectively. The shading of the boxes indicates the *H. verticillata* eDNA concentrations: white = undetected, light gray = <100 copies/L, dark gray > 100 copies/L. The map was constructed from geospatial data published by the Geographical Survey Institute, Japan.

was inserted into a pMD20-T vector (Takara, Shiga, Japan), and the vector was digested with EcoRI. A standard curve was constructed with 15,000, 1500, 150, 15, and 1.5 copies of the standard DNA per PCR reaction. The dilution series was used in triplicate for each reaction. The lowest copies of the standard DNA were always detected. The R^2 values of the standard curves ranged from 0.978 to 0.992 (PCR efficiencies = 75.4–85.9%). All PCR plates contained 3 no-template controls of ultrapure water in place of template DNA.

Statistics

To estimate the effect of plant phenology on eDNA concentration and detection rate, we examined whether the eDNA concentration and detection rate are different: 1) between 2 phenological groups (growth and dormant period, see the next paragraph), 2) among the sampling events (not considering length of time between events), and 3) with the temporal change of days and variation in water environments. All analyses were conducted with the software R version 3.3.2 (R Development Core Team 2016).

Table 1. Locations (decimal degrees) and topological information of 5 ponds.

Pond ID	Latitude (°N)	Longitude (°E)	Max Depth (m)	Surface area (m^2)
1	34.4734	132.8239	0.3	83
2	34.4236	132.6965	1.2	1021
3	34.4062	132.7345	0.7	998
4	34.4108	132.7549	1.5	1521
5	34.3806	132.7549	1.1	6017

First, we compared the 2 phenological groups. *Hydrilla verticillata* grows and blooms during summer and autumn, especially from Jul to Oct (grass form, Haramoto and Ikusima 1988). Thus, we divided the data into 2 groups: the growth period (Jul–Oct: 5 ponds × 3 sampling dates) and the dormant period (Apr and Dec: 5 ponds × 2 sampling dates). We tested whether differences in eDNA concentration and detection rate existed between the 2 groups with the Brunner–Munzel test and Fisher's exact test, respectively. The tests were conducted with the *brunner.munzel.test* function of the *lawstat* package and the *fisher.test* function of the R *stats* package.

Second, we pooled the data from all ponds and tested whether the eDNA concentration and detection rate differed among the 5 sampling dates (Apr 25, Jul 10, Aug 21, Oct 5, and Dec 11). We tested this with Friedman's test (at $\alpha = 0.05$) and Fisher's exact test, respectively. We used the functions *friedman.test* and *fisher.test* in the R *stats* package to conduct these tests.

Third, we tested whether the eDNA concentration and detection rate were correlated with days since 1st sampling, water temperature, and pH. We did this analysis with a generalized linear mixed model (GLMM) with a Gaussian distribution and identity link with the R function *lme* of the R *nlme* package. In this analysis, eDNA concentration was the response variable, days since 1st sampling, water temperature, and pH were the explanatory variables, and pond was a random variable. When we did a test with the same structure but a binomial distribution and detection rate as the response variable, the GLMM did not converge. Thus, we used a generalized linear model (GLM) with a binomial distribution and logit link in the *glm* function. We calculated the variance inflation factor (VIF) to check multicollinearity among the explanatory variables and confirmed that all the variables could be included in the same model (VIF < 2). The best models were selected with downward model selection (with Akaike's information criterion [AIC]). To compare among models, we calculated ΔAIC.

RESULTS

We analyzed a total of 25 samples (5 sampling times × 5 ponds) and detected *H. verticillata* DNA in 19 samples. Pond 3 did not contain *H. verticillata* DNA on Apr 25 or Dec 11, and Pond 4 had *H. verticillata* DNA only on Jul 10. Ponds 1, 2, and 5 had *H. verticillata* eDNA in every sample. We visually observed *H. verticillata* only 5× during sampling: in Jul, Aug, and Oct in Pond 1, and Aug and Oct in Pond 5 (Fig. 1). We did not detect *H. verticillata* DNA in any of the negative controls, including field, equipment, and non-template controls.

eDNA concentrations were significantly different between the growth period and the dormant period. eDNA concentrations were marginally significantly different on different dates (Friedman's $\chi^2 = 8.32$, df = 4, $p = 0.08$), and the eDNA concentrations between the growth period and the dormant period were significantly different (Brunner–Munzel Test Statistic = –2.50, df = 22.98, $p = 0.02$). The maximum eDNA concentration in every pond was found between Jul and Oct (Fig. 2A), which corresponds to the results of the differences between the growth and dormant periods.

The GLMM showed that the effects of number of days from 1st sampling, water temperature, and pH were selected as the explanatory variables with downward model selection with AIC. Both water temperature and pH were included in the 2 models that had ΔAIC < 2. The number of days from the 1st sampling was included in 1 of the best models, so it is not clear whether this variable helps explain eDNA concentration. The water temperature changed throughout the season in a similar manner in each pond (Fig. 2B). The minimum temperature in every pond occurred in Dec (8.0–9.5°C), whereas the maximum occurred in Jul or Aug (22.7–30.6°C). The temperatures in Apr and Oct were also similar to each other in all study ponds (range: 16.3–19.8°C). eDNA concentrations seemed to be lower in all study ponds when temperatures were the lowest, which corresponds with our GLMM result that water temperature significantly improves the model fit and is positively correlated with eDNA concentration (Table 2A). The measurements and ranges of pH were also different among the ponds (Fig. 2C). The lowest and highest values were pH 5.8 and 8.9, respectively. The pH values in Pond 3 remained constant throughout the year (range: pH 6.4–6.6), whereas those in Pond 5 changed mark-

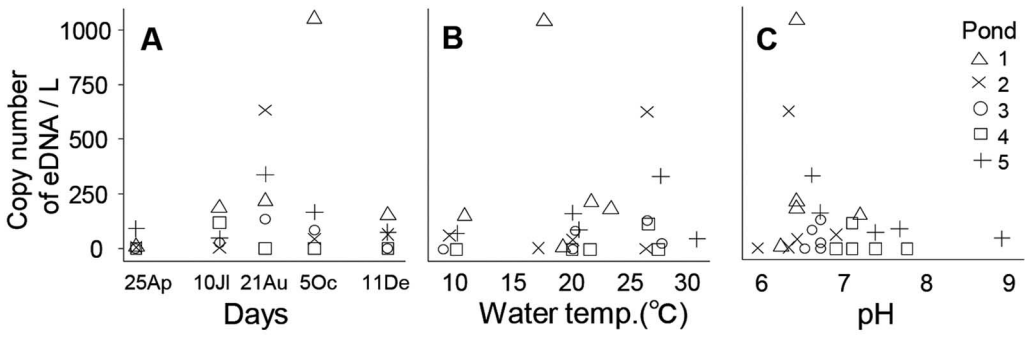

Figure 2. Relation between eDNA concentration of *Hydrilla verticillata* and sampling date (A), water temperature (B), and pH (C).

Table 2. Akaike's information criterion (AIC) and coefficient values for the generalized linear mixed model (GLMM) of the effects of water quality and the number of experimental days on eDNA concentrations (A) and for the generalized linear model (GLM) of the effects of water quality and the number of experimental days on the eDNA detection rate (B) in *Hydrilla verticillata*.

A					
(Intercept)	Days	Water temp	pH	AIC	ΔAIC
420.9	0.9766	11.29	−91.75	324.6	0
655.8		4.282	−89.19	325.6	1
722.2			−86.56	329.7	5.1
669.7	0.4416		−86.46	330.4	5.8
−190.5	0.9632	10.96		334.5	9.9
61.0		3.908		335.5	10.9
85.6	0.4438			340.2	15.6
B					
(Intercept)	Days	Water temp	pH	AIC	ΔAIC
−0.286		0.076		30.4	0
5.455			−0.63	30.7	0.3
4.948		0.084	−0.79	31.4	0.9
1.312	−0.0013			31.5	1.1
−1.318	0.0038	0.108		32.2	1.7
5.775	−0.0016		−0.65	32.7	2.2
3.974	0.0044	0.121	−0.82	33.0	2.6

edly (range: pH 6.5–8.9). Our GLMM model suggests that increases in pH are correlated with decreases in eDNA concentrations (Table 2A).

The differences in the detection rate of eDNA among time periods or sampling dates were not significant in Fisher's exact test. The *p*-values of Fisher's exact test were 0.18 and 0.82 in the analyses for the differences between the periods and among the 5 sampling dates, respectively. Our best GLM model included only the effect of water temperature, but not pH or the number of days from 1st sampling. However, each of these variables was included in at least 1 model with ΔAIC < 2, and no single variable or combination of variables best predicted the detection of eDNA in these ponds (Table 2B). Thus, the influence of these variables on eDNA detection rate was unclear.

DISCUSSION

Our results showed that eDNA concentration of *H. verticillata* changed seasonally in the ponds and suggested that phenology affected the eDNA concentration. The eDNA concentrations were higher during the growth period than during the dormant period. However, we expected to see an increase in eDNA concentrations at the beginning of dormant period, but the results suggested that this did not occur. The effect of the difference in eDNA concentrations among the sampling dates was smaller than that of the difference between the growth and the dormant period.

We found that both water temperature and pH influenced eDNA concentration in the research ponds. These results might suggest that the seasonality of eDNA concentration is influenced by the water environments we assessed. Water temperature could influence the growth and photosynthesis of macrophytes. Additionally, photosynthesis could influence water pH. This study did not address the relationships between phenological changes and changes in environmental conditions, but clarifying these relationships might allow us to better understand the relationships among eDNA concentration, phenology, and water environments.

Higher eDNA concentration is expected to contribute to the detection of target species. In animals, higher levels of activity driven by seasonal events such as breeding (Bylemans et al. 2017, Takahashi et al. 2017), seasonal migration (Yamanaka and Minamoto 2016), and seasonal increases in population size (Buxton et al. 2017) influence eDNA concentration. Our results may indicate that drastic phenological changes also influence the eDNA concentrations in aquatic plant species. For aquatic plants, pollen dispersal and activity of herbivores are also expected to increase during the growth period and affect eDNA concentrations. Thus, fully understanding these mechanisms will require further research on the observed seasonal differ-

ences in eDNA concentrations. In particular, the season in which eDNA concentration is highest or lowest needs to be confirmed with accurate analyses of natural aquatic plant populations.

eDNA concentration and detection rate may be affected by PCR inhibitors such as humic acid and ultraviolet (UV) radiation. PCR inhibitors in water environments may change seasonally because of changes in animal activity, or because of plant growth and death. The Environmental Master Mix 2.0 we used for quantitative PCR is suitable for use with environmental samples that contain PCR inhibitors (Doi et al. 2017b), so this reagent should reduce the effect of PCR inhibition in our results. However, future research should identify PCR inhibitors in the environment and determine their effect on eDNA concentration. UV radiation can also decrease eDNA concentrations (Strickler et al. 2015), even in the water, but we detected more eDNA in the summer when UV radiation is highest. Thus, eDNA concentrations of submerged plants such as *H. verticillata* might not be strongly influenced by UV radiation. We therefore do not believe that PCR inhibitors had a large effect on the results of this study.

We detected the *H. verticillata* eDNA in 3 ponds (Ponds 1, 2, and 5) during the dormant period when we did not observe any *H. verticillata* individuals. Our previous study found that most of the eDNA degraded within only 1 or 2 d (Matsuhashi et al. 2016), indicating that eDNA detections are a reliable method for identifying ponds with contemporary *H. verticillata* populations. Thus, eDNA detection in this study suggests the presence of the target species even if we did not observe it visually. The eDNA detection rate was lower during the dormant period than the growth period, but eDNA analysis still detected *H. verticillata* at higher rates than the visual observations. *H. verticillata* has been threatened with local extinction in native areas but has damaged the ecosystem and biodiversity severely as an invasive species in introduced areas, and accurately monitoring this species is important for effective management in both of these situations. This study indicates that sampling during the growth period with eDNA methods is most effective for distribution surveys. eDNA studies of aquatic plant species are still at an early stage, but eDNA methods for more species will be available in the future. Our findings suggest that considering phenology is important for future studies in this growing field.

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