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Note

Potential utility of environmental DNA for early detection of Eurasian watermilfoil (*Myriophyllum spicatum*)

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INTRODUCTION

Considering the harmful and irreversible consequences of many biological invasions, early detection of an invasive species is an important step toward protecting ecosystems (Sepulveda et al. 2012). Early detection increases the probability that suppression or eradication efforts will be successful because invasive populations are small and localized (Vander Zanden et al. 2010). However, most invasive species are not detected early because current tools have low detection probabilities when target species are rare and the sampling effort required to achieve acceptable detection capabilities with current tools is seldom tractable (Jerde et al. 2011). As a result, many invasive species go undetected until they are abundant and suppression efforts become costly.

Novel DNA-based surveillance tools have recently revolutionized early detection abilities using environmental DNA (eDNA) present in the water (Darling and Mahon 2011, Bohmann et al. 2014). In brief, eDNA monitoring enables the identification of organisms from DNA present and collected in water samples. Aquatic and semiaquatic organisms release DNA contained in sloughed, damaged, or partially decomposed tissue and waste products into the water and molecular techniques allow this eDNA in the water column to be identified from simple and easy-to-collect water samples (Darling and Mahon 2011). Despite limited understanding of the production, persistence, and spread of DNA in water (Barnes et al. 2014), eDNA monitoring has been applied not only to invasive species (Jerde et al. 2011), but also to species that are rare, endangered, or highly elusive (Spear et al. 2014). However, most eDNA research and monitoring has focused on detection of invertebrates and vertebrates and less attention has been given to developing eDNA techniques for detecting aquatic invasive plants.

Eurasian watermilfoil (EWM; *Myriophyllum spicatum* L.) is an invasive species for which improved early detection would be particularly helpful. Advanced EWM invasions have negative impacts on native biodiversity, recreational boating, fishing, and other types of aquatic tourism (e.g., Eiswerth et al. 2000). On a broader scale, EWM can also be harmful to man-made aquatic infrastructure, such as hydroelectric dams. If an EWM invasion can be detected in an early stage where eradication is still a possibility, many of these negative consequences can be limited or prevented altogether (e.g., Madsen et al. 2002).

The purpose of this research was to develop and validate a traditional polymerase chain reaction (PCR) assay for the detection of pure and hybridized EWM DNA using both laboratory and field experiments. We performed a pilot experiment in outdoor tanks to determine the basic functionality and sensitivity of the assay. Following this initial test, we collected field samples from Michigan and Montana lakes with and without known EWM populations. Taken together, our findings suggest that eDNA techniques have potential to be a useful strategy for the early detection of EWM.

MATERIALS AND METHODS

I. Marker development

We developed a PCR assay designed to detect EWM eDNA in water samples. Because eDNA techniques are not yet widely used for detecting aquatic plants, we used traditional PCR to test a proof of concept and minimize costs that would be associated with developing a more sophisticated quantitative PCR (qPCR) assay. We designed primers to amplify a 133-base-pair (bp) region of the internal transcribed spacer (ITS) region of the ribosomal DNA. This locus has been used for *Myriophyllum* taxonomy because it contains numerous polymorphisms that can distinguish watermilfoil species (Thum et al. 2006, Moody and Les 2007). Our primer locations were optimized for binding to EWM DNA sequences, and the amplicon contains

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multiple species-specific polymorphisms that can be examined via Sanger DNA sequencing. We confirmed the specificity of this assay in silico using the Basic Local Alignment Search Tool (BLAST) algorithm in GenBank. Importantly, our assay cannot distinguish if the origin of the EWM DNA is from hybrid EWM or from pure EWM, so individuals shedding EWM DNA might be hybrids. This limitation is minor because hybrid and pure EWM are both aquatic invasives.

Because extracellular DNA is rapidly degraded by hydrolytic cleavage and other forms of oxidative damage (Gilbert et al. 2003, Dejean et al. 2011, Barnes et al. 2014), even large-volume water samples might only contain a limited number of viable template molecules, which are likely to be very small (relative to DNA recovered from a tissue sample from a living organism). Therefore, our PCR amplicon of 133-bp was intentionally kept small to increase the chances of successful amplification when EWM eDNA is present.

II. Detection of Eurasian watermilfoil eDNA

Laboratory experiment. In order to test the functionality of our eDNA assay, we set up a series of outdoor 376-L (100-gallon) cattle tanks containing variable amounts of EWM (0, 1, 5, 10, 50 plants) at the Robert B. Annis Water Resources Institute (AWRI; Muskegon, MI). The experiment was run in triplicate. Each tank contained a soil–sand mixture capped with a layer of sterilized sand and was filled with tap water. The tanks were allowed to sit empty for several days to allow residual chlorine to evaporate from the water prior to planting. One-liter water samples were then taken from each tank and analyzed for EWM eDNA to confirm no contaminating DNA was present prior to planting. Cuttings were taken of EWM stem tips, which included the apical meristem, and the base of each stem was firmly pressed into the sand-soil mixture at the bottom of each tank. Once planting was completed, the tanks were allowed to equilibrate for approximately 3 h before 1-L samples were collected in sterile Nalgene bottles. One-liter field blanks of deionized water were collected for each sampling event and analyzed alongside the samples taken from the tanks (Goldberg et al. 2013). Sampling was carried out at the beginning of the day prior to entering any areas of the lab where EWM specimens or PCR products were present. These samples were immediately taken into a clean lab, where no EWM specimens or PCR products had ever been processed, and filtered through 0.45 µM PVDF filters (Millipore). The filters were placed in 50-ml conical tubes containing 100% ethanol, double-bagged, and stored at −20 C.

DNA extraction and PCR analysis. Prior to DNA extraction, filters containing eDNA were removed from their tubes under sterile conditions and dried in a laminar flow hood for at least 2 h. Dried filters were transferred into 1.7-ml microcentrifuge tubes using sterile instrumentation. For samples requiring multiple filters, each filter was extracted in its own tube. DNA was extracted using a Qiagen Plant Mini Kit and eluted into 100 µl AE buffer.

PCR was carried out in a 25-µl reaction containing 1U GoTaq Hot-Start DNA polymerase (Promega), 1× GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer (F: 5′-CCACCCTTCAAGGATAAGGC-3′; R: 5′-AGGCTGAGTTATCAACCACC-3′), and 5 µl of DNA extract. Triplicate PCR reactions were set up for each DNA extract and run on an Eppendorf Mastercycler for 55 cycles with an annealing temperature of 58 C, and the finished PCR reaction was checked on a 2.0% agarose gel. In the event that a set of triplicate PCRs gave an ambiguous result (only one or two out of three reactions amplified), we performed additional rounds of PCR following the method developed by Goldberg et al. (2013) to declare each sample positive or negative. For samples that required multiple filters, a positive result for at least one filter extract was required for the sample to be declared positive. This method attempts to maintain a useful level of sensitivity for the assay while providing a mechanism for dealing with occasional false positives. Finally, the DNA sequence of any positive reactions was checked to confirm EWM eDNA was amplified, and not that of a different milfoil species (see Results and Discussion section).

Field evaluation. Water samples were collected from four water bodies to test the eDNA assay in natural settings. EWM was known to be present at Half Moon Lake (Muskegon County, MI) and Jefferson Slough (Jefferson County, MT). EWM has not been detected previously from our two other field sites, Lake Alva and Georgetown Lake (Deer Lodge and Granite counties, MT), despite extensive sampling efforts. However, Lake Alva and Georgetown Lake contain the native watermilfoil species northern watermilfoil (*M. sibiricum* Komarov) and western watermilfoil (*M. hippuroides* Nutt. ex Torr & A. Gray), which provided an opportunity to test the specificity of our assay.

In Half Moon Lake, we collected six 15-ml water samples along a horizontal transect at 10-m intervals and a depth of approximately 20 cm, starting over a known bed of hybrid EWM. Although there might be some parental EWM remaining in the lake, the lake is dominated by hybrid watermilfoil (unpublished data). Water samples were returned to AWRI for filtration, preserved in 100% ethanol, and processed according to the methods of Ficetola et al. (2008).

We collected 1-L water samples from the subsurface of 15 sites in Jefferson Slough, which is a flowing side-channel of the Jefferson River. Discharge in the Jefferson Slough is heavily influenced by irrigation withdrawals and returns and varied from 0.11 $m^3\ s^{-1}$ to 0.35 $m^3\ s^{-1}$ across our study area on 2 August 2013. Collection sites were spaced at ∼1 km intervals and occurred upstream, within, and downstream of a population of hybrid and pure EWM that was mapped in 2012 by Montana Department of Agriculture (C. McLane, unpub. data). The proportion of hybrid to pure EWM is not known. We sampled in triplicate at three of the 15 sites for a total of 21 samples. We also collected four 1-L water samples from Lake Alva and 25 1-L samples from Georgetown Lake; these samples were collected at the subsurface adjacent to boat ramps, docks, and other public access sites that are vulnerable to AIS introductions (Johnson et al. 2001).

Table 1. Results of Eurasian watermilfoil (EWM) lab and field eDNA sampling. Up to three replicate (Rep) water samples were analyzed for each experiment. Samples that were scored as EWM positive are boldface. Up to six PCR reactions were analyzed for each sample and additional PCR reactions (shown within parentheses) were performed to clarify ambiguous results. For Half Moon Lake, sample indicates distance (m) away from a known bed of hybrid EWM. For Jefferson Slough, we sampled above, within, or below the mapped distribution of EWM. We collected 25 and 4 samples from Georgetown Lake and Lake Alva, respectively, near public access sites. All lab and field blanks were scored negative (0/3) for EWM.

			Positive PCRs		
Trial	Treatment	Sample	Rep 1	Rep 2	Rep 3
Lab	0 plants		0/3	0/3	2/3 (0/3, 0/3)
	1 plant		**3/3**	**3/3**	1/3 (0/3)
	5 plants		**3/3**	**3/3**	**3/3**
	10 plants		**3/3**	**3/3**	**3/3**
	50 plants		**3/3**	**3/3**	**3/3**
	100 plants		**3/3**	**3/3**	**3/3**
Field	Half Moon Lake	0 m	**3/3**		
		10 m	**3/3**		
		20 m	**3/3**		
		30 m	**3/3**		
		40 m	**2/3**		
		50 m	**2/3**		
		60 m	**3/3**		
	Jefferson Slough	Above 1	0/3	0/3	0/3
		Above 2	0/3		
		Above 3	0/3		
		Above 4	0/3		
		Above 5	0/3	0/3	0/3
		Above 6	0/3		
		Within 1	0/3		
		Within 2	0/3		
		Within 3	0/3		
		Within 4	**4/6**	**5/6**	**4/6**
		Within 5	**4/6**		
		Within 6	**4/6**		
		Within 7	**3/3**		
		Below 1	0/3		
		Below 2	0/3		
	Georgetown Lake	1–2, 4–25	0/3		
		3	1/3 (0/3)		
	Lake Alva	1–4	0/3		

All water samples were filtered in the field, preserved in 100% ethanol, and shipped to AWRI. Appropriate field blanks using deionized water were also collected at each site. If field samples could not be analyzed immediately, they were stored at −20 C until DNA extraction was performed. All DNA extraction and PCR steps were identical to those used for the laboratory experiment outlined above.

RESULTS AND DISCUSSION

The primary goal of our laboratory experiment was to evaluate the basic utility and sensitivity of our eDNA PCR assay. The results of our laboratory experiment indicate that the assay is functional and suggests that eDNA could be used to detect EWM at low densities, because we had positive eDNA detection from a single stem in as few as 3 h after introduction of the plant to the tank (Table 1). We found no evidence for contamination or false positives because all blanks and samples from tanks without milfoil were scored negative for EWM, although one of these samples required additional PCR reactions to clarify ambiguous results (Table 1).

We have also demonstrated that our PCR assay can detect EWM in field settings and our eDNA results are consistent with field surveys of these same lakes. We detected EWM eDNA in two water bodies where it was known to occur. In Half Moon Lake, we detected EWM eDNA in all samples, which ranged from directly over the location of a known hybrid EWM bed out to 60 m away from the bed (Table 1). In Jefferson Slough, our assay detected eDNA in four of six sites located within the mapped distribution of hybrid and pure EWM, including one site that was sampled in triplicate and all three subsamples were positive (Table 1). All six sites, including two sites that were sampled in triplicate, located upstream of the mapped EWM population were negative. The positive detections in Half Moon Lake and Jefferson Slough confirm that eDNA can be detected in water bodies with hybrid and pure EWM. However, we do not know how the presence or abundance of hybrid EWM complicates the field sensitivity of our assay. A better understanding of how EWM hybridization might influence false negatives is warranted given the increased invasiveness of hybrid EWM (LaRue et al. 2013).

Two important aspects of an eDNA assay are sensitivity and specificity. An eDNA assay will have the greatest value when it can detect the target species at low abundance, and when the rates of false negatives are low. In this study, we were unable to directly determine the ability of our assay to detect EWM eDNA across a range of abundances in field settings. Future studies should utilize our (or a similar) assay in a number of lentic and lotic waters representing a gradient of EWM abundances quantified through traditional survey methods.

In terms of false negatives, we failed to detect EWM at Jefferson Slough in two sites at the upstream edge of the mapped distribution and in two sites that were downstream of the mapped distribution (Table 1). The reliability of eDNA techniques in moving waters, such as Jefferson Slough, can be more variable than standing waters because of the reduced exposure time between a given quantity of water and an individual organism, DNA settling, and turbulence and dilution effects from increased water volume (Thomsen et al. 2011, Jane et al. 2015). For example, the detection success of weather loach [*Misgurnus fossilis* (Linnaeus, 1758)] in ponds with confirmed presence was 100%, but detection rates were only 54% in a flowing system known to contain this species (Thomsen et al. 2011). For brook trout [*Salvelinus fontinalis* (Mitchill, 1814)] in moving waters, eDNA concentration varied as a function of stream flow (L s^{-1}); eDNA was highest closest to the source and quickly trailed off over distance at the lowest flows but remained relatively constant at near and far distances from the source at higher flows (Jane et al. 2015). Similar to this brook trout study, stream flow varied greatly among sampling sites in Jefferson Slough due to irrigation withdrawals and returns. Thus, moving waters can complicate the reliability of eDNA techniques, and future work on this issue is warranted. Nevertheless, EWM eDNA was detected in most of our field samples, including those in flowing water,

indicating that eDNA can be an effective monitoring tool when multiple samples are collected.

It is also important that an eDNA assay be highly specific to the target species (Wilcox et al. 2013). In particular, false positives can occur if the eDNA assay amplifies DNA from species that are closely related and/or genetically similar at the marker locus. We did not detect eDNA in two lakes (Lake Alva and Georgetown Lake) known to contain native watermilfoils (northern and western watermilfoil) but not EWM (Table 1). In particular, northern watermilfoil is the sister species to EWM, and the two species are distinguished by only two nucleotide substitutions within our marker. This species is thus the most likely to generate false positive detections via cross-amplification in our assay. Furthermore, we confirmed that every PCR product generated from our field samples was from EWM, as opposed to other closely related and genetically similar species, by performing an FspI restriction digestion on every PCR product. FspI is utilized because the recognition site for this enzyme occurs at an area of the PCR product where EWM and NWM have different sequences. The results from our field samples provide evidence that our assay is specific to EWM; however, further testing of a larger number of water bodies with a range of abundance of different watermilfoil species is still warranted.

In conclusion, we have demonstrated in concept that eDNA could be a useful method for the early detection of EWM. Although many eDNA studies have focused on aquatic animals (Bohmann et al. 2014), to our knowledge this is the first study to demonstrate its potential utility for aquatic plants. We recommend further research and development of eDNA methods for aquatic plants. For example, our assay could be refined to increase its sensitivity, and potentially to detect multiple species simultaneously through development of a quantitative PCR approach. Such assays, when operational, could greatly improve the detection of invasive and rare aquatic plant species.

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