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Molecular techniques to distinguish morphologically similar *Hydrilla verticillata*, *Egeria densa*, *Elodea nuttallii*, and *Elodea canadensis*

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ABSTRACT

The four submerged aquatic species, hydrilla (*Hydrilla verticillata* [monoecious and dioecious]), Brazilian waterweed (*Egeria densa*), Canadian waterweed (*Elodea canadensis*), and western waterweed (*Elodea nuttallii*), are difficult to positively identify because of their morphological similarity to each other, resulting in possible misidentification. This limits our ability to understand their past and present distribution, which is important in aquatic plant management. We investigated a molecular technique to identify these species, which are problematic because of their invasive nature on multiple continents. Approximately 100 samples of these species, ranging in age from 40-yr-old herbarium samples to recently collected plants, were collected from regions across the United States. The distribution and range of the samples collected in this research were compared to those reported in the literature. We confirmed information on the current wide distribution of both hydrilla biotypes in the United States and discovered that hydrilla had actually invaded the waterways near Washington, DC 6 yr earlier than originally reported. In addition, we found evidence of the confusion, dating back to the 1980s, between Canadian waterweed and western waterweed in the mid-Atlantic region of the United States. Canadian waterweed was previously reported as common and western waterweed as rare; however, our samples indicate the opposite is true. This information indicates there is a need for investigators to anticipate the spread of hydrilla populations to northern U.S. waterways, where it will compete with existing plant species, including Canadian and western waterweeds. Our ability to confirm distribution and pace of spread of invasive and noninvasive species will improve with increased application of molecular techniques.

Key words: Chesapeake Bay, exotic, misidentification, polymerase chain reaction, submerged aquatic vegetation.

INTRODUCTION

Investigators use field surveys to determine plant conservation and wildlife management actions, and use herbarium records to predict and monitor the geography of species invasions. How accurate are morphologically-based identification records for a set of plant species with similar morphology? Molecular techniques can be used to reduce errors in identification, which in turn can improve descriptions of plant communities, identification of neophytes, or predictions of range and of species interactions.

The purpose of this study was to apply molecular techniques to reveal new information on the range and recent history of four related and morphologically similar species, hydrilla (*Hydrilla verticillata* (L. f.) Royle), Brazilian waterweed (*Egeria densa* Planchon), Canadian waterweed (*Elodea canadensis* Michx.), and western waterweed (*Elodea nuttallii* (Planch.) H. St. John) (family Hydrocharitaceae). These species are submerged macrophytes, a group of plants commonly referred to as submerged aquatic vegetation (SAV). These four species are similar in reproductive strategy in that they rely on vegetative propagation and reproduce via fragmentation. They look similar because each has nondissected leaves occurring in whorls (Cook and Lüönd 1982, Cook and Urmi-König 1984, Cook and Urmi-König 1985, Herault et al. 2008). Hydrilla and Brazilian waterweed have invasive status and the *Elodea* spp. have native status in North America. However, the Canadian and western waterweeds are considered invasive in Europe; thus, the four are considered nuisance invasive species on multiple continents (de Winton and Clayton 1996, Feijoo et al. 1996, Les and Mehrhoff 1999, Hérault et al. 2008). Two distinct biotypes of hydrilla occur in the United States. The dioecious southern introduction that was first found in Florida appears to have originated from the Indian subcontinent, while the monoecious northern introduction, first found in Delaware and in Washington, DC, appears to have originated from Korea (Madeira et al. 1997, Madeira et al. 2004). Despite many similarities in morphology, the two biotypes are unique in some of their reproductive and growth characteristics such that they may differ in their competitive ability and response to management strategies at different latitudes and in different environmental conditions (Van and Vandiver 1992, Netherland 1997, Michel et al. 2004, Mony et al. 2007, Owens et al. 2012).

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Building on existing molecular techniques (Taberlet et al. 1991, Nickrent et al. 1994, Liston et al. 1996, Madeira et al. 2004), we used a simple protocol to distinguish these four species. First, the preliminary protocol was developed using DNA sequenced samples as a standard for each species. Then, we tested and confirmed the protocol on 11 reference samples and 95 other samples of these four species from herbarium and live specimens collected from across the United States. We examined implications of the confirmed distribution relative to previous records and demonstrated the regional and national need for verification of these species collected recently or as much as a half century ago.

MATERIALS AND METHODS

Between 2006 and 2009 we acquired putative samples of each species and biotype as fresh individual plants (collected from 2006 to 2009) or leaflets from individual herbarium specimens (collected from: 1949 to 2003). The majority of samples were from the United States approximately 30% were from the Chesapeake Bay region, a few (ca. 10%) were from herbarium specimens collected outside the United States, and the remaining samples were store bought or were from ornamental ponds (Table 1). The fresh pressed samples are available by request from the corresponding author. Herbarium samples were acquired with permission from the United States National Herbarium, George Mason University, North Carolina State University, University of Florida, University of Alabama, Missouri Botanical Garden, University of Zurich, and the Patuxent Research Refuge (herbaria abbreviations are US, GMUF, NCSU, FLAS, UNA, MO, Z, and PRR, respectively).

Several herbaria provided us with reference samples that were identified (or classified) by experts and were included among our samples (Table 1). Three Brazilian waterweed reference samples were either determined morphologically by C. D. Cook, and collected in Italy, 11 September 1982, or by R. R. Haynes, and collected in Hawaii, 10 May 2001 and in South Carolina, 3 August 2001. R. R. Haynes determined two western waterweed samples that were collected in New York, 5 September 1998, or in South Dakota, 3 July 1998. D. Webb determined a monoecious and a dioecious hydrilla sample that he collected in Alabama, 27 September 2007. Also, a hydrilla sample was determined by C. D. Cook and collected in India, 28 November 1993, and it was shown in this study to be dioecious. We included a fresh monoecious hydrilla sample collected in 2006 from the Potomac River at Dyke Marsh, Virginia. This is a population that was previously verified using molecular techniques (Madeira et al. 1997). E. Gross provided freeze dried samples of reference material for Canadian waterweed that she had sequenced previously (Erhard 2005).

DNA extractions were performed by one of two methods, depending on the type of preservation. Dried samples were extracted using the DNeasy® plant mini kit[1] according to manufacturer's instructions. Fresh or frozen samples were sometimes extracted as above, or alternately were pressed onto an FTA plant DNA preservation card[2] and processed according to manufacturer's instructions.

PCR to distinguish hydrilla from Brazilian, Canadian, and western waterweeds by amplified length heterogeneity (ALH)

We evaluated a polymerase chain reaction (PCR) assay using a previously developed primer pair for potential in discriminating hydrilla from the other three species. The primer pair ITS5[3] and 26S25R[3] (Table 2) targets the nuclear rRNA internal transcribed spacer (ITS) region (ITS1, 5.8srRNA, ITS2). Based on sequence information available from these species, a smaller size amplicon (PCR product) is to be expected from hydrilla compared to Brazilian, Canadian, and western waterweeds. Briefly, a 35 cycle PCR was performed with 50uL reactions, a 55 C anneal step and 0.4 μM final primer concentration, 0.2mM dNTPs[4], 1.2mg/mL BSA[4], 2mM $MgCl_2$[4], 1X GoTaq® flexibuffer[4] and 1.25U GoTaq® polymerase[4]. Five μL of each PCR reaction were electrophoresed for 1 h at 100V on a 1% agarose gel in 1XSB electrophoresis buffer (Brody and Kern 2004). Bands were visualized by staining gels with ethidium bromide, UV illuminating gels, and photographing them using a KODAK® Gel Logic 100 imaging system[5]. The bands were visually classified as either hydrilla or as one of the other three species. Additional steps (shown below) were needed to determine the hydrilla biotype or differentiate among the waterweeds.

Restriction fragment length polymorphism (RFLP) analysis to distinguish Brazilian waterweed from Canadian or western waterweeds

Based on *in silico* digest simulations of sequences available from the amplified region described above, different sized DNA fragments should result from restriction digests of Brazilian waterweed versus Canadian or western waterweeds amplicons using either the enzymes, *Hha*I or *Alu*I[6]. Therefore, we tested both enzymes individually on our amplicons. Six μL of the PCR product, generated using the ITS5 and 26S25R primer pair, was digested with *Hha*I or *Alu*I for 4 h at 37 C in 20 μL reactions according to the enzyme manufacturer's instructions[6]. Digestion products were evaluated by electrophoresis for 1 h at 100V on a 1% agarose gel in 1XSB electrophoresis buffer (Brody and Kern 2004), and the banding patterns were visualized and photographed as described above. Banding patterns were classified by visual inspection as either Brazilian waterweed (*Egeria* sp.) or an *Elodea* spp. Additional steps (shown below) were used to differentiate between the *Elodea* spp.

RFLP analysis to distinguish between the Canadian and western waterweeds

A comparative study of Canadian and western waterweeds by Erhard (2005) included sequencing of the nuclear ITS region and revealed distinct nucleotide polymorphisms between the two species. Based on *in silico* digest simulations of those sequences, different sized DNA fragments should result from restriction digests of Canadian waterweed versus western waterweed amplicons using the enzyme *Mnl*I. Therefore, PCR products that had been determined to be

Table 1. Verified species, putative species, date collected, location, source herbarium (if applicable), and sample identification number (SAV#) used in this study.

Species[1]	Putative Species[2]	Date Collected	State or Country	County if United States	Location	Source[3]	S[4]	SAV#[5]
H. v (mon)	*E. can*	17 May 1976	VA	Fairfax	Potomac River, River Bend Park	GMUF	c	14
H. v (mon)	*Elodea*	20 September1980	DE	Sussex	Record's pond, Willow St., Laurel	MO	n	101
H. v (mon)	*H. v*	23 October 1984	NC	Wake	Lake Benson	NCSU		21
H. v (mon)	*H. v*	26 October 1984	NC	Wake	Lake Raleigh	NCSU		23
H. v (mon)	*H. v*	20 August 1990	VA	Fairfax	Potomac River at Alexandria	GMUF		10
H. v (mon)	*E. nutt*	5 September 1994	VA	Stafford	Potomac River at Brent Marsh	GMUF	c	13
H. v (mon)	*H. v*	9 September1999	VA	Page	Lake Stanley	GMUF		9
H. v (mon)	*H. v*	5 August 2003	NJ	Burlington	Lake Mallard, Medford	FLAS		44
H. v (mon)	*H. v*	4 January 2006	MD	Anne Arundel	Patuxent River at Jug Bay	live		17
H. v (mon)	*H. v*	18 August 2006	VA	Fairfax	Potomac River at Dyke Marsh	live	n	2[R]
H. v (mon)	*H. v*	11 September 2006	VA	Fairfax	Lake Fairfax	live		3
H. v (mon)	*H. v*	15 January 2007	NC	Moore	Woodlake Resort in Vass	live		20
H. v (mon)	*H. v*	28 January 2007	IN	Fulton	Manitou Lake	live		32
H. v (mon)	*H. v*	28 January 2007	OH	Jefferson	Ohio River, near Madison	live		31
H. v (mon)	*H. v*	11 May 2007	MD	Charles	Potomac River at Pomonkey Creek	live		51
H. v (mon)	*H. v*	9 July 2007	MD	Cecil	Chesapeake Bay at VA Hospital	live		53
H. v (mon)	*H. v*	9 July 2007	MD	Cecil	Chesapeake Bay at Stump Point	live		54
H. v (mon)	*H. v*	11 July 2007	MD	Charles	Potomac River at Pomonkey Creek	live		55
H. v (mon)	*H. v*	23 July 2007	WI	Marinette	Privately owned pond	live	c,n	73
H. v (mon)	*H. v*	18 September 2007	MD	Washington	Potomac River at McCoys Ferry	live		61
H. v (mon)	*H. v*	24 September 2007	CA	Yuba	Luban Pond, Merrysville	live		64
H. v (mon)	*H. v*	27 September 2007	AL	Limestone	Wheeler Reservoir, NW of Decatur	UNA	c	82[R]
H. v (mon)	*H. v*	31 October 2007	MD	Anne Arundel	Privately owned ornamental pond	live		69
H. v (mon)	*H. v*	18 September 2008	MD	Cecil	Susquehanna Flats- station 1	live	n	105
H. v (mon)	*H. v*	20 September 2006	VA	Fairfax	Potomac River at Gunston Cove	live		4
H. v (dio)	*H. v*	5 September 1979	GA	Seminole	Lake Seminole State Park	NCSU		24
H. v (dio)	*H. v*	20 May 1988	FL	Alachua	Paynes Prairie State Preserve	FLAS		45
H. v (dio)	*H. v*	28 November 1993	India	none	India, Kerala, Distr, Kottayam	Z		47[R]
H. v (dio)	*H. v*	1 January 2007	TX	unknown	unknown	live		18
H. v (dio)	*H. v*	28 January 2007	FL	Alachua	Orange Lake	live	n	33
H. v (dio)	*H. v*	28 January 2007	FL	Putnam	Rodman Lake	live	n	34
H. v (dio)	*H. v*	19 February 2007	FL	Okeechobee	Kissimmee River	live		35
H. v (dio)	*H. v*	27 September 2007	AL	Morgan	Wheeler Reservoir	UNA	c	83[R]
H. v (dio)	*H. v*	12 December 2007	ID	Owyhee	Bruneau River	live	c,n	72
H. v (dio)	*H. v*	2 June 2008	ID	Owyhee	Geothermal drain ditch, Boise	live		84
H. v (dio)	*H. v*	25 August 2008	KY	Johnson	Paintsville Lake	live		96
E. nutt	*Elodea*	22 October 1981	DE	Sussex	Burton Pond, Angola	MO	n	98
E. nutt	*Elodea*	30 May 1985	DE	Sussex	Wagoners Mill pond	MO		99
E. nutt	*E. nutt*	3 July 1998	SD	Custer	Horsethief Lake	UNA		81[R]
E. nutt	*E. nutt*	5 September 1998	NY	Dutchess	Fishkill Creek mouth	UNA		80[R]
E. nutt	*E. can*	16 October 2006	MD	Anne Arundel	Upper Chesapeake Bay	live	n	5
E. nutt	*E. nutt*	21 November 2006	MD	Anne Arundel	Severn River at Weems Creek	live	n	6
E. nutt	*E. can*	9 July 2007	MD	Cecil	Chesapeake Bay at Stump Point	live		52
E. nutt	*E. can*	18 September 2007	MD	Washington	Potomac River at McCoys Ferry	live		62
E. nutt	*E. can*	26 September 2007	WA	Yakima	Yakima River, Chandler's Return	live		65
E. nutt	*E. can*	26 September 2007	WA	Yakima	Yakima River, river mile 103	live		66
E. nutt	*E. can*	5 October 2007	MD	Cecil	Susquehanna Flats	live	n	68
E. nutt	*E. can*	18 November 2007	VA	Rockingham	Purcell Park, Harrisonburg	live	n	71
E. nutt	*E. can*	8 June 2008	VA	Page	Lake Arrowhead, Luray	live	n	85
E. nutt	*E. can*	20 June 2008	MD	Charles	Potomac R., Chicamuxen Creek	live	n	86
E. nutt	*E. can*	18 September 2008	MD	Harford	Susquehanna Flats- station 524	live		106
E. nutt	*E. can*	18 September 2008	MD	Harford	Susquehanna Flats-station 25	live	n	107
E. nutt	*E. can*	18 September 2008	MD	Cecil	Susquehanna Flats-station 118	live		108
E. nutt	*E. can*	24 July 2009	NY	Washington	Upper Hudson River, W. River Rd	live		111
E. nutt	*E. nutt*	15 September 2009	VA	Fairfax	Difficult Run Creek	live	n	113
E. nutt	*E. nutt*	10 August 2007	VA	Giles	Mountain Lake	live	n	57
E. nutt	*E. nutt*	31 August 1966	MD	Anne Arundel	Upper Patuxent River	PRR	n	109
E. nutt	*E. nutt*	unknown	Germany	none	Lake Rohrsee	UKON		91
E. nutt	*E. nutt*	unknown	Germany	none	Lake Rohrsee	UKON	c	92
E. nutt	*E. nutt*	unknown	Germany	none	Lake Rohrsee	UKON	c	93
E. nutt	*E. can*	unknown	VA	unknown	unknown	GMUF	n	15
E. can	*E. can*	18 September 1969	VT	Chittendam	Sandbar Waterfowl Refuge	NCSU		28
E. can	*H. v*	12 May 1983	NC	Wake	Weaver's pond	NCSU	n	22
E. can	*E. can*	29 September 2007	OR	Washington	Forest Park, Jay trail pond	live		67
E. can	*E. can*	unknown	Germany	none	Botanical Garden, Halle	UKON	c	94[R]
E. can	*E. can*	unknown	Germany	none	Botanical Garden, Regensburg	UKON	c	95[R]
Elodea	*E. nutt*	1 October 1966	NC	Tyrell	Alligator River at Fort Landing	NCSU		30
Elodea	*E. can*	22 August 1979	MA	Hampden	Connecticut River	NCSU		29
Elodea	*E. can*	18 July 1995	WV	Mason	Letart: Spring Run	UNA		77

Table 1. Continued.

Species[1]	Putative Species[2]	Date Collected	State or Country	County if United States	Location	Source[3]	S[4]	SAV#[5]
Eg. den	*Eg. den*	1 August 1982	NC	Wake	Futrells' pond	NCSU		26
Eg. den	*Eg. den*	11 September 1982	Italy	none	Italy, Lago d' Iseo, Sarnico	Z	n	49[R]
Eg. den	*Eg. den*	1 October 1990	VA	Fairfax	Brookfield Park pond	GMUF		11
Eg. den	*Eg. den*	10 May 2001	HI	Honolulu	O'ahu, Kaneohe Stream	UNA		74[R]
Eg. den	*Eg. den*	3 August 2001	SC	Georgetown	Waccamaw River	UNA		75[R]
Eg. den	*Eg. den*	11 September 2006	VA	Fairfax	Lake Fairfax	live	n	1
Eg. den	*Eg. den*	16 November 2006	MD	Caroline	Upper Nanticoke River	live		7
Eg. den	*Eg. den*	16 November 2006	unknown	unknown	Pet supply store in Virginia	live		8
Eg. den	*Eg. den*	1 January 2007	VA	Mecklenburg	Lake Gaston	live		19
Eg. den	*Eg. den*	24 September 2007	CA	Contra Costa	San Jaquin Delta at Frank's Tract	live	c	63
Eg. den	*Eg. den*	2 November 2007	MD	Frederick	Lilypons Aquatic Garden	live		70
Eg. den	*Eg. den*	22 July 2008	MD	Anne Arundel	Privately owned ornamental pond	live		89
Eg. den	*Eg. den*	4 August 2008	MD	Anne Arundel	Privately owned ornamental pond	live		90
Eg. den	*Eg. den*	unknown	VA	unknown	unknown	GMUF		12

[1]Species identification using fingerprinting protocol, abbreviations: *H. v*, hydrilla: mon, monoecious: dio, dioecious: *E. can*, Canadian waterweed: *E. nutt*, western waterweed: *Eg. dens*, Brazilian waterweed.
[2]Original morphological identification.
[3]Sources of dried samples include the United States National Herbarium, George Mason University, North Carolina State University, University of Florida, University of Alabama, Missouri Botanical Garden, University of Zurich, and the Patuxent Research Refuge (herbaria abbreviations are US, GMUF, NCSU, FLAS, UNA, MO, Z, and PRR, respectively) and the University of Konstanz (UKON).
[4]In column S, letters indicate the target gene sequenced in that sample (see NCBI Genbank database http://blast.ncbi.nlm.nih.gov/Blast.cgi; search the nucleotide database by species and SAV#): n, the nuclear rRNA ITS region (GenBank accession numbers JF703253 to JF703273): c, the chloroplast (plastid) *trn*L-*trn*F intron and intergenic spacer region (GenBank accession numbers JF703274 to JF703284).
[5]SAV#: Submerged aquatic vegetation sample identification number: R, reference samples, determined by experts and/or molecular techniques.

Elodea by the previous RFLP analysis were digested with *Mnl*I for >3 h at 37 C, according to manufacturer's instructions[6]. Digested fragments were then electrophoresed for 2 h on a 1% agarose gel in 1XSB, stained with ethidium bromide and imaged as described above. Standard samples from both species were included. Banding patterns were visually classified as Canadian or western waterweed.

Distinguishing hydrilla biotypes

Two PCR reactions were performed on all hydrilla DNA samples. We used the primer pair *trnL-trnF* e and f which separates monoecious and dioecious hydrilla and we used the monoecious specific primer pair h[3] and f[3] (Table 2). This method was developed by Madeira and others (2004).

Sequencing

During protocol development, we sequenced and confirmed the identity of several samples of each species and used these as positive controls or standards. Standards for western waterweed and Canadian waterweed were previously sequenced by Erhard (2005) and provided to us as freeze dried plant samples.

PCR products were purified with Wizard® PCR Preps[4]. Cycle sequencing was performed using BigDye® v3.1 according to manufacturer's instructions[7]. Samples were run on an ABI 310 sequencer and analyzed with ABI sequencing software[7]. Closest sequence identities were determined by comparison with those at the National Center for Biotechnology Information (NCBI; Zhang et al. 2000). The Basic Local Alignment Search Tool (BLAST) was used to determine the percent similarity between sequences of our specimen and those in the NCBI Genbank database (see http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

Molecular methods such as these allowed us to identify morphologically similar plants that may otherwise be misidentified. Of the 105 samples collected (including reference and standard samples), 83 yielded amplifiable,

Table 2. PCR primer pair sequences used to generate PCR products that were evaluated for their potential in discriminating among hydrilla and Brazilian, Canadian, and western waterweeds: ITS5/26S-25R primer pair is used in amplification to discriminate between the four species of plants, and the *trn*L/*trn*F primer pairs are used in the hydrilla biotyping.

Primer Pair	Forward Primer	Reverse Primer	Target Gene	Primer Reference
ITS5/26S-25R	5′-GGAAGGAGAAGTCGTAACAAGG-3′	5′-TATGCTTAAACTCAGCGGGT-3′	Nuclear rRNA ITS region, (ITS1, 5.8srRNA, ITS2)	Liston et al., 1996; Nickrent et al., 1994
*trn*L e/ *trn*F f	5′-GGTTCAAGTCCCTCTATCCC-3′	5′-ATTTGAACTGGTGACACGAG-3′	Chloroplast (plastid) *trn*L-*trn*F intron and intergenic spacer region	Taberlet et al., 1991
*trn*L h/ *trn*F f	5′-CCCTCTATCCCCAATAAAAATCC-3′	5′-ATTTGAACTGGTGACACGAG-3′	Chloroplast (plastid) *trn*L-*trn*F intron and intergenic spacer region	Madeira et al., 2004

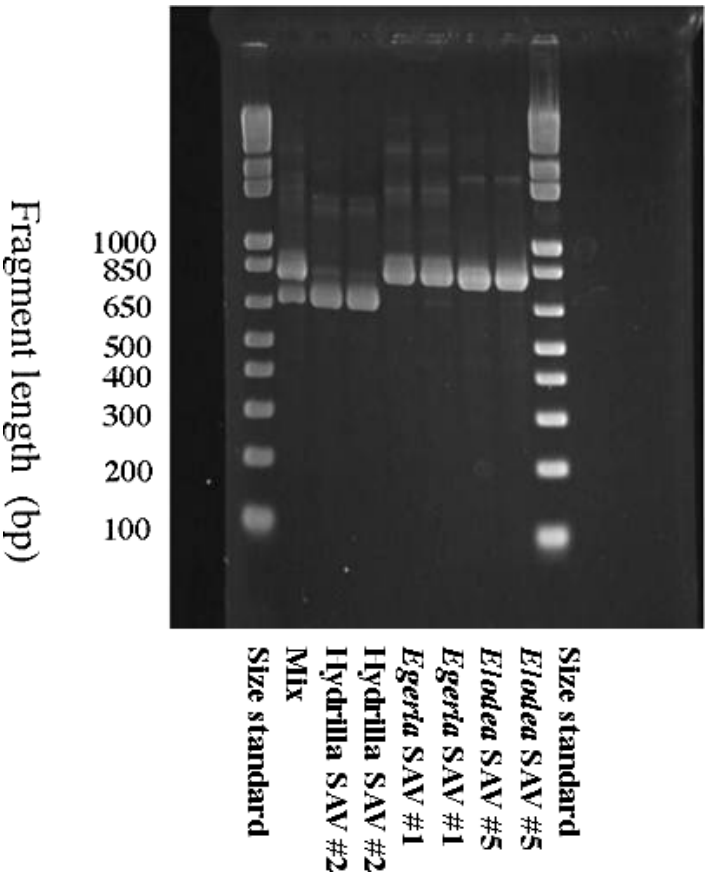

Figure 1. ALH-PCR with ITS5/26S-25R primer pair-gel showing differentiation in fragment length (base pairs) between PCR products of hydrilla and the other two genera, *Egeria and Elodea*. Abbreviations: mix is a mix of hydrilla and Brazilian waterweed, *Egeria* is Brazilian waterweed, and *Elodea* is *Elodea* sp. SAV # is in reference to Table 1.

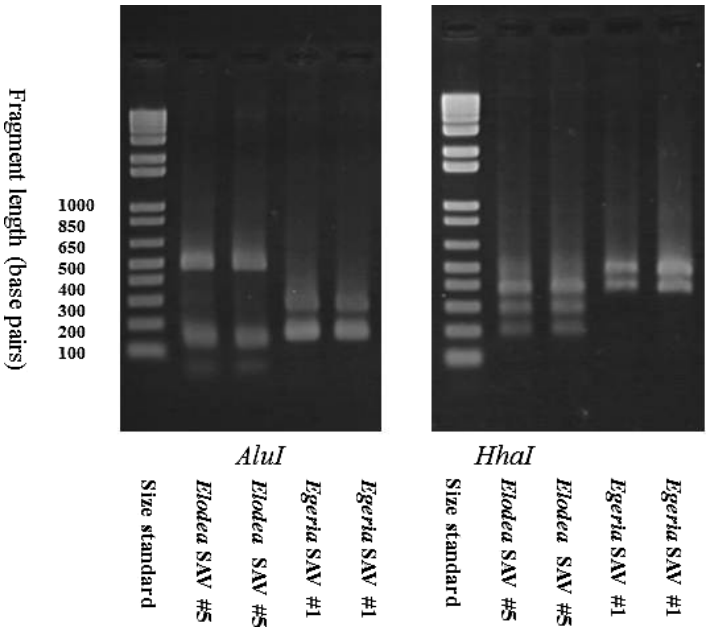

Figure 2. RFLP gel showing differentiation of *Elodea* sp. and Brazilian waterweed (*Egeria*) with restriction patterns (fingerprints) generated by *Alu*I and *Hha*I restriction enzyme digests of PCR product shown in Figure 1. SAV # is in reference to Table 1.

uncontaminated DNA (Table 1). The best templates were DNA from plants that were frozen fresh, pressed fresh onto FTA cards, or freeze dried such that green color was retained. Twenty-four herbarium samples that were collected between 1943 and 2002 did not yield DNA, while thirty herbarium samples collected between 1966 and 2007 yielded DNA. In contrast, all but one fresh sample produced clear results. In this study, older dried samples, particularly those which were brown and lacked any green pigment were most likely to have degraded DNA. The successfully identified samples ranged in age from fresh to approximately 40 yrs old. The use of molecular methods with older samples is in part contingent upon a handling procedure that ensures no cross-contamination or extensive, undue degradation of the DNA.

PCR to distinguish hydrilla from Brazilian, Canadian, and western waterweeds

The initial PCR using the ITS5 and 26S25R primer pair resulted in different fragment length products for specimens of hydrilla compared to *Egeria* and *Elodea*. Hydrilla produced a PCR product ca. 650 bp (base pairs) and *Egeria* and *Elodea* both produced a product of about 800 bp (Figure 1). This step verified that 36 samples were hydrilla. We used an additional PCR method to determine the biotype and found 25 samples were monoecious and 11 were dioecious. The remaining 47 nonhydrilla samples were then subjected to further analysis.

RFLP analysis to distinguish between *Elodea* and *Egeria*

We saw size variation in the RFLP pattern from PCR products of *Elodea* and *Egeria* with restriction enzymes *Hha*I and *Alu*I as expected by comparison to *in silico* digests of published *Elodea* and *Egeria* ITS region sequences, so either enzyme can be chosen for use (Figure 2). Of the 47 samples tested, 14 samples were confirmed to be Brazilian waterweed, while 33 samples were identified as *Elodea* and subjected to further analysis.

We saw two distinct patterns in *Mnl*I enzyme digests of ITS5/26S25R PCR products from standards of Canadian waterweed and western waterweed. Furthermore, when all specimens of *Elodea* were tested, a third pattern was also observed (Figure 3). Several examples of each pattern were sequenced, and the first two patterns corresponded to the appropriate Canadian waterweed and western waterweed sequences as expected. The third pattern showed >99% sequence similarity to published western waterweed sequences (nine of the fourteen with pattern 3 were sequenced), indicating that these samples were western waterweed. A single base change compared to published sequences resulted in the pattern difference. Of the 33 *Elodea* samples, 25 samples were identified as western waterweed and 5 as Canadian waterweed by this method

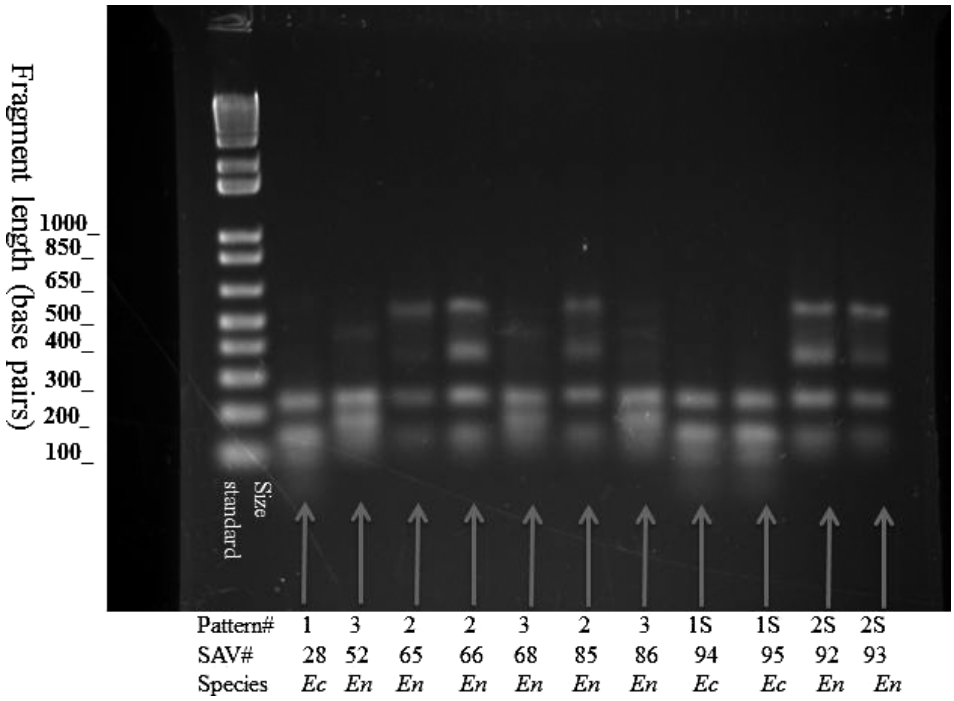

Figure 3. RFLP gel showing differentiation of Canadian waterweed (*Ec*) and western waterweed (*En*) with restriction patterns (fingerprints) generated by *Mnl*I restriction enzyme digests of PCR product shown in Figure 1. Note 3 patterns. Pattern 1 is *Ec*, pattern 2 is *En*, and pattern 3 is also *En*. 1S and 2S are sequenced standards for *En* and *Ec*, respectively. SAV # is in reference to Table 1.

(Table 1). Three of the *Elodea* samples were determined only to the genus level.

Sequencing

We sequenced 31 representative samples for the two gene areas utilized in this study (Table 1), the nuclear rRNA ITS region (GenBank accession numbers JF703253 to JF703273) and the chloroplast (plastid) *trn*L-*trn*F intron and intergenic spacer region (GenBank accession numbers JF703274 to JF703284). Comparison to the NCBI GenBank database confirmed the fingerprinting results for samples. Our procedure was to sequence at least three of each species for standards. We also sequenced samples for verification when the species determination by our fingerprint method did not agree with a published determination or distribution range. In addition, as mentioned an unpredicted RFLP pattern was observed for several *Elodea* spp. and representatives of that pattern were also sequenced and confirmed as western waterweed. Lastly, samples of Brazilian waterweed, Canadian waterweed, and western waterweed were also sequenced for the chloroplast (plastid) *trn*L-*trn*F intron and intergenic spacer region because there were no conspecifics in GenBank. These sequences are the first sequence deposits for these plants for this gene (GenBank accession numbers JF703281 to JF703284). The results of sequencing with the primers targeting the *trn*L-*trn*F intron corresponded with the results of Erhard (2005) that showed differences between these organisms based on the ITS gene.

Single step PCR methods to distinguish species or biotypes of one species can be very useful and simple tools. Our strategy included a several step process with the potential to use just the first step for hydrilla identification, and further steps to identify a nonhydrilla plant as one of the other three species. The time needed to differentiate the hydrilla from nonhydrilla is about 4 h. If more information is desired (biotyping hydrilla, and further identification of nonhydrilla), the additional RFLP or PCR steps may be added and require an additional day to complete.

In the case of species level identification in *Elodea*, we did discover a single mutation that changed the expected RFLP pattern in western waterweed. Now we know that both patterns are western waterweed. This suggests some population variation in the western waterweed sequences. The ecological significance of this variation is unknown, but being able to detect this difference could prove of significant value in the future.

We verified the results of this study with a sequencing effort, and are confident in the species assignments. RFLP analysis is limited in its ability to pick up sequence variations outside of the restriction site. However, as more and geographically diverse samples are interrogated, it is possible that new genotypes would occur and not be detected. It is also possible that minor variation at a restriction site could result in a new banding pattern for the same species. Therefore, in any study, a small sequencing effort to confirm some of the identifications should be done to reduce the likelihood of identification errors. The cost of sequencing is much lower and much more accessible now

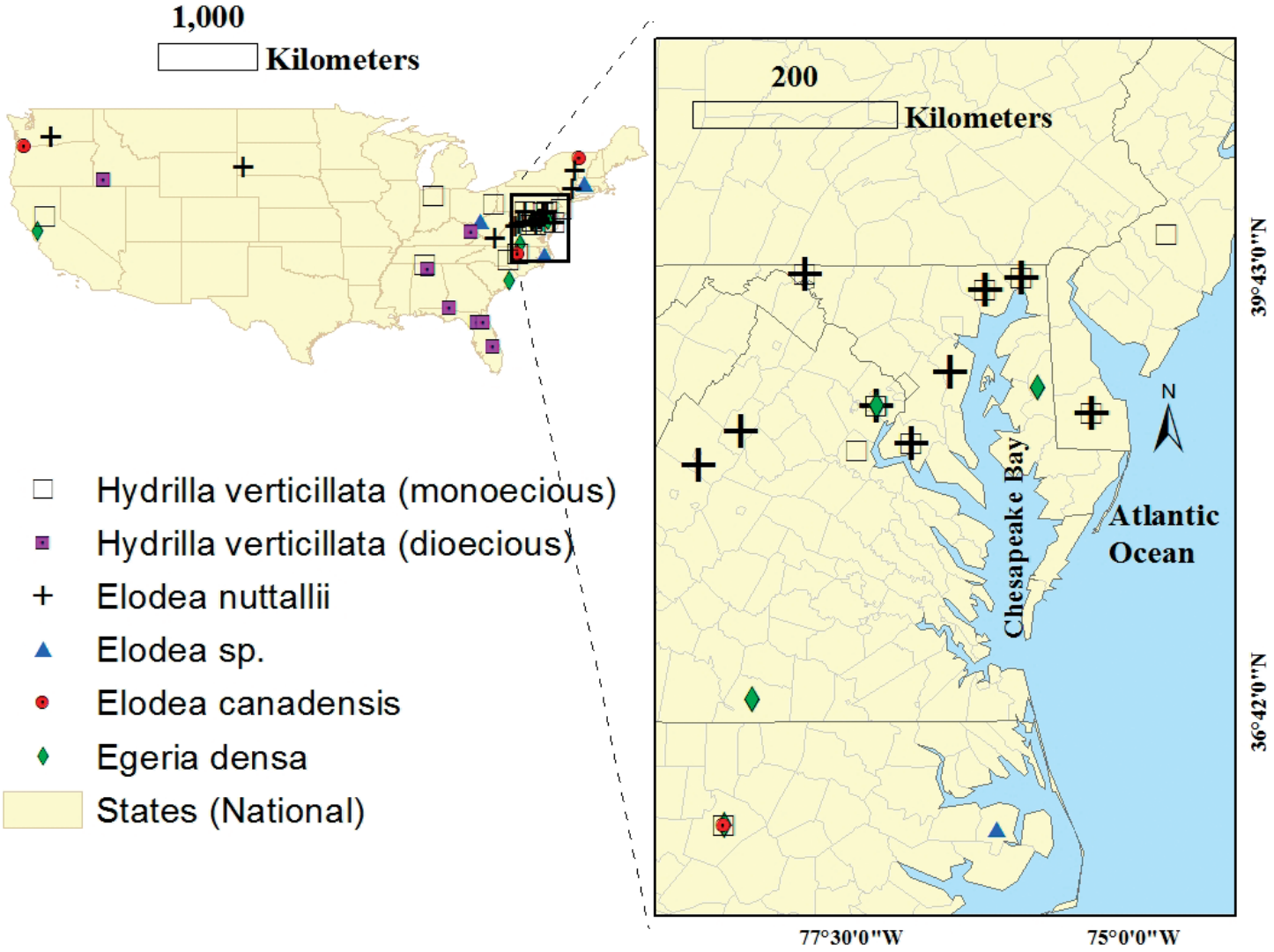

Figure 4. Map showing location of the 63 samples collected throughout the U.S. Note, some locations had several samples or species, so symbols overlap in those cases, and some samples not shown were acquired from outside the contiguous U.S., a pet store, an ornamental pond, or an unknown location.

than it was even 5 yr ago and this makes sequencing a more viable method for verifying the identity of plant tissues now, and in the future.

Biogeographical and ecological implications of samples redetermined by fingerprinting

In applying this protocol, we improved our understanding of the past and present distribution of hydrilla (monoecious and dioecious), Brazilian waterweed, Canadian waterweed, and western waterweed. To test the protocol we included numerous samples from a wide geographic and temporal gradient and they revealed new information on distribution patterns and misidentification potential among the species we tested (Figure 4, Table 1). The verified species occurrence data shown in Figure 4 are available on the Internet via the Global Biodiversity Information Facility (GBIF: http://www.gbif.org/).

The original epicenter for hydrilla in the United States was near Washington, DC for monoecious and in Florida for dioecious hydrilla and their spread from those locations has been documented (Owens et al. 2012). In re-evaluating herbarium samples, we discovered new information that monoecious hydrilla had invaded the area near Washington, DC 6 yr earlier than previously thought. Fingerprinting of herbarium specimens showed that monoecious hydrilla (originally identified as *Elodea* sp.) was in the nontidal Potomac River in 1976, and confirms a personal communication that monoecious hydrilla was present in Delaware by 1980 (Steward et al. 1984). This revised date of introduction shows that the 1983 expansion of hydrilla in the tidal Potomac may have been from colonies upstream, rather than from transplant experiments conducted in the tidal Potomac River (Dyke Marsh) in 1980 with plants thought to be *Elodea* sp. Much later, after the expansion of hydrilla in the Potomac River, an additional misidentified herbarium sample indicated that hydrilla was still being confused with *Elodea*. In redetermining the herbarium sample we found that rather than western waterweed, it was hydrilla that was

present during a vegetation survey of Brent Marsh on the Potomac River in Virginia in 1994 (Strong and Kelloff 1994).

Though seemingly rare, we confirmed a location where the biotypes co-occurred on the Tennessee River in Wheeler Reservoir in Alabama. Known locations where the biotypes co-occur include four in the southern Atlantic and Gulf of Mexico water basins as well as one in Northern California (Ryan et al. 1995, Madeira et al. 2000, Owens et al. 2012). Knowledge of the biotype is important since dioecious hydrilla is now showing a genetic resistance to the aquatic herbicide fluridone (Michel et al. 2004). Furthermore, to our knowledge, there are no long-term studies and little is known about competition or the temporal change in abundance in the two biotypes in locations where they co-occur.

Our samples were only opportunistically collected to verify the protocol, yet this set of widely spaced samples extended the information on the distribution of both hydrilla biotypes (Figure 4, Table 1) compared to the latest published distribution (Michel et al. 2004, Owens et al. 2012). We verified the dioecious biotype in Idaho (Bruneau River, in a geothermal area) and in Kentucky (Paintsville Lake), and the monoecious biotype in Indiana (Manitou Lake), Ohio (Ohio River), and New Jersey (Tamarac Lakes, Medford, NJ) from samples collected between 2007 and 2008. Idaho represents an area disjunct from the other areas and survival in this location is thought to be related to geothermal influences. Kentucky, Ohio, and Indiana sample locations (Figure 4) show that hydrilla's potential range is farther north than was predicted by an ecological niche model based on the ecological characteristics of thirty known occurrences of hydrilla in southern and eastern Asia (Peterson 2003).

The current northern spread of hydrilla accentuates the need for managers in northern states to be vigilant and prepare for hydrilla to survive conditions in northern latitudes. There is a misconception that hydrilla is a tropical species incapable of spread into northward latitudes, despite the fact that herbarium specimens of hydrilla show it is common in northern China where average January temperatures are 0 to −10 C (Balciunas and Chen 1993). It is also known to occur 9 degrees latitude below the Arctic Circle (Cook and Luond 1982, Les et al. 1997). Future investigations that predict relative invasiveness and competition between biotypes at different latitudes may be useful for managers.

This study demonstrated the troubling and re-occurring confusion in morphologically identifying these four species, especially the *Elodea* spp. (Table 1). Canadian waterweed was frequently given as the putative species of our samples, yet our results indicated that only western waterweed commonly occurred (Figure 4). We had hypothesized that the majority of *Elodea* spp. in the Chesapeake Bay region was Canadian waterweed based on a literature review of the estuarine distribution of *Elodea*. Vegetation surveys after 1970 list only Canadian waterweed in the tidal Chesapeake Bay (Stevenson and Confer 1978, Carter et al. 1983, Carter et al. 1985, Moore et al. 2000). We found that, in actuality, western waterweed had a wide distribution in the Chesapeake Bay region. In the Chesapeake Bay watershed, we found samples of western waterweed from the freshwater upper Chesapeake Bay (Susquehanna Flats) and tidal and nontidal freshwater Potomac River (in 2006 to 2009) as well as samples of western waterweed from the nontidal Patuxent River (in 1966), and the tidal Severn River (in 2006). In fact, we found no samples of Canadian waterweed in the Chesapeake Bay watershed, indicating an investigation is needed to verify reports of Canadian waterweed since the 1970s and to determine if Canadian waterweed and western waterweed still co-occur in this region, as was reported in the past (Hitchcock and Standley 1919).

There were few actual Canadian waterweed samples in this study (Table 1, Figure 4). Our Canadian waterweed samples included two standards from Europe and three samples from North Carolina (1983), Vermont (1969), and Oregon (2007). One Canadian waterweed sample from North Carolina was originally misidentified as hydrilla (1983). The misidentifications occurred during the time when the invasion of hydrilla into more northern waters came to national attention (Steward et al. 1984). Prior knowledge of the "expected" species, if used to identify ambiguous specimens, may lead to misidentification, as seems to have occurred in these instances in North Carolina and in the Chesapeake Bay region.

We now suspect there is a wider distribution of western waterweed and narrower distribution of Canadian waterweed than previously thought, as well as a possible displacement of Canadian waterweed with western waterweed in the tidal Chesapeake Bay. To confirm the numerous published surveys that report solely Canadian waterweed, we searched each of the herbaria that provided samples to this study trying to find samples of 1960s to 1980s' Canadian waterweed (or western waterweed) from the Chesapeake Bay. In our herbarium search, we found no Chesapeake Bay samples of Canadian waterweed; instead, we verified a herbarium specimen of western waterweed from the Patuxent River in 1966. Further verification of the distribution of these two species currently and historically is warranted. In Europe the displacement of Canadian waterweed with western waterweed has been proposed (Cook and Urmi-König, 1985; Erhard, 2005; Herault et al. 2008), making it more imperative to investigate this possibility in the United States

Our samples reveal that Brazilian waterweed continues to be available for purchase in stores, and four samples in this study were from stores (2006 to 2008) and collected in ornamental ponds in the Chesapeake watershed. In addition, one sample from an ornamental pond in 2007 was hydrilla. Because the pond owner purchased other aquatic plants but never knowingly purchased that species, the fingerprinting was useful to confirm unintentional transport of invasive species in the horticultural trade.

We have reinforced the fact that these species are co-occurring and that hydrilla is occurring in northern states. Given the spread of hydrilla farther north, where *Elodea* is firmly established (Gross et al. 2001, Hudon 2004), positive identification will be the key first step in any discussion of management options. In the future, quick and accurate identification of invasive plants is needed for early detection (Moody et al. 2008) if the intention is to prevent the spread of invasive species before they establish a beachhead population from which to spread farther.

SOURCES OF MATERIALS

[1]Qiagen®, 27227 Turnberry Lane , Suite 200, Valencia, California 91355

[2]Whatman®, 800 Centennial Avenue, Bldg 1, Piscataway, New Jersey 08854

[3]Eurofins MWG Operon®, 2211 Seminole Drive, Huntsville, Alabama 35805

[4]Promega®, 2800 Woods Hollow Road, Madison, Wisconsin 53711

[5]Eastman Kodak Company®, U.S., no longer in business

[6]New England Biolabs®, 240 County Road, Ipswich, Massachusetts 01938

[7]Applied Biosystems®, 850 Lincoln Centre Drive, Foster City, California 94404

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