A molecular genetic approach to evaluate herbicide resistance, geographic origins, and vectors of spread for populations of the invasive aquatic plant *Hydrilla verticillata* (Hydrocharitaceae) in the Northeastern United States

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ABSTRACT

Hydrilla verticillata (hydrilla) is a non-native and highly invasive aquatic plant that continues to expand its range in the northeastern United States. Despite the threat that hydrilla presents, there is a lack of technical scientific information on hydrilla in the Northeast. This study utilized a variety of molecular genetic techniques (DNA sequencing, microsatellite analyses) to evaluate where Northeast hydrilla originated geographically, to determine vectors of spread, and to determine whether any of the plants were potentially resistant to a specific herbicide (fluridone), which is used to manage weedy populations. We found all Northeast hydrilla populations examined to be monoecious and to lack any of the three known genetic mutations associated with herbicide resistance. Genetic data also indicated that Northeast hydrilla reproduces solely asexually by means of modified buds (tubers) and plant fragments. However, the low level of genetic diversity that we detected in this clonal species also limited our ability to reconstruct an historical geographic pattern of its spread or to identify specific vectors of its dispersal. From our genetic analyses, it is most likely that Connecticut hydrilla populations descended directly from mid-Atlantic region hydrilla populations. A rare genetic marker (allele) closely links the Mystic pond hydrilla to hydrilla in the Patuxent River in Maryland. Genetic variation was highest in Lake Gaston hydrilla where both monoecious and dioecious hydrilla has been introduced. Mixtures of microsatellite alleles characteristic of both monoecious and dioecious hydrilla were found in Lake Gaston individuals, a result suggesting that monoecious and dioecious hydrilla may have sexually reproduced at this location. Analyzing additional microsatellite alleles and plant samples may improve our ability to identify source populations of hydrilla and vectors of spread.

Introduction

Hydrilla verticillata (L. f.) Royle (Hydrocharitaceae) (commonly "hydrilla") is a highly invasive aquatic weed of fresh and low salinity waters that can cause serious ecological and economic harm. Native to Southeast Asia (Cook and Luond 1982) and Australia, it also occurs in Africa, Europe, China, the Pacific Islands, and South and North America. Hydrilla infestations reduce aquatic plant diversity (Haller 1978, Balciunas et al. 2002) and hinder recreational activities such as fishing, boating and swimming (Langeland 1996). Studies of waterfront property have shown that infestations of invasive aquatic weeds and the associated loss of recreational use can greatly reduce property values (Fishman et al. 1998, Halstead et al. 2003). Costs of controlling hydrilla in the United States run in the millions of dollars annually (Langeland 1996).

Two general "strains" or "biotypes" of *Hydrilla* (dioecious and monoecious) have been recognized (Cook and Luond 1982), and both have been introduced into the United States. Populations throughout the Southeast, Gulf Coast states, Idaho and California consist only of female plants and are believed to represent a single introduction of the dioecious (male and female flowers on different plants) form, which has spread vegetatively after its initial introduction. Monoecious plants (male and female flowers on the same plant) were discovered in Delaware in 1976 and then in 1982 found also in Kenilworth Aquatic Gardens, Washington D.C., and the Potomac River just south of Alexandria, Virginia (Steward et al. 1984). Subsequently, monoecious hydrilla has spread to every state from North Carolina to Maine (Les et al. 1997, Madeira et al. 2000), including New York and Pennsylvania, as well as to Georgia, Wisconsin, Indiana, Washington State and California (NAS database http://nas.er.usgs.gov).

Hydrilla possesses certain life history strategies and adaptations to aquatic environments that make it a successful invader capable of outcompeting native plants and dispersing readily from initial sites of introduction. Hydrilla grows under very low light conditions and branches out densely at the surface of the water, a growth habit that is effective in reducing light to other species of submerged plants (Langeland 1996). Hydrilla reproduces effectively by small vegetative fragments and also via asexual propagules called axillary turions and subterranean turions ("tubers") that can survive in bottom sediments for four years or more (Van and Steward 1990), and remain viable even after regurgitation by waterfowl (Joyce et al. 1980). Vectors of spread for hydrilla include transport by boat (e.g., fragments on propellers), waterfowl (e.g., fragments and turions), and accidental or intentional introduction by people (e.g., aquarium dumping, planting wetland plants contaminated with hydrilla). Of particular concern for managers of estuarine areas is this species' ability to tolerate salinities up to 11-13 ppt (Steward and Van 1987). For example, hydrilla is well established in fresh and oligohaline tidal waters of the Potomac River estuary and its tributaries where growth can be so dense that aquatic weed harvesters must be used to cut swathes through the plants to provide for boat passage at marinas (http://www.dnr.state.md.us/Bay/sav/key/hydrilla.asp).

Hydrilla populations in parts of the country where herbicide use has been extensive (e.g., Florida) have shown an alarming increase in herbicide resistance. Fluridone (brand name Sonar) is the only systemic herbicide approved for whole-lake treatments that is effective in controlling hydrilla. Fluridone acts by inhibiting the plant enzyme PDS (phytoene desaturase). Michel et al. (2004) sampled hydrilla from 200 Florida lakes, sequenced the PDS gene and identified three independent point mutations at codon 304 that confer

fluridone resistance. Because the plants are dispersed so readily, these herbicide-resistant strains inevitably will spread to other parts of the country. The over-use of fluridone in regions outside of Florida also has the potential to induce evolution of new resistant populations. To date, herbicide treatments of hydrilla in the Northeast (Mason Island Pond, CT, Long Pond, MA, Pickerel Pond, ME etc.) have been effective; however, treatment with dredging and herbicide has been unsuccessful at the Mystic Seaport pond in Connecticut. For the effective management of hydrilla, it is critical to know whether populations possess any of these genetic PDS mutations, which would render herbicide treatments ineffective.

Despite the threat that hydrilla presents, there is a lack of genetic or ecological data on hydrilla. It is imperative to learn more about this threat to water bodies of the northeastern U.S. in order to prevent further spread and to effectively manage the few existing populations. The objectives of this project are to assess the genetic composition of Northeast hydrilla populations in order to elucidate 1) whether herbicide-resistant strains occur in the Northeast, 2) the origin of populations including genetic relationship to other U.S. populations, and 3) vectors of spread.

Methods

Sample Collection

In summer 2007, multiple samples of hydrilla per site were collected from Virginia, Washington D.C., Maryland, Delaware, and Connecticut. In 2008, hydrilla was collected from North Carolina (Lake Gaston), Pennsylvania, and Connecticut. Lake Gaston, a man-made reservoir between Virginia and North Carolina, is the only known location where the two biotypes of hydrilla co-occur in the same water body. Hydrilla was collected previously (2006) from Florida, Georgia, South Carolina, and North Carolina (one sample per water body). Colleagues sent hydrilla samples (one sample per water body) -- fresh and/or in CTAB preservative -- from Massachusetts, Maine, New York, Wisconsin, Australia and South Korea. Appendix 1 lists all hydrilla specimens collected or received to date.

Molecular Genetic Analyses

Total genomic DNA was extracted from fresh samples of hydrilla, or from samples preserved in CTAB (Doyle and Dickson 1987), using the standard protocol for CTAB DNA extraction (Doyle and Doyle 1987).

To identify possible herbicide resistant genotypes, we cloned and sequenced the 5' region of the PDS gene. PDS was amplified using 819F and 1219R primers, modified to match the Genbank sequence for hydrilla PDS rather than the strictly degenerate primers described by Michel et al. 2004. Amplicons consistently had multiple bands and could not be sequenced directly. Amplicons were cloned and sequenced as described for the ITS except PDS primers were used. We noted the sequence of codon 304 where the 'wild-type' sequence CGT, coding for the amino acid arginine, produces a phytoene desaturase enzyme that is very susceptible to the herbicide fluridone. We looked for the presence of any of the three point mutations, AGT (serine), TGT (cysteine) and CAT (histidine), identified by Michel et al. 2004, which confer low, intermediate and high (respectively) herbicide resistance.

The nuclear ribosomal internal transcribed spacer (ITS) regions were amplified by polymerase chain reaction (PCR) and sequenced using the ITS4 and ITS5 primers (Baldwin

1992). ITS amplicons were cloned using a TopoTA cloning kit (Invitrogen). Six to 10 positive clones were amplified and sequenced using ITS4 and ITS5 primers. All sequencing reactions were analyzed on an ABI 3100 sequencer (Applied Biosystems).

Microsatellite regions of DNA consist of short nucleotide repeating units (i.e., usually two or three nucleotides) of rapidly evolving, non-coding DNA. These rapid genetic changes make the microsatellite regions suitable to distinguish individual populations from one another and to assess genetic relationships among populations of a species. We purchased the services of microsatellite library development for *Hydrilla verticillata* from Genetic Identification Services (GIS)(http://www.genetic-id-services.com/). Using this library, we identified those microsatellite regions suitable for amplification and screened 20 loci using primers designed by GIS. Eight loci amplified cleanly and consistently. Of these eight loci, four were variable (polymorphic) and used for analyses. Microsatellite amplicons were analyzed on an ABI3100 sequencer. Alleles were scored using the software Genemarker 1.7 (SoftGenetics LLC 2007).

Data Analysis and Interpretation

DNA sequences were aligned manually using the computer programs Sequencher 4.1.2 (Gene Codes, Ann Arbor, MI), Codon Code Aligner (Codon Code Corp.), and MacClade 4 (Maddison & Maddison, 2000). ITS sequences for hydrilla from India and for *Najas marina* were obtained from Les et al. 2006. The program PAUP* 4.0b10 (Swofford, 2002) was used for phylogenetic tree construction using maximum parsimony analyses (exhaustive search, random taxon addition, tree bisection reconnection, characters unordered, weighted equally). Insertion-deletions were treated as missing data. The degree of internal support for all nodes of resulting trees was determined by bootstrap analysis (500 replicates; full heuristic search) as implemented by PAUP*.

To assess genetic diversity using microsatellite marker data, we calculated total number of alleles per locus (allelic richness), observed heterozygosity, and frequency of dominant genotypes. Observed heterozygosity is the proportion of individuals with more than one allele at a locus. Without knowing the ploidy level of the samples, allele frequencies could not be calculated. To determine genetic relationships among East Coast U.S. hydrilla populations, we analyzed microsatellite sequence data using pairwise allele differences to construct a UPGMA (i.e., unweighted pairgroup method with arithmetic mean) phenogram, as implemented by the software program PAUP* 4.0b10 (Swofford, 2002).

Results

Herbicide resistance

All sequences of PDS codon 304 from hydrilla populations were the wild-type CGT sequence. Therefore, no herbicide resistant mutations were detected in any of the samples tested: Connecticut (two populations), Maine, Massachusetts, Pennsylvania, New York, Florida (three populations), Australia and Korea.

ITS Phylogeny

ITS sequences of all U.S. monoecious hydrilla were identical. However, seven point mutations and four polymorphisms were identified in the ITS sequences that clearly

distinguished the U. S. monoecious from U.S. dioecious strains of hydrilla. Maximum parsimony analysis of the ITS data (Fig. 1) resolved two distinct groups that correspond to monoecious (upper clade, 53% bootstrap support) and dioecious (lower clade, 74% support) hydrilla biotypes. The three Korean accessions form a strongly

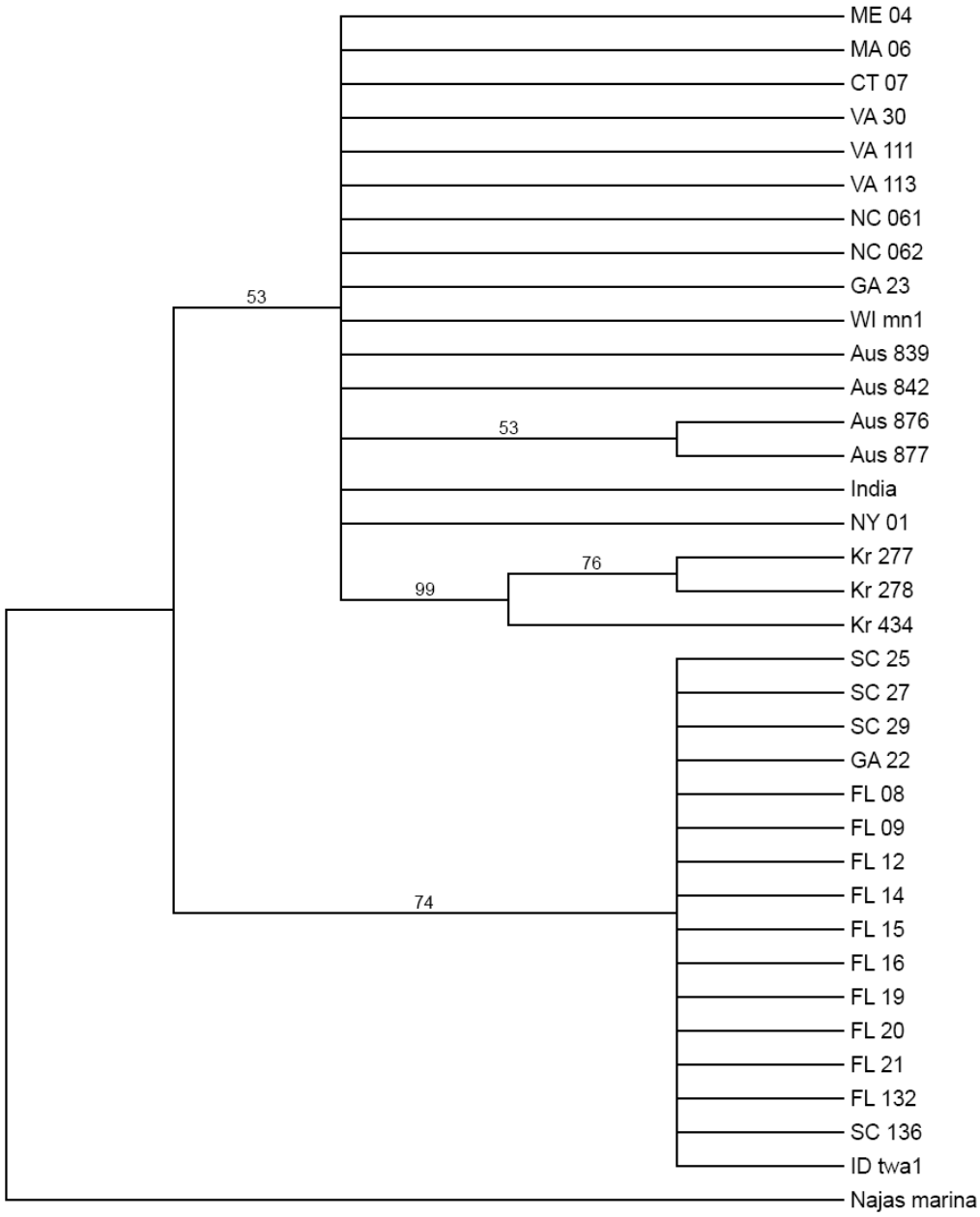

Fig. 1. Maximum parsimony cladogram (50% majority rule consensus) constructed from nrITS sequence data showing phylogenetic relationships of U.S. monoecious and dioecious accessions, and Australian, Korean and Indian accessions. Numbers above branches are bootstrap values. Abbreviations are for state or country (if other than U.S.), followed by collection number. Tree is rooted with outgroup *Najas marina.*

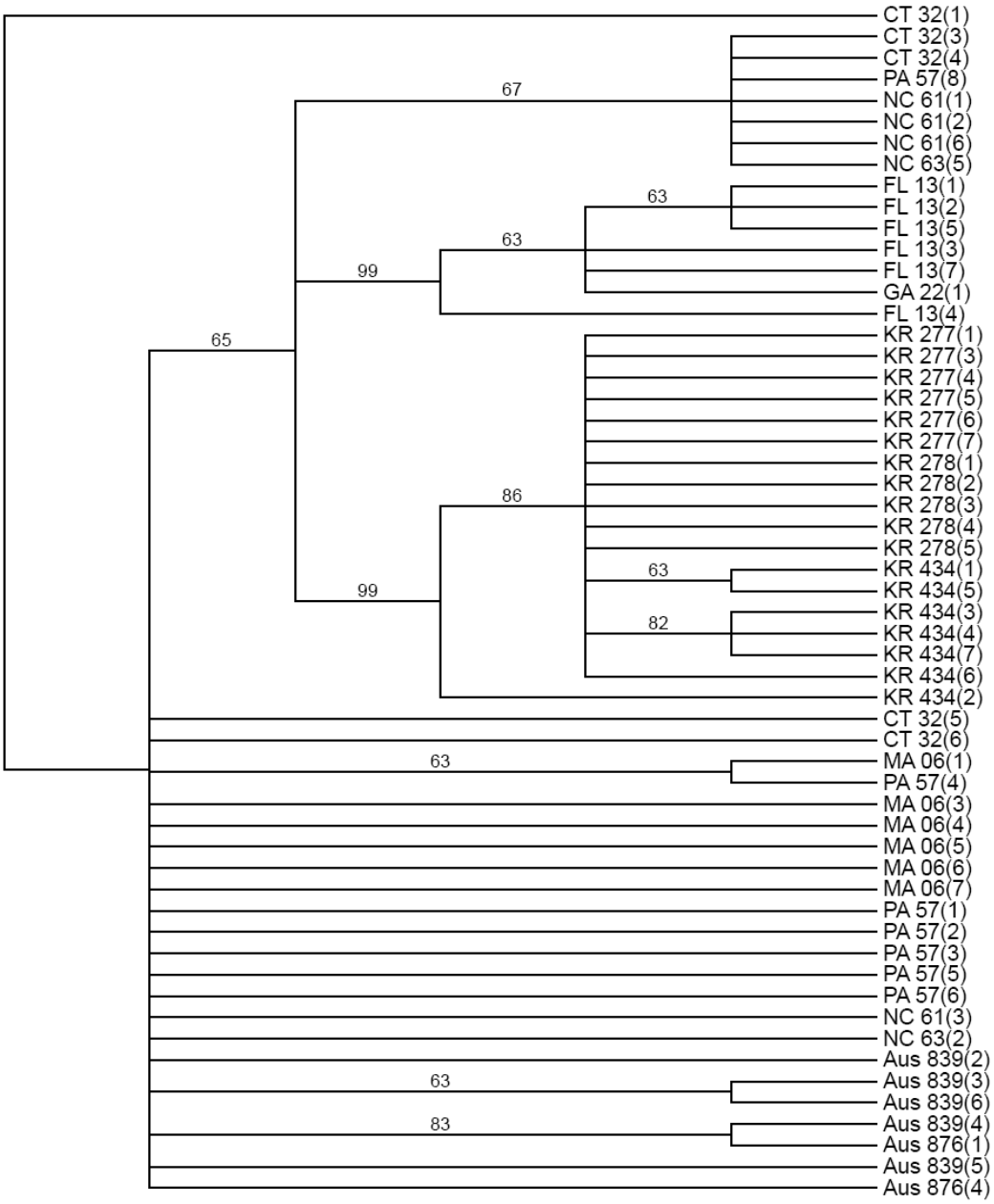

Fig. 2. Maximum parsimony cladogram (50% majority rule consensus) constructed from sequence data of cloned ITS genes. Numbers above branches are bootstrap values. Abbreviations are for state or country (if other than U.S.), followed by collection number, and clone number in parentheses.

supported clade that occurs within a clade of monoecious U.S., Australian and Indian accessions. The accessions from South Carolina, Georgia, Florida and Idaho form a well-supported clade of dioecious U.S. hydrilla.

The DNA polymorphisms in the U.S. monoecious ITS sequences indicate a possible hybridization event occurred in this biotype before it was introduced to the U.S. Cloning ITS amplicons can be used to identify the putative parental lineages of the hybrid, since one allele sequence will cluster with one parent and the second allele sequence with the other parent in a phylogenetic tree. Sequences of cloned ITS revealed a number of small indels in ITS (data not shown). Sequences of cloned ITS were used to construct a tree based on maximum parsimony (Fig. 2). Monoecious and dioecious clades are supported with 67% and 99%, respectively, bootstrap support. The Korean accessions form a well-supported clade with 99% bootstrap support. A number of Australian and U.S. monoecious clones are unresolved. Cloned ITS sequences from US monoecious hydrilla do not form clear associations with any of the other accessions that were tested.

Microsatellite analyses

The number of alleles per locus ranged from 9-12, while the number of heterozygous genotypes ranged from 6-13 (Table 1). Observed heterozygosity was higher for the two loci, HvB116 and HvA106, which exhibited frequently occurring heterozygous genotypes in the monoecious hydrilla samples. Since monoecious samples far outnumber dioecious samples, the measures of heterozygosity are more strongly influenced by the dominant genotypes of the monoecious samples. The dominant genotypes of the HvA118 and HvB108 loci, in contrast, are both homozygous.

Table 1. Diversity measures for four polymorphic microsatellite loci of *Hydrilla verticillata* (n=112). H_O: Observed heterozygosity.

Locus	Nucleotide repeats	Allele size range (bp)	Total no. alleles	No. heterozygous genotypes	H_o
HvA118	$(CA)_{11}$	144-179	12	6	0.42
HvA106	$(CA)_{12}$	269-284	9	6	0.76
HvB108	$(CT)_{13}$	250-270	12	10	0.34
HvB116	$(GA)_6GG(GA)_{10}$	178-206	11	13	0.86

Number of alleles per loci per hydrilla population varied from one to six, and observed heterozygosity ranged from zero (all homozygous genotypes) to one (all heterozygous). The Kenilworth Aquatic Gardens population had the fewest alleles, and the highest occurrence of fixed heterozygosity (all genotypes are the same heterozygous allele pair). Monoecious biotypes (all populations except southeastern U.S. dioecious) had few genotypes in common with the pooled dioecious hydrilla individuals. Although the Connecticut and Kenilworth populations had no genotypes in common with dioecious populations, each of the three locations had at least one sample with a dioecious biotype allele (data not shown). Lake Gaston hydrilla had genotypes in common with both monoecious and dioecious populations, as well as heterozygous genotypes that were a mixture of monoecious and dioecious alleles.

In the UPMGA cluster analysis, the U.S. dioecious populations (cluster of FL, ID, GA and SC) form a distinct group separate from the monoecious hydrilla populations (Fig. 3). The one exception to this dichotomy is the location of sample FL_14 which possesses mostly monoecious biotype microsatellite alleles, yet the ITS sequence is clearly the dioecious type. Hydrilla from this site, Lake Howard in Florida, may be a hybrid of monoecious and dioecious biotypes. Overall, the dioecious samples show almost no genetic variation.

Table 2. Measures of allelic richness, heterozygosity, and genotype frequency for four *H. verticillata* microsatellite loci in five U.S. populations. n: Number of individuals; H_O: Observed heterozygosity; dominant genotypes: allele size (bp) and frequency for the two most common genotypes

Locus		Mystic CT (CT 55)	Silvermine River CT (CT32, CT56)	Kenilworth Aquatic Gardens, D.C. (DC 48)	Lake Gaston, VA, NC (VA 30, NC 61, 62, 63)	Southeastern U.S. dioecious (FL 8, 12-15,19, 21, GA 22, SC 29)
HvA118	n	5	7	5	12	7
	#alleles	3	4	3	4	3
	H_o	0.40	0.43	0.00	0.58	0.86
dominant	146	0.60	0.57	0.60	0.25	0.14
genotypes	157,161	0.00	0.00	0.00	0.33	0.86
HvA106	n	5	7	6	14	9
	#alleles	2	2	2	3	3
	H_o	1.00	1.00	1.00	1.00	0.11
dominant	280,284	1.00	1.00	1.00	0.93	0.11
genotypes	279	0.00	0.00	0.00	0.00	0.89
HvB108	n	6	5	6	14	9
	#alleles	3	3	1	4	3
	H_o	0.33	0.40	0.00	0.29	0.89
dominant	258	0.67	0.60	1.00	0.71	0.11
genotypes	254,261	0.00	0.00	0.00	0.00	0.89
HvB116	n	6	7	6	13	9
	#alleles	3	6	2	4	4
	H_o	1.00	0.86	1.00	1.00	0.22
dominant	202,204	0.83	0.14	1.00	0.77	0.00
genotypes	180	0.00	0.00	0.00	0.00	0.78

Three Mystic Connecticut samples and one from the Silvermine River share the same alleles at all four loci with samples from Massachusetts, Lake Gaston, Kenilworth and Georgia. These samples all possess the dominant (most frequent) monoecious genotype at all four loci. Other samples from the two Connecticut sites cluster, at least once and often multiple times, with hydrilla from the other monoecious populations. Therefore, no clear geographic patterns of allele distribution are shown by this analysis. However, genetic structure does exist and indicates there is some genetic diversity in the monoecious biotype of this highly clonal species.

Shared rare genotypes and alleles can indicate close genetic relationships. The sample DLLM was collected by Donald Les and Les Mehrhoff from the Mystic Seaport pond location in 1996. This hydrilla sample has a rare allele (179 bp) at the HvA118 locus that is found in only one other sample from the Patuxent River (MD_49_3). As seen on the phenogram (Fig. 3), the DLLM hydrilla has the same genotypes as the hydrilla from the Patuxent River.

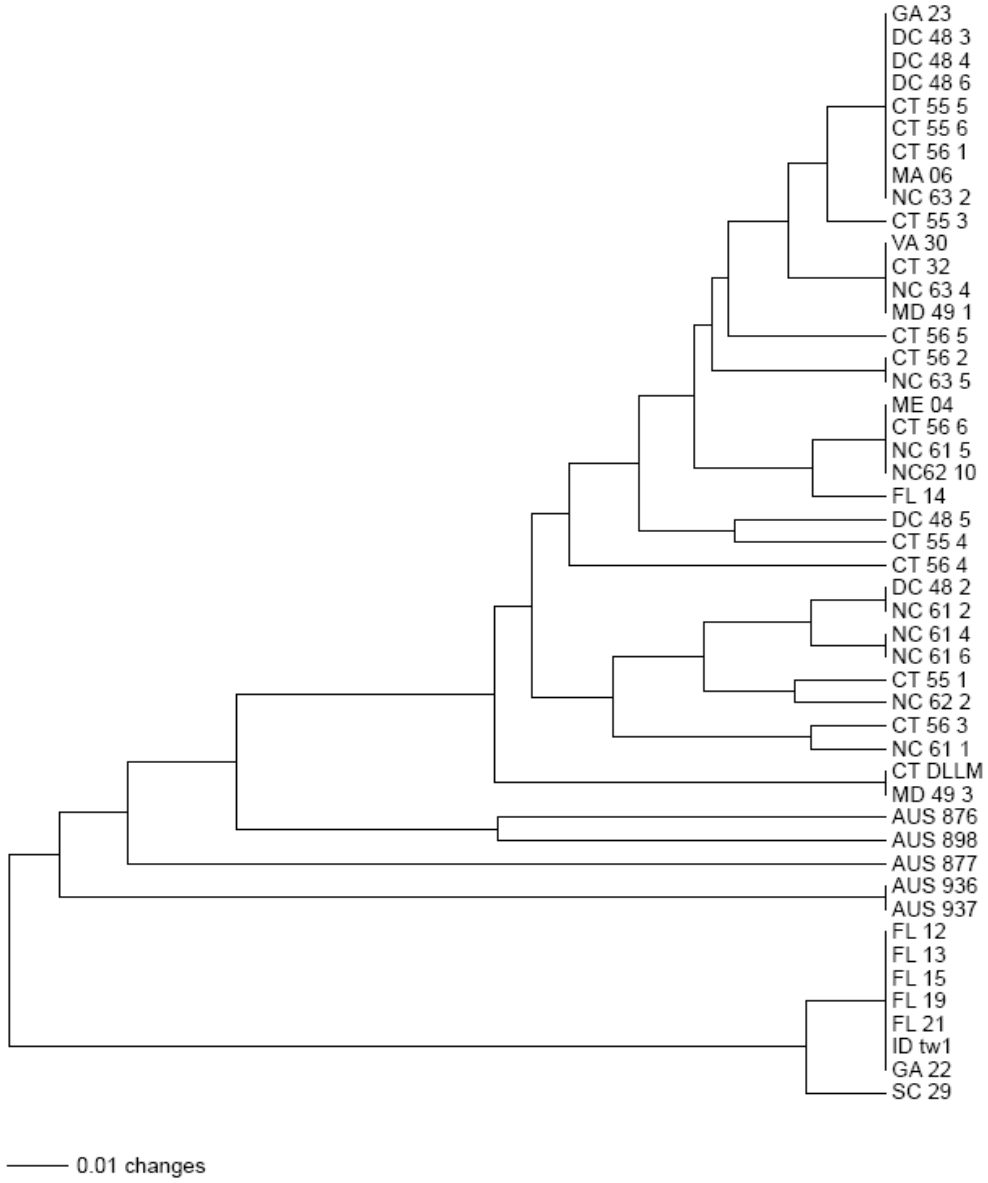

Fig. 3. UPGMA phenogram based on pairwise genetic distances among four polymorphic microsatellite loci in *Hydrilla verticillata*. Samples are labeled with state or country of origin, and sample number.

Discussion

Genetic analysis of the phytoene desaturase gene in northeast and three Florida hydrilla populations revealed none of the known mutations that confer herbicide resistance. In Florida, where use of herbicides has been extensive, 20 water bodies out of 200 tested were found to have herbicide resistant hydrilla plants (Michel et al. 2004). Dioecious hydrilla in Florida consists of only female plants and, lacking sexual reproduction, evolution of herbicide resistance was likely due to selection of somatic mutations (Michel et al. 2004). Monoecious plants in the Northeast have the potential to evolve resistant mutations via the germ line through sexual reproduction as well as by somatic mutation. In contrast to Florida, where hydrilla growth and herbicide applications occur year round, the climate in the Northeast supports a shorter growing season and fewer opportunities for applying herbicide. Minimizing the frequency of fluridone use, and alternating with other control methods, may decrease the likelihood of evolution of herbicide resistant mutations.

Monoecious and dioecious hydrilla biotypes are clearly differentiated by ITS DNA sequences. Korean hydrilla may be monoecious, but it forms a clade distinct from U.S. monoecious hydrilla. Overall, resolution of the cladogram is limited by the few parsimony informative loci (28) in the ITS region. However, these results do indicate that the vast geographical range of hydrilla is associated with informative genetic divergence, which has occurred over time and distance. Future work should include hydrilla samples from Southeast Asia where hydrilla is most likely native. Increasing the number of gene regions sequenced could improve the resolution of phylogenetic relationships.

Monoecious U.S. hydrilla has four ITS sequence polymorphisms that are indicative of a previous hybridization event. Hybrids can be identified by such polymorphisms, as was shown with the hybridization of the invasive Eurasian watermilfoil *Myriophyllum spicatum* with the native *M. sibiricum* (Moody and Les 2002). In order to identify the parental lineages, samples of the putative parents must be obtained and their sequences included in a phylogenetic tree. The U.S. monoecious cloned ITS sequences do not group with the Korean accessions, and therefore hydrilla from Korea is not likely one of the parents. Some Australian and U.S. monoecious sequences are associated in the unresolved part of the cladogram, but these data are inconclusive. Based on the samples we have, we cannot draw any conclusions about the parental lineage of U.S. monoecious hydrilla at this time. Additional hydrilla samples from around the world are needed to pursue this line of investigation further.

Distribution of microsatellite genotypes reflects the highly asexual reproductive history of hydrilla. The distribution is characterized by high frequencies of a single dominant genotype at each locus in most populations, while other genotypes are lacking, or found at very low frequencies. Dioecious hydrilla in particular has very low genetic diversity which may be attributed to a complete lack of sexual reproduction since only female plants have been introduced. Any genetic variation in dioecious hydrilla, such as the mutations in the PDS gene, is likely the result of somatic mutations. However, it should be noted that sampling of dioecious hydrilla lacked intrapopulation samples, so genetic variation may be underestimated.

Monoecious hydrilla can reproduce sexually since both sexes are present. Male and female flowers were found on samples from the Potomac River (personal observation), while samples from Kenilworth Gardens and from Trap Pond in Delaware developed male and female flowers in cultivation (Steward et al. 1984). However, no flowering has ever been

observed in Connecticut hydrilla populations (personal observations, and personal communication, N. Murray, CT DEP). In other New England and New York populations, aggressive treatments with fluridone will prevent flowering as plants are either killed or stressed. Additionally, the growing season in New England and New York may be too short to support flowering. Therefore, hydrilla in the Northeast most likely reproduces solely by asexual means via tubers and fragments, and the genetic data is consistent with this conclusion.

Genetic variation was highest in Lake Gaston hydrilla where both monoecious and dioecious hydrilla has been introduced. The high genetic diversity and presence of both monoecious and dioecious alleles and genotypes within individuals suggest sexual reproduction occurred between the biotypes at this location. However, U.S. dioecious hydrilla is triploid (Langeland 1989, Verkleij and Pieterse 1991), and U.S. monoecious may be either diploid (Verkleij and Pieterse 1991) or triploid (Langeland 1989, Les et al. 1997), and triploid plants of any species have very low fertility. Alternatively, the low frequency dioecious alleles in monoecious populations may be a result of hybridization between parental lineages in the Old World if one parent was dioecious. Connecticut hydrilla populations have a lower incidence of dioecious alleles than Lake Gaston hydrilla, and Kenilworth has none. When hydrilla is transported to a new location, the new population contains only a limited subset of the parent population's total genetic diversity, a condition known as a founder effect. Therefore, Connecticut hydrilla, a likely descendent of mid-Atlantic region hydrilla, may have fewer rare alleles, including dioecious ones, than the progenitor population. Active management of hydrilla at Kenilworth Aquatic Gardens may prevent sexual reproduction and induce a genetic "bottleneck" where dioecious alleles have been eliminated and only the most common alleles, those typical of monoecious plants, remain.

Genetic diversity in the Mystic Seaport pond is lower than that of the Silvermine River and Lake Gaston, and some genotypes differ between samples collected at the pond before and after management treatments. Similar to Kenilworth Aquatic Gardens, genetic diversity in the Mystic site may have been reduced following a bottleneck induced by management. Management of hydrilla at the Mystic pond consisted of treatment with the systemic herbicide fluridone, followed by dredging a foot or more of the sediments to remove any subterranean turions that were not killed by the herbicide. The year following these treatments, the pond was planted with wetland species from a commercial grower (location unknown). Subsequently, hydrilla was found growing in the pond and within 1-2 years post-treatment had returned to approximately 100% coverage (personal observations). The DLLM sample from this pond was collected in 1996, prior to the treatments. Samples CT_55 (1-6) were collected post treatment in 2007. The DLLM sample has a rare allele (HvA118 179 bp) found only in one other sample from the Patuxent River in Maryland, and not found in the samples collected post treatment. Two plausible scenarios can explain the differences in genetic composition between samples collected before and after treatment. The initial hydrilla infestation may have been incompletely killed by the treatments, and the population underwent a genetic bottleneck, where rare alleles, such as the HvA118 179 bp allele, were lost, and only common ones remained. Alternatively, the treatments were effective in eradicating the initial infestation, but the pond was re-infested with a new genetic stock of hydrilla from contaminated wetland plants introduced post treatment.

The genetic diversity of rapidly evolving microsatellite loci in sexually reproducing species is sufficient to distinguish different populations of the same species, similar to genetic fingerprinting. Low genetic diversity of hydrilla, a clonal species, has limited our ability to determine a geographic pattern of spread. Hence we cannot draw conclusions, based on

our genetic data, about vectors of spread. Increasing the number of microsatellite loci assessed may identify additional markers that will improve our ability to track the spread of hydrilla and identify source populations and vectors.

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