EVIDENCE OF CLONAL REPRODUCTION AND HIGH GENETIC VARIATION IN THE EXOTIC WEED *HYDRILLA VERTICILLATA*

by

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Introduction

Invasive species currently pose great economic and ecological challenges. Each year in the United States alone they cost $125-140 billion in control methods and repair expenses. In addition, they threaten biodiversity, destroy natural ecosystems, and outcompete native species (Mack *et al.* 2000; Baker 2001; Balciunas *et al.* 2002; Pimentel *et al.* 2005). In Florida, it has cost over $10 million annually to shut down recreational activities in two Florida lakes to clear them of hydrilla infestations (Pimentel *et al.* 2005). Certain characteristics including the ability to rapidly spread in new environments through vegetative or asexual reproduction, grow in disturbed areas, and competitively exclude native species by establishing monocultures, can make some plant species more invasive than others. Wetland habitats are especially susceptible to invasive aquatic plants and other organisms in large part due to the ability of these organisms to float to new locations and establish residence (Eckert *et al.* 2003; Zedler *et al.* 2004).

Invasive, clonally reproducing plants are expected to be less likely to develop resistance to herbicides or biological control agents compared to sexually reproducing species due to the accumulation of deleterious mutations and their presumed lower levels of genetic diversity resulting from founder events and asexual reproduction (Burdon and Marshall 1981; Maxwell and Mortimer 1994; Chapman *et al.* 2000, 2004). Now, with the development of more sensitive DNA markers, it is becoming apparent that the assertion of low genetic diversity may not generally be true for clonally reproducing organisms (Loxdale and Lushai 2003; Lushai *et al.* 2003). Studies have revealed that species which reproduce predominantly through vegetative spread and apomixes (reproduction by an unfertilized seed) can have intermediate to high levels of genetic diversity (Ellstrand and Roose 1987; Widen *et al.* 1994). High levels of genetic variation in populations of clonal organisms can occur due to multiple colonization events by

different clonal lineages, cryptic sexual reproduction, mitotic recombination, variable ploidy levels, and somatic mutation (Ellstrand and Roose 1987; Chapman *et al.* 2000, 2004; Mes *et al.* 2002; Eckert *et al.* 2003; Rottenberg and Parker 2004; Paun *et al.* 2006; Ren and Zhang 2007; Dodet *et al.* 2008).

Hydrilla verticillata is an invasive aquatic weed that costs millions of dollars in control efforts and now covers much of the eastern, southern, and far western continental United States (Balciunas *et al.* 2002). Hydrilla infestations are a major economic concern as they block irrigation systems, clog boat motors, decrease water quality, and cause damage to hydroelectric power plants (Balciunas *et al.* 2002). Hydrilla is also an ecological concern since it displaces native vegetation, alters the native ecosystem, and decreases biodiversity (Colle and Shireman 1980; Schmitz and Osborne 1984; Schmitz *et al*. 1993; Bates and Smith 1994; Balciunas *et al*. 2002; MacDonald *et al.*2008).

Historical accounts suggest this noxious weed was introduced twice into the United States. The first introduction was from Sri Lanka and was introduced into Tampa, Florida in 1959 by a tropical fish and plant dealer dumping his stock (Schmitz 1990). This first introduction was the dioecious biotype (male and female reproductive structures are on separate plants of hydrilla), and now covers much of the southern U.S. from Florida to Texas as well as California. Only female plants of the dioecious biotype have ever been found in the wild. The second introduction, this time of the monoecious hydrilla biotype (male and female reproductive structures are on the same plant), occurred in 1976 in Delaware and the Potomac River (Haller 1982; Steward *et al.* 1984; Ryan and Hommberg 1994). This biotype has spread over much of the central Atlantic states and has been found along the Columbia River in Washington, in California, and recently in Indiana (Kay 1992; Langeland 1996; Madiera *et al.* 2000, 2004;

Keller 2007). Studies using RAPDs and an intergenic region of chloroplast DNA have largely corroborated the historical accounts of two introductions (Madiera *et al.* 1997, 1999, 2000, 2007). The dioecious biotype is most similar to hydrilla from the Indian subcontinent, and the monoecious biotype is most similar to hydrilla from South Korea.

Hydrilla is invasive because of its ability to form monocultures, thrive in all types of aquatic environments, and grow in limited amounts of light (Balciunas *et al.* 2002). Its most invasive characteristic is the ability to spread vegetatively through turions and stem fragments (Arias *et al.* 2005). Turions are dormant buds that can resist desiccation by remaining under the hydrosoil following the drainage of a lake for up to five years. Once the area refills with water, turions establish a new hydrilla infestation (Balciunas *et al.* 2002; Arias *et al.* 2005). In addition to dispersal by floating, small fragments and turions can become attached to boats, people, and waterfowl and then carried to new freshwater environments where a single node can establish a mature plant with as many as 16 shoot tips (Langeland and Sutton 1980; Sutton *et al.* 1992; Arias *et al.* 2005). There has been no record of the introduced dioecious biotype ever sexually reproducing in the wild and only a few instances have been recorded for the monoecious biotype (Michel *et al.* 2004; Puri *et al.* 2005). Steward (1993) conducted controlled crosses of both dioecious and monoecious plants and found that both biotypes have the ability to produce viable seed. He concluded that the lack of sexual reproduction in the dioecious biotype is not due to a lack of ability to produce viable seed, but a lack of opportunity due to the absence of the opposite sex (Langeland and Smith 1984; Conant *et al.* 1984; Steward 1993).

Many control methods have been used to slow the spread of hydrilla, including mechanical removal, lake drainage, and the introduction of natural enemies (Poovey and Kay 1998; Doyle and Smart 2001; Balciunas *et al.* 2002; Arias *et al.* 2005). The herbicide fluridone,

however, has provided the most cost-effective control of hydrilla in lakes and ponds (Doong *et al.* 1993; MacDonald *et al.* 1993; Pimentel *et al.* 2005). This herbicide inhibits phytoene desaturase (*pds*), an important enzyme in the biosynthesis of carotenoids, and causes photobleaching of the plant (Chamovitz *et al.* 1993). Resistant strains of hydrilla to fluridone have recently been found in Florida lakes (Michel *et al.* 2004; Puri *et al.* 2005; unpub. data). Although several mutations within the nuclear *pds* gene have been identified that confer fluridone resistance, it is still not clear why resistance varies along a continuum from low to very high (Puri et al. 2005). The development of variable levels of resistance was surprising given the presumed single introduction and clonal spread of hydrilla (Michel *et al.* 2004; Puri *et al.* 2005).

In this study, we utilized nuclear microsatellite markers to determine levels of genetic diversity within invasive populations of *Hydrilla verticillata* in Florida and Texas. We specifically ask 1) if patterns of genetic diversity indicate that invasive hydrilla only spreads vegetatively or if sexual reproduction also occurs, and 2) if observed levels of genetic diversity can best be explained by sexual reproduction or by somatic mutations which have been proposed as a likely explanation for the development of resistance to fluridone (Michel *et al.* 2004).

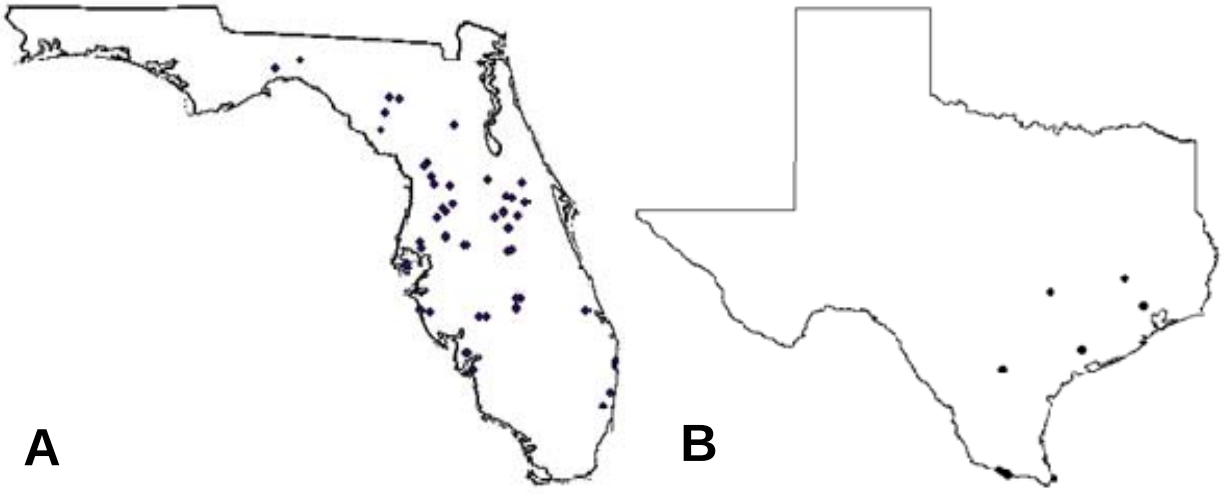

Figure 1. Map of hydrilla collection sites in Florida (A) and Texas (B).

Materials and Methods

Sample Collections

We sampled leaves from the tips of 262 individuals from across Florida and 42 samples from Texas (Fig. 1). We also collected nine samples from a lake in Louisiana. The plant samples were stored in silica and extracted following the methods of Kim *et al.* (1997). All samples were then further cleaned of PCR inhibitors using the Wizard DNA clean-up system (Promega USA).

Primer design

Genomic DNA for library construction was extracted using a modified protocol of Kim *et al.* (1997). All samples were then further cleaned using the Wizard DNA clean-up system (Promega USA). Microsatellite loci were isolated using the protocol of Glenn and Schable (2005). DNA was co-digested with *RsaI*, *AluI*, and *XmnI* and the resulting fragments were ligated to SuperSNX linkers. Fragments were then hybridized to groups of biotinylated oligos (ATC_8, ACT_{12}, GAA_8, GA_{12}) and (TGC_8, CCA_8) and captured with streptavidin coated magnetic beads (Promega USA). The enriched fragments were cloned with the pGEM-T Easy Vector System (Promega USA). We sequenced 300 clones using primers SP6 and T7 and ABI Big Dye Terminator Cycle Sequencing v 3.1 chemistry (Applied Biosystems USA). Sequences were electrophoresed on an ABI 3130 Genetic Analyzer (Applied Biosystems USA). We screened all sequences with MSATCOMMANDER (Faircloth 2008) for dinucleotide loci that had >8 repeats and trinucleotide loci that had >6 repeats and found 44 AG_n loci and nine GAA_n or ATC_n loci. There were another 13 dinucleotide and 39 trinucleotide loci with fewer repeat numbers (6-7 dinucleotide and 4-6 trinucleotide repeats). We designed primers for 34 clones that contained sufficient flanking regions using Primer 3 (Rozen and Skaletsky 2000). From these 34 loci, nine failed to amplify, 15 did not produce a specific product, two produced too much stutter to be

Table 1. Polymorphic microsatellite loci developed for *Hydrilla verticillata*.(n=550 individuals).

Locus name	Sequence (5'-3')	T_a (°C)	Repeat in original clone	Size of original clone (bp)	Size range (bp)	No. of Alleles
HvGA43	F: PET/TAGGGGTTCAATTCGCTCTG R:CCTGCAACAACCTCTGACAA	55	GA_9	218	211-238	13
HvGA04	F: GCTTCTTGTACCGCCTTCAC R: VIC/CCACCATCTCAAGGAGGAAA	55	GA_{16}	189	170-188	8
HvGA12	F: NED/TTCTACGGACCCGTTTGAAG R: ATCGATCGCAATTGGTTTTC	55	GA_{33}	225	164-180	4
HvGA45	F: VIC/TCGTTGGCCTGTAGATGAGA R: TTTCTTGGCCTGAACTGGAT	60	GA_{20}	250	224-253	9
HvLb18	F: VIC/AGATTCATCGGTGGTGCTTC R: CAAATTCCCGCAATGTTTTT	55	GA_{21}	202	165-207	15
HvLb19	F: 6FAM/TGATGGTGGTTTTCCACAAA R: ACAAGCAAAAAGCCATGCTC	55	GA_{25}	164	131-185	17
HvLb43	F: GACTTGCAACCAGCAACAAA R: 6FAM/AGCCCCCTTCTCAATAGGAC	60	$(GA)_6N_{25}(GA)_{27}$	250	169-318	25
HvLb44	F: GCCCACCTGAAAACTCTTTG R: PET/AATGCACGAGGAGGAAAATG	55	GA_{17}	240	239-334	31

T_a , annealing temperature

reliably genotyped, and eight were polymorphic, amplified consistently, and had scorable profiles (Table 1).

Genotyping

We screened all eight loci on 550 individuals from the United States (n = 384), Africa (61), Europe (1), Australia (18) and Asia (86). Polymerase chain reactions (PCR) (10 μL) contained 50 ng DNA, 50mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Tween, 200 μM each dNTP, 0.5 μM primers, and 0.4 U *Taq* DNA polymerase. Each locus was amplified separately and then co-loaded into two groups for genotyping. Coload A consisted of four loci: *HvGA04*, *HvGA43*, *HvGA45*, and *HvLb19* and coload B contained four loci: *HvGA12*, *HvLb18*, *HvLb43*, and *HvLb44*. Reactions were cycled in an ABI 2720 thermal cycler. The cycling parameters were one cycle at 94°C for 2 min, followed by 30 cycles of 15 s at 94° C, 15 s at 55° C (*HvGA04, HvGA43, HvLb19, HvGA12, HvLb44*) or 60°C (*HvGA45, HvLb43*), 30 s at 72°C, and one cycle at 72°C for 5 min. Genotypes were electrophoresed on an ABI 3130 Genetic Analyzer and scored using GENEMAPPER 4.0 (Applied Biosystems).

Data analysis

Hydrilla populations can contain a mix of individuals with different ploidy levels, including diploid (2n = 16), triploid (3n = 24), and tetraploid (4n = 32) individuals (Cook and Lüönd 1982; Verkleij *et al*. 1983; Harlan *et al*. 1985; Langeland 1989, Langeland *et al.* 1992). We could not accurately determine ploidy levels in the plants we collected. We used GenoDive v 2.0 (Meirmans and Van Tienderen 2004) for most analyses since it can analyze populations of clonal organisms that contain variable ploidy levels. For some analyses, such as the initial spatial autocorrelation analysis (see below) we scored specific microsatellite alleles as present or absent in each individual as if they were dominate markers.

We used GenoDive v 2.0 to estimate pair-wise genetic distances, assign clones based on defined thresholds of genetic distance, calculate diversity estimates, and test for clonal reproduction. We used a pair-wise distance measure between individuals that consisted of the number of mutations that would be required to transform one genotype into another genotype assuming a stepwise model of mutation. A histogram of the frequency distribution of the distances was then constructed to look for a potential bimodal distribution that might indicate the presence of both clonal and sexual reproduction (Rogstad *et al*. 2002; Douhovnikoff and Dodd 2003; Meirmans and Van Tienderen 2004, Arnaud-Haond *et al*. 2007). Individuals were assigned to clones using threshold distances which are the number of pair-wise differences that are allowed between individuals to still call them clone mates. Both genotyping error and somatic mutation can result in apparent differences between individuals of the same clonal lineage. Nei's corrected genetic diversity measure within populations was then calculated for a given threshold. Alleles were randomized among individuals 1,000 times to calculate the expected gene diversity under sexual recombination (Gomez and Carvalho 2000). The observed gene diversity was then compared to the expected distribution to determine if it was significantly lower as expected for clonal reproduction. We evaluated different thresholds for the assignment of clones and for clonal reproduction.

We calculated the number and frequency of alleles per locus using GenAlEx v 6.2 (Peakall and Smouse 2006). GenoDive v 2.0 was used to calculate Nei's genetic diversity index and evenness of genotypes within Florida and Texas. We used a bootstrap test, with subsampling to match population sizes, to test whether genetic diversity measures were different between Florida and Texas. Using the raw genotypes before clonal assignment, we computed

jackknife sampling curves using 1,000 permutations of the data to determine whether our sample sizes in Florida and Texas captured all genotypes and their evenness.

We compared allele sizes and frequencies for each locus to look for evidence of clonal reproduction. Under an expectation of clonal spread with variation arising mainly through somatic mutation we would expect to see some alleles at each locus at very high frequencies and others at very low frequencies that have resulted from somatic mutation. These alleles would also be expected to have repeat lengths relatively close to the common alleles under the assumption that somatic mutations involving microsatellite loci will follow the step-wise mutation model and have occurred relatively recently.

Spatial Structure

We tested for genetic spatial autocorrelation among clones using GenAlEx v 6.2. We first calculated the pair-wise genetic distance between all individuals in Florida and Texas using the method of Huff *et al.* (1993) (using the binary haplotype option in GenAlEx v 6.2). We created 11 defined distance classes within populations. An upper and lower 95% confidence limit was set around $r = 0$ (i.e. no correlation) by permuting individuals among distance classes and recalculating r 1,000 times. To calculate 95% confidence limits around each r value, each coefficient was bootstrapped within each distance class 1,000 times. If these confidence limits do not encompass $r = 0$ then the calculated r value is significant. Spatial autocorrelation analyses were also conducted using clonal assignments for the first several pair-wise distance thresholds.

We used K-means clustering to group individuals based on their pair-wise Euclidean distance into clusters in a way that maximizes the among-groups sum of squares. We used simulated annealing with 10,000 steps to cluster individuals into 2 – 20 clusters. The highest

Calinski-Harabasz (1974) pseudo F-statistic was used to determine which number of clusters was optimal. We tested for population differentiation between all individuals in Florida and Texas and all clones at thresholds zero and one using AMOVA in GenAlEx v 6.2.

Genotyping Error Estimates

All samples successfully amplified at all eight loci. To check for amplification and genotyping error we re-extracted DNA from 44 random samples from our world-wide hydrilla collection (N = 489 individuals), and reamplified all eight loci using the same conditions as above. There were two errors out of a total of 694 alleles across these individuals resulting in an allele error rate of 0.3%. These differences were distributed in two different individuals at two different loci resulting in a 4.5% genotypic error rate (i.e. 4.5% of individuals are expected to contain at least one allele error). We also selected 44 Florida individuals with unique genotypes to reamplify and genotype them at the locus that contained the unique allele. This allowed us to determine if these individuals truly had unique alleles or if the differences were due to amplification or scoring errors. There were four errors from a total of 470 alleles that occurred in three different individuals at four different loci. This resulted in an allelic error rate of 1.0% and a genotypic error rate of 6.8%. When combining these two measures of error, we have a final allelic error rate of 0.7% and a genotypic error rate of 5.7% (i.e. 5.7% of individuals are expected to contain at least one allele error).

Results

Genetic diversity

All eight loci were highly polymorphic in worldwide samples with an average of 17.4 alleles per locus (range 4 - 33 alleles) (Table 1). Genetic diversity across Florida and Texas was high. Out of 313 individuals there were a total of 54 alleles across eight loci. Each locus had an

average of 7.5 alleles (range 2 - 15 alleles); while each individual had on average 20 alleles. Most individuals (80.8%, n = 313 individuals) had a minimum of three alleles at several loci ($\bar{X}$ = 5.1 loci with three alleles, range: 4 - 6), while 19.2% had four alleles for at least one locus ($\bar{X}$ = 1.4 loci with four alleles, range: 1 - 3) suggesting all individuals were either triploid (n = 206 in Florida, 38 in Texas, and 9 in Louisiana) or tetraploid (n = 57 in Florida, 3 in Texas, and 0 in Louisiana). There were a total of 151 unique genotypes (n = 87 in Florida, 12 in Texas, and 0 in Louisiana) and genetic diversity in Florida (0.98) was similar to genetic diversity in Texas (0.96) (P > 0.35).

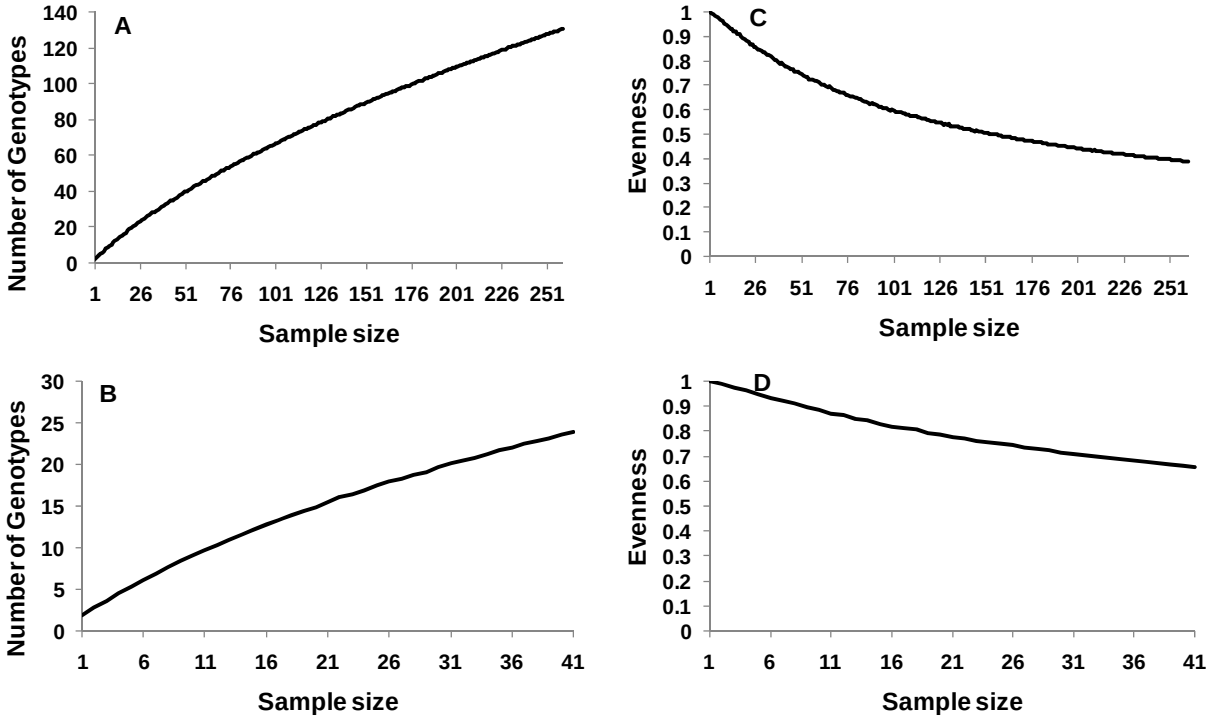

Figure 2. Jackknife sampling curves for *Hydrilla verticillata* genotypes in Florida (**A**) and Texas (**B**) and evenness of genotypes for Florida (**C**) and Texas (**D**).

Some of these individual genotypes are expected to be due to genotyping errors; however, even if we conservatively assume all errors resulted in a unique genotype we would expect only 9 - 10

of the 151 genotypes to be due to an error occurring at one allele. Evenness was 0.39 in Florida and 0.66 in Texas.

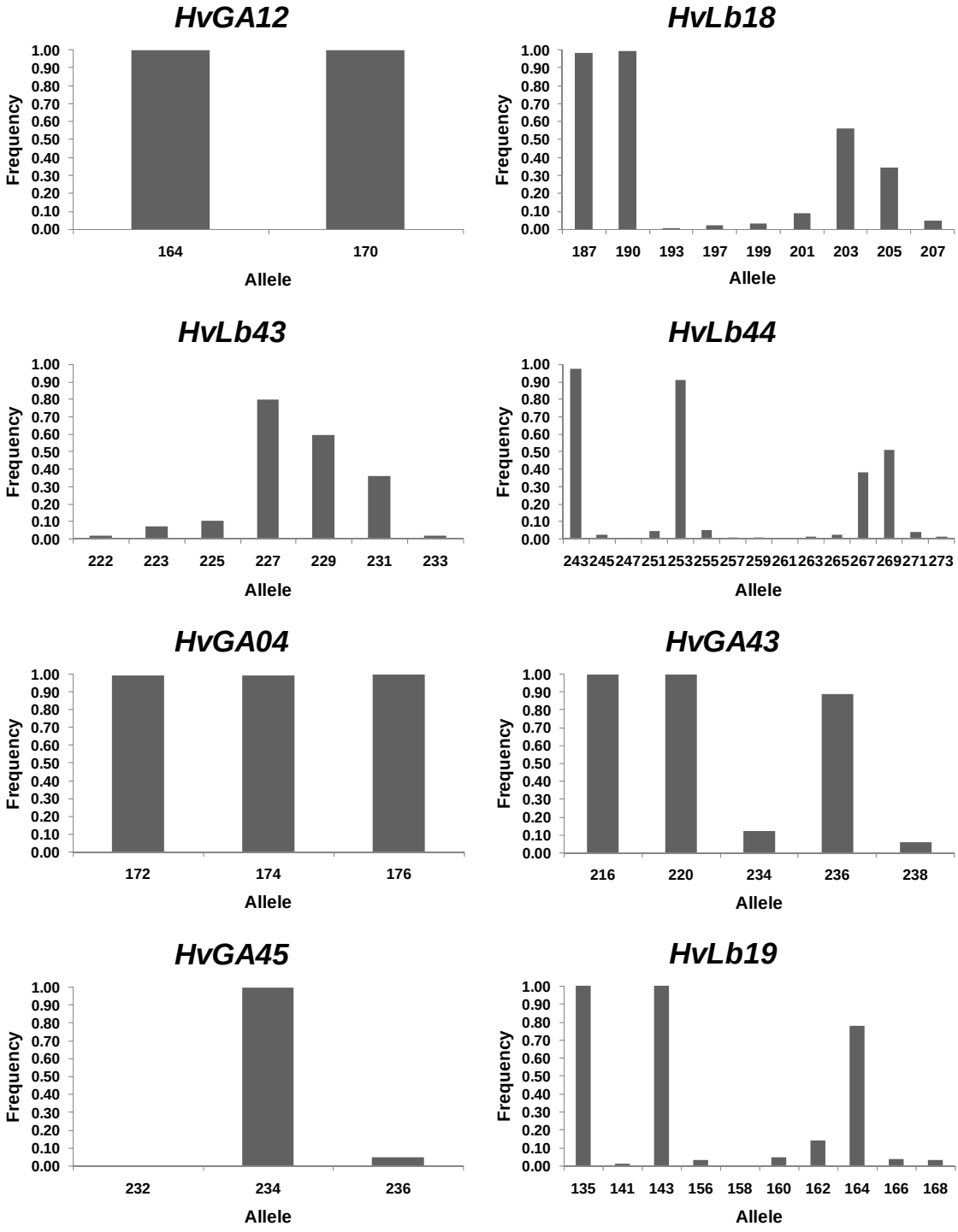

Figure 3. Allele sizes (bp) and frequency of occurrence in 313 individual *Hydrilla verticillata* samples for eight microsatellite loci.

Sampling curves suggest that we have not sampled all of the unique genotypes present in Florida and Texas nor have the estimates of evenness of these genotypes decreased to their final values (Fig. 2).

At three loci there were seven alleles that occurred in every individual while the remaining alleles varied among individuals (Fig. 3). Five of the fixed alleles were found in two loci, *HvGA12* and *HvGA04*, both of which were fixed for all alleles. The remaining loci contained low frequency alleles ($\bar{X} = 3.9\%$, range: 0.3 - 14.4%) that occurred within four repeat lengths of the most common alleles ($\bar{X} = 83.0\%$, range: 34.5 - 100%) (Fig. 3).

Clonality

Pair-wise differences between genotypes ranged from 0 to 22 with no evidence for a bimodal distribution (Fig. 4). A threshold of zero corresponded to either identical genotypes or genotypes that differed by only one stepwise mutation. At this threshold there were 70 clones and the levels of observed diversity were significantly lower than expected under simulated sexual recombination (P = 0.001). At thresholds 1-4 the number of clones decreased from 12 to 2 with a corresponding decrease in genetic diversity (Table 2). In all cases, observed genetic diversity using these clonal assignments was lower than expected under sexual recombination (P = 0.001 in all cases) (Table 1). The number of clones decreased to one at a threshold of five and remained there for increasing threshold values. At all thresholds the majority of individuals ($\bar{X} = 83.0\%$, range: 31.6 – 98.4%) were assigned to a single clone, while all other assigned clones occurred at very low frequencies ($\bar{X} = 1.0\%$, range: 0.3 – 10.2%) (Fig. 5).

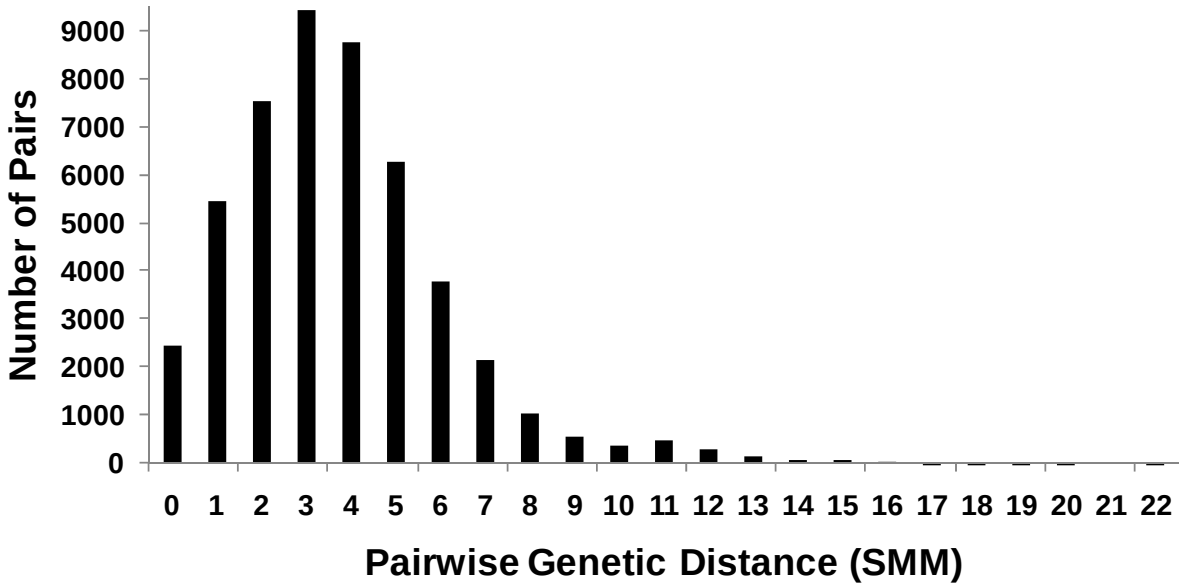

Figure 4. Histogram of the frequency of pair-wise genetic distances between *Hydrilla verticillata* samples using the step-wise mutation model

Table 2. Measures of genetic diversity for a given threshold of similarity between *Hydrilla verticillata* samples. Observed Nei's corrected genetic diversity (Obs. Div.) is compared to the expected diversity under sexual reproduction (see Methods).

Threshold	No. Clones	Population	Obs. Div.	Div. exp.
0	70	Overall	0.88	1.00
	60	FL	0.88	1.00
	18	TX	0.88	1.00
1	12	Overall	0.20	1.00
	11	FL	0.23	1.00
	3	TX	0.09	1.00
2	4	Overall	0.04	1.00
	3	FL	0.05	1.00
	2	TX	0.05	1.00
3	2	Overall	0.03	1.00
	2	FL	0.04	1.00
	1	TX	0.00	1.00
4	2	Overall	0.03	0.99
	2	FL	0.04	0.98
	1	TX	0.00	0.99
5	1	Overall	0.00	0.94
	1	FL	0.00	0.94
	1	TX	0.00	0.99

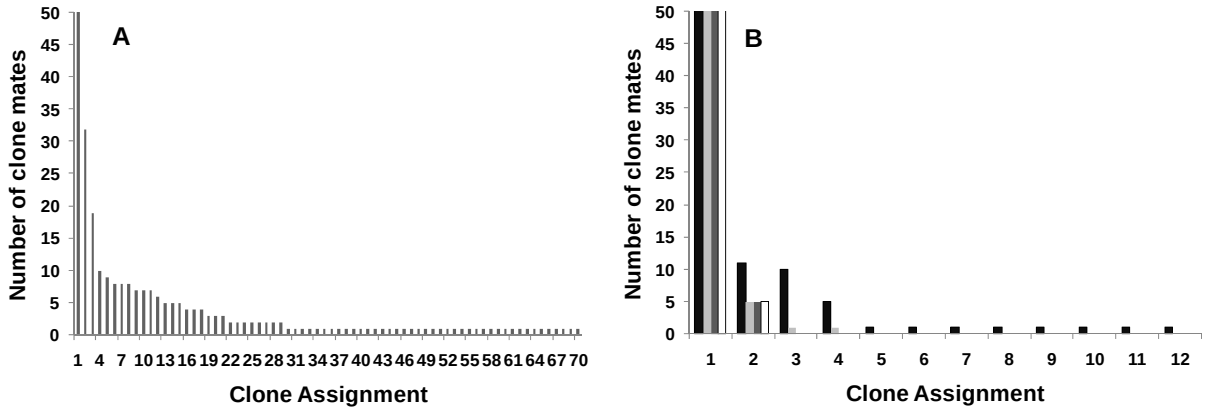

Figure 5. Number of clone mates assigned to each *Hydrilla verticillata* clone using thresholds 0 (A) and 1-4 (B). For clone assignment 1, threshold 0 has 99 clone mates, threshold 1 has 279 clone mates, threshold 2 has 306 clone mates, and thresholds 3-4 have 308 clone mates.

Spatial Autocorrelation

In Florida, there was significant positive spatial autocorrelation from 1 - 10 km (Fig. 6). The correlogram crosses the y-axis at 23 km after which the spatial correlation remains close to 0 until it becomes significantly negative after 300 km (Table 3). The spatial structure in Texas

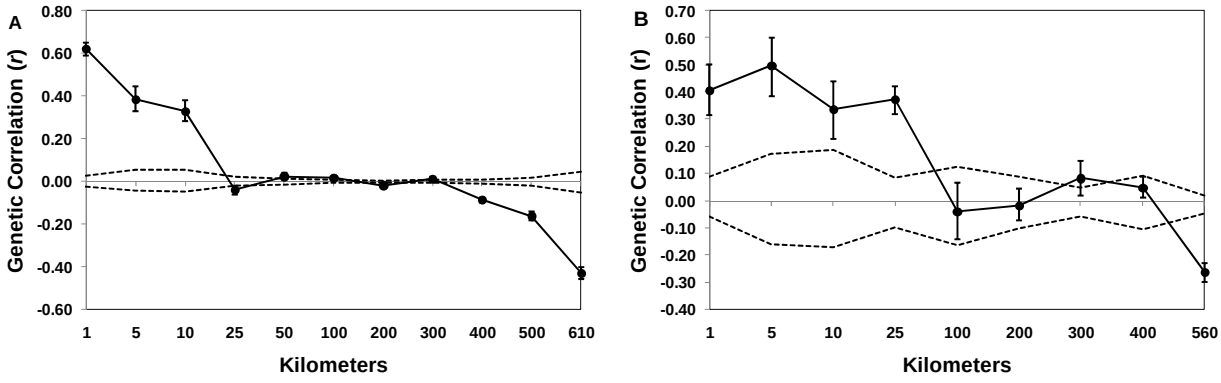

Figure 6. Correlograms of the genetic correlation coefficient *r* as a function of distance for all pair-wise comparisons of *Hydrilla verticillata* in Florida (N=262) (**A**) and Texas (N=42) (**B**). Dashed lines represent permuted 95% confidence intervals around *r*=0 and error bars represent the 95% confidence interval around *r* for each distance class.

was similar to the spatial structure in Florida with significant positive genetic correlations between 1 - 25 km, and *r* crossing the y-axis at 93 km and becoming significantly negative after

400 km (Fig. 6, Table 4). When using clonal assignments at different thresholds, both Texas and Florida have a significant positive correlation in the first distance class. The correlogram for clones assigned at threshold zero in Florida crosses the y-axis at approximately 76 km after which it remains close to zero until becoming significantly less similar than expected at 400 km. Clones with a threshold of zero in Texas remain close to zero after crossing the y-axis at approximately 4 km.

Table 3. Genetic correlation coefficient (*r*) between all pair-wise comparisons of *Hydrilla verticillata* in Florida within 11 defined distance classes using all individual genotypes (All) and clonal assignments at threshold 0 (T0).

Km	**1**	**5**	**10**	**25**	**50**	**100**	**200**	**300**	**400**	**500**	**610**
Pairs	**604**	**171**	**164**	**966**	**2040**	**6255**	**12727**	**6896**	**3132**	**1038**	**198**
All	0.62*	0.39*	0.33*	-0.04*	0.02	0.02*	-0.02*	0.01*	-0.03*	-0.16*	-0.43*
T0	0.50*	0.24*	0.23*	-0.01	0.01	-0.01	-0.01*	-0.01*	-0.06*	-0.07*	-0.19*

*p=0.001

Table 4. Genetic correlation coefficient (*r*) between all pair-wise comparisons of *Hydrilla verticillata* in Texas within nine defined distance classes using all individual genotypes (All) and clonal assignments at threshold 0 (T0).

Km	**1**	**5**	**10**	**25**	**100**	**200**	**300**	**400**	**560**
Pairs	**104**	**27**	**20**	**70**	**30**	**50**	**143**	**43**	**374**
All	0.41*	0.50*	0.34	0.37*	-0.04	-0.02	0.08	0.05	-0.26*
T0	0.21*	-0.09	-0.10	0.07	-0.06	0.02	0.01	-0.01	-0.06*

*p=0.001

Using AMOVA to compare Florida and Texas populations we found 96% of the genetic variation occurred within populations and 4% of the variation occurred between Texas and Florida (P = 0.001). At threshold zero variation between populations decreased to 2% (P=0.03) and at threshold one variation was not significantly different between the populations. The most likely number of k-means clusters was two across all samples from Florida, Texas and Louisiana with 204 individuals in cluster one and 109 individuals in cluster two. These clusters appear to result from six strong allele frequency differences between the clusters at loci *HvLb18* (203, 205), *HvLb43* (227, 229) and *HvLb44* (267, 269). The number of individuals in these clusters

were not different between Florida and Texas (χ^2_1=2.76, P=0.10) or between putative ploidy levels (χ^2_1 = 2.34, P = 0.13) (Table 5).

Table 5. K-means clusters for Florida and Texas. Number of individuals from Florida and Texas in cluster 1 and 2 and number of triploid and tetraploid individuals in clusters 1 and 2.

K-means	Pop.	No.	K-means	Ploidy	No.
1	FL	172	1	Triploid	159
	TX	22		Tetraploid	44
2	FL	90	2	Triploid	94
	TX	20		Tetraploid	16

The genetic signal from these two clusters was responsible for the significant negative spatial autocorrelation past 300 km in Florida and 560 km in Texas. Removing these six alleles from the spatial autocorrelation analysis results in the same short distance (1-25 km) positive spatial autocorrelation as when theses six alleles are retained but correlation values then decrease to levels near zero and remain there for all remaining distance classes. Mapping of individuals from the different clusters revealed that individuals from both clusters occurred over most of the state; however, the most geographically distant pair-wise comparisons in Florida and Texas contained individuals from the separate clusters, thus resulting in significant negative autocorrelation at these distances.

Discussion

Clonal, invasive weeds can quickly establish monocultures in new environments and remove native competition (Chapman *et al*. 2000, 2004; Eckert *et al.* 2003; Rottenburg and Parker 2004; Grimsby *et al.* 2007; Ren and Zhang 2007; Dodet *et al*. 2008). Invasive clonally reproducing plants were originally expected to have very low levels of genetic diversity and decreased evolutionary potential due to clonal reproduction and founder events. These populations were not expected to develop resistance to herbicides or biological control agents making them easier to control (Burdon and Marshall 1981; Chapman *et al*. 2000, 2004). Contrary to this expectation, some invasive clonally reproducing species have exhibited high genetic diversity

and adaptive genotypes in their new environments (Chapman *et al.* 2000, 2004; Dodet *et al*. 2008; Rottenberg and Parker 2009).

The dioecious biotype of *Hydrilla verticillata* was introduced once into Florida, and only female plants have been detected. The invasive spread of this aquatic weed is believed to have occurred vegetatively and so we might expect this weed to have low levels of allelic and genotypic diversity. Our results suggest, however, that the dioecious form of hydrilla is genetically diverse at neutral microsatellite loci. The source of this variation could potentially be due to cryptic sexual reproduction, multiple introductions, variable ploidy levels, or somatic mutation.

There are no known examples of dioecious hydrilla sexually reproducing in the southern United States, although successful reproduction could potentially take place if the dioecious biotype was crossed with the monecious biotype or there were cryptic males in the population (Steward 1993). Our data do not support the presence of sexual reproduction in the populations of the dioecious biotype that we sampled. Genetic diversity was always significantly less than expected under sexual recombination, there was no evidence of a bimodal distribution of pair-wise individual differences, and clonal assignments at varying thresholds resulted in most individuals being assigned to a single clone with very low frequencies of other clones. Patterns of spatial autocorrelation were also consistent with a pattern of vegetative spread with individual samples being more similar to each other than expected at random for the shortest distance classes.

Genetic variation could be due to multiple genotypes being present in the initial introduction. This might have been a possibility since the introduction description states that an aquarium dealer dumped a large batch of hydrilla into a canal behind his business. Genetic

markers support a single introduction event from a single source region, but we do not know if more than one multilocus genotype was released (Madeira *et al*. 1997, 1999, 2000, 2004). K-means clustering revealed the presence of two clusters in the samples that were the result of three pairs of alleles across three loci. The pairs of alleles were only one repeat apart which could suggest that genotyping error may have resulted in the frequency differences; however, our error checking did not indicate problems at these loci due to stutter and mis-scored alleles. These clusters may indicate that two common multilocus genotypes were originally introduced and have since been spread across the southern United States and have undergone further somatic mutations. Clonal assignments, however, result in most individuals being placed in a single clone. This suggests that these clusters are not related to clonal assignments. AMOVA analysis revealed a low (4%) but significant amount of genetic variation occurred between Florida and Texas which may simply be a result of a founder event when hydrilla from Florida was moved west.

The nature of the genotypic variation (low frequency alleles distributed one to four repeat lengths away from very common alleles), evidence against sexual reproduction, and short distance positive spatial autocorrelation all suggest somatic mutation is generating most of the genetic diversity we detected. The low frequency of individuals within most clonal assignments also suggests somatic mutation is responsible for most of the lineages across different thresholds. If multiple clonal lineages were present we might expect to have relatively high frequencies of individuals across multiple clonal assignments. Somatic mutation also best explains the recent development of new mutations and the step-wise distribution of alleles. .

A hydrilla colony originating from a single node can expand 4 cm per day and produce at least one new ramet per day, while the production of tubers can range from 1,250– 5,366 tubers

m^{-2} of lake sediment (Sutton *et al*. 1992; Sutton and Portier 1995; Madsen and Smith 1999). A single branching stem can grow up to 15 m in length with leaf whorls and nodes every 12 mm, each of which can give rise to a new plant (Langeland 1990; Ryan *et al*. 1995). With such rapid vegetative spread and hydrilla's ability to grow from fragments and turions, we would expect ample opportunities for mutations to arise from mitotic replication (e.g. Caetano-Anollés 1999) and to be potentially dispersed from their point of origin. Mutation levels are expected to be high for microsatellite loci (e.g. 10^{-4}, Schlötterer 2000), and since these loci are neutral their variation should have little adaptive or detrimental value for hydrilla. If mutation also generates high diversity in coding regions, then there would be opportunities for selection to act on this variation. Hydrilla could be described as a genetic mosaic (sensu Gill *et al*. 1995) in which advantageous somatic mutations have the ability to be spread due to the modular nature of hydrilla plants. Although most mutations are expected to be detrimental and therefore ramets containing them would be selected against, favorable variants residing in separate ramets of the same plant in a single lake or in different lakes could be selected for and spread through the dispersal of turions and stem fragments. This scenario could explain the recent emergence and spread of fluridone resistance in hydrilla. Sequence data from the *pds* gene suggests that this coding region also has relatively high nucleotide variation that has presumably been generated through somatic mutation (Michel *et al*. 2004; Puri *et al*. 2006; unpub. data). Nevertheless, organisms that reproduce by vegetative and asexual reproduction are predicted to have higher mutation loads in the long term compared to sexually reproducing organisms (Kondrashow 1988, 1994; Caetano-Anollés 1999). It is currently unknown how the accumulation of deleterious versus beneficial mutations (e.g. in the *pds* gene) might impact long term invasion success of clonal organisms.

Variation in ploidy levels has also been shown to increase levels of genetic diversity in clonally reproducing species and potentially contribute to their success in colonizing new habitats (Soltis and Soltis 2000; Eckert 2001; Van der Strate *et al*. 2002; Eckert *et al*. 2003; Chapman *et al*. 2004; Comai 2005; Paun *et al*. 2006; Paun and Horandl 2006). Studies using allozymes, chromosome counts and quantification of nuclear DNA content have indicated that diploid, triploid and tetraploid hydrilla can exist together in the same populations in the United States and in the native range (Cook and Lüönd 1982; Verkleij *et al*. 1983; Harlan *et al*. 1985; Langeland 1989, Langeland *et al*. 1992; Nakamura and Kadono 1993; Puri *et al*. 2007b). It is not known whether variation in ploidy levels in the United States developed after introduction or if the original introduction contained individuals with varying ploidy levels. Ploidy levels have been difficult to determine for hydrilla due to the presence of endoreduplication (resulting from chromosome replication without cytokinesis) (Langeland *et al*. 1992; Les *et al*. 1997; Puri *et al*. 2007b). Our study revealed evidence of triploid and tetraploid individuals and no evidence of diploid individuals in Florida or Texas. Although some of the putative triploid individuals may have been tetraploids in this study, triploidy appears to be the most common ploidy level in Texas and Florida as suggested by previous studies (Verkleij *et al.* 1983; Harlan *et al*. 1985; Langeland 1989; Puri *et al.* 2007b). One possibility for us not detecting diploid individuals is that some of our primer pairs may have actually amplified more than one locus. To check this possibility we cloned some of the microsatellite alleles and compared the flanking regions for individuals (results not presented). The flanking regions were highly similar to each other and the number of repeats was consistent with our scored alleles suggesting the multiple alleles were all part of the same locus. Polyploidy may confer an advantage to invasive hydrilla and so diploid individuals currently exist at very low levels in Florida and Texas.

The apparent absence of diploid individuals raises the question of whether the dioecious form of hydrilla in the United States is capable of sexual reproduction. Although the study of Steward (1993) suggests the dioecious form is fertile, the ploidy level of the particular individual that was used in these test crosses was unknown. The monoecious and dioecious forms are known to come into contact in Virginia and North Carolina and California (Davenport 1980; Yeo *et al.* 1984; Ryan *et al.* 1995). If successful sexual reproduction is possible, even at a low level, between the monoecious and dioecious forms it would increase levels of genetic diversity further and could remove some of the negative consequences of pure vegetative reproduction such as mutation accumulation. Further experimental crosses are needed to fully evaluate the ability of the dioecious form to reproduce successfully.

Our data suggest a single clone of the dioecious biotype of hydrilla introduced into Florida 50 years ago appears to have spread throughout Florida and the southern United States. Somatic mutation and variable ploidy levels appear to be the main reasons hydrilla has high variability at microsatellite loci. Our study also suggests that variable resistance levels to fluridone are not due to sexual recombination. Recent studies of invasive species have suggested that species are often introduced multiple times and that these introductions often have come from distinct source regions (Novak and March 2005; Roman and Darling 2007; Kolbe *et al*. 2007; Suarez and Tsutsui 2008). The subsequent hybridization between these introductions increases genetic diversity and the evolutionary potential of these populations making it more likely they will expand their range and develop resistance to chemical and biological control efforts. Invasive clonal species such as hydrilla may ultimately prove to be as adaptable as sexually reproducing species because they effectively produce their own variation through mutation and variable ploidy levels. Future studies should focus on genetic variation occurring

in coding regions to increase the understanding of the potential for somatic mutation to increase invasion success.

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ABSTRACT

EVIDENCE OF CLONAL REPRODUCTION AND HIGH GENETIC VARIATION IN THE EXOTIC WEED *HYDRILLA VERTICILLATA*

by
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Hydrilla verticillata is an invasive, aquatic weed that ranges from Florida to California. Hydrilla is believed to only spread vegetatively. Invasive clonal plants are expected to have low genetic variation limiting their ability to develop resistance to herbicides. Contrary to expectations, hydrilla has recently developed resistance to the herbicide used to control the plant. We used microsatellite loci to determine whether hydrilla is a single clone with low genetic diversity. We found high levels of genetic variation within populations of hydrilla. Spatial patterns of genetic diversity, patterns of genetic differences between individuals, and tests for sexual reproduction, indicate that hydrilla has originated from a single clone. Patterns of genetic diversity suggest that somatic mutations arising during vegetative growth are the main source of genetic diversity. Our study provides an example of how a clonally reproducing organism can potentially develop resistance to herbicides by effectively creating its own genetic diversity.