Genetic variation in the *pds* gene and its relation to fluridone resistance in *Hydrilla verticillata*

by

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Introduction

Invasive or non-native plant species have increased the problem of weed management in terrestrial and aquatic ecosystems. Early efforts to control weeds were limited to basic methods such as physical removal or destruction of plants roots (Tranel and Horvath 2009). In the mid-1900s the first synthetic herbicides were used for weed control. Today, herbicides are used on over 90% of the area of US crops totaling 87 million ha of cropland (Gianessi and Reigner 2007). Despite the continued use of herbicides, weeds still pose a significant problem in agriculture. Weedy and invasive species cost up to $100 billion dollars in crop losses and ecosystem damage each year (Pimentel et al. 2005).

Herbicide resistance has become a major problem in agriculture and management of invasive weedy species. Resistance to herbicides was first discovered in the common groundsel (*Senecio vulgaris*) to the herbicide triazine in 1968 (Ryan 1970). Currently, it has been reported that 358 biotypes of 197 angiosperm species worldwide have evolved resistance to herbicides (Heap 2011). In areas where herbicide use is persistent and widespread, resistance in populations can develop quickly.

The observed mechanisms by which weeds have evolved resistance can be grouped into two broad categories known as target-site resistance and non-target site resistance (Powels and Yu 2010). Target-site resistance describes changes to a target-site that limit the effectiveness of an herbicide that would normally be lethal. Non-target site resistance involves mechanisms that prevent the full dose of an herbicide from reaching a target-site (Powels and Yu 2010). Target-site resistance can include something as basic as a single point mutation. An example of this is the resistance of many weeds to the herbicide triazine which inhibits photosynthesis. A single point mutation in the chloroplastic psbA gene, which encodes the D1 protein of the photosystem

two (PSII) complex, results in a sharply decreased efficacy of the herbicide to compete with the substrate for binding on the D1 protein (Hirschberg et al. 1984). Another form of target-site resistance is an increase in copy number of the target gene. For instance, the herbicide glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that is involved in the shikimate pathway, a biosynthetic pathway that generates aromatic amino acids. The weed Palmer amaranth (*Amaranthus palmeri*) has evolved multiple copies of the EPSPS gene resulting in enough EPSPS production to allow the production of amino acids despite inhibition by glyphosate (Gaines et al. 2010). Non-target resistance is much less well understood and can involve Cytochrome P450 monooxygenases (P450s) that can better detoxify herbicides and mechanisms that reduce the rate of herbicide translocation in the plant (Werck-Reichhart et al. 2000, Siminszky 2006, Shaner 2009).

Hydrilla verticillata is an invasive aquatic weed that has a vast geographic range which currently extends to most continents (Cook and Lüönd 1982). Two hydrilla biotypes have been introduced to the United States on separate occasions (Madeira et al. 2000). During the 1950's, the dioecious biotype (male and female flowers on separate plants) was brought to Florida from Sri Lanka via the aquarium trade. Only female plants of the dioecious biotype have been found in the United States. The monoecious biotype (male and female flowers the same plant) was introduced to the United States in the 1970s from South Korea in the central Atlantic states (Madeira et al. 2000). Currently, the female dioecious form is predominant in the Southeastern U.S., while the monoecious form is found predominantly in the Northeastern and Middle Atlantic States and in Washington. Both biotypes have been found in California and they occur together in some Southeastern locations (Ryan et al. 1995, Michael Grodowitz pers comm.).

The main method of spread for hydrilla in its invasive range is through asexual, vegetative reproduction including regeneration from stem fragments, tubers, rhizomes, and specialized buds called turions (Arias et al. 2005). These structures can be dispersed by boats, water fowl, and water flow. Hydrilla can invade water bodies with a wide range of nutrient, pH, and salinity levels (Balciunas et al. 2002). Once hydrilla has become established in a new water body, the weed quickly forms dense, thick mats. These monocultures can negatively impact the economic and recreational value of water bodies and affect ecosystem dynamics (Balciunas et al. 2002, Arias et al. 2005).

The speed and efficiency of hydrilla dispersal and establishment has resulted in the development of mechanical, biological, and chemical control methods. The most cost-effective method currently in use is the herbicide, fluridone. Fluridone is effective against hydrilla in small doses, which are sub-lethal to native aquatic plants (Arias et al. 2005). Introduced in 1986, and used extensively in Florida water bodies since, fluridone is the only systemic herbicide approved by the EPA for use on large water bodies. Fluridone acts as a noncompetitive inhibitor of the enzyme phytoene desaturase that is coded by the *pds* gene in hydrilla (reviewed in Arias et al. 2006). Phytoene desaturase is located in the chloroplast and is important for the production of carotenoids. Carotenoids help protect chlorophyll from excess energy by capturing excess electrons that are released when chlorophyll returns to its ground state (Arias et al. 2006). When fluridone inhibits phytoene desaturase, carotenoids are no longer produced to protect the chlorophylls. The chlorophylls are destroyed and the plant tissue bleaches from photodegradation (Arias et al. 2006).

Fluridone resistance was first noticed in dioecious hydrilla about 14 years after the herbicide was introduced. Michel et al. (2004) discovered three point mutations in Arg304 of the

pds gene in populations of hydrilla with resistance to fluridone. The development of herbicide resistance was surprising given the lack of sexual reproduction in these populations and so these point mutations are likely due to somatic mutation (Michel et al. 2004). The sensitive codon is arginine (CGT), while the resistant codons include serine (AGT), cysteine (TGT) and histidine (CAT). These single base pair mutations were shown to confer low, intermediate, and high resistance to fluridone, respectively (Michel et al. 2004). There is evidence, however, that resistance may vary across a more continuous range than these three categories and may even vary between individuals with the same mutation (e.g. AGT; Puri et al. 2007a, Netherland unpub. data).

Dioecious hydrilla in the United States is believed to be predominantly triploid, although diploid and tetraploid individuals have been found (Langeland 1989, Langeland et al. 1992; Puri et al. 2007b). Triploid or tetraploid hydrilla could have up to three or four different alleles of the *pds* gene with mutations that confer resistance. Plants with multiple fluridone resistant alleles could potentially have increased resistance to fluridone and this may explain some of the variation in resistance. An increase in ploidy level has been observed to positively correlate with gene expression for some genes (Guo et al. 1996, Broz et al. 2009).

In this study, I describe the sequence diversity of the *pds* gene in hydrilla samples collected from five water bodies in Florida that vary in resistance from none to very high. I asked if the previously described amino acids found at codon 304 were correlated with the estimated fluridone resistance levels of each lake and whether there was evidence of other amino acid changes that might also be correlated with the observed levels of resistance. I also asked if there was evidence of multiple copies of the previously described mutant *pds* alleles within individual samples and whether this was associated with differences in fluridone resistance.

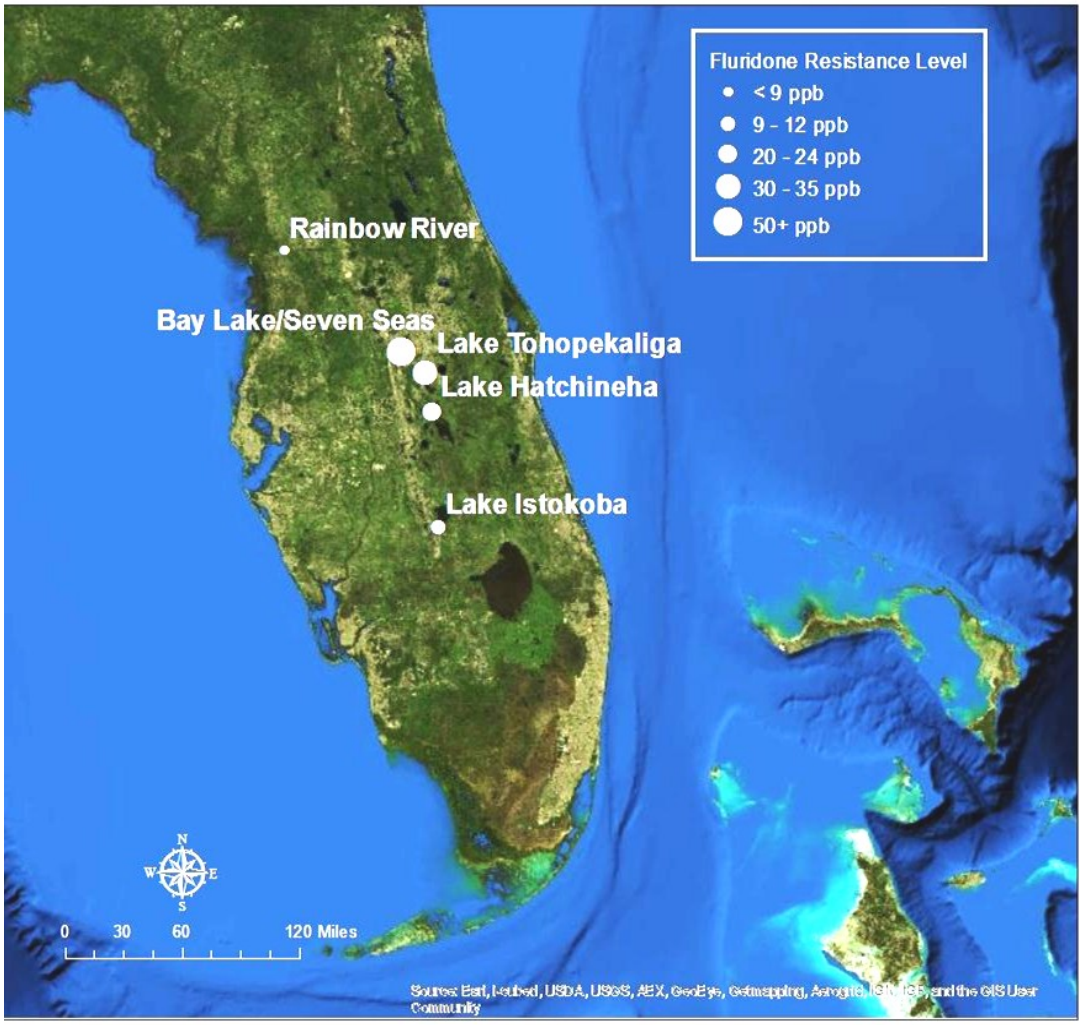


Figure 1. Sampling sites for hydrilla and their observed resistance to fluridone.

Materials and Methods

Sample Collections

Michael Netherland collected forty-nine hydrilla samples from five water bodies in Florida and stored them in silica gel. Samples were collected from Lake Hatchineha and Lake Tohopekaliga in December of 2008, and from Rainbow River, Lake Istokoba, and Bay Lake/Seven Seas in March of 2009 (Fig. 1). More detailed maps of individual sampling sites within each water body are listed in Appendix 1. The fluridone resistance values for Rainbow

River, Lake Istokoba, Lake Hatchineha, Lake Tohopekaliga, and Bay Lake/Seven Seas were < 9, 9-12, 20-24, 30-35, and 50+ ppb, respectively. All sites except Rainbow River had experienced fluridone applications in the past, but had not experienced herbicide treatments in the three years prior to collection for this study.

Genetic Analyses

DNA was extracted using the IBI Plant Genomic Mini Kit following the manufacturer's instructions. I amplified genomic DNA that included an approximately 970 bp region of the *pds* gene with introns and flanking codon 304. A custom designed "I_*pds*n1F" forward primer (5'-CAT ATG TTG AAG CTC AGG ATG G-3') and an "I_*pds*n1R" reverse primer (5'-GTA CGC CAA CCA ACT TGT CC-3') were used to amplify the target region. Polymerase chain reactions (PCR) (20 µL) contained 10-50 ng DNA, 0.2 µM of each primer, 1X Qiagen Multiplex PCR Master Mix with HotStar Taq, Multiplex PCR buffer with 3 mM $MgCl_2$ pH8.7, and dNTPs. Reactions were cycled on an ABI 2720 thermal cycler. The cycling parameters were one cycle at 94°C for 15 min, followed by 30 cycles of 30 s at 94°C, 15 s at 60°C, 1 min at 72°C, and one cycle at 72°C for 30 min. PCR products were gel purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA).

PCR products were cloned with the pGEM-T Easy Vector System (Promega USA). I sequenced 16 clones per sample using ABI Big Dye Terminator Cycle Sequencing v 3.1 chemistry (Applied Biosystems USA). Sequences were electrophoresed on an ABI 3130XL Genetic Analyzer (Applied Biosystems USA). Sequences were trimmed, edited, and contiged using Sequencher v. 5.0. Polymorphisms were checked by eye and then aligned in BioEdit (Hall 2004). Introns and exons were determined by matching sequences to a reference cDNA sequence from Genbank (GenBank AY639658.1) by Michel et al. 2004.

I used GenAlEx v6.3 (Peakall and Smouse 2006) to determine the number of alleles found in each sample for exons. There were initially 269 unique single sequences found in a single clone, 24 sequences found in two clones, and two unique sequences found in three clones. Six unique sequences were found both across individuals and water bodies. Haplotype networks constructed using TCS v 1.21 (Clement et al. 2000) revealed that the singleton sequences were usually one to two base pairs away from one of the six common sequences that occurred across water bodies. The singletons were arranged in a star-like pattern from these six alleles which were predicted by the networks to be the ancestral sequences. I therefore conservatively assumed that sequences that occurred only 1 – 3 times were due to *Taq* polymerase error which has been estimated to be 4×10^{-5} per nucleotide. This would mean I would expect on average ~1 error every two clone sequences of 473 bp of sequenced *pds* exon consistent with the large number of singletons I found (expected error rate per nucleotide = $4 \text{ X } 10^{-5}$ x 30 PCR cycles x 473 nucleotides). I then categorized each of the rare sequences (1-3 clones) as one of the six alleles based on their connections in the haplotype network.

Genetic Diversity

Because individual samples had from two to four different *pds* alleles, I converted their combination of alleles to a single allele combination for analyses. For instance, allele combinations 72/160/206, 86/206, and 86/129/206 would each be a different allele combination. I calculated haplotype frequencies and diversity (h) in GenAlEx. AMOVA estimates of pair-wise differentiation between water bodies (PhiPT) and a mantel test for isolation-by-distance were also performed in GenAlEx. Additionally, I calculated expected heterozygosity in GENODIVE 2.0b23 (Meirmans and Van Tienderen 2004) which can analyze polyploid data and data sets with mixed ploidy.

I used Fisher exact tests to determine if the number of individuals with herbicide resistance alleles in a water body differed between water bodies. I used Fisher exact tests to determine if the number of individuals with two versus three unique alleles in a water body differed between water bodies. I also calculated the proportion of clones that contained each allele in an individual then arcsine square root transformed the values before using a one-way ANOVA to determine if particular alleles were cloned more frequently than others within individuals.

Results

The sequenced region was 970 bp and contained 473 bp of *pds* exons and 497 bp of introns (Fig. 2). The *pds* gene was amplified from codon 256 to 398 (Michel et al. 2004, GenBank AY639658.1). Screening of 768 clones across 50 individuals and five water bodies

GCAGCCATATGTTGAAGCTCAGGATGGCTTAACAGTTCAAGAGTGGATGAGAAAACAGGTAAAATGCGATGC
TTTTCATCCTTGCAGTTAATCATCATCCAAGTAGAATTCCCAAGGACCTTTTCCCTGCAGGGCTCATCATTATAAGTA
TTGGGCTTACTTGGTTGTAATTTGCACTCTTTTTCTTAACTTTCTTAACAGGTTCTCTATTAATTGATCTGTACTACTA
TATACTTCAGGGTGTGCCGGATCGAGTCAATGACGAGGTTTTCATTGCAATGTCAAAGGCTCTTAACTTCATAAAC
CCTGATGAACTTTCCATGCAATGCATCCTGATTGCCTTAAAC**CGT**TTCCTTCAGGTTTACCTTCAATGGAAGTCAT
TTTTTGTTTTTTTTATGGAGCAATATCGTATGAGCACCATTAGTTGCAAAATGACCATATTCAAGTAAATATTCCAAA
TGCATTGACTTTTTAGTTTTTATTCGTGTTGATTATCAAAGGTCCAACATTAAACCATTATTTTTTTAGTACTTCCATT
TATTAAAAACTCCATCTTTTTGATTTCACCAGGAAAAGCATGGGTCGAAGATGGCCTTTTTAGATGGTAATCCACCT
GAAAGATTATGTAAGCCAATTGCTGATCACATCGAGTCATTGGGTGGCCAAGTCATCCTTAATTCCCGAATACAGA
AGATTGAGCTGAATGCAGACAAATCCGTCAAGCATTTTGTGCTCACCAATGGAAATATAATAACAGGAGATGCAT
ATGTATTTGCAACACCTGGTATGTTATGAACTATGTTTACATCTGTTATGTGATTTTTAGTACCTTTCCATTTTCTATT
AAGTCTTTGAGGCACAATATACTGTGATGTTTTCTTTGCATCAGTTGATATCTTGAAGCTTCTGTTACCTGAAGATT
GGAAGGAGATTTCATATTTCAAAAAATT**GGACAAGTTGGTTGGCGTAC**

Figure 2. A 970 bp portion of the *pds* gene with introns in hydrilla. The shaded areas represent exons and the unshaded areas are introns. Primer sites are in bold, and the mutation site conferring herbicide resistance is in larger bold font.

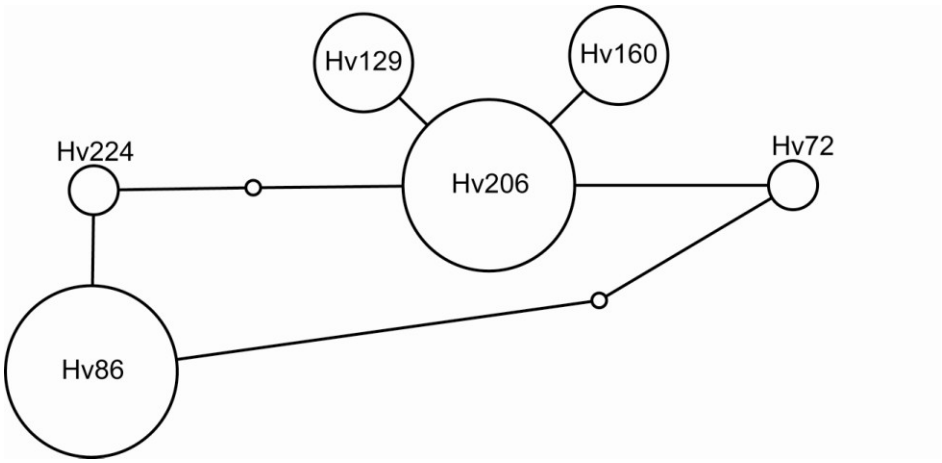


Figure 3. Haplotype network of six *pds* alleles. The susceptible codon (CGT) occurred in alleles 72, 86, 206, and 224. The low resistance mutation AGT occurred in allele 129 and the high resistance mutation CAT occurred in allele 160. The relative sizes of the circles represent the number of individuals that had that particular allele and each line represents a one base pair difference.

revealed six different *pds* alleles. The alleles were from 1 to 4 bp different from one another (Fig. 3). Amino acid polymorphisms were found at three different codon positions. Two amino acid changes were found at codon 304, the site of interest for mutations conferring fluridone resistance. Allele 129 had the AGT (serine) mutation, whereas allele 160 had the CAT (histidine) mutation. I did not find the TGT resistance mutation. Additionally, alleles 86 and 224 both had an Ala-Asp substitution at codon 331 and an Ile-Val substitution at codon 341.

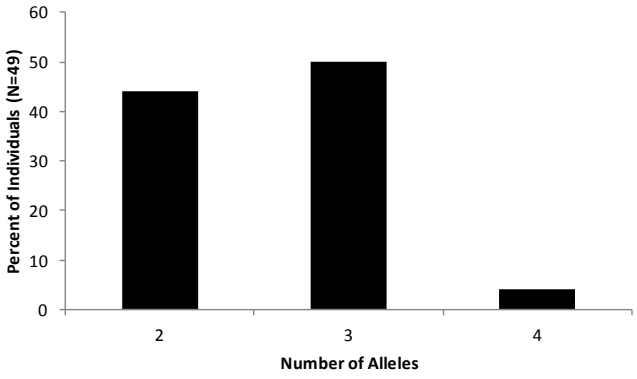


Figure 4. The percent of individuals (N = 49) containing from two to four different *pds* alleles.

Sampled individuals across water bodies had two to four different *pds* alleles. A majority of individuals had either two or three alleles accounting for 44% and 50% of individuals, respectively (Fig. 4). Lakes Tohopekaliga and Hatchineha had a higher frequency of individuals with three alleles, whereas Lake Istokoba, Bay Lake/Seven Seas, and Rainbow River had more individuals with two alleles (Fisher exact test p = 0.0004). Only two individuals (Hv 201, RCID 2-3) had four different alleles.

There was a total of 10 different allele combinations of the six alleles found in individuals (Table 1). All but two allele combinations had one resistance allele. A, B, D, and E were the most common allele combinations (Table 1, Fig. 5). The five water bodies

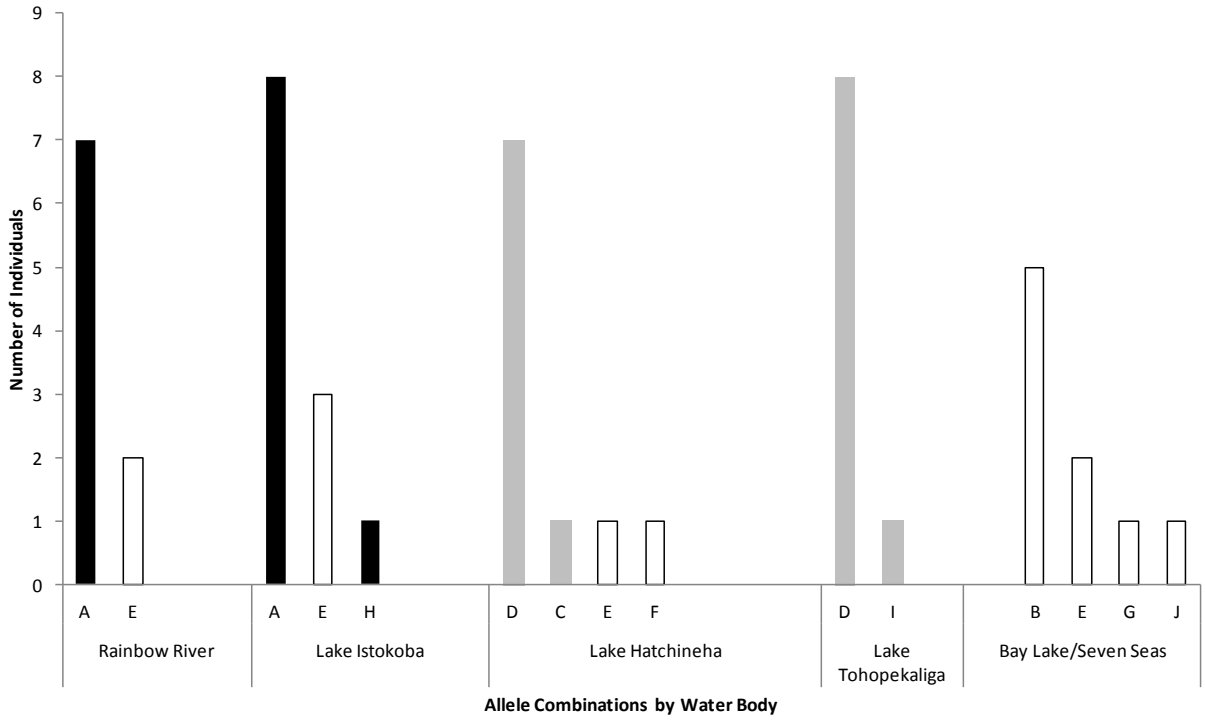


Figure 5. The number of individuals with each allele combination across water bodies. From left to right, the water bodies go from no resistance to very high resistance. Black bars contain only susceptible alleles. Grey bars contain low resistance allele 129. White bars contain the high resistance allele 160.

Table 1. Allele combinations found in individuals across five Florida water bodies.

Combination	Allele	Resistance Mutation	Number of Individuals
A	86, 206	None	16
B	86, 160	CAT	5
C	86, 129	AGT	1
D	86, 129, 206	AGT	14
E	86, 160, 206	CAT	7
F	160, 206, 224	CAT	1
G	72, 86, 160	CAT	1
H	86, 206, 224	None	1
I	72, 86, 129, 206	AGT	1
J	72, 86, 160, 224	CAT	1

ranged from having two to four different allele combinations. Combination A was most common in Rainbow River and Lake Istokoba, whereas combination D was most common in Lake Hatchineha and Lake Tohopekaliga (Fig. 5). A map of Florida with bar graphs of the number of individuals with each allele combination at each water body is in Appendix 3.

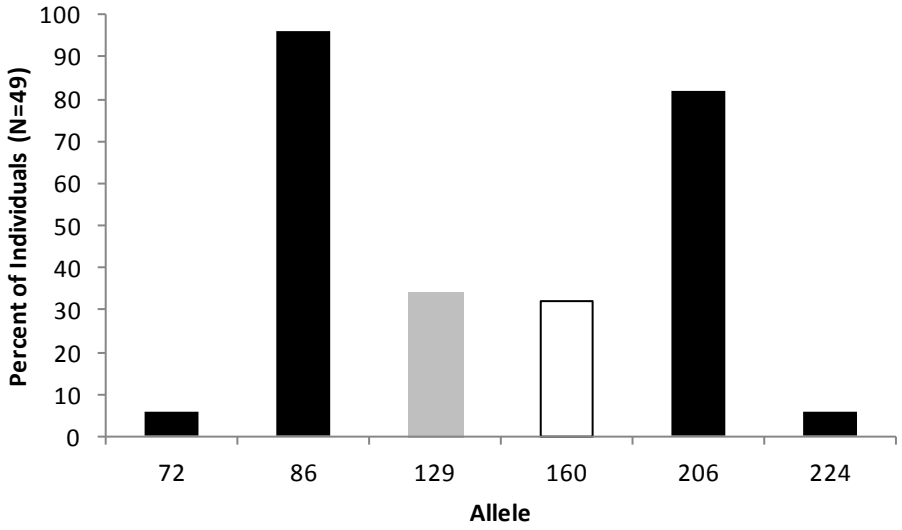


Figure 6. Percent of individuals from five Florida water bodies containing six *pds* alleles. The grey bar contains the low resistance mutation and the white bar contains the high resistance mutation.

The overall diversity (h) of allele combinations was 0.129 (SE = 0.030). Diversities (h) for Rainbow River, Lake Istokoba, Lake Hatchineha, Lake Tohopekaliga, and Bay Lake/Seven Seas were 0.065, 0.096, 0.244, 0.037, and 0.204, respectively. Expected heterozygosity (H_s) overall was 0.693 and within water bodies ranged from 0.602 to 0.771.

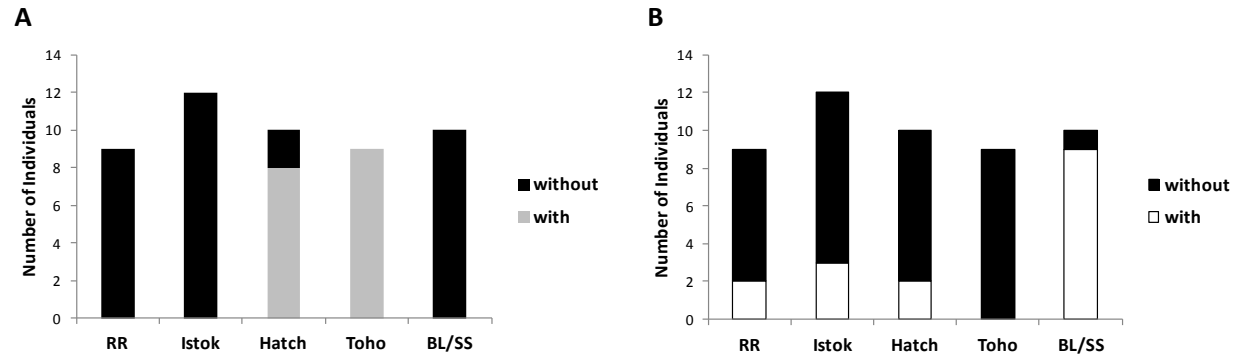


Figure 7. A) The number of individuals from each water body with and without the low resistance AGT mutation present. B) The number of individuals from each lake with and without the high resistance CAT mutation present.

Alleles 86 and 206 were the most common alleles and were found in 96% and 84% of the individuals, respectively (Fig. 6). Allele 206 was found across all water bodies, but appeared in lowest frequency in Bay Lake/Seven Seas with only 3 of 10 individuals containing the allele (Appendix 2). Allele 129, containing the AGT resistance mutation, was found in all individuals from Lake Tohopekaliga and most (78%) of the individuals from Lake Hatchineha and none of the individuals from the other water bodies (Fisher exact test, p = 0.001) (Fig. 7A). Allele 160, containing the CAT resistance mutation, was present in all but one individual in Bay Lake/Seven Seas and 2-3 individuals in the other water bodies except Lake Tohopekaliga (Fisher exact test, p = 0.0002) (Figure 7B). Alleles 72 and 224 occurred at the lowest frequencies across water bodies.

Table 2. Pairwise population PhiPT values. PhiPT values are below diagonal and p-values are above diagonal.

	Toho	Hatch	Istok	BL/SS	RR
Toho	-	0.184	0.001	0.001	0.001
Hatch	0.063	-	0.001	0.001	0.001
Istok	0.709	0.382	-	0.001	0.311
BL/SS	0.749	0.516	0.502	-	0.002
RR	0.771	0.384	0.000	0.523	-

Allele 72 was found in only one individual in Lake Tohopekaliga and two individuals in Bay Lake/Seven Seas, while allele 224 was found in one individual each in Lake Hatchineha, Lake Istokoba, and Bay Lake/Seven Seas (Appendix 2).

AMOVA revealed that 52% (PhiPT = 0.521) of the genetic variance in haplotypes was partitioned among the five water bodies and 48% was found among individuals within water bodies (p = 0.001 in both cases). Pairwise population PhiPT values ranged from 0.000 to 0.771 (Table 2).

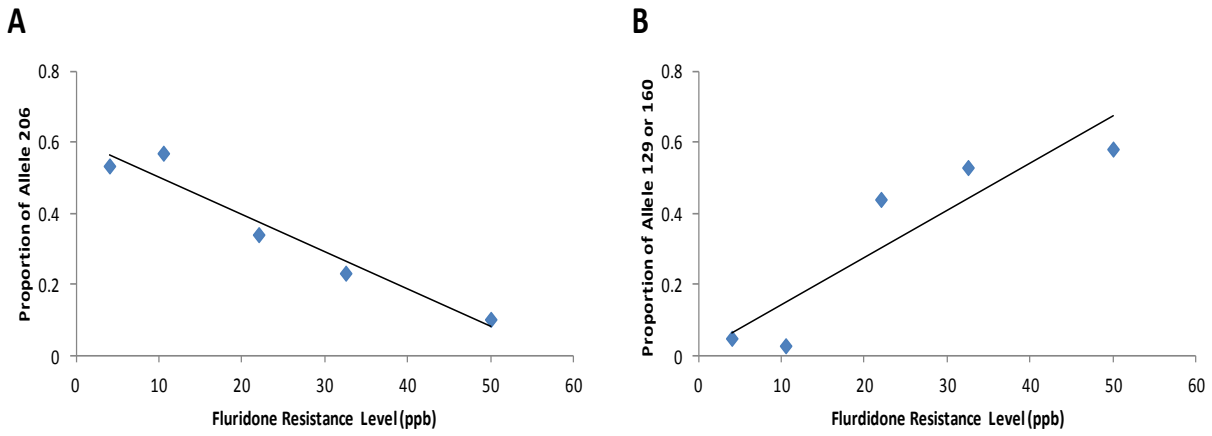


Figure 8. Regression analysis of observed resistance to fluridone versus A) the average proportion of individuals with allele 206 (y = -0.011x+0.061, R^2=0.94, F=49.03, df = 1, p-value = 0.006); B) average proportion of individuals with allele 129 or 160 (y=0.013x+0.01, R^2 = 0.82, F = 14.05, df = 1, p-value = 0.03).

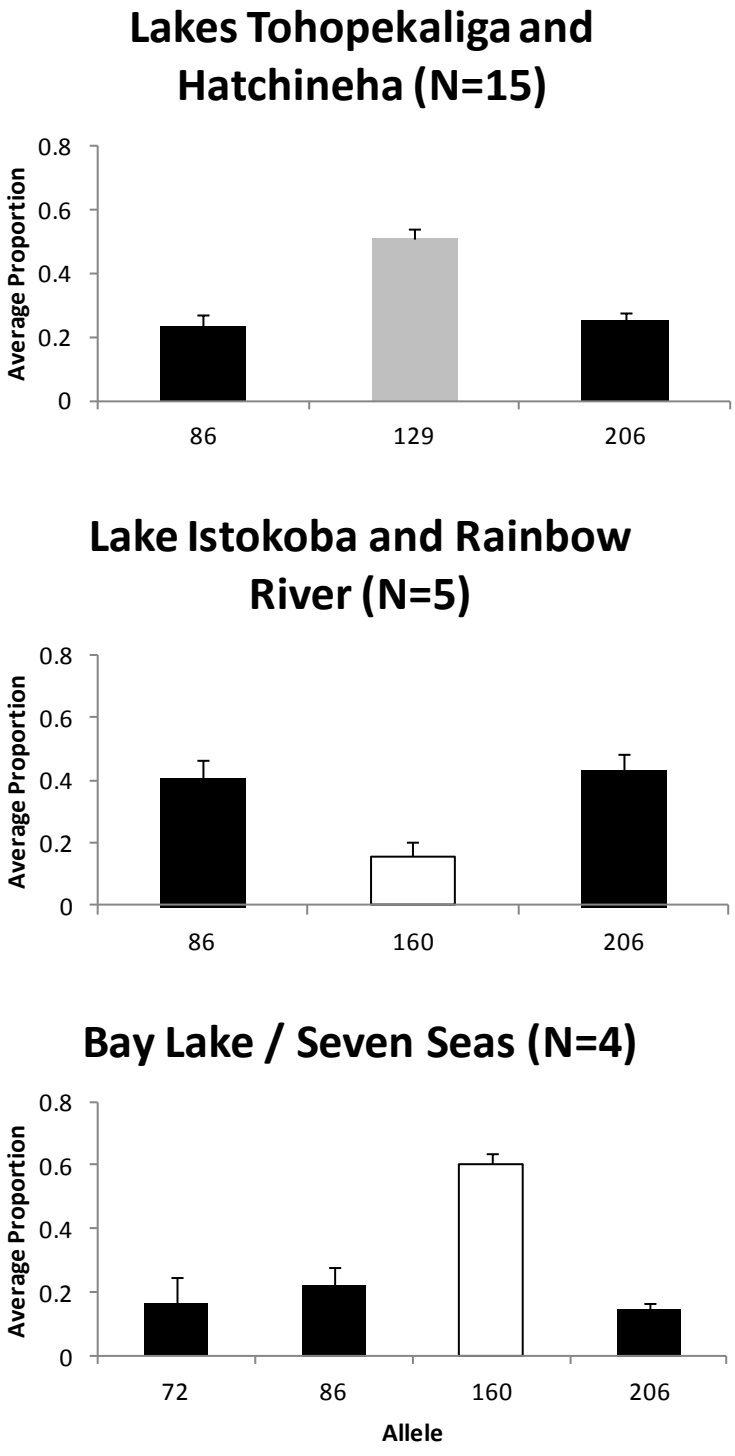


Figure 9. The average proportion of clones containing each pds allele within individuals. All individuals from each water body had three alleles. Individuals in Bay Lake/Seven Seas had alleles 86 and 160 and either allele 72 or allele 206. Alleles containing the AGT and CAT mutation have grey and white bars, respectively.

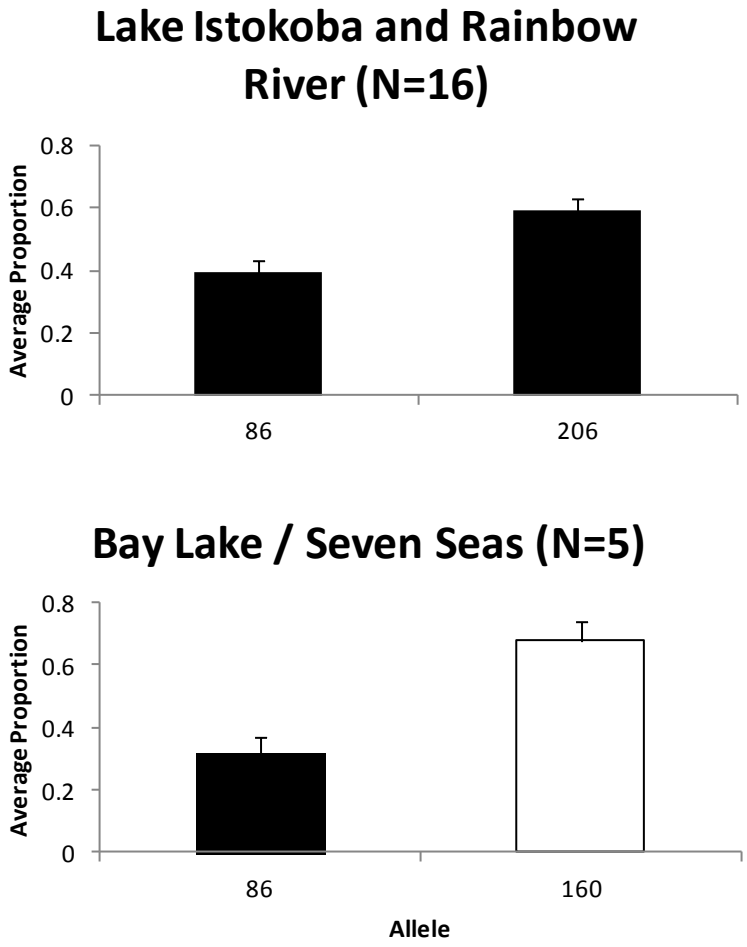


Figure 10. The average proportion of clones with each allele within individuals. All individuals from each lake had two alleles.

Only two pairwise population PhiPT values (Lake Tohopekaliga and Hatchineha and Lake Istokoba and Rainbow River) were not significantly different from zero (Table 2). There was no relationship between geographic distance and genetic differentiation between water bodies (Mantel test, $r = -0.31$, $p = 0.19$).

There was no significant correlation between Hs or diversity (h) and observed resistance to fluridone ($r = 0.62$ and 0.58, respectively). There was a strong negative relationship between the average proportion of allele 206 and observed resistance to fluridone and a strong positive

relationship between observed resistance to fluridone and the proportion of individuals that had either allele 129 or 160 (Fig. 8A and 8B).

A resistant allele (129 or 160) was cloned more often than the other two alleles from individuals with three different alleles in Lake Tohopekaliga combined with individuals from Lake Hatchineha, and Bay Lake/Seven Seas (ANOVA $F_{2, 42} = 3.22$, $p = 3.9 \times 10^{-7}$; $F_{3, 12} = 27.14$, $P = 1.2 \times 10^{-5}$, respectively) (Fig. 9). The resistance allele 160 was cloned with a lower frequency in Lake Istokoba and Rainbow River than alleles 86 and 206 (ANOVA $F_{2, 12} = 3.89$, $p = 0.0038$). There was a trend for the resistance allele (160) to be cloned more frequently in Bay Lake/Seven Seas individuals with two different alleles ($t = 1.99$, $df = 10$, $p = 0.074$) (Fig. 10). Allele 206 was more common in individuals with two different alleles from Rainbow River and Lake Istokoba ($t = 3.78$, $df = 30$, $p = 0.0007$) (Fig. 10).

Discussion

Michel et al. (2004) described three mutations conferring low, medium, and high resistance. In this study, I found two of the resistance mutations previously described by Michel et al. (2004) and their presence was consistent with the observed levels of fluridone resistance. The AGT mutation was found only in Lakes Tohopekaliga and Hatchineha, which displayed higher resistance levels compared to Rainbow River and Lake Istokoba. The high resistance CAT mutation was found in greatest frequency in Bay Lake/Seven Seas, which had the highest observed fluridone resistance compared to the other water bodies. The CAT mutation was also the most widespread mutation and was found in four of the five water bodies. There was a strong positive relationship between having either the AGT or CAT resistance alleles and observed fluridone resistance. There was also a strong negative relationship with having one of the most common susceptible alleles (206) and observed fluridone resistance. This pattern is

consistent with the results of the haplotype network which suggests that alleles 129 and 160 both arose as mutations from allele 206.

The AGT and CAT mutations were always found on the same alleles (129 or 160) but were not always found with the same combination of other alleles. Given the clonal nature of reproduction in hydrilla, the presence of resistance alleles in these different allele combinations suggests that multiple independent mutations have taken place. Previous authors have suggested that the dioecious form of hydrilla may have a high rate of mutation at codon 304, although the mechanism resulting in this high mutation rate is unknown (Arias et al. 2005).

There was not the expected negative correlation between resistance level and either haplotype diversity or heterozygosity. This was surprising in light of the fact that hydrilla spreads clonally which would make all genetic variation in an individual tightly linked. Strong selection by a herbicide, would be expected to result in a single resistant type within a lake. The lack of a relationship may have been due to the fact that diversity of allele combinations was generally low and did not vary widely between the water bodies. Nevertheless, the most resistant lake, Bay Lake/Seven Seas, had the highest diversity, while Rainbow River which is susceptible to fluridone and has not had a history of fluridone treatments had the second lowest genetic diversity. Interestingly, all four of the allele combinations in Bay Lake/Seven Seas had the highest resistance allele (CAT). Presumably these were all due to independent mutations from standing genetic variation and they have persisted in the absence of further herbicide use. Mutations at codon 304 appear to be stable and persist long after fluridone use has ceased (Puri et al. 2006). Two of the allele combinations from Bay Lake/Seven Seas were also found in other water bodies at low frequencies suggesting dispersal of the high resistance allele to other water bodies.

Patterns of genetic differentiation between water bodies were roughly concordant with similarities in resistance and not with the geographic distance between water bodies suggesting that differentiation may in part be due to differences in herbicide selective regimes. For instance, Lakes Tohopekaliga versus Hatchineha and Lake Istokoba versus Rainbow River which have similar levels of resistance were not significantly different from each other (PhiPT = 0.06 and 0.0, respectively), while water bodies that differed greatly in resistance were significantly differentiated from each other (e.g. Bay Lake/Seven Seas vs all other water bodies, Table 2).

Although the broad patterns found in this study are consistent with what is known about the Arg304 mutations and fluridone resistance it is not clear why Lakes Tohopekaliga and Hatchineha differ in resistance, or why Lake Istokoba has a higher level of resistance than Rainbow River. Most sampled individuals in both Lakes Tohopekaliga and Hatchineha have the AGT mutation, and Lake Istokoba and Rainbow River genotype composition is virtually identical. Puri et al. (2007a) described a similar situation in which two hydrilla biotypes with the AGT mutation differed in their resistance to fluridone. These results suggest that factors other than mutations at Arg304 may also confer resistance. The two other amino acid changes found in this study were on alleles 86 and 224 and were not correlated with observed resistance levels; allele 86 occurred in virtually all samples and allele 224 was not associated with any particular level of resistance. Since I only sequenced a portion of the *pds* gene, there may have been other mutations that were not detected in this study. Previous studies by Michel et al. (2000) and Puri et al. (2007a) found the amino acid changes described here as well as other mutations in the full length *pds* gene, but none of these mutations was clearly associated with fluridone resistance.

Ploidy level and gene duplication could potentially contribute to an individual's resistance level. A previous study suggests that most hydrilla in these five water bodies are

triploid since all samples contained three alleles at multiple microsatellite loci (Grajczyk 2009). The ploidy level of individuals is still uncertain; however, and needs to be corroborated through chromosome counts or flow cytometry (e.g. Puri et al. 2007b). Most of the individuals in this study had 3-4 different *pds* alleles, but a fair number (44%) only had two different alleles. The two water bodies with the lowest levels of resistance were predominantly composed of samples with two different *pds* alleles, whereas samples from the intermediate levels of resistance almost always had three different alleles. Bay Lake/Seven Seas had the highest resistance and samples often had only two different alleles one of which had the CAT mutation.

Certain alleles were cloned more frequently across individual samples in a water body. If each allele occurs only once in an individual I would expect that each allele would be cloned about equally when comparing across multiple samples. PCR amplification bias for particular alleles might explain these results; however; this explanation seems unlikely since for instance, allele 160 was found in higher frequency in Bay Lake/Seven Seas and at a lower frequency in Lake Istokoba/Rainbow River. The nucleotide composition of the different alleles is also very similar with identical priming sites and so it seems unlikely that a particular allele would be preferentially amplified or cloned across multiple samples. Another possibility is that alleles vary in their copy number either as a result of polyploidy or duplication events. Alleles that occurred multiple times in an individual should be amplified in higher numbers and so should then be cloned more often.

In Lake Istokoba and Rainbow River, allele 206 was cloned significantly more than allele 86 in individuals with these two alleles. This pattern suggests there may be multiple copies of allele 206 consistent with these individuals being triploid. Interestingly, resistance alleles were cloned at higher frequencies than the susceptible alleles in water bodies with intermediate and

high resistance to fluridone. Samples from Bay Lake/Seven Seas that had two different alleles had the CAT (allele 160) allele at two times the frequency of the wild-type allele. If these individuals are triploid then this would suggest there are two CAT alleles in these individuals.

Individuals with three different alleles in Lake Tohopekaliga/Lake Hatchineha, and Bay Lake/Seven Seas all had resistance alleles (alleles 129 or 160) cloned significantly more often than the other two alleles present. This may mean that either these samples are tetraploid with two copies of the resistance allele or alternatively that the *pds* gene has been amplified in triploid individuals. Tetraploid hydrilla is thought to be rare, although in-depth ploidy studies of populations have not been conducted in the introduced range (Langeland 1989, Langeland et al. 1992; Puri et al. 2007b). A study of glyphosate resistance in the weed Palmer amaranth (*Amaranthus palmeri*) has shown that weeds can evolve multiple copies of target genes to allow normal gene functioning despite the presence of herbicides (Gaines et. al 2010). This phenomenon has also been described in the mosquitoe *Culex pipens* which has evolved resistance to organophosphate insecticides via gene amplification, resulting in esterase overproduction (Lenormand et al. 1998).

Lake Istokoba/Rainbow River had the CAT resistance allele occur at a significantly lower frequency than the other two wild-type alleles. This pattern is more difficult to explain since there are three alleles and two of them occur at similar frequencies. Both of the susceptible alleles (86 and 206) occur at about two times the frequency of the resistant allele 160, which might suggest that these alleles have been duplicated in Lake Istokoba and Rainbow River.

If hydrilla has multiple copies of the *pds* resistance alleles, as suggested by this study, then there may be implications for the observed levels of resistance found in hydrilla. For instance, even though the AGT mutation confers a low level of resistance, having multiple copies

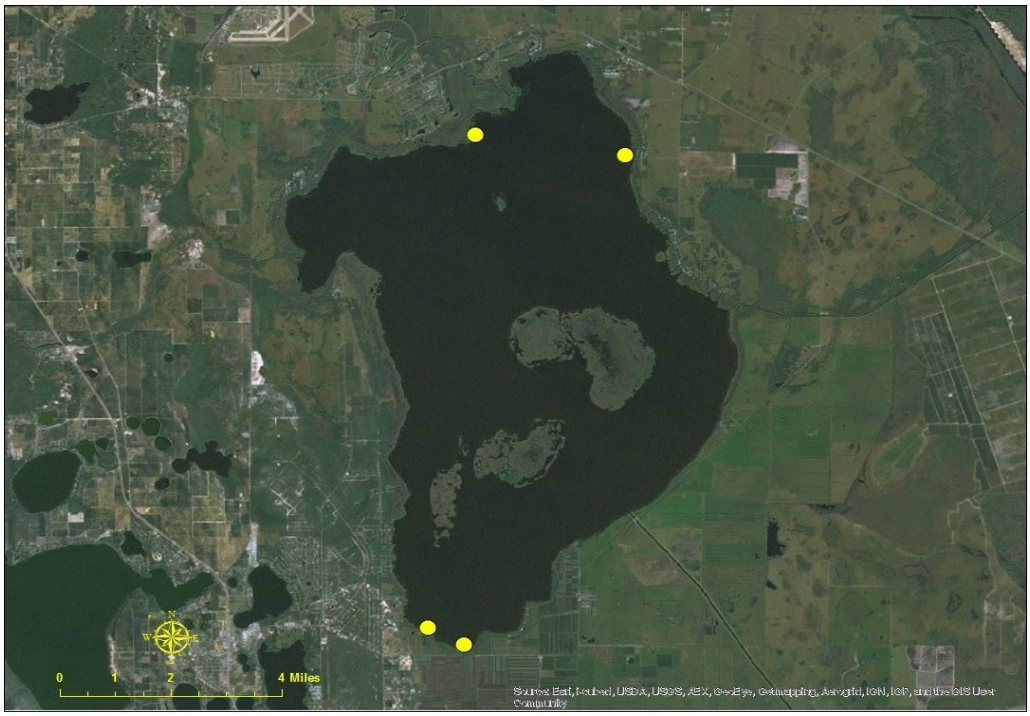
of this allele in Lakes Tohopekaliga and Hatchineha may confer a higher level of resistance than simply containing one resistance allele. This may explain why both of these water bodies have an intermediate level of resistance even though they do not contain the intermediate resistance mutation TGT at codon 304. Similarly, having multiple copies of the CAT allele may result in very high resistance in Bay Lake/Seven Seas. These results do not however, explain the differences in resistance between Lakes Tohopekaliga and Hatchineha or between Rainbow River and Lake Istokoba.

Future studies using ploidy determination and qPCR should be conducted to confirm the results from this study and to determine relative copy number of the different *pds* alleles. If individual plants do in fact have multiple resistance alleles then plants differing in copy number should be tested for resistance to fluridone to determine if increased copy number confers higher reistance. An emerging pattern in herbicide resistance is that weeds often have multiple avenues of resistance (Powels and Yu 2010). This study also suggests that resistance to fluridone in hydrilla is probably due to multiple factors including mutations at the Arg304 site and possibly copy number of resistance alleles, due either to ploidy differences or to gene duplication events. Other possibilities not addressed in this study could include changes in the promoter regions of the *pds* gene, or non-target site resistance factors that are limiting the amount of fluridone that reaches the *pds* gene, or epigenetic factors that affect gene regulation (Puri et al. 2006, 2007a, Vaillant and Paszkowski 2007).

Appendix 1. Maps of hydrilla sampling areas in five Florida water bodies. Yellow dots represent sampling areas.

Rainbow River

Lake Istokoba



Lake Hatchineha

Lake Tohopekaliga

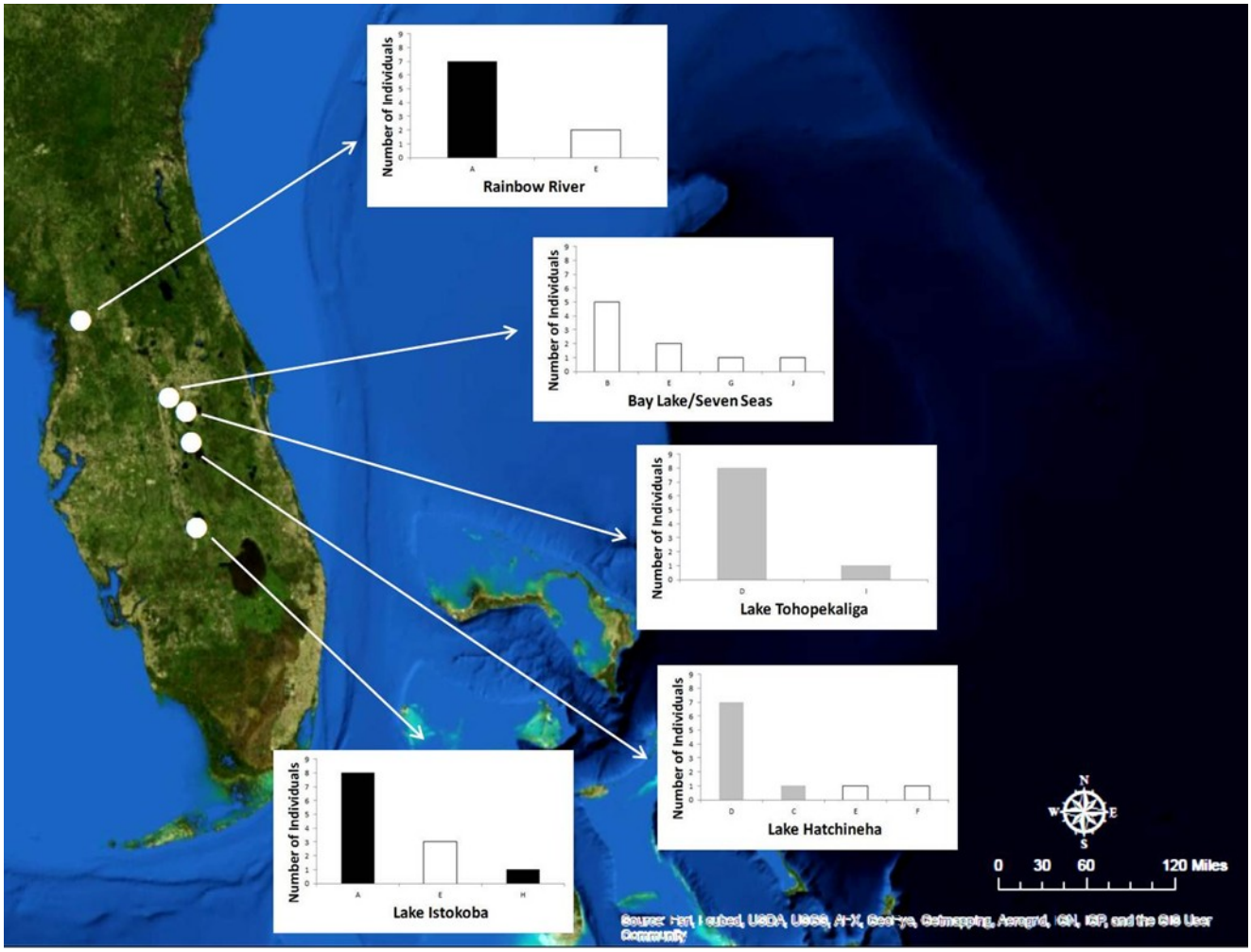
Bay Lake / Seven Seas

Appendix 2. The presence (1) or absence (0) of six alleles in individuals from five Florida water bodies and the total number of clones sequenced for each individual.

Individual	Lake	72	86	129	160	206	224	Total unique alleles	Number of clones
Hv190	Toho	0	1	1	0	1	0	3	15
Hv191	Toho	0	1	1	0	1	0	3	16
Hv193	Toho	0	1	1	0	1	0	3	20
Hv194	Toho	0	1	1	0	1	0	3	9
Hv197	Toho	0	1	1	0	1	0	3	8
Hv198	Toho	0	1	1	0	1	0	3	14
Hv199	Toho	0	1	1	0	1	0	3	15
Hv200	Toho	0	1	1	0	1	0	3	16
Hv201	Toho	1	1	1	0	1	0	4	16
Hv202	Hatch	0	1	1	0	1	0	3	22
Hv203	Hatch	0	1	1	0	0	0	2	10
Hv204	Hatch	0	1	1	0	1	0	3	17
Hv206	Hatch	0	1	1	0	1	0	3	11
Hv207	Hatch	0	1	1	0	1	0	3	14
Hv208	Hatch	0	0	0	1	1	1	3	7
Hv210	Hatch	0	1	1	0	1	0	3	10
Hv212	Hatch	0	1	1	0	1	0	3	13
Hv213	Hatch	0	1	0	1	1	0	3	11
IST11	Istok	0	1	0	1	1	0	3	15
IST12	Istok	0	1	0	0	1	0	2	13
IST13	Istok	0	1	0	0	1	0	2	11
IST21	Istok	0	1	0	0	1	0	2	14
IST22	Istok	0	1	0	1	1	0	3	14
IST23	Istok	0	1	0	0	1	0	2	16
IST31	Istok	0	1	0	1	1	0	3	34
IST32	Istok	0	1	0	0	1	0	2	16
IST33	Istok	0	1	0	0	1	0	2	12
IST41	Istok	0	1	0	0	1	1	3	20
IST42	Istok	0	1	0	0	1	0	2	20
IST43	Istok	0	1	0	0	1	0	2	11
RCID11	BL/SS	1	1	0	1	0	0	3	12
RCID12	BL/SS	0	1	0	1	0	0	2	12
RCID13	BL/SS	0	1	0	1	1	0	3	16
RCID21	BL/SS	0	1	0	1	0	0	2	15
RCID23	BL/SS	1	1	0	1	0	1	4	16
RCID31	BL/SS	0	1	0	0	1	0	2	15
RCID32	BL/SS	0	1	0	1	0	0	2	16
RCID33	BL/SS	0	1	0	1	0	0	2	16

RCID41	BL/SS	0	1	0	1	1	0	3	12
RCID43	BL/SS	0	1	0	1	0	0	2	8
RR11	RR	0	1	0	0	1	0	2	15
RR12	RR	0	1	0	0	1	0	2	14
RR21	RR	0	1	0	1	1	0	3	30
RR22	RR	0	1	0	0	1	0	2	16
RR31	RR	0	1	0	0	1	0	2	12
RR32	RR	0	1	0	0	1	0	2	14
RR41	RR	0	1	0	0	1	0	2	13
RR42	RR	0	1	0	0	1	0	2	9
RR43	RR	0	1	0	1	1	0	3	7

Appendix 3. Map of Florida sampling sites with bar graphs of the number of individuals with each allele combination found in each water body.



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VITA

Daniel Edward Tallent was born on December 10, 1985 in Seoul, South Korea. He is the son of Colleen Anna Wall and Frederick Edward Tallent and brother of Hana Marie Tallent. Daniel is a 2004 graduate of Elk Grove High School, Elk Grove, Illinois and he received a Bachelor of Arts with a degree in biology from the University of Texas at Arlington, Arlington, Texas in 2008.

In August 2010 he enrolled in graduate study at Texas Christian University, where he received his Master of Science in biology in 2012. During that time Daniel held a Teaching Assistantship while working on his master's degree.

ABSTRACT

GENETIC VARIATION IN THE *PDS* GENE AND ITS RELATION TO FLURIDONE RESISTANCE IN *HYDRILLA VERTICILLATA*

by
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Hydrilla verticillata is an invasive aquatic weed in the United States that has recently developed resistance to the herbicide fluridone due to point mutations in the *pds* gene. I amplified and sequenced the *pds* gene of 49 hydrilla samples from five Florida water bodies that vary in resistance to fluridone from none to very high. Genetic diversity within individuals was high with most individuals having 2-3 different *pds* alleles. I found two previously described resistance alleles and their occurrence across water bodies was broadly consistent with the observed levels of fluridone resistance. Fluridone resistance alleles were also cloned at higher frequencies within individuals than the susceptible alleles in water bodies with intermediate and high resistance to fluridone suggesting there are multiple copies of these alleles. Our study suggests that point mutations and allele copy number may both be responsible for fluridone resistance in hydrilla.