EMPLOYING A NEW TESTING METHOD FOR IDENTIFYING FLURIDONE RESISTANCE IN *HYDRILLA VERTICILLATA*

by

ULYSSES SEBASTIAN OLES

Bachelor of Arts, 2021

Texas Christian University

Fort Worth, Texas

Submitted to the Graduate Faculty of the College of Science and Engineering Texas Christian University in partial fulfillment of the requirements for the degree of

Master of Science

2024

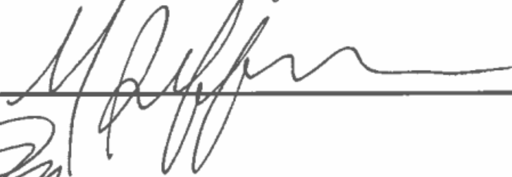
EMPLOYING A NEW TESTING METHOD FOR IDENTIFYING FLURIDONE RESISTANCE IN HYDRILLA VERTICILLATA

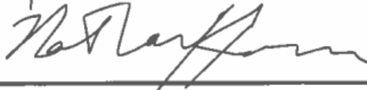
By

ULYSSES SEBASTIAN OLES

Thesis approved:

Major Professor





For the College of Science and Engineering

ACKNOWLEDGEMENTS

I would like to thank Dr. Dean Williams for his guidance on this project and for the initial method suggestions. I would like to thank Dr. Matthew Hale for his insight regarding the genetics components of this project, as well as for being generally supportive and for allowing me to use his lab and equipment. I would like to thank Dr. Marlo Jeffries for providing me with valuable information in the field of ecotoxicology and academic guidance. I would like to thank Dr. Nathan Harms for making the time to serve on my committee, for bringing indispensable hydrilla knowledge to the project, and for being constantly willing to connect me to useful resources.

I would like to thank my fellow current and former graduate students for their help making this project possible. I would like to thank Bridey Brown for guidance throughout the project and for making the process of learning DMAS infinitely easier. I would like to thank Kira Gangbin for assistance with DNA extractions and for being constantly supportive throughout the project. I would like to thank Evan Barfuss, whose previous research and willingness to teach made this project possible.

I would also like to thank my family for their unwavering support. My mother, father, brother, and sister were willing to sit through more practice presentations and weed science conversations than anyone should ever have to.

TABLE OF CONTENTS

Acknowledgements	ii
List of Figures	iv
List of Tables	v
Introduction	6
Methods	10
Results	14
Discussion	17
References	20
Vita	
Abstract	

LIST OF FIGURES

1. **Figure 1.** Location of tested samples 11

2. **Figure 2.** Average Cq scores for every sample run with the wildtype AGT 14

3. **Figure 3.** Average Cq scores for every sample run with the wildtype and CAT 15

4. **Figure 4.** Location of hydrilla with wildtype and fluridone resistant mutants 17

LIST OF TABLES

1. **Table 1.** The list of primer pairs designed and tested 24

Introduction

Invasive aquatic plants are a serious issue for recreational and commercial freshwater use in the United States. Plants introduced to a new environment occasionally establish and reproduce successfully in spite of the challenges that come from being in an unfamiliar place (Mack et al. 2000). This is often made possible by a lack of predators or other limiting environmental factors and availability of resources that equals or surpasses that of the native range. The presence of a new plant species can decrease native wildlife (Gerber et al. 2008), outcompete native plants, and even fundamentally change nutrient cycles (Havel et al. 2015). Beyond the negative impacts to nature, human recreational and commercial activities can be disrupted. Aquatic plant infestations can reduce the functionality of a body of water as a natural resource and impact the aesthetic appeal by overtaking the water and causing die offs and fish kills. The negative impacts of invasive aquatic plants cost billions of dollars in damages globally. (Cuthbert et al. 2021).

Hydrilla (*Hydrilla verticillata* (L.f.) Royle) is native to Asia and Asia (Cook and Lüönd 1982). Three distinct subspecies of hydrilla (Tippery 2023) have been introduced into the U.S. The dioecious subspecies was introduced into Florida through the aquarium trade in 1959 from Sri Lanka (Madeira et al. 2000; Madeira et al. 2004; Schmitz 1990). This subspecies spread throughout the lower latitudes of the US since cold temperatures have a strong negative impact on its vegetative growth (Chen et al. 1994; Van et al. 1978). A closely related monoecious subspecies was introduced into the Potomac River in 1976 and probably originated in South Korea (Madeira et al. 2000; Madeira et al. 2004; Steward et al. 1984). Monoecious hydrilla are found in colder environments than the dioecious biotype and are similarly spread in more northern areas of the U.S. (Madeira et al. 2004; Maki and Calatowitsch 2008). In 2016, a new introduction of hydrilla was found in the Connecticut

River (Tippery et al. 2020). This hydrilla belongs to a separate genetic clade than the previous two introductions and is found in northern areas of China in the native range (Harms et al. 2021; Tippery et al. 2020; Zhu et al. 2015). This subspecies may pose a risk of spreading to areas previously thought inaccessible in North America like parts of Canada due to it occupying a native range much colder than the other subspecies in North America (Harms et al. 2021).

Hydrilla invasions pose a serious risk to waterways due to ecosystem disruption and infrastructural concerns (Balciunas et al. 2002). Hydrilla forms a densely woven canopy near the surface of the water when left unchecked, and these mats are capable of impeding watercraft and getting caught up in the propellers of boats. Additionally, hydrilla outcompetes other plants in the water and can become so dense that it renders waterways hypoxic or limits the presence of large fish and reduces biodiversity in the ecosystem (Bates and Smith 1994; Bradshaw et al. 2015; Schmitz and Osborne 1984). So important is the management of hydrilla that millions of dollars are spent annually by states seeking to control problematic populations, with Florida alone estimated to spend $5-15 million each year (Weber et al. 2021). With more cold tolerant varieties present in the northeastern United States it seems likely that this figure will increase as states are required to combat cold-tolerant populations.

Two factors contributing to the spread of hydrilla are the way it reproduces and its ability to survive harsh environmental conditions (Cook and Lüönd 1982; Patrick and Florentine 2021). Hydrilla can reproduce asexually through fragmentation and through submerged turions and tubers (Cook and Lüönd 1982). When the plant is physically agitated, stem fragments break off and grow into entirely new individuals (Miller et al.

1993). Turions and tubers are dormant buds and root like structures that can remain dormant in the sediment during unfavorable environmental conditions and grow into new plants when conditions are favorable.

Hydrilla in the United States has historically been controlled with a variety of herbicides, and the fluridone is one of the most widely used. (Netherland and Getsinger 1995). This chemical is an inhibitor of carotenoid biosynthesis, a process integral to photosynthesis. Carotenoids are responsible for the protection of plant cells from damage due to exposure to UV rays from the sun. In the absence of carotenoids, plants become bleached and incapable of photosynthesis, resulting in death (Doong et al. 1993). Fluridone is an effective and safe herbicide for humans, animals, and other plants when used in properly controlled doses (Magnone et al. 2013; Sprecher et al. 1998).

When a control effort is heavily dependent on a single chemical there is a risk of emerging resistance. Three distinct mutations in the same codon (amino acid 304) of the phytoene desaturase gene (*pds*) have emerged in dioecious hydrilla conferring varying degrees of fluridone resistance (Michel et al. 2004). The wildtype CGT (arginine) is replaced with CAT (histidine), TGT (cysteine), and AGT (serine) in hydrilla plants found to be resistant to fluridone, with CAT plants exhibiting the greatest resistance and AGT the least (Michel et al. 2004). Because all dioecious plants in the US are female, it is very likely these mutations arose through somatic mutation (Michel et al. 2004). Furthermore, it has been shown that these mutants will linger in populations for years after fluridone exposure has ceased, indicating that there is likely little to no fitness tradeoff that would lead to these mutants being selected against in the absence of fluridone (Netherland and Jones 2015).

These mutations are particularly problematic since fluridone is considered a safe and effective way to control hydrilla populations. Most chemical control agents, including bispyribac-sodium, are nonspecific and pose a risk to other plants in the same waterways (Lycan and Hart 2005). Other common chemical agents for treatment of algal outbreaks and general aquatic pest control such as copper sulfate are toxic to fish (Mastin and Rodgers 2000). Mechanical control of hydrilla through physical removal can be time consuming and risky due to fragmentation. Stem fragments can develop into fully grown clones and cause the population to increase (Madsen and Smith 1999; Miller et al. 1993). Biological control agents such as triploid grass carp (Hanlon et al. 2000) and a variety of insect and fungal candidates (Weeks et al. 2022) have been considered for hydrilla management, but these potential solutions have ranged from inconsistent on their own to entirely ineffective. Even the most widely accepted fluridone replacement, endothall, has faced its own resistance concerns (Sperry et al. 2021). With all the complexities of controlling hydrilla in the absence of fluridone, it is important for aquatic systems managers to be aware of the presence of fluridone resistant mutants. This could potentially serve to reduce unnecessary waste of ineffective herbicide and allow managers to make informed decisions. However, little is known about the geographic distribution of these mutants since mutants are cryptic and require lengthy (~ 2 weeks) laboratory herbicide screening on live plants. Another method involves amplifying and sequencing a portion of the *pds* gene to detect the mutants that confer resistance (Benoit and Les 2013; Tallent 2012). Since dioecious hydrilla are usually triploid, sequencing results can sometimes be ambiguous, necessitating cloning to determine the sequence for all three copies of the *pds* gene.

My goal was to develop a quick, cost-effective, and reliable test to identify levels of hydrilla herbicide resistance in the context of aquatic systems management that does not require laboratory screening or sequencing part of the *pds* gene. A relatively new tool for genotyping called DMAS (double-mismatch allele-specific qPCR) is a promising candidate method for screening for alleles of interest that has a low cost and time investment and can work with low amounts of DNA (Lefever et al. 2019). This strategy involves designing sets of primers for each mutant consisting of a forward primer specific to the target allele, a forward primer specific to the wildtype allele, and a common reverse primer. Each of the forward primers also has an introduced mutation four base pairs upstream of the 3' terminal end in order to increase specificity (Lefever et al. 2019). Samples are run in an assay with both the mutant and wildtype primers, and the resulting output can be plotted graphically for quick and easy interpretation. My goal was to take this method and determine if it could be used on hydrilla to reliably identify fluridone resistant mutants.

Methods

Sample Selection – Sample collections were made by the United States Army Corps of Engineers United States Department of Agriculture, and a variety of aquatic systems managers throughout the state of Florida. Samples were dried and preserved in silica beads upon collection and mailed to the lab.

I tested 28 samples from five waterbodies (Lake Tohopekaliga, Lake Hatch, Lake Istokpoga, Bay Lake, and Rainbow River) that were studied by Tallent (2012)(Fig 1.). In that study, all samples had a portion of the *pds* gene amplified, cloned, and sequenced to

determine the presence of fluridone resistant alleles and so provided genetically characterized samples to validate the DMAS method.

I then tested 3-4 samples from 31 waterbodies across Florida for a total of 114 samples (Fig. 1). Large lakes such as Lake Okeechobee were divided into smaller sections (i.e. Lake Okeechobee North) to test 3-4 samples for each part of the lake.

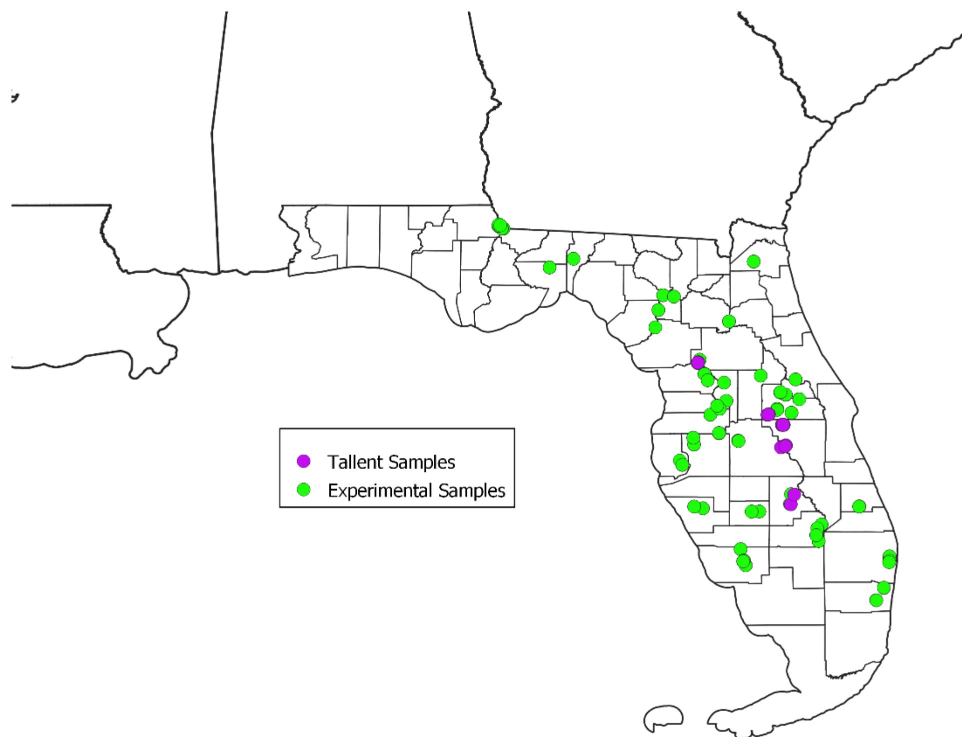


Fig 1. Location of tested samples. Samples in purple are from (Tallent 2012) with known genotypes at the phytoene desaturase (*pds* gene) and samples in green have unknown genotypes at the *pds* gene.

DNA Extraction – DNA extractions were performed using the MP Biomedicals Fast-96 Plant/Seed DNA kit following manufacturer recommendations. DNA quantity and quality were assessed using a Nanodrop One/OneC and gel electrophoreses. DNA concentrations averaged 30.525 ng/µl ± 3.416 and ranged from 1.8ng/µl to 235.2ng/µl. Most samples were below 100ng/µl, with only have having a greater concentration. All samples had high molecular weight as indicated by gel electrophoreses.

SNP Selection – Of the three SNPs known to confer resistance (Michel et al. 2004), two (CAT and AGT) were selected based on known positive samples previously sequenced in our lab. Of the original samples held in the lab, none were known to have the TGT mutation.

Primer Design – SNP specific qPCR primers were designed using Primer3 Web and the basic steps outlined previously (Lefever et al. 2019). Sets of primers consisted of two forward primers and a common reverse. One forward primer in each pair was specific to the wildtype (CGT) and the other to the mutant (CAT or AGT). The 3' terminal nucleotide of the primer overlaps with the mutant SNP. Additionally, a shared mutation was added to the fourth base pair from the 3' terminal end in order to increase specificity. This introduced mutation – determined by testing all possible introduced mismatches in the order of success outlined by Hirotsu *et al.* (2010) is the second mismatch that defines double mismatch allele-specific PCR primers (Hirotsu et al. 2010). Final primers used for DMAS were "AGT2F" forward (5'- AATGCATCCTGATTGCCTTACACA-3'), "AGT2WTF" forward (5'- AATGCATCCTGATTGCCTTACACC -3'), "AGT2R" reverse (5'- TGCAACTAATGGTGCTCATACGA -3'), "CAT3F" forward (5'- AATGCATCCTGATTGCCTTAATCCA -3'), "CAT3WTF" forward (5'-

AATGCATCCTGATTGCCTTAATCCG-3'), "CAT3R" reverse (5'-TGCAACTAATGGTGCTCATACG -3').

qPCR - Runs consisted of 96-well plates with a negative control (water), positive controls for the three genotypes with known samples (CGT, AGT, CAT – only in early runs), and 12-15 experimental samples run in triplicate with both the mutant and wildtype forward primers. We ran two 96-well plates of samples with known genotypes and five more sets of samples with each primer set (10 plates). The reaction mixture in each well contained a volume of 10µl consisting of 1µl of the DNA sample (~10-25 ng/ul), 5.0µl PerfeCTa SYBR Green FastMix, 0.36µl ROX passive reference dye, 0.4µl of primers (5 µM), and 3.24µl NFH2O. Plates were run on the Long Run setting (roughly 2 hours) with annealing temperatures of 56.5 (CAT) and 57 (AGT) degrees on the Applied Biosystems StepOnePlus Real-Time PCR System. Outputs were represented by Cq scores, which are determined by measuring the number of cycles required for the fluorescence of a sample to reach a specified threshold. The threshold values (0.016862 for AGT and 0.058963 for CAT) used were determined by running plates of positive controls. Samples with low Cq scores (below 25) for at least one primer were considered the strongest candidates for clear genotyping. If a sample had a Cq score of less than 25 for a mutant primer it was called as the associated genotype. Scores greater than or equal to 30 were considered unscorable. This determination was made due to occasional formation of curves for water blanks occuring outside of this range, with Cq scores averaging between 32.47 and 33.08 for each forward primer. This means that samples with scores in this range for mutant primers would be considered wildtypes. In the event that all primers resulted in scores greater than or equal to 30, the sample was not called. Samples that were called had consistently low standard

deviations for their associated forward primer averaging .21 ± .02. These criteria resulted in the removal of five samples (one Tallent and four unknown) from the final analysis. Additionally, melt curves were inspected for all samples run.

Validation of Results – Sanger sequencing was used to confirm the the genotypes of samples that gave conflicting results between the samples studied by Tallent (2012) and the DMAS-qPCR results in the current study using primers described in Benoit and Les 2013 and an additional newly developed internal primer I4 (5'-CCATCTTCGACCCATGCTTTTC-3'). Samples were cleaned after PCR using an ExoSap reaction consisting of 1.19µl dH2O, 0.01µl *Exo I*, 0.2µl rSAP, 0.7µl NEB2 Buffer 10X, and 5.0µl of the PCR product. Samples were then sequenced using a reaction that consisted of 5.75µl dH2O, 1.0µl of primer, 1.75µl 5x buffer (350mM Tris pH 9.0, 2.5mM MgCl2), 0.5µl BrightDye® (MCLAB), and 1.0µl of the post-ExoSap reaction DNA sample. Samples were then run on an ABI 3130XL Genetic Analyzer (ThermoFisher).

Standardization Assay – In order to determine whether DNA concentration variety would impact the ability to call samples accurately, a subset of samples (2 Wildtype, 2 AGT, and 2 CAT) were diluted to 20ng/µl, 10ng/µl, and 5ng/µl and run with both primer sets. Additionally, three of those samples were run at 40ng/µl due to an abundance of available material.

Statistical Analysis – Welch two sample t-test was used to check whether DNA concentration of the sample impacted the likelihood of being identified as a mutant. The average DNA concentration of wildtype samples were compared to the average concentration of mutant samples from the samples characterized by Tallent (2012). Following this, a series of five tests were performed comparing a random sample of 25

wildtype samples with DNA concentrations below 100ng/µl to all 25 mutants. The samples above 100ng/µl were removed because they were not representaive of the population as a whole and would have heavily skewed the analysis.

Results

The Cq scores output by the DMAS runs were averaged and plotted on two graphs, one for the AGT primer set (Fig 3.) and one for the CAT primer set (Fig 4.). Points clustered into two groups representing the wildtype and heterozygous mutants. Additionally, the AGT mutants were found to cluster with the wildtype samples when run with the CAT primer set. A DMAS setup with two possible alleles (ie. wildtype and CAT) would traditionally result in three distinct point clusters with two for homozygotes and a third for heterozygotes, but only two clusters are seen in these data since there are no documented homozygous mutant hydrilla.

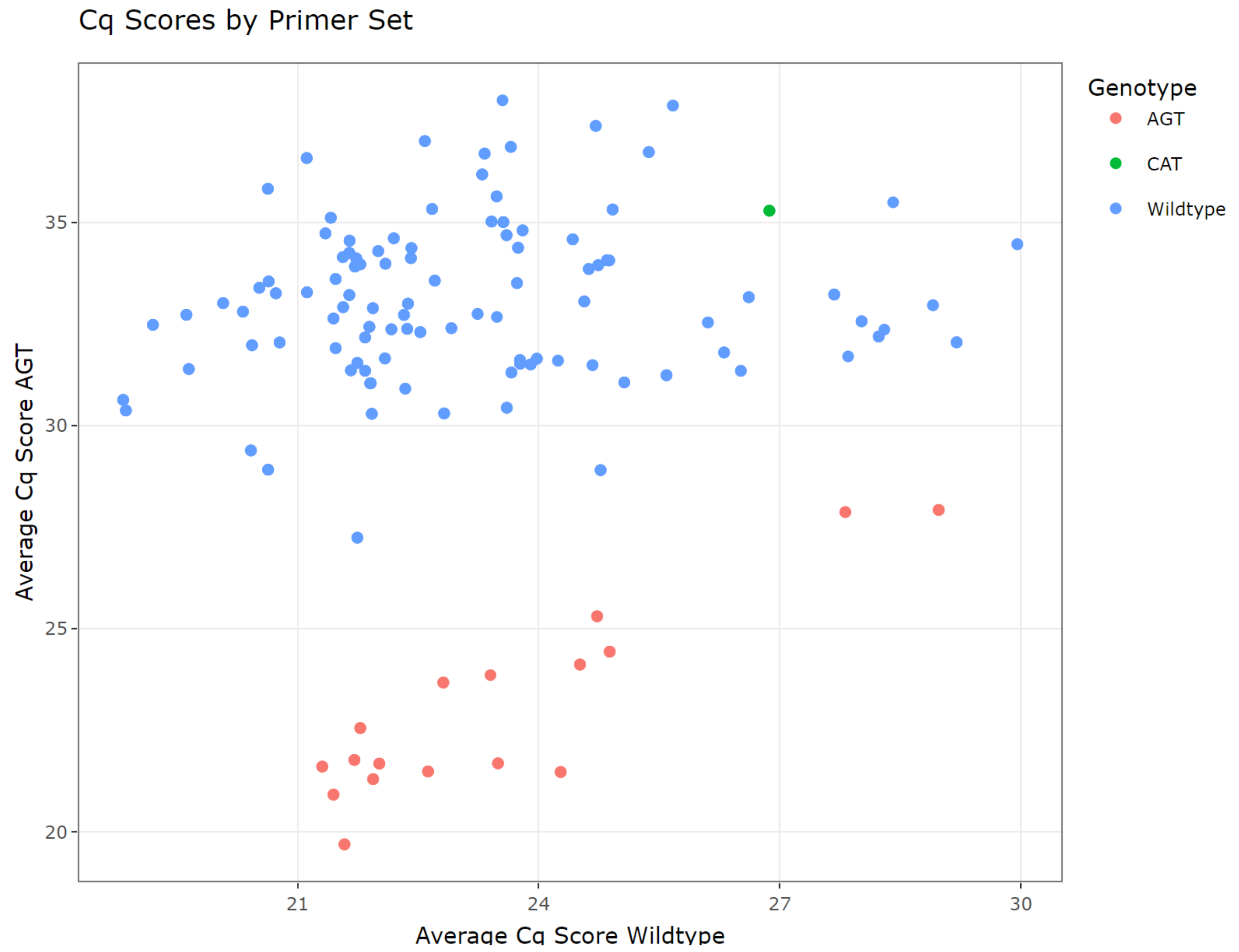


Fig 2. Average Cq scores for every sample run with the wildtype AGT primer sets. Wildtype and AGT samples are in two separate clusters.

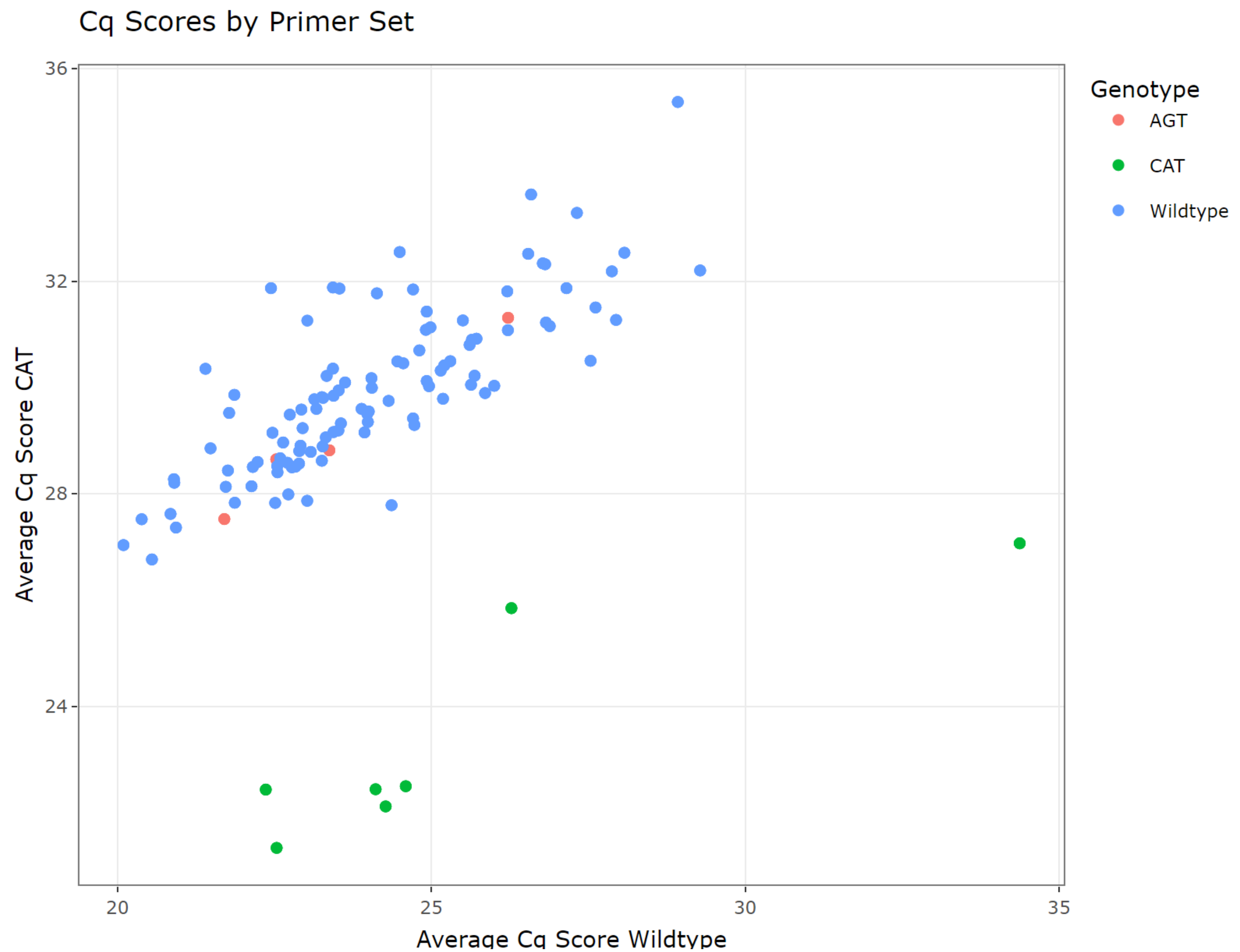


Fig 3. Average Cq scores for every sample run with the wildtype and CAT primer sets. The four AGT samples present in the plot were identified with the AGT primer set Cq scores. The wildtype and CAT samples are in two separate clusters.

Out of 28 samples from Tallent (2012) that had known genotypes, 8 were previously identified as wildtype, 7 had the CAT mutation and 12 had the AGT mutation. The results from DMAS-qPCR matched all 8 wildtypes, two CAT samples, and 10 AGT samples. Of the seven samples that did not match between Tallent 2012 and DMAS-qPCR, two AGT and 5 CAT samples were identified as wildtype by DMAS. Of the seven samples that were not

scored the same between the Tallent thesis and the DMAS results, six were successfully sequenced again with the Benoit primers and the internal I4 primer. All these sequences confirmed calls made based on the DMAS-qPCR results.

For the 110 samples with unknown genotypes, 104 were wildtype, while five samples had the AGT mutation, and one had the CAT mutation. The wildtypes were found without the presence of either mutant in 27 bodies of water. The AGT samples were found in three waterbodies, one in Hernando, FL (3 samples), one near Royal Palm Square Boulevard in Fort Myers, FL (1 sample), and at Big Gant Canal in Big Gant Lake (1 sample). The CAT positive sample was found in the Wacissa River (Fig 4.).

DNA concentrations were the same between samples called as a wildype and samples called as mutants for the samples from Tallent (2012) ($t = -.19$, $df = 13.10$, $p = .85$) Additionally, the five t-tests comparing a random subsection of wildtype samples to the mutants failed to reject the hypothesis that there was no difference in means with p-values ranging from 0.06 to 0.63. The dilution tests revealed that reductions in DNA concentration result in a shifting of Cq scores to the right, but there was no reduction in the ability to call mutants versus wildtype. These results suggest it is not necessary to standardize DNA concentrations to accurately call *pds* genotypes.

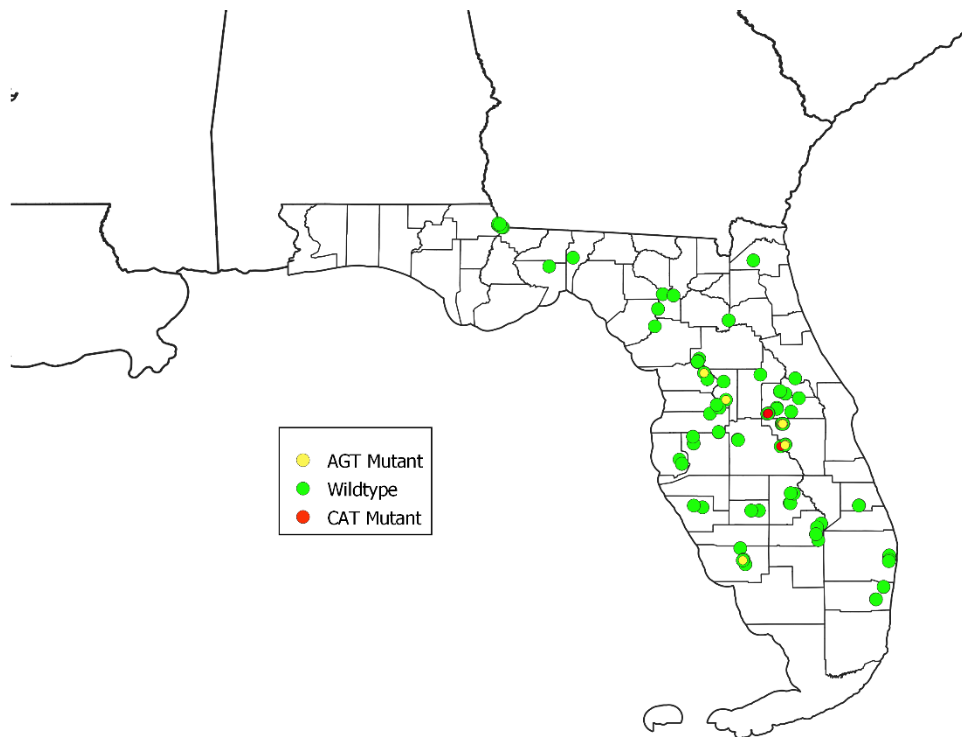


Fig 4. Location of hydrilla with wildtype and fluridone resistant mutants (CAT and AGT) at the phytoene desaturase (*pds*) gene.

Discussion

The principal goal of this work was to develop an alternative diagnostic tool for fluridone resistant hydrilla and based on my results I believe that DMAS-qPCR has strong potential for identifying fluridone resistance in hydrilla and could help to streamline the process of control. The results from running DMAS on known samples from Tallent (2012) generally aligned with the original findings, but with some exceptions. Sequencing of these

samples suggests that they were misidentified in the earlier study, supporting the need for more refined approaches to resistance screening.

Of the 31 unknown bodies of water tested, four were found to contain fluridone resistant hydrilla. Although a small sample size, this ratio of roughly 13 percent of waterways is similar to what was found in a previous study (Michel et al. 2004). In terms of practical application, these results suggest that fluridone resistance is present in multiple locations in Florida. Conversely, with further testing it may still be feasible to use fluridone as a control strategy in most waterways. The presence of fluridone resistant populations has been heavily documented in the Kissimmee Chain of Lakes (Netherland and Jones 2015) and connected waterways. A review of readily available documentation failed to uncover detailed records of resistant hydrilla in the Hernando area, Royal Palm Square, Big Gant Lake, or the Wacissa River. This supports the idea that fluridone resistant strains of hydrilla have been expanding their range in recent years and the need for a quick and effective test is still needed (Gaines et al. 2021). My sample was limited in not being able to test all bodies of water in Florida and in only having a limited number of samples from each location. More work likely needs to be done assessing the scope of sampling required to accurately determine whether or not resistant mutants are present in a system.

Standardizing DNA concentrations between samples does not appear to be necessary since it was always possible to correctly call mutants versus wildtypes. Q-PCR is capable of functioning with lower quantities of DNA than those present in our samples and it was always clear if a sample was heterozygous for the mutant.

Overall speed of testing compared to other lengthier processes such as NGS and Sanger sequencing allows DMAS to stand out, as a requirement of only 2-4 hours to run a plate is

considerably faster than the time required by other existing strategies (Benoit and Les 2013; Tallent 2012). Additionally, the overall cost involved with the DMAS process is much lower than utilizing common NGS techniques such as Illumina sequencing and there is a lower barrier to entry (Li and Harkess 2018). With a per sample cost of $12.26 per sample including the cost of DNA extraction and running samples in triplicate, the DMAS method of genotyping hydrilla samples is more affordable even when factoring in costs associated with trial and error early in the process and running old samples to help validate results.

Having a reliable, cost-effective, and practical test for detecting fluridone resistant hydrilla populations is an important step towards combatting invasions. With the slow rate at which new chemical control options become available and the risk of invaders developing resistance to those currently in use, combining chemical treatments is becoming an increasingly popular way to get extra mileage out of compounds before resistance can develop (Norsworthy et al. 2012). This practice could be made even more effective with more access to reliable methods for identifying resistance such as this method for identifying fluridone resistance in hydrilla.

REFERENCES

Balciunas JK, Grodowitz MJ, Cofrancesco AF, and Shearer JF (2002) Hydrilla. Biological control of invasive plants in the Eastern United States: 91-114

Bates AL and Smith CS (1994) Submersed plant invasions and declines in the southeastern United States. Lake and Reservoir Management 10: 53-55

Benoit LK and Les DH (2013) Rapid identification and molecular characterization of phytoene desaturase mutations in fluridone-resistant hydrilla (*Hydrilla verticillata*). Weed Science 61: 32-40

Bradshaw EL, Allen MS, and Netherland M (2015) Spatial and temporal occurrence of hypoxia influences fish habitat quality in dense *Hydrilla verticillata*. Journal of Freshwater Ecology 30: 491-502

Chen DX, Coughenour MB, Eberts D, and Thullen JS (1994) Interactive effects of CO2 enrichment and temperature on the growth of dioecious *Hydrilla verticillata*. Environmental and Experimental Botany 34: 345-353

Cook CDK and Lüönd R (1982) A revision of the genus hydrilla (Hydrocharitaceae). Aquatic Botany 13: 485-504

Cuthbert RN, Pattison Z, Taylor NG, Verbrugge L, Diagne C, Ahmed DA, Leroy B, Angulo E, Briski E, and Capinha C (2021) Global economic costs of aquatic invasive alien species. Science of the total environment 775: 145238

Doong RL, Macdonald GE, and Shilling DG (1993) Effect of fluridone on chlorophyll, carotenoid and anthocyanin content of hydrilla. Journal of Aquatic Plant Management 31: 55-59

Gaines TA, Busi R, and Küpper A (2021) Can new herbicide discovery allow weed management to outpace resistance evolution? Pest Management Science 77: 3036-3041

Gerber E, Krebs C, Murrell C, Moretti M, Rocklin R, and Schaffner U (2008) Exotic invasive knotweeds (*Fallopia spp.*) negatively affect native plant and invertebrate assemblages in European riparian habitats. Biological conservation 141: 646-654

Hanlon SG, Hoyer MV, Cichra CE, and Canfield DE (2000) Evaluation of macrophyte control in 38 Florida lakes using triploid grass carp. Journal of Aquatic Plant Management 38: 48-54

Harms NE, Williams DA, and Purcell MF (2021) The role of overseas genetic surveys to potentially accelerate biological control development for a new *Hydrilla verticillata* introduction in the USA. BioControl 66: 271-280

Havel JE, Kovalenko KE, Thomaz SM, Amalfitano S, and Kats LB (2015) Aquatic invasive species: challenges for the future. Hydrobiologia 750: 147-170

Hirotsu N, Murakami N, Kashiwagi T, Ujiie K, and Ishimaru K (2010) Protocol: a simple gel-free method for SNP genotyping using allele-specific primers in rice and other plant species. Plant Methods 6: 12

Lefever S, Rihani A, Van der Meulen J, Pattyn F, Van Maerken T, Van Dorpe J, Hellemans J, and Vandesompele J (2019) Cost-effective and robust genotyping using double-mismatch allele-specific quantitative PCR. Scientific Reports 9: 2150

Li FW and Harkess A (2018) A guide to sequence your favorite plant genomes. Applications in Plant Sciences 6: e1030

Lycan DW and Hart SE (2005) Cool-season turfgrass response to bispyribac-sodium. HortScience HortSci 40: 1552-1555
Mack RN, Simberloff D, Lonsdale WM, Evans H, Clout M, and Bazzaz FA (2000) Biotic invasions: causes, epidemiology, global consequences, and control. Ecological Applications 10: 689-710
Madeira PT, Jacono CC, and Van TK (2000) Monitoring hydrilla using two RAPD procedures and the nonindigenous aquatic species database. Journal of Aquatic Plant Management 38: 33-40
Madeira PT, Van TK, and Center TD (2004) An improved molecular tool for distinguishing monoecious and dioecious hydrilla. Journal of Aquatic Plant Management 42: 28-32
Madsen JD and Smith DH (1999) Vegetative spread of dioecious hydrilla colonies in experimental ponds. Journal of Aquatic Plant Management 37: 25-29
Magnone M, Scarfi S, Sturla L, Guida L, Cuzzocrea S, Di Paola R, Bruzzone S, Salis A, De Flora A, and Zocchi E (2013) Fluridone as a new anti-inflammatory drug. European Journal of Pharmacology 720: 7-15
Maki KC and Calatowitsch SM (2008) Cold tolerance of the axillary turions of two biotypes of hydrilla and northern watermilfoil. Journal of Aquatic Plant Management 46: 42-50
Mastin BJ and Rodgers JH (2000) Toxicity and bioavailability of copper herbicides (clearigate, cutrine-plus, and copper sulfate) to freshwater animals. Archives of Environmental Contamination and Toxicology 39: 445-451
Michel A, Arias RS, Scheffler BE, Duke SO, Netherland M, and Dayan FE (2004) Somatic mutation-mediated evolution of herbicide resistance in the nonindigenous invasive plant hydrilla (*Hydrilla verticillata*). Molecular Ecology 13: 3229-3237
Miller JD, Haller WT, and Glenn MS (1993) Turion production by dioecious hydrilla in north florida. Journal of Aquatic Plant Management 31: 101-105
Netherland MD and Getsinger KD (1995) Laboratory evaluation of threshold fluridone concentrations under static conditions for controlling hydrilla and Eurasian watermilfoil. Journal of Aquatic Plant Management 33: 33-36
Netherland MD and Jones D (2015) Fluridone-resistant hydrilla (*Hydrilla verticillata*) is still dominant in the Kissimmee chain of lakes, FL. Invasive Plant Science and Management 8: 212-218
Norsworthy JK, Ward SM, Shaw DR, Llewellyn RS, Nichols RL, Webster TM, Bradley KW, Frisvold G, Powles SB, Burgos NR, Witt WW, and Barrett M (2012) Reducing the risks of herbicide resistance: best management practices and recommendations. Weed Science 60: 31-62, 32
Patrick AES and Florentine S (2021) Factors affecting the global distribution of *Hydrilla verticillata* (L. fil.) Royle: A review. Weed Research 61: 253-271
Schmitz D (1990) The invasion of exotic aquatic and wetland plants into Florida: history and efforts to prevent new introductions. Aquatics 12: 6-24
Schmitz DC and Osborne JA (1984) Zooplankton densities in a hydrilla infested lake. Hydrobiologia 111: 127-132
Sperry BP, Leary JK, Jones KD, and Ferrell JA (2021) Observations of a submersed field application of florpyrauxifen-benzyl suppressing hydrilla in a small lake in central Florida. Journal of Aquatic Plant Management 59: 20-26

Sprecher SL, Netherland MD, and Stewart AB (1998) Phytoene and carotene response of aquatic plants to fluridone under laboratory conditions. Journal of Aquatic Plant Management 36: 111-120

Steward KK, Van TK, Carter V, and Pieterse AH (1984) Hydrilla invades Washington, D.C. and the Potomac. American Journal of Botany 71: 162-163

Tallent DE (2012) Genetic variation in the pds gene and its relation to fluridone resistance in *Hydrilla verticillata* Master's Thesis: Texas Christian University.

Tippery NP (2023) Subspecies classification of *Hydrilla verticillata* (Hydrocharitaceae). Journal of the Botanical Research Institute of Texas 17: 401-412

Tippery NP, Bugbee GJ, and Stebbins SE (2020) Evidence for a genetically distinct strain of introduced *Hydrilla verticillata* (Hydrocharitaceae) in North America. Journal of Aquatic Plant Management 58: 1-6

Van TK, Haller WT, and Garrard LA (1978) The effect of day length and temperature on hydrilla growth and tuber production. Journal of Aquatic Plant Management 16: 57-59

Weber MA, Wainger LA, Harms NE, and Nesslage GM (2021) The economic value of research in managing invasive hydrilla in Florida public lakes. Lake and Reservoir Management 37: 63-76

Weeks ENI, Hoyer MV, Bennett JP, Jackson MA, and Cuda JP (2022) Integrated management of hydrilla with two biological control agents and a herbicide. Journal of Aquatic Plant Management 60: 23-33

Zhu J, Yu D, and Xu X (2015) The phylogeographic structure of *Hydrilla verticillata* (Hydrocharitaceae) in China and its implications for the biogeographic history of this worldwide-distributed submerged macrophyte. BMC Evolutionary Biology 15: 95

Appendix

Table 1. The list of primer pairs designed and tested as candidates for the DMAS process. In bold print is the introduced mutation three base pairs upstream of the target SNP as a part of the design process.

Primer Pair	Forward Primer (5'-3')	Reverse Primer (5'-3')
AGT_A1 Wildtype	AATGCATCCTGATTGCCTTA**T**ACC	TGCAACTAATGGTGCTCATACG
AGT_A1 Mutant	AATGCATCCTGATTGCCTTA**T**ACA	TGCAACTAATGGTGCTCATACG
AGT_A2 Wildtype	AATGCATCCTGATTGCCTTA**C**ACC	TGCAACTAATGGTGCTCATACGA
AGT_A2 Mutant	AATGCATCCTGATTGCCTTA**C**ACA	TGCAACTAATGGTGCTCATACGA
AGT_A3 Wildtype	AATGCATCCTGATTGCCTTA**G**ACC	TGCAACTAATGGTGCTCATACG
AGT_A3 Mutant	AATGCATCCTGATTGCCTTA**G**ACA	TGCAACTAATGGTGCTCATACG
CAT_C1 Wildtype	AATGCATCCTGATTGCCTTAA**C**CCG	TGCAACTAATGGTGCTCATACGA
CAT_C1 Mutant	AATGCATCCTGATTGCCTTAA**C**CCA	TGCAACTAATGGTGCTCATACGA
CAT_C2 Wildtype	AATGCATCCTGATTGCCTTAA**G**CCG	TGGTGCTCATACGATATTGCTCCA
CAT_C2 Mutant	AATGCATCCTGATTGCCTTAA**G**CCA	TGGTGCTCATACGATATTGCTCCA
CAT_C3 Wildtype	AATGCATCCTGATTGCCTTAA**T**CCG	TGCAACTAATGGTGCTCATACG
CAT_C3 Mutant	AATGCATCCTGATTGCCTTAA**T**CCA	TGCAACTAATGGTGCTCATACG

VITA

Ulysses Sebastian Oles was born on April 12, 2000 in Pontiac, Michigan to Amber D. Oles and Dylan C. Oles. He is the eldest of three siblings with a younger brother in Paris X. Oles and a younger sister in Aletris S. Oles. Ulysses is a 2018 graduate of Keller High School, Keller, Texas and he received a Bachelor of Arts degree in biology from Texas Christian University, Fort Worth, Texas in 2021.

In August 2022 he enrolled in graduate study at Texas Christian University. During that time Ulysses held a Teaching Assistantship.

ABSTRACT

EMPLOYING A NEW TESTING METHOD FOR IDENTIFYING FLURIDONE RESISTANCE IN *HYDRILLA VERTICILLATA*

Ulysses Sebastian Oles, B.A., 2021

Department of Biology

Texas Christian University

Thesis Advisor: Dean Williams, Professor of Biology

The problem of herbicide resistance in aquatic invasive plants poses a consistent threat to the biota in invaded ecosystems, as well as to infrastructure and human water use. *Hydrilla verticillata* is an invasive aquatic plant that has developed resistance to the carotenoid biosynthesis inhibitor fluridone. I used the method of double-mismatch allele-specific qPCR in order to design a new test for identifying resistant mutants of two different genotypes. After testing 138 samples from across Florida using two sets of primers corresponding to each mutant genotype, I found clear and consistent results for most samples and validated these results through sequencing and comparison to old data. In addition to suggesting that this is an effective testing method, my data found resistant mutant samples in four bodies of water outside of our positive control samples.