USING GENETIC AND GENOMIC TECHNIQUES TO UNCOVER CRYPTIC DIVERSITY FOR IMPROVING AQUATIC INVASIVE PLANT MANAGEMENT

by

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of

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DEDICATION

To my wife, Audrey.

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It is difficult to put into words the amount of admiration and respect that I have for my advisor, Dr. Ryan A. Thum. I met Dr. Thum when I took one of his classes as an undergraduate. I was in the last year of a degree in biological science, and I had never worked in a lab nor did I have the slightest clue about getting a job in science. That summer, Dr. Thum offered me a job in his lab which allowed me to realize my path in science moving forward. We parted ways for my master's degree, but he remained a close advisor and committee member. When I decided to pursue my PhD, working with him seemed like a perfect fit, and that turned out to be the case. He has given me so much support over the years and I can honestly say that I would not be where I am today without his dedication to the students he takes on. All I can say is, thank you.

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LIST OF ABBREVIATIONS

EWM: Eurasian watermilfoil (*Myriophyllum spicatum* L.)
NWM: northern watermilfoil (*Myriophyllum sibricum* Kom.)
Hybrid watermilfoil: *Myriophyllum sibiricum* × *M. spicatum*
2,4-D: 2,4-dichlorophenoxyacetic acid
RNA-seq: Whole RNA sequencing

ABSTRACT

Genetic diversity can be important at many levels of invasive species management. And, for different questions, it matters at which level we measure diversity to understand its relevance. Some invaders may look similar to other species, so identifying the species to be managed may be difficult without genetic tools. Once the species has been identified, understanding the diversity in that species may be important to identify management units, invasive traits, and the possibility of spread. Finally, understanding how the alleles an individual possesses determine the traits expressed can give managers the tools to control for unwanted traits of an invasive species. In this body of work, I uncover diversity at the species/taxon level, the genotype/clone level, and finally at the gene level in invasive aquatic weed species. At the taxon level, I found that one invasion of aquatic weeds in the northeastern US was actually two or more separate invasions and taxa. At the genotype level, I found that the same genotype responds the same to a common herbicide management regardless of where it is found, and that different genotypes have varying responses to a common herbicide treatment. And, at the gene level, I found that different genotypes with different growth rates have different gene expression in the control and transcriptional response to a common herbicide treatment. At each of these levels, managers have questions and concerns about management decisions. Understanding that there were two unique taxa in what was considered one invasion informed managers that there may be variance in management relevant traits between the two. In the genotype level study, we learned that determining which clones are present in a lake slated for herbicide management may inform which herbicides to use. And, at the gene level, we are starting to understand the molecular process of management relevant phenotypes so that one day managers can screen for molecular markers that will reveal herbicide response of individuals slated for management.

CHAPTER ONE

INTRODUCTION

Intra- and interspecific genetic diversity has been revealed at an increasing rate since the incorporation of polymerase chain reaction (PCR) and DNA sequencing into studies (Bickford et al. 2006). Until the mainstream use of molecular techniques, species taxonomy relied mostly on morphological features. Since then, we have seen that taxonomically distant species can have very similar morphological traits (Bickford et al. 2006), potentially due to parallel evolution (Oke et al. 2017; Stuart et al. 2017). Therefore, if only morphology is used to determine taxonomy, researchers may falsely conclude that taxa are more closely related than they actually are and vice versa. This “hidden” diversity that is potentially overlooked by traditional methods is known as cryptic diversity.

Cryptic diversity can occur at many levels and diversity at all levels may be relevant to natural resource management. At the species or taxonomic level, I have already mentioned that different species may look the same, and managers will need to know which species they are managing. Within a species or taxa, there may be distinct breeding populations. For example, Chorak et al. (2019) used genotyping methods to show that a fish species residing in waterbodies connected to Lake Michigan were actually multiple cryptic stocks (i.e., breeding populations) that were being differentially harvested. This is important information because managers of harvested species want to make sure that one stock or population is not harvested to extinction. Different genotypes within species or populations may also have different phenotypes, either because of the genetic variants they possess or the way those genes are expressed. Managers could then use

this information to determine long term responses of populations to management. For instance, if there is a lot of variation in a management relevant trait (e.g., herbicide response) then over the management period, the population may shift to being dominated by individuals with the best trait for management (e.g., herbicide resistance evolution).

The overarching theme of my research has been to uncover diversity in invasive aquatic plant species using genetic and genomic techniques. I did this by conducting research projects at the species/taxon level (chapter 2), the genotype/clone level (chapter 4), and finally at the gene level (chapter 5). This chapter serves as a road map for my dissertation. I begin each section by presenting the question asked in that study. I then give some general background, followed by the rationale for the question and briefly state the results from each study. Each chapter is a self-contained publication with more in-depth background, rationale, results, and conclusions.

Chapter Two

In this chapter, I ask whether there were cryptic invasions of the already recognized and managed invasive water chestnut (*Trapa spp.*) in the northeastern United States. Understanding cryptic diversity can be extremely valuable in invasive species management. For example, many invaders are misidentified and known as cryptic invasions (see Morais and Reichard 2018). Cryptic invasions can be interspecific, whereby a non-native species introduction is not recognized because it is misidentified as a native or other invasive species. Or, cryptic invasions can be intraspecific, whereby a distinct lineage of a species is introduced into an area that contains a lineage of the same species (Morais and Reichard 2018). Understanding when alien species, or even new lineages of an already established invader appear is important to

management, because this period of time can be critical in controlling the outbreak. Most successful invasions maintain high genetic diversity, likely because of multiple lineage introductions or hybridization events (Barrett 2015; Cristescu 2015). This high genetic diversity allows the invaders to adapt quickly to the new environment. Therefore, the identification of these cryptic invasions through molecular data may give managers the information needed to control for additional introductions and the spread of the invader.

Researchers at the Army Corps of Engineers have been managing the invasive water chestnut (*Trapa natans*) in the Potomac River (Virginia, USA) for decades. In 2014, they noticed that some of the water chestnut only had two horns on their fruits, whereas the morphotype of water chestnut that was being managed usually had four horns. Managers wanted to know if new lineages had invaded the northeastern US or if there was simply morphological variability in the species. Invasive water chestnut is usually managed to prevent flowering and additional spread. Therefore, identifying different lineages or taxa is an important first step in determining trait differences between them. I addressed these questions by genotyping samples of the two invading morphotypes and samples from the native range. I was able to conclude that the new two-horn morphotype was indeed a new introduction, because it was genetically distinct from the four-horn morphotype, and likely a different species (*T. bispinosa*).

Chapter Three

Aquatic plant managers are increasingly concerned about cryptic genetic diversity that may lead to herbicide resistance evolution. This is because herbicides are frequently used to manage aquatic plants in the United States (Bartodziej and Ludlow 1998), and variation in

response within and among managed populations can lead to herbicide resistance evolution. There are also examples of different lineages or clones of aquatic plants that have been shown to respond differently to herbicides. For example, genotypes of hydrilla (*Hydrilla verticillata* L.f. Royle) were found to be resistant to fluridone (phytoene desaturase inhibitor) in Florida, USA (Arias et al. 2005; Dayan and Netherland 2007). Also, duckweed (*Landoltia punctate*) genotypes have been shown to exhibit resistance to diquat (photosystem I inhibitor; Koschnick et al. 2006). Hybrid watermilfoil in Townline Lake (Michigan, USA) was also shown to be resistant to fluridone (Berger et al. 2012, Thum et al. 2012, Berger et al. 2015). However, herbicide efficacy and resistance monitoring are rarely conducted in aquatic plant management, potentially because it is difficult to observe submerged aquatic plants (Morais and Reichard 2018; Bickford et al. 2006). Therefore, despite the few examples listed above, and the concern of herbicide resistance evolution in aquatic plant management, the frequency of herbicide resistance and variation in herbicide response among genotypes and within clones of aquatic plants is widely unknown.

In this chapter, I present a synthetic review of herbicide resistance in aquatic plant management. It was published as a best practices guide for aquatic plant managers to combat herbicide resistance evolution and was included in a larger book titled "Biology and Control of Aquatic Plants: A Best Management Practices Handbook 4th Ed." It serves as background for the studies in the following chapters (4 and 5), that both deal with herbicide response in watermilfoil.

Chapter Four

In this chapter, I ask how much inter- and intra-genotype variation there is in watermilfoil in response to the commonly used herbicide, fluridone. As stated above, the frequency and

repeatability of herbicide resistance in aquatic plant populations is widely unknown. We have very few examples of herbicide resistance in aquatic plant species relative to terrestrial systems (Heap 2021). This may be due to the fact that submersed aquatic plants are not easily observed in the field both before and after herbicide treatments (Morais and Reichard 2018; Bickford et al. 2006). And, in controlled conditions, herbicide trials that identify herbicide resistance are constrained by the amount of space available to house large tanks of water with adequate growth conditions.

Watermilfoil is one aquatic plant species where herbicide resistance evolution is of great concern, because it has already been documented in this species once (Berger et al. 2012, Thum et al. 2012, Berger et al. 2015). Watermilfoil is capable of both clonal and sexual propagation (Hartleb et al. 1993). Meaning, it has the ability to both make new genotypes (i.e., combinations of genes) with sexual reproduction and then maintain fit genotypes once it has found a good match for the environment. Therefore, an herbicide resistant genotype/clone may be maintained by selection with the corresponding herbicide and even spread to other lakes that are managed the same.

Hybrid watermilfoil in Townline Lake (Michigan, USA) was already found to be resistant to fluridone before genotyping of watermilfoil became common (Berger et al. 2012, Thum et al. 2012, Berger et al. 2015). And, since then, the “Townline” genotype has been found in at least nine other lakes in Michigan (Thum et al. 2020). I tested whether the same genotype of watermilfoil found in different lakes exhibited the same response to fluridone and, what the response of a handful of unique genotypes was to fluridone. I found that the same resistant genotype was reliably less sensitive than other genotypes to the fluridone treatment, exhibiting

that clones should be managed the same way regardless of the waterbody they are found in. Further, I identified another genotype that also shows resistance to fluridone.

Chapter Five

In this last research chapter, I ask whether there is variation in gene expression between two genotypes of invasive watermilfoil that show variation in their growth. The identification of resistant and susceptible populations and/or clones of aquatic plants may also allow for researchers to uncover the causal gene mutations of these traits through detailed breeding and assay studies. Resistance mutations can be classified as target site resistance (TSR) and non-target site resistance (NTSR). TSR are genetic mutations that lead to changes in the molecule(s) where the herbicide typically binds, leading to less effective binding, and thus less impact on the weed (Delye 2013). NTSR refers to genetic changes that occur not at the herbicide's site of action, but at other genes that are related to herbicide uptake, translocation, sequestration, detoxification, metabolism, etc. (Delye 2013). While NTSR could be the result of a single gene mutation (e.g., a gene that increases metabolism of the herbicide), it is generally thought that most cases of NTSR are due to multiple genes (Delye 2013; Neve and Powles 2005). Another genetic mechanism that can confer NTSR may be mutations in the regulatory regions of the genome that alter the number of times genes are being converted into active proteins (i.e. **gene expression**- a measure of the rate at which genes are translated into active proteins). For example, expression of transporter genes is increased in glyphosate resistant individuals of horseweed (Conyza Canadensis) but not in susceptible individuals when treated with glyphosate (Nol et al. 2012). Therefore, uncovering gene expression differences among populations and/or

clones of aquatic plants may be key to understanding why some are susceptible to herbicide management and others are not. For example, some genotypes may be more likely to establish and spread because they over-express key genes related to vegetative growth. Similarly, some genotypes may be more resistant to an herbicide because they over-express the gene(s) targeted by the herbicide and/or over-express key genes related to the metabolism or detoxification of the herbicide.

Experimental studies of different genotypes of watermilfoil have shown that genotypes vary in their growth rates in untreated and herbicide environments (Hoff and Thum *accepted*; Taylor et al. 2017). Therefore, I compared the molecular response between a genotype each of Eurasian and hybrid watermilfoil that differ in their growth rates to the commonly used auxinic herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D). I found that the molecular response of the EWM genotype to 2,4-D treatment was much stronger than the hybrid genotype. Further, I found that the EWM genotype expressed the genes in the putative 2,4-D response pathway as would be predicted by the currently know auxin herbicide pathway, but the hybrid genotype did not, suggesting that the hybrid genotype is less affected by 2,4-D treatment.

Chapter Six

In this final chapter, I wrap up this dissertation by giving overall conclusions. I also present the next steps that came from each chapter of my dissertation work. And, where applicable, present preliminary data from experiments that have already followed from my dissertation research.

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CHAPTER TWO

CRYPTIC INTRODUCTION OF WATER CHESTNUT (TRAPA) IN THE NORTHEASTERN UNITED STATES

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Cryptic introduction of water chestnut (*Trapa*) in the northeastern United States

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ABSTRACT

Trapa natans, characterized by four-horned fruits, has been recognized as an introduced species in the northeastern United States since the 1920's. However, in 2014 a two-horned morphotype of *Trapa* was discovered in the Potomac River in Virginia. As such, we hypothesize the two-horned variety represents a cryptic introduction of a *Trapa* taxon distinct from the four-horned *T. natans* previously identified in North America. We tested this hypothesis by genotyping 129 amplified fragment length polymorphism loci for 304 *Trapa* individuals collected from across the Northeast US and several populations from Asia and Africa. The two-horned and four-horned morphotypes in the northeastern US were found to be genetically and morphologically distinct. The two-horned *Trapa* taxon was most genetically and morphologically similar to samples from Taiwan identified as *T. bispinosa* Roxb. var *iinumai* Nakano. The four-horned *Trapa* taxon previously identified as *T. natans* was most genetically and morphologically similar to *T. natans* collected from Japan, but were genetically distinct. Therefore, it is likely that the US four-horned *Trapa* was introduced from somewhere outside of Japan, an unsampled area in Japan, or have genetically diverged since introduction. Distinguishing these two US *Trapa* taxa will be important for documenting spread and identifying new populations, and for further study on the timing and efficacy of physical, chemical, and biological control options. Our study also highlights the need for a comprehensive geographic survey of *Trapa* morphology and genetics to clarify the taxonomy.

Keywords

cryptic invasion; AFLP; Trapa natans; Trapa bispinosa; STRUCTURE; PCAmixdata; Trapa japonica; Trapa quadrispinosa; Trapa incisa

1 INTRODUCTION

Cryptic invasions occur when introduced taxa go undetected because they appear similar to taxa already established in that range (Morais and Reichard 2018). Cryptic invasions can be interspecific, whereby a non-native species introduction is not recognized because it is misidentified as a native or other invasive species. And, cryptic invasions can be intraspecific, whereby a distinct lineage of a species is introduced into an area that contains a lineage of the same species (native or non-native). Cryptic invasions are likely more common than currently recognized, and are particularly hard to detect when the taxa are closely related and/or their taxonomy is unclear (Morais and Reichard 2018).

Water chestnut (Myrtales: Lythraceae: *Trapa*) is a genus of aquatic plants native to temperate and tropical regions of Europe, Asia, and Africa (see USDA 2019), but is introduced and considered problematic in the northeastern United States and Canada (Madsen 1993; Methe et al. 1993; Hummel and Kiviat 2004). Dense growth of *Trapa* can have detrimental effects on ecosystem services, such as low dissolved oxygen, obstruction of water flow, and recreational impediments (Strayer 2010). Additionally, the floating-leaved growth habit of *Trapa* may outcompete native submerged aquatic vegetation, that is preferable waterfowl habitat, through shading (Kiviat 1939; Gwathmey 1945; Martin and Uhler 1939; Groth et al. 1996; Kadono 2004; Hummel and Kiviat 2004).

The identification of *Trapa* to species is difficult, and the taxonomic literature regarding the number of *Trapa* species worldwide is conflicting (Hummel and Kiviat 2004). Species have traditionally been defined morphologically (Van Driesche et al. 2002; Suriyagoda et al. 2007) on the basis of fruit characteristics such as the size and number of spines and protuberances (Kadono

1987; Hoque et al. 2009). In Europe, for example, some authors use variation in fruit morphology to define up to twelve distinct *Trapa* taxa, while others only recognize a single *Trapa* species (*T. natans* L.) with morphological variation (Frey et al. 2017). The source of overlapping and variable morphologies is unknown and may be due to phenotypic plasticity or lineages independently evolving the same traits (Kadono 2004, Frey et al. 2017). Since overlapping morphologies and unclear taxonomy are common characteristics in cryptic invaders (Morais and Reichard 2018), *Trapa* species are candidates for cryptic invasion.

Trapa was likely first introduced to North America in the 1870s, but was not recognized as a problem until the 1920s (Davenport 1879; Les and Mehrhoff 1999; Pemberton 1999). Until now, only a four-horned morphotype of *Trapa* has been recognized in the Northeast United States, which has been identified as *Trapa natans* L. (Fassett 1957; Britton and Brown 1970; Gray et al. 1970; Cronquist 1981; Cook 1990; Gleason and Cronquist 1991; Crow and Hellquist 2000). However, in August 2014 Virginia Game and Inland Fisheries biologists observed a colony of a two-horned morphotype of *Trapa* growing in the tidal Potomac River, Virginia, USA (Dodd et al. 2019). We hypothesize the two-horned *Trapa* sp. represents a cryptic introduction distinct from the four-horned *T. natans* previously identified in North America.

Given the varying identification methods and taxonomy of *Trapa*, we combined molecular (amplified fragment length polymorphism markers; AFLP) and morphological clustering analyses to determine the likelihood that the two-horned *Trapa* morphotype was a new cryptic introduction into the northeastern United States. In addition, we aimed to identify the origins of the two morphotypes by collecting a broad sample of *Trapa* from its native range.

2 METHODS

2.1 Sample Collection

Samples were collected in 2016 and 2017 from several water bodies in the northeastern US as well as in the People's Republic of China – Hubei and Taiwan, Japan, and South Africa (Supp. Fig. 1 & Supp. Table 1). The scientific names used here provisionally follow the usage of the flora of the respective country or regions: *T. quadrispinosa* Roxb. from the People's Republic of China as in Li et al (2017) and Wan (2000); *T. bispinosa* Roxb. var. *iinumai* Nakano as in Hsieh (1994) from the People's Republic of China – Taiwan; *T. incisa* Sieb. et Zucc., *T. japonica* Flerov sensu Kadono, and *T. natans* L. from Japan as in Takono and Kadono (2005); *T. natans* L. from South Africa as in Cook (2004); *T. natans* L. (USA) as in Crow and Hellquist (2000); and two-horn *Trapa* sp. as described in this study. All collection sites for the US two-horned *Trapa* sp. were from Virginia, US (Supp. Fig. 1 & Supp. Table 1). The collection sites of the US four-horned *T. natans* colonies included the rest of the sampling locations in the northeastern US (Supp. Fig. 1 & Supp. Table 1).

Varying numbers of rosettes with leaves and fruits attached were collected in lakes and ponds (11 to 30, depending on availability) at least one to two meters apart to avoid sampling the same plant, with at least one mature, fully ripe fruit (when available) for each population (Dodd et al. 2019). To our knowledge none of the samples were cultivated as agricultural products. For populations within the US, each rosette was rinsed and placed into a labeled plastic bag and fresh biomass was shipped overnight in coolers to the lab for morphological evaluation. Specimens from outside the US were dried in envelopes with silica beads prior to shipment to the US. For all

specimens, one to three leaves from each rosette was dried in silica gel for molecular analysis; fruits were oven dried to a constant weight for morphological analysis (Dodd et al. 2019).

2.2 Molecular Analysis

We used amplified fragment length polymorphism markers (AFLPs) to test whether the unknown two-horned *Trapa* sp. found in Virginia and the four-horned *Trapa* sp. from the US were genetically distinct. In addition, we tested to see what samples from around the globe the two US *Trapa* species were most related to, in an effort to better understand where they came from.

We extracted whole DNA from dried leaf tissue using Qiagen DNEasy extraction kits (Qiagen Valencia, CA). Next, AFLPs were prepared for each sample following the methods of Vos et al (1995). We digested ~200 ng whole DNA with 5 Units of both EcoRI and MseI enzymes (New England Biolabs) in 40 µL total reaction volume of 1X T4 buffer with ATP including 2 µg of BSA, (New England Biolabs) and 0.05 M NaCl (Fisher Sci). Restriction reactions were incubated at 37° C for one hour. We ligated adapters onto the cut ends of the fragments by adding 10 µL of ligation master mix to the 40 µL of restriction product, above. The ligation master mix included 1X T4 buffer with ATP, 0.5 µg BSA (New England Biolabs), 0.05 M NaCl (Fisher Sci), 0.5 µM EcoRI adapter and 5 µM MseI adapter with 70 Units of T4 DNA Ligase (New England Biolabs). The 50 µL of total ligation reaction (40 µL of restriction + 10 µL ligation master mix) was incubated at 37° C for three hours. We used two steps of PCR amplification: preselective and selective. Both PCR reactions took place in 20 µL total volume of 1X GoTaq Buffer (Promega), 0.2 mM of each dNTP (Fisher Sci) and 1.875 mM MgCl (Promega). The preselective amplification included 0.25 µM of each EcoRI - A and MseI - C primer, 1 Unit of GoTaq (Promega), and 3 µL of 1:3 dilution of the ligation product. We used two primer combinations

(MseI-CTG/EcoRI-AGG and MseI-CGA/EcoRI-AGG) for selective amplification. Each included 0.25 µM EcoRI - AGG - labeled with VIC (Applied Biosystems) and either MseI - CTG or MseI – CGA, 1 Unit of GoTaq Hot Start (Promega), and 3 µL of 1:20 dilution of the preselective amplification PCR product. For thermocycler conditions, we ran the preselective PCR for two min. at 72° C, and then 21 cycles at 94° C for 30 sec, 56° C for one min., and 72° C for one min., followed by a final extension at 60° C for 30 min. The selective PCR was run at 94° C for two min., and then ten cycles at 94° C for 20 sec., 66° C for 30 sec., and 72° C for two min. then 20 cycles at 94° C for 20 sec., 56° C for 30 sec., and 72° C for two min., followed by a final extension at 60° C for 30 min. Finally, we submitted 1:3 dilution of the selective amplification products to the University of Illinois Sequencing Core (Urbana-Champagne, IL) for fragment analysis on a 3730xl using LIZ 500 size standard (Applied Biosystems).

We used GeneMapper v5.0 (Applied Biosystems) to score polymorphic peaks between 100 and 500 bp lengths using all samples in the project as reference for auto binning. Poor quality chromatogram and size standard samples were removed. Next, we used methods from Ley and Hardy (2013) to assess the reliability of allele calls at each locus. We ran full duplicates of approximately 15% of the individuals in the dataset and only duplicated samples were run through SPAGeDi v1.5 (Hardy and Vekemans 2002), which was used to assign a heritability (h^2) score to each locus based on whether it is reproducible in the duplicate sample. We then removed all loci with an h^2 score < 0.80. Duplicates were also removed from the final dataset. We also removed samples with poor sequence quality and non-polymorphic and unrepeatable loci. AFLP data are available from the authors upon request.

We analyzed the final AFLP dataset using two methods to examine genetic differentiation and relatedness. We generated a neighbor joining tree in PAUP* v4.0a using 5,000 bootstrap replicates (Swofford 2002). We then visualized the 50% majority-rule consensus tree in FigTree v1.4.3 (Rambaut 2012). We also visualized genetic groups using the Bayesian clustering program STRUCTURE v2.3.4 (Pritchard et al. 2000). Each *Trapa* individual was clustered using the admixture model with a burn-in period of 20,000 and a run time of 50,000 Markov Chain Monte Carlo (MCMC) reps. Seven iterations were ran at each value of K (1 - 10). We ran STRUCTURE with flat priors. We found the most likely values of K using the ΔK method from Evanno et al. (2005) calculated in STRUCTURE HARVESTER v0.6.93 (Earl and vonHoldt 2012). We found consensus clusters across iterations of STRUCTURE by permuting and matching clusters using the "greedy algorithm" with a random input and 1000 repeats in CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007), and we used *distruct* v1.1 (Rosenberg 2004) to draw the final STRUCTURE plots.

2.3 Morphological Analysis

We tested for morphological differences between the putatively distinct US *Trapa*, and compared their morphology to populations from the native range. Because of the large geographic distribution of our sample, measuring fresh fruits was not feasible based on the logistics of sending and receiving them (preservation, permitting, etc.). Therefore, we used dried fruit specimens for consistency in protocol among sites worldwide. Fruits that were not shriveled during drying, or did not have damage affecting morphological measurements, were considered quality fruit. The dried fruit and leaves from the first 11 or 12 rosettes collected from each population that had quality fruits were archived and a subset were selected and used in

morphological and genetic analysis. Archived plant materials used in the analysis are at the US National Arboretum herbarium (NA) and George Mason University herbarium (GMUF), and photos are available from the authors upon request.

We measured morphological attributes following the procedure of Kadono (1987) for each of 22 populations from which we were able to obtain quality fruits (Supp. Table 2). We recorded the following observations for the dried fruits: presence or absence of a crown, number of spines (with or without barbed tips), orientation of upper spines, shape at apex of lower projections, orientation of lower projections, width across upper spines, width across lower projections, fruit height, and fruit thickness. For the US *Trapa* samples, we additionally measured color on the underside of the leaf (categorical: green or red). This trait was not measured on specimens collected from several populations outside the US, so this variable was not included in our analysis of worldwide samples.

We conducted Principal Components Analyses (PCA) using the package PCAmixdata (Chavent et al. 2018) in R, which can handle combinations of numerical and categorical variables. Samples missing any of the morphological variables were removed from analysis. Some populations were excluded from the morphological analysis since they did not have quality fruits to measure (see Supp. Table 1 & 2). We performed two mixed PCA analyses of *Trapa* morphology: one that included all 254 samples collected worldwide, and one including only the 128 samples from the US.

3 RESULTS

Our final genetic dataset contained 304 *Trapa* samples genotyped at 129 loci (Supp. Table 1). The Bayesian clustering implemented in STRUCTURE revealed an optimum ΔK at K = 3, with secondary peaks at K = 5 and K = 7 (Fig. 1, Supp. Fig. 2). The groups at these three K values were hierarchically structured. At K = 3, the major groups evident were: Group 1) *T. natans* (US), *T. natans* (Japan), and *T. quadrispinosa* (People's Republic of China - Hubei); Group 2) *T. japonica* (Japan), *T. incisa* (Japan), and *T. natans* L. (South Africa); and, Group 3) *T. bispinosa* var. *iinumai* (People's Republic of China - Taiwan) and the unknown two-horned *Trapa* sp. (US) (Fig. 1). At K = 5, Groups 1 and 2 showed additional structure: US versus Japanese *T. natans* grouped separately within Group 1, and *T. japonica* grouped separately from *T. incisa* (Japan) and *T. natans* (South Africa) within Group 2 (Fig. 1). Finally, at K = 7, Groups 1 and 2 showed additional structure: *T. japonica*, *T. incisa*, and *T. natans* (South Africa) all grouped separately in Group 2, and some additional genetic structuring within US *T. natans* appeared (Fig. 1). For each K, individual proportions of membership were high for each group.

The neighbor-joining tree results comported with the STRUCTURE results. All major groups were evident in the tree, and these corresponded to the groups delineated in the STRUCTURE analysis at K = 3, K = 5, and K = 7 and with bootstrap support (Fig. 2).

Clustering of *Trapa* based on morphology was generally consistent with the AFLP results in that distinct genetic groups also tended to be morphologically distinct. Groups were distinguished primarily by fruit size (height, width, and thickness variables), number of spines, shape at apex of lower projections, and presence or absence of a crown (see Fig. 3 and Supp. Table 3). AFLP Group 1 (US and Japan *T. natans*, and *T. quadrispinosa*) was morphologically distinct from

Group 3 (US two-horned *Trapa* and Taiwan *T. bispinosa*). As in the molecular analysis, the two-horned US *Trapa* sp. was most similar morphologically to *T. bispinosa* var. *iinumai* from Taiwan, and the four-horned US *T. natans* was most morphologically similar to *T. natans* from Japan (Fig. 3A). Additionally, the three species in Group 2 of the molecular analysis (*T. incisa* and *T. japonica* from Japan, and *T. natans* from South Africa) were all morphologically distinct (Fig. 3A).

Because we were specifically interested in the two genetically distinct US *Trapa*, we ran a mixed PCA to identify traits to distinguish them. The *T. natans* (US) and two-horn (US) were morphologically distinct (Fig. 3B). These taxa were distinguished by their number of spines, the shape at apex of lower projections, presence/absence of a crown, color of the underside of the leaf, and fruit size (see Supp. Table 3).

Three populations of *T. japonica* in Japan exhibited individual plants with four-horned fruits instead of two-horned fruits characteristic of the species (MIK-J, OHM-J, and ONO-J). Similarly, two populations of *T. natans* in Japan (KO-J and TEM-J) and four populations (5 of 96 individuals) of *T. natans* in the US (CH-RI, MRC-NY, SC-NY, and WL-NY) exhibited individual plants with two-horned fruits instead of the four-horned fruits characteristic of these species (Supp. Table 2). In all of these cases, all of the individuals in the population grouped together genetically, but they did not group together in the morphological analysis because horn number and was an important character driving morphological separation among samples (Fig. 3 and Supp. Table 3).

4 DISCUSSION

It is clear that there has been a cryptic introduction of *Trapa* into the northeastern US. The unknown two-horned *Trapa* sp. found in the Potomac River, VA is morphologically and genetically distinct from the previously established four-horned *Trapa* taxon in the US (Figs. 1-3). Several morphological traits can be used to distinguish these two species in the US. *T. natans* has four horns and a prominent crown, whereas the newly-recognized *Trapa* has two horns and lacks a crown. Additionally, the two can be distinguished by the color on the underside of the leaf; *T. natans* is green, whereas the two-horned *Trapa* tends to be reddish. *T. natans* also has white flower petals, whereas the two-horned *Trapa* has pink flowers. Although we were not able to formally include this trait in our morphological analysis, this was true for all samples that were flowering at the time of collection.

Based on the genetic and morphological clustering of samples (Figs. 1 - 3), the unknown two-horned *Trapa* sp. from the US is most genetically and morphologically similar to what is currently recognized in Taiwan as *Trapa bispinosa* var. *iinumai*. We therefore tentatively identify the US two-horned *Trapa* sp. as this species. Since this species is known in the northern part of Taiwan, mainland China, Korea, and Japan (Hsieh, 1994), it is unclear what the precise origin of this species in the US is, and further sampling across the known geographic range would be necessary to pinpoint its exact origin.

The four-horned *Trapa* in the US has historically been recognized as *T. natans* (Fassett 1957; Britton and Brown 1970; Gray et al. 1970; Cronquist 1981; Cook 1990; Gleason and Cronquist 1991; Crow and Hellquist 2000). Indeed, the four-horned US *Trapa* is most genetically and morphologically similar to *T. natans* sampled in Japan (Figs. 1 – 3). However, the US four-

horned *Trapa* and Japan *T. natans* are genetically distinct from one another in the STRUCTURE analysis with K=5 and K = 7 (Fig. 1). It is therefore unclear whether the US four-horned *Trapa* was introduced from a source not captured in our sampling, or whether the genetic differences result from genetic divergence following introduction from Japan. Further sampling of *Trapa* from across its native range may be able to resolve the geographic origin of the US four-horned species (tentatively *T. natans*).

While it was not our immediate focus, our study suggests that further taxonomic investigation of the genus based on a comprehensive geographic survey of molecular and morphological characters is warranted. The number of species recognized differs according to different authors and geographic regions (e.g., see Li et al. 2016; Frey et al. 2017), and therefore distinct taxa may be recognized under the same name, and *vice versa*. For example, using the nine fruit characteristics in this study, the samples identified as *T. natans* L. from South Africa were most morphologically similar to samples identified as *T. natans* from the US and Japan, but were genetically distinct from them, and instead grouped most closely with samples identified as *T. japonica* and *T. incisa* from Japan based on AFLPs (Figs. 1 - 3). We also observed within-population variation in horn number in several Japanese and US populations, although individuals within populations were genetically similar. Takano and Kadono (2005) made similar observations in Japan, and therefore suggested that fruit horn number can exhibit intraspecific variation and is therefore not a reliable diagnostic character. Molecular approaches such as the present study, and previous studies by Takano and Kadono (2005) and Li et al. (2016), can be helpful for identifying genetic clusters with which to stratify comprehensive morphological studies across the entire geographic range of *Trapa* to clarify its taxonomy and identification.

4.1 Management Implications

Invasive *Trapa* populations in the US are typically managed with hand-pulling or treatment with the herbicides 2,4-dicholorophenoxy acid (2,4-D) and triclopyr (Hummel and Kiviat 2004, Poovey and Getsinger 2007, GLIMRIS 2012). In both cases, the ideal timing of control is before seed set in order to reduce the number of seeds available for recolonization the following growing season. Distinguishing the two US *Trapa* taxa is important for determining whether they exhibit any differences in phenology or response to different herbicide formulations and rates that would influence the development and implementation of control strategies. There is also increasing interest in the potential for biocontrol of *Trapa*, with two promising beetle candidates (Pemberton 1999, Pemberton 2002, Ding and Blossey 2005). Since biocontrol typically involves matching specific herbivores to plant lineages as specifically as possible, distinguishing the two US *Trapa* taxa is important for studies evaluating possible biocontrol agents. Finally, distinguishing the two *Trapa* taxa will also be important for identifying new populations, and monitoring the spread of *Trapa* over time. Although the two taxa can be distinguished morphologically, the distinguishing features are primarily in their flowers and fruits (Fig. 3 and Supp. Table 3). So, genetic identification methods will be particularly useful for populations that have not yet flowered or set fruits, or for which the management strategy includes limiting the development of fruits.

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Figure Legends

Figure 1. STRUCTURE plot of cluster assignments for all *Trapa* samples (*n* = 304) into three genetic groups (K = 3; top), five genetic groups (K = 5; middle), and seven genetic groups (K = 7; bottom). Each vertical bar represents one individual, and color indicates the probability of each individual belonging to each genetic cluster.

Figure 2. Plot of consensus neighbor-joining tree for 304 *Trapa* individuals based on 129 AFLP markers. Bootstrap scores were generated with 5,000 iterations. Groups and individuals are color coded by species/collection location, and correspond to the colors in Figure 1.

Figure 3. **A**) Plot of the first two Principal Components Axes of numerical (width across upper spines, height, thickness across main fruit body, width across lower projections) and categorical (presence or absence of a crown, number of upper spines, orientation of upper spines, angle, and orientation of lower projections) morphological variables measured for 254 *Trapa* individuals collected worldwide. **B**) Plot of first two Principal Components Axes of numerical (width across upper spines, height, thickness across main fruit body, width across lower projections) and categorical (presence or absence of a crown, number of upper spines, orientation of upper spines, angle, orientation of lower projections, and underside of leaf color) morphological variables measured for 128 *Trapa* individuals collected in the United States. Taxa and populations are color coded as in Figure 1.

Figure 1

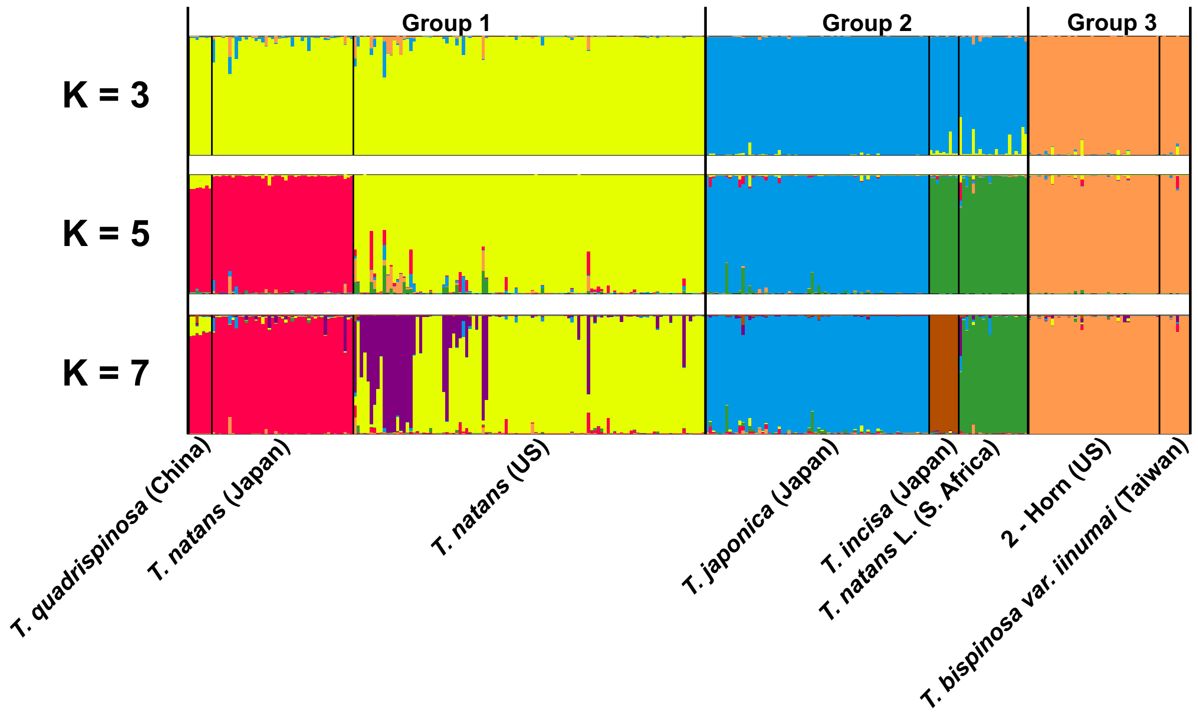


Figure 2

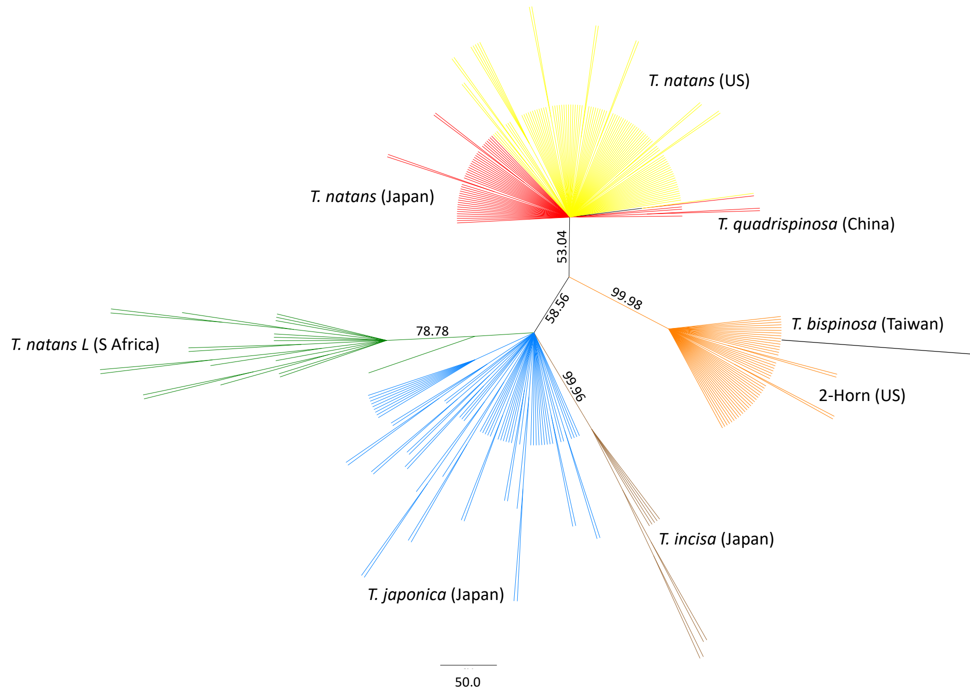
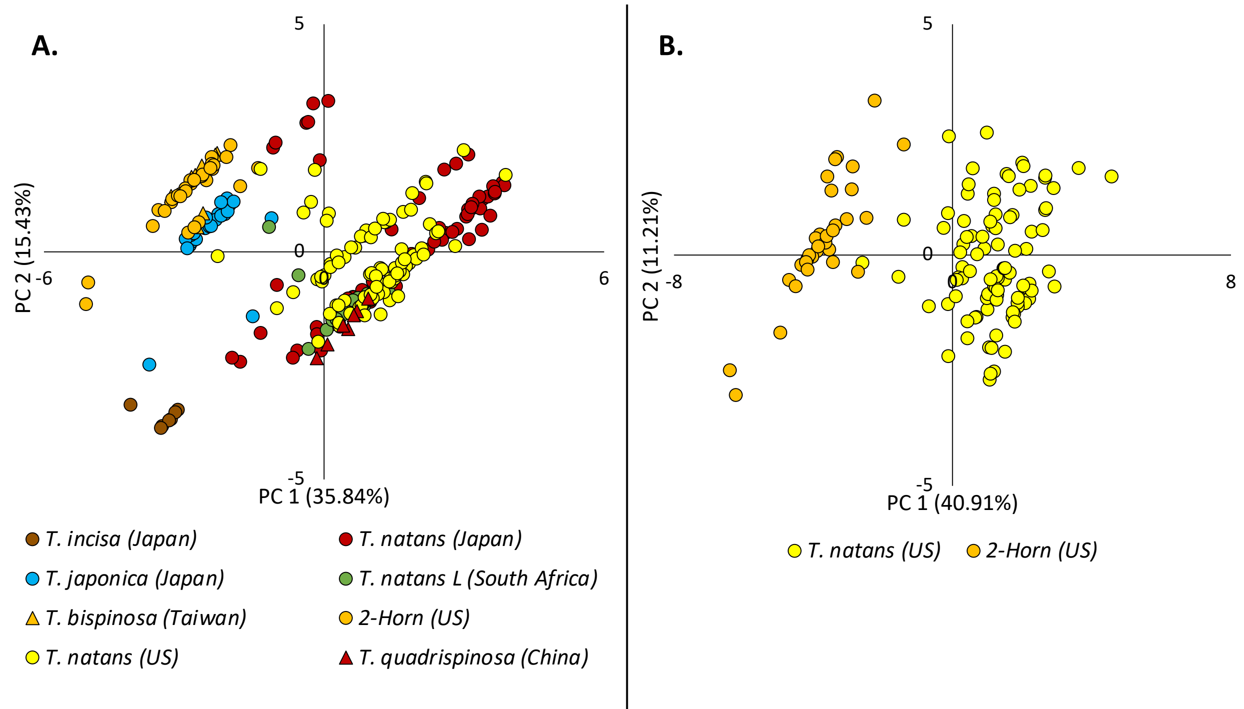


Figure 3



CHAPTER THREE

HERBICIDE RESISTANCE AND RESISTANCE MANAGEMENT OF AQUATIC PLANTS

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HERBICIDE RESISTANCE AND RESISTANCE MANAGEMENT OF AQUATIC PLANTS

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What is resistance?

The Aquatic Plant Management Society accepts the definition of herbicide resistance formulated by the Herbicide Resistant Plants Committee of the Weed Science Society of America (WSSA 1998). In this definition, herbicide resistance is, "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis." In the following paragraphs, we dissect this definition into its key terms.

The term "inherited" means the trait is passed down from parents to offspring, at least in part. This means that offspring tend to resemble their parents (because offspring inherit their genes from their parents). More specifically, this means that variation among individuals for some phenotypic trait (e.g., how a plant responds to different doses of an herbicide) arises partly

because those individuals possess different genes that influence the trait. This is in contrast to variation among individuals that arise solely from environmental factors, such as how much light or nutrients they receive, or whether or not they are under attack by natural enemies such as herbivores or pathogens.

Geneticists measure inheritance (or, heritability) in different ways (see Thum 2018 for a review). Since many aquatic plants can reproduce clonally, heritability can be demonstrated and estimated as differences among clones (genotypes) in a trait of interest (i.e., phenotype) in a common garden experiment. For example, Taylor et al. (2017) demonstrated heritable differences in vegetative growth in the presence and absence of 2,4-D among hybrid watermilfoil genotypes. Similarly, a hybrid watermilfoil genotype from Townline Lake, Michigan, has been vegetatively propagated and repeatedly shown a reduced response to fluridone compared to other genotypes (Thum et al. 2012; Berger et al. 2012, 2015). Therefore, we can confidently conclude that the Townline Lake fluridone response is inherited. For organisms that reproduce sexually, heritability can be estimated as the slope of a regression between the trait values of parents and their offspring, or through various breeding designs employed by quantitative geneticists. Heritability can also be demonstrated if specific DNA sequences can be shown to determine particular traits of interest, since organisms pass their DNA to their offspring. An example of this is the mutations in the phytoene desaturase gene that confer different levels of fluridone resistance in hydrilla (Michel et al. 2004). However, since the genes that determine most phenotypes are unknown, this method is uncommon, and it is important to recognize that inheritance can be demonstrated without knowing the specific gene(s) involved in determining a phenotype.

Next, let’s break down the phrase “dose of herbicide normally lethal to the wild type”. A “wild type” is a prevailing characteristic of individuals of a species in natural conditions. However,

atypical, mutant types can be found in most species. For example, most of us envision the familiar gray squirrel as having a grayish-brown coat on top, with a white coat on bottom. However, occasionally many of us have seen all black or all white squirrels of this same species. In this case, the grayish-brown squirrels are the wild type, and the black or white squirrels are mutants with respect to the wild type. For a given trait, as long as there is a reference for what constitutes a normal, expected response of a species to an herbicide, then any herbicide response that is elevated over that reference would be considered resistance. For example, if four parts per billion (ppb) of herbicide X is normally lethal to the wild type for a species, then the ability of a specific genotype of that species to survive and reproduce at four ppb of herbicide X would constitute resistance, so long as it can be demonstrated that the ability to survive and reproduce at four ppb of herbicide X can be passed down from parents to offspring (Fig. 1).

Resistance arises because mutations occur naturally and randomly when DNA replicates. In some cases, these mutations happen to provide an advantage in survival and/or growth of a plant in the presence of a certain amount of an herbicide. Thus, when a population is exposed to the herbicide, the individuals that happen to have mutations that confer some level of resistance are more likely to survive and reproduce than the wild type individuals. Over time, the population can become dominated by the mutant genotype(s). And, these genotypes could also spread to other lakes.

Herbicide tolerance: The WSSA and APMS distinguish herbicide resistance from herbicide tolerance. Herbicide tolerance is defined as, “the inherent ability of a species to survive and reproduce after herbicide treatment. This implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant.”

A key difference between resistance and tolerance is that the latter is a characteristic “of a species”. For example, many monocots are naturally tolerant to doses of 2,4-D that impact Eurasian watermilfoil, and therefore repeated use of 2,4-D may result in increased abundances of these naturally tolerant species. Therefore, the repeated use of a given herbicide may result in a shift in species composition of an aquatic plant community towards species that are naturally tolerant to that herbicide. This phenomenon is not the same as resistance, because resistance arises from genetic variation among individuals within a species that has a susceptible wild type. Nevertheless, the impacts of selecting for tolerant species via herbicide use can be of management concern.

Examples of herbicide resistance in aquatic plants

As of this writing, there are currently 498 cases of documented weed resistance to 166 different herbicides (International Survey of Herbicide Resistant Weeds, http://www.weedscience.org accessed February 18, 2019.). Most documented cases of herbicide resistant weeds come from terrestrial agriculture. Several aquatic plant species are documented as exhibiting resistance to herbicides in rice agriculture in Australia and Asia. For example, populations of starfruit - *Damasonium minus* R.Br. Buch., dirty dora - *Cyperus difformis*, and arrowhead - *Saggitaria montevidensis* Cham. & Schlechter have evolved resistance to bensulfuron (Graham et al. 1996). Pervasive weed resistance in agricultural systems understandably fuels concerns regarding herbicide resistance in aquatic plant management of private and public waters.

Documented cases of herbicide resistance in aquatic plant management of natural environments are rare compared to the large number of documented cases in terrestrial agriculture. The most well-known case of herbicide resistance in aquatic plants is fluridone resistance in dioecious

hydrilla (*Hydrilla verticillata* L.f. Royle; Michel et al. 2004). In addition, a population of duckweed (*Landoltia punctata*) in a Florida canal was shown to exhibit a high level of resistance to diquat and paraquat compared to the wild-type (Koschnick et al. 2006). Similarly, a genotype of hybrid Eurasian × northern watermilfoil (*Myriophyllum sibiricum* × *M. spicatum*) from Townline Lake, Michigan, has been documented to be resistant to fluridone compared to other Eurasian and hybrid watermilfoil genotypes (Berger et al. 2012, 2015, Thum et al. 2012).

The dearth of documented cases of herbicide resistance in managed aquatic plants in private and public waters in the US begs the question of whether herbicide resistance is truly rare in managed aquatic plants, or whether it isn't explicitly tested for or reported enough. It is possible that the factors that promote herbicide resistance (see below) are commonly lacking in aquatic plant management, leading to very low occurrences of resistance evolution. However, it is also possible that reduced efficacy in some populations goes undetected because of a lack of quantitative pre- and post-treatment monitoring, or because explanations for reduced efficacy do not consider the possibility of resistance (e.g., are explained by environmental factors that may have limited the dosage of herbicide). Thus, it is important to quantitatively monitor herbicide efficacy, and to consider herbicide resistance when efficacy is lower than expected. Conclusive testing for resistance would then come from laboratory dose-response curves comparing putative resistant to known wild-type, susceptible genotypes.

Factors that may influence the probability of herbicide resistance

It is important to recognize that there are different physiological and genetic mechanisms for herbicide resistance. **Target-site resistance** refers to mutations that lead to changes in the molecule(s) where the herbicide typically binds, leading to less effective binding, and thus less

impact on the weed. For example, amino acid changes in the phytoene desaturase gene in hydrilla cause resistance to fluridone (Michel et al. 2004). It is generally thought that herbicides with a single site of action will be more prone to the evolution of resistance, since mutations occurring at the target enzyme can directly confer resistance. It is therefore important to note that many of the herbicides registered for aquatic plant control target a single enzyme.

In contrast, **non-target site resistance** refers to genetic changes that occur not at the herbicide's site of action, but at other genes that are related to herbicide uptake, translocation, sequestering, detoxification, or metabolism. Whether herbicide resistance results from target versus non-target site mutations should be contingent upon the supply of mutations available within a population when an herbicide is applied.

Genetic mechanisms for herbicide resistance can also be broadly classified into single-gene versus polygenic (multiple genes). In single-gene resistance, the level of resistance observed is conferred by mutation at a single gene. Most likely, this would occur at the target site. For example, fluridone resistance in hydrilla appears to be conferred by mutations at the gene that codes the target enzyme (phytoene desaturase). However, resistance can occur via changes at multiple genes, each of which confers some fraction of the overall resistance observed. Thus, a given level of resistance could reflect mutations at a single gene that have a large effect on resistance, or by mutations at many genes that each have a small effect on resistance.

In general, high rates of herbicides should select for mutations of large effect at one gene; most likely, the target enzyme. However, low rates of herbicides should select for polygenic resistance. At low rates, and in genetically diverse populations, there may be alleles at different genes that can each allow higher survivorship and reproduction at low rates. For example, some individuals may carry genes that are capable of metabolizing small amounts of herbicide, allowing their

continued growth in the presence of low doses of herbicide. And, other individuals may carry genes that are capable of sequestering small amounts of herbicide. The population of surviving individuals are then enriched for these two different genes, both of which confer a small degree of herbicide resistance (e.g., survival and continued growth at low doses). Subsequent intercrossing of individuals carrying these two different genes can allow "gene stacking" that confers resistance to a higher dose of herbicide because the new genotypes can both metabolize and sequester. Examples of this phenomenon can be found in the terrestrial agricultural literature, but it is unknown whether or how commonly this occurs in aquatic plants. Certainly, the exposure of plants to low rates of herbicides is inevitable in many operational aquatic plant management projects that utilize spot treatments in large water bodies, where herbicide will rapidly dissipate. Gene stacking of low-level resistance alleles will depend on the extent of genetic variation within and among populations, and the extent to which sexual reproduction occurs. Thus, different control tactics (e.g., whole lake applications maintaining long contact times versus spot applications that likely dissipate to sublethal doses) may interact with plant life histories (e.g., exclusively asexual versus sexual or mixed reproductive strategies) to influence the probability and magnitude of herbicide resistance. Strong academic research on this issue could be helpful in the future for building better models of when, where, and how resistance could occur, as well as developing efficient tools to detect it (e.g., genetic).

Resistance Management

Traditionally, the idea behind resistance management is to prevent or delay the evolution of resistance. Some commonly used methods of resistance management are listed below:

Rotations and/or combinations of herbicides with different modes of action can limit resistance evolution since individuals are less likely to be resistant to all of the herbicides in the mixture or rotation. The premise is that resistance to different herbicides will likely require mutations in different genes. And, since mutations are random and rare, it is unlikely that an individual will happen to have multiple mutations that confer resistance to multiple herbicides. However, if populations are large enough and/or genetic diversity and mutations are plentiful, populations can be resistant to multiple modes of action (see http://www.weedscience.org).

Another resistance management strategy is to allow a sufficient amount of time in between applications of the same herbicide. This idea is based on the premise that resistant genotypes that survive treatment may exhibit a trade-off whereby they are less competitive growers in the absence of the herbicide (i.e., there is cost to resistance; Hendry et al. 2011). Therefore, by waiting between treatments, the number of resistant individuals should go down if there is a cost to being resistant in the untreated environment. However, it is important to recognize that the success of this strategy depends on sufficient costs of resistance, which isn't necessarily true. In addition, it isn't clear how long in between treatments is sufficient. Therefore, this strategy relies on having detailed information on the level of resistance, costs, and dynamics of competition over time.

One method that is used to reduce resistance evolution in agricultural systems is to maintain untreated populations adjacent to treated populations (Hendry et al. 2011). The hope is that interbreeding with an untreated population where there is no selection for resistance will keep resistance genes from becoming dominant in the treated population. This strategy has not been intentionally implemented in aquatic plant management to our knowledge. However, it is possible that this strategy is implemented de facto because across the landscape there is a mosaic of

treated and untreated lakes. Furthermore, spot treatments are common within many lakes, which may ultimately have this same effect. However, it is generally thought that sexual reproduction plays a small role in the overall reproduction of most managed aquatic plants, so it is unclear whether the intentional implementation of this strategy would work, unless resistant genotypes are frequently replaced by wild-type genotypes due to a cost of resistance (see above).

In addition to resistance management strategies, it is important that managers implement best management practices to maximize the impact of any control implementation. First, early detection and rapid response methods are recommended so that populations are treated when they are small and before they become a problem. The probability that a population harbors a resistance mutation will be proportional to its size, and therefore small populations are less likely to harbor a resistance mutation, unless the population was initiated by a resistant genotype to begin with (e.g., colonized from a nearby source that is resistant). Second, it is generally recommended to treat with the maximum allowable herbicide rate to minimize the number of surviving plants. Finally, management should include quantitative monitoring efforts so that surviving plants are identified and targeted for appropriate follow-up management, and so that changes in efficacy over time can be identified (see below).

Limitations to implementing resistance management in aquatic plant control operations –

Most stakeholders in aquatic plant management recognize the potential for herbicide resistance, and therefore the benefits of practices that prevent or delay the evolution of resistance. However, it is also important to recognize that there are logistical realities that will limit the implementation of resistance management practices in many locations.

Probably the most limited options to practice resistance management will be for large, public, multiple use water bodies where numerous factors influence the control options available. In these water bodies, the choice of herbicide, dose, and timing is influenced by a variety of factors including balancing different uses of the water body, cost of management, selectivity and efficacy, and hydrology. Given the limited number of herbicides available for aquatic use, the upshot is that resistance management practices that involve rotations or mixtures of different products may be infeasible in certain water bodies, and limited to non-herbicidal practices.

A second limitation to resistance management practices is justification for implementing them if there is no evidence that repeated use of an herbicide in a water body is selecting for resistant genotypes. It remains to be seen whether the risk of herbicide resistance in aquatic plant management is high, and to date there are few documented examples of resistance. The evolution of resistance will be limited by the supply of mutations available to confer enhanced survival and reproduction in the face of herbicides. Thus, herbicide resistance involves a "waiting time" for mutations, which may or may not occur over the life of a management project. Given the regulatory demands for demonstrating selectivity and efficacy, managers may be resistant to adopting resistance management strategies if they have no evidence that their favored strategy and tactics for a water body are deemed efficacious, especially if alternatives employed in resistance management practices are more costly and/or have less public support for their use.

Finally, a geographic region may represent a mosaic of water bodies that do and do not implement resistance management practices. In this case, it would be reasonable for managers that voluntarily implement resistance management practices (or are required to do so) at additional costs to ask themselves why they are doing it if others are not. Furthermore, because many aquatic plants can spread vegetatively among water bodies, locations with resistance

management practices could potentially be colonized by resistant genotypes from other areas that do not practice resistance management. The upshot is that it is important to be looking for resistance, and understanding its origins, even when resistance management is practiced.

Recommendations for addressing the potential for herbicide resistance –

As a practical matter then, how should aquatic plant managers address the issue of resistance? Managers should consider all available options to implement resistance management practices wherever and whenever possible. However, the likely reality is that in many situations, resistance management practices will be limited. Our opinion is therefore that an immediate priority should be to develop and implement methods that objectively and definitively identify whether control efficacy is lower than expected, and the likelihood that reduced efficacy is due to the presence of resistant genotypes.

Lake management plans should have monitoring protocols that can objectively and definitively separate out factors influencing the variation in control efficacy. For example, temporal data on control efficacy when the same control tactic is repeatedly employed should be informative for identifying trajectories of reduced efficacy over time within a water body. In such a case, laboratory determinations of dose-responses to the current practice and alternatives could be used to determine whether a change in management strategy is warranted. This kind of objective data could provide compelling evidence to warrant changes in management strategies or tactics that may otherwise be resisted due to increased costs, regulatory hurdles, or unfavorable public opinion.

Genetic tools hold promise to increase the efficiency in which we are able to test for resistant genotypes, and identify resistance evolution. Since resistance evolution occurs as the

displacement of genotypes that have wild type sensitivity to herbicides by mutant genotypes that are resistant, genetic information could assist in the identification of resistance. More specifically, we expect to see shifts in the genetic composition of populations over time if resistance is developing within a water body. Moreover, since most aquatic plants reproduce primarily by asexual means (e.g., fragments, turions, etc.), we may expect lakes that have quantitative data demonstrating lower than expected control efficacy to be dominated by a single clone. Thus, these genetic signatures can be used to prompt laboratory dose-response studies of specific genotypes suspected of exhibiting herbicide resistance.

Genetic tools currently available for hydrilla and Eurasian watermilfoil illustrate the promise of these tools. In the case of hydrilla, fluridone-resistant biotypes can be identified with genetic data (Benoit and Les 2013). These tests can help managers determine the dose of fluridone that would be required for efficacy given the genetic composition of the population, or whether fluridone is an inappropriate control option for that particular population. Similarly, a fluridone-resistant genotype has been identified in hybrid Eurasian watermilfoil (Thum et al. 2012; Berger et al. 2012, 2015). Molecular markers can distinguish this genotype from other genotypes, and this genotype has been discovered to have spread to at least several populations in Michigan (Pashnick and Thum submitted). Thus, genetic surveys of hybrid Eurasian watermilfoil can be used to determine whether fluridone should be applied to particular lakes. The characterization of genotypes in other species could be employed in the same way.

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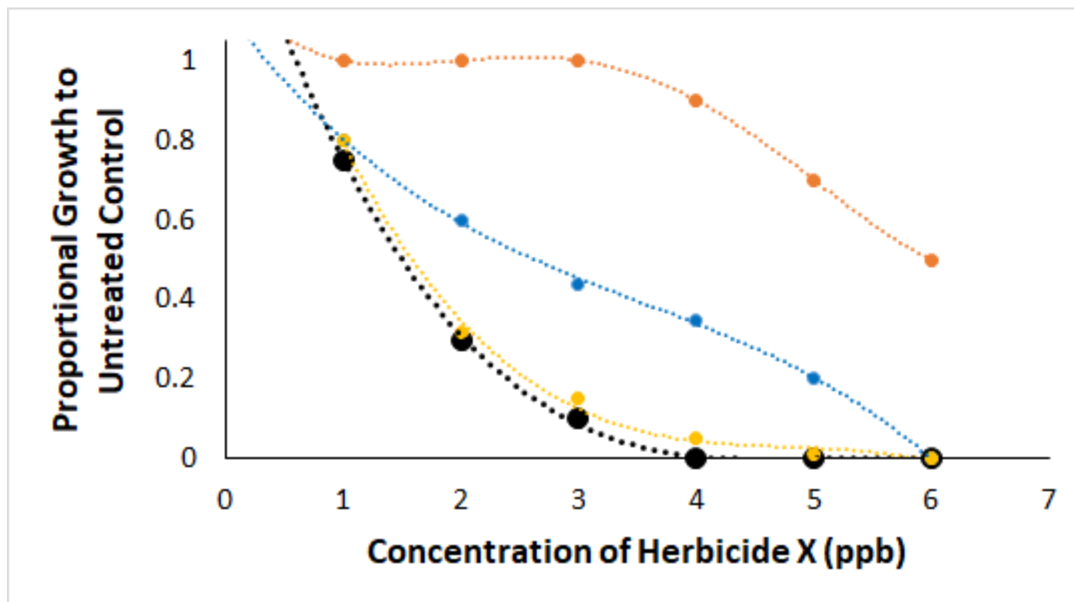

Figure 1. Example of genetic variation for herbicide response. The wild type (large black circles) is normally killed by four parts per billion of herbicide X. However, other genotypes have different responses. In some cases, response to the herbicide may only be slightly elevated for a different genotype (small yellow circles). However, other genotypes may exhibit a high level of resistance at concentrations normally lethal to the wild type (small blue circles and small orange circles).

CHAPTER FOUR

IDENTIFICATION OF RESISTANT CLONES OF EURASIAN (*MYRIOPHYLLUM SPICATUM*) AND HYBRID (*M. SPICATUM* X *M. SIBRICUM*) WATERMILFOIL TO AN OPERATIONAL RATE OF FLURIDONE

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Contributions: RAT contributed to study design and writing.

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Identification of resistant clones of Eurasian (*Myriophyllum spicatum*) and hybrid (*M. spicatum* X *M. sibricum*) watermilfoil to an operational rate of fluridone

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Abstract

Genetic assays to identify herbicide resistant plants are a promising tool to reduce herbicide failures. However, the genetic basis of herbicide resistance is frequently unknown. In clonal weed species, DNA fingerprinting could be a useful tool to identify known resistant versus susceptible genets (clones) that occur in multiple locations, without an immediate need for understanding the genetic mutation(s) conferring resistance. Eurasian watermilfoil (*Myriophyllum spicatum* L.) is a mostly clonal invasive aquatic plant, and the same clones can be found in multiple waterbodies. Previously, a clone was confirmed as resistant to the commonly used herbicide, fluridone, and a recent genetic survey in Michigan identified this genotype (MG-237) in at least seven other lakes. We hypothesized MG-237 collected from different lakes would also exhibit fluridone resistance.

However, MG-237 may have accumulated resistance mutations at different times during its spread across Michigan, resulting in fluridone resistant and susceptible MG-237 clones distributed in different lakes. We used an herbicide assay to test the response of several accessions, including MG-237 accessions from multiple lakes, to the Michigan operational rate of 6 $\mu g\ L^{-1}$ fluridone. We found that all accessions of MG-237 exhibited resistance to 6 $\mu g\ L^{-1}$ fluridone. A second genotype (MG-377) was also resistant to 6 $\mu g\ L^{-1}$ fluridone. The rest of the accessions were found to be significantly injured by 6 $\mu g\ L^{-1}$ fluridone. Our results suggest that 6 $\mu g\ L^{-1}$ fluridone would not effectively control waterbodies dominated by MG-237 or MG-377, whereas waterbodies dominated by the other genotypes in our study would likely be controlled. Although more studies are needed to identify the variation in sensitivity of the accessions tested here, and the genetic basis of fluridone resistance in watermilfoil, our results suggest that multilocus genotype data may be an effective tool to identify and track herbicide-resistant genotypes of watermilfoil in the short-term.

Management Implications

This study demonstrates that pre-treatment genetic monitoring combined with herbicide characterization of a prioritized set of clones could aid herbicide management decisions for the invasive aquatic plants, Eurasian (*Myriophyllum spicatum* L.) and hybrid (*M. spicatum* X *M. sibricum*) watermilfoil. For example, our study confirmed resistance to the operational rate of fluridone used in Michigan to control watermilfoil (6 µg L^{-1}) in accessions from five different lakes containing the same microsatellite multilocus genotype (MG-237), and from a second lake with a different genotype (MG-377). Therefore, we suggest that fluridone would not be effective treatment for invasive watermilfoil control in lakes where MG-237 or MG-377 occurs at a high frequency. Conversely, our study identified several multilocus microsatellite genotypes that appeared susceptible to 6 µg L^{-1} fluridone, and some of these genotypes have been identified in multiple Michigan lakes. Therefore, we suggest that fluridone would likely be an effective treatment in lakes that consist mostly of one or more of the susceptible genotypes identified in our study. More generally, our study suggests that building a 'catalog' of prioritized genotypes (e.g., those that occur most commonly across the landscape) and their response to commonly used herbicides could be used to predict herbicide outcomes without an immediate need for identifying the genetic basis of herbicide resistance. Further, since clones are frequently shared across nearby waterbodies, managers may be able to pool resources to identify herbicide responses to common clones in their region.

Key Words: Herbicide resistance, Aquatic plant management, Herbicide assay

Introduction

When used effectively, herbicides are estimated to do the work of approximately 70 million workers (Gianessi and Reigner 2007). However, the effectiveness of herbicides is threatened by the evolution of herbicide resistance (Oreke 2006; Baucom 2019). Currently there are 262 species of weeds resistant to at least one mode of herbicide action (Heap 2020). One potential tool to help maintain the efficacy of herbicides is pretreatment screening for molecular markers that identify resistant versus susceptible individuals. Identification of individuals in a population slated for herbicide treatment that are resistant to certain herbicides can reduce the evolution and spread of resistance. There are currently genetic marker assays in some species of managed weeds for genes that confer herbicide resistance. For example, alleles of acetyl-CoA carboxylase (ACCase; Menchari et al. 2006) and phytoene desaturase (PDS; Benoit and Les 2013) are known to confer resistance to ACCase inhibiting herbicides and fluridone (1-methyl-3-phenyl-5-[3-(trifluromethyl) phenyl]-4(1*H)*-pyridinone), respectively. EPSPS (5-enolypyruvyl-shikamate-3-phosphate synthase) copy number can also be used to determine resistance to glyphosate (Chathamet al. 2015). Pretreatment identification of resistant individuals using these types of genetic markers can then inform herbicide decisions in order to maintain resistant individuals at a low frequency in managed weed populations.

For clonal organisms, characterizing herbicide response of widespread clones would potentially inform herbicide response of the same clones in all locations they are found. Therefore, genetic markers that are able to identify ramets of the same genet and distinguish unique genets may also accurately predict herbicide response. Because all genes of a clone are essentially linked, clone

identification may be as good a predictor of herbicide response as causal herbicide resistance alleles in the short-term.

Eurasian watermilfoil (*Myriophyllum spicatum* L.) including its hybrid with native northern watermilfoil (*Myriophyllum sibiricum* × *M. spicatum*) are heavily managed with herbicides in the US (Bartodziej and Ludlow 1998). Experimental studies of watermilfoil clearly indicate that distinct genotypes can differ in vegetative growth and herbicide response properties (Thum et al. 2012; Netherland and Willey 2017; Taylor et al. 2017). Although watermilfoil is capable of sexual reproduction, clonal reproduction is common (Hartleb et al. 1993), and the same clones of watermilfoil have been distinguished using multilocus microsatellite genotyping (MG) within and among regions (Taylor et al. 2017; Thum et al. 2020). Therefore, if herbicide response were characterized for widespread watermilfoil genotypes, managers may be able to make informed herbicide treatment decisions based on the genotypes that are present in a lake.

The herbicide, fluridone (WSSA Group 12) is commonly used in aquatic plant management and is typically an effective tool to reduce watermilfoil populations (Berger et al. 2012; Thum et al. 2012). However, a failed treatment of hybrid watermilfoil using fluridone in Townline Lake, Michigan, raised concerns for the possibility of herbicide resistance. Laboratory testing found that hybrid watermilfoil collected from Townline Lake exhibited resistance to the operational rate of fluridone used in Michigan (Berger et al. 2012; Thum et al. 2012; Berger et al. 2015).

A recent genetic survey of watermilfoils across the state of Michigan identified the same genotype found in Townline Lake (hereafter referred to as MG-237) in at least seven other lakes

in Michigan (Thum et al. 2020; Thum additional unpublished data). Given that it is unlikely that the same genotypes arose independently through sexual reproduction, it follows that all MG-237 individuals are clones of the same lineage. We therefore expect that the same genotypes will exhibit the same resistance phenotype in response to fluridone, because they are the same clonal lineage that has spread across the landscape. However, genotypes that share ancestry through clonal reproduction can still differ in their fluridone response because of somatic mutations (e.g., Michel et al. 2004). For example, if mutation(s) conferring fluridone resistance arose before MG-237 spread to other lakes, then we would expect all lakes where MG-237 clones occur to exhibit fluridone resistance. Alternatively, it is possible that an ancestral, fluridone-sensitive MG-237 spread across the landscape, and that subsequent somatic mutation(s) conferred fluridone resistance in one or a subset of lakes where it occurs.

In this study we used a 6 $\mu g\ L^{-1}$ fluridone response assay to determine the susceptibility of the same and different watermilfoil genotypes to the operational rate of fluridone used in Michigan. Specifically, we tested whether MG-237 clones sampled from different lakes in Michigan exhibited resistance to the Michigan operational rate of fluridone, as would be expected if the genotype spread across the landscape after fluridone resistance evolved. While studies of Townline Lake watermilfoil have shown resistance to fluridone (Berger et al. 2012; Thum et al. 2012), it is unknown how common fluridone resistance is in watermilfoil. For this reason, we tested the response of several other genotypes to begin building a ‘catalog’ of fluridone responses for different watermilfoil genotypes. This catalog may also be used to inform fluridone treatment decisions in the future when managers see a characterized genotype in their lakes.

Methods

We tested the response to fluridone of 13 watermilfoil accessions. Here, we define an accession by the combination of its taxon (Eurasian vs hybrid), multilocus microsatellite genotype, and where/when it was collected, as the same multilocus microsatellite genotype can be found in different lakes (Table 1). A culture of each accession was initiated from a single meristem collected from the field and vegetatively propagated at Montana State University's Plant Growth Center (Bozeman, MT). The multilocus microsatellite genotypes for each accession were determined in a previous study (Thum et al. 2020).

In order to determine the extent of fluridone resistance in clones of MG-237 found in multiple waterbodies, seven accessions of MG-237 were included in our fluridone assays. Accessions one through five were all MG-237 but were collected in different lakes (Table 1). Accessions five and six were MG-237 collected in the originally-identified fluridone resistant lake (Townline Lake, MI), but in different years; the accession collected in 2008 preceded the documentation of fluridone resistance (Berger et al. 2012; Thum et al. 2012), whereas the accession collected in 2017 represents a recolonization of that same genotype after several years of management with alternative herbicides (Table 1). To begin building a catalog of fluridone responses, seven additional accessions with unique multilocus microsatellite genotypes were also included (accessions 7-13) in our fluridone assays (13 total accessions) (Table 1).

To measure the response of each accession to fluridone, we used a treatment vs control assay. While the initial identification of herbicide resistance in a species should use a dose-response assay with several herbicide concentrations above and below the operational field rate (Burgos et

al. 2013; Burgos 2015), fluridone resistance has previously been confirmed using a dose-response assay in Townline Lake, MI watermilfoil (MG-237; Berger et al. 2012; Thum et al. 2012). As is common in herbicide resistance screens (Burgos 2015), we were logistically constrained by space for housing large tanks suitable for growing and testing multiple accessions at multiple treatment levels. We therefore decided to prioritize the number of accessions to assay over the number of treatment levels. Because regulation in Michigan, where MG-237 is widespread, restricts the use of fluridone above 6 $\mu g\ L^{-1}$, we chose this as the discriminating dose for our fluridone resistance assays. Our assays directly followed the recommendations of Burgos (2013; 2015) for confirming herbicide resistance and included three replicate tanks of both the discriminating dose (6 $\mu g\ L^{-1}$) and untreated control.

We replicated the whole fluridone assay twice on all 13 accessions of watermilfoil (hereafter referred to as trial one and two). For each trial, we vegetatively propagated each accession in separate tanks to generate enough meristems for the assay. We planted 8 cm meristems of each accession into cone-tainers with soil (1:1:1 peat:topsoil:sand) capped with pure sand. For each of the two trials, we used six 378.5 L steel cattle tanks with approximately 10 cm of soil capped with sand on the bottom and filled with approximately 296.5 L of Smart and Barko (1985) buffered water. To account for tank variance, three of each cone-tainer planted accession was randomly arranged into each of the six tanks by inserting the cone-tainer approximately 7.5 cm deep into the soil at the bottom of each tank. Only one meristem of accession six and eight were included per tank in trial one because there were not enough meristems in culture to draw from. Both trials were conducted under greenhouse conditions with ambient and supplemental

incandescent lighting to maintain a 16-hour light:8-hour dark period. Adequate aeration of the water in each tank was maintained with an air bubbler for both trials.

Our target concentration exposure time (CET) for both trials was 6 µg L^{-1} fluridone for 50 days. After a 12 day establishment period, we randomly selected three of the six tanks in each trial to receive a treatment of fluridone (Sonar Genesis®, SePRO Corp. 11550 N. Meridian St. Carmel, IN 46032). Approximately two weeks after treatment, we measured fluridone concentration in each tank (SePRO Corp. FasTEST®) to determine if fluridone concentration needed to be adjusted in the treated tanks. Based on the fluridone concentration measurements, we discovered a clerical error in the first trial regarding the stock concentration of the fluridone used, which resulted in an initial concentration of only 3 µg L^{-1}. Therefore, 20 days after the initial fluridone treatment, the concentration in treated tanks was increased to 6 µg L^{-1} fluridone. Because our target CET was 6 µg L^{-1} fluridone for 50 days, we extended the first trial for 10 days (60 days total treatment time) to equal the same total fluridone exposure for the two trials (3 µg L^{-1} * 20 days + 6 µg L^{-1} * 40 days = 300 µg L^{-1}days = 6 µg L^{-1} * 50 days). In trial two, the target 6 µg L^{-1} fluridone concentration was achieved and maintained for 50 days in treated tanks. While the two trials differed in the above regards, we saw an overall fluridone treatment effect (Table 2). Over the treatment period in both trials, water temperatures ranged from 18-22° C and pure water was replaced weekly as it evaporated. At the end of the trial, plants were harvested and dried to a constant mass. We then measured dry biomass of each plant.

We used a linear mixed effects model (*lme4* package in R) to test for fixed effects of Treatment and Accession while removing random effects of Tank and Trial. Trials also included three replicates of each accessions planted within a tank, but these tank accession replicates were not included as a random effect in the model because the model fit (using the Akaike information criterion) was higher without them. We then used the *emmeans* package in R to calculate estimated marginal means (emmeans) and pairwise contrasts between the control and 6 µg L^{-1} fluridone treatments within each accession (Supplementary Appendix S1).

Results and Discussion

Overall tank dry biomass was reduced in the 6 µg L^{-1} fluridone treated tanks when compared to the control tanks (effect of treatment in Table 2; see also accession responses in Figure 1), indicating that 6 µg L^{-1} fluridone was effective at decreasing overall watermilfoil biomass. However, an analysis of variance on the linear mixed effects model of the data also showed a significant accession by treatment interaction (Table 2), indicating that different accessions responded differently to the 6 µg L^{-1} fluridone treatment. This trend can also be visually seen in the qualitatively differing slopes if the interaction plot of control and 6 µg L^{-1} treatment estimated marginal means in each accession (Figure 1).

None of the MG-237 accessions were significantly negatively affected by 6 µg L^{-1} fluridone treatments (Figure 1). Accessions one through five showed a non-significant difference in dry biomass emmeans between control and 6 µg L^{-1} fluridone treatments ($P = 0.1323 – 0.7831$; Figure 1). Accession six showed a significant difference in emmean biomass between control and 6 µg L^{-1} fluridone treatments ($P < 0.0001$), but mean dry biomass was larger in 6 µg L^{-1} fluridone

than in control treatments for that accession (Figure 1). These results align with the previous dose-response assays that showed MG-237 is resistant to 6 µg L^{-1} fluridone (Berger et al. 2012; Thum et al. 2012) and a failed 6 µg L^{-1} fluridone treatment in Townline Lake, Michigan (Thum et al. 2012). Therefore, we conclude that 6 µg L^{-1} fluridone treatments would not be effective at reducing MG-237 biomass, because MG-237 accessions tested here also showed resistance to the Michigan field rate of 6 µg L^{-1} fluridone. These results also align with the expected pattern of a clonal lineage that evolved resistance before its spread across the landscape. We therefore recommend that any lakes found to be dominated by MG-237 individuals should not be treated with fluridone, as they will likely exhibit resistance to the typical 6 µg L^{-1} fluridone treatment for Eurasian watermilfoil.

Accession seven also showed resistance to 6 µg L^{-1} fluridone treatments because emmean dry biomass in 6 µg L^{-1} fluridone treatments was significantly larger than in control treatments for that accession ($P = 0.0069$; Figure 1). Accession seven is a unique genotype (MG-377) from Lake Lansing, Michigan. We therefore conclude that the Michigan field rate of 6 µg L^{-1} fluridone treatments are likely to be ineffective in the long term at controlling watermilfoil biomass in Lake Lansing. Further genetic monitoring is needed to determine whether MG-377 is present in other lakes, and if so, fluridone is likely to be an ineffective treatment method in those lakes as well.

As predicted, several accessions were significantly reduced with 6 µg L^{-1} fluridone (Figure 1). Accessions eight through thirteen all showed a significant decrease in emmean dry biomass in 6 µg L^{-1} fluridone treated versus control treatments ($P = <0.0001 – 0.0167$; Figure 1). This significant decrease in emmean dry biomass in 6 µg L^{-1} treated versus control indicates that these

accessions are more likely to be effectively controlled by 6 μg L^{-1} fluridone treatments than MG-237 and MG-377. However, when looking at the slopes of the lines between control and treatment emmeans in Figure 1, they suggest that significantly reduced accessions identified here likely exhibit variation in the amount of dry biomass reduction with 6 μg L^{-1} fluridone. Our data suggest that there is variation in the response to fluridone of significantly reduced accessions tested here. Dose response assays on these accessions are therefore warranted in order to determine their variation in sensitivity to fluridone treatments.

One interesting trend that came out in this study is that accession six and seven showed an increase in growth in the fluridone treatment compared to the untreated control (Figure 1). Previous dose response studies of Townline Lake watermilfoil also found more growth in 6 μg L^{-1} fluridone treatment when compared to the untreated control. (Berger et al. 2012; Thum et al. 2012). This increased growth in 6 μg L^{-1} fluridone treatment may indicate some density regulation of growth in resistant accessions. Further investigation into the mechanism/s of fluridone resistance in these accessions of watermilfoil may elucidate the cause of this and whether it is a potential resistance trade-off.

This study also demonstrates that the characterization of herbicide response for common and/or putatively problematic watermilfoil genotypes is likely to predict efficacy outcomes for managers who perform pretreatment genetic monitoring. For instance, if managers detect MG-237 at a high frequency in their lake, then according to the results here, fluridone would not be an effective control tactic for reducing biomass of watermilfoil in that lake. Conversely, if lakes are mostly dominated by one of the significantly reduced genotypes identified here, then fluridone may be an

effective control tactic to reduce watermilfoil biomass. Preliminary data suggest that watermilfoil clones are frequently shared across nearby lakes (Thum et al. 2020). Therefore, the continuation of assays like these and dose response assays to build a 'catalog' of herbicide responses for genotypes of watermilfoil found across the managed landscape may be an effective short-term management tool for watermilfoil with a region-wide benefit to managers.

While the above catalog will be invaluable for lakes harboring characterized genotypes, there may be too many genotypes to exhaustively characterize them all. Thus, in the long-term, the genetic basis of fluridone resistance in watermilfoil should be explored. Distinguishing the (or closely linked) gene/s resulting in fluridone resistance would ultimately be a more effective marker to scan for in populations. Similar to the mutation that indicates fluridone resistance in the aquatic plant hydrilla (*Hydrilla verticillata*), this type of marker could predict resistance regardless of genetic background (Michel et al. 2004; Benoit and Les 2013). However, in the short-term, identifying and prioritizing specific genotypes for herbicide characterization could help preserve the effectiveness of currently used herbicides for watermilfoil control.

Supplementary Materials

Supplementary Appendix S1. Raw dataset and R based statistical analyses of data.

Acknowledgments

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Tables and Legends

Table 1. Description of the thirteen watermilfoil accessions in this study, including their multilocus microsatellite genotype ("Genotype"), taxon (EWM = *Myriophyllum spicatum* L. and hybrid = *Myriophyllum sibiricum* × *M. spicatum*), and the lake, US state, and year it was collected from the field.

Accession	Genotype	Taxon	Waterbody, State	Year Collected
1	MG-237	hybrid	Indian, MI	2018
2	MG-237	hybrid	Tamarack, MI	2018
3	MG-237	hybrid	Templene, MI	2018
4	MG-237	hybrid	Muskellunge, MI	2018
5	MG-237	hybrid	Townline, MI	2008
6	MG-237	hybrid	Townline, MI	2017
7	MG-377	EWM	Lansing, MI	2018
8	MG-429	hybrid	Hayden, ID	2016
9	MG-268	EWM	Jefferson, MT	2015
10	MG-457	hybrid	Muskellunge, MI	2018
11	MG-5650	EWM	Coeur d'Alene, ID	2015
12	MG-1282	hybrid	Baseline, MI	2018
13	MG-231	hybrid	White, MI	2018

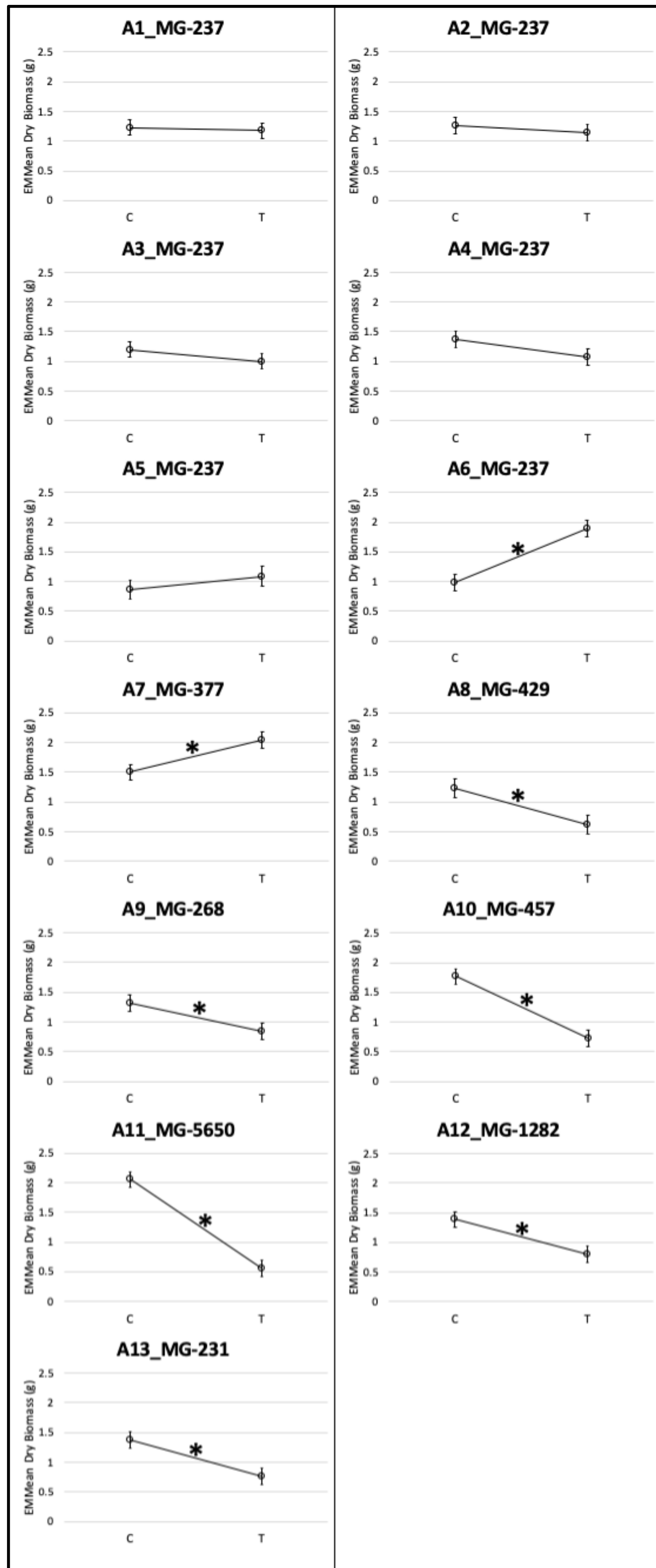
Table 2. Analysis of variance (Type II Satterthwaite's method) table for the linear mixed effects regression model (Dry Biomass ~ Treatment*Accession + (1 | Trial : Tank) + (1 | Tank)) determining the effects of the 'Accession' of watermilfoil exposed to control and 6 µg L^-1 fluridone 'Treatments' on 'Dry Biomass' over a 50day exposure period.

	Sums of Squares	Mean Square	Degrees of Freedom	Denominator Degrees of Freedom	F-Value	P-Value
Treatment	2.485	2.485	1	13.150	9.505	0.009
Accession	18.717	1.560	12	407.150	5.966	<0.001
Treatment*Accession	42.366	3.531	12	407.890	13.505	<0.001

Figure Legends

Figure 1. Interaction plot of the estimated marginal means (emmeans) of dry biomass for each accession (A) in both control (C) and 6 μg L^{-1} fluridone (T) treatment environments. Error bars around the emmeans represent the standard error and "*" represents significant ($P < 0.05$) differences between control and 6 μg L^{-1} fluridone treatment emmeans within that accession. The slopes of the lines between the control and 6 μg L^{-1} treatment emmeans indicate how much an accession was affected by 6 μg L^{-1} fluridone treatment. The title of each plot also includes the multilocus genotype (MG) of the accession plotted.

Figure 1



CHAPTER FIVE

TRANSCRIPTIONAL RESPONSES OF EURASIAN (MYRIOPHYLLUM SPICATUM) AND HYBRID (M. SPICATUM × M. SIBRICUM) WATERMILFOIL WITH DIFFERING GROWTH RATES TO 2,4-D HERBICIDE TREATMENT

Contribution of Authors and Co-Authors

Manuscript in Chapter 5

Author: Gregory M. Chorak

Contributions: GMC designed and executed all aspects of this project and led the writing of the manuscript.

Co-Author: Jennifer Lachowiec

Contributions: JL contributed to the design of the experiment, the analysis of the data, and writing of the manuscript.

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Contributions: RAT contributed to the design of the experiment, sample collection, and writing of the manuscript.

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Transcriptional responses of Eurasian (*Myriophyllum spicatum*) and hybrid (*M. spicatum* × *M. sibricum*) watermilfoil with differing growth rates to 2,4-D herbicide treatment

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ABSTRACT

BACKGROUND: Aquatic plant managers frequently treat Eurasian watermilfoil (*Myriophyllum spicatum* L.; EWM) and hybrid watermilfoil (*Myriophyllum spicatum* L. × *Myriophyllum sibiricum* Komarov) with herbicides. One of the most common modes of action in aquatic plant management are synthetic auxins. However, in experimental studies, different genotypes of the

two taxa have been shown to differ in their growth rates in untreated and herbicide environments. In this project, we focused on comparing the molecular response between a genotype each of Eurasian and hybrid watermilfoil that differ in their growth rates to the commonly used auxinic herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D). We compared gene expression differences between control and $0.5 mg/kg^{-1}$ 2,4-D treated plants at several times after treatment.

RESULTS: We found that the molecular response of the EWM genotype to $0.5 mg/kg^{-1}$ 2,4-D treatment was much stronger than the hybrid genotype, indicated by a greater number of differentially expressed contigs at all time points after treatment. Further, we found that the EWM genotype expressed the genes in the putative 2,4-D response pathway as we would expect, but the hybrid genotype did not, suggesting that the hybrid genotype is less affected by $0.5 mg/kg^{-1}$ 2,4-D treatment.

CONCLUSIONS: The results of this study lead us to hypothesize that this hybrid genotype may be able to mitigate the effects of 2,4-D treatment. Future studies should further investigate the mechanisms of mitigation and whether they are a feature of hybridity, or a genotype-level response.

Key Words: Herbicide response, Aquatic plant management, RNA-Seq

1 INTRODUCTION

Worldwide, approximately 366 million hectares of weeds are treated with synthetic auxin herbicides every year.[1] The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was the first synthetic auxin herbicide developed (in 1945) and is still commonly used for weed control.[2] Like the rest of the synthetic auxins, 2,4-D mimics the natural plant hormone indole-3-acetic acid (IAA).[1-2] There are currently 41 species of weeds resistant to synthetic auxins, and of those 25 are resistant to 2,4-D.[3]

Understanding the mechanism(s) of 2,4-D response is complicated because it induces the same signaling pathways as the naturally occurring hormone IAA. In fact, in high concentrations IAA stimulates the same herbicide-like effect as 2,4-D.[4] There are several auxin/IAA receptors in plants, and it is possible that mutations in any one of them may confer resistance to 2,4-D[2]; however, to date only one example of this has been found.[5] Studies in *Arabidopsis thaliana* and other species have found several non-target site resistance pathways for reduced sensitivity to 2,4-D, including differences in absorption, translocation, metabolism, and gene expression versus the wildtype susceptible plants.[1-2, 6-7]

Historically, ethylene accumulation and subsequent hydrogen cyanide production was thought to be the cause of plant necrosis and death from auxinic herbicides.[4] However, a more recent study found that not all synthetic auxin herbicides induced ethylene production and instead proposed that increased levels of abscisic acid (ABA) alone are sufficient for the downregulation of photosynthesis and growth that ultimately kills the plants.[8-9] While the mechanism of plant death from auxinic herbicides is still unclear, there is consensus of the events that take place after

a plant is exposed to auxinic herbicides. Once auxins enter the plant cell, they bind to and remove the repressor of the 9-cis-epoxycarotenoid dioxygenase (NCED) gene and, with 2,4-D treatment specifically, the 1-aminocyclopropane-1-carboxylic acid (ACS) gene.[4, 8] Both NCED and ACS are then upregulated and are the rate limiting steps in the production of the hormones, ABA and ethylene, respectively. Finally, there is downregulation of photosynthesis in the plant (Fig 1).[4, 8-9]

The invasive aquatic macrophyte Eurasian watermilfoil (*Myriophyllum spicatum* L. [EWM]) and its hybrids with native northern watermilfoil (*Myriophyllum sibiricum* Komarov × *M. spicatum*; both invasive EWM *sensu lato*) are heavily managed with herbicides in the US.[10] Synthetic auxin herbicides, including 2,4-D, are one of the most common modes of action used to control invasive watermilfoil populations across the US. But, experimental studies in watermilfoil show that there is variation in growth rates between genotypes of invasive watermilfoil in control and 2,4-D treated environments.[11-16] For example, Taylor et al.[14] found that genotype was a significant predictor of growth rate in both 2,4-D and untreated environments. Therefore, we hypothesized that genotypes that vary in their growth rates in untreated and 2,4-D environments may be responding differentially at the molecular level, specifically in the genes they express.

Gene expression differences between watermilfoils may be constitutive, occurring in the presence and absence of herbicide, or facultatively stimulated by herbicide treatment. A review of the literature on gene expression and herbicide resistance concluded that expression differences between herbicide susceptible and resistant genotypes tended to be constitutive.[17] However, there are well-defined cases of facultative expression conferring resistance to herbicides. For example, expression of transporter genes is increased in glyphosate resistant individuals of horseweed (*Conyza canadensis*) but not in susceptible individuals when treated with glyphosate.[18] While

there has not been a documented case of true resistance (genotype by treatment interaction) to 2,4-D in watermilfoil, variation in growth rates between genotypes in 2,4-D environments means that some genotypes are physiologically different.[14-15] Here, we specifically tested for differences in constitutive expression and differences in facultative gene expression in known 2,4-D/auxin response genes (Fig. 1) between two genotypes of invasive watermilfoil that differ in their growth rates. To do this, we performed RNA sequencing (RNA-seq) of control and treated replicates at several time points after treatment with 0.5mg/kg^{-1} 2,4-D.

2 METHODS

2.1 Sample collection

To determine gene expression differences between two genotypes of watermilfoil with differing growth rates, we used a hybrid watermilfoil genotype collected from Hayden Lake, Idaho, and a Eurasian genotype collected from Coeur D'Alene Lake, Idaho (from here on referred to as 'hybrid' and 'EWM' genotypes). These two genotypes were chosen because preliminary data suggests that they differ in their growth rates. In Taylor et al.[14] the Hayden Lake hybrid genotype exhibited relatively fast growth compared to the other genotypes in the study. In contrast, the Coeur D'Alene Lake EWM genotype was a relatively slow grower compared to the other genotypes in Hoff and Thum.[15] Each genotype was vegetatively propagated from a single meristem collected from the field in 208L drums in the Montana State University Plant Growth Center (Bozeman, MT), until there were enough meristems for the number of experimental units used below. We planted 30 - 8cm meristems of each genotype into individual 50mL glass beakers (VWR International Radnor, PA) with sediment described in Organisation for Economic and Co-

operation Development.[19] Each 50mL beaker with a watermilfoil tip was then placed into a 1.5L glass beaker (VWR International Radnor, PA) filled with Smart and Barko[20] buffered deionized water.

To reduce variation among experimental units, we grew the beaker-planted watermilfoil tips and performed herbicide treatments in an incubator in the Plant Growth Center at Montana State University (Bozeman, MT). The incubator was equipped with LED lights and was set to a photoperiod of 16hr light:8hr dark, a light intensity of 275 ¡ 27 mmol m^{-2} s^{-1}, and a constant temperature of 21°C. All 30 beakers of each genotype (n = 60 total) were randomly arranged on the same plane in the incubator and were rotated throughout the experiment to reduce positional effects. After a nine-day establishment period, six of each genotype were harvested to check for roots which ensured the plants had established.

To capture facultative expression response of the two genotypes, half (n = 12) of the beakers of each genotype were treated with 0.5mg/kg^{-1} 2,4-D. We used a powdered chemical grade 2,4-D (Fisher Scientific Waltham, MA) solubilized in 100% ethanol before adding to water. Because of this, the control beakers were also given the same dose of 100% ethanol. We then harvested three beakers of each genotype and treatment level at 12, 24, 48, and 96 hours after treatment (HAT) with 2,4-D. Because auxins stimulate growth and 2,4-D is a synthetic auxin, we wanted to determine gene expression in the most actively growing tissues – the meristems. We harvested meristem tissue at each of the time points after treatment by separating the meristem from the rest of the plant and immediately flash freezing it in liquid nitrogen to preserve total RNA.

2.2 Molecular methods

We then extracted total RNA from each frozen meristem using Qiagen RNeasy RNA extraction kits (Valencia, CA). Total RNA of each sample was quantified on a Nanodrop spectrophotometer (ThermoFisher Waltham, MA) and Qubit 4 flurometer (ThermoFisher Waltham, MA) before sending for sequencing. We sent 2µg of RNA for each sample to Novogene Corp. (Sacramento, CA) for quantification on an Agilent 2100 Bio Analyzer (Santa Clara, CA), after which they performed the library prep using poly-A selection and cDNA synthesis. The 150bp size selected fragments were then paired end sequenced using Illumina sequencing technologies (San Diego, CA). Each sample per direction (paired end) was sequenced to a minimum depth of 20 million raw reads, and two control samples from each genotype were also sequenced to a minimum depth of 40 million raw reads per direction for *de novo* transcriptome assembly.

2.3 De novo transcriptome assembly and annotation

Because there is not a sequenced genome for watermilfoil or any closely related species, we assembled a watermilfoil transcriptome *de novo*. We started by removing adaptors and common Illumina sequencing artifacts (e.g. phi X) from the four samples sequenced to 40 million raw reads using BBDuk (http://jgi.doe.gov/data-and-tools/bb-tools/). Next, we verified fragment sizing and quality using FastQC v0.11.8.[21] To ensure only watermilfoil reads were being assembled in the *de novo* transcriptome, we filtered any contaminant reads using BBMap (http://jgi.doe.gov/data-and-tools/bb-tools/) by removing reads that aligned to non-plant species'

reference files included in BBMap. After filtering, samples ranged from 43,356,672 to 50,529,019 cleaned reads with an average 150bp size.

We assembled the cleaned reads into a reference transcriptome using Trinity v2.8.4 and default parameters.[22] To ensure that differential expression between treatments and genotypes was reliable, and not due reference alignment biases, we constructed transcriptomes from EWM only reads, hybrid only reads, and combined EWM and hybrid reads. We then aligned both taxa back to all transcriptomes and determined that both taxa aligned equally to all transcriptomes (Supp. Table 1). We used the combined transcriptome, assembled from both taxa reads, for differential expression analyses because it ensured we were looking at the same contig between taxa. For the final combined transcriptome, Trinity assembled 494,628,791 bases into 334,917 contigs (535,628 including isoforms) averaging 923 bases. Next, we annotated the Trinity assembled transcriptome by aligning the assembled contigs to the Uniprot Swiss-Prot plant database using BlastX v2.8.1+. Of the 334,917 unique contigs in the transcriptome, 209,074 mapped to a functional gene in the Uniprot Swiss-Prot database. We then used this annotated watermilfoil transcriptome to align experimentally treated transcripts for differential gene expression.

2.4 Differential expression analyses

For the 48 experimental samples, we removed sequencing adaptors with BBDuk and verified their quality with FastQC v0.11.8. We then used STAR v2.7.0 to align each set of paired end sample sequences to the reference transcriptome generated above.[23] The STAR aligned sequences were then processed with eXpress v1.5.1 (http://bio.math.berkeley.edu/eXpress) to get

a normalized count for each contig per sample. Finally, we used this normalized count file as input into edgeR v3.28.1 to determine differential expression between treatment groups.[24] For facultative and temporal differential expression we compared the three replicates of each treatment (0.5mg/kg^{-1} 2,4-D) at all timepoints (12, 24, 48, and 96 HAT) relative to the control. For constitutive expression in the control, we compared the log fold change of control samples from all four time points (12 total/genotype) between the two genotypes to determine whether each contig was significantly higher, lower, or the same between in each of the two genotypes.

To visualize the differential expression of potentially important auxin herbicide pathway genes, we filtered the data to include only significantly differentially expressed contigs (FDR < 0.05). Because the 2,4-D response is complex, we searched for terms in the annotated transcriptome that included '9-cis-epoxycarotenoid dioxygenase (NCED)', 'abscisic acid (ABA)', '1-aminocyclopropane-1-carboxylic acid (ACS)', 'ethylene' and the 'photosystem' to build gene sets that show differential expression of known genes in the auxin pathway (Gene descriptions in Supp. Table 2).[4, 8] We then used the SciPy package in Python for contingency table tests of the number of differentially expressed contigs per gene set between the two genotypes.[25] Finally, we plotted the log-fold-change of each contig in these gene sets using the pheatmaps v1.0.12 package in R.[26]

3 RESULTS and DISCUSSION

In this study we compared differential expression in response to 0.5mg/kg^{-1} 2,4-D treatment over time between two genotypes that preliminary data suggest exhibit different growth rates.[14-15] We also constructed the first transcriptome (*de novo*) in Eurasian watermilfoil which is

now the first genomic resource available for the species (transcriptome available at: Dryad.link when accepted).

An interesting trend that appeared in our analyses is that the hybrid had a higher number of total contigs aligned to the transcriptome at all time points than the EWM genotype (Table 1). Because we used both hybrid and pure EWM reads to construct the *de novo* assembled transcriptome, the transcriptome likely contains genes that are unique to the hybrid genotype, because the hybrid's genome is half northern watermilfoil. This is likely the cause of the discrepancy in the total aligned contigs between hybrid and EWM (Table 1). This means that the hybrid genotype has more opportunity for contigs to show differential expression between control and treatment. However, as described below we observe the opposite pattern throughout our study.

At the constitutive level, contigs tended to be expressed higher in the hybrid genotype (~75% of aligned contigs; n = 52,752) than in the EWM (~25% of aligned contigs; n = 17,122) in the control environment. It is possible that this is a feature of hybridity due to the increased heterozygosity and reregulation of the genome when two divergent genomes come back together.[27-28] It may also be a feature of this specific genotype of hybrid watermilfoil, and future studies should examine gene expression levels of a collection of hybrid and parental genotypes of watermilfoil to determine whether hybrid watermilfoil exhibit heterosis.

At the facultative level, both hybrid and EWM exhibited a response to the 0.5mg/kg^{-1} 2,4-D treatment. Both genotypes showed differential expression between the untreated control and treated groups at all time points after treatment (Table 1; Fig. 2). Further, both genotypes showed

a similar trend in their pattern of response to 0.5mg/kg^{-1} 2,4-D treatment over time (Table 1; Fig. 2). At 12 HAT, 0.4% of the aligned contigs in the hybrid and 1.6% of the aligned contigs in EWM showed differential expression. Differential expression increased in both genotypes going from 12 to 24 HAT (Table 1; Fig. 2). Differential expression peaked at 48 HAT with both genotypes showing the greatest number of differentially expressed contigs at that time point (Table 1; Fig. 2). And, by 96 HAT both genotypes show a reduction in differential expression between the treated and control groups relative to the 48 HAT timepoint (Table 1; Fig. 2). Although both genotypes showed a similar pattern of differential gene expression over time, the 0.5mg/kg^{-1} 2,4-D treatment did not affect the hybrid genotype's gene expression as much as the EWM genotype (Table 1; Fig. 2). The EWM genotype differentially expressed a greater proportion of contigs relative to the hybrid genotype at all time points after treatment (Fisher's Exact $P < 0.001$).

The proportion of contigs up – versus down-regulated in response to the 2,4-D treatment was relatively even at all time points in both hybrid and EWM. However, both genotypes showed greater up-regulation of contigs in the treatment relative to the control, except for the hybrid genotype at 96 HAT (Table 1; Fig. 2).

Because the hybrid genotype did not respond as aggressively in differential expression to 2,4-D treatment relative to the EWM genotype, we questioned whether the hybrid genotype was absorbing 2,4-D to the same extent as the EWM genotype. To test this, we examined differential expression of the two genotypes in contigs annotated as 'auxin response' genes (Fig. 3; Gene descriptions in Supp. Table 2). Indeed, both genotypes showed a number of contigs annotated as 'auxin response' genes up-regulated in response to the 0.5mg/kg^{-1} 2,4-D treatment (Fig. 3).

However, within these ‘auxin response’ genes there was only a marginally significant difference between the hybrid and EWM genotype in the number of contigs showing differential expression at each HAT (2x4 ChiSquare contingency P = 0.077). While both genotypes likely did respond specifically to 2,4-D rather than a general stress response, studies that specifically measure 2,4-D absorption by the two genotypes are needed to determine if the reduced response may be related to absorption differences between them.

Because the hybrid genotype’s gene expression was less affected by 2,4-D treatment, we hypothesized that it may naturally have higher concentrations of auxin hormone. If the hybrid constitutively has greater auxin biosynthesis and regulation it may not be as affected when more auxin is added (e.g., 2,4-D treatment). To test this, we compared the total number of contigs annotated as ‘auxin response’ that were higher expressed in each genotype in the control (Fig. 3 ‘Constitutive’). However, when comparing the total numbers of ‘auxin response’ genes that had greater log fold change in the hybrid versus the EWM (and vice versa) using a Fisher’s Exact test, they were not significantly different (P = 0.836). Given that the genes we would expect to be expressed if auxins were present in different concentrations were not different between genotypes, we conclude that auxinic hormone concentrations are likely not higher in the hybrid genotype (Fig. 3 ‘Constitutive’). However, studies that measure auxin concentrations between genotypes in control conditions is warranted to confirm this.

The normal progression of an auxin herbicide response should begin with a general auxin response (Fig. 3), then NCED and ACS up-regulation, followed by an ABA and ethylene response as ABA and ethylene accumulate in the plant, and finally, down-regulation of the photosystem (Fig. 1).[4, 8-9] We see this expected response in the EWM genotype but not in the

hybrid (Figs. 3-5). Both the hybrid and EWM show a general auxin response (Fig. 3 and described above) and NCED up-regulation (Fig. 4 'NCED'), and the number of differentially expressed contigs in these gene groups is not significantly different between genotypes (NCED 2x3 [12-48 HAT] ChiSquare contingency $P = 0.737$). However, the hybrid genotype diverges from the predicted auxin herbicide response after these first steps and even seems to recover the NCED response by 96 HAT (Fig. 4 'NCED'). In the ACS, ABA, ethylene, and photosynthesis gene groups, the numbers of differentially expressed contigs at each HAT between the hybrid and EWM vary significantly (Fig. 4 and 5; ChiSquare contingency $P < 0.050$). This pattern suggests that the hybrid is mitigating the 2,4-D treatment and molecular response in some way, possibly through metabolism, sequestration, or lower growth restriction. This would be consistent with other studies that have found 2,4-D resistance of weeds through metabolism or reduced growth deregulation.[7, 29]

While the recently proposed pathway of auxin-mediated plant death does not include ethylene accumulation[8-9], we found several contigs annotated as 'ethylene response' genes upregulated in the treatment groups (Fig. 5 'Ethylene'). Similar to the response of the other auxin herbicide-induced genes, we found that the hybrid and EWM genotypes were significantly different in the number of contigs annotated as ethylene response genes (2x4 ChiSquare contingency $P < 0.010$); Fig. 5 'Ethylene'). This pattern fits with the results from previous studies on Dicamba resistant *Kochia scoparia* that showed ethylene did not accumulate in the resistant biotype like in the susceptible biotype.[30] There may be a similar mechanism taking place in the genotype of hybrid watermilfoil we used. While McCauly et al.[8] found that not all synthetic auxin herbicides stimulated ethylene production, 2,4-D was one of the herbicides that did. Though we

did not measure ethylene accumulation in this study, our data suggest that there is a difference in ethylene production between the hybrid and EWM genotypes tested here (Fig. 5 'Ethylene'), and future studies should examine the correlation as it relates to 2,4-D sensitivity.

The pattern described above suggests that the hybrid is absorbing the 2,4-D and responding to it but may be able to regulate excess auxin better than the EWM genotype, possibly through metabolism or sequestration of the herbicide or reduced growth deregulation.[7, 29, 31] We searched for contigs annotated as common herbicide metabolism genes (e.g. Cytochrome P450, Glutathione S-transferase, etc) in both control and treated comparisons, but did not find a pattern. While the exact mechanism is unclear, the hybrid genotype seems to have a reduced 2,4-D molecular response when compared to the EWM genotype, which is consistent with the phenotypic difference we see between the two genotypes in the lab (i.e. growth rates). The hybrid genotype used here tends to have a higher growth rate in both control and 2,4-D treated environments than the EWM genotype.[14-15] To this end, it may be that the hybrid genotype is better at auxin homeostasis regulation because it grows faster.

Finally, it is unclear if all hybrid genotypes of watermilfoil have a reduced response to 2,4-D when compared to EWM genotypes or if this is a specific feature of the genotypes in this study. Previous 2,4-D experimental studies in watermilfoil typically show that hybrids grow better in control and 2,4-D environments.[11-16] For example, Taylor et al.[14] found that all hybrid genotypes grew better in 2,4-D than the two EWM genotypes tested. However, Hoff and Thum[15] showed that there can be overlap in growth rates between the two taxa in both control and treatment environments. As such, this avenue of study into gene expression differences would benefit from comparing multiple hybrid genotypes and their parents (i.e., Eurasian and northern

watermilfoil) to determine if hybrid gene expression is always constitutively higher and less affected by 2,4-D treatment than their parents (i.e. heterosis), or if gene expression distributions overlap between taxa.

3.1 Conclusion

In conclusion, overall differential gene expression in response to 0.5mg/kg^{-1} 2,4-D treatment was different between the hybrid and EWM genotype. The hybrid differentially expressed a much smaller proportion of contigs when compared to the EWM genome at time point 24, 48, and 96 HAT (Table 1). The hybrid also expressed most (~75%) aligned contigs higher than the EWM genotype (~25% aligned contigs) constitutively. Our data suggest that both hybrid and EWM responded specifically to the 2,4-D treatment by up-regulating genes related to auxin response and NCED, the catalyst for ABA production (Figs. 3-5). However, the hybrid seems to stop the auxin response pathway at this step, whereas the EWM genotype continues the cascade of signaling induced by high auxin concentrations. The EWM genotype up-regulates ACS, ABA, and ethylene, and downregulates photosystem genes, completing the currently known auxin response pathway (Fig. 1).[4, 8] Conversely, we did not see this clear pattern of differential expression in key auxin response genes in the hybrid genotype and the hybrid recovered all differential expression of NCED by 96 HAT. These differences in facultative gene expression between the genotypes tested here shows that there are molecular response differences among genotypes of invasive watermilfoil in their response to 2,4-D herbicide treatments. This may become helpful information for and managers in the future to optimize herbicide treatments based on the genotypes that are present in a lake. Future studies should determine the extent to

which gene expression varies among genotype and among taxa of invasive watermilfoil to better understand best management practices of this costly invader.

Acknowledgments

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No conflicts of interest have been declared.

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Table Legends

Table 1. A summary of the number and proportion of contigs that were aligned and significantly differentially expressed either up or down in the 0.5mg/kg^{-1} 2,4-D treated groups relative to the control groups in both genotypes of watermilfoil (hybrid and EWM) at all timepoints sampled after treatment.

Tables

Hours after treatment	Total aligned contigs		differentially expressed contigs (FDR<0.05)		Proportion differentially expressed		Down-regulated in treatment		Up-regulated in treatment	
	hybrid	EWM	hybrid	EWM	hybrid	EWM	hybrid	EWM	hybrid	EWM
12	115259	97760	407	1533	0.004	0.016	87	378	320	1155
24	112759	94521	1703	4947	0.015	0.052	396	1855	1307	3092
48	117792	94943	3639	17712	0.031	0.187	1840	7669	1799	10043
96	119584	98146	1516	8713	0.013	0.089	875	4105	641	4608

Table I

Figure Legends

Figure 1. Putative response pathway to 2,4-D treatment in plants. Redrawn from Grossman et al. 2010 and Gaines 2020 using Biorender.com.

Figure 2. Plot of the number of contigs that are significantly (FDR < 0.05) differentially expressed in the control vs treatment group for both 'hybrid' and 'Eurasian' genotypes of watermilfoil at all time points after treatment with 0.5mg/kg^{-1} 2,4-D. Total number of contigs differentially expressed is broken into up- and down-regulated in treatment relative to control for each genotype at each time point.

Figure 3. Plot of log fold change in expression between treatment levels and genotypes (Eurasian vs hybrid) for indole-3-acetic acid/auxin response genes. Each row represents a contig and each column represents a control vs treatment comparison for a genotype at a timepoint in hours after treatment with 0.5mg/kg^{-1} 2,4-D except for the 'Constitutive' comparison which represents the EWM genotype vs hybrid genotype in the control environment. Negative values ('red' colors) indicate that a contig was expressed higher in the 2,4-D treatment or in the hybrid genotype for 'Constitutive', and positive numbers ('blue' colors) indicate that contig was expressed lower in the treatment relative to the control or higher in the EWM genotype for the 'Constitutive' comparison. The more saturated the color the greater the log fold change between the control and treatment or EWM and hybrid genotypes. The 'Gene' column and legend contains abbreviations from the Uniprot Swiss-Prot plant database that each contig was aligned to, and more information on each gene's function can be found by searching the abbreviation on https://www.uniprot.org.

Figure 4 Plot of log fold change in expression between treatment levels and genotypes (Eurasian vs hybrid). Each row represents a contig and each column represents a control vs treatment comparison for a genotype at a timepoint in hours after treatment with 0.5mg/kg^{-1} 2,4-D. Negative values ('red' colors) indicate that a contig was expressed higher in the 2,4-D treatment and positive numbers ('blue' colors) indicate that contig was expressed lower in the treatment relative to the control and the more saturated the color the greater the log fold change between the control and treatment. The 'Gene' column and legend contains abbreviations from the Uniprot Swiss-Prot plant database that each contig was aligned to, and more information on each gene's function can be found by searching the abbreviation on https://www.uniprot.org. Panes show contigs grouped by annotation as 9-cis-epoxycarotenoid dioxygenase 'NCED', 1-aminocyclopropane-1-carboxylic acid 'ACS', and Abscisic acid 'ABA' response genes.

Figure 5 Plot of log fold change in expression between treatment levels and genotypes (Eurasian vs hybrid). Each row represents a contig and each column represents a control vs treatment comparison for a genotype at a timepoint in hours after treatment with 0.5mg/kg^{-1} 2,4-D. Negative values ('red' colors) indicate that a contig was expressed higher in the 2,4-D treatment and positive numbers ('blue' colors) indicate that contig was expressed lower in the treatment relative to the control and the more saturated the color the greater the log fold change between the control and treatment. The 'Gene' column and legend contains abbreviations from the Uniprot Swiss-Prot plant database that each contig was aligned to, and more information on each gene's function can be found by searching the abbreviation on https://www.uniprot.org. Panes show contigs grouped by annotation as 'Ethylene' response and 'Photosystem' regulation genes.

Figures

Figure I

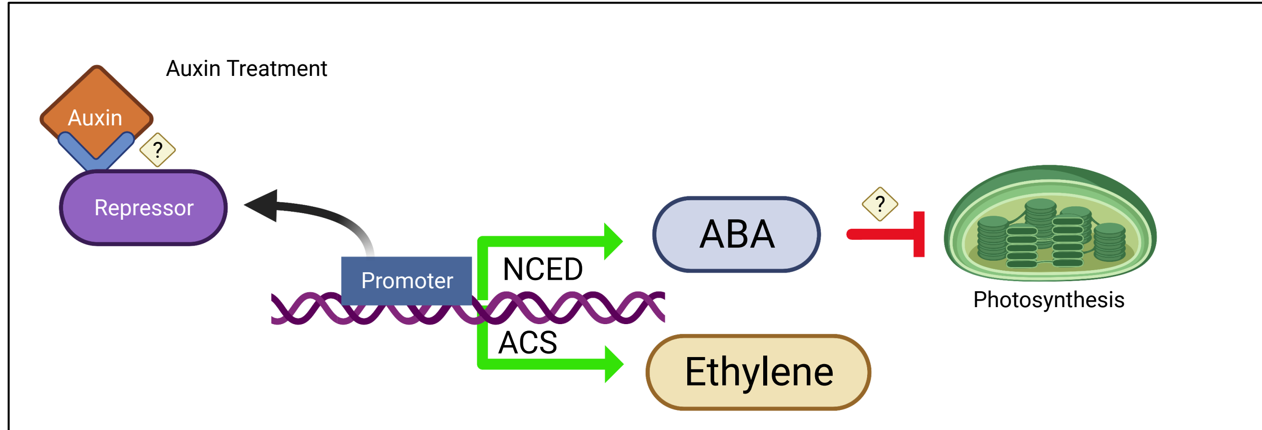


Figure II

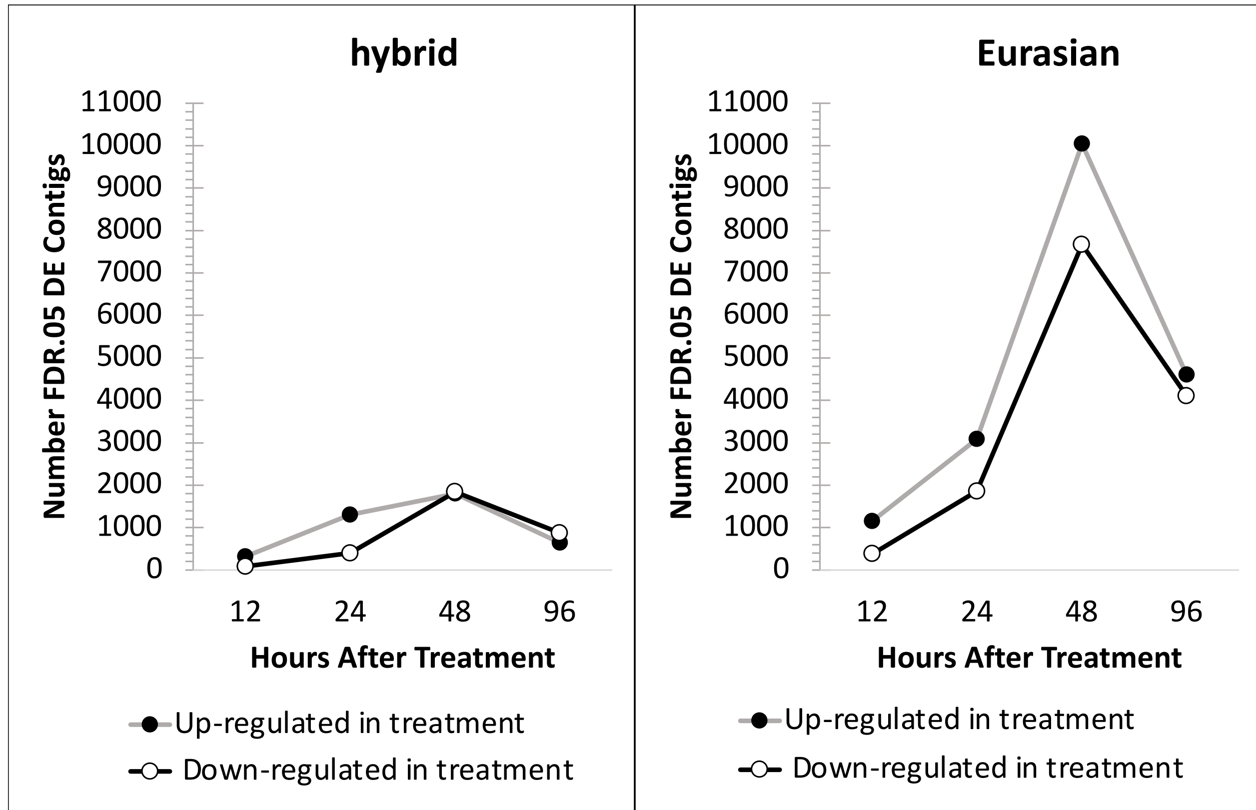


Figure III

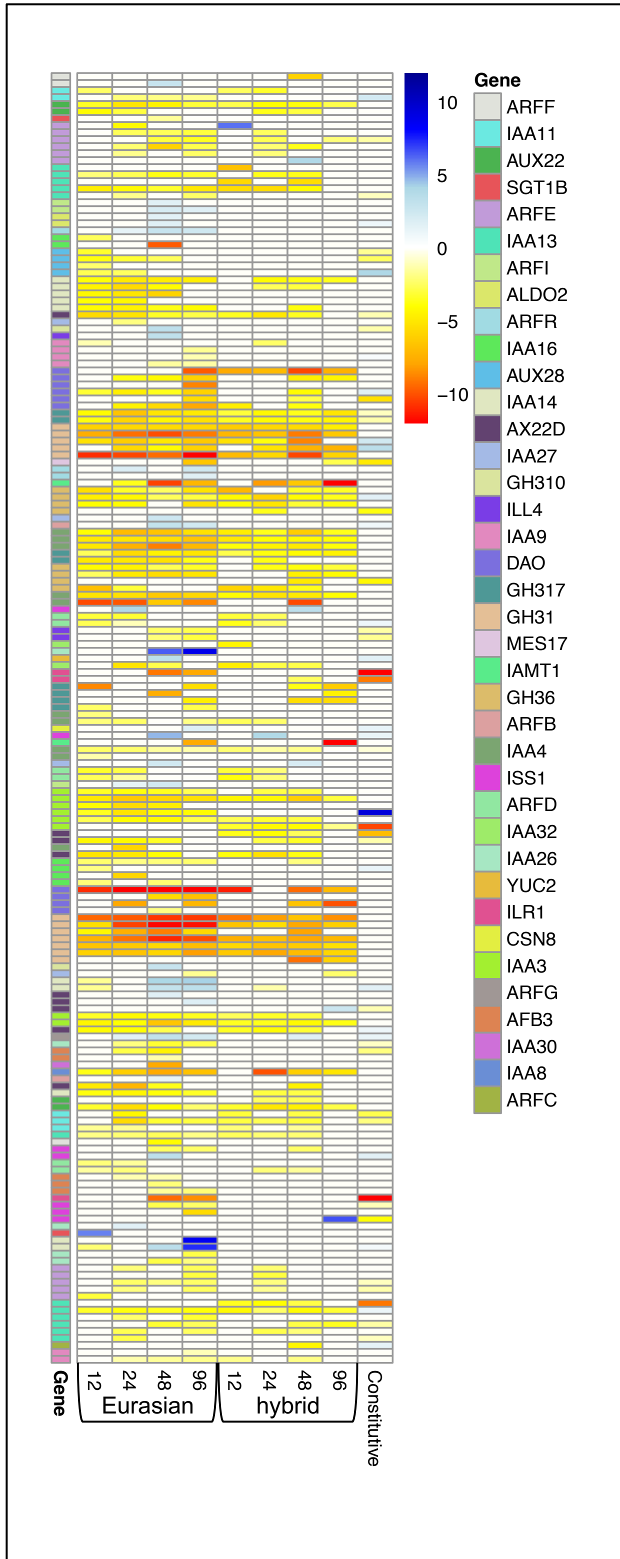


Figure IV

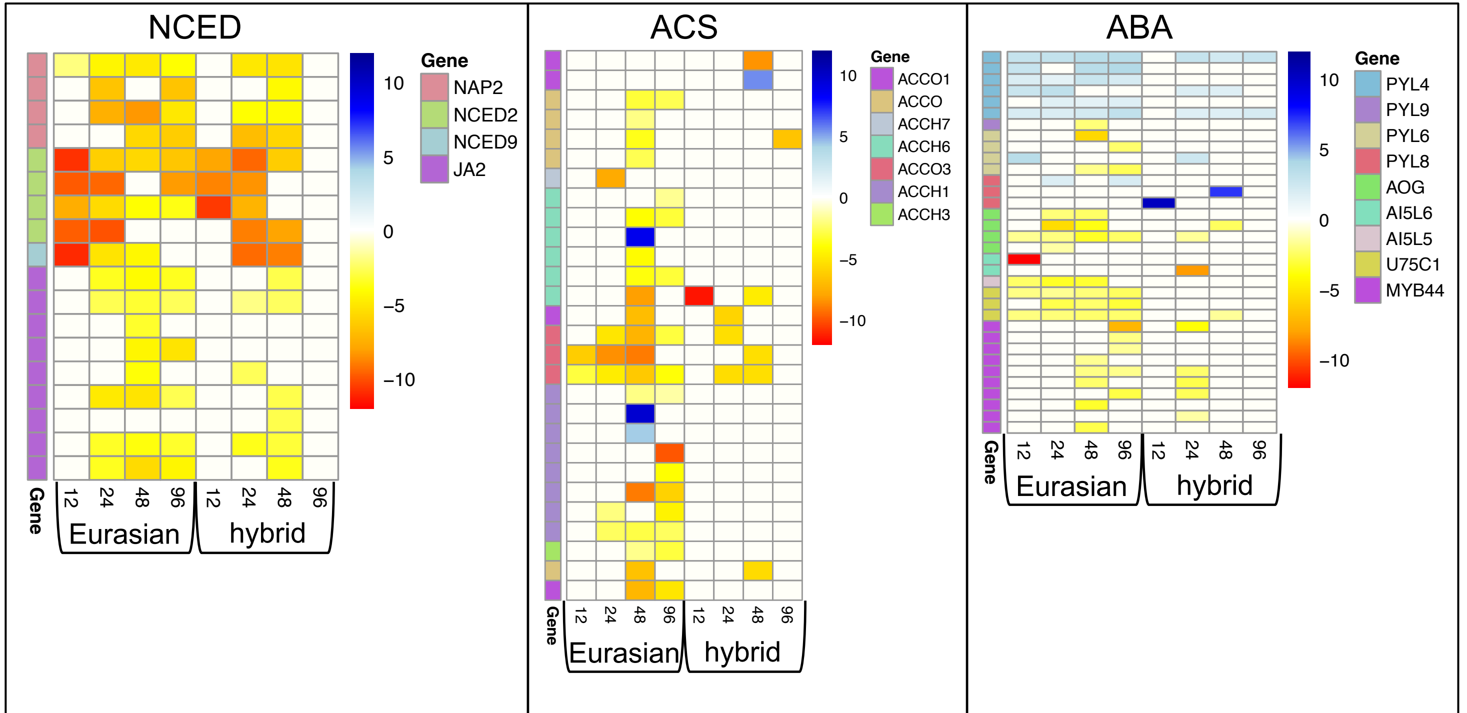
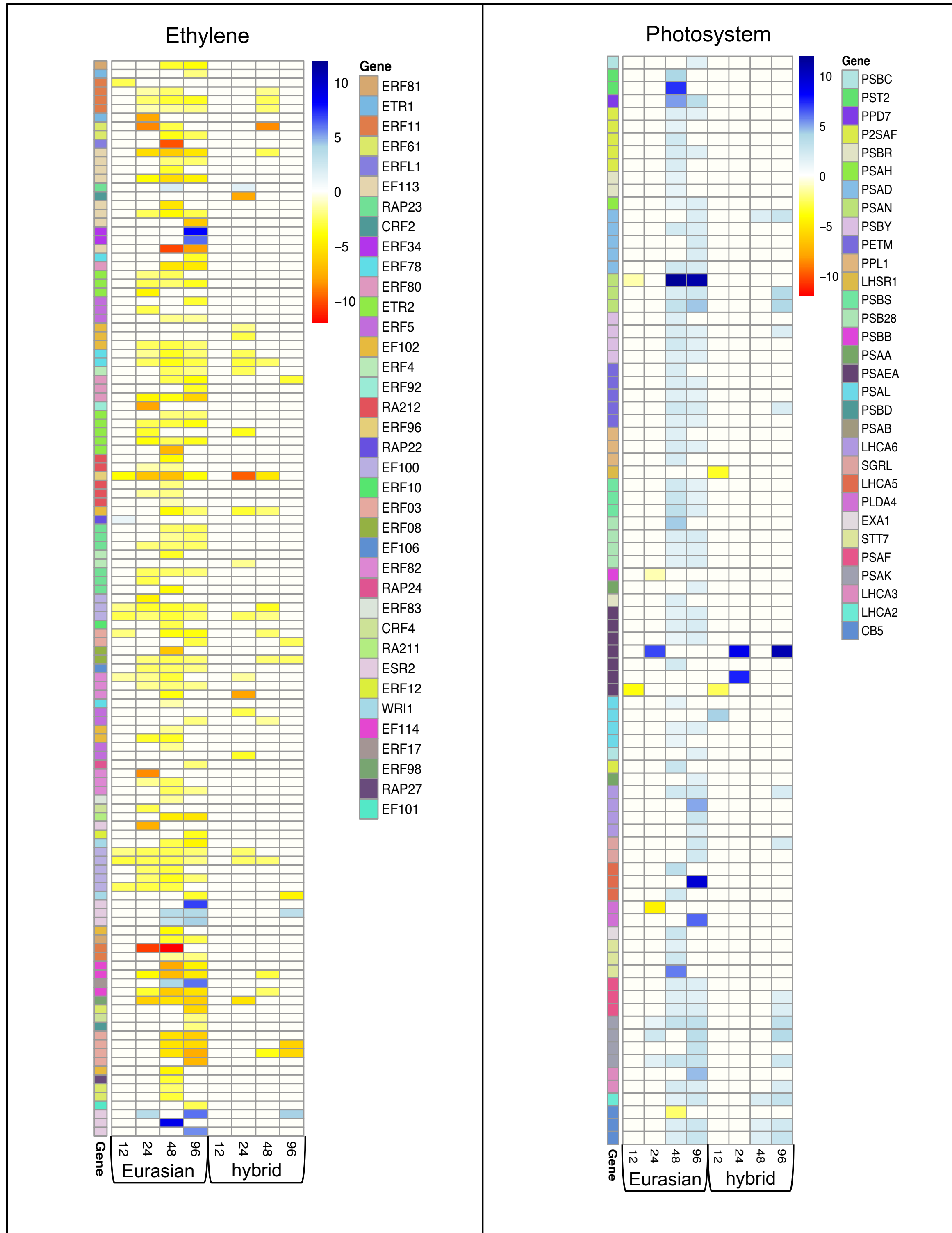


Figure V



CHAPTER SIX

CONCLUSIONS AND FUTURE WORK

Each of the following sections will walk through the conclusions from each of the research chapters presented above. Where applicable, I will also present any preliminary data from projects that have come out of the previous research chapters. As with any research program, this dissertation is just a piece of the overall research that is being conducted in this area, and I am excited to see where things go from here. Fortunately, I have the opportunity to continue driving this research forward as a postdoctoral researcher, and any future work presented hereafter will be part of my postdoctoral research.

Chapter Two – A cryptic invasion of *Trapa*

The main conclusion from the genetic study of invasive water chestnut (*Trapa* spp.) in the northeastern US was that there are two distinct lineages of water chestnut that are being managed. The new lineage is likely a unique species of water chestnut introduced from a different native range than what was previously being managed in the Northeast US. I say likely because the taxonomy of *Trapa* is still unresolved.

Regardless of the unresolved taxonomy, there are several implications from the results of this research. Invasive *Trapa* populations in the US are typically managed with hand-pulling or treatment with the herbicides 2,4-dicholorophenoxy acid (2,4-D) and triclopyr (Hummel and Kiviat 2004, Poovey and Getsinger 2007, GLIMRIS 2012). In both cases, the ideal timing of control is before seed set in order to reduce the number of seeds available for recolonization the following growing season. Distinguishing the two US *Trapa* taxa is important for determining

whether they exhibit any differences in phenology or response to different herbicide formulations and rates that would influence the development and implementation of control strategies. There is also increasing interest in the potential for biocontrol of *Trapa*, with two promising beetle candidates (Pemberton 1999, Pemberton 2002, Ding and Blossey 2005). Since biocontrol typically involves matching herbivores to plant lineages as specifically as possible, distinguishing the two US *Trapa* taxa is important for studies evaluating possible biocontrol agents. Finally, distinguishing the two *Trapa* taxa will also be important for identifying new populations, and monitoring the spread of *Trapa* over time. Although the two taxa can be distinguished morphologically, the distinguishing features are primarily in their flowers and fruits. So, genetic identification methods will be particularly useful for populations that have not yet flowered or set fruits, or for which the management strategy includes limiting the development of fruits.

Because of the implications for management, the United States Army Corps of Engineers have decided that this is a valuable avenue of research to fund. A new grant was recently awarded to the Thum Lab to investigate whether the distinct *Trapa* that were identified in the above study can hybridize with each other. Hybridization can be important for management because invaders successfully expanded their range by increasing genetic diversity through hybridization (Hovick and Whitney 2014; Schierenbeck and Ellstrand 2009; Ellstrand and Schierenbeck 2000). For example, Larue et al. (2013) found that hybrids of watermilfoil (*Myriophyllum spicatum* L. × *M. sibricum* Komarov) were more invasive than the invasive parent (*M. spicatum*). Moody and Les (2002) showed that genetic data is required to reliably distinguish hybrid watermilfoil from the parental species. While I will not be directly involved in this further research, I am excited to see where it goes.

Chapter Four – Fluridone response of watermilfoil clones

From this research, it was clear that the same clone (i.e. multi-locus genotype) of watermilfoil responded the same to fluridone herbicide treatment regardless of the waterbody it was found in. Therefore, managers should not use fluridone on the two clones that were not injured by fluridone in this study. However, the other six clones used in this study that were injured would be effectively managed with fluridone treatment.

The results from this study demonstrate that genetic monitoring before treatment, combined with herbicide characterization of widespread/prioritized clones could inform management decisions. If there was a database of clones that have been characterized for their response to commonly used herbicides, genotyping of the clones in a lake could inform decisions about the herbicide to use for best control when a lake is slated for management. Out of this research, Dr. Ryan Thum and myself were able to secure funding from the United States Army Corps of Engineers to begin to build a database of characterized clones.

The first thing we want to improve for tracking clones is the genotyping process that we use. While the previous genotyping method (8 microsatellite markers) has enough resolution to determine clones, genotyping results from microsatellite-based genotyping is not easily shared across labs or projects. To this end, a panel of microhaplotype markers was developed by a previous graduate student in the lab using restriction site-associated DNA sequencing data. From the original 128 microhaplotype markers, I selected 62 that seemed to work well in a pilot run of 12 individuals. I then ran another test run of 620 individuals that had previously been genotyped with microsatellite markers. While the trial run showed that these markers amplified reliably in the 12 pilot individuals, I am seeing a lot of missing data in the new dataset. Therefore, although I

can distinguish most individuals, I don't have enough resolution to distinguish closely related genotypes (Fig 1 different colored points closer than same color points in upper left). This resolution issue may also be compounded by polyploidy. Figure 2 shows that most markers align across homoeologous chromosomes in the genome, so genotypes may be combinations of homoeologous alleles instead of homologous alleles. I will continue to work on this project in my postdoctoral research and am currently troubleshooting polymerase chain conditions for more reliable amplification of all microhaplotype markers.

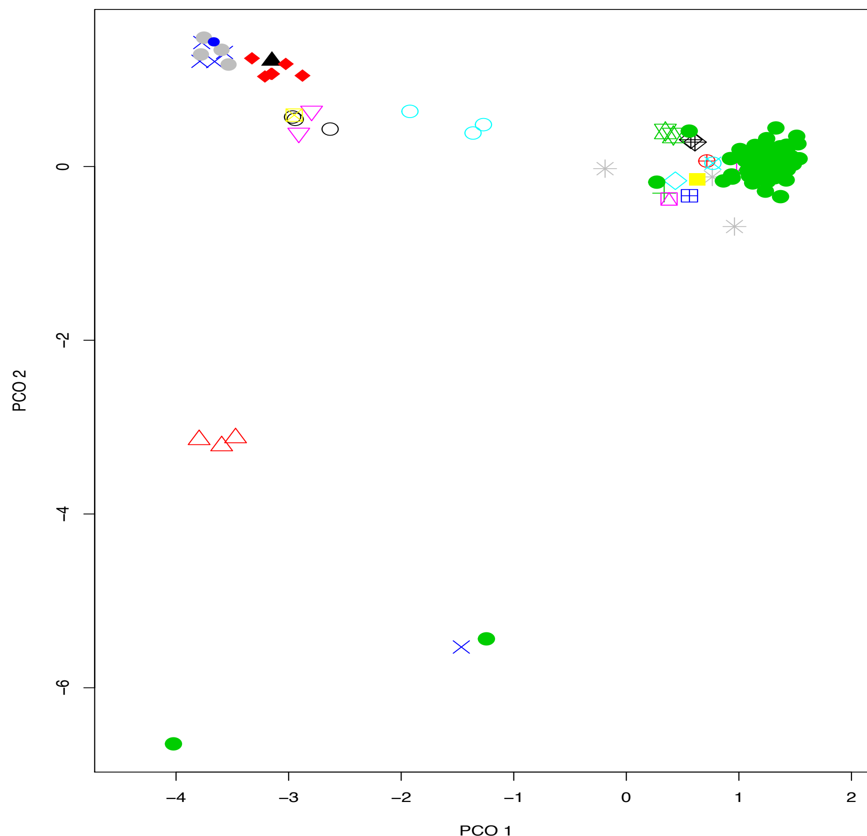


Figure 1. First two axes of a principal coordinates analysis of microhaplotype genotypes analyzed as binary. Only the Eurasian watermilfoil individuals plotted here. Each point represents an individual and point shapes and colors code the microsatellite genotype they were previously assigned. The same shape and color point is the same genotype according to microsatellite genotyping. Points are ordinated based on genetic distance of microhaplotype genotypes.

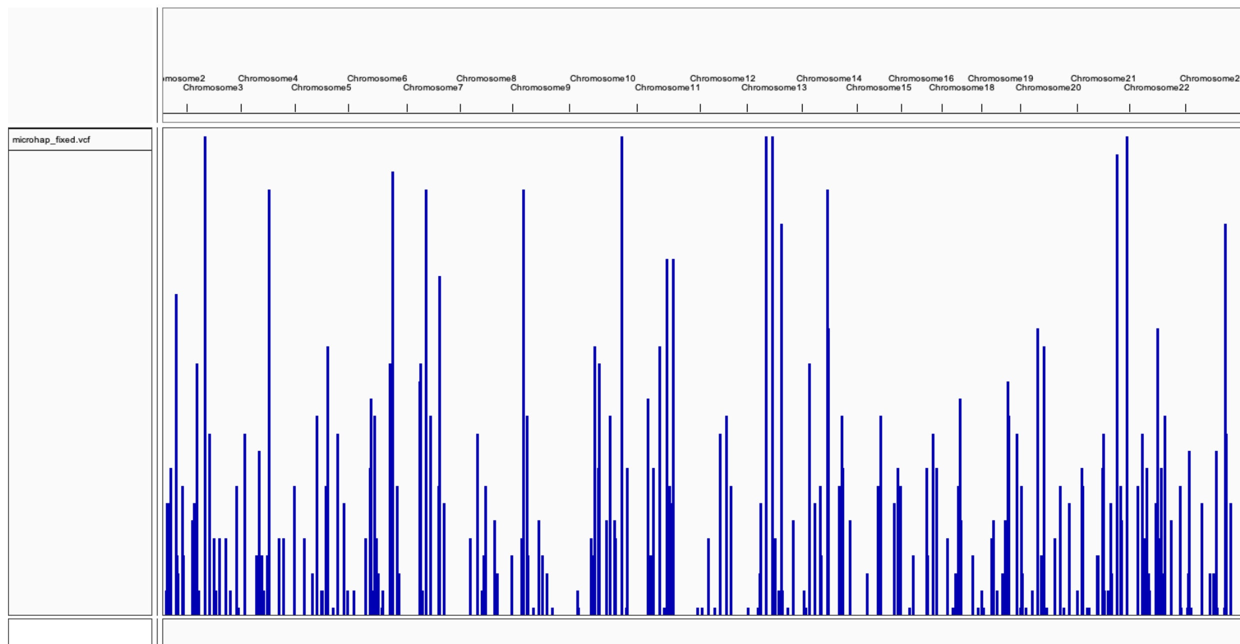


Figure 2. Screen shot from Integrative Genome Viewer. The draft Eurasian watermilfoil genome is organized by chromosome along the top of the pane and individuals are stacked along the vertical. If an individual was genotyped at a marker, it gets a blue mark. Eurasian watermilfoil is a hexaploid, so there are three sets of seven homoeologous chromosomes represented. There are markers in all chromosome scaffolds, and many individuals are missing data (no blue mark at sites).

While clone tracking may be sufficient in the short term, in order to predict susceptibility versus resistance using molecular markers, we ultimately need to understand the genetic architecture of resistance. For example, fluridone resistance in hydrilla is determined by point mutations at a single amino acid position in the phytoene desaturase gene (PDS; Michel et al. 2004), and assays can screen for these mutations instead of tracking clones. Therefore, to determine potential genes associated with herbicide resistance, we needed to construct a ‘genetic mapping population’ between a known sensitive and resistant genotype. Because we identified genotypes that are fluridone resistant (MG-377) versus susceptible (MG-1282) in the study in chapter four, I strategically crossed them to form an F_1 hybrid line. Because the resistant parent is likely a heterozygote, I then screened the F_1 population to check for segregation of growth in

fluridone. I did see segregation in this F_1 population (Fig. 3), and this population is now being sequenced using low coverage whole genome sequencing at Hudson Alpha (Huntsville, AL). The progeny from the F_1 cross are also being intercrossed with one another to create an F_2 mapping population that segregates for fluridone resistance and the other resistant genotype (MG-237) from the chapter four study is being crossed with itself and susceptible genotypes (MG-1282; MG-429) to create more mapping populations for fluridone resistance.

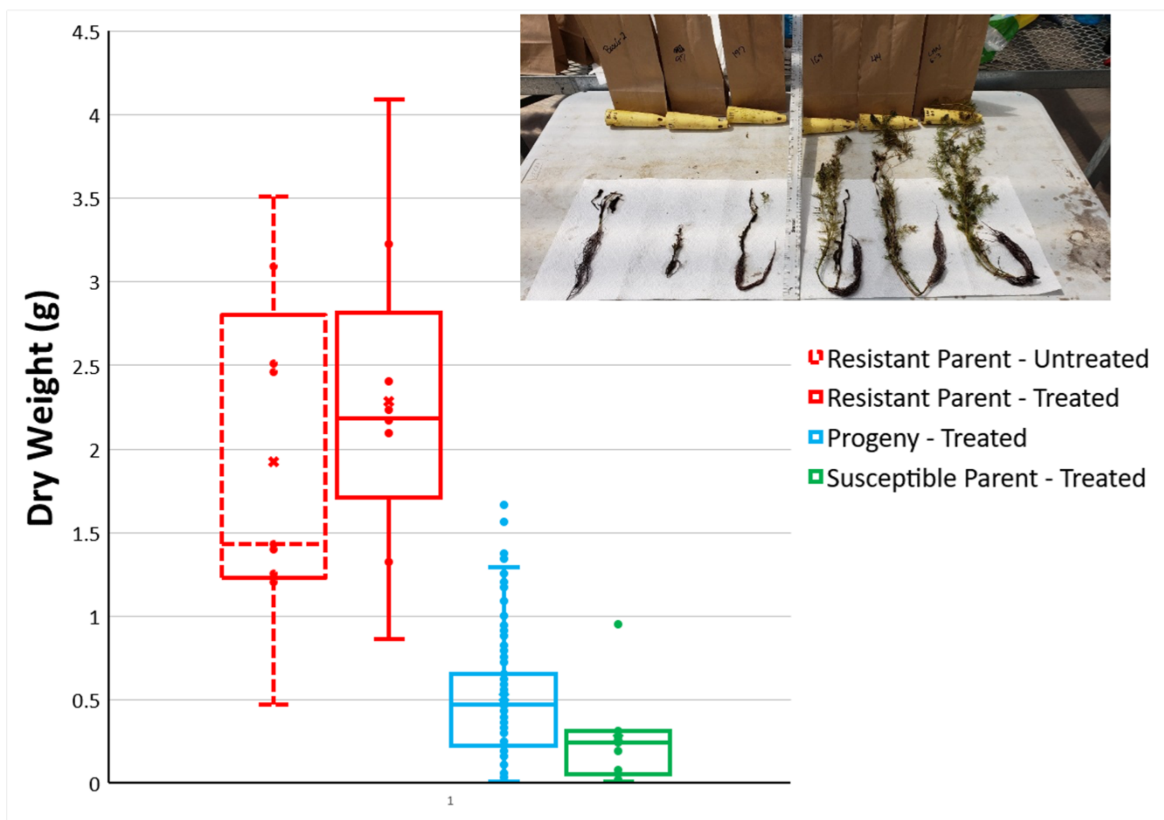


Figure 3. Boxplot of dry weight biomass in grams of resistant parent in control (red dash), resistant parent in 6ppg fluridone (solid red), susceptible parent in 6ppb fluridone (solid green), and F_1 progeny between the two parents in 6ppb fluridone (solid blue). Picture in upper right corner shows plants from 6ppb fluridone treatment from left to right: susceptible parent, four progeny with varying sensitivities, and the resistant parent.

Chapter Five – Gene expression response of watermilfoils

RNA sequencing experiments tend to be hypothesis generating experiments. Therefore, many hypotheses have come out of this study (see chapter five "Results and Discussion"). Arguably one of the most interesting conclusions from this study was that the hybrid genotype expressed more genes in the control environment and up regulated less genes when treated with 2,4-D herbicide than the Eurasian genotype. Because the two genotypes in this study were selected because of growth response differences, the difference between hybrid and Eurasian was smuggled in but with only one genotype of each there isn't much inference to draw from that comparison. As a result, the next step is to determine variation in gene expression between the two taxa. By comparing multiple genotypes of hybrid and Eurasian watermilfoil constitutive gene expression and expression response to 2,4-D, then we will have a better understanding of whether genes are always expressed higher in hybrid watermilfoil (heterosis) or if there is overlapping variation between the two taxa. Further, by comparing genotypes with varying growth rates we may be able to associate gene expression with growth traits in control and treatment environments. This is the exact experiment that is being run by Research Associates in the Thum Lab, and I look forward to helping analyze the data.

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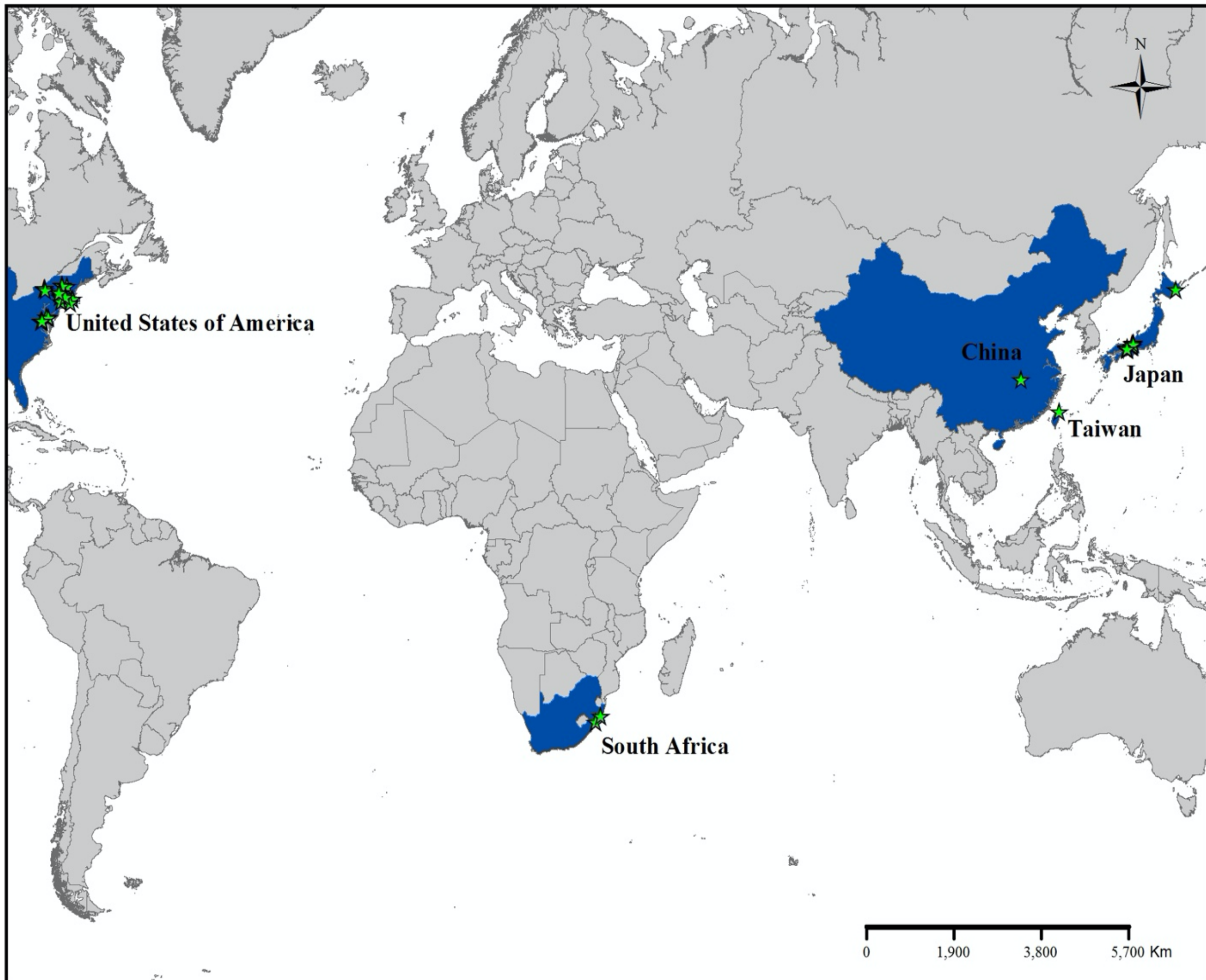
APPENDICES

APPENDIX A

SUPPLEMENTARY MATERIAL FOR CHAPTER TWO



Supplementary Figure 1. Map of all sampling locations of *Trapa* used in this study

Supplementary Table 1. *Trapa* populations included in the AFLP analysis. Putative species identifications are based on regional taxonomic keys. Sample size (n) indicates the number of individuals included in the AFLP analysis; in some cases this is different from the numbers of individuals included in the morphology analysis. Population codes for morphological analysis are as in Supp. Table 2; an asterisk indicates populations that were excluded from morphology analyses because quality fruits were unavailable (See Supp. Table 2).

Putative Species	Country	Location (locality; county, district or city; state, province or prefecture)	n	Population Code
T. bispinosa	People's Republic of China - Taiwan	Shuanglian Reserve; Yilan; Taiwan	9	SLR-TW
T. incisa	Japan	Nakaikemi Marsh; Tsuruga; Fukui	9	NAK-J
T. japonica	Japan	Irrigation Pond, Kashiyama-cho; Ono; Hyogo	9	ONO-J
T. japonica	Japan	Oh-Ike Pond, Iwaoka-cho; Kobe; Hyogo	10	IWA-J
T. japonica	Japan	Lake Mikata, Wakasa-cho; Mikatakaminaka; Fukui	11	MIK-J
T. japonica	Japan	Takaoka; Katoh; Hyogo	8	KAT-J
T. japonica	Japan	Kiri-ike Pond, Nagasaka-dera, Uozumi-cho; Akashi; Hyogo	7	KIR-J
T. japonica	Japan	Lake Biwa, Minamihama Park; Kusatsu; Shiga	6	KUS-J*
T. japonica	Japan	Lake Biwa, Choumyou-ji; Ohmihachiman; Shiga	8	OHM-J*
T. japonica	Japan	Lake Shirarutoro; Shibecha-cho, Kushiro–shicho; Hokkaido Island	9	SHI-J
T. natans	Japan	Ohtoribami-ike Pond; Kande-cho, Kobe; Hyogo	8	KO-J
T. natans	Japan	Temma-Oh-ike; Inami-cho; Hyogo	9	TEM-J
T. natans	Japan	Lake Biwa; Kohoku-cho, Nagahama; Shiga	7	BIWA-J
T. natans	Japan	Ima-ike Pond; Kitano, Noguchi-cho, Kakogawa; Hyogo	9	IMA-J
T. natans	Japan	Uchiga-ike; Oka, Inami; Hyogo	10	UCH-J
T. natans L.	South Africa	Durban Botanic Garden Pond; Durban; KwaZulu-Natal	10	DBG-KZN*
T. natans L.	South Africa	Farm Dam; Empangeni; KwaZulu-Natal	11	EM-KZN
T. natans	United States	South Sodus Bay; Wayne County; New York	5	SSB-NY
T. natans	United States	Red Creek; Wayne County; New York	5	RC-NY
T. natans	United States	Montezuma Marsh; Cayuga County; New York	8	MM-NY
T. natans	United States	Drowned Lands, Lake Champlain; Rutland County; Vermont	4	CDL-VT

T. natans	United States	Mill Bay, Lake Champlain; Warren County; New York	2	CMB-NY
T. natans	United States	Chubbs Dock, Lake Champlain; Washington County; New York	1	CCD-NY
T. natans	United States	Tivoli Lake Park; Albany County; New York	6	TP-NY
T. natans	United States	Inbocht Bay, Hudson River; Columbia County; New York	5	IB-NY
T. natans	United States	Stockport Creek, Hudson River; Columbia County; New York	5	SC-NY
T. natans	United States	Central Pond; Providence County; Rhode Island	5	CP-RI
T. natans	United States	Chapman Pond; Washinton County; Rhode Island	6	CH-RI
T. natans	United States	Bird River; Baltimore County; Maryland	6	BR-MD*
T. natans	United States	Honstanic River; Litchfield County; Connecticut	10	HR-CT
T. natans	United States	Mohawk River; Saratoga County; New York	10	MRC-NY
T. natans	United States	Sassafras River; Kent County; Maryland	9	SR-MD
T. natans	United States	Brickyard Ponds; Westfield County; Massachusetts	10	WF-MA
T. natans	United States	Watervliet Reservoir; Albany County; New York	10	WL-NY
T. quadrispinosa	People's Republic of China - Hubei	Yujiazui; Jiayu; Hubei	7	HB-C
T. sp. (2-horn)	United States	Myrtle Leaf Drive Pond; Fairfax County; Virginia	10	ML-VA
T. sp. (2-horn)	United States	Waples Mill Pond; Fairfax County; Virginia	9	WP-VA
T. sp. (2-horn)	United States	Nutley Pond, Virginia Center Blvd; Fairfax County; Virginia	10	VCB-VA
T. sp. (2-horn)	United States	Wellington Contractors Pond; Prince William County; Virginia	10	WC-VA
T. sp. (2-horn)	United States	Willow Oak Corporate Drive Pond; Fairfax County; Virginia	1	WO-VA

Supplementary Table 2. Raw data used in the morphological mixed PCA analysis. Categorical variables include: underside of leaf color (**GR**een, **R**ed/**P**urple/**B**rown, **G**reen/**R**ed/**P**urple/**P**ink/**B**rown), fruit crown (**Y**es/**N**o), spines (**T**wo/**F**our), upper spine orientation (**A**scending, **D**escending, **H**orizontal), lower spine shape at apex (**P**ointed/**B**lunted), lower spine direction (**A**scending, **D**escending, **H**orizontal, **N/A** [too small to tell]). Quantitative variables include: upper fruit width (mm), lower fruit width (mm), fruit height (mm), and fruit

thickness (mm). Individual identifications include population codes (as in Appendix B) and individual rosette numbers. This list of samples is a subset of those rosettes with quality fruits and only includes those with DNA and genotyping quality.

Individual	Leaf Underside Color	Putative Species	Crown	Spines	Spine Orientation	Lower Shape at Apex	Lower Direction	Upper Fruit Width (mm)	Lower Fruit Width (mm)	Fruit Height (mm)	Fruit Thickness (mm)
BIWA-J_1	-	*natans*	Y	F	D	P	D	53.5	46	24	14
BIWA-J_12	-	*natans*	Y	F	H	P	D	48.5	45	25	14
BIWA-J_16	-	*natans*	Y	F	H	P	D	54.5	49	23	16
BIWA-J_17	-	*natans*	Y	F	H	P	D	47.5	43	21.5	14
BIWA-J_4	-	*natans*	Y	F	H	P	D	59	52	26	15
BIWA-J_7	-	*natans*	Y	F	D	P	D	48	41	23.5	15
BIWA-J_8	-	*natans*	Y	F	H	P	D	53.5	50	24	16
CCD-NY_9	GR	*natans*	Y	F	A	P	H	41.5	30.75	21.5	12
CDL-VT_11	GR	*natans*	Y	F	A	P	A	44.9	35.1	20.1	12
CDL-VT_19	GR	*natans*	Y	F	A	P	A	41.1	29.5	21	12
CDL-VT_9	GR	*natans*	Y	F	A	P	A	35	28	19.1	11
CH-RI_10	GR	*natans*	Y	F	A	P	A	44	37	20.1	12
CH-RI_11	GR	*natans*	Y	T	A	P	H	42	27	18	11.5
CH-RI_3	GR	*natans*	Y	F	A	P	H	40	33.1	22	12
CH-RI_4	GR	*natans*	Y	T	A	P	H	44	24	20	11.5
CH-RI_7	GR	*natans*	Y	F	A	P	H	37.2	31.9	20.6	12
CMB-NY_15	GR	*natans*	Y	F	A	P	D	32.8	26.5	19.2	11
CMB-NY_7	GR	*natans*	Y	F	A	P	A	34.5	25	18.5	11
CP-RI_11	GR	*natans*	Y	F	A	P	H	38.4	32	19	11
CP-RI_15	GR	*natans*	Y	F	A	P	A	39	29	21	13
CP-RI_17	GR	*natans*	Y	F	A	P	D	36.1	31.1	21.25	13
CP-RI_23	GR	*natans*	Y	F	A	P	H	30	24	18.5	11
CP-RI_27	GR	*natans L.*	Y	F	A	P	D	33	30	18.1	13
EM-KZN_10	-	*natans L.*	Y	F	A	P	A	33.5	34	19	9.5
EM-KZN_11	-	*natans L.*	Y	F	A	P	D	40.5	22	20.5	10
EM-KZN_12	-	*natans L.*	Y	F	A	B	D	37	17.5	19	9.5
EM-KZN_13	-	*natans L.*	Y	F	A	P	D	41.5	26.5	21	10
EM-KZN_14	-	*natans L.*	Y	F	A	P	D	40.5	44.5	20	10
EM-KZN_15	-	*natans L.*	Y	F	A	P	D	32.1	18.5	19	9
EM-KZN_16	-	*natans L.*	Y	F	A	P	D	38	24.5	19	10

EM-KZN_18	-	*natans L.*	Y	F	A	P	D	37	17.5	19	9
EM-KZN_2	-	*natans L.*	Y	F	A	P	D	33	20	20	10.5
EM-KZN_20	-	*natans L.*	Y	F	A	P	D	43.5	23	20	9.5
EM-KZN_21	-	*natans L.*	Y	F	A	P	D	36	20.5	18	10
EM-KZN_3	-	*natans L.*	Y	F	A	P	D	47.5	21.5	21.5	11
EM-KZN_4	-	*natans L.*	Y	T	A	B	D	40	18	19.5	9.5
EM-KZN_5	-	*natans L.*	Y	F	A	P	D	28	18.5	17	8.5
EM-KZN_6	-	*natans L.*	Y	F	A	P	D	39.5	34.5	18	9
EM-KZN_7	-	*natans L.*	Y	F	A	P	D	39.5	31.5	19	9.3
EM-KZN_8	-	*natans L.*	Y	F	A	P	D	41.5	34	19.5	8.5
EM-KZN_9	-	*natans L.*	Y	F	A	P	D	40.5	19.5	20.5	10
HB-C_10	-	*quadrispinosa*	Y	F	A	P	D	25	34.5	12.5	10
HB-C_12	-	*quadrispinosa*	Y	F	A	P	D	30.5	37.5	16	10.5
HB-C_4	-	*quadrispinosa*	Y	F	A	P	D	38	36.5	11.5	14
HB-C_5	-	*quadrispinosa*	Y	F	A	P	D	41	37.5	12.5	11.5
HB-C_6	-	*quadrispinosa*	Y	F	A	P	D	31	36	10.5	11.5
HB-C_8	-	*quadrispinosa*	Y	F	A	P	D	38.5	33.5	15.5	13.5
HB-C_9	-	*quadrispinosa*	Y	F	A	P	D	31.5	34	11.5	14
HR-CT_1	GR	*natans*	Y	F	H	P	H	41	34	22	16
HR-CT_10	GR	*natans*	Y	F	D	P	D	44	34	23	16
HR-CT_12	GR	*natans*	Y	F	D	P	D	35	26	17	12
HR-CT_14	GR	*natans*	Y	F	A	P	D	36	35	17	13
HR-CT_15	GR	*natans*	Y	F	H	P	H	44	37	23	16
HR-CT_22	GR	*natans*	Y	F	H	P	D	38	30	20	15
HR-CT_23	GR	*natans*	Y	F	D	P	D	35	22	18	14
HR-CT_24	GR	*natans*	Y	F	A	P	H	40	38	22	14
HR-CT_3	GR	*natans*	Y	F	H	P	D	40	31	21	13
HR-CT_4	GR	*natans*	Y	F	H	P	D	36	31	20	16
IB-NY_10	GR	*natans*	Y	F	A	P	H	38	26	18	11
IB-NY_13	GR	*natans*	Y	F	A	P	H	36	28.2	19	9
IB-NY_2	GR	*natans*	Y	F	A	P	D	36	30.5	23	13
IB-NY_3	GR	*natans*	Y	F	A	P	A	42.75	35.5	23	14
IB-NY_9	GR	*natans*	Y	F	A	P	D	40	32.5	19	11
IMA-J_1	-	*natans*	Y	F	A	P	D	58	52.5	22	16.5
IMA-J_13	-	*natans*	Y	F	A	P	D	52	56	24	17
IMA-J_16	-	*natans*	Y	F	H	P	D	64	59	23.5	14
IMA-J_19	-	*natans*	Y	F	H	P	D	56	55	23	16
IMA-J_23	-	*natans*	Y	F	A	P	D	53	53.5	23	14

IMA-J_26	-	*natans*	Y	F	H	P	D	58	46	22	16
IMA-J_27	-	*natans*	Y	F	A	P	D	54	47	23.5	18
IMA-J_3	-	*natans*	Y	F	A	P	H	58	58	25	16
IMA-J_8	-	*natans*	Y	F	D	P	D	57	44	23	16
IWA-J_1	-	*japonica*	Y	T	A	B	NA	43	9.75	15	11
IWA-J_10	-	*japonica*	Y	T	A	B	NA	44	10.2	17.5	10
IWA-J_13	-	*japonica*	Y	T	A	B	NA	42	11.3	15.1	9.8
IWA-J_19	-	*japonica*	Y	T	A	B	H	35.2	13.1	18	10
IWA-J_2	-	*japonica*	Y	T	A	B	NA	41	11.5	17.5	10
IWA-J_3	-	*japonica*	Y	T	A	B	NA	37	8	16.2	8.8
IWA-J_4	-	*japonica*	Y	T	A	B	NA	39.2	13	19	10.5
IWA-J_6	-	*japonica*	Y	T	A	B	NA	36	10	15	9.1
IWA-J_7	-	*japonica*	Y	T	A	B	NA	36	9.25	14.5	10.2
KAT-J_18	-	*japonica*	Y	T	A	B	NA	37.5	10	16	9
KAT-J_8	-	*japonica*	Y	T	A	B	D	41.5	14.5	19	11
KIR-J_1	-	*japonica*	Y	T	A	B	NA	41.5	12	17	9
KIR-J_11	-	*japonica*	Y	T	H	B	NA	43	10	16	10
KIR-J_13	-	*japonica*	Y	T	A	B	NA	37	11	18	10
KIR-J_2	-	*japonica*	Y	T	A	B	NA	41	12	18.5	10
KIR-J_3	-	*japonica*	Y	T	A	B	NA	40	13	20	10
KIR-J_9	-	*japonica*	Y	T	A	B	NA	45	12	20	10
KO-J_12	-	*natans*	Y	T	A	B	H	45.5	21.1	22.2	16
KO-J_14	-	*natans*	Y	F	A	P	D	55.5	38.5	19	12.5
KO-J_15	-	*natans*	Y	T	A	B	H	57.6	27.5	20	13
KO-J_16	-	*natans*	Y	T	A	B	H	56	24	25	14
KO-J_19	-	*natans*	Y	F	A	P	A	47.8	45	13	5.5
KO-J_23	-	*natans*	Y	T	A	B	H	55	17	20.1	10.1
KO-J_25	-	*natans*	Y	F	A	P	A	47.5	47	17.1	7
KO-J_7	-	*natans*	Y	T	A	B	D	61	16.1	19	14
MIK-J_1	-	*japonica*	Y	F	A	P	NA	35	19	12	5
MIK-J_13	-	*japonica*	Y	F	A	P	NA	35.25	12	14	9
MIK-J_15	-	*japonica*	Y	F	A	P	D	34.2	14	14.5	8
MIK-J_19	-	*japonica*	Y	T	A	P	NA	27.25	5	11	2
MIK-J_2	-	*japonica*	Y	F	A	P	D	37	16.8	16	9.25
MIK-J_25	-	*japonica*	Y	F	A	P	NA	39	7.2	16	2.4
MIK-J_28	-	*japonica*	Y	T	A	P	D	31.2	11.5	14	8.6
MIK-J_3	-	*japonica*	Y	F	A	P	D	39.8	12.75	16.2	9
MIK-J_30	-	*japonica*	Y	F	A	P	D	37.6	18.6	15	9

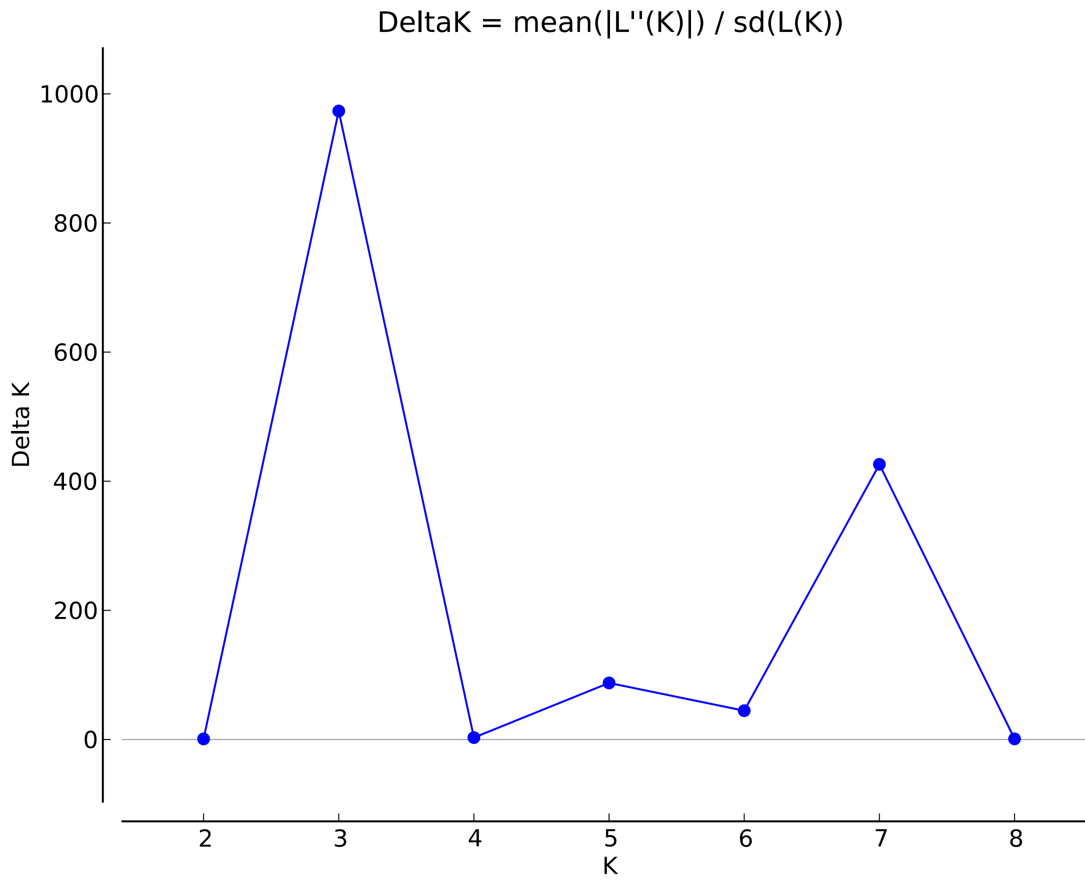
MIK-J_4	-	*japonica*	Y	F	A	P	D	29.8	14	15	8
ML-VA_12	RPB	*sp (US 2-horn)*	N	T	A	B	H	35	19	15	11.1
ML-VA_14	RPB	*sp (US 2-horn)*	N	T	A	B	H	39.5	19	15.1	10
ML-VA_16	RPB	*sp (US 2-horn)*	N	T	A	B	H	32	16	13	18.25
ML-VA_18	RPB	*sp (US 2-horn)*	N	T	A	B	H	38.5	16	16	10
ML-VA_8	RPB	*sp (US 2-horn)*	N	T	A	B	H	34.6	14	12.1	9
MM-NY_11	GR	*natans*	N	F	A	P	H	46	32	20	12
MM-NY_13	GR	*natans*	N	F	A	P	H	38.5	33	20.5	13
MM-NY_16	GR	*natans*	Y	F	A	P	D	30	23.1	15.1	10
MM-NY_17	GR	*natans*	Y	F	A	P	H	34.8	27.5	18	11.5
MM-NY_4	GR	*natans*	Y	F	A	P	H	42.1	31	18.9	11.5
MM-NY_5	GR	*natans*	Y	F	A	P	D	37	30.9	18	11
MM-NY_7	GR	*natans*	Y	F	A	P	H	31.5	30.1	18.1	10
MM-NY_9	GR	*natans*	N	F	A	P	A	38.1	31.5	21	13
MRC-NY_10	GR	*natans*	Y	F	A	P	D	41	33	20	11
MRC-NY_14	GR	*natans*	Y	F	H	P	D	41	34	21	12
MRC-NY_16	GR	*natans*	Y	F	A	P	D	37	30	20	10
MRC-NY_19	GR	*natans*	Y	F	H	P	A	43	35	25	14
MRC-NY_21	GR	*natans*	Y	F	H	P	D	33	26	17	12
MRC-NY_22	GR	*natans*	Y	F	A	P	D	40	36	22	14
MRC-NY_23	GR	*natans*	Y	F	A	P	D	38	30	19	12
MRC-NY_4	GR	*natans*	Y	F	A	P	D	30	28	17	11
MRC-NY_7	GR	*natans*	Y	T	A	B	A	43	27	23	14
MRC-NY_8	GR	*natans*	Y	F	A	P	D	37	32	20	11
NAK-J_17	-	*incisa*	N	F	A	P	NA	19.5	3.4	8.25	3.5
NAK-J_18	-	*incisa*	N	F	A	P	D	15.4	4.75	9	4
NAK-J_21	-	*incisa*	N	F	A	P	D	21.2	5	9	3.6
NAK-J_23	-	*incisa*	N	F	A	P	D	17	4	8	3
NAK-J_25	-	*incisa*	N	F	A	P	D	19	4	9	4
NAK-J_28	-	*incisa*	N	F	A	P	D	15	4	9	4
NAK-J_30	-	*incisa*	N	F	A	P	D	16	4	8	3
ONO-J_1	-	*japonica*	Y	T	A	B	H	29	11	15.1	8
ONO-J_12	-	*japonica*	Y	T	A	B	H	26.25	7.8	16.5	7.6
ONO-J_19	-	*japonica*	Y	F	A	P	D	37	35	19	11
ONO-J_25	-	*japonica*	Y	T	A	B	NA	35	9.6	15	9
ONO-J_3	-	*japonica*	Y	T	A	B	H	36	11.25	18	9.2
ONO-J_6	-	*japonica*	Y	F	A	P	D	24	28.75	17	9
ONO-J_7	-	*japonica*	Y	T	A	B	H	28.5	12.1	15	8.5

ONO-J_8	-	*japonica*	Y	T	A	B	H	31	8.5	18.5	8.2
RC-NY_13	GRPPB	*natans*	Y	F	A	P	D	39	39	20	11
RC-NY_14	GRPPB	*natans*	Y	F	A	P	D	43.5	35.1	24	14.5
RC-NY_16	GRPPB	*natans*	Y	F	A	P	H	39	23	27.9	10
RC-NY_5	GRPPB	*natans*	Y	F	A	P	A	41	35.5	21.5	11
RC-NY_8	GRPPB	*natans*	Y	F	A	P	H	32	17	13	9.5
SC-NY_2	GR	*natans*	Y	F	A	P	H	35.2	30.5	18	12
SC-NY_3	GR	*natans*	Y	F	A	P	H	44	30.5	20	12
SC-NY_5	GR	*natans*	Y	F	A	P	H	35	36	21	14
SC-NY_8	GR	*natans*	Y	T	A	B	H	40.5	19.1	19.2	12
SC-NY_9	GR	*natans*	Y	F	A	P	H	39.5	36	22.5	14
SHI-J_18	-	*japonica*	Y	F	A	P	A	38	33	19.5	10
SHI-J_19	-	*japonica*	Y	F	A	P	A	42	36.5	17	10
SHI-J_20	-	*japonica*	Y	F	A	P	A	34.5	35	15.5	10
SHI-J_24	-	*japonica*	Y	F	A	P	A	43.5	39	20	10
SHI-J_25	-	*japonica*	Y	F	A	P	A	40	35.5	20	10
SHI-J_6	-	*japonica*	Y	F	A	P	A	34	37	17	10
SHI-J_8	-	*japonica*	Y	F	A	P	A	42	33	17	10
SLR-TW_1	-	*bispinosa var iinumai*	N	T	H	B	A	35	12.1	14.5	10
SLR-TW_11	-	*bispinosa var iinumai*	N	T	H	B	H	40	14	16.5	11
SLR-TW_16	-	*bispinosa var iinumai*	N	T	H	B	H	41	14.5	14	9.5
SLR-TW_18	-	*bispinosa var iinumai*	N	T	A	B	H	35.25	12.5	14	10
SLR-TW_20	-	*bispinosa var iinumai*	N	T	H	B	H	41	13	14	10.25
SLR-TW_21	-	*bispinosa var iinumai*	N	T	A	B	H	40	14.5	16.25	10
SLR-TW_23	-	*bispinosa var iinumai*	N	T	H	B	H	40.25	14	16	11
SLR-TW_4	-	*bispinosa var iinumai*	N	T	H	B	D	35.5	3	13.5	10
SLR-TW_9	-	*bispinosa var iinumai*	N	T	H	B	H	37	13.5	14	10
SR-MD 2017_1	GR	*natans*	Y	F	H	P	D	26	35	17	11
SR-MD 2017_14	GR	*natans*	Y	F	H	P	D	37	37	20	12
SR-MD 2017_16	GR	*natans*	Y	F	H	P	D	35	32	21	11
SR-MD 2017_23	GR	*natans*	Y	F	A	P	D	33	26	19	11

SR-MD 2017_3	GR	*natans*	Y	F	H	P	D	36	30	22	12
SR-MD 2017_4	GR	*natans*	Y	F	H	P	D	36	32	25	12
SR-MD 2017_5	GR	*natans*	Y	F	A	P	D	40	30	20	11
SR-MD 2017_9	GR	*natans*	Y	F	H	P	D	35	31	22	11
SSB-NY_11	GR	*natans*	Y	F	A	P	H	44	44	24.2	12.5
SSB-NY_13	GR	*natans*	Y	F	A	P	H	46	40.5	23	11.5
SSB-NY_16	GR	*natans*	Y	F	A	P	D	40	34	22.5	9
SSB-NY_17	GR	*natans*	Y	F	A	P	D	50	37	25	14
SSB-NY_20	GR	*natans*	Y	F	A	P	D	41	40.5	24	14
TEM-J_1	-	*natans*	Y	T	A	B	H	60	16	23	14
TEM-J_11	-	*natans*	Y	F	A	P	D	60.6	48.5	25.2	13.5
TEM-J_13	-	*natans*	Y	F	A	P	H	63	58	23	14
TEM-J_16	-	*natans*	Y	T	A	B	H	58.1	15.5	21.5	9
TEM-J_19	-	*natans*	Y	F	A	P	D	59.8	39.25	24	12
TEM-J_25	-	*natans*	Y	T	A	P	D	57	15	15	4
TEM-J_3	-	*natans*	Y	F	A	P	H	51.5	50	23.2	12.5
TEM-J_7	-	*natans*	Y	F	A	P	H	48	53	19	11
TEM-J_9	-	*natans*	Y	F	A	P	H	58.5	51.2	23	15
TP-NY_13	GR	*natans*	Y	F	A	P	D	42	29.9	21	14
TP-NY_19	GR	*natans*	Y	F	A	P	A	34.5	28	16	11
TP-NY_2	GR	*natans*	Y	F	A	P	D	35	26	17	10
TP-NY_20	GR	*natans*	Y	F	A	P	D	33.5	33	19	13
TP-NY_7	GR	*natans*	Y	F	A	P	H	37	29	16.2	10.2
TP-NY_9	GR	*natans*	Y	F	A	P	D	41	28	18.8	11
UCH-J_1	-	*natans*	Y	F	A	P	D	58.5	52	24	14
UCH-J_16	-	*natans*	Y	F	A	P	D	58	54	19	13
UCH-J_17	-	*natans*	Y	F	A	P	D	49	46	20.5	14
UCH-J_21	-	*natans*	Y	F	H	P	D	59	49	20	15
UCH-J_23	-	*natans*	Y	F	A	P	D	48	48	19	9
UCH-J_3	-	*natans*	Y	F	H	P	D	64	51	22.5	15
UCH-J_4	-	*natans*	Y	F	A	P	D	53	46.5	20	14.25
UCH-J_5	-	*natans*	Y	F	A	P	D	52.5	53.25	21	15
UCH-J_6	-	*natans*	Y	F	H	P	D	59	56.25	21	16.5
UCH-J_9	-	*natans*	Y	F	A	P	D	52	39	17	15
VCB-VA_12	RPB	*sp (US 2-horn)*	N	T	A	B	H	20	15.75	14	10
VCB-VA_14	RPB	*sp (US 2-horn)*	N	T	A	B	H	35	18.5	14	11

VCB-VA_16	RPB	*sp (US 2-horn)*	N	T	A	B	H	35.5	17.5	14.2	8.4
VCB-VA_19	RPB	*sp (US 2-horn)*	N	T	A	B	H	24.5	10.2	9	2.5
VCB-VA_25	RPB	*sp (US 2-horn)*	N	T	A	B	H	31.5	19	12.25	9.5
VCB-VA_6	RPB	*sp (US 2-horn)*	N	T	A	B	H	35.25	18.2	15	9
VCB-VA_8	RPB	*sp (US 2-horn)*	N	T	A	B	H	36	18.6	15	9
WC-VA_1	GRPPB	*sp (US 2-horn)*	N	T	A	B	A	41	24	20	11
WC-VA_11	GR	*sp (US 2-horn)*	N	T	A	B	A	41	25	20	14
WC-VA_14	GR	*sp (US 2-horn)*	N	T	A	B	H	36	16	14	9
WC-VA_15	GR	*sp (US 2-horn)*	N	T	A	B	H	39	20	18	11
WC-VA_16	GRPPB	*sp (US 2-horn)*	N	T	A	B	H	37	23	19	10
WC-VA_17	GR	*sp (US 2-horn)*	N	T	A	B	H	24	22	20	12
WC-VA_20	RPB	*sp (US 2-horn)*	N	T	A	B	H	43	22	19	12
WC-VA_21	GRPPB	*sp (US 2-horn)*	N	T	A	B	A	32	17	15	10
WC-VA_24	GRPPB	*sp (US 2-horn)*	N	T	A	B	H	37	21	15	9
WC-VA_25	GRPPB	*sp (US 2-horn)*	N	T	A	B	A	35	18	16	10
WF-MA_1	GR	*natans*	Y	F	H	P	D	39	33	19	12
WF-MA_16	GR	*natans*	Y	F	H	P	H	44	38	24	15
WF-MA_18	GR	*natans*	Y	F	H	P	D	38	26	22	13
WF-MA_19	GR	*natans*	Y	F	H	P	H	47	43	26	18
WF-MA_21	GR	*natans*	Y	F	H	P	D	49	44	28	19
WF-MA_23	GR	*natans*	Y	F	D	P	D	39	27	18	13
WF-MA_25	GR	*natans*	Y	F	A	P	NA	50	41	23	17
WF-MA_5	GR	*natans*	Y	F	H	P	D	42	29	19	14
WF-MA_7	GR	*natans*	Y	F	H	P	D	38	33	18	12
WL-NY_1	GR	*natans*	Y	F	A	P	A	36	27	19	10
WL-NY_10	GR	*natans*	Y	F	H	P	D	38	32	22	18
WL-NY_11	GR	*natans*	Y	F	H	P	H	33	30	19	15
WL-NY_12	GR	*natans*	Y	F	A	P	H	36	19	14	10
WL-NY_2	GR	*natans*	Y	F	H	P	D	33	30	20	14
WL-NY_3	GR	*natans*	Y	F	H	P	D	32	26	17	13
WL-NY_4	GR	*natans*	Y	F	H	P	H	37	29	21	14
WL-NY_8	GR	*natans*	Y	T	A	B	A	30	14	15	10
WL-NY_9	GR	*natans*	Y	F	H	P	D	34	25	18	13
WO-VA_11	GRPPB	*sp (US 2-horn)*	N	T	A	B	A	36	20	14	10
WP-VA_1	RPB	*sp (US 2-horn)*	N	T	A	B	H	34.5	20.25	17.2	11
WP-VA_13	RPB	*sp (US 2-horn)*	N	T	A	B	H	36.25	21	19	11
WP-VA_19	RPB	*sp (US 2-horn)*	N	T	A	B	H	36	20	17.6	10.5
WP-VA_2	RPB	*sp (US 2-horn)*	N	T	A	B	H	35	17.8	14	9.8

WP-VA_25	RPB	*sp (US 2-horn)*	N	T	A	B	NA	21	11	12.25	3
WP-VA_3	RPB	*sp (US 2-horn)*	N	T	A	B	H	35	17.2	17	10.5
WP-VA_4	RPB	*sp (US 2-horn)*	N	T	A	B	H	35.6	20.8	18.5	11.1
WP-VA_6	RPB	*sp (US 2-horn)*	N	T	A	B	H	32	18.8	15	10
WP-VA_7	RPB	*sp (US 2-horn)*	N	T	A	B	H	35	24.2	20	12



Supplementary Figure 2. Delta K plot for iterations of K in STRUCTURE (as in Evanno et al. 2005).

Supplementary Table 3. Square loadings for each morphological variation on the first two axes of each Principle Component Analysis shown in Fig. 3.

	A) Worldwide		B) USA	
Variable	**PC 1**	**PC 2**	**PC 1**	**PC 2**
Upper.Fruit.Width..mm.	0.3752	0.3121	0.2863	0.4037
Lower.Fruit.Width..mm.	0.7711	0.0206	0.7909	0.0466
Fruit.Height..mm.	0.5733	0.1978	0.6060	0.1681
Fruit.Thickness..mm.	0.5087	0.2693	0.4337	0.0635
Under Leaf Color	-	-	0.7032	0.1866
Crown	0.4398	0.0482	0.7519	0.0428
Spines	0.5834	0.3072	0.7986	0.0475
Spine.Orientation	0.1545	0.0390	0.2317	0.1566
Lower.Shape.at.Apex	0.5597	0.3317	0.8210	0.0403
Lower.Direction	0.3350	0.3258	0.3044	0.4136

APPENDIX B

SUPPLEMENTARY MATERIAL FROM CHAPTER FOUR

Identification of resistant and susceptible clones of Eurasian watermilfoil (Myriophyllum spicatum) to the operational rate of fluridone - Data Analysis

Gregory M. Chorak
08/03/2020

1. Loading the data into R.

```
library(readr)
Fluridone_data <- read_csv("~/Desktop/Fluridone_Full.csv")
## Parsed with column specification:
## cols(
## ID = col_character(),
## Accession_Genotype = col_character(),
## Treat = col_character(),
## Tank = col_character(),
## Rep = col_character(),
## Wt = col_double(),
## Block = col_double()
## )
print.data.frame(Fluridone_data)
## ID Accession_Genotype Treat Tank Rep Wt Block
## 1 Ind.1 1_MG-237 C Tank.1 ATank.1 1.47 1
## 2 Ind.2 1_MG-237 C Tank.1 BTank.1 1.50 1
## 3 Ind.3 1_MG-237 C Tank.1 CTank.1 1.77 1
## 4 Ind.4 1_MG-237 C Tank.2 ATank.2 0.94 1
## 5 Ind.5 1_MG-237 C Tank.2 BTank.2 1.10 1
## 6 Ind.6 1_MG-237 C Tank.2 CTank.2 1.07 1
## 7 Ind.7 1_MG-237 C Tank.5 ATank.5 1.20 1
## 8 Ind.8 1_MG-237 C Tank.5 BTank.5 1.30 1
## 9 Ind.9 1_MG-237 C Tank.5 CTank.5 1.26 1
## 10 Ind.10 1_MG-237 T Tank.3 ATank.3 1.08 1
## 11 Ind.11 1_MG-237 T Tank.3 BTank.3 1.29 1
## 12 Ind.12 1_MG-237 T Tank.3 CTank.3 0.89 1
## 13 Ind.13 1_MG-237 T Tank.4 ATank.4 1.14 1
## 14 Ind.14 1_MG-237 T Tank.4 BTank.4 0.69 1
## 15 Ind.15 1_MG-237 T Tank.4 CTank.4 1.43 1
## 16 Ind.16 1_MG-237 T Tank.6 ATank.6 1.11 1
## 17 Ind.17 1_MG-237 T Tank.6 BTank.6 0.71 1
## 18 Ind.18 1_MG-237 T Tank.6 CTank.6 1.62 1
## 19 Ind.19 1_MG-237 C Tank.1 ATank.1 1.03 2
## 20 Ind.20 1_MG-237 C Tank.1 BTank.1 1.41 2
## 21 Ind.21 1_MG-237 C Tank.1 CTank.1 1.11 2
## 22 Ind.22 1_MG-237 C Tank.4 ATank.4 0.70 2
## 23 Ind.23 1_MG-237 C Tank.4 BTank.4 0.84 2
## 24 Ind.24 1_MG-237 C Tank.4 CTank.4 1.87 2
## 25 Ind.25 1_MG-237 C Tank.6 ATank.6 0.99 2
## 26 Ind.26 1_MG-237 C Tank.6 BTank.6 2.15 2
## 27 Ind.27 1_MG-237 C Tank.6 CTank.6 0.66 2
```

```
## 28 Ind.28 1_MG-237 T Tank.2 ATank.2 0.71 2
## 29 Ind.29 1_MG-237 T Tank.2 BTank.2 0.76 2
## 30 Ind.30 1_MG-237 T Tank.2 CTank.2 1.84 2
## 31 Ind.31 1_MG-237 T Tank.3 ATank.3 0.56 2
## 32 Ind.32 1_MG-237 T Tank.3 BTank.3 2.09 2
## 33 Ind.33 1_MG-237 T Tank.3 CTank.3 0.81 2
## 34 Ind.34 1_MG-237 T Tank.5 ATank.5 1.24 2
## 35 Ind.35 1_MG-237 T Tank.5 BTank.5 0.79 2
## 36 Ind.36 1_MG-237 T Tank.5 CTank.5 2.15 2
## 37 Ind.37 2_MG-237 C Tank.1 ATank.1 1.85 1
## 38 Ind.38 2_MG-237 C Tank.1 BTank.1 1.52 1
## 39 Ind.39 2_MG-237 C Tank.1 CTank.1 1.68 1
## 40 Ind.40 2_MG-237 C Tank.2 ATank.2 1.43 1
## 41 Ind.41 2_MG-237 C Tank.2 BTank.2 1.14 1
## 42 Ind.42 2_MG-237 C Tank.2 CTank.2 1.28 1
## 43 Ind.43 2_MG-237 C Tank.5 ATank.5 0.97 1
## 44 Ind.44 2_MG-237 C Tank.5 BTank.5 1.53 1
## 45 Ind.45 2_MG-237 C Tank.5 CTank.5 1.20 1
## 46 Ind.46 2_MG-237 T Tank.3 ATank.3 1.34 1
## 47 Ind.47 2_MG-237 T Tank.3 BTank.3 0.62 1
## 48 Ind.48 2_MG-237 T Tank.3 CTank.3 1.42 1
## 49 Ind.49 2_MG-237 T Tank.4 ATank.4 0.84 1
## 50 Ind.50 2_MG-237 T Tank.4 BTank.4 0.76 1
## 51 Ind.51 2_MG-237 T Tank.4 CTank.4 0.78 1
## 52 Ind.52 2_MG-237 T Tank.6 ATank.6 1.50 1
## 53 Ind.53 2_MG-237 T Tank.6 BTank.6 1.72 1
## 54 Ind.54 2_MG-237 T Tank.6 CTank.6 1.51 1
## 55 Ind.55 2_MG-237 C Tank.1 ATank.1 1.50 2
## 56 Ind.56 2_MG-237 C Tank.1 BTank.1 1.13 2
## 57 Ind.57 2_MG-237 C Tank.1 CTank.1 1.03 2
## 58 Ind.58 2_MG-237 C Tank.4 ATank.4 1.88 2
## 59 Ind.59 2_MG-237 C Tank.4 BTank.4 1.00 2
## 60 Ind.60 2_MG-237 C Tank.4 CTank.4 0.61 2
## 61 Ind.61 2_MG-237 C Tank.6 ATank.6 0.52 2
## 62 Ind.62 2_MG-237 C Tank.6 BTank.6 1.09 2
## 63 Ind.63 2_MG-237 C Tank.6 CTank.6 1.64 2
## 64 Ind.64 2_MG-237 T Tank.2 ATank.2 0.97 2
## 65 Ind.65 2_MG-237 T Tank.2 BTank.2 1.26 2
## 66 Ind.66 2_MG-237 T Tank.2 CTank.2 0.80 2
## 67 Ind.67 2_MG-237 T Tank.3 ATank.3 1.24 2
## 68 Ind.68 2_MG-237 T Tank.3 BTank.3 0.87 2
## 69 Ind.69 2_MG-237 T Tank.3 CTank.3 0.73 2
## 70 Ind.70 2_MG-237 T Tank.5 ATank.5 1.30 2
## 71 Ind.71 2_MG-237 T Tank.5 BTank.5 0.88 2
## 72 Ind.72 2_MG-237 T Tank.5 CTank.5 1.88 2
## 73 Ind.73 3_MG-237 C Tank.1 ATank.1 1.39 1
## 74 Ind.74 3_MG-237 C Tank.1 BTank.1 1.31 1
## 75 Ind.75 3_MG-237 C Tank.1 CTank.1 1.57 1
## 76 Ind.76 3_MG-237 C Tank.2 ATank.2 1.70 1
## 77 Ind.77 3_MG-237 C Tank.2 BTank.2 1.34 1
## 78 Ind.78 3_MG-237 C Tank.2 CTank.2 1.22 1
## 79 Ind.79 3_MG-237 C Tank.5 ATank.5 1.03 1
## 80 Ind.80 3_MG-237 C Tank.5 BTank.5 0.89 1
```

```
## 81 Ind.81 3_MG-237 C Tank.5 CTank.5 0.99 1
## 82 Ind.82 3_MG-237 T Tank.3 ATank.3 1.32 1
## 83 Ind.83 3_MG-237 T Tank.3 BTank.3 1.20 1
## 84 Ind.84 3_MG-237 T Tank.3 CTank.3 1.22 1
## 85 Ind.85 3_MG-237 T Tank.4 ATank.4 0.60 1
## 86 Ind.86 3_MG-237 T Tank.4 BTank.4 0.34 1
## 87 Ind.87 3_MG-237 T Tank.4 CTank.4 0.88 1
## 88 Ind.88 3_MG-237 T Tank.6 ATank.6 0.99 1
## 89 Ind.89 3_MG-237 T Tank.6 BTank.6 1.24 1
## 90 Ind.90 3_MG-237 T Tank.6 CTank.6 0.89 1
## 91 Ind.91 3_MG-237 C Tank.1 ATank.1 1.48 2
## 92 Ind.92 3_MG-237 C Tank.1 BTank.1 0.16 2
## 93 Ind.93 3_MG-237 C Tank.1 CTank.1 0.48 2
## 94 Ind.94 3_MG-237 C Tank.4 ATank.4 1.36 2
## 95 Ind.95 3_MG-237 C Tank.4 BTank.4 0.83 2
## 96 Ind.96 3_MG-237 C Tank.4 CTank.4 1.45 2
## 97 Ind.97 3_MG-237 C Tank.6 ATank.6 1.44 2
## 98 Ind.98 3_MG-237 C Tank.6 BTank.6 0.93 2
## 99 Ind.99 3_MG-237 C Tank.6 CTank.6 2.27 2
## 100 Ind.100 3_MG-237 T Tank.2 ATank.2 1.00 2
## 101 Ind.101 3_MG-237 T Tank.2 BTank.2 0.94 2
## 102 Ind.102 3_MG-237 T Tank.2 CTank.2 0.89 2
## 103 Ind.103 3_MG-237 T Tank.3 ATank.3 1.48 2
## 104 Ind.104 3_MG-237 T Tank.3 BTank.3 0.91 2
## 105 Ind.105 3_MG-237 T Tank.3 CTank.3 1.63 2
## 106 Ind.106 3_MG-237 T Tank.5 ATank.5 0.23 2
## 107 Ind.107 3_MG-237 T Tank.5 BTank.5 1.74 2
## 108 Ind.108 3_MG-237 T Tank.5 CTank.5 0.33 2
## 109 Ind.109 4_MG-237 C Tank.1 ATank.1 1.40 1
## 110 Ind.110 4_MG-237 C Tank.1 BTank.1 1.21 1
## 111 Ind.111 4_MG-237 C Tank.1 CTank.1 1.84 1
## 112 Ind.112 4_MG-237 C Tank.2 ATank.2 1.43 1
## 113 Ind.113 4_MG-237 C Tank.2 BTank.2 1.20 1
## 114 Ind.114 4_MG-237 C Tank.2 CTank.2 1.17 1
## 115 Ind.115 4_MG-237 C Tank.5 ATank.5 1.35 1
## 116 Ind.116 4_MG-237 C Tank.5 BTank.5 1.47 1
## 117 Ind.117 4_MG-237 C Tank.5 CTank.5 1.09 1
## 118 Ind.118 4_MG-237 T Tank.3 ATank.3 0.80 1
## 119 Ind.119 4_MG-237 T Tank.3 BTank.3 0.94 1
## 120 Ind.120 4_MG-237 T Tank.3 CTank.3 0.43 1
## 121 Ind.121 4_MG-237 T Tank.4 ATank.4 0.73 1
## 122 Ind.122 4_MG-237 T Tank.4 BTank.4 0.71 1
## 123 Ind.123 4_MG-237 T Tank.4 CTank.4 0.65 1
## 124 Ind.124 4_MG-237 T Tank.6 ATank.6 0.66 1
## 125 Ind.125 4_MG-237 T Tank.6 BTank.6 0.84 1
## 126 Ind.126 4_MG-237 T Tank.6 CTank.6 1.08 1
## 127 Ind.127 4_MG-237 C Tank.1 ATank.1 0.51 2
## 128 Ind.128 4_MG-237 C Tank.1 BTank.1 1.49 2
## 129 Ind.129 4_MG-237 C Tank.1 CTank.1 1.28 2
## 130 Ind.130 4_MG-237 C Tank.4 ATank.4 1.28 2
## 131 Ind.131 4_MG-237 C Tank.4 BTank.4 1.94 2
## 132 Ind.132 4_MG-237 C Tank.4 CTank.4 2.34 2
## 133 Ind.133 4_MG-237 C Tank.6 ATank.6 0.76 2
```

```
## 134 Ind.134 4_MG-237 C Tank.6 BTank.6 1.38 2
## 135 Ind.135 4_MG-237 C Tank.6 CTank.6 1.83 2
## 136 Ind.136 4_MG-237 T Tank.2 ATank.2 0.93 2
## 137 Ind.137 4_MG-237 T Tank.2 BTank.2 1.81 2
## 138 Ind.138 4_MG-237 T Tank.2 CTank.2 0.76 2
## 139 Ind.139 4_MG-237 T Tank.3 ATank.3 1.38 2
## 140 Ind.140 4_MG-237 T Tank.3 BTank.3 1.79 2
## 141 Ind.141 4_MG-237 T Tank.3 CTank.3 0.36 2
## 142 Ind.142 4_MG-237 T Tank.5 ATank.5 2.02 2
## 143 Ind.143 4_MG-237 T Tank.5 BTank.5 1.13 2
## 144 Ind.144 4_MG-237 T Tank.5 CTank.5 2.03 2
## 145 Ind.145 6_MG-237 C Tank.1 ATank.1 1.91 1
## 146 Ind.146 6_MG-237 C Tank.1 BTank.1 0.89 1
## 147 Ind.147 6_MG-237 C Tank.1 CTank.1 1.23 1
## 148 Ind.148 6_MG-237 C Tank.2 ATank.2 0.89 1
## 149 Ind.149 6_MG-237 C Tank.2 BTank.2 1.04 1
## 150 Ind.150 6_MG-237 C Tank.2 CTank.2 1.03 1
## 151 Ind.151 6_MG-237 C Tank.5 ATank.5 1.12 1
## 152 Ind.152 6_MG-237 C Tank.5 BTank.5 1.17 1
## 153 Ind.153 6_MG-237 C Tank.5 CTank.5 0.72 1
## 154 Ind.154 6_MG-237 T Tank.3 ATank.3 1.33 1
## 155 Ind.155 6_MG-237 T Tank.3 BTank.3 0.72 1
## 156 Ind.156 6_MG-237 T Tank.3 CTank.3 1.55 1
## 157 Ind.157 6_MG-237 T Tank.4 ATank.4 1.18 1
## 158 Ind.158 6_MG-237 T Tank.4 BTank.4 1.25 1
## 159 Ind.159 6_MG-237 T Tank.4 CTank.4 1.53 1
## 160 Ind.160 6_MG-237 T Tank.6 ATank.6 1.46 1
## 161 Ind.161 6_MG-237 T Tank.6 BTank.6 1.38 1
## 162 Ind.162 6_MG-237 T Tank.6 CTank.6 1.74 1
## 163 Ind.163 6_MG-237 C Tank.1 ATank.1 0.55 2
## 164 Ind.164 6_MG-237 C Tank.1 BTank.1 0.58 2
## 165 Ind.165 6_MG-237 C Tank.1 CTank.1 0.43 2
## 166 Ind.166 6_MG-237 C Tank.4 ATank.4 1.42 2
## 167 Ind.167 6_MG-237 C Tank.4 BTank.4 0.92 2
## 168 Ind.168 6_MG-237 C Tank.4 CTank.4 1.29 2
## 169 Ind.169 6_MG-237 C Tank.6 ATank.6 0.73 2
## 170 Ind.170 6_MG-237 C Tank.6 BTank.6 0.90 2
## 171 Ind.171 6_MG-237 C Tank.6 CTank.6 1.02 2
## 172 Ind.172 6_MG-237 T Tank.2 ATank.2 1.56 2
## 173 Ind.173 6_MG-237 T Tank.2 BTank.2 1.28 2
## 174 Ind.174 6_MG-237 T Tank.2 CTank.2 2.84 2
## 175 Ind.175 6_MG-237 T Tank.3 ATank.3 3.05 2
## 176 Ind.176 6_MG-237 T Tank.3 BTank.3 2.33 2
## 177 Ind.177 6_MG-237 T Tank.3 CTank.3 2.83 2
## 178 Ind.178 6_MG-237 T Tank.5 ATank.5 1.88 2
## 179 Ind.179 6_MG-237 T Tank.5 BTank.5 1.99 2
## 180 Ind.180 6_MG-237 T Tank.5 CTank.5 3.88 2
## 181 Ind.181 5_MG-237 C Tank.1 ATank.1 1.22 1
## 182 Ind.182 5_MG-237 C Tank.1 BTank.1 1.29 1
## 183 Ind.183 5_MG-237 C Tank.2 ATank.2 0.83 1
## 184 Ind.184 5_MG-237 C Tank.2 BTank.2 0.81 1
## 185 Ind.185 5_MG-237 C Tank.2 CTank.2 1.67 1
## 186 Ind.186 5_MG-237 C Tank.5 ATank.5 0.60 1
```

```
## 187 Ind.187 5_MG-237 C Tank.5 BTank.5 0.59 1
## 188 Ind.188 5_MG-237 C Tank.5 CTank.5 0.81 1
## 189 Ind.189 5_MG-237 T Tank.3 ATank.3 0.89 1
## 190 Ind.190 5_MG-237 T Tank.3 BTank.3 1.10 1
## 191 Ind.191 5_MG-237 T Tank.3 CTank.3 0.95 1
## 192 Ind.192 5_MG-237 T Tank.4 ATank.4 0.98 1
## 193 Ind.193 5_MG-237 T Tank.4 BTank.4 0.71 1
## 194 Ind.194 5_MG-237 T Tank.4 CTank.4 0.59 1
## 195 Ind.195 5_MG-237 T Tank.6 ATank.6 1.24 1
## 196 Ind.196 5_MG-237 T Tank.6 BTank.6 1.23 1
## 197 Ind.197 5_MG-237 T Tank.6 CTank.6 0.90 1
## 198 Ind.198 5_MG-237 C Tank.4 ATank.4 0.45 2
## 199 Ind.199 5_MG-237 C Tank.5 ATank.5 0.79 2
## 200 Ind.200 5_MG-237 C Tank.6 ATank.6 0.43 2
## 201 Ind.201 5_MG-237 T Tank.1 ATank.1 1.45 2
## 202 Ind.202 5_MG-237 T Tank.2 ATank.2 1.11 2
## 203 Ind.203 5_MG-237 T Tank.3 ATank.3 0.95 2
## 204 Ind.204 7_MG-377 C Tank.1 ATank.1 1.42 1
## 205 Ind.205 7_MG-377 C Tank.1 BTank.1 1.25 1
## 206 Ind.206 7_MG-377 C Tank.1 CTank.1 1.00 1
## 207 Ind.207 7_MG-377 C Tank.2 ATank.2 1.63 1
## 208 Ind.208 7_MG-377 C Tank.2 BTank.2 1.73 1
## 209 Ind.209 7_MG-377 C Tank.2 CTank.2 1.37 1
## 210 Ind.210 7_MG-377 C Tank.5 ATank.5 1.18 1
## 211 Ind.211 7_MG-377 C Tank.5 BTank.5 1.32 1
## 212 Ind.212 7_MG-377 C Tank.5 CTank.5 1.07 1
## 213 Ind.213 7_MG-377 T Tank.3 ATank.3 1.57 1
## 214 Ind.214 7_MG-377 T Tank.3 BTank.3 1.45 1
## 215 Ind.215 7_MG-377 T Tank.3 CTank.3 2.12 1
## 216 Ind.216 7_MG-377 T Tank.4 ATank.4 1.08 1
## 217 Ind.217 7_MG-377 T Tank.4 BTank.4 1.31 1
## 218 Ind.218 7_MG-377 T Tank.4 CTank.4 0.84 1
## 219 Ind.219 7_MG-377 T Tank.6 ATank.6 1.88 1
## 220 Ind.220 7_MG-377 T Tank.6 BTank.6 1.32 1
## 221 Ind.221 7_MG-377 T Tank.6 CTank.6 2.07 1
## 222 Ind.222 7_MG-377 C Tank.1 ATank.1 2.45 2
## 223 Ind.223 7_MG-377 C Tank.1 BTank.1 0.90 2
## 224 Ind.224 7_MG-377 C Tank.1 CTank.1 1.21 2
## 225 Ind.225 7_MG-377 C Tank.4 ATank.4 2.70 2
## 226 Ind.226 7_MG-377 C Tank.4 BTank.4 2.01 2
## 227 Ind.227 7_MG-377 C Tank.4 CTank.4 1.81 2
## 228 Ind.228 7_MG-377 C Tank.6 ATank.6 1.94 2
## 229 Ind.229 7_MG-377 C Tank.6 BTank.6 1.51 2
## 230 Ind.230 7_MG-377 C Tank.6 CTank.6 0.70 2
## 231 Ind.231 7_MG-377 T Tank.2 ATank.2 3.19 2
## 232 Ind.232 7_MG-377 T Tank.2 BTank.2 1.09 2
## 233 Ind.233 7_MG-377 T Tank.2 CTank.2 1.42 2
## 234 Ind.234 7_MG-377 T Tank.3 ATank.3 2.04 2
## 235 Ind.235 7_MG-377 T Tank.3 BTank.3 4.96 2
## 236 Ind.236 7_MG-377 T Tank.3 CTank.3 2.09 2
## 237 Ind.237 7_MG-377 T Tank.5 ATank.5 3.27 2
## 238 Ind.238 7_MG-377 T Tank.5 BTank.5 2.75 2
## 239 Ind.239 7_MG-377 T Tank.5 CTank.5 2.17 2
```

```
## 240 Ind.240 8_MG-429 C Tank.1 ATank.1 1.80 1
## 241 Ind.241 8_MG-429 C Tank.1 BTank.1 1.02 1
## 242 Ind.242 8_MG-429 C Tank.1 CTank.1 1.86 1
## 243 Ind.243 8_MG-429 C Tank.2 ATank.2 0.96 1
## 244 Ind.244 8_MG-429 C Tank.2 BTank.2 0.52 1
## 245 Ind.245 8_MG-429 C Tank.2 CTank.2 1.05 1
## 246 Ind.246 8_MG-429 C Tank.5 ATank.5 1.14 1
## 247 Ind.247 8_MG-429 C Tank.5 BTank.5 0.96 1
## 248 Ind.248 8_MG-429 C Tank.5 CTank.5 1.41 1
## 249 Ind.249 8_MG-429 T Tank.3 ATank.3 0.68 1
## 250 Ind.250 8_MG-429 T Tank.3 BTank.3 0.71 1
## 251 Ind.251 8_MG-429 T Tank.3 CTank.3 0.65 1
## 252 Ind.252 8_MG-429 T Tank.4 ATank.4 0.34 1
## 253 Ind.253 8_MG-429 T Tank.4 BTank.4 0.24 1
## 254 Ind.254 8_MG-429 T Tank.4 CTank.4 0.38 1
## 255 Ind.255 8_MG-429 T Tank.6 ATank.6 0.74 1
## 256 Ind.256 8_MG-429 T Tank.6 BTank.6 0.62 1
## 257 Ind.257 8_MG-429 T Tank.6 CTank.6 0.85 1
## 258 Ind.258 8_MG-429 C Tank.4 ATank.4 1.66 2
## 259 Ind.259 8_MG-429 C Tank.5 ATank.5 0.69 2
## 260 Ind.260 8_MG-429 C Tank.6 ATank.6 1.84 2
## 261 Ind.261 8_MG-429 T Tank.1 ATank.1 0.20 2
## 262 Ind.262 8_MG-429 T Tank.2 ATank.2 0.79 2
## 263 Ind.263 8_MG-429 T Tank.3 ATank.3 0.34 2
## 264 Ind.264 9_MG-268 C Tank.1 ATank.1 0.90 1
## 265 Ind.265 9_MG-268 C Tank.1 BTank.1 0.80 1
## 266 Ind.266 9_MG-268 C Tank.1 CTank.1 1.09 1
## 267 Ind.267 9_MG-268 C Tank.2 ATank.2 0.71 1
## 268 Ind.268 9_MG-268 C Tank.2 BTank.2 0.60 1
## 269 Ind.269 9_MG-268 C Tank.2 CTank.2 1.75 1
## 270 Ind.270 9_MG-268 C Tank.5 ATank.5 0.97 1
## 271 Ind.271 9_MG-268 C Tank.5 BTank.5 1.09 1
## 272 Ind.272 9_MG-268 C Tank.5 CTank.5 1.25 1
## 273 Ind.273 9_MG-268 T Tank.3 ATank.3 1.00 1
## 274 Ind.274 9_MG-268 T Tank.3 BTank.3 0.39 1
## 275 Ind.275 9_MG-268 T Tank.3 CTank.3 0.83 1
## 276 Ind.276 9_MG-268 T Tank.4 ATank.4 0.47 1
## 277 Ind.277 9_MG-268 T Tank.4 BTank.4 0.37 1
## 278 Ind.278 9_MG-268 T Tank.4 CTank.4 0.45 1
## 279 Ind.279 9_MG-268 T Tank.6 ATank.6 0.77 1
## 280 Ind.280 9_MG-268 T Tank.6 BTank.6 0.87 1
## 281 Ind.281 9_MG-268 T Tank.6 CTank.6 1.09 1
## 282 Ind.282 9_MG-268 C Tank.1 ATank.1 0.75 2
## 283 Ind.283 9_MG-268 C Tank.1 BTank.1 1.43 2
## 284 Ind.284 9_MG-268 C Tank.1 CTank.1 1.05 2
## 285 Ind.285 9_MG-268 C Tank.4 ATank.4 2.32 2
## 286 Ind.286 9_MG-268 C Tank.4 BTank.4 1.39 2
## 287 Ind.287 9_MG-268 C Tank.4 CTank.4 1.93 2
## 288 Ind.288 9_MG-268 C Tank.6 ATank.6 1.53 2
## 289 Ind.289 9_MG-268 C Tank.6 BTank.6 3.67 2
## 290 Ind.290 9_MG-268 C Tank.6 CTank.6 0.79 2
## 291 Ind.291 9_MG-268 T Tank.2 ATank.2 1.00 2
## 292 Ind.292 9_MG-268 T Tank.2 BTank.2 0.57 2
```

```
## 293 Ind.293 9_MG-268 T Tank.2 CTank.2 1.80 2
## 294 Ind.294 9_MG-268 T Tank.3 ATank.3 0.61 2
## 295 Ind.295 9_MG-268 T Tank.3 BTank.3 1.23 2
## 296 Ind.296 9_MG-268 T Tank.3 CTank.3 1.28 2
## 297 Ind.297 9_MG-268 T Tank.5 ATank.5 0.31 2
## 298 Ind.298 9_MG-268 T Tank.5 BTank.5 0.78 2
## 299 Ind.299 9_MG-268 T Tank.5 CTank.5 1.01 2
## 300 Ind.300 10_MG-457 C Tank.1 ATank.1 2.06 1
## 301 Ind.301 10_MG-457 C Tank.1 BTank.1 2.22 1
## 302 Ind.302 10_MG-457 C Tank.1 CTank.1 2.04 1
## 303 Ind.303 10_MG-457 C Tank.2 ATank.2 1.35 1
## 304 Ind.304 10_MG-457 C Tank.2 BTank.2 1.41 1
## 305 Ind.305 10_MG-457 C Tank.2 CTank.2 1.91 1
## 306 Ind.306 10_MG-457 C Tank.5 ATank.5 2.14 1
## 307 Ind.307 10_MG-457 C Tank.5 BTank.5 1.95 1
## 308 Ind.308 10_MG-457 C Tank.5 CTank.5 2.26 1
## 309 Ind.309 10_MG-457 T Tank.3 ATank.3 0.78 1
## 310 Ind.310 10_MG-457 T Tank.3 BTank.3 0.79 1
## 311 Ind.311 10_MG-457 T Tank.3 CTank.3 0.80 1
## 312 Ind.312 10_MG-457 T Tank.4 ATank.4 0.55 1
## 313 Ind.313 10_MG-457 T Tank.4 BTank.4 0.16 1
## 314 Ind.314 10_MG-457 T Tank.4 CTank.4 0.43 1
## 315 Ind.315 10_MG-457 T Tank.6 ATank.6 0.61 1
## 316 Ind.316 10_MG-457 T Tank.6 BTank.6 0.58 1
## 317 Ind.317 10_MG-457 T Tank.6 CTank.6 0.43 1
## 318 Ind.318 10_MG-457 C Tank.1 ATank.1 2.28 2
## 319 Ind.319 10_MG-457 C Tank.1 BTank.1 1.25 2
## 320 Ind.320 10_MG-457 C Tank.1 CTank.1 1.90 2
## 321 Ind.321 10_MG-457 C Tank.4 ATank.4 2.33 2
## 322 Ind.322 10_MG-457 C Tank.4 BTank.4 1.34 2
## 323 Ind.323 10_MG-457 C Tank.4 CTank.4 2.05 2
## 324 Ind.324 10_MG-457 C Tank.6 ATank.6 1.61 2
## 325 Ind.325 10_MG-457 C Tank.6 BTank.6 1.20 2
## 326 Ind.326 10_MG-457 C Tank.6 CTank.6 0.67 2
## 327 Ind.327 10_MG-457 T Tank.2 ATank.2 0.72 2
## 328 Ind.328 10_MG-457 T Tank.2 BTank.2 1.93 2
## 329 Ind.329 10_MG-457 T Tank.2 CTank.2 0.21 2
## 330 Ind.330 10_MG-457 T Tank.3 ATank.3 1.11 2
## 331 Ind.331 10_MG-457 T Tank.3 BTank.3 1.67 2
## 332 Ind.332 10_MG-457 T Tank.3 CTank.3 0.62 2
## 333 Ind.333 10_MG-457 T Tank.5 ATank.5 0.20 2
## 334 Ind.334 10_MG-457 T Tank.5 BTank.5 1.04 2
## 335 Ind.335 10_MG-457 T Tank.5 CTank.5 0.29 2
## 336 Ind.336 11_MG-5650 C Tank.1 ATank.1 1.49 1
## 337 Ind.337 11_MG-5650 C Tank.1 BTank.1 2.38 1
## 338 Ind.338 11_MG-5650 C Tank.1 CTank.1 0.97 1
## 339 Ind.339 11_MG-5650 C Tank.2 ATank.2 1.49 1
## 340 Ind.340 11_MG-5650 C Tank.2 BTank.2 1.17 1
## 341 Ind.341 11_MG-5650 C Tank.2 CTank.2 1.20 1
## 342 Ind.342 11_MG-5650 C Tank.5 ATank.5 1.35 1
## 343 Ind.343 11_MG-5650 C Tank.5 BTank.5 1.16 1
## 344 Ind.344 11_MG-5650 C Tank.5 CTank.5 1.05 1
## 345 Ind.345 11_MG-5650 T Tank.3 ATank.3 0.24 1
```

```
## 346 Ind.346 11_MG-5650 T Tank.3 BTank.3 0.32 1
## 347 Ind.347 11_MG-5650 T Tank.3 CTank.3 0.62 1
## 348 Ind.348 11_MG-5650 T Tank.4 ATank.4 0.37 1
## 349 Ind.349 11_MG-5650 T Tank.4 BTank.4 0.58 1
## 350 Ind.350 11_MG-5650 T Tank.4 CTank.4 0.33 1
## 351 Ind.351 11_MG-5650 T Tank.6 ATank.6 0.33 1
## 352 Ind.352 11_MG-5650 T Tank.6 BTank.6 0.66 1
## 353 Ind.353 11_MG-5650 T Tank.6 CTank.6 0.54 1
## 354 Ind.354 11_MG-5650 C Tank.1 ATank.1 2.38 2
## 355 Ind.355 11_MG-5650 C Tank.1 BTank.1 5.03 2
## 356 Ind.356 11_MG-5650 C Tank.1 CTank.1 2.86 2
## 357 Ind.357 11_MG-5650 C Tank.4 ATank.4 2.73 2
## 358 Ind.358 11_MG-5650 C Tank.4 BTank.4 1.97 2
## 359 Ind.359 11_MG-5650 C Tank.4 CTank.4 1.88 2
## 360 Ind.360 11_MG-5650 C Tank.6 ATank.6 1.18 2
## 361 Ind.361 11_MG-5650 C Tank.6 BTank.6 2.82 2
## 362 Ind.362 11_MG-5650 C Tank.6 CTank.6 4.03 2
## 363 Ind.363 11_MG-5650 T Tank.2 ATank.2 0.98 2
## 364 Ind.364 11_MG-5650 T Tank.2 BTank.2 0.09 2
## 365 Ind.365 11_MG-5650 T Tank.2 CTank.2 0.78 2
## 366 Ind.366 11_MG-5650 T Tank.3 ATank.3 0.51 2
## 367 Ind.367 11_MG-5650 T Tank.3 BTank.3 0.27 2
## 368 Ind.368 11_MG-5650 T Tank.3 CTank.3 0.88 2
## 369 Ind.369 11_MG-5650 T Tank.5 ATank.5 0.22 2
## 370 Ind.370 11_MG-5650 T Tank.5 BTank.5 1.35 2
## 371 Ind.371 11_MG-5650 T Tank.5 CTank.5 0.69 2
## 372 Ind.372 12_MG-1282 C Tank.1 ATank.1 1.39 1
## 373 Ind.373 12_MG-1282 C Tank.1 BTank.1 1.83 1
## 374 Ind.374 12_MG-1282 C Tank.1 CTank.1 1.11 1
## 375 Ind.375 12_MG-1282 C Tank.2 ATank.2 1.15 1
## 376 Ind.376 12_MG-1282 C Tank.2 BTank.2 1.06 1
## 377 Ind.377 12_MG-1282 C Tank.2 CTank.2 1.21 1
## 378 Ind.378 12_MG-1282 C Tank.5 ATank.5 1.57 1
## 379 Ind.379 12_MG-1282 C Tank.5 BTank.5 1.73 1
## 380 Ind.380 12_MG-1282 C Tank.5 CTank.5 1.54 1
## 381 Ind.381 12_MG-1282 T Tank.3 ATank.3 0.80 1
## 382 Ind.382 12_MG-1282 T Tank.3 BTank.3 0.84 1
## 383 Ind.383 12_MG-1282 T Tank.3 CTank.3 1.07 1
## 384 Ind.384 12_MG-1282 T Tank.4 ATank.4 0.20 1
## 385 Ind.385 12_MG-1282 T Tank.4 BTank.4 0.24 1
## 386 Ind.386 12_MG-1282 T Tank.4 CTank.4 0.35 1
## 387 Ind.387 12_MG-1282 T Tank.6 ATank.6 0.86 1
## 388 Ind.388 12_MG-1282 T Tank.6 BTank.6 1.35 1
## 389 Ind.389 12_MG-1282 T Tank.6 CTank.6 0.87 1
## 390 Ind.390 12_MG-1282 C Tank.1 ATank.1 0.79 2
## 391 Ind.391 12_MG-1282 C Tank.1 BTank.1 1.75 2
## 392 Ind.392 12_MG-1282 C Tank.1 CTank.1 1.57 2
## 393 Ind.393 12_MG-1282 C Tank.4 ATank.4 1.23 2
## 394 Ind.394 12_MG-1282 C Tank.4 BTank.4 1.91 2
## 395 Ind.395 12_MG-1282 C Tank.4 CTank.4 1.10 2
## 396 Ind.396 12_MG-1282 C Tank.6 ATank.6 1.25 2
## 397 Ind.397 12_MG-1282 C Tank.6 BTank.6 1.25 2
## 398 Ind.398 12_MG-1282 C Tank.6 CTank.6 1.69 2
```

```
## 399 Ind.399 12_MG-1282 T Tank.2 ATank.2 0.93 2
## 400 Ind.400 12_MG-1282 T Tank.2 BTank.2 1.00 2
## 401 Ind.401 12_MG-1282 T Tank.2 CTank.2 0.44 2
## 402 Ind.402 12_MG-1282 T Tank.3 ATank.3 0.99 2
## 403 Ind.403 12_MG-1282 T Tank.3 BTank.3 1.17 2
## 404 Ind.404 12_MG-1282 T Tank.3 CTank.3 0.96 2
## 405 Ind.405 12_MG-1282 T Tank.5 ATank.5 0.58 2
## 406 Ind.406 12_MG-1282 T Tank.5 BTank.5 0.75 2
## 407 Ind.407 12_MG-1282 T Tank.5 CTank.5 0.70 2
## 408 Ind.408 13_MG-231 C Tank.1 ATank.1 2.01 1
## 409 Ind.409 13_MG-231 C Tank.1 BTank.1 0.89 1
## 410 Ind.410 13_MG-231 C Tank.1 CTank.1 1.67 1
## 411 Ind.411 13_MG-231 C Tank.2 ATank.2 1.87 1
## 412 Ind.412 13_MG-231 C Tank.2 BTank.2 0.80 1
## 413 Ind.413 13_MG-231 C Tank.2 CTank.2 1.13 1
## 414 Ind.414 13_MG-231 C Tank.5 ATank.5 1.36 1
## 415 Ind.415 13_MG-231 C Tank.5 BTank.5 1.88 1
## 416 Ind.416 13_MG-231 C Tank.5 CTank.5 1.71 1
## 417 Ind.417 13_MG-231 T Tank.3 ATank.3 0.56 1
## 418 Ind.418 13_MG-231 T Tank.3 BTank.3 0.88 1
## 419 Ind.419 13_MG-231 T Tank.3 CTank.3 1.07 1
## 420 Ind.420 13_MG-231 T Tank.4 ATank.4 0.75 1
## 421 Ind.421 13_MG-231 T Tank.4 BTank.4 0.31 1
## 422 Ind.422 13_MG-231 T Tank.4 CTank.4 0.28 1
## 423 Ind.423 13_MG-231 T Tank.6 ATank.6 0.88 1
## 424 Ind.424 13_MG-231 T Tank.6 BTank.6 1.10 1
## 425 Ind.425 13_MG-231 T Tank.6 CTank.6 0.99 1
## 426 Ind.426 13_MG-231 C Tank.1 ATank.1 0.96 2
## 427 Ind.427 13_MG-231 C Tank.1 BTank.1 2.09 2
## 428 Ind.428 13_MG-231 C Tank.1 CTank.1 0.79 2
## 429 Ind.429 13_MG-231 C Tank.4 ATank.4 0.61 2
## 430 Ind.430 13_MG-231 C Tank.4 BTank.4 2.03 2
## 431 Ind.431 13_MG-231 C Tank.4 CTank.4 1.27 2
## 432 Ind.432 13_MG-231 C Tank.6 ATank.6 1.33 2
## 433 Ind.433 13_MG-231 C Tank.6 BTank.6 1.73 2
## 434 Ind.434 13_MG-231 C Tank.6 CTank.6 0.81 2
## 435 Ind.435 13_MG-231 T Tank.2 ATank.2 0.65 2
## 436 Ind.436 13_MG-231 T Tank.2 BTank.2 0.84 2
## 437 Ind.437 13_MG-231 T Tank.2 CTank.2 0.66 2
## 438 Ind.438 13_MG-231 T Tank.3 ATank.3 0.60 2
## 439 Ind.439 13_MG-231 T Tank.3 BTank.3 0.37 2
## 440 Ind.440 13_MG-231 T Tank.3 CTank.3 0.72 2
## 441 Ind.441 13_MG-231 T Tank.5 ATank.5 0.61 2
## 442 Ind.442 13_MG-231 T Tank.5 BTank.5 1.46 2
## 443 Ind.443 13_MG-231 T Tank.5 CTank.5 0.83 2
```

2. Determining the best fit linear mixed e ects model.

```
library(lme4)
## Loading required package: Matrix
Fl.lmer <- lmer(Wt ~ Treat * Accession_Genotype + (1 | Block:Tank:Rep) +
(1 | Tank:Rep) + (1 | Rep), data = Fluridone_data)
## boundary (singular) fit: see ?isSingular
Fl.lmer2 <- lmer(Wt ~ Treat * Accession_Genotype + (1 | Block:Tank) +
```

```
(1 | Tank), data = Fluridone_data)
## boundary (singular) fit: see ?isSingular
anova(Fl.lmer, Fl.lmer2)
## refitting model(s) with ML (instead of REML)
## Data: Fluridone_data
## Models:
## Fl.lmer2: Wt ~ Treat * Accession_Genotype + (1 | Block:Tank) + (1 | Tank)
## Fl.lmer: Wt ~ Treat * Accession_Genotype + (1 | Block:Tank:Rep) + (1 |
## Fl.lmer: Tank:Rep) + (1 | Rep)
## npar AIC BIC logLik deviance Chisq Df Pr(>Chisq)
## Fl.lmer2 29 711.81 830.53 -326.91 653.81
## Fl.lmer 30 722.89 845.70 -331.45 662.89 0 1 1
Fl.lmer2
## Linear mixed model fit by REML [ lmerMod ]
## Formula: Wt ~ Treat * Accession_Genotype + (1 | Block:Tank) + (1 | Tank)
## Data: Fluridone_data
## REML criterion at convergence: 712.2855
## Random effects:
## Groups Name Std.Dev.
## Block:Tank (Intercept) 0.1557
## Tank (Intercept) 0.0000
## Residual 0.5113
## Number of obs: 443, groups: Block:Tank, 12; Tank, 6
## Fixed Effects:
## (Intercept) TreatT
## 1.229810 -0.055175
## Accession_Genotype10_MG-457 Accession_Genotype11_MG-5650
## 0.533333 0.820556
## Accession_Genotype12_MG-1282 Accession_Genotype13_MG-231
## 0.153333 0.142778
## Accession_Genotype2_MG-237 Accession_Genotype3_MG-237
## 0.035000 -0.029444
## Accession_Genotype4_MG-237 Accession_Genotype5_MG-237
## 0.144444 -0.364571
## Accession_Genotype6_MG-237 Accession_Genotype7_MG-377
## -0.251667 0.268333
## Accession_Genotype8_MG-429 Accession_Genotype9_MG-268
## 0.007307 0.091667
## TreatT:Accession_Genotype10_MG-457 TreatT:Accession_Genotype11_MG-5650
## -0.977222 -1.440000
## TreatT:Accession_Genotype12_MG-1282 TreatT:Accession_Genotype13_MG-231
## -0.531667 -0.551111
## TreatT:Accession_Genotype2_MG-237 TreatT:Accession_Genotype3_MG-237
## -0.062222 -0.141667
## TreatT:Accession_Genotype4_MG-237 TreatT:Accession_Genotype5_MG-237
## -0.247778 0.281449
## TreatT:Accession_Genotype6_MG-237 TreatT:Accession_Genotype7_MG-377
## 0.966667 0.604444
## TreatT:Accession_Genotype8_MG-429 TreatT:Accession_Genotype9_MG-268
## -0.553763 -0.429444
## convergence code 0; 0 optimizer warnings; 1 lme4 warnings
```

3. ANOVA of the best fit linear mixed e ects model.

```
require(lmerTest)
## Loading required package: lmerTest
##
## Attaching package:   lmerTest
## The following object is masked from   package:lme4  :
##
## lmer
## The following object is masked from   package:stats  :
##
## step
anova(Fl.lmer2, type = 2)
## Warning in anova.merMod(Fl.lmer2, type = 2): additional arguments ignored:
##   type
## Analysis of Variance Table
## npar Sum Sq Mean Sq F value
## Treat 1 2.431 2.4306 9.2973
## Accession_Genotype 12 18.719 1.5599 5.9670
## Treat:Accession_Genotype 12 42.366 3.5305 13.5047
```

4. Calculating pairwise least-squares means and contrasts between control and 6ppb fluridone treatment means.

```
library(emmeans)
Fl.emm <- emmeans(Fl.lmer2, ~ Treat | Accession_Genotype)
pairs(Fl.emm)
## Accession_Genotype = 1_MG-237:
## contrast estimate SE df t.ratio p.value
## C - T 0.0552 0.200 136 0.276 0.7831
##
## Accession_Genotype = 10_MG-457:
## contrast estimate SE df t.ratio p.value
## C - T 1.0324 0.200 136 5.160 <.0001
##
## Accession_Genotype = 11_MG-5650:
## contrast estimate SE df t.ratio p.value
## C - T 1.4952 0.200 136 7.473 <.0001
##
## Accession_Genotype = 12_MG-1282:
## contrast estimate SE df t.ratio p.value
## C - T 0.5868 0.200 136 2.933 0.0039
##
## Accession_Genotype = 13_MG-231:
## contrast estimate SE df t.ratio p.value
## C - T 0.6063 0.200 136 3.030 0.0029
##
## Accession_Genotype = 2_MG-237:
## contrast estimate SE df t.ratio p.value
## C - T 0.1174 0.200 136 0.587 0.5583
##
## Accession_Genotype = 3_MG-237:
## contrast estimate SE df t.ratio p.value
## C - T 0.1968 0.200 136 0.984 0.3269
##
```

```
## Accession_Genotype = 4_MG-237:
## contrast estimate SE df t.ratio p.value
## C - T 0.3030 0.200 136 1.514 0.1323
##
## Accession_Genotype = 5_MG-237:
## contrast estimate SE df t.ratio p.value
## C - T -0.2263 0.234 283 -0.969 0.3335
##
## Accession_Genotype = 6_MG-237:
## contrast estimate SE df t.ratio p.value
## C - T -0.9115 0.200 136 -4.556 <.0001
##
## Accession_Genotype = 7_MG-377:
## contrast estimate SE df t.ratio p.value
## C - T -0.5493 0.200 136 -2.745 0.0069
##
## Accession_Genotype = 8_MG-429:
## contrast estimate SE df t.ratio p.value
## C - T 0.6089 0.230 273 2.652 0.0085
##
## Accession_Genotype = 9_MG-268:
## contrast estimate SE df t.ratio p.value
## C - T 0.4846 0.200 136 2.422 0.0167
##
## Degrees-of-freedom method: kenward-roger
```

APPENDIX C

SUPPLEMENTARY MATERIAL FROM CHAPTER FIVE

Transcriptome	Sample	% Unique Mapped	% Multi-Mapped
EWM	hybrid-17	20.86	63.17
hybrid	hybrid-17	21.94	64.14
combined	hybrid-17	21.96	62.99
EWM	hybrid-22	19.64	66.72
hybrid	hybrid-22	21.23	65.48
combined	hybrid-22	20.99	64.52
EWM	hybrid-27	20.54	67.80
hybrid	hybrid-27	21.57	66.12
combined	hybrid-27	21.32	64.85
EWM	EWM-47	20.23	67.53
hybrid	EWM-47	20.20	64.29
combined	EWM-47	19.90	64.23
EWM	EWM-54	20.41	68.35
hybrid	EWM-54	20.63	65.52
combined	EWM-54	20.01	65.66
EWM	EWM-60	20.33	68.86
hybrid	EWM-60	20.68	65.12
combined	EWM-60	20.17	65.76

Supplemental Table S1. Mapping statistics between transcriptomes

Uniprot gene	Gene Description	Source Species
ILL4	IAA-amino acid hydrolase ILR1-like 4 (EC 3.5.1.-) (jasmonoyl-L-amino acid hydrolase) (EC 3.5.1.127)	Arabidopsis thaliana (Mouse-ear cress)
IAA16	Auxin-responsive protein IAA16 (Indoleacetic acid-induced protein 16)	Arabidopsis thaliana (Mouse-ear cress)
AUX22D	Auxin-induced protein 22D (Indole-3-acetic acid-induced protein ARG13)	Vigna radiata var. radiata (Mung bean) (Phaseolus aureus)
GH31	Probable indole-3-acetic acid-amido synthetase GH3.1 (EC 6.3.2.-) (Auxin-responsive GH3-like protein 1) (AtGH3-1)	Arabidopsis thaliana (Mouse-ear cress)
AUX22	Auxin-induced protein AUX22	Glycine max (Soybean) (Glycine hispida)
AUX28	Auxin-induced protein AUX28	Glycine max (Soybean) (Glycine hispida)
IAA4	Auxin-responsive protein IAA4 (Auxin-induced protein AUX2-11) (Indoleacetic acid-induced protein 4)	Arabidopsis thaliana (Mouse-ear cress)
CSN8	COP9 signalosome complex subunit 8 (CSN complex subunit 8) (Constitutive photomorphogenesis protein 9) (Protein FUSCA 7)	Arabidopsis thaliana (Mouse-ear cress)
IAA4/5	Auxin-induced protein IAA4	Pisum sativum (Garden pea)
ILR1	IAA-amino acid hydrolase ILR1 (EC 3.5.1.-)	Arabidopsis thaliana (Mouse-ear cress)

ARF5	Auxin response factor 5 (Auxin-responsive protein IAA24) (Transcription factor MONOPTEROS)	Arabidopsis thaliana (Mouse-ear cress)
IAA3	Auxin-responsive protein IAA3 (Indoleacetic acid-induced protein 3) (Short hypocotyl) (Suppressor of HY2)	Arabidopsis thaliana (Mouse-ear cress)
IAA11	Auxin-responsive protein IAA11 (Indoleacetic acid-induced protein 11)	Arabidopsis thaliana (Mouse-ear cress)
IAA13	Auxin-responsive protein IAA13 (Indoleacetic acid-induced protein 13)	Arabidopsis thaliana (Mouse-ear cress)
IAA14	Auxin-responsive protein IAA14 (Indoleacetic acid-induced protein 14) (Protein SOLITARY ROOT)	Arabidopsis thaliana (Mouse-ear cress)
IAA8	Auxin-responsive protein IAA8 (Indoleacetic acid-induced protein 8)	Oryza sativa subsp. japonica (Rice)
IAA32	Auxin-responsive protein IAA32 (Indoleacetic acid-induced protein 32)	Arabidopsis thaliana (Mouse-ear cress)
ARF2	Auxin response factor 2 (ARF1-binding protein) (ARF1-BP) (Protein MEGAINTEGUMENTA)	Arabidopsis thaliana (Mouse-ear cress)
ARF18	Auxin response factor 18	Arabidopsis thaliana (Mouse-ear cress)

ISS1	Aromatic aminotransferase ISS1 (EC 2.6.1.27) (EC 2.6.1.5) (EC 2.6.1.88) (Methionine aminotransferase ISS1) (Phenylalanine aminotransferase ISS1) (Protein INDOLE SEVERE SENSITIVE 1) (Protein REVERSAL OF SAV3 PHENOTYPE 1) (Tryptophan aminotransferase ISS1) (Tyrosine aminotransferase ISS1)	Arabidopsis thaliana (Mouse-ear cress)
GH317	Indole-3-acetic acid-amido synthetase GH3.17 (EC 6.3.2.-) (Auxin-responsive GH3-like protein 17) (AtGH3-17)	Arabidopsis thaliana (Mouse-ear cress)
GH36	Indole-3-acetic acid-amido synthetase GH3.6 (EC 6.3.2.-) (Auxin-responsive GH3-like protein 6) (AtGH3-6) (Protein DWARF IN LIGHT 1) (DFL-1)	Arabidopsis thaliana (Mouse-ear cress)
MES17	Methylesterase 17 (AtMES17) (EC 3.1.1.-) (Methyl indole-3-acetic acid esterase)	Arabidopsis thaliana (Mouse-ear cress)
SGT1B	Protein SGT1 homolog B (AtSGT1b) (Protein ENHANCED DOWNY MILDEW 1) (Protein ENHANCER OF TIR1-1 AUXIN RESISTANCE 3) (Suppressor of G2 allele of SKP1 homolog B)	Arabidopsis thaliana (Mouse-ear cress)
ARF9	Auxin response factor 9	Arabidopsis thaliana (Mouse-ear cress)

GH3.10	Indole-3-acetic acid-amido synthetase GH3.10 (EC 6.3.2.-) (Auxin-responsive GH3-like protein 10) (Protein DWARF IN LIGHT 2)	Arabidopsis thaliana (Mouse-ear cress)
IAA27	Auxin-responsive protein IAA27 (Auxin-induced protein 27) (Indoleacetic acid-induced protein 27) (Phytochrome-associated protein 2)	Arabidopsis thaliana (Mouse-ear cress)
ARF6	Auxin response factor 6	Arabidopsis thaliana (Mouse-ear cress)
ARF4	Auxin response factor 4	Arabidopsis thaliana (Mouse-ear cress)
SGT1B	Protein SGT1 homolog B (AtSGT1b) (Protein ENHANCED DOWNY MILDEW 1) (Protein ENHANCER OF TIR1-1 AUXIN RESISTANCE 3) (Suppressor of G2 allele of SKP1 homolog B)	Arabidopsis thaliana (Mouse-ear cress)

Supplemental Table S2. Auxin/IAA gene list.

Uniprot Gene	Gene Description	Source Species
NAP2	NAC domain-containing protein 2 (SlNAP2)	Solanum lycopersicum (Tomato) (Lycopersicon esculentum)
NCED2	9-cis-epoxycarotenoid dioxygenase NCED2, chloroplastic (LeNCED2) (SlNCED2) (EC 1.13.11.51) (Nine-cis-epoxycarotenoid dioxygenase 2)	Solanum lycopersicum (Tomato) (Lycopersicon esculentum)
NCED9	9-cis-epoxycarotenoid dioxygenase NCED9, chloroplastic (AtNCED9) (EC 1.13.11.51)	Arabidopsis thaliana (Mouse-ear cress)
JA2	NAC domain-containing protein JA2 (Protein JASMONIC ACID 2)	Solanum lycopersicum (Tomato) (Lycopersicon esculentum)

Supplemental Table S3. NCED gene list.

Uniprot gene	Gene Description	Source Species
PYL4	Abscisic acid receptor PYL4 (ABI1-binding protein 2) (PYR1-like protein 4) (Regulatory components of ABA receptor 10)	Arabidopsis thaliana (Mouse-ear cress)
PYL9	Abscisic acid receptor PYL9 (ABI1-binding protein 4) (PYR1-like protein 9) (Regulatory components of ABA receptor 1)	Arabidopsis thaliana (Mouse-ear cress)
PYL6	Abscisic acid receptor PYL6 (ABI1-binding protein 5) (PYR1-like protein 6) (Regulatory components of ABA receptor 9)	Arabidopsis thaliana (Mouse-ear cress)
PYL8	Abscisic acid receptor PYL8 (ABI1-binding protein 1) (PYR1-like protein 8) (Regulatory components of ABA receptor 3)	Arabidopsis thaliana (Mouse-ear cress)
AOG	Abscisate beta-glucosyltransferase (EC 2.4.1.263) (ABA-glucosyltransferase)	Phaseolus angularis (Azuki bean) (Vigna angularis)
AI5L6	ABSCISIC ACID-INSENSITIVE 5-like protein 6 (Abscisic acid responsive elements-binding factor 3) (ABRE-binding factor 3) (Dc3 promoter-binding factor 5) (AtDPBF5) (bZIP transcription factor 37) (AtbZIP37)	Arabidopsis thaliana (Mouse-ear cress)
AI5LF	ABSCISIC ACID-INSENSITIVE 5-like protein 5 (ABA-responsive element-binding protein 1) (Abscisic acid responsive elements-binding factor 2) (ABRE-binding factor 2) (bZIP transcription factor 36) (AtbZIP36)	Arabidopsis thaliana (Mouse-ear cress)
U75C1	UDP-glycosyltransferase 75C1 (Abscisic acid beta-glucosyltransferase) (Indole-3-acetate beta-glucosyltransferase) (SlUGT75C1) (EC 2.4.1.121) (EC 2.4.1.263)	Solanum lycopersicum (Tomato) (Lycopersicon esculentum)
MYB44	Transcription factor MYB44 (Myb-related protein 44) (AtMYB44) (Myb-related protein R1) (AtMYBR1)	Arabidopsis thaliana (Mouse-ear cress)

Supplemental Table S4. ABA gene list.

Uniprot gene	Gene Description	Source Species
CRF2	Ethylene-responsive transcription factor CRF2 (Protein CYTOKININ RESPONSE FACTOR 2)	Arabidopsis thaliana (Mouse-ear cress)
CRF4	Ethylene-responsive transcription factor CRF4 (Protein CYTOKININ RESPONSE FACTOR 4)	Arabidopsis thaliana (Mouse-ear cress)
EF100	Ethylene-responsive transcription factor 1A (AtERF1A) (Ethylene-responsive element-binding factor 1A) (EREBP-1A)	Arabidopsis thaliana (Mouse-ear cress)
EF101	Ethylene-responsive transcription factor 2 (AtERF2) (Ethylene-responsive element-binding factor 2) (EREBP-2)	Arabidopsis thaliana (Mouse-ear cress)
EF102	Ethylene-responsive transcription factor 5 (AtERF5) (Ethylene-responsive element-binding factor 5) (EREBP-5)	Arabidopsis thaliana (Mouse-ear cress)
EF106	Ethylene-responsive transcription factor ERF106	Arabidopsis thaliana (Mouse-ear cress)
EF113	Ethylene-responsive transcription factor ERF113 (Protein RELATED TO AP2 6L)	Arabidopsis thaliana (Mouse-ear cress)
EF114	Ethylene-responsive transcription factor ERF114 (ERF bud enhancer)	Arabidopsis thaliana (Mouse-ear cress)
EIN4	Protein EIN4 (AtEIN4) (EC 2.7.11.-) (Protein ETHYLENE INSENSITIVE 4)	Arabidopsis thaliana (Mouse-ear cress)
ERF03	Ethylene-responsive transcription factor ERF003	Arabidopsis thaliana (Mouse-ear cress)
ERF08	Ethylene-responsive transcription factor ERF008	Arabidopsis thaliana (Mouse-ear cress)
ERF10	Ethylene-responsive transcription factor ERF010	Arabidopsis thaliana (Mouse-ear cress)
ERF11	Ethylene-responsive transcription factor ERF011	Arabidopsis thaliana (Mouse-ear cress)

ERF12	Ethylene-responsive transcription factor ERF012 (Dehydration response element-binding protein 26)	Arabidopsis thaliana (Mouse-ear cress)
ERF17	Ethylene-responsive transcription factor ERF017	Arabidopsis thaliana (Mouse-ear cress)
ERF34	Ethylene-responsive transcription factor ERF034	Arabidopsis thaliana (Mouse-ear cress)
ERF4	Ethylene-responsive transcription factor 4 (Ethylene-responsive element-binding factor 3) (EREBP-3) (Ethylene-responsive element-binding factor 4 homolog) (NsERF3)	Nicotiana sylvestris (Wood tobacco) (South American tobacco)
ERF5	Ethylene-responsive transcription factor 5 (Ethylene-responsive element-binding factor 4) (EREBP-4) (Ethylene-responsive element-binding factor 5 homolog) (NsERF4)	Nicotiana sylvestris (Wood tobacco) (South American tobacco)
ERF5	Ethylene-responsive transcription factor 5 (Ethylene-responsive element-binding factor 4) (EREBP-4) (Ethylene-responsive element-binding factor 5 homolog) (NtERF4)	Nicotiana tabacum (Common tobacco)
ERF61	Ethylene-responsive transcription factor ERF061	Arabidopsis thaliana (Mouse-ear cress)
ERF78	Ethylene-responsive transcription factor 4 (AtERF4) (Ethylene-responsive element-binding factor 4) (EREBP-4) (Protein RELATED TO APETALA2 5)	Arabidopsis thaliana (Mouse-ear cress)

ERF80	Ethylene-responsive transcription factor 9 (AtERF9) (Ethylene-responsive element-binding factor 9) (EREBP-9)	Arabidopsis thaliana (Mouse-ear cress)
ERF81	Ethylene-responsive transcription factor 12 (AtERF12) (Ethylene-responsive element-binding factor 12) (EREBP-12)	Arabidopsis thaliana (Mouse-ear cress)
ERF82	Ethylene-responsive transcription factor 3 (AtERF3) (Ethylene-responsive element-binding factor 3) (EREBP-3)	Arabidopsis thaliana (Mouse-ear cress)
ERF83	Ethylene-responsive transcription factor 7 (AtERF7) (Ethylene-responsive element-binding factor 7) (EREBP-7)	Arabidopsis thaliana (Mouse-ear cress)
ERF92	Ethylene-responsive transcription factor 1B (AtERF1B) (Ethylene-responsive element-binding factor 1B) (EREBP-1B)	Arabidopsis thaliana (Mouse-ear cress)
ERF96	Ethylene-responsive transcription factor ERF096	Arabidopsis thaliana (Mouse-ear cress)
ERF98	Ethylene-responsive transcription factor ERF098	Arabidopsis thaliana (Mouse-ear cress)
ERFL1	Ethylene-responsive transcription factor-like protein At4g13040	Arabidopsis thaliana (Mouse-ear cress)
ESR2	Ethylene-responsive transcription factor ESR2 (Protein DORNROESCHEN-LIKE) (Protein DRN-LIKE) (Protein ENHANCER OF SHOOT REGENERATION 2) (Protein SUPPRESSOR OF PHYTOCHROMEB 2)	Arabidopsis thaliana (Mouse-ear cress)
ETR1	Ethylene receptor (EC 2.7.13.3)	Prunus persica (Peach) (Amygdalus persica)

ETR2	Ethylene receptor 2 (AtETR2) (EC 2.7.11.-) (Protein ETHYLENE RESPONSE 2) (Protein ETR2)	Arabidopsis thaliana (Mouse-ear cress)
HLS1	Probable N-acetyltransferase HLS1 (EC 2.3.1.-) (Protein CONSTITUTIVE PHOTOMORPHOGENIC 3) (Protein HOOKLESS 1) (Protein UNUSUAL SUGAR RESPONSE 2)	Arabidopsis thaliana (Mouse-ear cress)
RA211	Ethylene-responsive transcription factor RAP2-11 (Protein RELATED TO APETALA2 11)	Arabidopsis thaliana (Mouse-ear cress)
RA212	Ethylene-responsive transcription factor RAP2-12 (Protein RELATED TO APETALA2 12)	Arabidopsis thaliana (Mouse-ear cress)
RAP22	Ethylene-responsive transcription factor RAP2-2 (AtRAP2.2) (Protein RELATED TO APETALA2 2)	Arabidopsis thaliana (Mouse-ear cress)
RAP23	Ethylene-responsive transcription factor RAP2-3 (Cadmium-induced protein AS30) (Ethylene response factor 72) (ERF72) (Ethylene-responsive element binding protein) (AtEBP) (Protein RELATED TO APETALA2 3) (Related to AP2 3)	Arabidopsis thaliana (Mouse-ear cress)
RAP24	Ethylene-responsive transcription factor RAP2-4 (Ethylene-responsive transcription factor ERF059) (Protein RELATED TO APETALA2 4)	Arabidopsis thaliana (Mouse-ear cress)
RAP27	Ethylene-responsive transcription factor RAP2-7 (Protein RELATED TO APETALA2 7) (Protein TARGET OF EAT 1)	Arabidopsis thaliana (Mouse-ear cress)

TIR1	Protein TRANSPORT INHIBITOR RESPONSE 1 (Weak ethylene-insensitive protein 1)	Arabidopsis thaliana (Mouse-ear cress)
WRI1	Ethylene-responsive transcription factor WRI1 (Protein ACTIVATOR OF SPORAMIN::LUC 1) (Protein WRINKLED 1)	Arabidopsis thaliana (Mouse-ear cress)

Supplemental Table S5. Ethylene gene list.

Uniprot gene	Gene Description	Source Species
PSBC	Photosystem II CP43 reaction center protein (PSII 43 kDa protein) (Protein CP-43)	Manihot esculenta (Cassava) (Jatropha manihot)
PST2	Photosystem II 5 kDa protein, chloroplastic	Petunia hybrida (Petunia)
PPD7	PsbP domain-containing protein 7, chloroplastic (Photosystem II reaction center PsbP family protein)	Arabidopsis thaliana (Mouse-ear cress)
P2SAF	Photosystem II stability/assembly factor HCF136, chloroplastic	Arabidopsis thaliana (Mouse-ear cress)
PSBR	Photosystem II 10 kDa polypeptide, chloroplastic (Light-inducible tissue-specific ST-LS1 protein)	Solanum tuberosum (Potato)
PSAH	Photosystem I reaction center subunit VI, chloroplastic (PSI-H) (Light-harvesting complex I 11 kDa protein)	Spinacia oleracea (Spinach)
PSAD	Photosystem I reaction center subunit II, chloroplastic (PS I subunit 5) (Photosystem I 20 kDa subunit) (PSI-D)	Cucumis sativus (Cucumber)
PSAN	Photosystem I reaction center subunit N, chloroplastic (PSI-N)	Arabidopsis thaliana (Mouse-ear cress)

PSBY	Photosystem II core complex proteins psbY, chloroplastic (L-arginine-metabolizing enzyme) (L-AME) [Cleaved into: Photosystem II protein psbY-1, chloroplastic (psbY-A1); Photosystem II protein psbY-2, chloroplastic (psbY-A2)]	Spinacia oleracea (Spinach)
PETM	Cytochrome b6-f complex subunit 7 (Cytochrome b6-f complex subunit PetM) (Cytochrome b6-f complex subunit VII) (Fragment)	Spinacia oleracea (Spinach)
PPL1	PsbP-like protein 1, chloroplastic (OEC23-like protein 4) (PsbP-related thylakoid lumenal protein 2)	Arabidopsis thaliana (Mouse-ear cress)
PPL1	PsbP-like protein 1, chloroplastic (OEC23-like protein 4) (PsbP-related thylakoid lumenal protein 2)	Arabidopsis thaliana (Mouse-ear cress)
LHSR1	Light-harvesting complex stress-related protein 1, chloroplastic (Chlorophyll a-b binding protein LHCSR1)	Chlamydomonas reinhardtii (Chlamydomonas smithii)
PSBS	Photosystem II 22 kDa protein, chloroplastic (CP22)	Spinacia oleracea (Spinach)
PSB28	Photosystem II reaction center PSB28 protein, chloroplastic (Photosystem II protein W-like)	Oryza sativa subsp. japonica (Rice)
PSBB	Photosystem II CP47 reaction center protein (PSII 47 kDa protein) (Protein CP-47)	Vitis vinifera (Grape)
PSBR	Photosystem II 10 kDa polypeptide, chloroplastic (PII10)	Nicotiana tabacum (Common tobacco)

PSAEA	Photosystem I reaction center subunit IV A, chloroplastic (PSI-E A) [Cleaved into: Photosystem I reaction center subunit IV A isoform 2]	Nicotiana sylvestris (Wood tobacco) (South American tobacco)
PSAL	Photosystem I reaction center subunit XI, chloroplastic (PSI-L) (PSI subunit V)	Spinacia oleracea (Spinach)
PSBD	Photosystem II D2 protein (PSII D2 protein) (EC 1.10.3.9) (Photosystem Q(A) protein)	Ranunculus macranthus (Large buttercup)
P2SAF	Photosystem II stability/assembly factor HCF136, chloroplastic	Oryza sativa subsp. japonica (Rice)
PSAB	Photosystem I P700 chlorophyll a apoprotein A2 (EC 1.97.1.12) (PSI-B) (PsaB)	Calycanthus floridus var. glaucus (Eastern sweetshrub) (Calycanthus fertilis var. ferax)
LHCA6	Photosystem I chlorophyll a/b-binding protein 6, chloroplastic (LHCI type III LHCA6)	Arabidopsis thaliana (Mouse-ear cress)
SGRL	Magnesium dechelatase SGRL, chloroplastic (EC 4.99.1.10) (Protein STAY-GREEN LIKE)	Arabidopsis thaliana (Mouse-ear cress)
LHCA5	Photosystem I chlorophyll a/b-binding protein 5, chloroplastic (Lhca5) (LHCI type III LHCA5)	Arabidopsis thaliana (Mouse-ear cress)
PLDA4	Phospholipase D alpha 4 (AtPLDalpha4) (PLD alpha 4) (EC 3.1.4.4) (PLDalpha3) (Phospholipase D epsilon) (AtPLDepsilon) (PLD epsilon)	Arabidopsis thaliana (Mouse-ear cress)

EXA1	Protein ESSENTIAL FOR POTEXVIRUS ACCUMULATION 1 (Plant SMY2-type ILE-GYF domain-containing protein 1) (Protein MUTANT, SNC1-ENHANCING 11)	Arabidopsis thaliana (Mouse-ear cress)
STT7	Serine/threonine-protein kinase STN7, chloroplastic (EC 2.7.11.1) (Protein STATE TRANSITION 7) (Stt7 homolog)	Arabidopsis thaliana (Mouse-ear cress)
PSAF	Photosystem I reaction center subunit III, chloroplastic (Light-harvesting complex I 17 kDa protein) (PSI-F)	Arabidopsis thaliana (Mouse-ear cress)
PSAK	Photosystem I reaction center subunit psaK, chloroplastic (PSI-K) (Photosystem I subunit X)	Arabidopsis thaliana (Mouse-ear cress)
LHCA3	Photosystem I chlorophyll a/b-binding protein 3-1, chloroplastic (Lhca3*1) (LHCI type III LHCA3)	Arabidopsis thaliana (Mouse-ear cress)
LHCA2	Photosystem I chlorophyll a/b-binding protein 2, chloroplastic (Lhca2) (LHCI type III LHCA2)	Arabidopsis thaliana (Mouse-ear cress)
CB5	Chlorophyll a-b binding protein CP26, chloroplastic (LHCB5) (LHCIIc) (Light-harvesting complex II protein 5)	Arabidopsis thaliana (Mouse-ear cress)

Supplemental Table S6. Photosystem gene list.

Uniprot gene	Gene Description	Source Species
PSBC	Photosystem II CP43 reaction center protein (PSII 43 kDa protein) (Protein CP-43)	Manihot esculenta (Cassava) (Jatropha manihot)
PST2	Photosystem II 5 kDa protein, chloroplastic	Petunia hybrida (Petunia)
PPD7	PsbP domain-containing protein 7, chloroplastic (Photosystem II reaction center PsbP family protein)	Arabidopsis thaliana (Mouse-ear cress)
P2SAF	Photosystem II stability/assembly factor HCF136, chloroplastic	Arabidopsis thaliana (Mouse-ear cress)
PSBR	Photosystem II 10 kDa polypeptide, chloroplastic (Light-inducible tissue-specific ST-LS1 protein)	Solanum tuberosum (Potato)
PSAH	Photosystem I reaction center subunit VI, chloroplastic (PSI-H) (Light-harvesting complex I 11 kDa protein)	Spinacia oleracea (Spinach)
PSAD	Photosystem I reaction center subunit II, chloroplastic (PS I subunit 5) (Photosystem I 20 kDa subunit) (PSI-D)	Cucumis sativus (Cucumber)
PSAN	Photosystem I reaction center subunit N, chloroplastic (PSI-N)	Arabidopsis thaliana (Mouse-ear cress)

PSBY	Photosystem II core complex proteins psbY, chloroplastic (L-arginine-metabolizing enzyme) (L-AME) [Cleaved into: Photosystem II protein psbY-1, chloroplastic (psbY-A1); Photosystem II protein psbY-2, chloroplastic (psbY-A2)]	Spinacia oleracea (Spinach)
PETM	Cytochrome b6-f complex subunit 7 (Cytochrome b6-f complex subunit PetM) (Cytochrome b6-f complex subunit VII) (Fragment)	Spinacia oleracea (Spinach)
PPL1	PsbP-like protein 1, chloroplastic (OEC23-like protein 4) (PsbP-related thylakoid lumenal protein 2)	Arabidopsis thaliana (Mouse-ear cress)
PPL1	PsbP-like protein 1, chloroplastic (OEC23-like protein 4) (PsbP-related thylakoid lumenal protein 2)	Arabidopsis thaliana (Mouse-ear cress)
LHSR1	Light-harvesting complex stress-related protein 1, chloroplastic (Chlorophyll a-b binding protein LHCSR1)	Chlamydomonas reinhardtii (Chlamydomonas smithii)
PSBS	Photosystem II 22 kDa protein, chloroplastic (CP22)	Spinacia oleracea (Spinach)
PSB28	Photosystem II reaction center PSB28 protein, chloroplastic (Photosystem II protein W-like)	Oryza sativa subsp. japonica (Rice)
PSBB	Photosystem II CP47 reaction center protein (PSII 47 kDa protein) (Protein CP-47)	Vitis vinifera (Grape)
PSBR	Photosystem II 10 kDa polypeptide, chloroplastic (PII10)	Nicotiana tabacum (Common tobacco)

PSAEA	Photosystem I reaction center subunit IV A, chloroplastic (PSI-E A) [Cleaved into: Photosystem I reaction center subunit IV A isoform 2]	Nicotiana sylvestris (Wood tobacco) (South American tobacco)
PSAL	Photosystem I reaction center subunit XI, chloroplastic (PSI-L) (PSI subunit V)	Spinacia oleracea (Spinach)
PSBD	Photosystem II D2 protein (PSII D2 protein) (EC 1.10.3.9) (Photosystem Q(A) protein)	Ranunculus macranthus (Large buttercup)
P2SAF	Photosystem II stability/assembly factor HCF136, chloroplastic	Oryza sativa subsp. japonica (Rice)
PSAB	Photosystem I P700 chlorophyll a apoprotein A2 (EC 1.97.1.12) (PSI-B) (PsaB)	Calycanthus floridus var. glaucus (Eastern sweetshrub) (Calycanthus fertilis var. ferax)
LHCA6	Photosystem I chlorophyll a/b-binding protein 6, chloroplastic (LHCI type III LHCA6)	Arabidopsis thaliana (Mouse-ear cress)
SGRL	Magnesium dechelatase SGRL, chloroplastic (EC 4.99.1.10) (Protein STAY-GREEN LIKE)	Arabidopsis thaliana (Mouse-ear cress)
LHCA5	Photosystem I chlorophyll a/b-binding protein 5, chloroplastic (Lhca5) (LHCI type III LHCA5)	Arabidopsis thaliana (Mouse-ear cress)
PLDA4	Phospholipase D alpha 4 (AtPLDalpha4) (PLD alpha 4) (EC 3.1.4.4) (PLDalpha3) (Phospholipase D epsilon) (AtPLDepsilon) (PLD epsilon)	Arabidopsis thaliana (Mouse-ear cress)

EXA1	Protein ESSENTIAL FOR POTEXVIRUS ACCUMULATION 1 (Plant SMY2-type ILE-GYF domain-containing protein 1) (Protein MUTANT, SNC1-ENHANCING 11)	Arabidopsis thaliana (Mouse-ear cress)
STT7	Serine/threonine-protein kinase STN7, chloroplastic (EC 2.7.11.1) (Protein STATE TRANSITION 7) (Stt7 homolog)	Arabidopsis thaliana (Mouse-ear cress)
PSAF	Photosystem I reaction center subunit III, chloroplastic (Light-harvesting complex I 17 kDa protein) (PSI-F)	Arabidopsis thaliana (Mouse-ear cress)
PSAK	Photosystem I reaction center subunit psaK, chloroplastic (PSI-K) (Photosystem I subunit X)	Arabidopsis thaliana (Mouse-ear cress)
LHCA3	Photosystem I chlorophyll a/b-binding protein 3-1, chloroplastic (Lhca3*1) (LHCI type III LHCA3)	Arabidopsis thaliana (Mouse-ear cress)
LHCA2	Photosystem I chlorophyll a/b-binding protein 2, chloroplastic (Lhca2) (LHCI type III LHCA2)	Arabidopsis thaliana (Mouse-ear cress)
CB5	Chlorophyll a-b binding protein CP26, chloroplastic (LHCB5) (LHCIIc) (Light-harvesting complex II protein 5)	Arabidopsis thaliana (Mouse-ear cress)

Supplemental Table S7. ACS gene list.