Summer 8-6-2018

Genetic Diversity in an Invasive Clonal Plant? A Historical and Contemporary Perspective

Elliot D. Weidow
University of New Orleans, edweidow@uno.edu

Recommended Citation

Genetic Diversity in an Invasive Clonal Plant? A Historical and Contemporary Perspective

A Thesis

Submitted to the Graduate Faculty of the

University of New Orleans

in partial fulfilment of the

requirements for the degree of

Master of Science
in
Biological Sciences

by

Elliot Weidow

B.S. Frostburg State University, 2012

August, 2018

Table of Contents

List of Figures

Abstract:

Introduced populations of *Eichhornia crassipes* (Pontederiaceae) possess extremely low levels of genetic diversity due to severe bottleneck events and clonal reproduction. While populations elsewhere have been well studied, North American populations of *E. crassipes* remain understudied. We used Amplified Fragment Length Polymorphism markers to assess genetic diversity and population structure of *E. crassipes* in the United States. Patterns of diversity over the past fifty years were analyzed using herbarium specimens. Furthermore, we sampled populations across the Gulf Coast of the United States throughout a year to determine contemporary genetic diversity and assess potential seasonal effects. Genetic diversity was found to be scant in the United States without population structure, agreeing with previous studies from other regions. Genetic diversity has remained consistently low over the past fifty years despite significant changes in selection pressure. Also, no evidence of population structure between seasons was found and the consequences of this are discussed.

Introduction:

Invasive species in new regions are typically constrained by bottleneck events that reduce the population size as well as their genetic diversity. Invasive species can overcome their low initial genetic diversity and prosper, despite the paradigm that low genetic diversity reduces the success of a population (Roman, 2007). Invasive species uniquely overcome this hurdle, but there are several archetypes for how populations overcome low genetic diversity and prosper. First, low diversity populations can exploit a widespread habitat that matches their abilities. An example of this would be *Procambarus virginalis*, Marbled Crayfish (Gutekunst, 2018) where the genetic diversity of introduced populations began and remained low restricting the organism to specific habitats, yet aggressively invading those habitats. Second, low diversity populations are able to exploit a wide range of habitats because they possess significant phenotypic plasticity. An example of this can be found in *Alternanthera philoxeroides,* Alligatorweed (Geng, 2007) where high levels of plasticity allowed this plant to circumnavigate reduced genetic diversity and thrive outside the initial introduction range. Finally, repeated introductions can inject genetic variation that allows introduced species to exploit new habitats and spread out of their initial introduction range. Two interesting examples of this are *Carcinus maenas*, the European Green Crab (Roman, 2006); and *Phragmities* sp., Giant Reed (Saltonstall, 2002). In both cases, the introduction of new genetic material facilitated the invasion of these organisms. Understanding how successful invasive species overcome the challenge of low initial genetic diversity gives insight into the history, ecology, and future of all invasive species.

Eichhornia crassipes (Pontederiaceae)*,* water hyacinth, is among the worst invasive species (Penfound, 1948; Vietmeyer, 1975; Villamagna, 2010; Zhang, 2010). Rapidly forming large floating mats, with a reported doubling time of 14 days, *E. crassipes* infestations can quickly overgrow freshwater systems (Penfound, 1948). These infestations cause serious biotic and abiotic problems and have been known to alter water quality (Penfound, 1948), alter composition of flora and fauna in aquatic communities (Penfound, 1948; Khanna, 2011; Gichuki, 2012), damage infrastructure (Pfingsten, 2018), and contribute to flooding (Penfound, 1948). The economic impacts of *E. crassipes* are substantial, and control efforts can be expensive and of limited impact (Villamagna, 2010).

Eichhornia crassipes is a free floating tristylous plant native to the upper Amazon Basin, and was spread anthropogenically across the globe in the 19th century for its ornamental lavender flowers

(Penfound, 1948; Vietmeyer, 1975; Barrett, 1977; Parolin, 2010).The initial introduction of *Eichhornia crassipes* to North America is commonly reported as an 1884 World Cotton Exposition in New Orleans, Louisiana where plants were given away as souvenirs (Penfound, 1948); however, there is evidence that this plant was commercially available from seed catalogs as early as 20 years prior to the 1884 convention (Mack, 1991). By 1890, *E. crassipes* had become an established pest plant in the United States and spread as far as Florida, the U.S. Congress declared the plant a hazard to navigation in 1897, and by 1920 *E. crassipes* could be found in all southern coastal states and as far north as Virginia (Penfound, 1948; Mack, 1991). As of 2018, *E. crassipes* is reported in 34 states in the United States (Pfingsten, 2018).

The biology and introduction history of *E. crassipes* are likely to promote limited genetic diversity in non-native populations. The species reproduces readily via asexual budding through stolons and this appears to be the primary means of reproduction in introduced populations (Penfound, 1948; Center, 1981). Although many plants produce flowers and seeds, successful sexual reproduction is rare due to specific germination and seedling growth requirements that are uncommon outside of the native range of *E. crassipes* (Barrett 1980a, Barrett, 1980b). In addition, introduced populations of E. *crassipes* have been subjected to extreme founder effects and genetic bottlenecks which are likely to restrict genetic diversity (Zhang, 2010). For instance, *Eichhornia crassipes* was introduced to Indonesia in 1884 (Parolin, 2010; Parolin, 2012). From the Indonesian population, *E. crassipes* spread to Taiwan in 1903 and was finally introduced to mainland China in the 1930's (Jianqiang, 2001). These repeated nested bottlenecks during introduction events have resulted in the worldwide distribution of a single clonal genotype (Zhang, 2010).

As expected from biology and introduction history, ecological and molecular studies have found that introduced populations of *Eichhornia crassipes* have extremely low levels of genetic diversity. *Eichhornia crassipes* is tristylous, and early studies by Barrett (1977; 1980a) determined that extreme founder events had severely restricted the frequency of floral morphs in introduced populations. More recently, Ren *et al.* (2005) surveyed populations in China (n = 1009) using Random Amplified Polymorphic DNA (RAPD) markers and found low genetic diversity with a single dominant genotype present across all sites. A study by Li *et al*. (2006) surveyed *E. crassipes* in Southern China (n=60) and was unable to find any polymorphic loci using both RAPD and Inter-Simple Sequence Repeats (ISSR) markers. Additionally, Zhang *et al.* (2010) analyzed the population structure and genetic diversity of *E. crassipes* using Amplified Fragment Length Polymorphism (AFLP) markers and found a single genotype accounted

for 75.9% of individuals sampled across the global invasive range. Specifically, 80% of introduced populations were found to be genetically uniform after surveying 1,140 samples across 54 sites (Zhang, 2010).

Despite consensus that introduced populations of *E. crassipes* lack genetic diversity, previous studies have been limited in the scope of their sampling range and focused on populations in China (Ren, 2005; Li, 2006). Zhang *et al.* (2010) expanded the sampling range to include Africa, Europe, and South America; however, roughly half the specimens analyzed in the study were from China, and only 6 samples were from North America.

The lack of detailed information on North American populations represents a major gap in our understanding of the genetic structure of this species. The date and location of initial introduction in North America is reasonably well established and supported by numerous herbarium specimens. Despite this, we are unaware of any attempts to use herbarium specimens of *E. crassipes* to quantify historic levels of genetic diversity or population structure. By analyzing herbarium specimens, it is possible to characterize changes in population structure and genetic diversity at a temporal scale (Saltonstall, 2002; Saltonstall, 2003); and, it may be possible to do characterize changes in populations of *E. crassipes* since the initial introduction to North America.

The United States has a robust trade in exotic plants, and it is possible that contemporary populations are derived from independent introductions, each potentially bearing a distinct genetic signature. North American populations are also likely to have experienced selective pressures with the potential to generate local adaptation. The warm temperate and subtropical habitats of the Gulf Coast are directly connected to cooler temperate habitats associated with river systems draining from north to south, offering the possibility of adaptation to colder temperatures not found in the native range. The United States has aggressively pursued chemical control of *E. crassipes* since the end of the 19th century (Penfound, 1948; Gettys, 2009) and introduced several biocontrol agents in the 1970's (see Center, 1999) that have seriously impacted populations of *E. crassipes* (Nesslage, 2016). These pressures may promote adaptations such as herbicide or biocontrol resistance. A detailed molecular analysis of North American populations may provide new insights into whether this species is represented by a single clone or multiple genotypes, is the product of individual or multiple introductions, is currently generating genetic variation through recombination during sexual reproduction, or has experienced selection for local adaptation.

Here, we analyze temporal and spatial patterns of genetic variation in United States populations of *Eichhornia crassipes* using samples from herbarium collections and 5 contemporary populations from the central Gulf States. We used historical herbarium specimens to determine if North American populations have changed since their introduction in the 19th century to the present day and hypothesize that the genetic diversity of populations of *E. crassipes* in the United States has changed from higher genetic diversity to lower genetic diversity due to the exotic plant trade initially boosting diversity early in the 20th century and the introduction of biocontrols in the 1970's reducing genetic diversity. We studied contemporary populations to quantify genetic variation among and within populations, and to ask if population structure of *E. crassipes* is affected by seasonal environmental change*.* We hypothesize that contemporary populations in the United States to have low genetic diversity but will not have the extremely low genetic diversity reported elsewhere. Further, we hypothesize that there will be a subtle change in genetic variation across seasons as a result of sexual reproduction and clonal competition. Finding high genetic diversity in North American populations would be consistent with derivation form multiple independent introductions, sexual reproduction, and possibly selection and adaptation in the introduced range. Alternatively, low genetic diversity would be consistent with derivation from a single source population, asexual reproduction, and low potential for adaptation in the introduced range.

Materials and Methods

Sampling:

Historic patterns of genetic diversity were assessed using herbarium specimens of *Eichhornia crassipes*. Specimens were found by searching the Southeast Regional Network of Expertise and Collections (SERNEC) database to locate relevant specimens and herbariums. Once herbarium specimens were obtained, approximately 2 cm^2 of leaf tissue was removed under the permission and guidance of the herbarium. To collect as many samples as possible, we sampled all specimens with documentation of sample age and collection location, that were not obviously degraded or damaged (e.g. blackened and/or moldy). We restricted herbarium samples to those collected in the United States since our contemporary collections were restricted to this region. Potential duplicate samples having identical collection information were eliminated from the analysis. Collected leaf tissue was preserved in a desiccator, relative humidity <50%, for genetic analysis. A full list of herbarium samples used in this study can be found in Appendix A.

Contemporary genetic diversity in the United States was quantified by sampling the central Gulf Coast region because this area encompasses one of the earliest documented introductions of *E. crassipes* in North America, the 1884 New Orleans, Louisiana introduction. The following locations were chosen for contemporary sampling because they had a consistent year-round infestation of *E. crassipes*: Laccasine National Wildlife Refuge, LA, part of the Chenier Plain approximately 50 km southeast of Lake Charles, LA; Jean Lafitte National Park, LA, a backwater swamp 20 km south of New Orleans, LA; New Orleans City Park, LA, a 1300 acre urban park; Pass Manchac, LA, a large swamp marsh system between Lake Ponchartrain and Lake Maurepas 50 km northwest of New Orleans, LA; and Eastabuchie, MS, a closed oxbow lake off the Leaf River 12 km north of Hattiesburg, MS. Lacassine, Jean Laffitte, New Orleans City Park, and Pass Manchac are warm coastal habitats, experiencing an average of 10 or fewer days per year with freezing conditions, while Eastabuchie experiences an average of 36 days per year with freezing conditions (National Climatic Data Center, 2018).

To quantify contemporary genetic diversity and to test for seasonal changes, ten samples were collected from each site at several times in 2016-2017: fall 2016 (October), spring 2017 (March), early summer 2017 (May), and late summer 2017 (July/August). Fall 2016 sampling did not occur at Eastabuchie or Pass Manchac. We attempted to sample at the genet level and avoid resampling ramets, by leaving a minimum of 10 meters between collected specimens. This issue was deemed unavoidable

by Zhang *et al.* (2010); however, we still maintained a 10m distance to prevent overtly resampling ramets, especially in the summer when clonal mats dominate. Plants were randomly collected from the shore with a 3m pool pole to which a grappling hook was attached. After leaf tissue was extracted, plants were released to prevent any removal bias. Leaf tissue was rinsed clean with water and dried in a desiccator, relative humidity <50%, upon returning to the lab to prepare samples for DNA extraction.

DNA Extraction and Marker Development:

Leaf cuttings were processed by shredding 0.015-0.020 g of desiccated material using MP Biomedical lysing tubes with matrix A. Given the toughness of leaves, three 15-second bursts of grinding in a homogenizer (MP-FastPrep 24) were required to thoroughly break down tissue. Samples were allowed to rest for approximately 15 seconds between bursts to prevent degradation from overheating. Genomic DNA was extracted using DNEasy Plant Minikits (Qiagen). DNA concentrations were quantified using QuBit Double Stranded High Sensitivity Assays (Invitrogen). Samples with a DNA concentration of less than 5 ng/μl were considered too low and excluded from analyses. If a poor DNA extraction was suspected, the extraction was redone and re-quantified to see if higher concentrations were possible.

Amplified Fragment Length Polymorphism (AFLP) markers were employed according to the protocol of Zhang *et al.* (2010) (complete protocol listed in Appendix B). This protocol was chosen for its proven success with developing markers for *E. crassipes*. AFLPs were first described by Vos *et al.* (1995) (for a thorough review see Meudt, 2007). This technique produces a genotype consisting of binary dominant loci (i.e. fragments are either present or absent) which individually contain relatively little information but produce a substantial number of markers which culminates in a robust dataset. AFLPs have long been used in plant studies and are noted for their relatively low cost, number of markers produced, and non-reliance on *a priori* sequence information (Meudt, 2007), which is important because *E. crassipes* currently lacks a sequenced genome.

Zhang *et al.* (2010), used the enzymes EcoRI and MseI for restriction and ligated the matching E-adaptor and M-adaptor (sequences listed in Appendix C). The following primers were used for pre-selective amplification: E-A and M-C (Appendix C); and, the following primers were used for selective amplification: E-AAC (fluorescently tagged with FAM) and M-CTG (Appendix C). Zhang *et al*. (2010) used 4 primer pairs, and it was shown that the primer combination E-AAC and M-CTG were the most polymorphic primer combinations and discriminated the most clones. This primer combination was

6

considered sufficient for our analyses. After AFLP markers were developed we then used ExoSap-It (Affymetrix Inc.) to purify PCR products. Fragment analysis was performed on finished products by GeneWiz, LLC on an ABI 3130 Analyzer (Applied Biosystems).

Electropherograms from the fragment analysis were visualized, ladders were sized, and peaks were called using PeakScanner V2 (Applied Biosystems). All electropherograms were manually checked and samples that failed to amplify were eliminated from the dataset. Negative controls were accounted for by PeakScanner and any false peaks were removed. Next, the R package RawGeno, developed by Arrigo *et al.* (2009), was used to bin and score the PeakScanner output (see also Arrigo, 2014). Since low quality samples were manually observed and removed in PeakScanner, the first filtering step of RawGeno which targets these issues was omitted. The following settings for RawGeno were used after customizing them to minimize error. Herbarium samples were scored with the following parameters: maximum bin size 1.5 base-pairs (bp), minimum bin size 1 bp, min. peak size 75 bp, max. peak size 300 bp, min. fluorescence 100 relative fluorescence units (rfu), low frequency bins 1, reproducibility 97.5%, and untested bins were eliminated. Contemporary samples were scored with the following parameters: max. bin size 1.75 bp, min. bin size 1 bp, min. peak size 75 bp, max. peak size 500 bp, min. fluorescence 200 rfu, low frequency bins 5, reproducibility 95%, and untested bins were eliminated. Once RawGeno had scored and binned our data, a genotype binary matrix was created, excluding replicates, for subsequent molecular analyses.

Given the age of herbarium samples, the chances of DNA degradation were higher; therefore, it is reasonable to assume herbarium samples will have a higher error rate. The herbarium samples were analyzed separately from collected samples to prevent low-quality herbarium samples from affecting the error rate of the contemporary analysis. The same analysis pipeline was used for herbarium specimens and collected samples, with some settings relaxed or reduced to account for the higher probability of degraded herbarium DNA. These different settings make a direct comparison between the genotypes of collected samples and herbarium samples difficult. However, we are comparing patterns of genetic diversity and avoid this issue.

Comparing samples against their replicates, RawGeno calculates the genotyping error rate and I_{bin}, the information content per bin. I_{bin} is an optimality criterion used as a proxy for error rate in optimizing binning and scoring parameters (Arrigo, 2009). We replicated the genotypes of 14 herbarium samples (21.9% of total samples) and 47 contemporary samples (27.9% of total samples). Replicates were spread across different plates and reactions as suggested by Crawford *et al.* (2012). Due to limited

material, all herbarium samples replicates and most contemporary sample replicates were not developed from separate DNA extractions; and, instead were developed from previously extracted DNA, so the same extractions were used for marker developments and replicates. All replicates were developed separately as AFLP markers from the original extraction, were randomly selected, were incorporated on different AFLP plates, and were well distributed among these samples.

Data Analysis:

We used the R package POPPR for molecular data analysis because it specializes in handling non-model populations, such as clonal organisms (Kamvar, 2014). The binary genotypic table, created by RawGeno, was imported allowing population statistics to be calculated. We used POPPR to quantify the following genetic diversity measurements: Simpson's Dominance (λ) and Simpson's Diversity (1-λ) (Simpson, 1949) as well as Nei's unbiased genetic diversity (Nei, 1978). Simpson's Dominance calculates the probability that at a single locus any two alleles taken at random represent the same type and Nei's incorporates the relative frequency of a sample to give the probability that at a single locus any two alleles are different. Using different measures of diversity gives a more complete understanding of what the historic and contemporary genetic diversity is for populations of *E. crassipes* in the United States. POPPR has the capability to perform some tests with clone corrected data which censors the data so only one individual per multilocus genotype is represented, and thereby removing any potential bias from calculations such as linkage disequilibrium and AMOVA (Kamvar, 2014; Grünwald, 2017).

Herbarium data was stratified into populations of 'decades' and subpopulations of the collection location (i.e. state). Contemporary sample data was stratified into populations of sampling locale and subpopulations of collection season. By stratifying the data this way, we were able to perform an Analysis of Molecular Variance to identify differences between our populations and subpopulations (Excoffier, 1992). AMOVA can give negative variance values which can be considered effectively 0. AMOVA, as implemented in POPPR, calculates the genetic variation (σ) in these subpopulations, the percentage of total variation, and Φ_{ST} (a proxy for F_{st}) that reflects the degree of population differentiation, with values close to 1 reflecting strong differentiation and values close to 0 meaning no differentiation (Kamvar, 2014). AMOVA significance values were determined by random permutation the dataset 999 times, randomly assigning individuals to populations, thereby creating a null dataset, and then comparing the observed variation against the null dataset (Excoffier, 1992). If the observed

8

variation lies outside of the null dataset these results are considered statistically significant and p-values are calculated by POPPR (Excoffier, 1992; Kamvar, 2014). Exploratory statistics of genetic distance were also calculated: Hendrick's G'_{ST} (Hendrick, 2005), which compares heterozygosity to calculate a standardized measure of differentiation ranging from 0 (no differentiation) to 1 (complete differentiation), with negative values considered 0; and Nei's genetic distance (Nei, 1978), which compares heterozygosity and is used here to generate a neighbor joining tree to visualize population structure. Dendrograms were checked for support by 200 bootstraps. Both Hendrick's G'st and Nei's genetic distance are implemented and interpreted through POPPR (Grünwald, 2017).

Linkage disequilibrium was calculated to test for evidence of sexual reproduction. Linkage among loci can be caused by clonal reproduction and selection events; and, as linkage increases populations fall into linkage disequilibrium, while recombination from sexual reproduction breaks up linkage among loci and generates linkage equilibrium. To quantify linkage, POPPR calculates the indices I_A and $\bar{r}_d$. High values of I_A, i.e. values that differ strongly from 0, can be interpreted as evidence of strong linkage and thus linkage disequilibrium (Grünwald, 2017). The value $\bar{r}_d$ has been shown to be a more reliable estimator of linkage equilibrium than I_A since it is not influenced by sample size (Agapow, 2001), but to be thorough both metrics were calculated. Significance was tested by creating a null dataset (999 random permutations) and if the observed $\bar{r}_d$ value lies outside the null dataset then the null hypothesis that no linkage exists would be rejected (Kamvar, 2014; Grünwald, 2017).

Results

Herbarium Analysis:
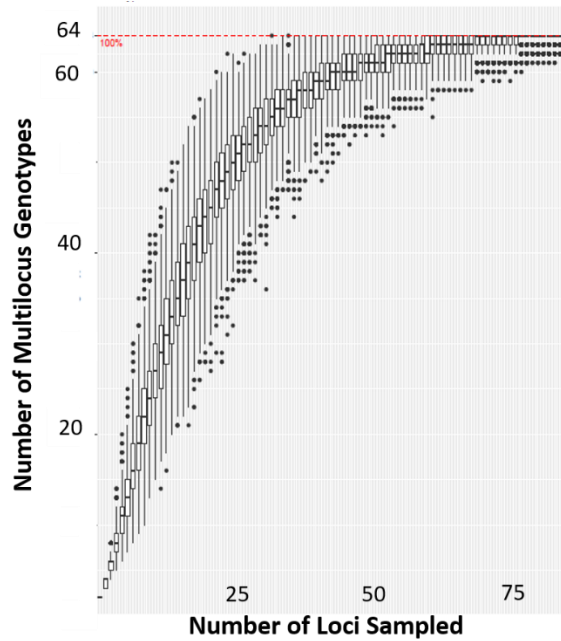
Figure 1: Genotype accumulation curve for herbarium samples

Herbarium sampling yielded 203 specimens obtained from 9 herbariums. After DNA extraction and marker development, 64 samples were deemed suitable for analysis. The 64 specimens used for analysis range in collection date from 1927-2011, have a mean collection date of 1981 (SD 16.9 years), and cover 8 states (see Appendix A). After fragment analysis, 89 dominant loci were found, incorporating 14 replicates (21.9% of the total), and yielding an error rate of 24.16% and I_{bin} of 18.88%. A genotype accumulation curve for herbarium multilocus genotypes (MLG's) suggests these loci have sufficient power to discriminate distinct genotypes (Figure 1). The resulting binary matrix was used for all further analysis.

Decade Stratification:
Pre- 1959 (n=3)
1960-1969 (n=9)
1970-1979 (n=21)
1980-1989 (n=7)
1990– 1999 (n=13)
2000– 2011 (n=11)

Figure 2: Temporal distribution of herbarium specimens
(n= sample size)

Table 1: Herbarium analysis results: genetic diversity and linkage

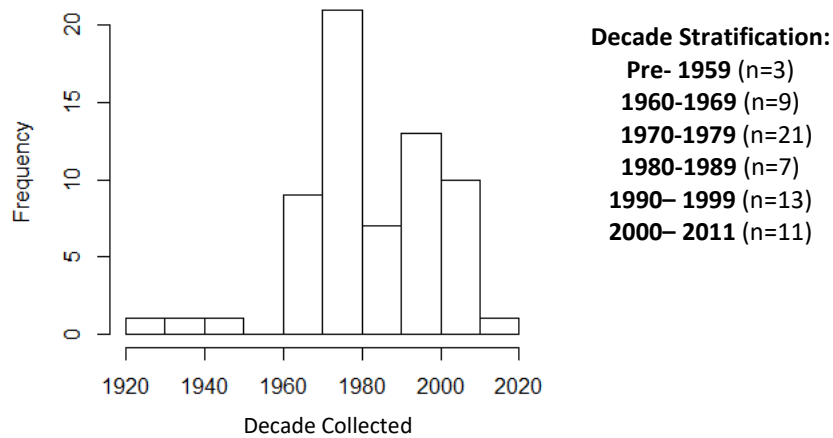|	n	MLG's	Simpson's Dominance (λ)	Nei's Unbiased Genetic Diversity	I_A	$\bar{r}_d$
Pre-1959	3	3	0.667	0.341	3.73	0.0867
1960-1969	9	9	0.889	0.166	1.96	0.0483
1970-1979	21	21	0.952	0.167	3.43	0.0594
1980-1989	7	7	0.857	0.217	4.35	0.0855
1990-1999	13	13	0.923	0.153	2.12	0.0431
2000-2011	11	11	0.909	0.293	5.34	0.0730
Herbarium Summary	64	64	0.984	0.200	5.08	0.0637

Herbarium samples were separated by locality and date of collection into populations and subpopulations (Figure 2). Populations were described by the decade in which they were collected in (Pre-1959, 1960-1969, 1970-1979, 1980-1989, 1990-1999, 2000-2011) and subpopulations were designated by the state samples were originally collected in. POPPR determined the herbarium data set to have an overall Simpson's Dominance (λ) of 0.984 and a Simpson's Diversity (1-λ) of 0.016, with Nei's Unbiased Genetic Diversity calculated as 0.200 (Table 1). A decade by decade breakdown is shown in Table 1. All three indices show that genetic diversity has been at extremely low levels since at least the 1960's. Linkage was calculated for each decade (Table 1), and the values of I_A and $\bar{r}_d$ support linkage disequilibrium, suggesting high levels of clonal reproduction. Due to the high error rate of the herbarium dataset, this was not analyzed further.

An AMOVA was performed to test variation and population structure from decade to decade (Table 2). Samples older than 1960 (n=3) were excluded from this analysis due to the small sample size. Subpopulations that contained only one individual (n=6) were excluded from the AMOVA as well. Nearly all variation was explained between samples (σ=8.674, 102.86% of the total variation) meaning that all samples were essentially in panmixia, with no differentiation between decades or sites. All Φ_{ST} values were close to 0 (negative values are treated as 0), so there is evidence of no population differentiation (Table 2), and there was no statistically significant difference between decades (Figure 3). This pattern was still present when tested with clone corrected data, showing that these patterns were not the result of clones in the dataset.

Table 2: Herbarium AMOVA results

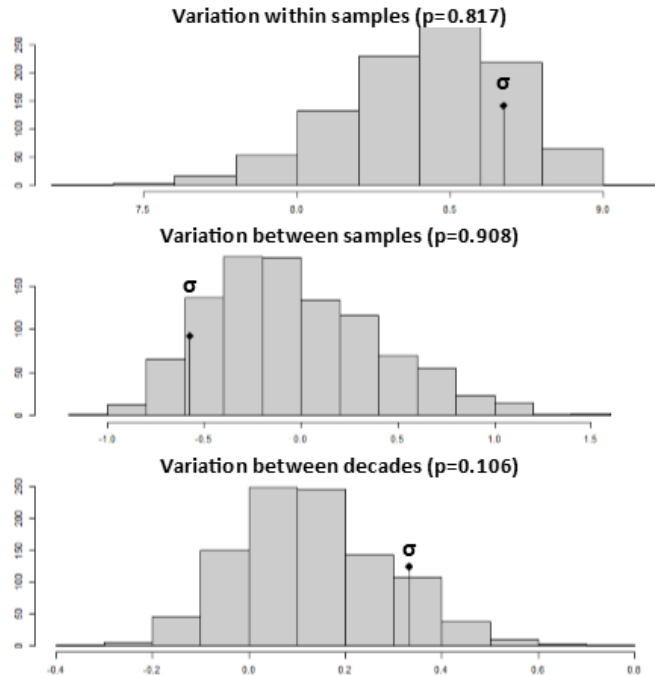|	Variation (σ)	Percent of Total Variation
Variation between decades	0.332	3.94 %
Var. between samples within decades	-0.573	-6.80 %
Var. within samples	8.674	102.86%
Total Variation	8.432	
Φ_{ST} Decades	0.0394	
Φ_{ST} Samples within decades	-0.0708	
Φ_{ST} Samples	-0.0286	

Figure 3: Herbarium AMOVA significance testing: observed variance (σ) compared to null dataset (grey bars represent null dataset and flag represents observed variation (σ))

Contemporary Analysis:

A total of 187 contemporary samples were collected across all 5 sites. DNA extraction and AFLP marker development was successful for 168 samples, with 19 samples being discarded for either poor DNA quality or poor marker development. After fragment analysis, 80 dominant loci were found, incorporating 47 replicates (27.9% of the total), and yielding an error rate of 7.50% and I_{bin} of 9.43%. A lower error rate was possible with 31 loci; but, AFLP markers contain relatively little information (i.e. presence/absence), therefore we opted for a slightly higher error rate which allowed for more loci (Zhang, 2012). These 80 loci provided sufficient power to distinguish genotypes as shown by the accumulation curve in Figure 4. The resulting binary matrix was used for all further analyses.
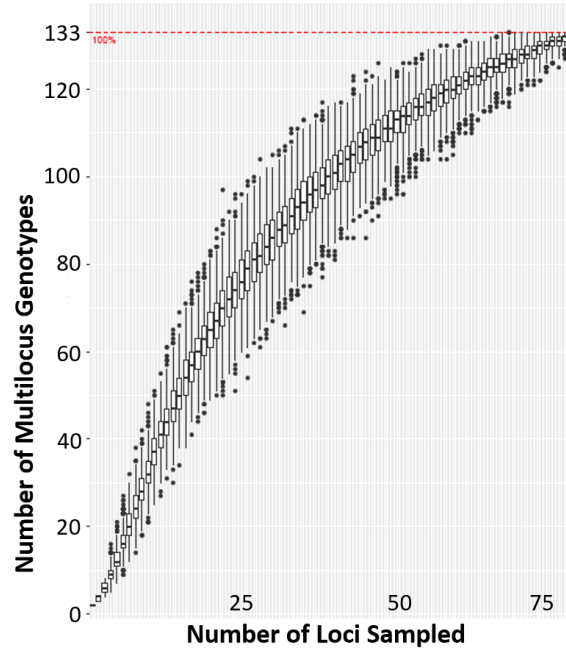
Figure 4: Genotype accumulation curve for contemporary samples

POPPR determined the contemporary data set to have an overall Simpson's Dominance (λ) of 0.970 and a Simpson's Diversity of 0.030 (1-λ) with Nei's Unbiased Genetic Diversity calculated as 0.1243 (Table 3). These results suggest a dearth of genetic diversity in Gulf Coast populations of *E. crassipes*. This trend was consistent across all 5 sites indicating that genetic diversity is low across the Gulf Coast region of the United States (Table 3).

Table 3: Contemporary analysis results: genetic diversity and linkage

	n	MLG's	Simpson's Dominance (λ)	Nei's Unbiased Genetic Diversity	I_A	$\bar{r}_d$
City Park, LA	39	35	0.996	0.1253	4.08	0.0618
Manchac, LA	27	19	0.900	0.0960	10.70	0.1987
Eastabuchie, MS	27	27	0.963	0.2164	5.42	0.0752
Lacassine NWR	41	31	0.921	0.0796	3.43	0.0619
Jean Lafitte NP	34	30	0.953	0.1176	4.14	0.0625
Contemporary Summary	168	133	0.970	0.1243	5.97	0.0783

To understand whether there was a phenological effect on genetic diversity, data was stratified first by site and then by season so an AMOVA could be performed (Table 4). Little variation was found between sampling sites (σ=0.0401, 0.81% of total variation). The bulk of variation was found within samples, (σ=4.7278, 94.7% of total var.). The variation between seasons (i.e. between samples within

14

sites) accounted for 4.49% of the total variation (σ=0.224). Variation between samples and variation between seasons were both significant (p=0.001) (Table 4). These patterns were consistent when tested with clone corrected data. However calculated values of Φ_{ST} were small and did not support strong population structure at any level (Table 4). This pattern was still present when tested with clone corrected data. This suggests that the difference detected between seasons, though small (Φ_{ST}= 0.045), is significant.

Table 4: Contemporary AMOVA results
(* denotes statistically significant values (p=0.001))

	Variation (σ)	Percent of Total Variation
Variation between sites	0.0401	0.81%
Var. between samples within site*	0.2241	4.49%
Var. within samples*	4.7278	94.70%
Total Variation	4.9921	
Φ_{ST} Site	0.00805	
Φ_{ST} Samples within Sites*	0.04526	
Φ_{ST} Samples *	0.05295	

The statistically significant population structure detected by AMOVA, was not supported by other measures of genetic distance. Hendrick's genetic distance was calculated across loci with G_{st} = -0.02178 suggesting no population structure. Hendrick's genetic distance was also calculated separately for each site and did not support any population structure (Appendix D), none of which support population structure. A pairwise matrix for Nei's genetic distance was calculated and a neighbor joining tree was created individually for each site to visualize if and how samples were clustering. These trees produced no recognizable pattern and did not result in individuals clustering or segregating based on season, thus suggesting no seasonal population structure (Appendix D). The City Park dendrogram did show weak clustering of fall samples, but this pattern was not supported by Hendrick's Gst nor was it found at any other site. Both Hebdrick's G_{ST} and Nei's genetic distance do not agree with the AMOVA results and do not support any population differentiation or structure.

The complete contemporary dataset was tested for linkage disequilibrium and evidence of sexual reproduction. We found I_A = 5.972 and $\bar{r}_d$ = 0.0783. The observed RD value fell significantly outside the null dataset, thus rejecting the null hypothesis of no linkage (p=0.001) and suggest that

these populations have high levels of linkage and are significantly in linkage disequilibrium. Our values of I_A, differ strongly from 0, further supporting strong linkage and linkage disequilibrium.

The potential population structure between seasons, and possible seasonal effect, was further explored. The contemporary dataset was restructured, and populations were only stratified by season. Simpson's Dominance (λ) values were calculated for each season: fall 0.942, spring 0.957, early summer 0.967, late summer 0.948. Linkage was calculated for each season (Figure 6), which provided further evidence that linkage was present in each season (p=0.001). Linkage disequilibrium dominates Gulf Coast populations of *E. crassipes* year-round.
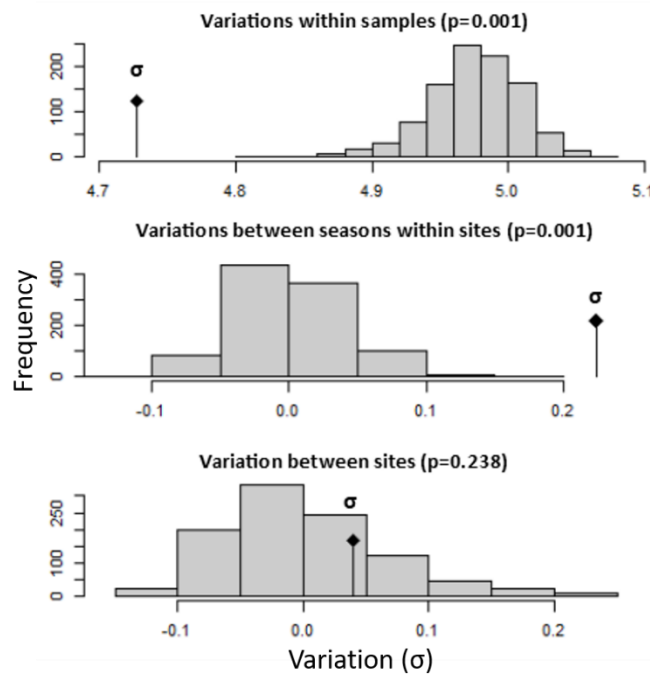

Figure 5: Contemporary AMOVA significance testing: observed variance (σ) compared to null dataset (grey bars represent null data set and flag represents observed variation (σ))
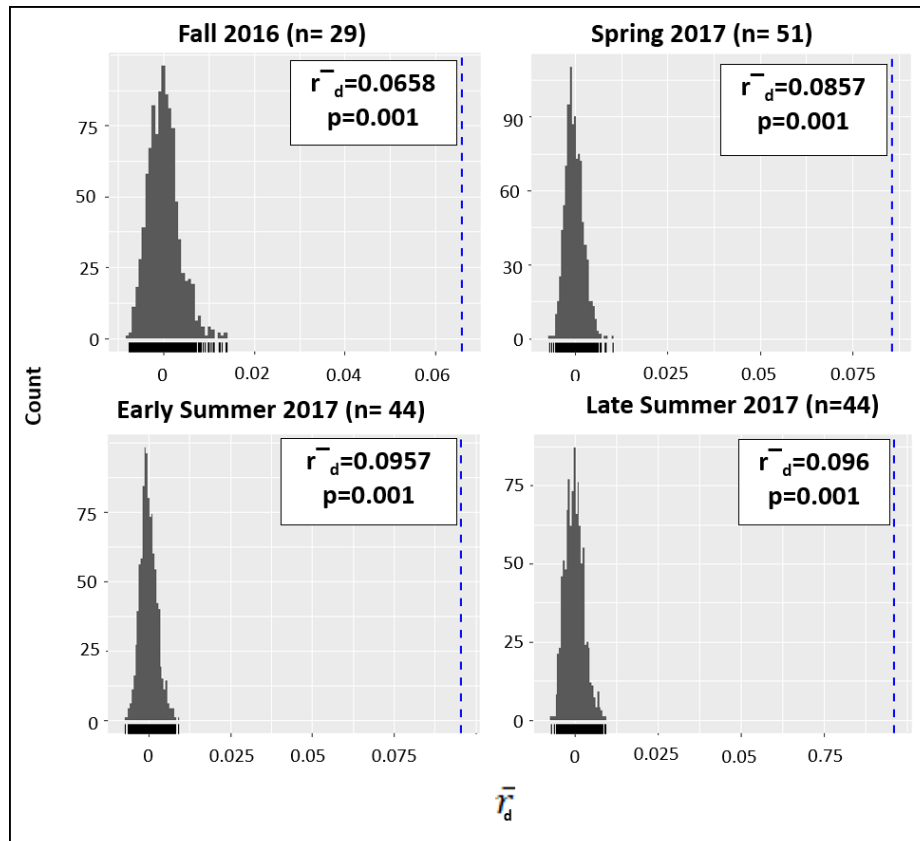
Figure 6: Contemporary linkage significance across seasons
(dashed lines show observed values and grey bars represent null data set)

Discussion:

Our results suggest that genetic diversity of *Eichhornia crassipes* populations in the Central Gulf States was and remains low and provides strong evidence of clonal reproduction. These results are consistent with studies of introduced populations of *E. crassipes* in other regions (Barrett, 1977; Barrett, 1980a; Ren, 2005; Li, 2006; Zhang, 2010) and suggest that North American populations are likely the product of introduction from a single genetic source. It is possible that the highly uniform populations detected in the United States are comprised of the same clonal genotype, clone W, that was detected by Zhang *et al* (2010), but because our binning procedures were different we cannot be certain we are detecting the same clonal genotype. Despite an active trade in exotic plants and potential selection for cold tolerance and herbicide and herbivore resistance, the low genetic diversity of our populations suggests that populations of *E. crassipes* in the United States are likely highly uniform and possess little potential for adaptive evolution at this time.

Our herbarium analyses are limited by a high error rate. Despite filtering out low quality samples during collection and DNA extraction, and manually checking our Electropherograms, we believe that some herbarium samples had suffered DNA degradation. This caused individual variation in AFLP marker quality and inflated the herbarium error rate. While this issue makes deeper analysis of herbarium data difficult, we are still confident in our broader conclusions because our herbarium analyses agree with our analysis of contemporary data and the published literature (Barrett, 1977; Barrett, 1980a; Ren, 2005; Li, 2006; Zhang, 2010).

The analysis of herbarium specimens suggests that genetic diversity of *E. crassipes* populations in the United States has consistently been low since the 1960's. In this time there have been many selection pressures imposed on *E. crassipes*. In the 1970's there were widespread introductions of biocontrol agents in the southern United States, such as the weevils *Neochetina eichhorniae* and *N. bruchi* (Manning, 1979; Center, 1999). These biocontrol releases combined with ongoing chemical and mechanical control and several harsh winters have led to an over 80% reduction in Louisiana populations of *E. crassipes* between 1976 and 2013 (Nesslage, 2016). Despite these major changes, genetic structure of United States populations of *E. crassipes* has not changed through time, and thus rejects our hypothesis that genetic diversity has decreased through time. This lack of change and diversity suggests that no new genetic material has taken hold in this region through either introductions, adaptation, or sexual reproduction, and supports the idea that these United States populations are derived from a single source population.

Overall, current populations of *E. crassipes* in the Gulf Coast region of the United States can be characterized by an extreme dearth of genetic diversity as revealed by an overall Simpson's Dominance (λ) of 0.970. This pattern was found across all 5 sampling sites and across all seasons. Furthermore, the AMOVA revealed there was no significant difference between sites, suggesting genetic diversity is low across the entire Gulf Coast region. Most variation was found within samples and accounts for 94.7% of all variation. This pattern was expected for an invasive species where anthropogenic spread from a single introduction produces homogeneous population structure across space. Our hypothesis of low genetic diversity in the United States was supported. However, our prediction that populations in the United States would not have the extreme lack of diversity found elsewhere was not supported with the populations surveyed here are just as genetically uniform as other introduced populations.

Conflicting evidence was found for population structure between seasons. AMOVA revealed small yet significant variation between seasons (i.e. between samples within sites, p=0.001) with 4.49% of the total variation explained. However, when analyzing Hendrick's G'$_{st}$ and Nei's genetic distance, no significant population differentiation was detected (Appendix D). As such we conclude that there is no population differentiation between seasons in populations of *E. crassipes*, but it is possible that phenology effects other characteristics of populations of *E. crassipes*.

Exploring any potential seasonal differences, we compared genetic diversity and linkage across seasons. Genetic diversity was consistently low when the data was separated by seasons (Simpson's Dominance (λ): fall 0.942, spring 0.957, early summer 0.967, late summer 0.948). Therefore, genetic diversity does not seem to vary meaningfully between seasons, but this may be the result of an extremely low baseline genetic diversity that prevents or obscures any phenological effects.

Linkage disequilibrium, and by implication clonal reproduction, dominate populations of *E. crassipes* in the United States, which agrees with previous research (Penfound, 1948; Barrett, 1980b; Ren, 2005; Li, 2006; Zhang, 2010). Despite high levels of linkage disequilibrium, there were small changes in linkage across seasons. Both early summer ($\bar{r}_d$=0.0957) and late summer ($\bar{r}_d$=0.0960) have the highest levels of linkage equilibrium, with spring ($\bar{r}_d$=0.0857) decreasing and with fall having the lowest levels of linkage ($\bar{r}_d$=0.0658). While small, this may be indicative of a change in the linkage rates, potentially reflecting changes in rates of sexual reproduction or selection pressures throughout a year.

Populations of *E. crassipes* undergo strong seasonal changes in abundance which may explain the observed fluctuations in $\bar{r}_d$. *Eichhornia crassipes* populations are reduced by winter freeze events, causing significant die offs (Center, 1981; Nesslage, 2016). Clonal growth resumes in the spring and intensifies in the summer producing large floating mats that dominate waterbodies in the late summer (Penfound, 1948; Center, 1981). This pattern of rapid clonal growth may explain the higher levels of linkage in the early and late summer. Higher summer linkage levels may be reinforced by selection events that occur during the summer, Center *et al*. (1981) found that during the early summer roughly three plants per m^2/day were lost, possibly due to clonal competition. Control measures such as herbicide application, biocontrol damage, and mechanical removal also intensify in the summer. Lower linkage during the fall may be the result of relaxed clonal growth allowing for the few individuals produced by sexual reproduction to grow, develop, and be detected. Interestingly, the seasonal variation in linkage disequilibrium does not appear to have contributed to changes in genetic diversity or population structure over the past 55 years. It is likely that initial low levels of genetic diversity have been maintained by low levels of sexual reproduction and strong selection pressure (Penfound, 1948; Barrett, 1980b; Nesslage, 2016). Despite no population structure across seasons, levels of linkage do change across seasons thus giving equivocal support for our prediction that populations of *E. crassipes* are affected by phenology.

Phenological changes across seasons have the potential to alter genetic features like linkage in meaningful ways. While these patterns are unclear in introduced populations of *E. crassipes,* this may be an artifact of low genetic diversity and may exist in less extreme organisms. This has important implications for future molecular assessments of clonal populations because changes in genetic diversity in conjunction with seasonal could lead to gross misrepresentations of genetic diversity, populations structure, and linkage equilibrium. For example, sampling during periods of rapid clonal growth would lead to an underestimation of diversity and vice versa. Populations of *E. crassipes* have extremely low levels of genetic diversity; and, if these seasonal variations were detectable in these populations then there is potential for seasonal variation to have a more pronounced impact on other clonal organisms. Sampling of clonal organisms should take this into account by incorporating phenology and life history with experimental design.

The extreme lack of genetic diversity in populations of *E. crassipes* is remarkable given that the plant has successfully spread and thrived globally. Its success in a wide variety of habitats is likely due to significant phenotypic plasticity of the dominant clonal genotype (Center, 1981; Zhang, 2010). The

strategy of high levels of plasticity, with the addition of clonal growth, appear to have allowed *E. crassipes* to overcome the invasive paradox of low initial genetic diversity (Roman, 2007). This plasticity is also likely responsible for the presence of outlier populations found in cool temperate climates such as the Laurentian Great Lakes in North America (Adebayo, 2011) and the Erft River in Northern Germany (Hussner, 2014). Aside from these published reports, we found herbarium samples from Wisconsin that appeared in multiple years in close locales (9 field specimens in the past 20 years found using SERNEC's herbarium database). These reports of northern infestations are troubling since harsh winters are one of the few effective killers of *E. crassipes* (Nesslage, 2016; Pfingsten, 2018). While some appear to be anomalies due to abnormal micro-climate conditions (Hussner, 2014), these new infestations warrant further investigation. These fluctuating environments would be expected to favor sexual over asexual reproduction since cool temperate populations may rely on sexually produced seeds to escape harsh winter conditions. Higher levels of sexual reproduction would facilitate the spread of new genetic variation (Muller, 1932) and cool temperate populations may serve as sources of genetic variation. Increased genetic variation gives natural selection and adaptive evolution more variation to act upon. We suggest that northern populations of *E. crassipes* would be particularly worthy of study to determine if they are as genetically homogeneous and reliant on clonal reproduction as more southern populations.

References:

Adebayo, A.A., Briski, E., Kalaci, O.,Hernandez, M., Ghabooli, S., Beric, B., Chan, F.T., Zhan, A., Fifield, E., Leadley, T., and MacIsaac, H.J. (2011). Water hyacinth (*Eichhornia crassipes*) and water lettuce (*Pistia stratiotes*) in the Great Lakes: Playing with fire? Aquatic Invasions. 6(1), 91-96.

Agapow, P., and Burt, A. (2001). Indices of multilocus linkage disequilibrium. Molecular Ecology. 1, 101-102.

Arrigo, N., Tuszynski, J.W., Ehrich, D., Gerdes, T., and Alvarez, N. (2009). Evaluating the impact of scoring parameters on the structure of intra-specific genetic variation using RawGeno, an R package for automating AFLP scoring. BMC Bioinformatics. 10(33).

Barrett, S.C. (1977) Tristyly in *Eichhornia crassipes* (Mart.) Solms (Water Hyacinth). Biotropica. 9(4), 230-238.

Barrett, S. (1980a) Sexual Reproduction in *Eichhornia crassipes* (Water Hyacinth) I. Journal of Applied Ecology. 17(1), 101-112.

Barrett, S. (1980b) Sexual Reproduction in *Eichhornia crassipes* (Water Hyacinth) II. Journal of Applied Ecology. 17(1), 113-124.

Brundu, G. Azzella, M.M., Blasi, C., Camarda, I., Iberite, M., and Celesti-Grapow, L. (2013). The silent invasion of *Eichhornia crassipes* (Mart.) Solms In Italy. Plant Biosystems. 147(4), 112-1127.

Center, T.D., Dray, F.A. JR., Jubinsky, G.P., and Grodowitz, M.J. (1999). Insects and Other Arthropods That Feed on Aquatic and Wetland Plants. U.S. Department of Agriculture, Agriculture Research Service, Technical Bulletin No. 1870, 110-123.

Center, T.D., amd Spencer, N.R. (1981). The Phenology and Growth of Water Hyacinth (*Eichhornia crassipes* (Mart.) Solms) in a Eutrophic North-Central Florida Lake. Aquatic Botany. 10, 1-32.

Crawford, L.A., Koscinski, D., and Keyghobadi, N. (2012). A call for more transparent reporting of error rates: The quality of AFLP data in ecological and evolutionary research. Molecular Ecology. 21(24), 5911-5917.

Excoffier, L., Smouse, P.E., and Quattro, J.M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics. 131(2), 479-491.

Hedrick, P.W. (2005) A standardized genetic differentiation measure. Evolution. 59, 1633–1638.

Gettys, L.A., Haller, W.T., and Bellaud, M. (2009). Biology and Control of Aquatic Plants: a best management practices handbook. Aquatic Ecosystem Restoration Foundation. 113-118.

Geng, Y.P., Pan, X.Y., Xu, C.Y., Zhang, W.J., Li, B., Chen, J.K., Lu, B.R., Song, Z.P. (2007). Phenotypic plasticity rather than locally adapted ecotypes allows the invasive alligator weed to colonize a wide range of habitat. Biol. Invasions. 9, 245-256.

Gichuki, J., Omondi, R., Boera, P., Okorut, T., Matano, A.S., Jembe, T.,and Ofulla, A. (2012). Water Hyacinth *Eichhornia crassipes* (Mart.) Solms-Laubach Dynamics and Succession in the Nyanza Gulf of Lake Victoria (East Africa): Implications for Water Quality and Biodiversity Conservation. The Scientific World Journal. 2012.

Grünwald, N.J., Kamvar, Z.N., Everhart, S.E., Tabima, J.F., and Knaus, B.J.(2017). Population genetic and genomics in R. http://grunwaldlab.github.io/Population_Genetics_in_R/. Accessed May 2018.

Gutekunst, J., Andriantsoa, R., Falckenhayn, C., Hanna, K., Stein, W., Rasamy, J., Lyko, F. (2018). Nature Ecology and Evolution. 2, 567-573.

Hussner, A. (2014). Long-term macrophyte mapping documents a continuously shift from native to non-native aquatic plant dominance in the thermally abnormal River Erft (North Rhine-Westphalia, Germany). Limnologica. 48. Pp. 29-45.

Jianqing, D., Ren, W. Weidong, F., and Guoliang, Z. (2000). Water Hyacinth in China: Its Distribution, Problems and Control Status. Proceedings of the Second Meeting of the Global Working Group for the Biological and Integrated Control of Water Hyacinth. ACIAR Proceedings. 102, 29-32.

Kamvar, Z.N., Tabima, J.F., and Grünwald, N.J. (2014) Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ. DOI: 10.7717/peerj.281.

Khanna, S., Santos, M.J., Hestir, E.L., and Ustin, S.L. (2011). Plant community dynamics relative to the changing distribution of a highly invasive species, *Eichhornia crassipes*: a remote sensing perspective. Biological Invasions. DOI: 10.1007/s10530-011-0112-X.

Li W., Wang, B., and Wang, J. (2006). Lack of genetic variation of an invasive clonal plant Eichhornia crassipes in China revealed by RAPD and ISSR markers. Aquatic Botany.   DOI: 10.1016/j.aquabot.2005.09.008.

Mack, R. (1991). The Commercial Seed Trade: An Early Disperser of Weeds in the United States. Economic Botany. 45(2), 257-273.

Manning, J. (1979). Establishment of Waterhyacinth Weevil Populations in Louisiana. Journal of Aquatic Plant Management. 17, 39-41.

Meudt, H.M., and Clarke, A.C. (2007). Almost Forgotten or Latest Practice? AFLP applications, analyses and advances. Trends in Plant Science. 12(3), 106-117.

Muller, H.J. (1932). Some Genetic Aspects of Sex. The American Naturalist. 66(703) 118-138.

National Climatic Data Center, 2018. https://www.ncdc.noaa.gov/cgi-bin/climatenormals/climatenormals.pl. Downloaded May 20, 2018.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics. 89(3), 583–590.

Nesslage, G.M., Wainger, L.A., Harms, N.E., and Cofrancesco, A.F. (2016) Quantifying the population response of invasive water hyacinth, *Eichhornia crassipes*, to biological control and winter weather in Louisiana, USA. Biological Invasions. 18, 2107-2115.

Parolin, P., Bartel, S., and Rudolph, B. (2010). The Beautiful Water Hyacinth *Eichhornia crassipes* and the Role of Botanic Gardens in the Spread of an Aggressive Invader. Bollettino dei Musei e degli Istituti Biologici Universitari di Genova. 72, 21-29.

Parolin, P. Rudolph, B., Bartel, S., Bresch, C., and Poncet, C. (2012). Worldwide Invasion Pathways of the South American *Eichhornia crassipes*. Acta Horticulturae. 937, 1133-1140.

Penfound, W.T., and Earle, T.T. (1948). The Biology of the Water Hyacinth. Ecological Monographs. 18(4), 447-472.

Pérez, J.E., Nirchio, M., Alfonsi, C., and Muñoz, C. (2006). The biology of invasions: the genetic adaptation paradox. Biological Invasions. 8(5), 1115-1121.

Pfingsten, I.A., Thayer, D.D., Jacono, C.C., Richerson, M.M., and Howard, V. (2018). *Eichhornia crassipes* (Mart.) Solms: U.S. Geological Survey. Nonindigenous Aquatic Species Database. https://nas.er.usgs.gov/queries/FactSheet.aspx?SpeciesID=1130, Revised: February 29, 2016, Peer Reviewed: February 25, 2016, Accessed: May 23, 2018.

Ren, M.X., Zhang, Q.G., and Zhang, D.Y. (2005). Random amplified polymorphic DNA markers reveal low genetic variation and a single dominant genotype in *Eichhornia crassipes* populations throughout China. Weed Research. 45, 236-244.

Roman, J. (2006). Diluting the founder effect: Cryptic invasions expand a marine invader's range. Proceeding of the Royal Society Biology. 273, 2453-2459. DOI:10.1098/rspb.2006.3597.

Roman, J., Darling, J. (2007). Paradox lost: genetic diversity and the success of aquatic invasions. Trends in Ecology and evolution. 22(9), 454-464. DOI:10.1016/j.tree.2007.07.002.

Saltonstall, K. (2002). Cryptic invasion by a non-native genotype of the common reed, *Phragmites australis*, into North America. PNAS. 99(4) 2445-2449.

Saltonstall, K. (2003). Microsatellite variation within and among North American lineage of *Phragmites australis*. Molecular Ecology. 12(7), 1689-1702.

Simpson, E.H. 1949. Measurement of diversity. Nature. 163(688). DOI: 10.1038/163688a0.

Vietmeyer, N. (1975). The Beautiful Blue Devil. Natural History. 84, 65-72.

Villamagna, A.M., and Murphy, B.R. (2010). Ecological and socio-economic impacts of invasive water hyacinth (*Eichhornia crassipes*): a review. Freshwater Biology. 55(2), 282-298.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research. 23(21), 4407-4414.

Zhang, Y., Zhang, D., and Barrett, S. (2010). Genetic uniformity characterizes the invasive spread of water hyacinth (*Eichhornia crassipes*), a clonal aquatic plant. Molecular Ecology. 19, 1774-1786.

Zhang, H., and Hare, M.P. (2012). Identifying and reducing AFLP genotyping error: An example of tradeoffs when comparing population structure in broadcast spawning versus brooding oysters. Heredity. 108(6), 616-625.

Appendix A: Herbarium Sample Details

Sample ID	Date	County/Parish, State	Herbarium	Specimen ID
A01	7/25/1981	Allen Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00010999
A03	9/21/1974	Assumption Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011001
A04	10/12/1974	Ascension Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011002
A05B	6/28/1939	Beauregard Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011003
A07	10/16/1976	Cameron Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011005
A08	9/17/1977	Cameron Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011006
A09	7/28/1972	East Baton Rouge Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011007
A10B	10/16/1927	East Baton Rouge Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011008
A12	1/22/1993	Iberville Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011010
A17	7/26/1990	Plaquemines Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU-00011015
A20	9/10/1986	Rapides Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU-00011018
A22	10/18/1969	St. Charles Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011020
A25	8/29/1971	St. Helena Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011023
A26	11/1/1992	St. John the Baptist Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011024
A27	9/8/1990	St. John the Baptist Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011025
A28	9/16/1989	St. Landry Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011026
A29	11/24/1974	St. Landry Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011027
A31	6/5/1986	St. Martin Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011029
A32	10/7/1973	St. Martin Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011030
A33	6/8/1968	St. Mary Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011031
A40	10/18/1992	Terrebonne Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011038
A41	5/20/1980	Terrebonne Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011039
A42B	2/20/1993	West Feliciana Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011040
A43B	8/12/1972	West Feliciana Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011042
A44B	10/7/1986	West Feliciana Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00011041
A47	3/12/1994	St. Martin Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00029369
A48	10/17/1977	Cameron Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00032499

A51	9/10/2001	Avoyelles Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00043323
A55	10/1/1977	Travis County, TX	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00077773
A60	7/12/2004	Iberia Parish, LA	Louisiana State University, Shirley C. Tucker Herbarium	LSU- 00097646
B04	7/29/1997	Terrebonne Parish, LA	Tulane University Herbarium	Tulane-NO-0039777
B15	11/7/1940	Orleans Parish, LA	Tulane University Herbarium	Tulane-NO-0039788
B22B	10/11/1978	St. Tammany Parish, LA	Tulane University Herbarium	Tulane-NO-0039795
B23	10/11/1978	St. Tammany Parish, LA	Tulane University Herbarium	Tulane-NO-0039795
B25	8/6/1995	Orleans Parish, LA	Tulane University Herbarium	Tulane-NO-0039797
B26	7/5/1967	Chesterfield, SC	Tulane University Herbarium	Tulane-NO-0039798
C05B	8/13/1991	Hancock County, MS	University of Southern Mississippi Herbarium	USMS-0-5239
C06B	8/9/1964	Forrest County, MS	University of Southern Mississippi Herbarium	USMS-0-5240
D08	10/30/2006	Leflore County, MS	Mississippi Museum of Natural Science Herbarium	MMNS-026876
D10	9/18/2008	Tate County, MS	Mississippi Museum of Natural Science Herbarium	MMNS-032065
D11	11/15/2011	Lawrence County, MS	Mississippi Museum of Natural Science Herbarium	MMNS-033233
E01	10/12/1974	Jasper County, TX	Howard Payne University Herbarium	HPU-19484
E02	9/30/1972	Gonzales County, TX	Howard Payne University Herbarium	HPU-2570
E03	5/5/1972	Hidalgo County, TX	Howard Payne University Herbarium	HPU-2569
E10B	2/3/1973	Val Verde County, TX	Howard Payne University Herbarium	HPU-19485
E11	5/23/1972	Rapides Parish, LA	Howard Payne University Herbarium	HPU-2573
F01	11/28/1975	Highlands County, FL	University of Wisconsin Madison, WI State Herbarium	WIS-V0311346
F02	6/10/1963	Hayes County, TX	University of Wisconsin Madison, WI State Herbarium	WIS-V0311332
F07	6/10/1971	Marion County, FL	University of Wisconsin Madison, WI State Herbarium	WIS-V0311341
F08	4/8/1969	Beach County, FL	University of Wisconsin Madison, WI State Herbarium	WIS-V0311333
F22	10/1/2002	Price County, WI	University of Wisconsin Madison, WI State Herbarium	WIS-V0200836
G02	9/1/1971	Harrison County, TX	University of Illinois at Urbana-Champaign	UIUC-03
H11	8/30/1981	Seminole County, FL	University of Central Florida Herbarium	FTU-0003198
H14	3/29/1997	Okeechobee County, FL	University of Central Florida Herbarium	FTU-0003201
H16	6/27/1991	Orange County, FL	University of Central Florida Herbarium	FTU-0003203
J02B	8/21/2002	Pike County, AL	University of Mississippi, Thomas M. Pullen Herbarium	Miss-0013804
J03	10/30/2006	Leflore County, MS	University of Mississippi, Thomas M. Pullen Herbarium	Miss-0013800
J04B	9/18/2008	Tate County, MS	University of Mississippi, Thomas M. Pullen Herbarium	Miss-0013801

J05	10/25/1995	Pickens County, AL	University of Mississippi, Thomas M. Pullen Herbarium	Miss-0013805
J07	5/14/1964	Lowndes County, GA	University of Mississippi, Thomas M. Pullen Herbarium	Miss-0013807
J10	6/22/1965	Forrest County, MS	University of Mississippi, Thomas M. Pullen Herbarium	Miss-0013795
J11	7/25/2003	Franklin County, MS	University of Mississippi, Thomas M. Pullen Herbarium	Miss-0013796
J13	10/30/2006	Issaquena County, MS	University of Mississippi, Thomas M. Pullen Herbarium	Miss-0013798
J14	10/22/1969	Jones County, MS	University of Mississippi, Thomas M. Pullen Herbarium	Miss-0013799

Adapted from:
Zhang, Y., Zhang, D., and Barrett, S. (2010). Genetic uniformity characterizes the invasive spread of water hyacinth (*Eichhornia crassipes*), a clonal aquatic plant. Molecular Ecology. 19, 1774-1786.

Adaptor Preparation:
Annealing was done by combining equal molar amounts of each adaptor in water, heating to $94°C$ for 4-5 minutes, and allowing to slowly cool to room temperature for 12 minutes.

Restriction and Ligation: (single step, 20 µL reaction)
- 200-400 ng genomic DNA, 8 U EcoRI (New England Biolabs (NEB)), 2 U MseI (NEB), 80 U T4 DNA Ligase (NEB), 1X NEBuffer2 (NEB), 2 µg BSA, 0.2 mM ATP (NEB), 0.2 µM EcoRI Adaptor, 0.16 µM MseI Adaptor, and H_2O

-Incubated at $37°C$ for 3h, denatured at $70°C$ for 10 min

Pre-Selective PCR: (20 µL reaction)
- 2 µL restriction-ligation product, 0.5 U Taq Polymerase (NEB), 1X standard pcr buffer (NEB), 2.0 mM $MgCl_2$, 025 mM mixed dNTP (NEB), 0.25 µM Primer-EA, 0.25 µM Primer-MC, and H_2O

- $94°C$ for 2 min, 28 cycles of $94°C$ for 45s, $56°C$ for 45s, $72°C$ for 1 min, and finished with $72°C$ for 10 min

Selective PCR: (20uL reaction)
- 3 µL pre-selective product, 0.5 U Taq Polymerase (NEB), 1 X PCR buffer (NEB), 2 mM $MgCL_2$, 0.25 mixed dNTP (NEB), 0.25 µM Primer-E-AAC, 0.30 µM Primer-M-CTG, and H_2O

- $94°C$ for 2 min, followed by 13 cycles of $94°C$ for 30 s, $65°C$ for 30s (decreasing by $0.7°C$ each cycle), $72°C$ for 1 min, 23 cycles of $94°C$ for 30s, $56°C$ for 30s, $72°C$ for 1 min, and finishing with $72°C$ for 5 min

Appendix C: Primer Sequences

Note- All primers were obtained from Integrated DNA Technologies

Ligation Adaptor Primers:
  E-adaptor-1 (5' CTC-GTA-GAC-TGC-GTA-CC)
  E-adaptor-2 (5' AAT-TGG-TAC-GCA-GTC-TAC)
  M-adaptor-1 (5' GAC-GAT-GAG-TCC-TGA-G)
  M-adaptor-2 (5' TAC-TCA-GGA-CTC-AT)
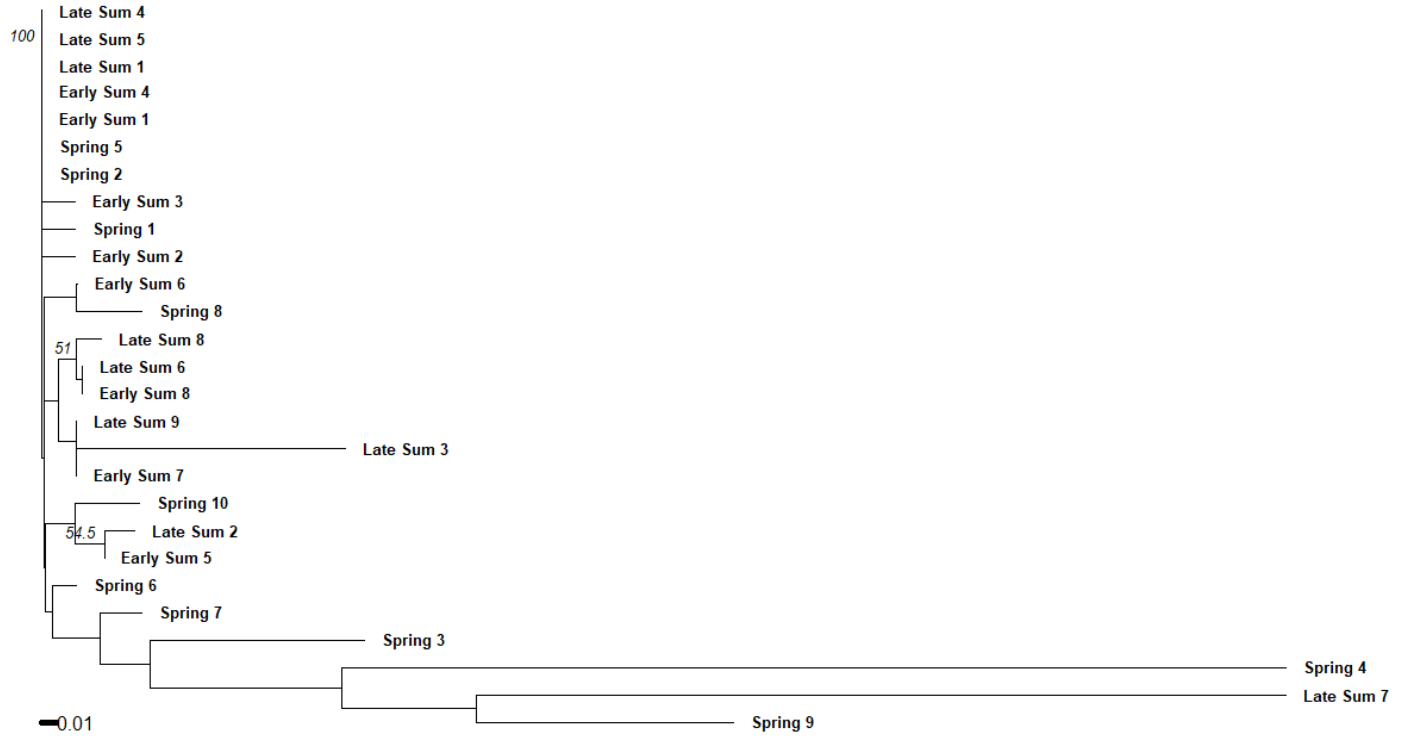
Pre-Selective Amplification Primers:
  E-A (5' GAC-TGC-GTA-CCA-ATT-CA)
  M-C (5' GAT-GAG-TCC-TGA-GTA-AC)

Selective Amplification Primers:
  E-AAC-Tagged (5' /6-FAM/GAC-TGC-GTA-CCA-ATT-CAA-C)
  M-CTG (5' GAT-GAG-TCC-TGA-GTA-ACT-G)

Appendix D: Genetic Distance Results

Manchac Nei's Genetic Distance Neighbor Joining Tree
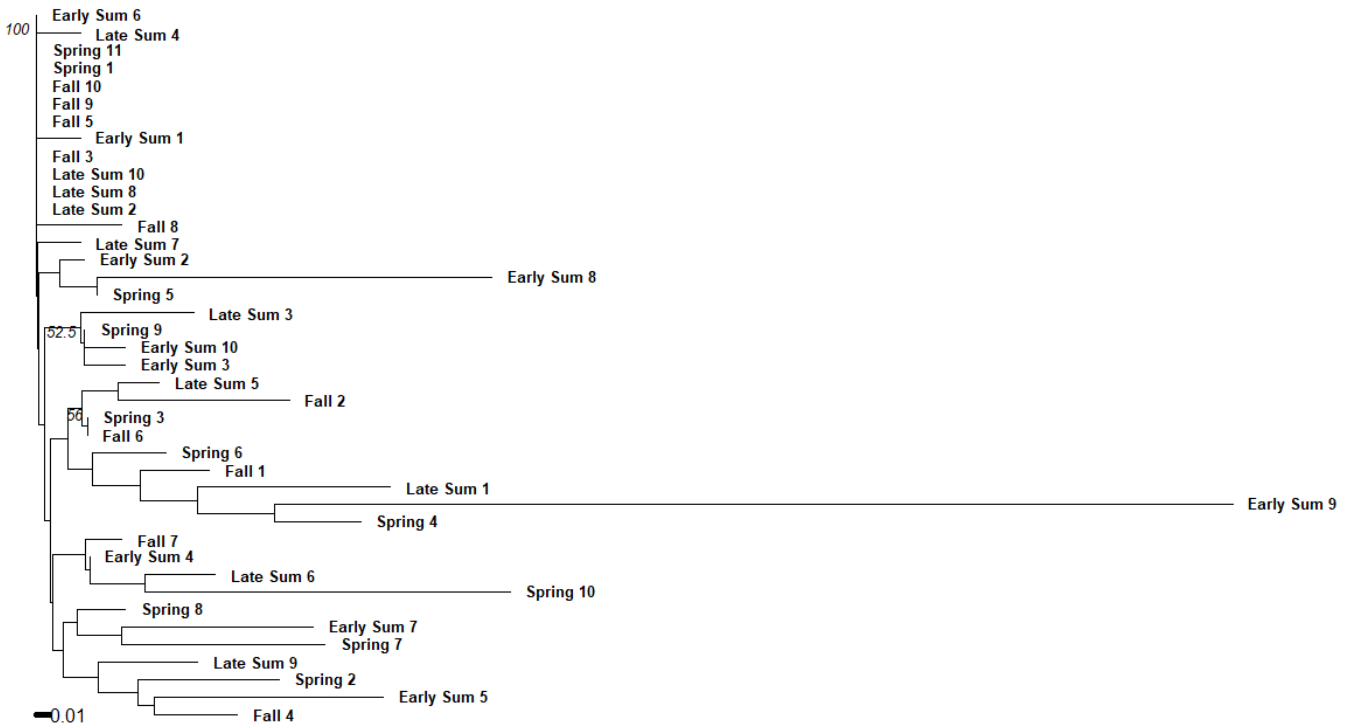Hendrick's Gst: -0.0189

(scale bar shows percent difference and bootstrap values shown)

Eastabuchie Nei's Genetic Distance Neighbor Joining Tree
Hendrick's Gst: -0.02986
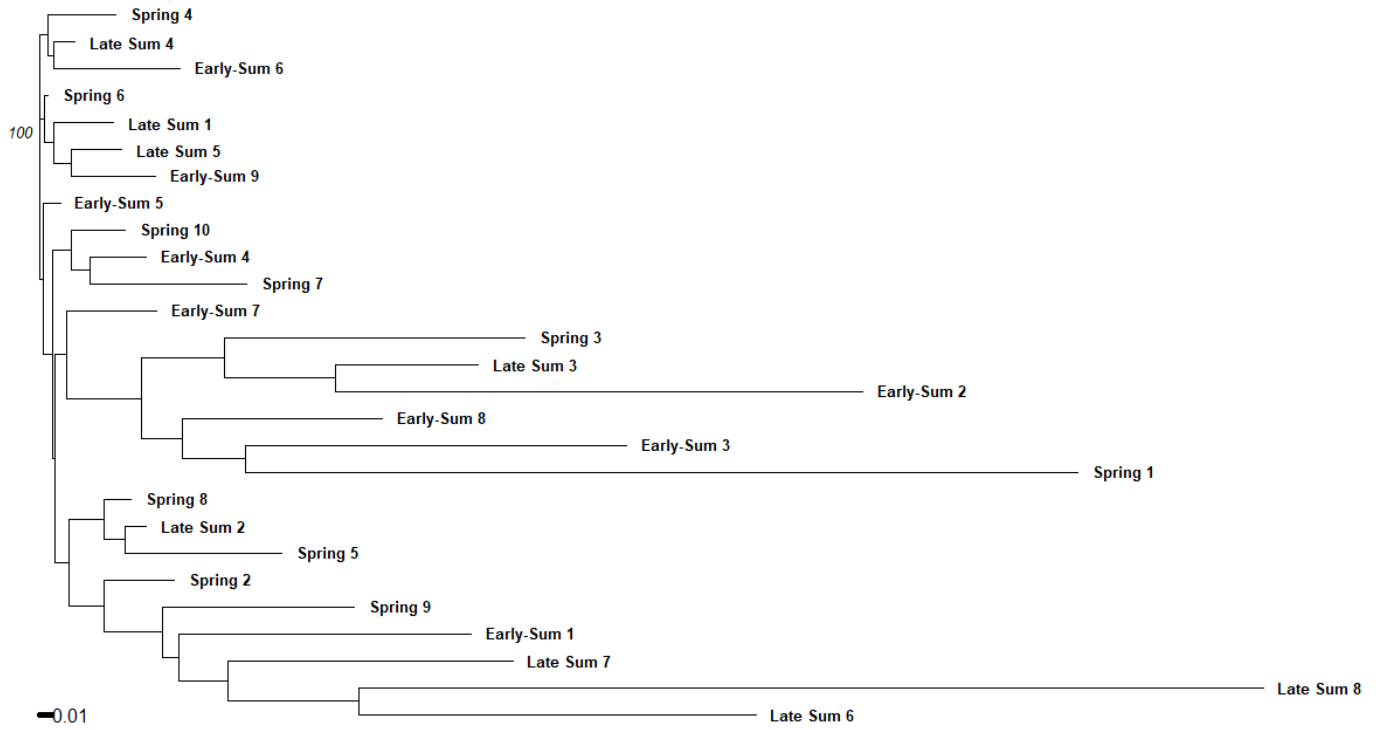
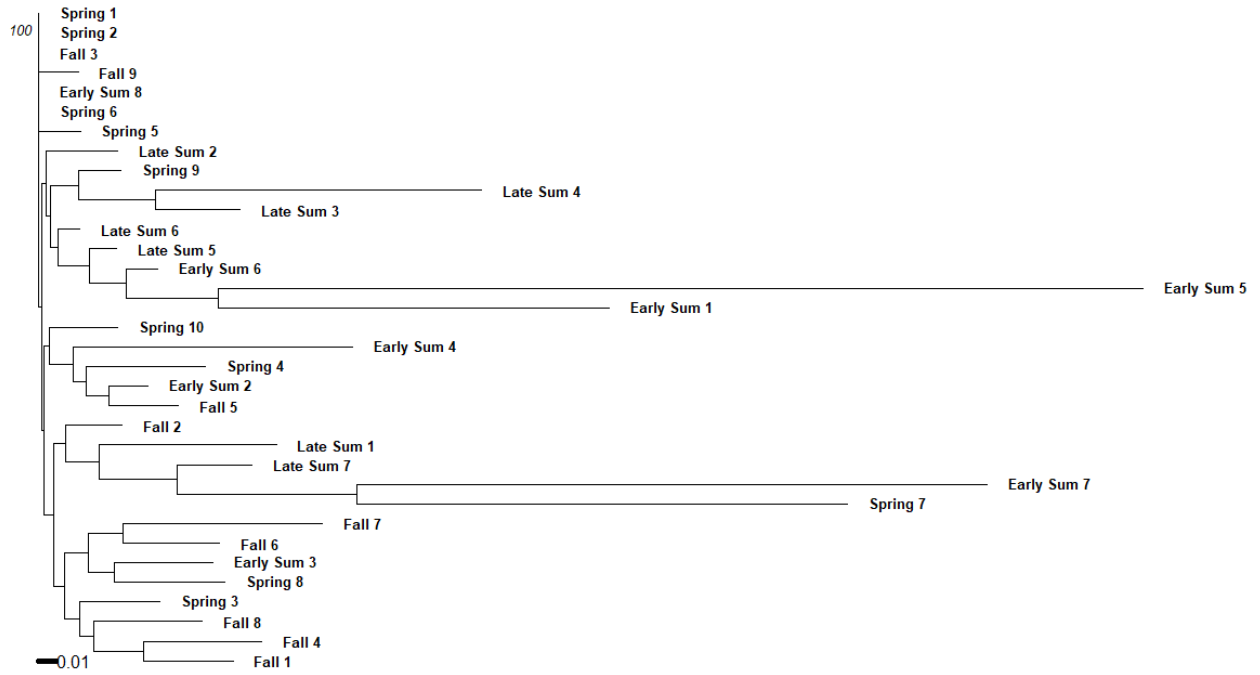(scale bar shows percent difference and bootstrap values shown)

Laccassine Nei's Genetic Distance Neighbor Joining Tree
Hendrick's Gst: -0.0235

(scale bar shows percent difference and bootstrap values shown)
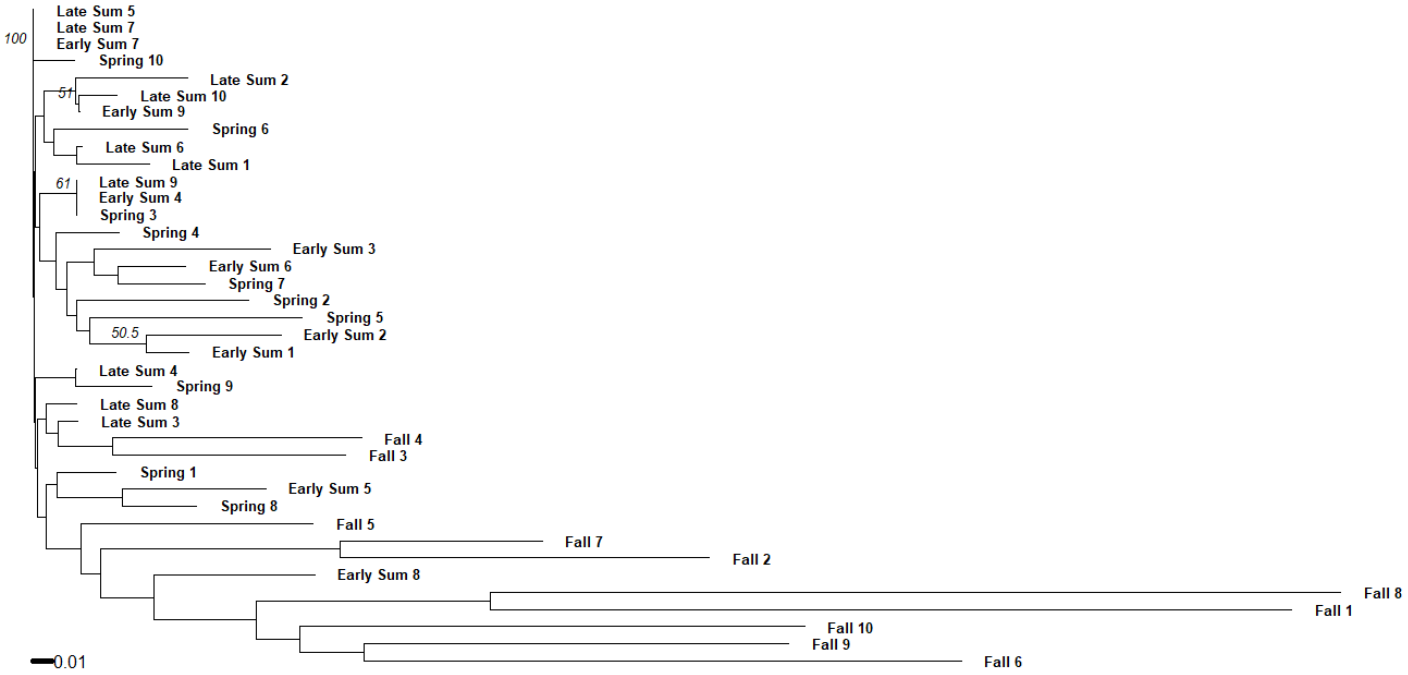
Jean Lafitte National Park

Hendrick's Gst: -0.0237

(scale bar shows percent difference and bootstrap values shown)

City Park

Hendrick's Gst: -0.0298

(scale bar shows percent difference and bootstrap values shown)

Vita

    Elliot Weidow was raised in Baltimore County, MD. He earned a Bachelor of Science degree from Frostburg State University where he also studied Philosophy. In 2016 he began his graduate studies at the University of New Orleans pursuing a M.S. in Biological Sciences in Dr. Jerome Howard's lab.