ASSESSMENT OF TEMPORAL AND GEOGRAPHIC POPULATION STRUCTURING OF *PHRAGMITES AUSTRALIS* ALONG THE HUDSON RIVER USING MICROSATELLITE DNA MARKERS

A Final Report of the Tibor T. Polgar Fellowship

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ABSTRACT

Phragmites australis is a perennial grass that has been spreading significantly throughout wetlands of the Northeastern United States within the last 100 years. For the Hudson River populations, previous studies have suggested high genetic variability within and among populations, as a reason for its dramatic spread. In this study, samples were collected from nine locations along the Hudson River and its surrounding areas. These, as well as samples collected in 2004 from the same locations, were analyzed for genetic variability using microsatellite primers for eight different loci. Results showed that populations growing in the northern regions of the Hudson River are genetically different from populations growing in the southern Hudson River regions. Comparison of 2004 and 2011 samples suggested that several populations have evolved over the last seven years. All samples were found to be of the invasive genotype suggesting that native populations are rare or not present in and around the Hudson River Valley. Based on high genetic variability, seed dispersal was proposed to be an important spread mechanism for Hudson River populations supporting previous studies.

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INTRODUCTION

Phragmites australis is a tall perennial grass of the Poacaea family and is also known as "giant reed" or "common reed." *Phragmites* is one of the most productive species of plants on earth (Hartog 1989). Today, it is distributed all over the mainland US as well as through southern Canada (Wilcox et al. 2003). In many areas, it has become the dominant plant of freshwater and brackish marshes at the exclusion of other genera such as *Typha* and *Spartina* (Stanne et al. 1996). Even though fossil records show that the plant has been in the U.S. for about 40,000 years, its abundance has increased dramatically since the early 1900's (Saltonstall 2002).

This plant has had great ecological success because it can colonize even small patches of disturbed soils very quickly (Kettenring et al. 2010). *Phragmites* alters the habitat to promote its own growth by modifying the sulfide, carbon, oxygen and moisture characteristics of soils as its populations become established (Osgood et al. 2003). Once established, *Phragmites* populations can maintain dense stands (200-300 culms/m^2) through the propagation of its rhizomes. This biomass can cause the shoots to exceed living plants resulting in a thick mat that not even new *Phragmites* can penetrate (Haslam 1972). Not only does *Phragmites* affect the type of plants that grow in its habitat, it also affects the animal life as well. A study done by Wells et al. (2008) suggests that *Phragmites* has greatly reduced the number avian species diversity in some tidal marshes along the Hudson River including Iona Island and Constitution Marshes. Some researchers agree that this plant is an invasive nuisance and reduces the biodiversity of

wetlands and thus many regions around the country have implemented programs to monitor and reduce its expansion (Marks et al. 1994; Chambers 1999; Meyerson et al. 2000). However, some scientists dispute the necessity of aggressive removal of *Phragmites*. Kiviat has illustrated that many species use *Phragmites* as a food source and habitat and that complete removal can have severe consequences to a community (Kiviat and MacDonald 2003; Kiviat 2010).

The Hudson River and the surrounding New York/New Jersey region provide a diverse array of habitats that *Phragmites* has been able to colonize. Soil habitats can range from polluted to non-polluted and salinity can range from brackish to freshwater. The distribution and abundance of *Phragmites* along the Hudson River has grown dramatically since the 1970s because it has been able to colonize a variety of habitats presented to it. It has been so successful that it is now predicted to become the dominant wetlands plant in the tidal Hudson River in the next few decades (Kiviat 2010). The mechanisms that have allowed it to spread so rapidly will not fully be understood without characterizing the genetics of its populations.

Saltonstall (2002; 2003a; 2003b) was the first researcher to genetically characterize *P. australis* using chloroplast and microsatellite DNA markers. She first characterized chloroplast DNA (cpDNA) from *Phragmites* samples collected all over the globe (Saltonstall 2002). From this study, Saltonstall characterized 27 cpDNA haplotypes, one of which, Haplotype M, was identified as the Eurasian variety that is now invasive in the Northeast United States. A Restriction Fragment Length Polymorphism (RFLP) technique was also developed to easily distinguish between native and invasive varieties (Saltonstall 2003b). This RFLP technique confirmed that the vast majority of

*Phragmite*s in the Northeast are descended from the Eurasian lineage. However, a recent study has identified native *P. australis* populations in freshwater and oligohaline marshes of Delaware and southern New Jersey (Meadows and Saltonstall 2007).

Saltonstall also developed a set of microsatellite primers to characterize the nuclear genome of *P. australis*. This method confirmed diversity among *P. australis* individuals and that native and invasive varieties did not form hybrids (Saltonstall 2003a). However, the focus of this study was to compare native, invasive and gulf populations of *Phragmites* and did not closely examine populations within a particular river system.

P. australis populations from the Rhode River, a brackish sub-estuary of the Chesapeake Bay, have been characterized using microsatellite DNA (McCormick et al. 2010). McCormick used eight primers (designed by Saltonstall 2003a) to amplify microsatellite regions in *P. australis*. Her analysis illustrated that *P. australis* populations followed an isolation-by-distance model. Populations in close proximity were more closely related than those at greater distances. The genetic analyses and mapping of *P. australis* populations supported the importance of seed dispersal rather than vegetative propagation. Patches growing in isolation were found to be genetically unique confirming the establishment by seed. Using satellite images, McCormick was able to analyze *P. australis* expansion over the last 35 years. It was found that *P. australis* populations have expanded rapidly since 1971. High genetic variation and the rapid spread of *P. australis* populations lead to the conclusion that the invasive varieties have responded to environmental changes (McCormick et al. 2010).

To date, only one study has examined the genetic structure of Hudson River *Phragmites* populations (Maltz and Stabile 2005). Three ISSR primers were used to

characterize 153 culms from a total of eight populations along the Hudson River and its surrounding areas including, Rye, NY, Berry's Creek, NJ, and Staten Island, NY. ISSR banding patterns indicated that there were high levels of genetic variation within and among populations. None of the collection sites were clonal, suggesting that seed dispersal may be a more important mechanism for dispersal than previously thought. A UPGMA phenogram also suggested that populations from brackish and saline sediments were more closely related to each other than those from freshwater environments. Other studies have illustrated ecotypic differentiation among sites based on salinity (Li et al. 2009; Gong et al. 2003; Takahashi et al. 2007). Unfortunately, the ISSR based results could not be used to quantify variation within and among populations using established population genetic calculations such as Hardy-Weinberg equilibrium and F-statistics (fixation indices). This study proposes to compare microsatellite regions so that the within and among population variance among Hudson River populations can be determined and better correlated with habitat type. Recent studies have illustrated that the Hudson River samples from 2004 have some chloroplast DNA sequence variation in the intergenic spacer region between the *trn K* and *Mat K* genes (Lipus and Stabile unpublished). However, the sequence variation did not lend itself to an RFLP analysis that could be used to easily characterize the populations. Microsatellites are currently the best option for analyzing the population genetics of Hudson River *Phragmites* populations.

It was hypothesized that high levels of genetic variability in *Phragmites* populations allow them to adapt to a variety of local conditions. Population genetic theory predicts that species that have large population sizes and reproduce rapidly should have high

levels of genetic variation. Genetic variation should allow *Phragmites* populations to adapt to the variety of environmental conditions found along the Hudson River. This hypothesis was tested using the microsatellite primer developed by Saltonstall (2003a) and recently used by McCormick et al. (2010) to characterize the 2004 sample set and compared it to new samples taken from the same Hudson River sites in 2011.

METHODS

Sample Collection

A minimum of 40 culms were collected at each site following transects through the center of the population and along the perimeter. Approximately 20 culms were amplified from each site and this appeared to be adequate to identify the different microsatellite alleles. The area occupied by each population was demarcated using a GPS for future reference.

Table 1. Sampling sites from 2004 and 2011 Collections

Sites	Habitat
Tivoli Bays	Fresh Water
Constitution Island/Cold Spring Marsh	Fresh Water
Iona Island Marsh	Fresh Water
Piermont Marsh	Fresh Water
Berry's Creek	Brackish
Passaic River	Brackish
Rye Marsh	Salt Water
Richmond	Salt Water
Albany	Fresh Water

Microsatellite Analysis

Table 2. Microsatellite primer sets and PCR conditions used by McCormick et al (2010) from Saltonstall. (2003a).

Primer	Annealing Temperature (^{o}C)	Alleles
PaGT4	50	5
PaGT9	50	7
PaGT12	56	5
PaGT13	50	6
PaGT14	58	5
PaGT16	56	8
PaGT21	58	11
PaGT22	50	8

For microsatellite analysis, PCR reactions were carried out with a volume of 12.5 ul, with 50 ng or less of template DNA, 1 X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) 2 mM $MgCl_2$, 0.25 mM dNTPs, 05 mM forward and reverse primers and 0.1 U *Taq* DNA polymerase. PCRs were performed as single reactions and then subsequently pooled prior for microsatellite analysis. Characterization of microsatellite genotypes was done on a Beckman Coulter CEQ^{TM}8000 capillary-based DNA sequencer. Multiplexed PCR reactions were diluted up to 1:3 with Sample Loading Solution (Beckman Coulter), 0.5 to 2 ul of diluted PCR reactions were loaded onto 96 well plates along with 0.5 ul of CEQ DNA Size Standard-400 (Beckman Coulter) and 40 ul of Sample Loading Solution (Beckman Coulter) and run with the FRAG 1 program. Microsatellite data was analyzed for population differentiation using GenePop (Raymond and Rousset 1995). An UPGMA tree was generated using Poptree (Takezaki et al. 2010).

RESULTS

About 90% of amplified samples yielded usable PCR products. The quality of PCR products was initially analyzed using agarose gel electrophoresis. Figure 1 illustrates the PCR products representative of those used in microsatellite analyses. 332 of the collected 372 culms (184 from 2004 and 188 from 2011) were analyzed. Sets of two or three loci were analyzed at the same time simultaneously in the DNA sequencer. Data output showed alleles of each locus in a different color. Each peak stood for one fragment size and one locus. Overall loci were observed to be either homozygous, heterozygous or had more than two alleles at each locus probably due to alloploidy. Figures 2a, b and c show typical DNA sequencer outputs for those three cases. Blue peaks represent size alleles for locus 12 alleles, green peaks represent the different size alleles locus 13 alleles and the black peaks represent the size for locus 22.

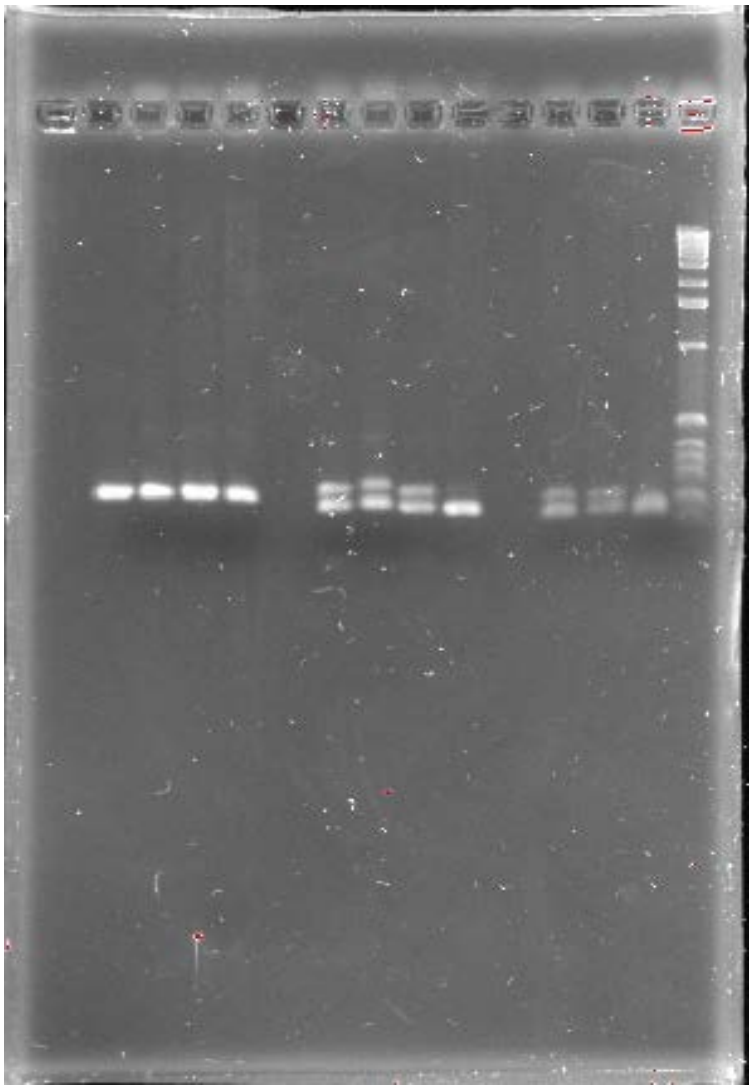

Figure 1. Ethidum bromide stained minigel of 11 PCR products using microsatellite primers PAGT-12, PAGT-13 and PAGT-22

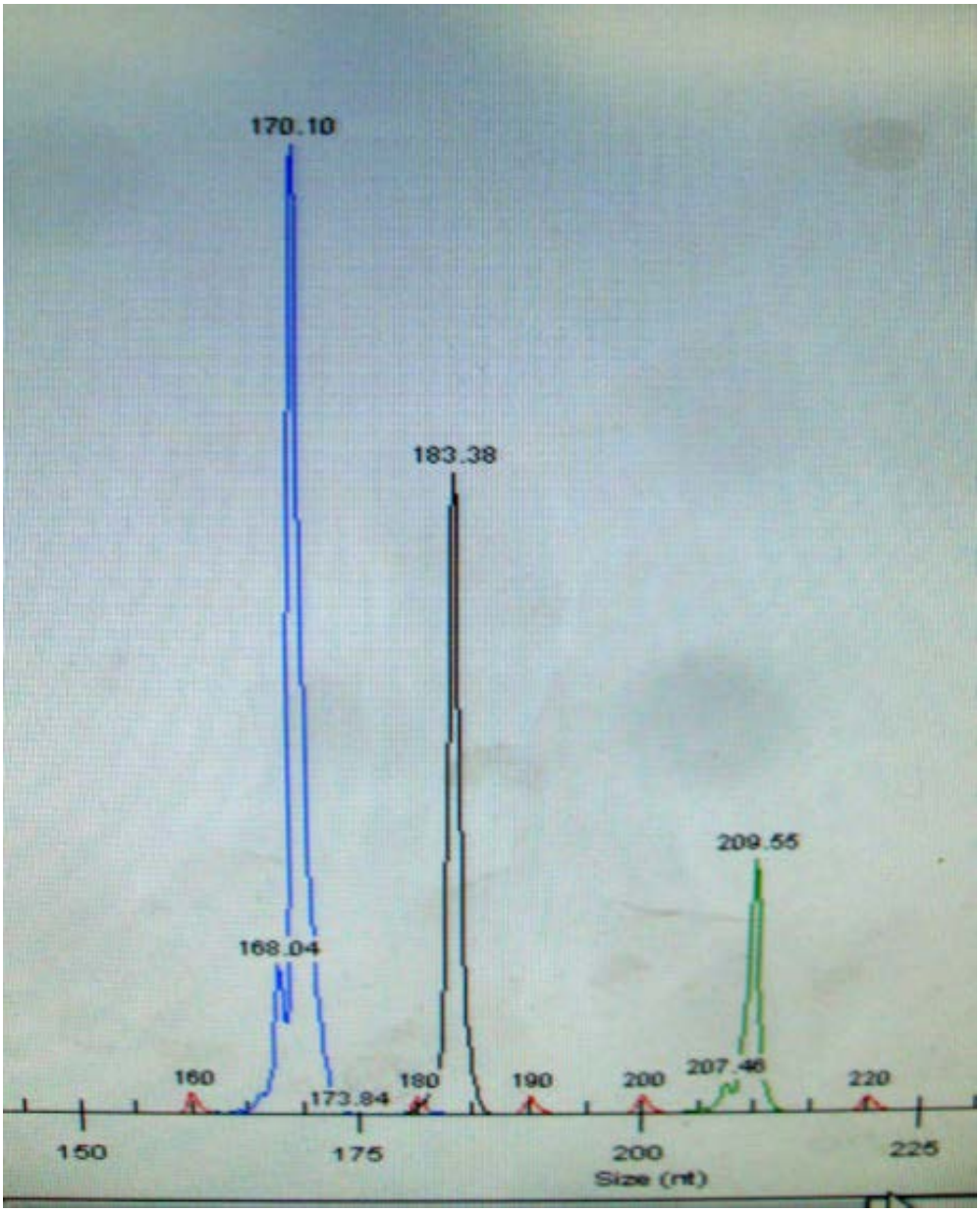

Figure 2a. DNA sequencer output showing homozygous alleles for loci 12, 13 and 22. Each colored peak represents the size of a DNA fragment for a particular locus.

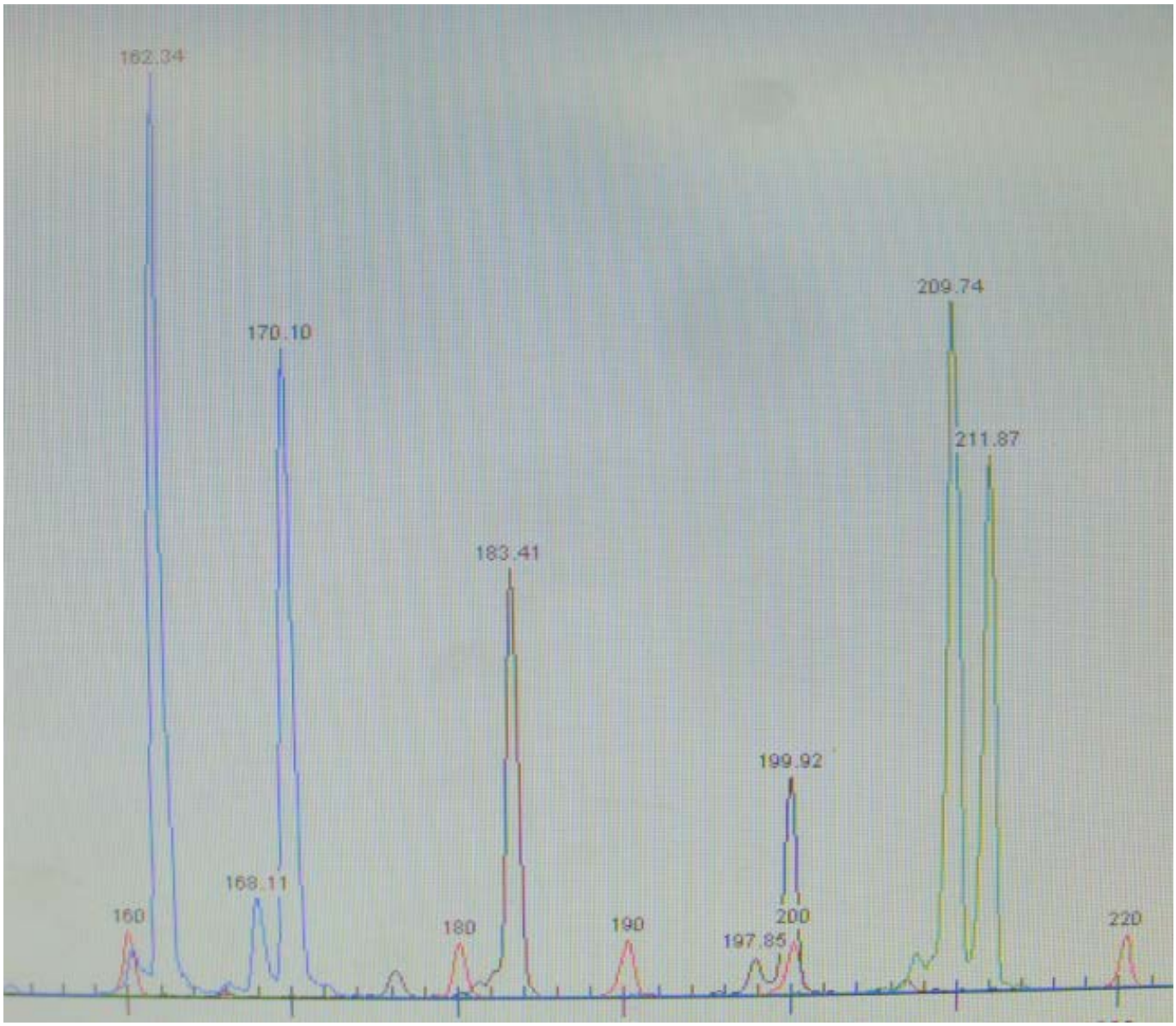

Figure 2b. DNA sequencer output showing heterozygous alleles for loci 12, 13 and 22. Each colored peak represents the size of a DNA fragment for a particular locus.

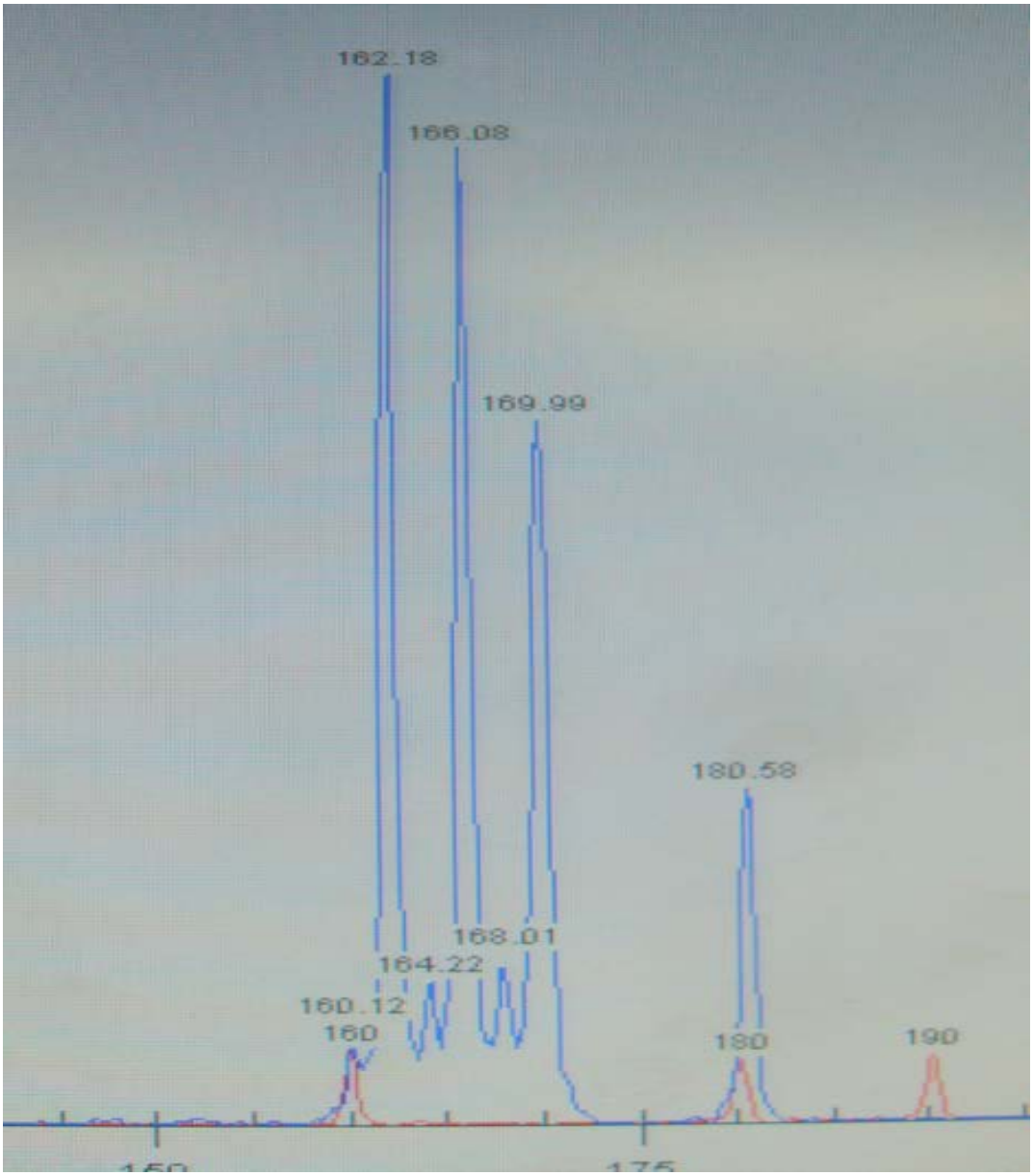

Figure 2c. DNA sequencer output showing multiple alleles for locus 12.

Raw data for each sample and locus were recorded for further data analysis. Table 3 shows the alleles for samples 471 to 490 (Berry's Creek populations) for all eight loci. The data showed that some loci had mulitple alleles (>2) and some loci had individual alleles for those samples. Microsatellite analysis of samples for loci PAGT-4 and PAGT-9 has not been concluded yet, and results for these loci were not included in

the data analysis of this paper. The number of alleles for each locus was determined and compared to the number of alleles per locus of the McCormick et al. study (2010). Table 4 shows differences marked in red.

Table 3. Raw data showing allele sizes of eight different loci for seven different samples.

	Loci (Fragment size):							
	PAGT-4	PAGT-9	PAGT-12	PAGT-13	PAGT-14	PAGT-16	PAGT-21	PAGT-22
Sample No:								
471	277	197,201	170	210	191	262,293	172,195	183
475	277	197,201	170	210	191	262,293	172,191,195	183,200
479	277,279	197,201	162,170	210,212	191	262,293	191	183,190
483	277,279	197	162,170	210	191	293	191	183
487	277,279	197	162,170	208,210	191	293	191	183
489	277,279	197	162,170	210	191	293	191	183
490	277,279	197	162,170	210	191	293	191	183

Table 4. Comparison for number of alleles determined in this study and by McCormick et al. (2010).

Locus	Number of alleles in current study	Number of alleles in McCormick study
PAGT-4	3	5
PAGT-9	3(4)	7
PAGT-12	5	5
PAGT-13	2	6
PAGT-14	5	5
PAGT-16	3	8
PAGT-21	4	11
PAGT-22	7	8

Table 5.1-5.3. Results of population differentiation analysis for 2011 sample (5.1) , 2004 samples (5.2) and combined data (5.3). Listed populations showed significant differences from each other. Populations were considered significantly different with a P value < 0.0001.

2004 Populations	VS	2004 Populations
Berry's Creek		Richmond
Passaic River		Cold Spring
Berry's Creek		Cold Spring
Tivoli Bays		Cold Spring
Richmond		Cold Spring
Rye Marshlands		Albany
Passaic River		Albany
Berry's Creek		Albany
Tivoli Bays		Albany
Richmond		Albany
Piermont		Albany

2011 Populations	VS	2011 Populations
Rye Marshlands		Rye Marshlands
Piermont		Iona Island
Albany		Iona Island
Iona Island		Piermont
Cold Spring		Piermont
Albany		Albany
Tivoli Bays		Albany
Albany		Albany
Rye Marshlands		Tivoli Bays
Passaic River		Tivoli Bays
Berry's Creek		Tivoli Bays
Tivoli Bays		Tivoli Bays
Piermont		Tivoli Bays
Cold Spring		Tivoli Bays
Albany		Tivoli Bays
Tivoli Bays		Cold Spring
Iona Island		Cold Spring
Piermont		Cold Spring
Tivoli Bays		Cold Spring

2004 Populations	VS	2011 Populations
Rye Marshlands		Berry's Creek
Piermont		Berry's Creek
Iona Island		Berry's Creek
Albany		Berry's Creek
Cold Spring		Passaic River
Albany		Passaic River
Tivoli Bays		Rye
Albany		Rye
Rye Marshlands		Iona Island
Passaic River		Iona Island
Berry's Creek		Iona Island
Tivoli Bays		Iona Island
Richmond		Iona Island
Piermont		Iona Island
Cold Spring		Piermont
Albany		Piermont
Tivoli bays		Richmond
Berry's Creek		Albany
Piermont		Albany
Cold Spring		Albany
Albany		Albany
Rye Marshlands		Tivoli Bays
Passaic River		Tivoli Bays
Berry's Creek		Tivoli Bays
Tivoli Bays		Tivoli Bays
Richmond		Tivoli Bays
Iona Island		Tivoli Bays
Cold Spring		Tivoli Bays
Albany		Tivoli Bays
Rye Marshlands		Cold Spring
Berry's Creek		Cold Spring
Tivoli Bays		Cold Spring
Cold Spring		Cold Spring
Albany		Cold Spring

Allele data for six loci (12, 13, 14, 16, 21 and 22) were analyzed using Genepop for population differentiation. Results showed highly significant differences in the frequencies of alleles among multiple pair-wise populations' comparisons. Comparison of 2004 samples indicated that the Albany and Cold Spring populations were most different from all the other populations. Highly significant temporal differences between populations were also observed in comparisons between populations from the 2004 and 2011 samples. Specifically, the allele frequencies of the Albany, Cold Spring and Tivoli Bays populations changed over the last seven years.

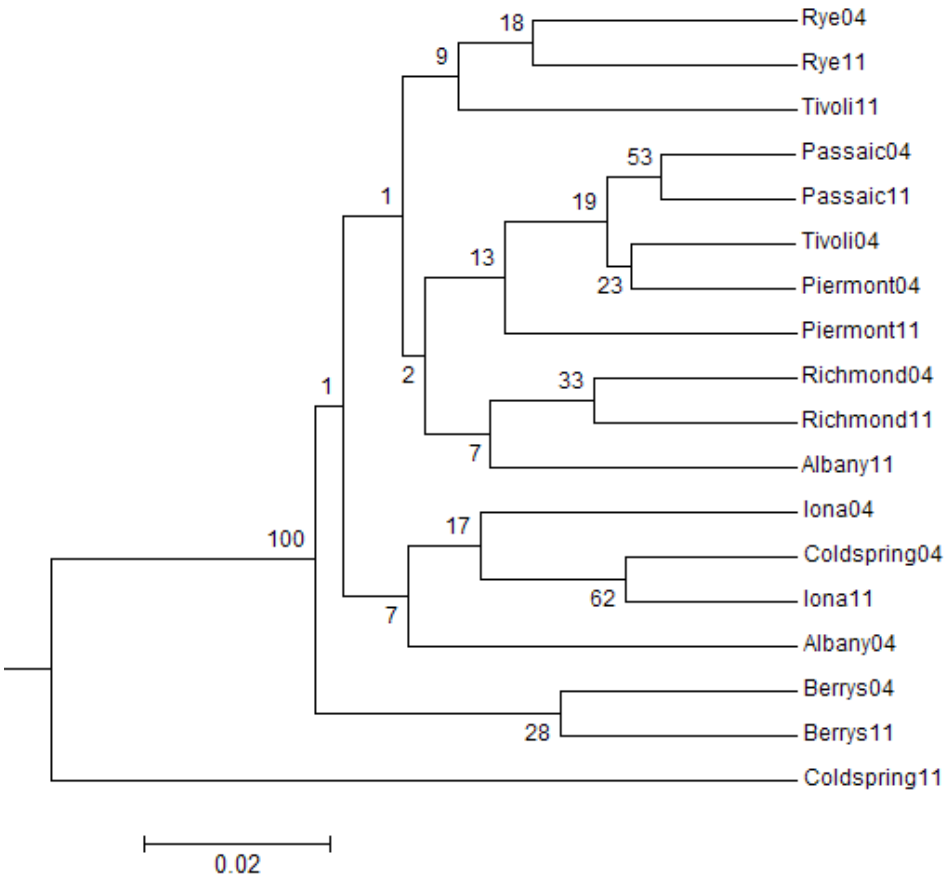

Figure 5. UPGMA tree for the analyzed Hudson River Populations. Bootstrapping values of 50 or greater are shown at the nodes.

Analysis of the 2011 samples suggested that Albany, Cold Spring and Tivoli Bays were most different from the remaining populations. Figure 3 lists the populations' comparisons showing highly significant differences (P-value < 0.05).

An UPGMA tree was constructed based on the population differentiation test results. Results showed that every patch, regardless of size, had multiple phenotypes. 2004 and 2011 *P. australis* populations from Rye, Passaic River, Piermont, Richmond and Berry's Creek were found to be genetically similar (Figure 5). 2011 Cold Spring, Albany and Tivoli bays populations were found to be genetically different from the 2004 populations based on the UPGAM analysis. Cold Spring was determined to be genetically isolated from the other populations.

Neither native microsatellite allele phenotypes nor endemic genotypes were present in the analyzed sample. All samples were shown to be of the invasive genotype.

DISCUSSION

Analysis of six microsatellite loci for samples from nine different locations collected at two different times supports the hypothesis that *P. australis* populations have high levels of genetic variability. Due to the fact that the analyzed populations grow in different habitats this project supports the theory that genetic variability allows populations to adapt and spread along a variety of local conditions along the Hudson River. Analysis of allele differences suggested that there is significant diversity within sites and patches. Variability among patches was determined to be greater than within patches.

High numbers of alleles in both homozygous and heterozygous conditions were also

observed. The number of different alleles determined in this study was smaller than those observed by McCormick et al. (2010). It is difficult to determine the reason that fewer genotypes were observed in the Hudson River populations as compared to the Rhode River. Some possible factors could be differences in habitat and climate between the two river systems. Additionally, Hudson River populations may have lost some genotypes due to stochastic mechanisms such genetic drift or founder effects. Allele sizes for most loci were found to be in the vicinity of the data reported by Saltonstall (2002). No samples were found to exhibit allele sizes common to native genotypes confirming that no or very few native *P. australis* populations exist in the Northeast (Saltonstall 2002) or along the Hudson River (Kiviat 2010).

Population differentiation analysis suggested a geographic pattern for population structuring. Microsatellite analysis of the 2004 sample set indicated significant differences between the populations located along the northern Hudson River (Albany, Cold Spring) and the remaining populations located closer towards New York City. Also, Albany and Cold Spring populations did not exhibit any significant differences to each other. Interestingly, Tivoli Bays populations, which are geographically located between the Albany and Cold Springs populations, were found to be significantly different from either but did not show any significant differences to the other analyzed populations. Richmond populations were found to be significantly different from Albany, Cold Spring and Berry's Creek populations. Based on these data, it was suggested that populations from fresh water regions (northern Hudson River Estuary) are genetically different from populations growing in brackish water regions. Richmond populations are exposed to the highest salinity levels due to their location near the New York Bight. Furthermore, Richmond populations are exposed to different and higher levels of pollutants than most of the other populations, which could be one of the reasons why they were found to be

genetically different. The presented data suggests a connection between the diverse environments and the genetic variability. Results regarding population clustering confirm the data reported by Maltz and Stabile (2005), yet the Cold Spring populations were shown to be significantly different from Iona Island and Tivoli Bays population, which was not reported in the 2005 study.

For the 2011 samples, a similar geographic pattern was observed. Freshwater populations located further north in the Hudson River Estuary(Tivoli Bays, Cold Spring and Albany) were significantly different from the other populations located closer to New York City. In addition, the Iona Island populations were significantly different both from populations located further north (Albany, Tivoli Bays and Cold Spring) and from populations located along the southern Hudson River Estuary (Piermont). Among the 2004 samples, Cold Spring and Albany populations were not significantly different from each other but were found to be significantly different from the Tivoli Bays populations, suggesting they must be genetically isolated. The general pattern of northern freshwater populations showing genetic differences in comparison to southern brackish water populations was confirmed; however, several populations seem to have evolved and show significant differences to the majority of the other populations contradicting the clustering proposed by Maltz and Stabile (2005).

The Iona Island, Piermont and Tivoli Bays populations seem to be evolving. They did not show any significant differences to their surrounding populations in 2004 but were found to be different in 2011. The 2011 samples also suggested significant differences between the Rye Marsh populations and most other analyzed populations, which is not surprising due to the population's location along the Long Island Sound and its geographical isolation from the Hudson River.

UPGMA tree analysis suggests that 2011 Cold Spring populations are genetically isolated from the other populations. This result is surprising because the population is not geographically isolated. It is located between Tivoli Bays and Albany. Reasons for the genetic differences could be the history of the Constitution marsh including its contamination in the 50's and 60's and the resulting remediation projects.

Direct allele comparison of the 2004 and 2011 samples showed many differences between populations. Most importantly it was determined that 2011 Albany, Tivoli Bay and Cold Spring populations were significantly different from 2004 populations from the same locations. UPGMA tree analysis supported this hypothesis. 2004 and 2011 populations from Albany, Tivoli Bays and Cold Spring were found to be genetically different. 2004 and 2011 from Rye, Passaic River, Staten Island and Berry's Creek Populations were clustered together in the tree and therefore considered genetically similar. This suggests that the northern Hudson River Estuary populations have changed within the last seven years. Reasons for the rapid change could be environmental or external factors such as pollution and habitat restoration. Genetic drift also has to be considered as one of the reasons for increased differences between populations. The remaining populations did not show any change within the last seven years suggesting that these populations have reached genetic equilibrium.

Previous studies have suggested that seed dispersal has to be considered an important mechanism by which *Phragmites* can spread. Researchers came to this conclusion due to high genetic variability within stands of *P. australis* and among populations (Maltz and Stabile 2005; McCormick et al. 2010). No single stand was observed to be monoclonal and individuals with different genotypes were collected in very close proximities within stands. Results of this study support this hypothesis based on the fact that *P. australis* populations growing along the Hudson

River were found to exhibit considerable genetic variability. Studies monitoring *P. australis* populations along the Rhode River reported that establishment by seed dispersal has been more common in populations influenced by human activities (McCormick et al. 2010). Furthermore, it was determined that seed viability was greater among *Phragmites* populations with greater genetic variation. This study did not examine the correlation between genetic variation and seed viability. Populations growing along the upper Hudson River Estuary showed more differences to each other than populations growing along the lower Hudson River Estuary, suggesting that seed dispersal could be the primary mechanism of spread within this area. To obtain more exact data regarding those parameters a more detailed analysis is necessary.

The project showed that *P. australis* populations along the Hudson River are diverse and have the ability to evolve. A general pattern shows population clustering with the populations growing along the upper Hudson River Estuary (Albany, Cold Spring, Tivoli Bays) being significantly different to populations growing along the lower Hudson River Estuary. Analysis of the 2011 samples showed that more populations started to evolve independently within the last seven years and show fewer similarities to populations in close proximity. No native phenotypes or genotypes were discovered supporting studies by Saltonstall (2002) and Kiviat (2010) which state that the invasive haplotype M is the most common *P. australis* genotype in the Northeast.

The data also suggest that seed dispersal may be an important colonization mechanism for *P. australis* and thus confirmed data of previous studies. These results suggest that these populations should be monitored continuously. It will be also necessary to look at seed viability. Monitoring program should include genetic analysis to be able to recognize aggressive phenotypes in an early stage. As stated previously, programs should also consider seed dispersal as an important mechanism for colonization by *P. australis*.

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