Distribution, rate of expansion and subspecific make-up of *Phragmites australis* at Pierce Cedar Creek Institute.

Cameron Poyner, Dylan Sterling, and Bradley J Swanson

Department of Biology, Central Michigan University, Mt. Pleasant, MI 48859

Abstract

Phragmites australis australis is reducing the ecological effectiveness of wetlands, reducing species diversity where it is found, and hybridizing with the native *Phragmites australis americanus*. If the spread of *P. a. australis* continues, the possibility of extinction of *P. a. americanus* is high. Our study investigated the stands of *P. australis* at Pierce Cedar Creek Institute (PCCI) to facilitate an effective restoration effort on the Pierce Cedar Creek Institute property. We found the number of stands of *P. australis* increased from 7 stands in 2007 to 23 in 2014. Additionally, the area covered by *P. australis* increased by 147% during this period. Amplified Fragment Length Polymorphism (AFLP) analysis suggests that all individuals at PCCI comprise a single genetic population. Given that two of the sample locations are the invasive subspecies *P. a. australis*, our results suggest that all of the *P. australis* stands at PCCI either contain invasive plants, or hybrids between the native *P. a. americanus* and *P. a. australis.* Given the rapid increase in area covered, and the apparent genetic make-up, we suggest strong control mechanisms be implemented on all *P. australis* sites.

Introduction

P. australis is the only species of *Phragmites* in North America. *P. australis* typically can be found in wetlands, ponds, and swamps that have been disturbed by anthropogenic constructions and is considered an indicator of tidal wetland disturbance (Chambers et al., 1999; Kiviat and Hamilton, 2001). Additionally, *P. australis* was found to grow abundantly in places with significant agricultural activity (Trebitz and Taylor, 2007). One of the difficulties associated with *P. australis* is the presence of a native and invasive subspecies. *P. a. americanus* is native to North America, including the Midwest (Lynch and Saltonstall, 2002) and has been

present since the Cretaceous (Chambers et al., 1999). The invasive subspecies *P. a. australis*, is suggested to have come from Europe as trade increased between the United States and Europe over the past few centuries (Meyerson and Cronin, 2013). *P. a. australis* expanded aggressively from the Atlantic coast throughout the 1900's and still continues to do so today; it spreads quickly by having seeds carried by wind and birds (Ailstock et al., 2001; Meyerson and Cronin, 2013). Once established, *P. australis* grows vigorously by making a complex system of rhizomes and stolons. The culms that grow out of the rhizomes are tough and resistant to decay (Hamilton and Kiviat, 2001). The large size, high reproductive potential, and high growth rate of *P. australis* can overwhelm other wetland plants producing large monoculture stands (Ailstock et al., 2001).

More recently, both the native and invasive subspecies of *P. australis* have started to grow out of control in many regions of the United States, the spread of *P. a. australis* being more rapid as it is more aggressive than the native genotype (Kiviat and Hamilton, 2001). The invasive subspecies, *P. a. australis*, has spread extensively, and become problematic, throughout the Gulf coast, East coast, and Great Lakes regions of the U.S. Lacking the salinity of the ocean that limits the expansion of *P. a. australis* along the Atlantic coast, the wetlands of the Great Lakes are highly susceptible to invasion (Tulbure and Johnston, 2010). The aggressive expansion of *P. a. australis* is leading to many adverse effects on North American wetlands, such as loss of plant and animal diversity (Taylor and Trebitz 2007; Meyerson and Cronin 2013).

P. a. australis invasion is a concern to wetland managers due to its rapid growth and ability to exclude other plant species while providing little usable wildlife habitat when present as dense stands (Ludwig et al., 2003) resulting in an overall decrease in functionality of the wetland (Silliman and Bertness, 2004; Tulbure and Johnston, 2010; Kirk et al., 2011). Given the

negative effects, and rapid spread of *P. a. australis*, many methods were developed to facilitate control, including burning, flooding, herbicides, and the introduction of herbivorous insects (Ailstock et al., 2001).

An additional ecological and evolutionary pressure of *P. a. australis* is its ability to hybridize with *P. a. americanus* (Meyerson et al., 2010). Only crosses with *P. a. australis* pollen donors and *P. a. americanus* produce a viable seed, which indicates that gene flow is unidirectional (Meyerson et al., 2010). Intraspecific hybridization can serve as a stimulus for evolution, increasing the invasive ability of the plant (Ellstrand and Schierenbeck, 2000). Intraspecific hybridization is an invasion mechanism that creates novel genetic combinations that increase the speed of the invasion and reduces the fitness of natives, causing a decline in the native populations (Meyerson et al., 2010). Hybridization and introgression among the different *P. australis* subspecies increases the genetic diversity of the hybrids (Lambertini et al., 2012; Paul et al., 2010) and produces hybrids, which are more aggressive in spreading then their parental lineages (Meyerson et al., 2010). Intraspecific hybridization can also result in altered epistatic interactions, increased genetic variance, and masking of the introgression of deleterious alleles (Culley and Hardiman, 2009). Although hybridization often results in hybrid vigor, it may also lead to outbreeding depression through the break-up of co-adapted gene complexes (Paul et al., 2010), reducing fitness and possibly leading to the extirpation of the outbred population (Meyerson et al., 2010; Meyerson et al., 2012).

Along with anthropogenic disturbance, competition with *P. a. australis* has caused a significant reduction in the number and size of *P. a. americanus* populations in the Great Lakes region (Meyerson et al., 2010). *P. a. australis* invasion can occur very rapidly (Tulbure and Johnston, 2010), suggesting that a major effort is needed to achieve effective restoration and

control of ecosystems affected by invasions (LeMaitre et al., 2011). Restoration of *P. a. americanus* can be achieved through treatment of *P. a. australis.* Ailstock et al. (2001) showed that after treatment of *P. a. australis,* there was an increased number of plant species and abundance at the test field, with the formerly *P. a. australis* dominated wetlands eventually returning to pre-invasion conditions.

While control of *P. a. australis* is necessary, it can be difficult to distinguish between the two subspecies morphologically; chloroplast DNA can be used to readily differentiate the two. The native subspecies P. *a. americanus* has 11 distinct haplotypes, while only two haplotypes (L1 and M) of *P. a. australis* have been found in North America. Of these two haplotypes, only one (haplotype M) appears to be found outside of a restricted area in Quebec (Meyerson and Cronin, 2013).

Our study investigated the rate of spread of the *Phragmites* species at PCCI as well as the composition of the stands (i.e., native, invasive, or hybrid individuals). The first hypothesis we tested is that there has been no expansion of *Phragmites* since 2007. Additionally, we tested the hypothesis that the number of subspecies of *P. australis* present on PCCI's property is one. Lastly, we tested the hypothesis that hybridization is not occurring between the two subspecies. Knowing the status of each stand will allow PCCI to make informed decisions regarding the management of each stand and facilitate the removal of an invasive species within the PCCI property.

Methods

We searched the entirety of the PCCI property to find all stands of *P. australis*. We outlined each stand of *Phragmites* by walking around the stand with MobileMapper^{TM} GPS

(Thales Navigation; San Dimas, CA, USA) set to continuously record waypoints. Once located and recorded, ArcMap10.2 (ESRI; Redlands, CA, USA) was used to upload the information, creating an updated map of individual stand size and total area occupied by *Phragmites* on PCCI's property.

Genetic samples were collected from all the *Phragmites* locations (Figure 1). We took tissue samples from the apical portion of individual blades. We sampled 16 individuals from each of the five large populations on the property; A, B, E, K and P (Figure 1), and 6 individuals from the remaining smaller populations. In an attempt to ensure that all of our samples are from different genets, all tissue samples were taken from individuals separated by > 3 m. Within 24 hours of collection we ground approximately 100 mg of leaf tissue in lysis buffer using a Tissue-Ruptor (Qiagen Inc., Valencia, California, USA) and extracted the DNA using a Nucleo-Spin Plant II kit (Macherey-Nagel Inc., Bethlehem, Pennsylvania, USA) according to the published protocol.

We sequenced two non-coding chloroplast gene regions using the *trnT– trnL* and rbc-L psaI primers (Saltonstall 2002; Meryerson and Cronin, 2013) to distinguish between *P. a. americanus* and *P. a. australis* (Saltonstall, 2003). All samples were run in a 10µl PCR reaction containing 1x PCR buffer, 2.5 mMol $MgCl_2$, 0.3 mMol dNTP, 0.4 µMol of each primer, 1 U of *Taq* polymerase, and 75 ng of template DNA. PCR conditions for the sequencing reactions were identical to those of Meyerson and Cronin (2013). Post-PCR cleaning was done with a Life Sciences PCR Cleaning kit (Life Technologies, Carlsbad, California, USA) following their protocols, followed by sequencing at MSU's Genome Facility. Sequence data was submitted to GenBank for comparison, through BLAST (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi), to determine which haplotype was present.

Time and monetary constraints limited our analysis to sites C, D, E, and K. We used AFLP analysis to compare the nuclear genetic make-up of individuals following the AFLP Plant Mapping Protocol (Life Technologies, Corp., Carlsbad, CA); we increased the number of cycles to 30 for the selective amplification polymerase chain reaction (PCR) to increase the number of amplicons. We used one primer-pair combination, E-TAG with M-CGC (Eurofins MWG Operon, Huntsville, AL) with the selective EcoR1 primer tagged with a fluorescent dye (5~HEX), to amplify DNA for PCR since AFLP profiles tend to display a large number of bands. The products were analyzed with an automated DNA sequencer (3130 Genetic Analyzer, Life Technologies, Inc., Foster City, CA), and the bands were scored using GeneMapper 4.0 software (Life Technologies, Inc., Foster City, CA). We only accepted peaks that were between 50-400 base-pairs in size and had a height that was above 70 fluorescent units. For each individual, the band presence was scored as either present (1) or absent (0). The Bayesian analysis program STRUCTURE (Pritchard et al., 2000) was used to group individuals together based on genotype similarities and to also construct genetic clusters based on the genotype similarities. STRUCTURE (Pritchard et al., 2000) settings included admixture and correlated allele frequencies with a burn-in period of 50,000 iterations followed by 100,000 iterations of data collection. Evanno's K (ΔK; Evanno et al. 2005), as implemented by STRUCTURE HARVESTER (Earl and vonHoldt, 2012), was used to estimate the most likely number of genetic clusters (K). The STRUCTURE Q-plot was used to display the proportion of an individual's genetic make-up deriving from each genetic cluster identified by STRUCTURE. Individuals are considered to be assigned to a cluster if >80% of their genetic make-up belongs to a single cluster. The two unique subspecies should exhibit different genetic make-up, which would be able to be separated out using genetic clustering programs based on nuclear markers.

In contrast, hybrid individuals should not be assigned to either the native or invasive genetic cluster, but should rather appear as a mixture of the two.

We performed an Analysis of Molecular Variance (AMOVA), as implemented in GenAlEx (Peakall and Smouse, 2012) to determine how genetic variation was portioned within and among the putative subspecies with 999 permutations to determine significance. We also estimated Nei's genetic distance (Nei and Roychoudhury, 1974) using GenAlEx (Peakall and Smouse, 2012).

Results

Once we had an updated 2014 map from ArcMAP, the areas of *P. australis* were compared to data from 2007 and 2012 (Figure 1; Table 1). In 2007 the total area occupied by *P. australis* on PCCI's property was 10,181m^2, whereas in 2012 the total area occupied was 13,717m^2 (Table 1). This represents a 31% increase. From 2012 to 2014 the area covered by *P. australis* increased by 87% to 25,126m^2. In total from 2007 to 2014, the total percent change of *Phragmites* on the property increased by 147%. Additionally, we found several new *P. australis* sites in the forests (sites Q – X).

We obtained 125 samples with at least 300 base-pairs of sequence from 220 individuals submitted for sequencing of the chloroplast DNA; an average of 87% of the sequence exceeded the Q20 quality standard. However, none of the sequences found a significant match in GenBank.

STRUCTURE suggested that two genetic clusters exist in our sample of *P. australis*. It is from this assumption that we chose to use AFLPs. Examination of the Q-plot for K = 2 (Figure 2) shows that four and five of the individuals sampled from sites C and D (Figure 1)

come from each of the two genetic clusters. One individual (10%) was unassignable to either genetic cluster as the percentage of their genetic make-up did not exceed 80% for either genetic cluster. Similarly, the distribution of the 22 individuals sampled from populations E and K (Figure 1) showed seven individuals assigning to one genetic cluster, 13 individuals assigning to the other genetic cluster and three individuals (9.4%) being unassignable.

The Analysis of Molecular Variance (AMOVA) found that 97% of the AFLP genetic variation was contained within the samples, while only 3% of the genetic variation was found amongst sample sites. Nei's genetic distance between the two genetic clusters was low at 0.05.

Discussion

The number and size of *P. australis* stands has increased dramatically over a seven-year period. One of the most worrying aspects of the expansion is that the rate of spread is increasing, going from an average increase of 6% per year from 2007 to 2012, to an average increase of 43% per year from 2012 to 2014. Additionally, it was not expected for us to locate so many new stands of *Phragmites* on PCCI's property, including an expansion in to the forest (sites Q – X, Figure 1). Those sites were very small pockets of *Phragmites* in the cedar swamp on the south western section of the property. Based on our observations, since *Phragmites* has made such significant expansions on PCCI's property, we believe some measure of management should be put in place regardless of it being *P. a. australis, P. a. americanus,* or hybrid.

Although we were not able to determine any haplotypes from the DNA sequencing, we continued to analyze a subset of our samples with the AFLP markers to try and determine if more than one genetic cluster occurred. Based on morphology, sites C and D were suggested to be pure stands of *P. a. australis*, while sites E and K appeared to be *P. a. americanus* (J. Howell pers. comm). If individuals at sites C and D were the invasive subspecies, and individuals at

sites E and K are the native subspecies, we should have seen two genetic clusters, representing the putative invasive and native subspecies with the genetic clusters separating along the sample locations. While we did find that K=2 was the most likely number of genetic populations, the individuals in the two genetic clusters were spread out across all four sample sites. Assuming that the two genetic clusters represent a genetic signal of the native *P. a. americanus* and the invasive *P. a. australis*, this suggests that all the stands are mixture of both native and invasive individuals. In addition, four (9.5%) of our samples could not be assigned to either genetic cluster, suggesting that they are hybrids of the two genetic clusters.

The mixture of the two genetic clusters across the four sites suggests that extensive gene flow is occurring throughout the system. Further, about 10% of our individuals proved to be unassignable to either genetic cluster, suggesting that hybridization is occurring between the subspecies. These results are further supported by the results of the AMOVA, concluding that little genetic differentiation exists amongst the sites. The majority of the genetic variation is contained within the populations, with only 3% unique genetic variation existing between the two sites. The low Nei's genetic distance found between the two groups further supports the conclusion that there is no significant genetic differentiation between the sites C and D and E and K.

The absence of any genetic structuring in the system suggests that gene flow is great enough among the sites such that all locations likely comprise a mixture of native, invasive, and hybrid individuals. Given the rate of expansion of the *Phragmites* at PCCI, and that all the stands appear to be mixtures, the *Phragmites* population needs to be managed before it becomes an ecological problem. Some possible ways PCCI can manage *Phragmites* includes an initial

herbicide treatment, monitored by follow up treatments as necessary such as prescribed fires, mechanical removal, or water level management.

References

Ailstock, M. S., C. M. Norman, and P. J. Bushmann. 2001. Common Reed *Phragmites australis*: Control and Effects Upon Biodiversity in Freshwater Nontidal Wetlands. Restoration Ecology 9:49-55.

Chambers, R. M., L. A. Meyerson, and K. Saltonstall. 1999. Expansion of *Phragmites australis* into tidal wetlands of North America. Aquatic Ecology 64:261-273.

Cronin, J. T. and L. A. Meyerson. 2013. Evidence for multiple introductions of *Phragmites australis* to North America: detection of a new non-native haplotype. Biological Invasions 15:2605-2608.

Culley, T.M., and N.A. Hardiman. 2009. The role of intraspecific hybridization in the evolution of invasiveness: a case study of the ornamental pear tree *Pyrus calleryana*. Biological Invasions 11:1107-1119.

Earl, Dent A. and vonHoldt, Bridgett M. (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4:359-361

Ellstrand, N.C., and K.A. Schierenbeck. 2000. Hybridization as a stimulus for the evolution of invasiveness in plants? Proceedings of the National Academy of Sciences of the United States of America 97:7043-7050.

Hamilton, E. and E. Kiviat. 2001. *Phragmites* use by Native North Americans. Aquatic Botany 69:341-357.

Johnston, C. A. and M. G. Tulbure. 2010. Environmental Conditions Promoting Non-native *Phragmites australis* Expansion in Great Lakes Coastal Wetlands. Wetlands 30:577-587.

Kirk, H., J. Paul, J. Straka, and J.R. Freeland. 2011. Long-distance dispersal and high genetic diversity are implicated in the invasive spread of the common reed, *Phragmites australis* (Poaceae), in northeastern North America. American Journal of Botany 98: 1180-1190.

Lambertini, C., Et Al.,. 2012. Tracing the origin of Gulf Coast *Phragmites* (Poaceae): A story of long-distance dispersal and hybridization. American Journal of Botany 99:538-551.

LeMaitre, D.C., Et Al., 2011. Impacts of invasive Australian acacias: implications for management and restoration. Diversity and Distributions 17:1015-1029.

Lynch, E.A. and K. Saltonstall. 2002. Paleoecological and Genetic Analyses Provide Evidence For Recent Colonization of Native *Phragmites australis* in a Lake Superior Wetland. Wetlands 22:637-646.

Ludwig, D.F., T.J. Iannuzzi, and A.N. Esposito. 2003. Phragmites and Environmental Management: A Question of Values. Estuaries 26:624-630.

Meyerson, L.A., and J. T. Cronin. 2013. Evidence for multiple introductions of *Phragmites australis* to North America: detection of a new non-native haplotype. Biological Invasions 15:2605-2608.

Meyerson LA, Lambertini C, McCormick MK, Whigham DF. 2012. Hybridization of common reed in North America? The answer is blowing in the wing. AoB PLANTS 2012: pls022; doi:10.1093/aobpla/ols022

Meyerson, L.A., D.V. Viola, and R.N.Brown. 2010. Hybridization of invasive *Phragmites australis* with a native subspecies in North America. Biological Invasions 12:103-111.

Nei, M., and A. K. Roychoudhury. 1974. Sampling variances of heterozygosity and genetic distance. Genetics 76: 379-390.

Paul, J., N. Vachon, C.J. Garroway, and J.R. Freeland. 2012. Molecular data provide strong evidence of natural hybridization between native and introduced lineages of *Phragmites australis* in North America. Biological Invasions 12:2967-2973.

Peakall, R., and Smouse P.E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28, 2537-2539.

Saltonstall, K. 2003. Genetic variation among North American populations of *Phragmites australis*: Implications for management. Esutaries 26: 444-451.

Silliman, B.R., and M.D. Bertness. 2004. Shoreline Development Drives Invasion of *Phragmites australis* and the Loss of Plant Diversity on New England Salt Marshes. Conservation Biology 18:1424-1434.

Taylor, D. L. and A. S. Trebitz. 2007. Exotic and Invasive Aquatic Plants in Great Lakes Coastal Wetlands: Distribution and Relation to Watershed Land Use and Plant Richness and Cover. Journal of Great Lakes Research. 33:705-721.

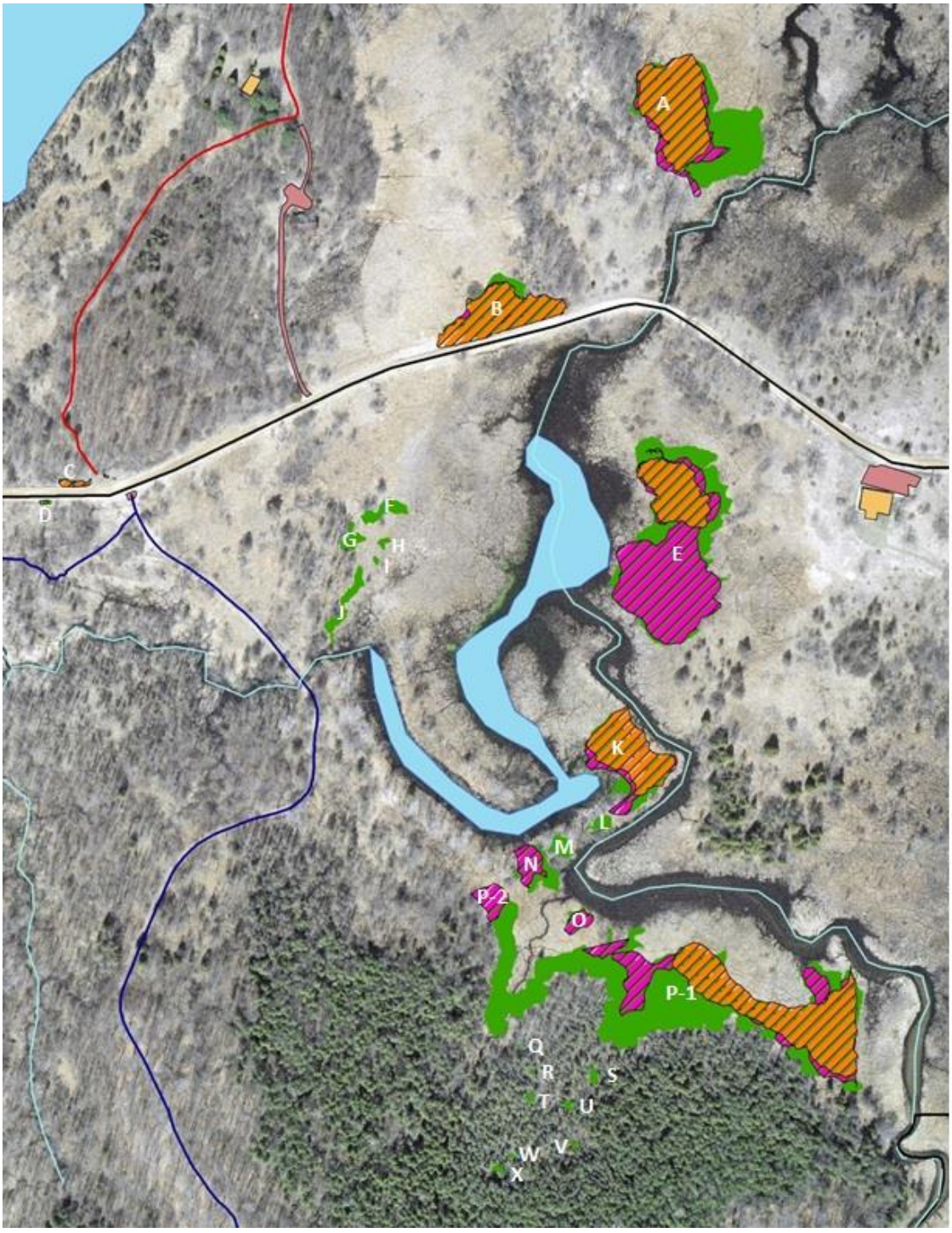

Figure 1. Stands of *Phragmite australis* overlay map from 2007 (orange), 2012 (purple) and 2014 (green) at Pierce Cedar Creek Institute.

Figure 2. Q-plot based on AFLP analysis showing the genetic clusters of 42 *Phragmites australis* individuals from Pierce Cedar Creek Institute. Population 1 represents all 10 individuals from sites C and D (Figure 1), putative *P. a. australis*. Population 2 represents 32 individuals from populations E and K (Figure 1), putative *P. a. americanus*.

Table 1. Individual stand size and total area covered by *Phragmites australis* at Pierce Cedar Creek Institute. All number are in m2.

Site	2007	2012	2014
A	2309.94	2504.87	4408.00
B	1597.81	703.87	1464.00
C	65.66	26.92	15.12
D	10.21		17.18
E	1325.03	5020.76	6502.00
F			215.30
G			135.80
H			35.55
I			15.08
J			257.50
K	1514.18	1287.12	1332.00
L			98.54
M			149.10
N		319.59	491.10
O		162.39	172.60
P-1	3357.94	3389.82	9642.00
P-2		301.33	
Q			>100
R			>100
S			>100
T			>100
U			>100
V			>100
W			>100
X			>100
Total	10,181 m^2	13,717 m^2	25,126 m^2