

Remnant native *Phragmites australis* maintains genetic diversity despite multiple threats

Kristin Saltonstall

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Abstract Over the past century, an increasing number of species have been negatively impacted by anthropogenic factors such as habitat disturbance and introduced species. One such plant, *Phragmites australis* subsp. *americanus* is a perennial emergent grass found in tidal and inland marshes of the Atlantic coast of the United States. While rarely dominant, it grows in mixed communities and across much of this area its distribution has been reduced dramatically, likely due to eutrophication and the invasion of conspecific *P. australis* introduced from Europe. In this study, two noncoding cpDNA markers and six microsatellite loci were used to characterize genetic diversity among 58 remnant native *P. australis* stands from North Carolina to Maine. Five chloroplast DNA haplotypes were identified along with 42 multilocus genotypes. Bayesian exploration detected no population structure (e.g., optimal $K = 1$), indicating that these individuals form a single population. The analysis also detected no presence of hybrids of native and introduced *P. australis* in the samples, despite the close proximity of individuals to each other in many cases. These results suggest that the genetic composition of native *P. australis* across the region remains homogeneous and pure, providing managers with justification for its conservation and a potentially large source of germplasm for use in restoration activities.

Keywords Common reed · Conservation · Hybridization · Marsh · Native · Invasive species · Restoration

Introduction

Anthropogenic habitat modification has become a leading threat to biodiversity over the past century, especially in wetland habitats (Vitousek et al. 1997; Wilcove et al. 1998; Zedler and Kercher 2004 and references therein). Along the Atlantic coast of North America, an estimated 38% of coastal salt marshes have been lost since European settlement, particularly in the Boston, MA–Washington, DC corridor (Gedan and Silliman 2009). Other anthropogenic threats include invasive species, nitrogen eutrophication, altered hydrologic and sedimentation regimes and sea level rise (Vitousek et al. 1997; Wilcove et al. 1998), many of which have far-reaching effects that are difficult to control. While many important habitats and ecosystems have been protected from further physical destruction, biological refugia can become increasingly isolated as urbanization and other changes occur around them as a result of human activities and often they cannot be fully insulated from many threats.

The potential demographic and genetic threats of many anthropogenic changes, such as habitat fragmentation and invasive species, on plant populations have been well documented (e.g. Ayres et al. 2004; Ellstrand and Elam 1993; Marchant 1967 and references therein). Plant species with small and isolated populations are already vulnerable to demographic, environmental and genetic stochasticity, and therefore face a higher risk of local extinction (Ellstrand and Elam 1993). Ecological and life history characteristics of a species, such as mating system and methods of pollination and dispersal, can influence how a species might be affected by anthropogenic threats (Hamrick and

K. Saltonstall (✉)
Smithsonian Tropical Research Institute, Apartado,
0843-03092 Panamá, Republic of Panamá
e-mail: kristin.saltonstall@aya.yale.edu

Godt 1996). Thus an understanding of the interactions between population demography and genetic connectivity is of great importance to biologists in formulating conservation strategies for rare and threatened species.

Phragmites australis (Cav.) Trin ex Steud is a perennial rhizomatous grass with a cosmopolitan distribution. It is both wind pollinated and dispersed and typically establishes in wetland habitats via either seed or vegetative propagules (Saltonstall et al. 2010). Once established, it grows rapidly and often forms monocultures. Along the Atlantic Coast of North America, two subspecies of *P. australis* are found: native *P. australis* subsp. *americanus* Saltonstall, P.M. Peterson & Soreng (hereafter native *P. australis*), found from North Carolina to southern Canada, and an introduced *P. australis*, which is most likely of Eurasian origin and today dominates many coastal communities in the region (Saltonstall 2002; Saltonstall et al. 2004). Both field and laboratory studies have demonstrated that introduced *P. australis* typically has higher biomass, higher growth rate, and may have a higher reproductive output than the native (League et al. 2006; Meyerson et al. 2010; Saltonstall and Stevenson 2007; Vasquez et al. 2005). The presence of both native and introduced lineages of the same species in close proximity to each other also raises the possibility of hybridization between the lineages and erosion of the genetic integrity of native *P. australis* (Meyerson et al. 2010; Saltonstall 2003a).

Native *P. australis* was historically distributed across much of North America but likely was not common across much of its range (Saltonstall 2002). Along the Atlantic seaboard, preserved rhizome tissues found in marsh sediments indicate that it has been a component of brackish and freshwater marsh communities for at least 4,000 years (Niering et al. 1977; Orson 1999). Today its distribution has been severely reduced, likely due to habitat modification and the invasion of introduced *P. australis* which has overtaken marsh communities across much of the Atlantic coast and today is probably the most aggressive introduced species across the region (Saltonstall 2002, 2003a).

Since native *P. australis* was formally described (Saltonstall 2002; Saltonstall et al. 2004), a number of remnant stands of this subspecies have been identified along the Atlantic coast of the United States, the majority of which are found in close proximity to introduced *P. australis*. This study characterizes the genetic diversity of these native stands at the regional level and looks for evidence of hybridization with introduced *P. australis*. While much attention has been paid to the dramatic invasion of introduced *P. australis* in North America, little is known about native *P. australis*. Here I provide baseline genetic data for these remnant individuals that, in addition to being representative of healthy native marsh communities, have important conservation priorities.

Materials and methods

Sampling and DNA methods

Leaf samples from 58 native *P. australis* plants across the Atlantic seaboard region were collected from 2003 to 2008 (Fig. 1). Sampling was opportunistic and occurred as novel stands were identified. Forty-three stands were located in the mid-Atlantic region (NJ, DE, MD, VA, NC) and 15 in New England (ME, NH, MA, RI, CT). In the mid-Atlantic, all were found growing in the tidal oligohaline portions of rivers. In New England, stands were found along the upland edges of brackish marshes, along the tidal portions of rivers, and at inland freshwater fens. All stands were initially identified as native in the field based on morphological characters distinguishing native from introduced *P. australis* (Saltonstall et al. 2004). A single leaf sample was collected from each stand and samples were air or oven dried. They were then stored dry at room temperature until processed in the laboratory. All DNA extractions were done using a CTAB extraction protocol (Doyle and Dickson 1987).

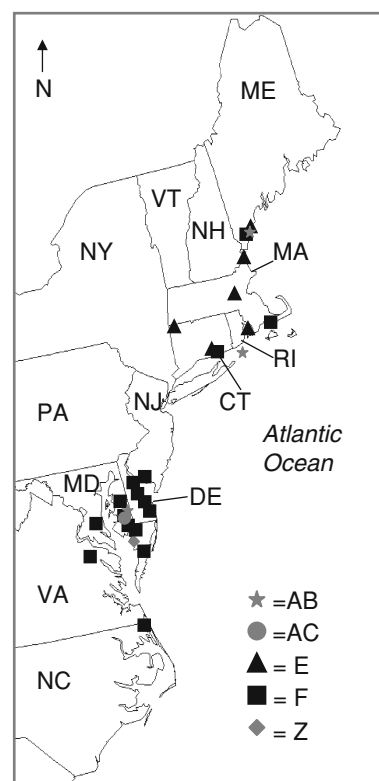


Fig. 1 Sampling locations from across the study area. Symbols (defined adjacent to the map) represent cpDNA haplotype codes for each sample. At some locations, multiple stands located in close proximity to one another and displaying the same haplotype are represented by a single symbol

Two noncoding chloroplast (cpDNA) gene regions, *trnT-trnL* (Taberlet et al. 1991) and *rbcL-psaI* (Saltonstall 2001) were PCR amplified, sequenced on an ABI 3100 sequencer, and analyzed, as described in Saltonstall (2002, 2003a), to identify the cpDNA haplotype. Six nuclear microsatellite loci (GT4, GT8, GT9, GT11, GT13, GT16) were also amplified, as described in Saltonstall (2003b). Samples were genotyped on an ABI 3100 sequencer using LIZ 500 as a size standard and allele sizes were estimated using GeneMapper version 3.7 (Applied Biosystems).

Although *P. australis* represents an allopolyploid complex (Raicu et al. 1972), both native and introduced *P. australis* are likely tetraploid (Saltonstall et al. 2007). Due to this polyploidy, it is usually not possible to determine the allelic dosage in heterozygotes (e.g. Aaaa versus AAaa). The six microsatellite loci analyzed here were chosen because they typically amplify only one or two alleles per individual (Saltonstall 2003b), displaying an inheritance pattern similar to diploids. Although this assumes that allelic dosage follows a fixed parental inheritance pattern, namely that the presence of two alleles represents two copies of each allele rather than one and three copies of each respectively, it facilitates a more detailed analysis of population structure.

Genetic diversity

As sampling at the stand level was limited (e.g. only one ramet per stand), within-site measures of genetic diversity were not calculated and the analysis focuses on diversity of the subspecies at the regional level. FSTAT version 2.9.3.2 (Goudet 2002) was used to calculate the total number of alleles (A), allelic richness (A_R), observed heterozygosity (H_O), and gene diversity (GD) per locus. Total numbers of observed genotypes (G_O) were also calculated for each locus. Samples displaying identical cpDNA haplotypes and multilocus genotypes that originated from nearby stands along a river were considered to be clonal offspring of a single parent and were represented by a single individual in these analyses. Such duplications reduced the total number of individuals analyzed to 52.

Population structure was analyzed using the five microsatellite loci showing variation (GT4, GT8, GT9, GT13, GT16) and the Bayesian clustering approach implemented in STRUCTURE version 2.3.3 (Pritchard et al. 2000). The admixture model with correlated allele frequencies was used with 10 replicates for each run. The number of genetic groups evaluated ranged from $K = 1$ to $K = 5$ and each run consisted of a burn-in period of 10,000 MCMC (Monte Carlo Markov chain) steps followed by 100,000 iterations. When interpreting the STRUCTURE analysis, both the ΔK method (Evanno et al. 2005) and plots of the log posterior probability of the data ($\ln P(D)$) for

each value of K (Pritchard et al. 2000) were applied to infer the number of genetic groups sampled.

Hybridization between native and introduced *P. australis*

A reference dataset of 235 introduced *P. australis* individuals collected throughout the study area (Saltonstall 2003b, unpub. data) was used to evaluate whether extant native *P. australis* individuals show evidence of interbreeding with introduced ones. The pairwise F_{ST} value between the two subspecies based on this dataset was 0.47. The possibility of hybridization between the two *P. australis* subspecies was explored in two ways: (1) using the Bayesian admixture analysis approach implemented in STRUCTURE, and (2) the partial Bayesian method (Rannala and Mountain 1997) implemented in GeneClass2 version 2.0 (Piry et al. 2004). Using all six loci, individual genetic assignments to either native or introduced *P. australis* were then obtained. These six microsatellite loci amplify alleles and genotypes that are diagnostic to both native and introduced *P. australis* facilitating identification of hybrids (Boecklen and Howard 1997; Saltonstall 2003b). Alleles known to display size homoplasy between the native and introduced lineages (Saltonstall 2003b) were treated as ambiguous data.

STRUCTURE was run with ten independent runs using 100,000 iterations, with a burn-in period of 10,000 iterations. Both the admixture and no admixture models were run (Falush et al. 2003) and the presence of two genetic clusters was assumed ($K = 2$). Each of the samples was then assigned to either the native cluster (when membership probability (q) was >0.90), the introduced cluster ($q < 0.10$) or to a potentially intraspecific hybrid group ($0.10 \leq q \leq 0.90$). Although the number of microsatellite markers used was low in this study, the high F_{ST} between the two subspecies permits the use of a q value threshold of 0.10 as an efficient means of identifying hybrids (Vähä and Primmer 2006).

Using GeneClass2, the assignment option was run first to check that samples were correctly assigned to the subspecies designation suggested by their cpDNA haplotypes. A probability computation using 10,000 MCMC simulations (Paetkau et al. 2004) was then used with an assignment threshold of the L_h/L_{max} P value greater than 0.05 as a cutoff for inclusion in a subspecies group.

Results

cpDNA haplotype diversity

Five native cpDNA haplotypes were identified (haplotypes E, F, Z, AB, AC, as in Saltonstall 2002; Fig. 1) in the 57

samples that were sequenced. These five haplotypes are closely related and differ due to variation in the number of copies of several indels, primarily in the *trnT-trnL* region. Today, haplotype E is the most common haplotype in the Northeast and haplotype F is most common in the mid-Atlantic. Haplotype F was also found in two samples from the Northeast (MA, CT), indicating that its distribution is not restricted to the mid-Atlantic. While less common, haplotype AB is widespread with samples found in ME, RI, and MD. Haplotype Z was found at two locations along the same watershed in MD and haplotype AC was found in MA.

Microsatellite diversity

A total of 42 multilocus genotypes were found across the six loci ($n = 58$ individuals). Observed heterozygosity was low, ranging from 0 to 0.58 across loci, with an overall mean of 0.26 ± 0.24 . Loci GT9 and GT13 showed the highest number of alleles and genotypes. Most alleles were shared across haplotypes and allelic diversity across the six loci studied was low, with the mean number of alleles per locus being 4.0 (Table 1).

The clustering analysis implemented in STRUCTURE showed no defined genetic clusters across the study area. This indicates that native *P. australis* individuals across the Atlantic coast likely belong to a single, interbreeding population ($K = 1$ was optimal).

Hybridization between native and introduced *P. australis*

Using STRUCTURE, all individuals were correctly assigned to their respective subspecies and none were designated as potential hybrids. Similarly, in the GeneClass2 simulation, all individuals were assigned correctly

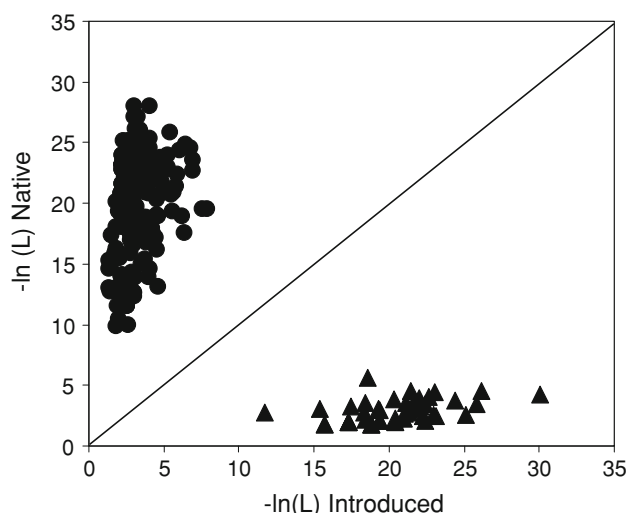


Fig. 2 Plot of $-\log$ of assignment values (L) for 52 native (filled triangle) and 235 introduced (filled circle) *P. australis* samples using six microsatellite loci as calculated with GeneClass2. The line of equality separates the assignment areas. Samples that fall above the line are assigned to introduced *P. australis*, those below the line are assigned to the native lineage

to the native or introduced groups, based on cpDNA haplotype, and all but one sample (with $P = 0.0869$) had a probability of assignment to the alternative subspecies of less than 0.05 (Fig. 2).

Discussion

Chloroplast DNA diversity remains high in Atlantic coast populations of native *P. australis*, with five of the 13 native haplotypes that have been reported across North America (Meadows and Saltonstall 2007; Saltonstall 2002) detected in this study of modern populations. If haplotype AA, which was previously identified in historical samples from

Table 1 Genetic diversity in 52 Atlantic coast native *P. australis* individuals at six microsatellite loci

	GT4	GT8	GT9	GT11	GT13	GT16
A_O	4	3	5	1	7	4
GD	0.25	0.35	0.62	0.00	0.49	0.56
A_R	4.0	3.0	5.0	1.0	6.9	4.0
G_O	5	4	8	1	8	5
DG_O	266 (4)	178 (12)	210 (33)	145 (100)	208 (38)	265 (40)
	266/274 (16)	178/180 (19)	210/212 (21)		208/218 (46)	265/290 (4)
	274 (76)	180 (67)	210/214 (21)		218 (4)	290 (50)
			212 (10)			
			214 (5)			
H_O	0.20	0.21	0.52	0.00	0.58	0.04

A_O observed number of alleles; GD gene diversity; A_R allelic richness; G_O observed number of genotypes; DG_O dominant genotypes observed, values in parentheses are the percentages of each genotype; H_O observed heterozygosity

CT (Saltonstall 2002), is included, nearly half (46%) of the known haplotypes of native *P. australis* are found within the study area. Although closely related, these Atlantic coast haplotypes are distinct from those found in other parts of North America and only haplotype E is known to occur outside of the study region, where its distribution extends across northern portions of North America from the east to the west coasts (Saltonstall 2002, 2003a).

In contrast, genetic diversity at the microsatellite loci tested here is relatively low with most loci exhibiting only a few common alleles and genotypes. However, observed heterozygosity across the six loci was higher than previously reported for these loci in native *P. australis* across its entire range (average $H_O = 0.17 \pm 0.11$; Saltonstall 2003b). This previous study also evaluated native *P. australis* herbarium specimens collected prior to 1910 in the Atlantic Coast region and found similar patterns of allelic diversity and genotypes and similar levels of observed heterozygosity ($n = 12\text{--}22$ samples per locus, average $H_O = 0.24 \pm 0.21$; Saltonstall 2003b). Importantly, in this study at least one novel allele per locus, in the five loci exhibiting multiple alleles, was identified that was not found in the historical samples. While all of these alleles, 11 in total, were in low frequency and the number of samples tested in this study was two to three times greater at each locus, this comparison suggests that modern native *P. australis* along the Atlantic coast is maintaining the genetic diversity that was historically present in the region prior to the widespread invasion of introduced *P. australis*. Further, as native *P. australis* has previously been shown to have lower levels of genetic diversity at many of these loci than introduced *P. australis* (average $H_O = 0.45 \pm 0.25$ at these loci; Saltonstall 2003b), the low levels of diversity found here may be due to the microsatellite loci having been developed from introduced *P. australis* DNA which may be different enough to have variation in the primer regions. Other studies have shown that cross-species amplifications with microsatellite markers can result in detection of lower levels of diversity due to null alleles, leading to high levels of homozygosity (Barbará et al. 2007; Pemberton et al. 1995). With loci developed specifically for native *P. australis* higher levels of nuclear diversity more concordant with the variation found in the cpDNA may be found.

Hybridization with introduced *P. australis*

Should it occur, hybridization and/or introgression between the two *P. australis* subspecies found along the Atlantic coast could pose a significant threat to the long-term viability of native *P. australis*. This native subspecies could effectively become extinct due to swamping of its gene pool by introduced *P. australis* (Ellstrand and Schierenbeck 2000; Novak

and Mack 2005) or hybrid individuals could show increased vigor and outcompete native parents (Ayres et al. 2004). Despite the close proximity of the native *P. australis* sampled to introduced *P. australis* throughout the study area, the previous finding that hybridization events between native and introduced *P. australis* are rare, if occurring at all (Saltonstall 2003b), is supported by this expanded data set.

Given that they are closely related and have existed in sympatry for at least 150 years, it is perhaps surprising to not detect interbreeding between the two subspecies (Saltonstall 2003b). Controlled crosses using hand pollination in the laboratory have been performed on populations originating from the study area and viable seed has been produced using pollen from introduced plants to fertilize native inflorescences (Meyerson et al. 2010). While it is possible that inter-subspecies fertilizations are occurring in natural populations as well, it is also possible that outbreeding depression may cause hybrid seedlings have lower survivorship and reproductive rates than pure native or introduced seedlings (Hufford and Mazer 2003; Lynch 1991 and references therein). Recent work suggests that hybrid *P. australis* may be present in the Great Lakes region, possibly due to clinal changes in flowering times of introduced *P. australis* or genetic compatibility of native and introduced plants in the area (Paul et al. 2010). Many questions remain unanswered and will require further research to determine the threat that introduced *P. australis* places on the genetic integrity of native *P. australis*. However, the high diagnostic ability of both the nuclear and chloroplast genomes in this expanded dataset lends credence to the hypothesis that native and introduced *P. australis* lineages continue to evolve independently along the Atlantic coast despite their modern overlap in geographic distribution (Saltonstall 2003b).

Conservation and management implications

Although the underlying mechanism as to when and where hybridization between native and introduced *P. australis* could occur is not well understood at this time, it is important to think about what can be done to prevent it in the future as it may be inevitable and must be considered when managing native *P. australis*. While hybridization can be beneficial to species, as new adaptive diversity can be generated (Ellstrand and Schierenbeck 2000; Seehausen 2004; Stebbins 1959), the persistence of “pure” native *P. australis* individuals is paramount wherever preservation of native biodiversity serves as a management goal. Preventing establishment of introduced *P. australis* by minimizing both upland disturbance and nutrient enrichment (Packett and Chambers 2006; Silliman and Bertness 2004) is key to protecting native *P. australis*. Control of introduced clones growing in close proximity to remnant

native ones should also be encouraged, particularly in locations where introduced clones are small and recently arrived. Where physical intermixing of the two subspecies occurs, control measures using manual techniques such as gloving can be used to selectively control introduced stems without damaging native ones (Meadows 2006). Further, accurate identification of hybrid individuals, which may resemble one of the parental subspecies morphologically, may become more important as their removal could become a priority to prevent backcrossing into the native population and pollution of this genetic pool (Vilà et al. 2003).

Marsh restoration

Although use of native *P. australis* in marsh restoration projects is not common at this time, it is a natural component of coastal marshes on the Atlantic coast and can be considered an indicator of a healthy marsh ecosystem (Meadows and Saltonstall 2007). These data suggest that across the region, native *P. australis* forms a single homogeneous population. Thus any clone, and especially large well-established ones in healthy marsh systems, could serve as germplasm for introduction to new or historically occupied sites. Although use of seeds to produce germplasm can be problematic as seedlings can experience high rates of mortality (Saltonstall and Stevenson 2007), *P. australis* stems are capable of sprouting at the nodes and plants will grow from stem cuttings. Thus large quantities of native germplasm could be produced for restoration purposes relatively easily without destruction of existing stands and marsh habitats to obtain rhizomes.

However, despite the lack of genetic structuring seen in the neutral markers used in this study, plants from New England and the mid-Atlantic region may have ecotypic differences between them which provide adaptation to different habitats (Holderegger et al. 2006). In the mid-Atlantic region, native *P. australis* plants are found in fresh-oligohaline tidal marshes along riverbanks (League et al. 2006; Meadows and Saltonstall 2007; Packett and Chambers 2006) while in New England, they are found along the upland edges of brackish marshes and in inland freshwater fens in addition to oligohaline tidal marshes (T. Rawinski, pers. comm.). Growth studies have noted differences in the appearance of aboveground growth between native haplotypes (Saltonstall and Stevenson 2007; Vasquez et al. 2005) and it is not known if these indicate differences in their competitive ability. This suggests that should native *P. australis* germplasm be used in restoration projects, attention should be paid to the origin of such germplasm to optimize its chances of establishment and, as with any species, local populations would be most desirable.

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