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ORIGINAL PAPER

Hybridization of invasive *Phragmites australis* with a native subspecies in North America

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Abstract Interspecific hybridization can lead to the extinction of native populations and increased aggressiveness in hybrid forms relative to their parental lineages. However, interbreeding among subspecies is less often recognized as a serious threat to native species. *Phragmites australis* offers an excellent opportunity to investigate intraspecific hybridization since both native and introduced lineages occur in North America. Introduced *Phragmites* is a highly successful estuarine plant invader throughout North America, but native *Phragmites* populations are declining in the eastern US. Despite range overlaps, hybridization has not yet been detected between the native and introduced lineages in the wild, suggesting that phenological or physiological barriers preclude cross-pollination. We demonstrate, for the first time, that native and introduced populations of *Phragmites* can hybridize. There is substantial overlap in flowering period between native and introduced populations from the same geographic locations. We manually cross-pollinated native individuals with pollen from introduced *Phragmites* and recovered viable offspring. We then used microsatellite markers to prove that alleles unique to the pollen parent were transferred to progeny. Our results imply a mechanism for the further decline of native *Phragmites* in North America and a potential for the formation of aggressive hybrid offspring.

Keywords *Phragmites australis* · Hybridization · Invasive species · Vigor · Microsatellites · Phenology

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Introduction

Recently published literature has highlighted the role of increased genetic variation in the success of biological invasions and in the resultant loss of native species (e.g., Ellstrand and Schierenbeck 2000; Lavergne and Molofsky 2007; Hufbauer 2008; Ayres et al. 2008). For many species, multiple introductions to a new range (a component of propagule pressure) can increase invasion success by increasing genetic diversity and reducing genetic bottlenecks (e.g., Lockwood et al. 2005; Dlugosch and Parker 2008). For example, repeated introductions of the invasive grass *Phalaris arundinacea* have resulted in higher genetic diversity and heritable phenotypic variation

in its invasive range than in parts of its native range (Lavergne and Molofsky 2007).

In other cases, interspecific hybridization has increased genetic diversity and led to aggressive hybrid forms relative to their parental lineages or has contributed to the extinction of natives through swamping of the gene pool (Rhymer and Simberloff 1996; Ellstrand and Schierenbeck 2000; Novak and Mack 2005; Vila et al. 2000; Pooler et al. 2002; Ayres et al. 2008). Numerous examples of interspecific hybridizations in the grasses are well described (Angelo and Boufford 1998; Cox et al. 2002). For example, the salt marsh species *Spartina alterniflora* and *S. maritima* have demonstrated interspecific hybridization in the wild to form an invasive hybrid, *S. anglica*. This hybrid species has rapidly invaded salt marshes on multiple continents with significant impacts on native biological diversity (Thompson 1991). *Spartina alterniflora* has also been introduced to California where it has hybridized with *S. foliosa*, creating a hybrid swarm that is threatening native populations with local extinction (Ayres et al. 2008). Such human-mediated invasions affect biodiversity dynamics and, in some cases, may lead to reverse speciation (i.e., genetic re-admixtures of species with a sympatric history) (Seehausen 2006; Seehausen et al. 2008).

Interbreeding among subspecies is less well studied than interspecific hybridization and has been virtually ignored as a serious threat to native species (D'Antonio et al. 2001). Nonetheless, intraspecific hybridization may in fact be an invasion mechanism that creates novel genetic combinations that have a greater potential to invade or that can reduce the fitness of and cause declines in native populations. This scenario is plausible for *Phragmites australis* which exists as distinct native and introduced subspecies in North America (*P. australis americanus* and *P. australis australis*, respectively) (Saltonstall 2002; Saltonstall et al. 2004). Introduced *Phragmites* is one of the most successful estuarine plant invaders in North America, but native *Phragmites* populations are declining in the eastern US, partly because they have been replaced by introduced *Phragmites*.

Despite range overlaps, hybridization between the native and introduced lineages was not been detected in nature by Saltonstall (2002, 2003). Although she searched for evidence of hybridization in her analysis of native and introduced lineages, she concluded that hybridization occurred "rarely, if at all" between the native and introduced lineages, possibly because of assortive mating or low rates of sexual reproduction (Saltonstall 2002, 2003). Therefore, one widely accepted hypothesis has been that a temporal phenological barrier precludes cross-pollination between the native and introduced types. The major goals of this research were to demonstrate coincident flowering phenologies of multiple populations of native and introduced *Phragmites* collected from a broad geographic range and to prove that hybridization between the native and introduced strains of *P. australis* was indeed possible.

Methods

Parent plants

We planted field-collected rhizome fragments from 12 populations of *Phragmites* (4 native, 8 introduced, Table 1) in standard greenhouse potting soil (Metro-mix®) in 5-gallon plastic containers at the University of Rhode Island (URI) in Kingston, RI. Plants were grown outside under ambient conditions in small plastic pools beginning in April 2006 until the onset of anthesis (pollen shedding and stigma receptivity) in late August. The plants were then moved inside a greenhouse for controlled pollen transfer. We recorded the days that at least one individual from a population was in anthesis to characterize any overlap in flowering phenology between native and introduced subspecies.

Table 1 Origins of the introduced and native parent populations of *Phragmites australis* used in hand crosses

Introduced	Native
Moncton, NB	Stratham, NH
Wells, ME	Falmouth, MA
South Kingstown, RI (MHI)	New Shoreham, RI
Narragansett, RI	Seneca Falls, NY
Charlestown, RI	
South Kingstown, RI (MSB)	
Stratham, NH	
New Shoreham, RI	

Cross-pollination

Due to natural variation in the timing of anthesis, the choice of individuals and populations for cross-pollination was necessarily opportunistic. However, we gave priority to crosses between native and introduced individuals originating from the same geographic location and attempted to perform a minimum of 10 crosses per population pair.

As flowers entered anthesis, we conducted manual cross-pollinations. Pollen was collected in paper corn pollen bags by covering a panicle and carefully shaking while angling the bag downward. We immediately transferred pollen to receptive individuals by placing the pollen bag over the entire panicle and shaking. Pollinated panicles were then enclosed in Reemay® bags (spun polypropylene), secured at the bottom to prevent accidental pollen transfer. Because all flowers on a panicle do not open simultaneously, we added supplemental pollen from the same source as the original cross-pollination on subsequent days when it was available. Control panicles were also enclosed in Remay bags to prevent any foreign pollen contamination. Panicles remained covered until seed set in mid-November when they were collected and stored for three months at 4°C.

We harvested seeds from flowers by hand the following spring with a goal of at least 100 seeds per individual cross. Seeds were planted in potting soil in germination trays and grown at a URI greenhouse. The percent germination of seed from native and introduced parents and from their offspring was quantified.

Naming protocol for hybrid *Phragmites*

The hybrid offspring lines of native-introduced *Phragmites* crosses bred have been named according to the following protocol: X indicates a hybrid cross, the male parent (pollen donor) is always listed first (e.g. BIM [Block Island haplotype M]), the female recipient (maternal donor) is always listed second (e.g., BIAB [Block Island haplotype AB]). The year that the cross was conducted follows the identification of the parental populations. Therefore the hybrid line for crosses of Block Island introduced and native *Phragmites* would be named XBIM-BIAB06.

Microsatellite analysis

Leaf tissue was collected from all parents and offspring and was frozen at −80°C. DNA was extracted from 50 mg of leaf tissue using a Qiagen DNeasy 96 Plant Kit®. Saltonstall (2003) identified microsatellite regions in native and introduced *Phragmites* and developed primers for PCR amplification of these regions. Because these primers did not amplify under our lab conditions, we designed additional primers using microsatellite clone sequences deposited in GenBank (AY230868–AY230876). Primer sequences were identified in DNAStar Lasergene and cross-evaluated in NetPrimer (Table 2). Each 15 µl

Table 2 Microsatellite loci and redesigned primer sequences. Redesigned primers were based on the microsatellite clone sequences identified by Saltonstall (2003) (GenBank accession numbers AY230868–AY230876). Primers for loci 4 and 16 were adopted from Saltonstall (2003)

Locus	Accession no.	Primer sequences (5′–3′)
*Pa*GT8	AY230868	F: TTTGCTTGCTGTTTGTTTGTCTGA
		R: GTTTGCGTCTTATCTGAACTTCT
*Pa*GT9	AY230869	F: AATCATCCAGCATACCTT
		R: GTTTATTTAGAGTGAACCGACAA
*Pa*GT11	AY230870	F: GAAGCAACTCCGTGAATGAC
		R: GTTTACTAATGGACTGCCCCTATGT
*Pa*GT12	AY230871	F: TGCCGAGCTACCAAAATACG
		R: GTTTAGCACGCTGTCCCCATAAG
*Pa*GT13	AY230872	F: TAGGTGCTCTCCAACTCAAC
		R: GTTTGACAGCCATTTTAGAACCACATTA
*Pa*GT16		F: TGCCACCAATCAGTCAGA
		R: TAACAGTGCCTCCCAAAGTA
*Pa*GT22	AY230876	F: GCTTTTGAGTGCCTGGTGT
		R: GCGACTTGCGTTTTCTGA

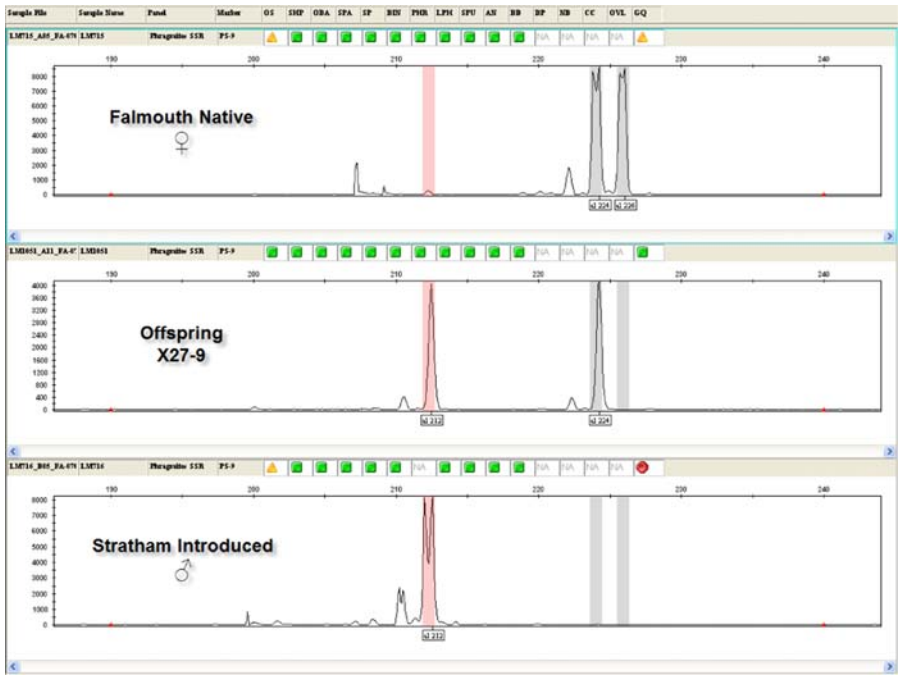

Fig. 1 Allele combinations for seed parent (Falmouth native, top), pollen parent (Stratham introduced, bottom), and progeny (Offspring X27-9, middle). Allele 212 is unique to the pollen parent and thus can be used to determine parentage

PCR reaction contained 15 ng template DNA, 1× PCR buffer (Thermopol buffer, New England Biolabs), 0.2 μM each dNTP, 0.2 μM each primer, and 0.75 units *Taq* DNA polymerase (New England Biolabs). The 5′ end of each forward primer was labeled with a fluorescent dye (6FAM, NED, PET, or VIC). We assigned dyes to each primer pair based on expected fragment size so that all PCR products for a sample could eventually be pooled for capillary electrophoresis (the minimum difference for primers labeled with the same dye was ∼100 bp).

PCR was carried out on an MJ Research Dyad multiblock thermalcycler using the following conditions: an initial denaturation at 94°C for 3 min, followed by 42 cycles of 94°C for 30 s, 52–60°C for 30 s, and 72°C for 30 s, and finally a 10 min extension at 72°C. Subsamples of PCR reactions were run on a 3% agarose gel to verify amplification. All remaining PCR products for a given sample were then pooled, unincorporated primers were removed with AMPure magnetic beads (Agencourt), and the resulting sample was diluted with Hi-Di formamide (Applied Biosystems). PCR fragment length was determined with single base pair resolution using an Applied Biosystems 3130xl Genetic Analyzer.

All parent plants and progeny resulting from manual cross-pollinations were genotyped using GeneMapper® v4.0 (Applied Biosystems) to determine whether hybrids were produced. This requires that the pollen parent (male) have unique alleles not present in the seed parent (female) (Fig. 1). If a progeny individual contains a unique allele present in the pollen parent but not in the seed parent, the allele must have come from the pollen parent. Conversely, if all of the pollen parent's alleles are also present in the seed parent, it is not possible to determine parentage of their progeny (i.e., progeny may be the result either of a cross between pollen and seed parents or of self-pollination by the seed parent).

Results

Flowering phenology

Native and introduced *Phragmites* populations exhibit considerable overlap in their flowering phenology (Fig. 2). Native populations were in anthesis for 10 ± 4.7 days (mean ± standard deviation) compared to 7 ± 5.4 days for introduced populations. There was an average of 5.25 days of overlap in anthesis between native and introduced populations from the same geographic origin. This represents a substantial fraction of the flowering

Fig. 2 *Phragmites* flowering phenology. *Bars* represent the time span over which at least one individual from a population was in anthesis (native populations are in *gray*, introduced in *black*). Overlap in anthesis between populations in close proximity suggests hybridization is possible in wild populations

period during which gene flow between populations could occur.

Cross-pollination

Seed set occurred only in those crosses where the pollen parents were introduced *Phragmites* and the seed parents were native populations. The reverse crosses where the pollen parents were native and the seed parents were introduced did not produce any seed. We therefore report results only for the crosses that set seed (i.e., introduced pollen parent–native seed parent). We used hand pollination methods in a greenhouse to interbreed native and introduced populations of *P. australis* (Table 1). Primer sequences for seven microsatellite loci were used to verify the hybrid status of the germinating seeds produced from these crosses (Table 2).

Outcrossing rates varied across population pairs from 100% (only intraspecific hybrids produced) to 0% (no intraspecific hybrids produced) (Table 3). The identity of the populations appears to be of some importance. For the six pairings with an outcrossing rate greater than 75%, all seed parents were from native populations in Stratham, NH or Falmouth, MA. Because the degree of allelic similarity between seed and pollen parents differed across pairings, the number of loci that could be used to determine if progeny were the result of cross-pollination varied from 1 to 6 loci. However, neither the number of loci nor the proportion of alleles unique to the pollen parent (both measures of our ability to determine parentage) explained the variation in outcrossing ($r^2 = 0.001$ and 0.024, respectively).

Outcrossing varied from 27 to 75% across microsatellite loci (Table 4). However, there were large differences in sample size for each locus due to

Table 3 Frequency of intraspecific outcrossing between native and introduced *Phragmites australis* for thirteen population pairs. *N*, Number of offspring individuals analyzed with the number of loci in parentheses; A_p, total number of parental alleles; A_{nm}/A_p, ratio of unique pollen parent alleles to the total number of parental alleles; FOC, proportion of samples with unique pollen parent alleles. Values for A_p and A_{nm}/A_p are means across loci ±SE

♂, introduced	♀, native	*N*	A_p	A_{nm}/A_p	FOC
Moncton, NB	Stratham, NH	15 (1)	4.0	0.8	1.00
Wells, ME	Falmouth, MA	3 (2)	4.0 ± 0.0	0.6 ± 0.1	1.00
South Kingstown, RI	Stratham, NH	55 (2)	5.0 ± 0.0	0.4 ± 0.0	1.00
Narragansett, RI	Stratham, NH	42 (2)	4.5 ± 0.5	0.3 ± 0.1	1.00
Charlestown, RI	Falmouth, MA	7 (6)	3.2 ± 0.2	0.4 ± 0.1	0.86
South Kingstown, RI	Falmouth, MA	5 (5)	3.2 ± 0.4	0.5 ± 0.1	0.80
Stratham, NH	Falmouth, MA	43 (3)	3.3 ± 0.3	0.5 ± 0.1	0.70
New Shoreham, RI	New Shoreham, RI	3 (2)	4.0 ± 1.0	0.4 ± 0.0	0.67
Narragansett, RI	Seneca Falls, NY	15 (2)	4.0 ± 0.0	0.6 ± 0.1	0.47
South Kingstown, RI	Seneca Falls, NY	46 (2)	4.0 ± 0.0	0.6 ± 0.1	0.43
South Kingstown, RI	New Shoreham, RI	8 (4)	4.3 ± 0.5	0.2 ± 0.0	0.13
Stratham, NH	Seneca Falls, NY	8 (1)	3.0	0.7	0.00
South Kingstown, RI	New Shoreham, RI	1 (3)	4.7 ± 0.3	0.3 ± 0.1	0.00

Table 4 Frequency of intraspecific outcrossing between native and introduced *Phragmites australis* for seven microsatellite loci. N = number of offspring individuals analyzed with the number of population pairs in parentheses. A_p, A_{nm}/A_p, and FOC are defined as in Table 3, except A_p and A_{nm}/A_p are averaged across population pairs

Locus	N	A_p	A_{nm}/A_p	FOC
*Pa*GT12	12 (2)	2.5 ± 0.5	0.4 ± 0.1	0.75
*Pa*GT4	3 (1)	3.0	0.3	0.67
*Pa*GT11	248 (13)	3.9 ± 0.2	0.6 ± 0.1	0.64
*Pa*GT8	16 (3)	3.0 ± 0.0	0.3 ± 0.0	0.38
*Pa*GT9	218 (10)	4.3 ± 0.2	0.4 ± 0.0	0.30
*Pa*GT13	7 (1)	3.0	0.3	0.29
*Pa*GT22	64 (5)	4.0 ± 0.3	0.3 ± 0.1	0.27

differences both in amplification success and in the number of pairings for which pollen parents' possessed unique alleles. For example, loci *Pa*GT9 and *Pa*GT11 amplified very well and contained unique pollen parent alleles in 10 and 13 of the pairings, respectively. In contrast, there were no unique pollen parent alleles at locus *Pa*GT16. Loci *Pa*GT4 and *Pa*GT13 amplified for the fewest individuals, indicating that these primer binding sites may be rare for the introduced populations used in this study.

Germination rates of seed collected from native, introduced and hand pollinated flowers (hybrids) varied between 9 and 55% for native populations and between 56 and 100% germination for introduced populations (Fig. 3). Seed produced from hand crosses between native and introduce *Phragmites* germinated between 17.5 and 88%.

Discussion

Our research conclusively demonstrates, for the first time, that native and introduced populations of *Phragmites* can hybridize and produce offspring with novel allelic combinations that may have significant effects on fitness.

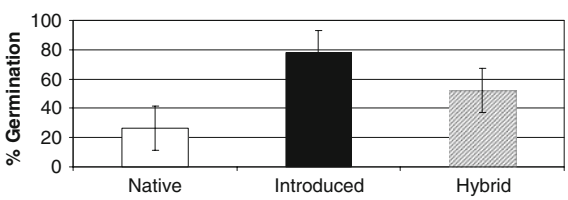

Fig. 3 Germination rates of native, introduced and hybrid seeds. Germination rate for introduced *Phragmites* seed was significantly higher ($P < 0.05$) than those for either native or hybrid *Phragmites* seed

Previous studies have not successfully detected hybrid populations in the field but have concluded that this was most likely because microsatellite analyses were infrequently applied (Saltonstall 2002, 2003), because analyses used mature stem tissues rather than possible hybrid seedlings germinated from field-collected seed, and/or because hybrid individuals may have a low chance of survival in an established clonal stand (see below). While the North American *Phragmites* literature generally reports low, if any, sexual reproduction, more recent evidence suggests that *Phragmites* does establish and spread by seed, particularly if seedlings survive the first winter (Brisson et al. 2008).

Our data demonstrates the ability of the native and introduced lineages to interbreed, but successful crosses were highly dependent on the identities of the parent populations (Table 2). In addition, using redesigned primers (Table 2), we could successfully amplify only 8 of 10 microsatellite loci identified by Saltonstall (2003), and not all 8 loci were useful in determining parentage in every cross-pollination pairing we conducted. It is therefore likely that our estimates of outcrossing between native and introduced *Phragmites* underestimate the rates that would be found if more loci had been examined.

Furthermore, only crosses with introduced *Phragmites* pollen donors and native recipients exhibited seed set, suggesting gene flow is unidirectional. Outbreeding depression that results in reduced fitness of the F1 generation may explain why intraspecific hybrids have not been found in the field (Hufford and Mazer 2003). Alternatively, because the identification of the native and introduced lineages has been relatively recent and new sites colonized by native and introduced *Phragmites* are currently being identified, the lack of detection of hybrids may simply reflect a lack of effort in field searching. Our data indicate that the germination rate of seed from introduced populations is greater than that from either native or hybrid populations (Fig. 3). However, the germination rate for F1 hybrid seed is not different from that of native seeds, but can be as high as 50% in some instances. Thus, if outbreeding depression is responsible for the absence of hybrid populations in the field, its effects likely arise after the germination

stage of the life cycle, such as death of the seedling in the first winter season (Brisson et al. 2008).

Since Saltonstall's initial genetic screening for native and introduced *Phragmites* (2002), dozens of native populations have been discovered growing adjacent to introduced *Phragmites* in New England and elsewhere (L.A. Meyerson unpublished data, E. Hazelton and B. Blossey, personal communication). Such knowledge greatly enhances our estimation of the likelihood that hybridization has occurred and, if screening efforts are intensified, that we will find instances of it in the field.

Our research results have pervasive and long-term consequences both for the management of introduced species and for the conservation of native populations. The primary motivation for control of *Phragmites* is that invasions lead to faunal habitat degradation and declines in the species richness of native plants (Farnsworth and Meyerson 1999; Benoit and Askins 1999; Meyerson et al. 2000, 2009; Gratton and Denno 2005, 2006). Along with anthropogenic disturbance, competition with introduced *Phragmites* has likely led to significant losses of native populations in the eastern US (Chambers et al. 1999; Saltonstall 2002).

If hybridization is occurring in the field, it will undoubtedly lead to further declines of the native subspecies through genetic swamping and, potentially, through increased competition if hybrids exhibit increased vigor. Therefore, the proximity of introduced *Phragmites* to remnant native populations could be a higher control priority than those introduced stands that are less likely to directly affect a native population (Meyerson 2007). While pollen cannot strictly be considered a propagule because it cannot independently give rise to a new plant, the impacts of an invasive species arising in part from hybridization with a native species is an important component of propagule pressure and should be considered in conservation and management efforts.

Conclusions

Our research demonstrates that no reproductive barrier exists between introduced and native *Phragmites* and proves that these subspecies can hybridize and produce viable offspring. As introduced *Phragmites* expands its range and comes into contact with novel populations of native *Phragmites*, the potential for interbreeding exists in the wild. Introduced *Phragmites* is already among the most prominent invaders in North America, but it continues to expand its range southward into Virginia (Chambers et al. 1999) and westward along canals in Utah, Arizona, California, and elsewhere (A. Lambert, personal communication) where native populations of *Phragmites* persist. Native *Phragmites* populations in both of these geographic locations should be high conservation priorities to protect the native gene pool including studies to determine their susceptibility to hybridization with the invasive *Phragmites* hapolotype.

Our results suggest that the conventional species-level approach to assessing the effects of biological invasions, in which all populations are considered roughly analogous, may be too coarse. The value of taking a population-level approach to biological invasions is supported in our study by the strong dependence of outcrossing rates on the identity of the native and introduced parent populations. Furthermore, native and introduced *Phragmites* may also be a good model plant system for hybridizations across species (interspecific hybridizations) because both lineages of *Phragmites* are widely found throughout North America in multiple habitat types under different environmental conditions and because interbreeding of well differentiated populations within a species (such as *P. australis*) are likely to display an evolutionary response similar to hybridization that occurs between species (Seehausen et al. 2008).

To date we have determined cross compatibility of different *Phragmites* lineages and geographically isolated populations using hand pollination techniques and confirmed hybridity using microsatellite techniques. Ongoing research is testing the resultant hybrid offspring (F1 generation) for differences in vigor over parental populations, and we will conduct backcrosses of F1 generation to both parents and quantify and compare changes in ability to interbreed and resultant vigor of successful crosses to F1 and original parent populations. We are also screening for natural hybrids in the field.

Over the last decade, *Phragmites* control efforts in wetland ecosystems have focused on eradication because introduced *Phragmites* provides poor habitat for many native species and alters natural ecosystem processes. However, funding resources for control, management, and restoration have not kept pace with

the rate of invasion and have not considered the loss of native *Phragmites* through hybridization with the introduced lineage. Our results suggest substantial population level differences in fitness and vigor, responses to disturbance and the ability to hybridize. Therefore, control, management, restoration and conservation efforts should consider individual population traits when determining the appropriate course of action.

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