

BRIEF COMMUNICATION

**CONFIRMED FIELD HYBRIDIZATION OF NATIVE AND INTRODUCED
PHRAGMITES AUSTRALIS (POACEAE) IN NORTH AMERICA¹**

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- *Premise of the study:* Intraspecific hybridization between native and introduced lineages of a species can increase invasiveness and may lead to the decline of native lineages. The introduction of Eurasian *Phragmites australis* has caused profound changes to wetland habitats across North America, yet evidence for hybridization between native and introduced *Phragmites australis* in North America is lacking and has puzzled researchers for over a decade. Here we present the first confirmed field hybridization event between the two lineages.
- *Methods:* Hybrid plants were initially recognized during field surveys by their intermediate morphology and distinct herbivore community. We verified hybrid status using chloroplast DNA haplotypes and microsatellite markers.
- *Key results:* Confirmed hybrid stems were restricted to one site and displayed morphological characteristics of both native and introduced *P. australis*. Based on their microsatellite profiles, all samples likely represent a single clone of a first generation hybrid. Sequencing of cpDNA indicates that the maternal parent is from the introduced lineage.
- *Conclusions:* Identification of hybrid *P. australis* in the field is complex and requires multiple characters. All suspected hybrids should be verified using genetic techniques. Preventing the spread of introduced genes and genotypes through North America will require recognition and rapid management response to hybrid plants.

Key words: cpDNA; hybrid; *Lasioptera hungarica*; microsatellite; morphology; *Phragmites australis*; Poaceae.

Hybridization between species is an important evolutionary process that is common in plants, involving at least 25% of species (Mallet, 2005). It can occur between species that may or may not be closely related and is important in the creation of novel species, often through polyploidization (Soltis and Soltis, 2000; Pandit et al., 2011). Hybridization has also been predicted to stimulate invasiveness and may contribute to it by generating new phenotypic variants (Gaskin et al., 2012), promoting hybrid vigor, and providing the genetic material for rapid evolutionary change (Schierenbeck and Ellstrand, 2009). In addition, hybridization of native with introduced species is an increasing conservation concern, threatening native species through the possibility of outbreeding depression or swamping of gene flow that may lead to loss of locally adapted populations (Bleeker et al., 2007). Regardless of the direction of gene flow, interspecific crosses may create novel species (e.g., *Spartina anglica* C.E. Hubbard [Marchant, 1967], *Fallopia ×bohemica* and *Typha ×glauca* [Schierenbeck and Ellstrand, 2009]) or invasive hybrid swarms (e.g., *S. alterniflora ×foliosa* [Ayres et al., 2004]; *Tamarisk* sp. [Gaskin et al., 2012]). Intraspecific crosses of previously isolated lineages may also create highly success-

ful hybrid swarms (e.g., *Phalaris arundinacea* L. [Lavergne and Molofsky, 2007]; *Pyrus calleryana* Decne. [Culley and Hardiman, 2007]).

Phragmites australis (Cav.) Trin. ex Steud is a widespread perennial grass, which over the past 150 yr, has rapidly expanded its distribution in wetlands across North America. While a native lineage, *Phragmites australis* subsp. *americanus* Saltonstall, P.M. Peterson, and Soreng, was historically present across much of the current range of the species, introduction of a nonnative European lineage has been responsible for dramatic increases in abundance and range expansion (Saltonstall, 2002). This introduction has dramatically altered estuarine and freshwater communities (Chambers et al., 1999) and likely caused reductions in the distribution of the native subspecies due to competitive exclusion (Saltonstall, 2002; Saltonstall et al., 2010).

Extensive molecular work confirms that native and introduced *P. australis* remain genetically distinct lineages despite growing in close proximity at many field sites (Saltonstall, 2002, 2003, 2011; Saltonstall et al., 2007; Kettenring and Mock, 2012). To explain lack of gene flow between the two lineages, Saltonstall (2003) earlier posited that low levels of sexual reproduction or differences in phenology may reduce chances of hybridization. However, recent studies show that both native and introduced *P. australis* regularly reproduce and establish new populations via seed dispersal (Brisson et al., 2008; McCormick et al., 2010; Saltonstall et al., 2010). Furthermore, flowering times of native and introduced populations in the Northeast overlap (Park and Blossey, 2008; Meyerson et al., 2010), yet extensive searches for potential hybrids in the field have come up empty or produced unconvincing data, leading to speculation about the accuracy or ability of the methods developed

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by Saltonstall (2003) to detect hybrid plants (Meyerson et al., 2010, 2012). Hand pollination has been used to create hybrids, suggesting the strong possibility for field hybridization, although its success was specific to certain parental lineages (Meyerson et al., 2010). Paul et al. (2010) identified putative hybrid plants in southern Ontario, but the microsatellite allele sizes presented in their study (Table 1 in Paul et al., 2010) show overlaps between native and introduced plants at several loci that do not correspond well with the much larger data set developed to distinguish the two lineages (Saltonstall, 2003; Saltonstall et al., 2010). This calls into question their ability to detect hybrids, which could be an artifact of their data set.

Here we describe a hybridization event for *P. australis* in the field in upstate New York where we found plants that were not clearly identifiable as native or introduced based on morphological evidence. To confirm whether these plants were hybrids of native *P. australis* subsp. *americanus* and introduced *P. australis*, we used two noncoding chloroplast regions and nine nuclear microsatellite markers to determine parentage and evaluate evidence for hybridization and backcrossing.

MATERIALS AND METHODS

Field surveys—The Montezuma National Wildlife Refuge (42°58'N, 76°44'W) and surrounding wetlands in Seneca Falls, New York, United States are a stronghold for native *P. australis* subsp. *americanus* in New York State, but the area also shows rapidly advancing populations of introduced *P. australis*. Native and introduced *P. australis*, their herbivore communities, and their management and impact on native biota in this area have been studied for well over a decade (Saltonstall, 2002; Blossey, 2003; Park and Blossey, 2008; Martin and Blossey, 2013). Thus, the area and its *P. australis* populations are well known and visited frequently. During a visit in early May 2009, we noticed stems with intermediate morphology and unusual herbivore attack, while nearby stems could be clearly classified as native or introduced based on morphology. These stems were found less than 200 m away from confirmed native and introduced clones (Saltonstall, 2002).

Not long after these plants were observed, the area underwent extensive marsh restoration efforts. In the process of reshaping marsh elevation, hydrology, and plant communities, all native and introduced clones as well as suspected hybrid individuals were eliminated. However, *P. australis* rhizome fragments were redistributed with surface materials, and soon, vigorously recruiting stems appeared throughout the area. Initially, we could not be certain of their status as native, introduced, or potential hybrid due to lack of clearly developed morphological characters in early recruiting stems. We were able to relocate suspected hybrid individuals in 2011, and in 2012 we surveyed for potential hybrid individuals throughout the Montezuma wetlands complex, which includes the wildlife refuge as well as the Montezuma Wildlife Management Area and private land holdings.

Morphological characteristics and biotic communities—A suite of traits, including morphological variation and differences in herbivore communities, can be used to distinguish the native and introduced lineages. Examples include characters such as glume length and ligule height, leaf-sheath adherence, stem smoothness, and sometimes stem color (Blossey, 2003; Saltonstall et al., 2004). Throughout the Northeast and Mid-Atlantic states, native *P. australis* subsp. *americanus* can be recognized by leaf sheaths that loosen over the course of the summer and drop off in late fall or winter, with only a few leaf sheaths remaining on the stems into the spring. In addition, stems develop a high gloss, producing a polished appearance. These characters become more pronounced as the growing season progresses, with stems in spring and early summer being the most difficult to identify. The characters remain on standing old stems from previous seasons, although native stems slowly lose their gloss and can be relied on even when plants are dormant or not flowering. In addition to leaf sheath adherence and stem gloss, most stems on native genotypes show an extensive maroon stem color by fall, which slowly fades over the winter months. This maroon coloration is particularly pronounced on stem portions exposed to sunlight or stems at the edge of clones and extends beyond the basal internodes, often up to the middle portions of a stem.

Introduced plants across North America can be identified throughout the year by tight-fitting leaf sheaths that remain firmly attached to the stems during the aerial decomposition process, which may last several years. Furthermore, if leaf sheaths are manually removed, stems are dull and do not show the gloss that characterizes native stems. While there are regional differences in expression of morphological characters for native lineages, introduced plants are morphologically identical for leaf sheath adherence and lack of stem gloss across their entire range in North America (B. Blossey, unpublished data). While red coloration can be found on the lowest internodes of introduced *P. australis*, it is less common and much less extensive than that observed in *P. australis* subsp. *americanus*.

Our sampling occurred through much of the growing season, and we collected leaves for molecular work, which also allowed us to assess ligule heights. We measured ligule heights using a Nikon SMZ645 dissecting microscope with a SC-W10XA/22 ocular micrometer, by measuring the ligule at the center of the leaf blade. Both the ligule membrane and the adjacent short fringe of hairs were measured, excluding any long hairs. Since previous work has shown that within-sample variation in ligule height is negligible between mature leaves (Saltonstall et al., 2004), only one leaf blade was examined for the majority of specimens.

Extensive surveys of herbivore communities of native and introduced *P. australis* show at least eight widespread stem-feeding herbivores in native and introduced *P. australis* in the Northeast (Blossey, 2003), although the entire herbivore community on *P. australis* is much more extensive (Tewksbury et al., 2002). Two native species, *Thrypticus willistoni* (Wheeler) and *Calamomyia phragmites* Felt., attack only native stems, while the introduced gall midge *Lasioptera hungarica* Möhn attacks introduced stems, even when native, and introduced stems grow in close proximity. All other species attack both native and introduced *P. australis* (Blossey, 2003; Park and Blossey, 2008).

Lasioptera hungarica is a univoltine gall midge with *P. australis* as the only recorded host plant. This Eurasian species was recognized in North America in 1999, but the species is now widespread throughout the Northeast and Midwest (Tewksbury et al., 2002). Larvae overwinter in the stem, 30–300 yellow-orange larvae can be found in a single internode, and multiple internodes on a stem can be attacked. The species is easily identified by its association with the black mycelia of the fungus *Sporothrix* that fill the internode and is consumed by larvae of *L. hungarica* (Rohfritsch, 2008).

Molecular analyses—To verify replicability of the data, we performed three independent DNA extractions on each of four putative hybrid samples. DNA was extracted from dried green leaf tissue using either a modified CTAB extraction protocol (two replicates; Doyle and Doyle, 1987) or a Qiagen 96-well plant DNA extraction kit (one replicate). Reference samples from both lineages ($n = 11$ putative native and $n = 12$ putative introduced individuals collected in and around Montezuma NWR specifically for this study, $n = 375$ other) were extracted only once, using CTAB. Following extraction, samples were stored at 4°C until further analysis.

To determine the lineage of the seed parent, we amplified two noncoding chloroplast DNA (cpDNA) regions, *trnT*(UGU)“a”-*trnL*(UAA)5' “b” (Taberlet et al., 1991) and *rbcL-psaI* (Saltonstall, 2001), sequenced them on an ABI 3100 sequencer, and identified the cpDNA haplotype of the plant, as described in Saltonstall (2002). All putative native plants collected for the study were also haplotyped.

We genotyped samples using nine microsatellite loci (*GT4*, *GT8*, *GT9*, *GT11*, *GT13*, *GT14*, *GT16*, *GT21*, *GT22*) on an ABI 3100 sequencer, using the methods of Saltonstall (2003). Alleles were sized using GeneMapper 4.0 (Applied Biosystems) and screened for lineage-specific alleles. Each replicate DNA extraction of putative hybrid samples was run three times to verify the presence of each allele. Because North American *P. australis* is tetraploid (Saltonstall et al., 2007) and more than two alleles were found at some loci, the allelic dosage could not be estimated. Allelic profiles are hereafter referred to as allelic phenotypes (Saltonstall, 2003). Two loci (*GT4*, *GT9*) are known to display size homoplasy and diagnostic base substitutions between the native and introduced *P. australis* lineages, and we sequenced alleles from these loci to verify their lineage of origin. Alleles were amplified as in Saltonstall (2003) and compared to reference sequences representing each lineage ($n = 19$ introduced, 23 native).

We used a reference data set of 295 introduced and 103 native (haplotypes E, F, G, S, Z, AA, AB, AC) individuals of *P. australis* collected throughout the northeastern portion of the USA and eastern Canada (Saltonstall, 2003; K. Saltonstall, unpublished data) to evaluate whether alleles displayed in putative hybrid samples were representative of either lineage. We calculated background allele frequencies of parental lineages based on the percentage of individuals

having an allele within the introduced and native lineages. We identified hybrids using a principle coordinates analysis based on the band-sharing Lynch distance metric (Lynch, 1990), as implemented in the R package Polysat (Clark and Jasieniuk, 2011) and Bayesian clustering methods implemented in STRUCTURE 2.3.3 (Pritchard et al., 2000). In STRUCTURE, the admixture model with correlated allele frequencies was used with 10 replicates for each run. Two genetic groups were assumed ($K = 2$), and each run consisted of a burn-in period of 10000 Markov chain Monte Carlo (MCMC) steps followed by 50000 iterations. Alleles known to display size homoplasy between native and introduced lineages were treated ambiguously and coded as the same allele size for both lineages. Each sample was then assigned to either the native cluster (when membership probability [q] was ≥ 0.95), the introduced cluster ($q \leq 0.05$) or to a potentially intraspecific hybrid group ($0.05 < q < 0.95$).

RESULTS AND DISCUSSION

We collected 27 samples for this study, of which 12 were identified as introduced and 11 as native *P. australis* (combined cpDNA haplotypes E and AB; GenBank no. AY016325/AY016333, AY714215/AY016333) based on both morphological and DNA analyses. Of the four putative hybrids identified in the field using morphological characters, three are likely hybrids based on their microsatellite allele phenotypes, and the other is introduced (cpDNA haplotype M and a 99.8% probability of assignment to the introduced cluster in STRUCTURE; GenBank no. AY016327/AY016335). These three hybrid samples were growing several meters apart and had identical allelic phenotypes, suggesting that they are clonal replicates of a single individual (Fig. 1). Soil movement at the site likely redistributed rhizome fragments that then gave rise to these identical individuals, also indicating a single recruiting event followed by vegetative spread. The fourth putative hybrid, mistakenly identified in the field using morphological characters, was a newly recruiting individual in a periodically flooded stream channel with loosely attached leaf sheaths and no herbivore attack. This misidentification in the field highlights the need for use of multiple traits in determining native, hybrid, or introduced status and indicates the difficulties associated with newly

recruiting clones. In some instances, genetic testing may be absolutely essential to reliably determine status of a clone.

The cpDNA haplotype of the hybrid clone was haplotype M, the most widespread introduced haplotype lineage in North America. Structure analysis gave the clone an assignment of 45% to the introduced cluster and 55% to the native cluster. Further, at nearly every locus, this clone displayed one allele that is common in introduced *P. australis* and one that is common in native *P. australis* subsp. *americanus* (Table 1). Sequencing of alleles at loci GT4 and GT9 revealed diagnostic substitutions in the microsatellite flanking regions indicative of alleles from both native and introduced lineages within the sample. This confirms that this specimen from Montezuma NWR is a hybrid of introduced and native *P. australis*, the first to be positively identified using this combination of genetic techniques, and verifying that these nine microsatellite loci are effective for detecting hybrid *P. australis* in North America despite concerns of other researchers (e.g., Meyerson et al., 2012). Given that alleles common to both parental lineages are present at nearly all loci, it is also likely that it represents an F1 or first generation hybrid.

Morphologically, this hybrid individual shows similarities to both the introduced and native lineages. The height of the ligule membrane was 0.7 mm, with the adjacent hair fringe also measuring 0.7 mm, giving a combined height of 1.4 mm. This combined height is similar to the native lineage (mean 1.4 ± 0.3 mm), which has a larger ligule than the introduced lineage (mean 0.8 ± 0.3 mm; Saltonstall et al., 2004; K. Saltonstall,

TABLE 1. Observed alleles in the hybrid sample from Seneca Falls, New York and the expected occurrence of these alleles in native and introduced *Phragmites australis* from eastern North America with the maternal lineage confirmed by cpDNA haplotype. Background allele frequencies represent the percent of individuals from each respective group displaying the allele. N = native ($n = 123\text{--}134$ individuals/locus), I = introduced ($n = 145\text{--}162$ individuals/locus).

| Locus | Observed alleles (bp) | Background allele frequencies (%) | |
|-------------------|-----------------------|-----------------------------------|-------|
| | | N | I |
| GT4 | 266 | 61.5 | 0.0 |
| | 276 | 0.0 | 84.6 |
| GT8 | 176 | 1.6 | 94.9 |
| | 178 | 61.8 | 25.6 |
| GT9 | 198 | 0.0 | 77.2 |
| | 210 | 83.2 | 0.0 |
| GT11 | 142 | 6.9 | 100.0 |
| | 145 | 97.7 | 0 |
| GT13 | 147 | 0.8 | 85.5 |
| | 210 | 0.8 | 46.8 |
| GT14 ^a | 218 | 61.9 | 0.0 |
| | 183 | 100.0 | 0.0 |
| GT16 | 191 | 0.0 | 75.0 |
| | 261 | 9.4 | 74.0 |
| GT21 ^b | 265 | 85.9 | 0.0 |
| | 155 | 96.3 | 1.6 |
| GT22 ^c | 171 | 0.0 | 62.3 |
| | 191 | NA | 28.3 |

^a Background allele frequencies are based on 58 native and 28 introduced individuals.

^b Background allele frequencies are based on 81 native and 61 introduced individuals.

^c Background allele frequencies are based on 99 introduced individuals. Because this locus does not amplify well in native samples, no calculation was made for natives.

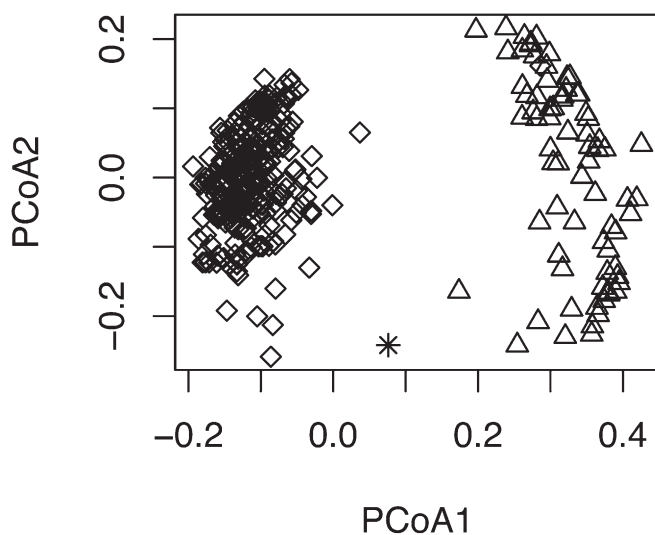


Fig. 1. Principle coordinate analysis plot of 399 individuals of *Phragmites australis* from North America based on microsatellite profiles at nine loci. Triangles represent native, diamonds represent introduced, and the star represents a hybrid individual.

unpublished data). Overwintered stems from the 2011 growing season showed stem gloss suggestive of the native lineage, but the vast majority of leaf sheaths remained on the stems, although they were not tightly attached and had opened widely and separated partially from the stem (adjacent native stems had lost all of their leaf sheaths; adjacent introduced stems retained all of their leaf sheaths firmly attached to the stem). All hybrid stems we could examine showed abundant attack by *L. hungarica* and contained its associated fungus. Native stems at the location did not show any *L. hungarica* attack, whereas the species was abundant in introduced *P. australis*. The number of hybrid stems was too low to do a full quantitative analysis of herbivore attack rates, but all hybrid stems we examined showed similar morphology and attack by *L. hungarica*. No evidence of the native herbivores that typically attack native *P. australis* (*T. willistoni* and *C. phragmites*) was found in any of the 20 examined stems.

Because this study is the first confirmed report of hybrid introduced *P. australis* × *P. australis* subsp. *americanus* in the United States, despite extensive overlap in the geographical distribution, habitat requirements, and phenology of the parental lineages, and analyses of samples by multiple researchers, it appears that compatibility between introduced and native lineages is low. The finding that we are likely dealing with a single individual and hence a single recruiting event with subsequent vegetative spread aided by soil movements at the refuge is supportive of this determination. As Meyerson et al. (2010) created hybrids with a native seed parent and we have determined the seed parent in our case to be introduced, it appears that hybridization between native and introduced *P. australis* can occur in either direction. However, given the low frequency of natural hybrids, incompatibilities between lineages may prevent widespread hybridization events. Further, the specimen discussed here represents an F1, and nothing is known about its reproductive ability, whether it can backcross and pose a threat to the genetic integrity of native *P. australis* or to the viability of any seeds it might produce. It is possible that hybrid individuals could remain infrequent and reproductively isolated and pose little threat to the distribution and abundance of native *P. australis*. This clone has been present at the site for at least 3 years and has not shown signs of rapid spread, and hybrid stems are interspersed with native and introduced stems. However, its present distribution should not be interpreted as indication of vigor of the existing hybrid or an indication of its potential invasiveness, which would require further studies. A better understanding of the genetic and morphological variation in native, introduced, and hybrid *P. australis* and the impact of each lineage on other members of biotic communities (e.g., plants, animals, microbial communities, and ecosystem processes) is needed.

Rapid detection and removal of hybrid *P. australis* stems/clones appears prudent wherever they are found, even where individuals do not appear to exhibit invasive spread—particularly if preventing spread of genetic material from introduced *P. australis* is a management goal. That hybrids, at least in this case, appear to show intermediate morphological and herbivore attack characteristics should greatly aid in surveying additional locations. However, it may also become more difficult to distinguish native and introduced plants due to the existence of morphologically intermediate hybrids. Additional scrutiny and use of multiple morphological characters will likely be required to identify different lineages and infer their origin. The most reliable characters may also vary in different parts of North America.

For example, the presence or absence of *L. hungarica* can only be used in areas where this gall midge has been located. While the species appears to be spreading rapidly from east to west (B. Blossey, unpublished data), *P. australis* populations west of Indiana and Illinois show very low attack rates and not all populations may be attacked. The use of genetic techniques appears prudent for evaluating any intermediate morphological variant.

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