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A simple screen to detect hybrids between native and introduced *Phragmites australis* in the United States and Canada

Douglas L. Wendell [a,*], Xinmei Huang [a], Brianne Gryspeerd [a], Joanna Freeland [b]

[a] Department of Biological Sciences, Oakland University, 118 Library Drive, Rochester, MI 48309-4479, USA
[b] Department of Biology, Trent University, 2140 East Bank Drive, Peterborough, ON, K9J 7B8, Canada

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ABSTRACT

In much of the United States and Canada, the common reed consists of both a native subspecies *Phragmites australis americanus,* and a highly invasive introduced subspecies *P. australis australis*. DNA testing is generally used to distinguish them definitively and is necessary to detect hybridization. We report a group of single nucleotide polymorphisms and indels in the nuclear *NRT2* gene of *Phragmites* that differentiate North American native and European-introduced populations. All native samples tested were identical in *NRT2* sequence over 1564 bases except for two positions. There were nine positions, consisting of seven base substitutions and two indels, at which all introduced samples were fixed for a different allele than the native samples. For the two indels, samples collected from northern Europe were also fixed for the same allele as the introduced samples collected across North America. One of the indels was easily detected by a PCR-RFLP assay and provides a rapid and inexpensive way to screen for hybrids between native and European-introduced populations of *Phragmites* and thus can facilitate more widespread surveillance for hybrids between native and introduced populations in North America.

Introduction

Invasive *Phragmites australis* subsp. *australis* in North America is widely sympatric with the native *Phragmites australis* subsp. *americanus.* The two lineages can be differentiated on the basis of morphological characteristics (Saltonstall et al., 2004; Catling et al., 2007; Saltonstall et al., 2014; Williams et al., 2019), but these can be difficult to differentiate in the field. Additionally, there have been multiple recent reports by land managers in Ontario, Canada, and Michigan, USA, of *Phragmites* with characteristics that are broadly intermediate to those characterized in native and introduced lineages (J. Gilbert, N. Cassel, pers. Comm.). As a result, DNA markers are often used for differentiating lineages. The available DNA markers for *Phragmites* are single nucleotide polymorphisms (SNPs) and indels in the chloroplast genome, and microsatellites in the nuclear genome. The chloroplast DNA (cpDNA) variants form haplotypes that definitively distinguish introduced (M haplotype) from native North American populations (Saltonstall, 2003b; Saltonstall, 2002). Although the cpDNA variants are powerful for identification of introduced *Phragmites* in North America, they cannot identify hybrids because, as with most angiosperms, their cpDNA is maternally inherited. Instead, microsatellite markers of nuclear genome sequences are used to identify hybrids. The available microsatellite markers in the nuclear genome are polymorphic both between and within introduced and native populations. Therefore, one use for them is to study ancestry within populations. However, although native and introduced *Phragmites* share some alleles, there are large enough differences in allele frequencies between populations for the microsatellites to also be used to distinguish these populations (Saltonstall, 2003a).

Hybridization between native and introduced populations of *Phragmites* had not been detected until genetic markers were used. Since 2010 there have been several reports of hybridization between native and the European-introduced populations of *Phragmites* (Paul et al., 2010; Saltonstall et al., 2014; Wu et al., 2015; Saltonstall et al., 2016; Lambert et al., 2016). Whether the low number of documented cases of hybridization is due to its rarity or reflects undersampling could help determine the extent to which hybridization occurs.

Expanding the molecular toolbox for *Phragmites* could inform the hybridization question. SNPs and indels allow for simple genotyping by methods like PCR-RFLP, TaqMan assays, and others, and could be used for the rapid identification of hybrids because their detection follows a codominant pattern of inheritance. In an initial

* Corresponding author.
E-mail address: wendell@oakland.edu (D.L. Wendell).

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screen for nuclear gene variants between native and introduced populations, we have identified a group of SNPs and indels for which native and European-introduced samples are fixed for alternate alleles. Here we present a rapid and inexpensive PCR-RFLP screen for potential hybrids. In contrast to microsatellite genotyping, which for native versus invasive *Phragmites* studies is typically based on at least seven loci and requires native and invasive reference databases with which to compare allele frequencies, the new diagnostic method that we report is based on a single nuclear gene region, and hence a single PCR amplification. Its ease of use can enable more widespread surveillance for hybrids of native and introduced *P australis*.

Methods

Phragmites samples

Phragmites leaf samples were collected from sites across Canada, the northeastern United States, and the southwestern United States (Table 1 and Fig. 1). We used samples from a broad geographical area as opposed to large sample sizes because the former is often more important for capturing intraspecific variation (Bergsten et al., 2012, Wyler and Naciri, 2016). The native plants (17 total) that we assayed were collected from sites separated by distances up to ~ 4700 km, and the sampled invasive plants (16 total) were from sites separated by up to ~ 4200 km.

DNA was purified from dried leaf samples using either a Macherey-Nagle Nucleospin Plant II kit (Bethlehem, PA, USA) or an E.Z.N.A. Plant DNA Kit (Omega Bio-Tek, Georgia, USA). All samples were tested using the cpDNA RFLP assay of Saltonstall (Saltonstall, 2003b) to confirm their native or introduced status. A subset of samples was further characterized as native or introduced *Phragmites* or their hybrids on the basis of nine microsatellite loci (Table 1), following the methods of Kirk et al. (2011). We also sequenced one sample from each of four European countries, representing part of the native range of the European-invasive lineage (Saltonstall, 2002).

DNA sequencing and analysis

We used cDNA sequences from nuclear genes that had been deposited in the NCBI Nucleotide database (Sayers et al., 2020) to design primers to target the corresponding gene. Nuclear genomic sequence was not available as the *Phragmites* genome has not been sequenced at the time of this study. From among the few nuclear sequences present, we found Genbank accession *AB096061*, which is the cDNA of the high affinity nitrate transporter gene *NRT2* (Araki et al., 2005), to be a promising target because preliminary analysis showed homozygosity within populations and several polymorphisms between populations. Primers to amplify the gene by PCR and nucleotide sequencing as well as additional internal primers to obtain complete sequence coverage (Table 2) were designed using the *NRT2* cDNA sequence and NCBI Primer Design Tool (Ye et al., 2012).

To produce amplicons for DNA sequencing, PCR reactions (100 μl) contained the following: 200 ng genomic DNA, 80 pmols each of *AB096061-F* and *AB096061-R* primers, and PCR master mix (either MeanGreen Master Mix from Empirical Bioscience, Grand Rapid, MI or DreamTaq Green PCR Master Mix from Thermo Fisher, Waltham, MA USA). Reactions were cycled with the following protocol: 94°for two minutes; 35 cycles of 94 °C for 45 s, 64 °C for 45 s, 72 °C for 60 s; 72 °C for 2 min.

Amplicons were purified for nucleotide sequencing using a GeneJET PCR Purification Kit (Thermo Fisher Scientific USA) and quantified using a Qubit Fluorometer (Thermo Fisher Scientific, USA). Template purity was confirmed by observation of a single band in agarose gels. Nucleotide sequencing of PCR amplicons was performed by GENEWIZ, LLC (South Plainfield, NJ, USA). Each sample was sequenced with all of the primers listed in Table 2 to obtain complete coverage on both strands and DNA sequencing traces were assembled using the CAP3 program (Huang and Madan, 1999). Nucleotide sequences were deposited into GenBank and accession numbers are given in Table 1.

Heterozygosity in individual samples was identified by manual inspection of DNA sequencing chromatograms using Applied Biosystems Sequence Scanner Software v2.0 (Applied Biosystems, Foster City, CA, USA). To identify DNA sequence polymorphisms, the sequence from native sample 11-001 (accession MT857023) was used as a reference sequence and individual sequences were aligned to it using EMBOSS Water (Madeira et al., 2019).

PCR-RFLP

To determine if any of the fixed variants detected could be assayed by PCR-RFLP, we compared the sequences surrounding the polymorphic sites. We found one unique restriction site in the native sequence that was disrupted by a deletion in the introduced and is described further in the results.

To detect variants by PCR-RFLP test, PCR reactions were performed as above except that the reaction volumes were 20 μl and contained 20–100 ng genomic DNA and 10 pmols each of *AB096061-F* and *AB096061-R* primers (expected fragment length 1668–1775 base pairs). After PCR each reaction was divided in half. One half was digested with *Xap*I (Thermo Fisher Scientific, USA) according to manufacturer's instructions and the other half was placed in the same buffer but without enzyme. After digestion, samples were loaded into 1.2% agarose gels containing SYBR Safe DNA stain (Invitrogen, Carlsbad, CA, USA) and subjected to electrophoresis at 150 V for 40 min.

Results and Discussion

There were 9 positions in the *NRT2* sequence that were polymorphic between native and introduced samples with each group being fixed for a different allele (Table 3). There were also ten positions that were polymorphic both within and between native and introduced samples (Table 4). At eight of these positions, all native samples were homozygous for the same allele while there was polymorphism within both the North American introduced population and the European population. There were two positions in the entire sequence that showed polymorphism within the native samples (Table 4).

A simple and quick method to assay a SNP or indel is PCR-RFLP (Konieczny and Ausubel, 1993) so we examined the sequences flanking all of the polymorphisms to determine if any were fortuitously located in restriction enzyme recognition sites. We found a unique AAATTT sequence in the native samples which is disrupted by the four-base deletion that is present in all introduced samples, as well as all European samples (henceforth referred to as *NRT2-Δ4*). This sequence is a cutting site for the enzymes *ApoI*, *AcsI*, and *XapI* (isoschizomers which all recognize RAATTY). *XapI* was used because of availability to us, but any of its isoschizomers should work as well. We used this to design a PCR-RFLP assay that clearly distinguishes native and introduced genotypes. Because native "cut" and introduced "uncut" are co-dominant, this PCR-RFLP can also identify heterozygotes. As expected a hybrid sample collected in Nevada (a region that has relatively high hybrid frequencies (Saltonstall et al., 2016)) and identified from microsatellite data by Lambert et al. (2016) shows both uncut and cut bands (Fig. 2).

Table 1
Phragmites samples used in this study. All samples were identified as either native or invasive based on the cpDNA PCR-RFLP method of Saltonstall (2003b). Additional information is provided here for samples that were further characterized on the basis of cpDNA sequences (cpDNA haplotype) or nuclear microsatellite genotypes (msat).

Accession	Sample ID	cpDNA haplotype	msat.	Location	Latitude, Longitude	Source
A. Native Lineage Phragmites samples						
MT857023	11-001			Fon Du Lac, WI	43.800912, −88.467925	1
MT903260	2-002			Au Train Lake, Alger County, MI	46.41033, −86.83371	2
MT903261	2-014			Ogontz Bay, Delta County, MI	45.827926, −86.775339	2
MT903262	3-001			Bullard Lake Fen, Hartland Twp, MI	42.642793, −83.702505	3
MT903263	10-001		Y	Buckton River, AB	58.29319, −111.87429	4
MT903264	10-002		Y	First Chainey Lake, AB	58.797127, −110.983094	4
MT903265	LP3	E2	Y	Long Point Provincial Park, ON	42.5818, −80.3907	5, 10
MT903266	LP4	E2	Y	Long Point Provincial Park, ON	42.5818, −80.3907	5, 10
MT903267	Sack3	E4	Y	Sackville, NB	45.9320, −64.3384	5, 10
MT903268	Sack4	E4	Y	Sackville, NB	45.9320, −64.3384	5, 10
MT903269	WC1		Y	Wascana Creek, SK	50.4315, −104.6082	4
MT903270	WC5		Y	Wascana Creek, SK	50.4315, −104.6082	4
MW960627	UT-3			San Pitch River, Sanpete Co., UT	39.546634, −111.516889	6
MW960628	CA-1	H		Santa Clara River; Santa Paula, CA	34.3553, −119.0064	7
MW960629	CA-3	B		Little Caliente Spring, Los Padres Nat. Forest, CA	34.54142, −119.61943	7
MW960630	NV-2			Virgin River, Mesquite, NV	36.7938, −114.0828	7
MW960631	NV-3			Northshore Rd Bridge, Las Vegas Wash, NV	36.12222, −114.90485	7
	NV-4		Y hyb	Below Pond 7, Las Vegas Wash, NV	36.0932, −115.0152	7
B. Introduced Lineage Phragmites samples						
MT903251	Eld2-1	M	Y	Eldridge, ME	43.2908, −70.5716	5
MT903252	Scar3-2	M	Y	Scarborough, ME	43.5767, −70.3772	5
MT903253	2-007			Ogontz Bay, Delta County, MI	45.8333, −86.7866	2
MT903254	3-002			Bullard Lake Fen, Hartland Twp, MI	42.642793, −83.702505	3
MT903255	C334S45			Black Lake, Mason County, MI	44.157, −86.151	8
MT903256	Poc1-3	M	Y	La Pocataire, QB	47.3779, −70.0501	5, 10
MT903257	Mon3	M	Y	Montreal, QB	45.5970, −33.1288	5, 10
MT903258	JC4		Y	Jackson Creek, Peterborough, ON	44.3112, −78.3398	4
MT903259	Rond1-14	M	Y	Rondeau Provincial Park, ON	42.3142, −81.8520	5, 10
MW960632	UT-1			Liberty Beach, Utah Lake, UT	40.142113, −111.802797	6
MW960633	UT-2			Canyon View Park, Spanish Fork, UT	40.082258, −111.602418	6
MW960634	CA-4	M		Salinas River, Atascadero, CA	35.4993, −120.6520	7
MW960635	SAPH1			riverside in Spaardam, Netherlands	52.417887, 4.691190	4
MW960636	ICP5			roadside near Camargue, France	43.572112, 4.307109	4
MW960637	WH12	M		riverside, in Milton Keynes, England	52.023365, −0.713200	9
MW960638	K09			highway stop between Kallmunz and Beratzhausen, Germany	49.114436, 11.873903	4
C. European Samples						
MW960635	SAPH1			riverside in Spaardam, Netherlands	52.417887, 4.691190	4
MW960636	ICP5			roadside near Camargue, France	43.572112, 4.307109	4
MW960637	WH12	M		riverside, in Milton Keynes, England	52.023365, −0.713200	9, 10
MW960638	K09			highway stop between Kallmunz and Beratzhausen, Germany	49.114436, 11.873903	4

Sources of samples
1 Conrad Bekta, Wisconsin Department of Natural Resources, USA.
2 Darcy Rutkowski, Upper Peninsula Resource Conservation and Development Council. Marquette, MI, USA.
3 Kurt Kowalski, U.S. Geological Survey – Great Lakes Science Center, Ann Arbor, MI, USA
4 This work.
5 (Kirk et al., 2011).
6 Lyle Bingham, author and citizen scientist, Payson, UT.
7 (Lambert et al., 2016).
8 Carolyn Henne, Huron-Manistee National Forest, Cadillac, MI, USA.
9 (Paul et al., 2011).
10 (Vachon and Freeland,2011).

Fig. 1. Locations of North American (A) native and (B) introduced samples used in this project. The star in part A shows the location of a hybrid sample with native seed parent. We also included four introduced samples from their native range in western Europe (not shown on map).

Table 2
Primers for PCR and DNA Sequencing of *NRT2*. Use refers to primers that were used for PCR amplification, sequencing, or both.

Primer	Sequence	Use
AB096061-F	AAGACTCGAGAGGCCAGCTA	PCR, sequencing
374F	CGTCTTCTGCATGTCCCTCA	sequencing
967F	CTACTACGACCACTTCGACCTA	sequencing
533R	GCCAGAGAGAAGCCGATCAA	sequencing
1035R	TTGGCCATTCCGAAGCAA	sequencing
AB096061-R	ACGTGCGTCTTATACGTGCT	PCR, sequencing

This screening assay is dependent on complete restriction digestion. An incomplete digestion of a native sample could resemble the profile of a hybrid (false positive), and a failed restriction digest on an actual hybrid DNA sample would give the same profile as homozygous introduced (false negative). Therefore, users should always include a confirmed native sample as a control for the restriction digest.

The nine nuclear gene variants (seven SNPs and two indels) that are fixed for different alleles in the native and introduced populations are powerful for differentiating native, introduced *Phragmites*, and their hybrids. The microsatellite markers currently available for *Phragmites* are more polymorphic but some loci have shared alleles between native and introduced populations (Saltonstall, 2003a; Paul et al., 2010). Use of the available microsatellites requires capillary electrophoresis and specialized software to distinguish alleles and involves testing with multiple markers followed by statistical analysis to determine the probability of a sample being native, introduced, or hybrid (Paul et al., 2010). While the suite of microsatellite markers is much more appropriate for studies of population genetics, the simple and relatively inexpensive PCR-RFLP test that we report here should be sufficient for simply differentiating native from invasive *Phragmites* and identifying their hybrids.

The first reports of hybridization between native and introduced populations of *Phragmites* in North America were in 2010 and there have so far been a total of five published reports (Paul et al., 2010; Saltonstall et al., 2014; Wu et al., 2015; Saltonstall et al., 2016, Lambert et al., 2016). Hybridization may be very rare, or the question may be suffering from undersampling because identification of hybrids depends on having investigators testing for them. The *NRT2-Δ4* PCR-RFLP can be used as an initial screen because it only requires a single PCR and digest, and then be confirmed with other established methods.

We have not yet surveyed the sequence of *NRT2* in the Gulf Coast (Pellegrin and Hauber, 1999) type of *Phragmites*. Therefore, the *NTRT2* polymorphisms should be used for screening for hybrids between native *Phragmites australis americanus* and the introduced strains of European origin, namely haplotype M as described (Saltonstall, 2002). Users should couple this test with the standard cpDNA tests for lineages (Saltonstall, 2003b) and consider it in the context of their local populations.

A *Phragmites* reference genome would be valuable to understanding the different populations of this organism. We used the *NRT2* gene in this study because it was one of few nuclear sequences available at the time and lent itself to our study. Given that we have identified interesting and useful polymorphisms out of the few nuclear single-copy sequences available, we wonder what riches can be found in the whole genome. A full-scale analysis that incorporated diverse populations would increase the understanding of differences between native and introduced *Phragmites* and the mechanisms of invasion.

Conclusions

The objective of this study was to identify simple nuclear DNA polymorphisms that can be used to screen for hybrids between North American native and introduced *Phragmites*. We identified nine such polymorphisms and developed a simple PCR-RFLP test for one of them. A simple and rapid screen will allow greater surveillance for hybrids.

CRediT authorship contribution statement

Douglas L. Wendell: Conceptualization, Methodology, Writing – original draft, Investigation, Resources. **Xinmei Huang:** Investi-

Table 3
NRT2 polymorphisms that show fixed differences between North American native and introduced/European *Phragmites* lineages.

Position	Flanking DNA	Native	Intr	Eur	Gene Structure
101	TGCCCGACGA[ ]GTTGCAGCGA	T	C	C,T	synonymous
103	CCCGACGATG[ ]TGCAGCGAGC	T	A	A,T	missense V > N
499–502	GCCCGATGAA[indel]ATATGTACAT	ATTT	del	del	intron
531–533	AATTACTGAA[indel]CGTTGCCGCC	CGT	del	del	intron
975	GCGTGGAGCT[ ]ACCACCGACA	T	G	G	synonymous
1287	TCGGCGTCAC[ ]CCTTTCGTCA	C	T	C,T	synonymous
1452	GCACGCTTCC[ ]GTGGTGTTCG	A	C	C	synonymous
1508	CCCAGCGCCG[ ]CGCCGTCGAG	G	A	A,G	missense G > D
1532	CACTACTACA[ ]CTCGGAGTGG	A	G	G	missense N > S

Table 4
NRT2 polymorphisms that vary within North American native, North American introduced, or European *Phragmites* lineages.

Position	Flanking DNA	Native	Introduced	European
260	CGCCGCTCGT[ ]CCCATCATCC	C	C,T	C,T
486	GTACGTCTCA[ ]GTGCCCGATG	C	C,T	C,T
514	TATGTACATT[ ]CAACATAATT	G	G,T	C,G
536	CTGAACGTCG[ ]TGCCGCCATG	T	C,T	C,T
547	TGCCGCCATG[ ]TGCATGCATG	T	C,T	C,T
726	ACGACGTCAT[ ]CGCAAGTGCG	C	C,T	C,T
849	ACGGCAACCT[ ]AGGAGCCTCC	C	C,G	C,G
1179	CTGGCGGCGC[ ]TTCTGCCTCT	C,T	C	C
1185	GCGCCTTCTG[ ]CTCTGGCTCG	C	C,T	C
1449	CGTGCACGCT[ ]CCAGTGGTGT	G,T	G	G

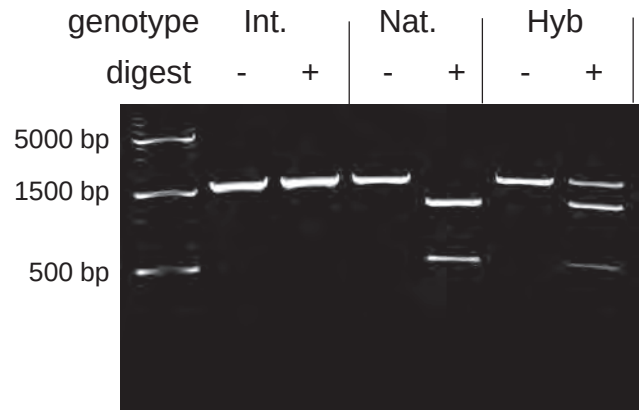

Fig. 2. Representative banding patterns from RFLP analysis of native, introduced and hybrid *P. australis* samples from North America using the nuclear marker *NRT2-Δ4*. Genomic DNA was amplified with primers *AB096061*-F and *AB096061-R* and then digested (+) with *XapI* or or was not digested (−) as described in Materials and Methods. Genomic DNA were introduced (Int), native (Nat), and a hybrid of the two (Hyb). The size standard was a GeneRuler 1 kb Plus Ladder (Fisher Thermo Scientific, Waltham, MA, USA).

gation. **Brianne Gryspeerd:** Investigation, Resources. **Joanna Freeland:** Conceptualization, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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