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Rapid Communication

Eurasian haplotype M *Phragmites australis* (Cav.) Trin. *ex* Steud., 1841 invasion in Minnesota, USA: a baseline for further monitoring in the upper Mississippi watershed

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Abstract

The cryptic invasion of North American aquatic ecosystems by non-native haplotypes of *Phragmites australis* (Cav.) Trin. *ex* Steud., 1841, has been well documented. Most research has focused on eastern Canada, and the eastern seaboard, St. Lawrence waterway, southwest (Utah), and Gulf coast regions of the United States. Less has been published on the extent of this invasion in the north central United States. In this report, 69 populations of *Phragmites australis* were identified and sampled within the Minnesota and Mississippi River corridors of Minnesota, as well as from roadway drainage ditches within the greater Minneapolis-St. Paul metropolitan region (MSP). Restriction fragment length polymorphism analysis and DNA sequencing were used to determine the cpDNA lineage of each population. All populations sampled within the river valleys were native North American haplotype E, as were most MSP populations. However, numerous isolated populations of Eurasian haplotype M *Phragmites australis* were identified along MSP transportation corridors. Nuclear microsatellites indicated that these M haplotype populations are not clonal. These results show that Eurasian haplotype M *Phragmites australis* has become established in at least one region of Minnesota, but has not yet widely invaded the riparian wetlands of Minnesota's two largest rivers. This provides a baseline for continued monitoring of the spread of this invasive plant.

Key words: *Phragmites*, Minnesota River, Mississippi River, invasion, transportation, ditch

Introduction

Common reed, *Phragmites australis* (Cav.) Trin. *ex* Steud., 1841 (hereafter *Phragmites*) is a cosmopolitan, hydrophytic grass found in wetlands and estuaries throughout North America. Plants grow to heights of up to four meters and occupy hydric soils ranging from occasionally saturated to continuously inundated. In recent decades, the aggressive expansion of *Phragmites* populations in many North American wetlands has been noted, and has caused concern for the stability of these ecosystems (Chambers et al. 1999; Galatowitsch et al. 1999). Because of the subtle differences in the morphology and phenology of *Phragmites* subspecies throughout the world, researchers surmised that native North American populations may have been cryptically invaded by a morphologically similar strain or subspecies of *Phragmites* not native to North America (Marks et al. 1994). Conclusive genetic evidence for this cryptic invasion of native North American haplotype *Phragmites australis* (hereafter native *Phragmites*) populations by introduced Eurasian haplotype M (hereafter Eurasian *Phragmites*) was reported by Saltonstall (2003a), and has since driven extensive studies of this important ecological phenomenon (Kettenring and Mock 2012; Paul et al. 2010; Meyerson et al. 2010; McCormick et al. 2010). In 2013, Meyerson and Cronin reported evidence of an additional invasion by a second introduced haplotype (L), further complicating the history of the non-native *Phragmites* spread in North America.

Eurasian *Phragmites* was recently identified in the Platte and Missouri River basins in the central US

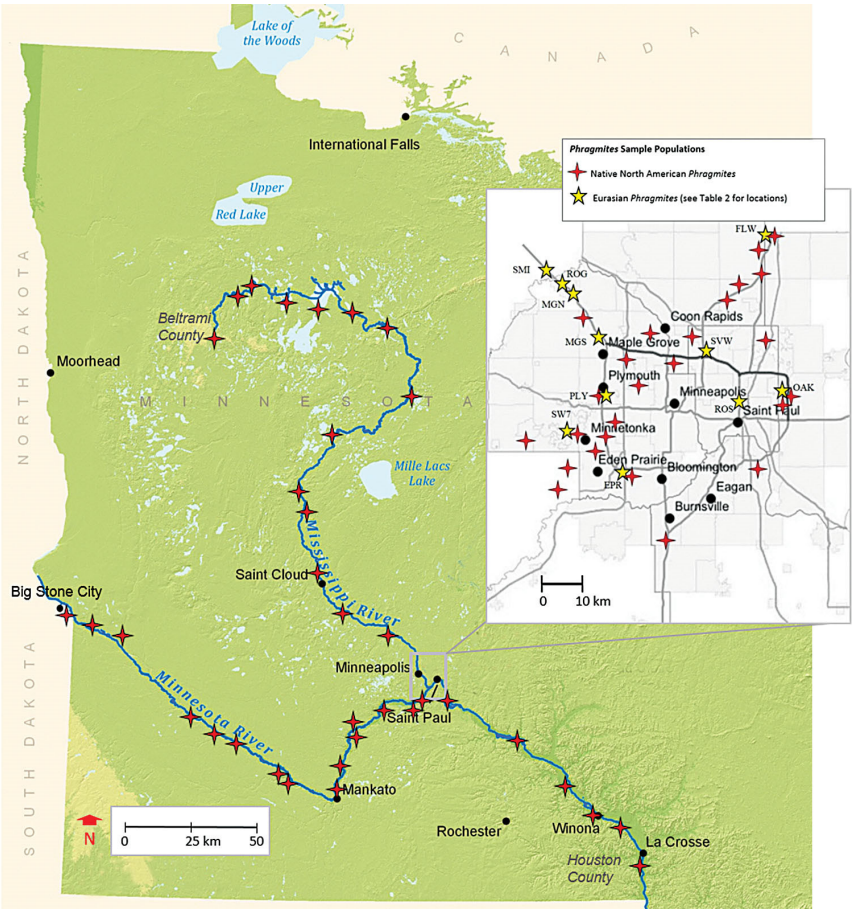

Figure 1. *Phragmites australis* population sample locations.

plains, and poses a significant threat of disruption to that ecosystem (Larson et al. 2011). However, little information about the spread of Eurasian *Phragmites* in the upper Mississippi River watershed has been published. The purpose of this study was to evaluate the extent of Eurasian *Phragmites* in the northern-most region of the Mississippi River watershed, including the Mississippi and Minnesota River corridors within Minnesota, and to establish a starting point from which to monitor the invasion of Eurasian and other non-native *Phragmites* subspecies into aquatic habitats in this region.

Material and methods

Sample collection

Phragmites populations (n=14) were identified and sampled at 15 to 25 km intervals within the Minnesota River valley from Big Stone City, South Dakota, to the confluence of the Minnesota and Mississippi Rivers in St. Paul, Minnesota. Populations (n=20) within the Mississippi River corridor from its headwaters in Beltrami County, Minnesota, to the Iowa state line in Houston County, Minnesota, were similarly identified and sampled. In addition, populations (n=36) within drainage ditches and wetlands associated with the major federal (US and Interstate) and Minnesota state highways in the greater Minneapolis-St. Paul metropolitan area (MSP) were also sampled. Population sizes ranged from very small, isolated ditch patches (100 – 600 m^2) to large expanses of marshes up to 200 ha. The small MSP patch populations were often in closer proximity to each other than those in the river corridors (Figure 1). In three locations (EPR, SW7, and FLW), two morphologically discrete populations (one identified in the field as probable-Eurasian, and the other as probable-native North American *Phragmites*) were discovered within 100 m of each other, and sampled accordingly. Presumptive field identification was based on recently published guidelines (Saltonstall et al. 2014).

Within each population, individual leaves were collected from mature shoots at 5 m intervals along a transect through the widest part of the stand. Leaf sheath morphology (loose or tight, color, texture) was recorded for each specimen. Specimens were then immediately rinsed with sterile water, blotted dry, placed in sterile collection tubes, labeled, and stored at 4°C during transport. All samples were maintained at -20°C until DNA extraction.

DNA extraction and chloroplast DNA lineage identification

DNA was extracted from each leaf specimen with MoBio Labs Ultraclean PowerPlant® Pro DNA extraction kits (MoBio Laboratories, Inc., Carlsbad, CA, USA) and stored at -20°C. Lineage (i.e. native or Eurasian haplotype) for each plant sample was determined through PCR amplification and subsequent Restriction Fragment Length Polymorphism (RFLP) analysis of the chloroplast DNA intergenic spacer regions *trnLb* (*trnT-trnL)* and *rbcL* (*rbcL-psaI)* as described by Saltonstall (2003a). PCR reactions for each region contained 1x PCR master mix (GoTaq® G2 Master Mix, Promega Corp., Madison, WI, USA), 10 pmol of each primer, and 0.5 – 1µg template DNA in a total reaction volume of 50 µl. The *trnLb* and *rbcL* primer pair sequences were those described by Saltonstall (2003a), and were generated by Integrated DNA Technologies, Inc., Coralville, IA, USA. Thermal cycling was performed with a BioRad T100 cycler (BioRad Corp., Hercules, CA, USA) using the following conditions: Initial denaturation for 2 min at 94ºC followed by 35 cycles of 94ºC for 45 sec, 52ºC for 45 sec, and 72ºC for 60 sec; followed by a final extension at 72ºC for 2 min. RFLP was performed exactly as indicated by Saltonstall (2003a). *Hha*I and *Rsa*I restriction enzymes for the RFLP analysis were purchased from New England Biolabs, Inc., Ipswich, MA, USA.

Additionally, *trnLb* and *rbcL* region amplicons (above) from one representative sample of each collected population were sent for sequencing to Functional Biosciences, Inc., Madison, Wisconsin, USA, on an Applied Biosystems 3730XL DNA analyzer (Big Dye V3.1 protocol). For more specific haplotype determination, sequences were aligned with and compared to Saltonstall's (2002) GenBank® type specimens (Popsets 13508465, 13508473, 18251930, 18307892) with BLAST® National Center for Biotechnology Information, Bethesda, MD, USA), and deposited in GenBank® (accession numbers in Supplementary material Table S1).

Microsatellite amplification and scoring

Six previously identified nuclear microsatellite loci (PaGT4, PaGT9, PaGT12, PaGT13, and PaGT16; (Saltonstall 2003b)) were also amplified from the extracted DNA of several plants from each of the 10 populations identified by RFLP analysis as Eurasian *Phragmites*. Primer sequences, PCR reaction mixtures and cycling conditions were as previously published (Saltonstall 2003b). PCR reactions for each region contained 1x PCR master mix (GoTaq® G2 Master Mix, Promega Corp.), 4 pmol of each primer (Integrated DNA Technologies, Inc., Coralville, IA, USA), and 100–200 ng template DNA in a total reaction volume of 20 µl. All forward primers contained a 5' Fluorescein (6-FAM) label. Thermal cycling was performed with a BioRad T100 cycler (BioRad Corp.) using the following conditions: Initial denaturation for 12 min at 94ºC followed by 35 cycles of 94ºC for 30 sec, 54ºC for 30 sec, and 72ºC for 4 sec; followed by a final extension at 72ºC for 2 min. Capillary electrophoresis to determine amplicon sizes was performed on an ABI 3730XL DNA analyzer by Genewiz, Inc. (Plainfield, NJ, USA), using GeneScan™ 500 LIZ™ dye size standards (Applied Biosystems, Foster City, CA, USA), and scored manually with Peak Scanner 2.0 software (Applied Biosystems).

Results

RFLP determination of chloroplast lineage

Individual shoots (n=365) from 69 isolated *Phragmites* populations within the sample areas were analyzed in this study (Table 1). RFLP analysis of the *trnLb* and *rbcL* cpDNA regions from these populations indicated that 11 populations (16%) were Eurasian *Phragmites*, while the remaining 58 populations (84%) were of native North American lineage. None of the populations contained a mixture of native and Eurasian specimens, although three of the Eurasian *Phragmites* stands (EPR, SW7, and FLW) were found within 100 m of native *Phragmites*. Furthermore, none of the Eurasian *Phragmites* populations were found in either the Minnesota River or Mississippi River sample corridors, and were only found in wet ditches or wetlands immediately adjacent to major roadways in the MSP collection area. As indicated in Figure 1, the 11 Eurasian *Phragmites* populations were distributed throughout the MSP area, where numerous native *Phragmites* stands were also located.

Table 1. cpDNA[a] lineage of *Phragmites australis* populations in Minnesota.

		Native Populations[b]		Eurasian (M) Populations[c]	
Corridor/Study Area	Populations (Samples)	n	%[d]	n	%[d]
Mississippi River Valley	14 (83)	14	*100%*	0	*0%*
Minnesota River Valley	20 (97)	20	*100%*	0	*0%*
MSP Transportation System	35 (185)	24	*69%*	11	*31%*
Total	69 (365)	58	*84%*	11	*16%*

[a]Based on *trn*b and *psa*I region RFLP analysis and sequencing
[b]North American *Phragmites australis* haplotype E (Saltonstall 2002)
[c]Eurasian *Phragmites australis* haplotype M
[d]Percent of populations within the corridor (e.g. Mississippi River valley); under totals, percentage indicates percent of stands in the entire study area

Table 2. Eurasian *Phragmites australis* haplotype M populations in MSP region.

Population Identity	Samples (n)	Patch Size (m^2)	Location[a]	Genotypes in Population[b]
EPR	3	400	44.890051, -93.401297	1
FLW	3	200	45.287306, -93.002484	1
MGN	3	500	45.163824,-93.521088	2
MGS	3	100	45.150978, -93.502260	2
OAK	3	120	44.998791,-92.957814	1
PLY	3	600	45.007746,-93.456775	2
ROG[c]	-	150	45.195167, -93.551673	-
ROS	3	260	45.009972, -93.088579	1
SMI	3	225	45.218649, -93.595774	2
SVW	3	600	45.080846, -93.184853	2
SW7	3	155	44.908946,-93.530571	1

[a]Population location coordinates (Global Spherical Mercator (WGS84) system).
[b]Genotype here is defined as a specimen with ≥2 well-defined microsatellite allele polymorphisms compared to all other specimens within the population at a location (e.g. EPR). All genotypes except one from the MGN site were unique to the entire study area (i.e. existed at no other locations).
[c]Not amplified for microsatellites

All plants exhibiting native cpDNA on RFLP had morphological features (loose leaf sheaths, glossy stems, maroon internodes), characteristic of native *Phragmites* haplotypes, while those identified with the molecular methods as the Eurasian haplotype had the tight leaf sheaths and dull stems common in the introduced haplotype M (Swearingen and Saltonstall 2010; Saltonstall et al. 2014). No morphologically intergrade specimens were observed, nor did any of the identified Eurasian specimens express microsatellite genotypes that suggested Eurasian × native hybridization (Saltonstall et al. 2014), including those from the three locations where the Eurasian and native *Phragmites* are essentially sympatric.

trnLb and rbcL cpDNA sequences

Sequences for the *trnLb* and *rbcL* cpDNA regions of representative samples from each of the 68 *Phragmites* populations were aligned and compared with homologous type specimen sequences in the GenBank popsets described above to confirm lineage and determine specific haplotype based on Saltonstall's (2002) classification system. The *trnLb* sequences for all *Phragmites* populations identified by RFLP as native *Phragmites* were identical to each other, as were sequences from the *rbcL* region. The sequences of both regions from this group matched Saltonstall's Haplotype E type specimens

(match identity 100% over a 254 base query; E-value 3×10^{-129}). Similarly, all *trnLb* and *rbcL* cpDNA region sequences from populations determined by RFLP analysis to be Eurasian *Phragmites* were identical, respectively. These sequences matched Saltonstall's haplotype M type specimens (match identity 100% over a 777 base query; E-value 3×10^{-160}).

Microsatellites

Table 2 displays the number of microsatellite genotypes identified within each Eurasian *Phragmites* population, as well as population size and geographic location. A total of 29 different alleles and 14 genotypes were identified over the six microsatellite loci in the ten Eurasian *Phragmites* populations.

Discussion

Prior to this report, the only published account of Eurasian *Phragmites* occurring in Minnesota was based on a single, preserved herbarium specimen collected in the early 1960's, suggesting that the invasive strain has been in the region for at least five decades (Saltonstall 2002). The results of this survey demonstrate that numerous, small patch populations of Eurasian *Phragmites* exist in the roadway drainage system of a 3,500 km^2 area of the MSP metropolitan region of the study area. However, *Phragmites* populations in the two major riparian corridors of Minnesota are composed primarily, if not exclusively, of native *Phragmites* haplotype E.

The absence of Eurasian *Phragmites* from wetlands associated with the two major rivers that traverse Minnesota initially appeared surprising, particularly because multiple populations of Eurasian *Phragmites* were found throughout the MSP highway ditch system, much of which ultimately drains to these rivers. While it is possible, or even likely, that Eurasian *Phragmites* has reached the major river corridor wetlands of Minnesota, the results reported here suggest that the Eurasian haplotype has either not yet been broadly disseminated to these riparian marshes, or has become established at such low frequency that the plants remain undetected. The pattern of Eurasian *Phragmites* becoming established in and disseminating from road ditches prior to expansion to inland marshes and waterways has been well documented in eastern Canada (Guo et al. 2013; Brisson et al. 2010; Jodoin et al. 2008; Lelong et al. 2007). Our data indicate that the upper Mississippi river watershed area may be in the early stages of a similar invasion pattern.

Plausible explanations exist for the current rarity of Eurasian *Phragmites* in the Minnesota and Mississippi river valleys in Minnesota, and its relatively higher frequency in urban road ditch wetlands. For example, greater salinity in urban ditches from winter road salt runoff has been implicated as a selective pressure favoring Eurasian *Phragmites* plants in this environment (Brisson et al. 2010; Vasquez et al. 2005; Richburg et al. 2001). Furthermore, hybrids between ovule-donor native and pollen-donor Eurasian *Phragmites* plants would produce native cpDNA haplotype plants, which would also carry Eurasian *Phragmites* nuclear DNA. These hybrids would be undetectable by current cpDNA methods. Such hybridization has been demonstrated in controlled environments (Meyerson et al. 2010), and in the field (Wu et al. 2015; Saltonstall et al. 2014). However, if hybrid populations exist in Minnesota, it is unlikely that all would develop exclusively as cpDNA natives (Saltonstall et al. 2014; Meyerson et al. 2012; Meyerson et al. 2010).

Nuclear microsatellites from a small number of samples of each Eurasian *Phragmites* population were used to determine whether or not the Eurasian *Phragmites* populations found in the study area were clonal. All of the Eurasian *Phragmites* populations in this study exist within a relatively small (3,500 km^2) geographic area. Genetically identical populations would suggest a single vegetative source for the MSP Eurasian *Phragmites* populations. However, each of these populations differed from all the others by at least one allele. Five of the Eurasian *Phragmites* populations (SMI, MGN, MGS, PLY and SVW) contained multiple genotypes. In four of those populations, different genotypes were evident at two or more microsatellite loci. Multiple genotypes within these small Eurasian *Phragmites* populations may be the result of seeds from multiple parent populations germinating in the same location. Alternatively, such variation may also be the result of sexual reproduction within or among these Eurasian stands, or a combination of both seed recruitment and sexual reproduction. Regardless, the results indicate that the Eurasian *Phragmites* stands in this study area are not clonal.

Considered together, these results show that although relatively few Eurasian *Phragmites* populations currently exist in the study region, and while those populations are small, they exhibit at least some genetic diversity. The ecological ramifications of this are significant. Long distance seed dispersal by *Phragmites* is well documented (Lambertini et al. 2012). Further, seed production by sexually reproductive populations of Eurasian *Phragmites* has been shown to be more robust than that of native *Phragmites*. These mechanisms afford Eurasian *Phragmites* the means to outcompete older native populations (Kettenring and Mock 2012; McCormick

et al. 2010). In addition, the role of roads and ditches in advancing the spread of invasive plants has been well established (Menuz and Kettenring 2013; Brisson et al. 2010). It is also clear that Eurasian *Phragmites*, once transported, can successfully establish itself in wetlands along large inland rivers (Larson et al. 2011). Therefore, it seems inevitable that Eurasian *Phragmites* within drainage ditches of this study area will be transported as either seeds or rhizome fragments to local tributaries, inland marshes, and both the Minnesota and upper Mississippi Rivers, if this has not already occurred.

Wetlands cover approximately four million hectares of Minnesota and are of critical importance to wildlife and commerce in this state (Kloiber and Norris 2013). Disruption of these resources by invasive non-native plants and animals threatens not only the biodiversity of aquatic ecosystems, but also their commercial value in sporting, tourism, and natural food industries (Mitsch and Gosselink 2015; Oslund et al. 2010). Understanding the dynamics of biological invasions as they are occurring is critical to any effort to minimize ecological damage. Our data can be used as a reference point for long-term *Phragmites* monitoring in the two largest watersheds in Minnesota. Moreover, the recurrent pattern of Eurasian *Phragmites* using establishment in road ditches as a springboard to invading a region's rivers, which is likely occurring in the upper Mississippi watershed, may provide incentive for conservation and natural resource agencies to act quickly before these sexually reproductive populations cast their viable seeds into new, unexploited habitats of the upper Midwestern United States.

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Supplementary material

The following supplementary material is available for this article:

Table S1. North American *Phragmites* population locations and cpDNA accession numbers (GenBank).

This material is available as part of online article from:
http://www.reabic.net/journals/bir/2016/Supplements/BIR_2016_Melchior_Weaver_Supplement.xls