COMPARISON OF MORPHOLOGICAL VARIATION INDICATIVE OF PLOIDY LEVEL IN *PHRAGMITES AUSTRALIS* (POACEAE) FROM EASTERN NORTH AMERICA

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ABSTRACT. Variation in ploidy levels in *Phragmites australis* is a well-documented phenomenon although North American populations are less studied than European ones. It has been suggested, based on morphological measurements, that native and introduced *P. australis* subspecies in North America represent different ploidy levels. The objectives of this study were to assess whether guard cell size and stomatal density, morphological differences indicative of variation in ploidy level between native and introduced *P. australis*, are truly associated with different ploidy levels as measured by flow cytometry. Significant differences in guard cell size and stomatal densities were found between subspecies, with native plants having larger guard cells and lower stomatal density. However, no differences in 2C DNA content were found. Although these morphological measurements are significantly correlated with subspecies and can be added to the list of useful morphological characters distinguishing the two subspecies, it does not appear that they are accurate indicators of ploidy levels. Potential implications of these differences on the invasion biology of introduced *P. australis* are discussed.

Key Words: common reed, invasive plant, guard cell size, polyploidy, stomatal density, wetland

Approximately 70% of grass species are polyploid (Masterson 1994; Stebbins 1956), and in many cases, polyploid complexes occur within species (Keeler 1998). The evolutionary success of polyploids has often been attributed to the consequences of having multiple genomes, with individuals of higher ploidy levels considered to be more adaptable to differing conditions due to genetic advantages

that facilitate their establishment and persistence (Comai 2005; Stebbins 1971). Once populations of different polyploid levels are established, mating among polyploids of different origins may further enrich the gene pool or mating may be limited by chromosomal incongruencies between ploidy levels (Soltis and Soltis 2000).

The physiological effects of changes in ploidy level have been investigated in several crop species (Madlung and Comai 2004; Tal 1980). Typically, plants of higher ploidy levels have larger but sparser stomata, reduced transpiration rates, higher relative water contents, and better water balance than their diploid progenitors (Warner and Edwards 1989). Polyploidization is thus a potential mechanism for changes in stomatal size and density in plant species, since with increased cell size, stomatal density typically decreases (Hetherington and Woodward 2003). This may be advantageous, for example, in arid habitats where water loss is of concern (Hunter et al. 2001). In wetland plants where water loss is typically not a problem, limitations of other resources, such as CO_2 in very dense plant communities, could make increased stomatal density advantageous.

Phragmites australis (Cav.) Trin. *ex* Steud. (Arundinoideae) is a perennial grass species found throughout the world (Haslam 1972). It is well recognized that this species forms a polyploid complex with tetraploids being most common worldwide and ploidy levels ranging from $3x$ to $22x$ (Clevering and Lissner 1999 and references therein). In North America, karyotypes of $3x$, $4x$, $6x$, and $8x$ plants have been documented with tetraploids in the majority (Gervais et al. 1993; Keller 2000; Pellegrin and Hauber 1999). In Europe, although the distribution of different ploidy levels has been documented (Clevering and Lissner 1999), no correlations with specific habitats or growing conditions have been noted for the different ploidy levels (Björk 1967; Djebrouni 1992), and sites where multiple ploidy levels grow adjacent to one another have been found (Björk 1967; Paucã-Comãnescu et al. 1999).

In North America, genetic evidence has demonstrated that populations of both native *Phragmites australis* subsp. *americanus* Saltonstall, P.M. Peterson & Soreng (hereafter referred to as native *P. australis*) and introduced *P. australis* of Eurasian origin (hereafter, introduced *P. australis*) are found across the continent, with introduced *P. australis* now being a dominant plant in many

emergent wetlands of the Atlantic coast region (Saltonstall 2002, 2003b). Studies of this aggressive invader have demonstrated that it can rapidly form a monoculture and will, in most cases, outcompete other native vegetation following establishment (Chambers et al. 1999). This process may be enhanced by eutrophication and physical disturbance of the environment (Minchinton and Bertness 2003; Silliman and Bertness 2004). In contrast, although it persists in other parts of the continent, native *P. australis* has disappeared from much of the Atlantic coast where it was found historically (Saltonstall 2002). Remnant populations from coastal regions, as well as paleobotanical specimens (Orson 1999), suggest that native *P. australis* typically grows in mixed plant communities (Meadows and Saltonstall 2007). Although the two subspecies are often found growing adjacent or in close proximity to one another, to date no evidence of hybridization between native and introduced *P. australis* exists (Saltonstall 2003c) and no plausible explanation for this lack of interbreeding has been shown.

Morphologically, native and introduced *Phragmites australis* can be difficult to distinguish due to high phenotypic variability, although ligule height, glume length, and several other morphological characters can be used to identify the two different lineages (Saltonstall et al. 2004). Besitka (1996) hypothesized that historical, and presumably native, *P. australis* populations on the Atlantic coast may have a higher ploidy level than modern ones, based on measurements of guard cell length, glume length, and pollen diameter. It is well recognized that guard cell length and stomatal density can be indicators of ploidy level in plant species (Hansen et al. 2007; Masterson 1994; Pozzobon and Valls 2000), although stomatal density may also be influenced by environmental factors such as light intensity, water availability, and CO_2 concentrations (Hetherington and Woodward 2003; Pazourek 1973).

We tested the hypothesis that native and introduced *Phragmites australis* populations in North America represent different ploidy levels by: 1) measuring stomatal density and sizes of guard cell pairs on both surfaces of leaves from native and introduced *P. australis* plants; and 2) estimating ploidy levels of both lineages using flow cytometry to see if cell size correlates with ploidy level. We compare these results with previous studies on *P. australis* and discuss their implications in the context of *P. australis* invasion biology.

MATERIALS AND METHODS

Guard cell size and stomatal density. Native and introduced *Phragmites australis* leaves were collected across eastern North America with a focus on the Chesapeake Bay region (Maryland, Delaware, and Virginia; Figure 1; sample tissues and DNA extractions are available from K. Hunter on request). Samples were air- or oven-dried, then stored dry at room temperature. Prior to guard cell analysis the subspecies of each population was confirmed using a restriction fragment length polymorphism (RFLP) assay, which distinguishes native from introduced *P. australis* based on non-coding chloroplast DNA (cpDNA) sequence (Saltonstall 2003a). The cpDNA haplotype of the majority of native samples was also determined using the methods of Saltonstall (2002).

Leaves from 69 *Phragmites australis* populations (36 introduced, 33 native) were sampled for guard cell measurement. The native populations included seven different cpDNA haplotypes [haplotypes E (n = 4), F (n = 19), G (n = 1), S (n = 1), Z (n = 2), AB (n = 2), and AC (n = 1)], as well as three samples from New Brunswick of unknown haplotype. All introduced populations sampled had a single cpDNA haplotype, haplotype M (Saltonstall 2002).

To verify that the method of preservation did not influence guard cell size, five populations were measured twice using both air-dried and oven-dried leaves. Also, multiple leaf samples were taken within nine of the populations to ensure that guard cell size did not vary significantly between different ramets of a population.

Guard cells were measured from epidermal peels, taken by painting leaves with clear fingernail polish, allowing it to dry, then removing the peel. Abaxial surface peels of 69 leaves (one leaf from each *Phragmites australis* population sampled; 36 introduced and 33 native) were used for the cell measurements. Lengths and widths of 20 guard cell pairs per leaf were measured on a light microscope with a calibrated optical micrometer (400× magnification). Guard cell areas were calculated using the formula of a rectangle, which best represents the bell-shaped structure of the guard cells: $L \times W$, where L is length and W is width of a pair of guard cells. Mean guard cell areas per sample were used for comparisons between native and introduced plants. Measurements were also taken on the adaxial leaf surface from a subset of 10 samples (five introduced,

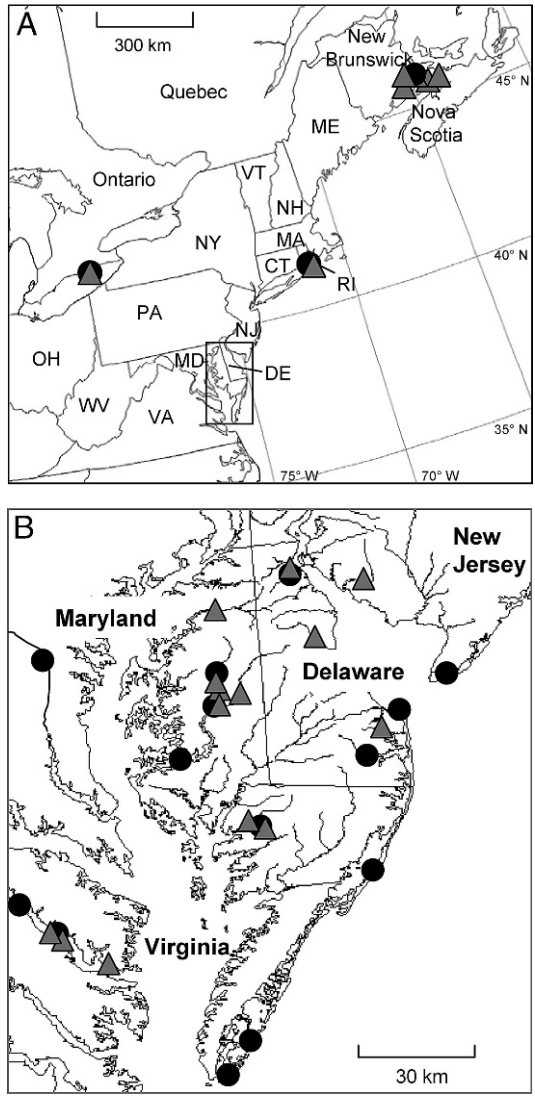

Figure 1. Sampling locations of native (▲) and introduced (●) *Phragmites australis* populations. A. Eastern North America. B. The Chesapeake Bay region and southern New Jersey. Some sampling points represent more than one population at a location.

five native) to see if differences in guard cell size exist between leaf surfaces.

Stomatal density was calculated for 20 populations—10 introduced (haplotype M), 10 native [haplotypes F (n = 8), Z (n = 1), and AB (n = 1)]—using three epidermal peels taken from the bottom quarter of both the abaxial and adaxial leaf surfaces of each specimen. Peels were made as described above at each leaf margin and in the middle of the leaf, at least 4 cm from the base of the leaf. Peels were photographed and numbers of stomata per unit area were counted directly from the photographs at approximately 3500×. The three counts were averaged to calculate the mean stomatal density per mm^2 of leaf surface area.

Flow cytometry. Rhizomes of a subset of *Phragmites australis* plants—six introduced (haplotype M) and seven native [haplotypes E (n = 1), F (n = 3), Z (n = 1), and AB (n = 2)]—were collected in spring 2003 and transplanted into 18-gallon rectangular tubs located outdoors in full sunlight at Horn Point Laboratory. Tubs contained washed quarry sand as substrate with 100 g Osmocote® (19-6-12 NPK; The Scotts Company, Marysville, OH) mixed in prior to transplanting. Plants were watered as needed with deionized water. Seeds of *Hordeum vulgare* cv. Sultan, which has a 2C DNA content of 11.12 pg (Johnston et al. 1999), were also planted and grown outdoors in full sunlight for use as a reference size standard in flow cytometry measurements.

In September after several months of growth and before plants began to senesce, the uppermost fully expanded leaves were collected from each plant and immediately prepared for analysis. Twenty mg of *Phragmites australis* and 10 mg of *Hordeum* tissues were excised and placed on ice in a 60- by 10-mm plastic Petri dish. The tissue was sliced into thin strips (0.25–0.5 mm wide) using a sterile razor blade in 1 ml ice-cold Tris-$MgCl_2$ buffer (0.2 M Tris, 4 mM $MgCl_2$. $6H_2O$, 0.5% Triton X-100; Pfosser et al. 1995) containing 50 µg/ml propidium iodide and 50 µg/ml RNase. The suspension was withdrawn using a pipette, filtered through a 60 µm nylon mesh into a 1.5 ml Eppendorf tube, and incubated on ice for 15 minutes prior to flow cytometric analysis. Samples were analyzed on a Becton Dickinson FACSCalibur flow cytometer using the FL-2 channel. Total event counts numbered 20,000 or greater for each sample. The mean positions of the G_0/G_1 (nuclei) peaks were calculated by CellQuest software. The 2C DNA content

was calculated using the formula:

$$\text{Nuclear DNA Content} = \frac{\text{Mean Position of } \textit{P. australis} \text{ Peak}}{\text{Mean Position of } \textit{Hordeum} \text{ Peak}} \times 11.12$$

Data analysis. Means of guard cell areas for native and introduced *Phragmites australis* were compared using t-tests in SAS 9.1.3 (SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was also used to compare means by haplotype and geographic origin for native and introduced *P. australis* using the PROC MIXED command in SAS 9.1.3. Geographic origin (Atlantic Coast or Midwest/Canada as designated by Saltonstall 2003b) and haplotype were treated as fixed effects in the analysis. Similarly, stomatal density was analyzed using ANOVA for comparisons between leaf surfaces. A Principal Components Analysis (PCA) was performed using PC-ORD (Version 4, MJM Software Design, Gleneden Beach, OR) using a correlation matrix of standardized data for the variables.

RESULTS

Leaf stomatal density differed significantly between native and introduced *Phragmites australis* ($F_{1,36} = 108.82$, $p < 0.0001$; Table 1). Introduced plants had approximately twice the stomatal density of native ones on both leaf surfaces. Both population types had significantly higher stomatal densities on the abaxial leaf surface ($F_{1,36} = 17.84$, $p = 0.0002$). Adaxial : abaxial stomatal ratios were not significantly different ($t = 1.28$, $df = 18$, $p = 0.2179$), but introduced plants tended to have a more equal ratio of stomata on the two leaf surfaces (Table 1).

The rectangular areas of guard cell pairs were significantly larger in native (range = 219.4–376.0 μm^2) than in introduced *Phragmites australis* plants (range = 116.9–195.0 μm^2; $t = -48.67$, $df = 1358$, $p < 0.0001$; Table 1). Intrapopulation variation conformed to these patterns with guard cell measurements taken from multiple stems within a population falling within these ranges (data not shown). Within the native samples, no significant differences were seen in cell sizes between the seven different haplotypes ($F_{6,23} = 0.34$, $p = 0.9085$). Similarly, no effect of geographic origin was seen ($F_{1,65} = 2.74$, $p = 0.1024$).

Table 1. Stomatal and guard cell characteristics for native and introduced *Phragmites australis* populations (means ± SD). Numbers in parentheses are the number of samples analyzed.

Characteristics	Native	Introduced
Stomata per mm^2 adaxial surface	804.3 ± 122.7 (10)	1725.7 ± 380.3 (10)
Stomata per mm^2 abaxial surface	1147.0 ± 283.8 (10)	2167.4 ± 533.3 (10)
Adaxial : abaxial stomatal density	0.72 ± 0.13 (10)	0.82 ± 0.21 (10)
Guard cell length (μm)	25.2 ± 3.1 (646)	18.7 ± 2.6 (714)
Guard cell width (μm)	11.3 ± 1.8 (646)	8.3 ± 1.5 (714)
Rectangular area of guard cell pairs (μm^2)	286.6 ± 62.1 (646)	155.4 ± 34.7 (714)

All differences in guard cell size occurred in both the length and width measurements, both being significantly greater in native than introduced samples (length: $t = -42.08$, $df = 1358$, $p < 0.0001$; width: $t = -34.36$, $df = 1358$, $p < 0.0001$; Table 1). Native guard cell lengths ranged from 15.0–37.5 μm while introduced ones ranged from 12.5–27.5 μm. Widths ranged from 7.5–17.5 μm in native samples and 5.0–12.5 μm in introduced ones. No difference was seen in guard cell areas of samples dried differently, confirming earlier evidence that preservation and environment do not confound cell size measurements (Hunter et al. 2001; Masterson 1994). The mean rectangular area of a guard cell pair showed no variation between leaf surfaces ($F_{1,15} = 0.09$, $p = 0.7674$). When determining the stomatal area per mm^2 leaf surface (mean guard cell pair area × stomatal density), native and introduced plants had nearly equivalent values (328,936 ± 86,557 $\mu m^2\ mm^{-2}$ and 348,631 ± 86,957 $\mu m^2\ mm^{-2}$, respectively).

The PCA confirmed and reinforced our interpretation of the abovementioned results (Figure 2). The first two PCs accounted for 90.09% of the total variation in the data. The first axis alone accounted for nearly 80% of the total variance (eigenvalue = 2.392) and had negative loadings for all native specimens and positive ones for all introduced specimens. Thus more negative values along PC1 indicate larger morphological structures and lower stomatal density, as seen in the native specimens. PC2 and PC3 had eigenvalues of 0.311 and 0.297, respectively.

No differences in 2C DNA contents were found between native and introduced *Phragmites australis*, with natives having a mean value of 7.29 ± 0.05 pg and introduced 7.25 ± 0.05 pg per cell ($t =$

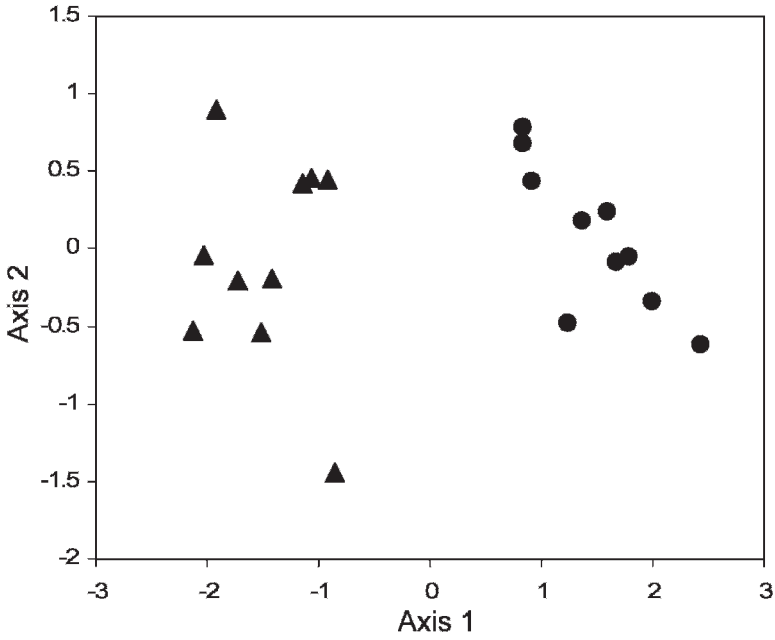

Figure 2. Principal component scores for stomatal density, guard cell length, and guard cell width for ten native (▲) and ten introduced (●) *Phragmites australis* samples on PC Axes 1 and 2.

−0.15, df = 11, p = 0.8856). No significant variation in 2C DNA content was seen among the four native haplotypes tested.

DISCUSSION

Morphological variation. Introduced *Phragmites australis* plants had smaller guard cells and denser stomata than native plants, which suggests that they represent a lower ploidy level (Hansen et al. 2007; Masterson 1994). In a similar analysis using values from a study done on *P. australis* of different ploidy levels in Sweden as reference (Björk 1967), Besitka (1996) suggested that the larger guard cell lengths seen in historical North American populations of *P. australis* (mean = 29.7 ± 2.0 μm; range = 27.2–33.3 μm) indicate that these herbarium specimens, which are presumably native, are hexaploid while modern *P. australis* populations (mean = 22.8 ± 1.6 μm; range = 19.6–24.7 μm), which are most likely to be introduced (Saltonstall 2002), are tetraploid. However, our

measurements of mean guard cell lengths in both subspecies fell within or near the range of both tetraploid and hexaploid plants observed by Björk ($4x$ = 23.2 μm ± 1.5, $6x$ = 29.0 μm ± 1.5, data from Tables 9, 19, 30, 39, 50 in Björk 1967; compare with Table 1). Further, our mean stomatal density for native plants is within the ranges of both tetraploid and hexaploid plants in Sweden as presented by Björk ($4x$ = 1213.4 mm^{-2} ± 182.0, $6x$ = 962.1 mm^{-2} ± 126.8, data from Tables 9, 19, 30, 39, 50 in Björk 1967) while that of introduced plants is nearly double (compare with Table 1). Although methodological differences may account for some of the discrepancies between our study and those of Besitka (1996) and Björk (1967), it is difficult to conclude that the differences that we have found between the two subspecies are indicative of ploidy level differences based on these measurement alone. A more likely explanation is that some factor other than ploidy level, such as a genetic difference distinguishing introduced North American *P. australis* from populations in Sweden, is controlling guard cell size and stomatal density in the populations we studied.

Flow cytometry. In contrast, our flow cytometry results showed no significant differences in 2C DNA content between the native and introduced subspecies, suggesting that they are of the same ploidy level. Using flow cytometry, Clevering and Lissner (1999) found that the majority of both North American and European *Phragmites australis* populations are tetraploid and all 18 populations that they tested, which originated from the same geographic area as our study, were tetraploid. However, no 2C DNA content values were reported in that study, making direct comparisons with our results difficult. Since chromosomal counts of North American populations have also found tetraploids to be in the majority, it is possible that all of the populations studied here are tetraploid. Further, Hauber et al. (1991) reported that two dominant morphological variants on the Mississippi River delta are tetraploids, based on karyotyping. Both chloroplast DNA sequencing and nuclear DNA microsatellite analysis of these same two populations identified by Hauber et al. (1991) found them to be introduced *P. australis* (Saltonstall 2002, 2003a). We measured guard cells on these samples and found them to be within the range of other introduced plants in this study (mean areas = 160.0 and 174.2 μm^2), thus, it is plausible that introduced plants are predominantly tetraploid. No chromosomal counts of verified

native *P. australis* populations exist in our data set so it is difficult to say if the populations studied here represent the same ploidy level or if other genomic differences, such as differences in the amount of repetitive DNA in the genome, are causing native plants of different ploidy levels to have the same 2C DNA content as introduced North American *P. australis*.

At this time we feel it is unlikely that native and introduced *Phragmites australis* populations represent different ploidy levels based on our flow cytometry results and those of Clevering and Lissner (1999). Additional studies verifying the actual karyotype of the two lineages are needed to fully understand the disparities in our morphological and genomic data sets. The issue of ploidy differences between native and introduced *P. australis* has important implications for management of the two subspecies, since differences in ploidy level could help to explain the lack of hybrid populations (Saltonstall 2003c). However, should they belong to the same ploidy level (most likely tetraploid), hybridization between native and introduced populations is more likely and efforts to prevent establishment of hybrid populations may be needed where preservation of native biodiversity is a management goal.

Ecological implications. The characters depicted here may also indicate ecophysiological differences between the two lineages of *Phragmites australis*, which may begin to explain their differences in invasiveness. Although the overall stomatal area per mm^2 of leaf surface did not differ between native and introduced *P. australis*, the guard cells of introduced *P. australis* were smaller and denser than those of native *P. australis*. Further, introduced *P. australis* typically has a higher stem density and higher leaf biomass than the native (League et al. 2006), and it has been observed to have earlier leaf emergence and longer leaf longevity relative to other native vegetation (Farnsworth and Meyerson 2003; League et al. 2006; Meadows and Saltonstall 2007). Since differences in ploidy level do not appear to account for the variation in guard cell size and stomatal density between these *P. australis* subspecies, the higher stomatal density of introduced *P. australis* suggests that there could be an adaptive significance to this higher density. Although we can only speculate about this, it is well recognized that stomatal structures strongly influence the ability of a plant to both take up CO_2 and retain water. In general, smaller stomata are able to open

and close faster and may provide the capacity for rapid increases in stomatal conductance of a leaf, thus maximizing CO_2 diffusion during favorable conditions for photosynthesis (Hetherington and Woodward 2003). In a dense monoculture where both light and CO_2 concentrations may be limited except for along the margins of a stand, smaller more dense stomata may be physiologically advantageous as they may enable plants to more efficiently conduct photosynthesis (Hetherington and Woodward 2003). Mozdzer (2005) found that introduced *P. australis* had significantly higher chlorophyll concentrations in leaf tissues and a higher photosynthetic rate than native *P. australis* under laboratory conditions. We also tested foliar chemistry of five introduced and four native specimens collected in the field and found that foliar nitrogen was significantly greater in introduced than native leaves (3.4% vs. 2.8%; $t = 2.36$, $df = 6$, $p = 0.05$), suggesting higher chlorophyll levels (K. Saltonstall, unpubl. data).

Although we have not measured stomatal conductance or photosynthetic rates in either lineage, this study suggests that introduced *Phragmites australis* has the potential to take better advantage of available light and CO_2 than native *P. australis*, possibly providing further insight into the rapid spread of this introduced subspecies throughout North America. Previous studies have suggested that introduced *P. australis* now dominates many marsh habitats because it is better able to take advantage of nutrients and disturbed sites than other species (Roman et al. 1984; Silliman and Bertness 2004; Windham and Meyerson 2003) but the physiological mechanism by which this can occur has not been studied. This is an area needing further study, in particular the effects of differing CO_2 levels on the stomatal densities and photosynthetic rates of native and introduced *P. australis* and differences seen with other plant species.

In conclusion, this study has shown that patterns of guard cell size and stomatal density are valid morphological characters distinguishing native and introduced *Phragmites australis*. Although overlaps in the range of guard cell lengths and widths occured between subspecies, when the area of a guard cell pair was calculated, no overlap was found, making this character a good diagnostic tool. In addition, our data set represents seven of the 13 known native *P. australis* haplotypes (Meadows and Saltonstall 2007; Saltonstall 2002) and the samples originate from sites across eastern North America. We also have measurements for three

specimens from western North America (haplotypes A and E), which follow the same patterns as our larger data set, thus, it is likely that these patterns in guard cell size are representative of differences between the native and introduced *P. australis* subspecies. Similarly, stomatal density showed a clear distinction without overlap between native and introduced plants. These characters may thus be added to the growing suite of characters useful in distinguishing native from introduced *P. australis* populations without genetic tests (Blossey 2002; Saltonstall et al. 2004). However, they do not appear to be useful for identifying ploidy levels of North American *P. australis* populations.

ACKNOWLEDGMENTS. Research support was provided by the Adkins Arboretum (to A.B. and K.S.) and Horn Point Laboratory (to K.S.). Thanks to Al Hanson, Robert Meadows, Tom Mozdzer, Alice Wellford, and Kerrie Wilcox for assistance with sample collections. Michael Scott assisted with map preparation. We thank two anonymous reviewers and Jefferson Hall for comments on earlier versions of the manuscript. This is Contribution #4053 from the University of Maryland Center for Environmental Sciences.

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