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ORIGINAL PAPER

Extent and Reproductive Mechanisms of *Phragmites australis* Spread in Brackish Wetlands in Chesapeake Bay, Maryland (USA)

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Abstract The number of patches of non-native *Phragmites australis* in brackish tidal wetlands in the Rhode River subestuary increased from 5 in 1971–72 to 212 in 2007, and the area covered by the patches increased more than 25 times during the same time interval. Genetic analysis of the patches showed that the expansion has primarily been from seed, and genetic similarities between patches indicate that most cross-pollination occurs within a distance of 50 m. Comparison of patches in different parts of the subestuary indicate that the expansion of *Phragmites australis* has occurred at the scale of the entire subestuary and not the scale of subsections of the subestuary dominated by differing upland land-uses.

Keywords Clonal propagation · Genetic diversity · Invasive species · Non-native genotype · Rhode River · Seeds

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Introduction

Phragmites australis (Cav.) Trin. Ex Steud., a widespread, perennial grass found on every continent except Antarctica, has been rapidly expanding into freshwater and brackish wetlands across North America (Marks et al. 1994). *Phragmites australis* is native to North America, but Saltonstall (2002) identified a non-native haplotype, likely introduced from Europe in the late 1700s, that is responsible for a recent widespread North American invasion (Saltonstall 2002, 2003a, b, Lelong et al. 2007). The non-native haplotype of *P. australis* has dramatically altered estuarine and freshwater wetland communities in the United States (Windham and Ehrenfeld 2003), especially along the east coast and Chesapeake Bay (Saltonstall 2003b).

Concern caused by the invasion of *P. australis* has prompted investigations into the mechanisms responsible for its expansion. Disturbances of upland habitats adjacent to tidal wetlands, shoreline development, and eutrophication of estuarine habitats have been shown to be positively correlated with concentrations of nitrogen in *P. australis* leaves and the abundance of *P. australis* in brackish wetlands (Bertness et al. 2002, Silliman and Bertness 2004, King et al. 2007, Chambers et al. 2008). However, to understand how eutrophication and disturbances associated with development result in increased abundance of *P. australis*, we first need to understand the underlying mechanisms responsible for its spread and invasion.

Most aquatic (Cronk and Fennessy 2001) and many invasive species (Barrett et al. 2008) are at least facultatively clonal, raising questions about the reproductive mode

responsible for their spread. *Phragmites australis* can reproduce both clonally and sexually. Pieces of *P. australis* rhizomes can break off and be transported by water to lodge in wrack piles where they sprout and start new patches (Minchinton 2002). *Phragmites australis* also produces windborne seeds that can travel long distances in the air and can float for extended distances in the water (Minchinton 2002, Soons 2006).

The extent to which seeds and rhizomes are responsible for the spread of the non-native haplotype of *P. australis* is unclear. Studies in the U.S. and Europe have indicated that establishment of *P. australis* is predominantly from rhizomes (e.g., Haslam 1972, Pellegrin and Hauber 1999, Keller 2000, Hudon et al. 2005) or a combination of establishment from rhizomes and seeds (e.g., Alvarez et al. 2005). We have found highly variable seed viability among *Phragmites* patches across the Chesapeake Bay (Kettenring et al. in press, Kettenring and Whigham 2009) and Gervais et al. (1993) suggested that generally poor seed set and slow development of seedlings made establishment of patches from seed unlikely.

More than 30 y of regular observations of wetlands in the Rhode River subestuary of Chesapeake Bay by one of the authors (DFW) found no evidence, through physical examination of newly observed individual plants or groups of shoots, that the establishment of new patches of *P. australis* was from rhizomes. Even if some of the new patches had established from rhizome fragments, the large increase in the number of new patches established between 1971 and 2007 would indicate that establishment from seed is the most likely mechanism. Much of the invasion of *P. australis* in the Rhode River has occurred in wetlands that are located in a part of the subestuary where the wetlands are surrounded by development (e.g., mostly housing and dock construction along with bulkheading of the shoreline). This pattern is consistent with the findings of Bertness et al. (2002) and Silliman and Bertness (2004) working in Rhode Island and King et al. (2007) in the Chesapeake Bay. The invasion of *P. australis*, however, has also occurred in wetlands in the largest portion of the Rhode River subestuary that is surrounded by undeveloped land owned by the Smithsonian Environmental Research Center (SERC). SERC owns approximately 26 km of the 39 km Rhode river subestuary shoreline and there has been almost no development of the SERC-owned property; there are few docks, and almost all of the shoreline is natural and buffered by forests. The Rhode River subestuary, thus provides an appropriate setting to examine the spread of *P. australis* under circumstances that differ from the scenarios described elsewhere (Bertness et al. 2002, Silliman and Bertness 2004, King et al. 2007).

This study had two goals. First, to quantify the increase of *P. australis* in the Rhode River subestuary we determined the number and aerial extent of patches in wetlands located in the developed and forested parts of the subestuary and compared it with *P. australis* distribution identified by McCormick and Somes (1982) in 1971–72. Second, to test the hypothesis that the expansion of *P. australis* within Rhode River wetlands was primarily the result of the establishment of plants from seeds, we used microsatellite analysis to distinguish sexual from clonal spread within and among patches.

Methods

Study Area

This study was conducted in brackish tidal wetlands of the Rhode River (Fig. 1), a subestuary of the Chesapeake Bay, near Edgewater, Maryland, USA (38°53'N, 76°32'W). The Rhode River watershed contains a mixture of forested, agricultural, and developed land. The subestuary has about 39 km of shoreline and it can be visually divided into two portions with one part having a watershed dominated by suburban development and the other a watershed dominated by forests and, to a lesser degree, agriculture (Fig. 1).

Defining and Mapping Patches of *P. australis*

From September to November 2007, we surveyed all of the shoreline and all intertidal wetlands in the Rhode River for patches of *P. australis*. We defined a *P. australis* “patch” as being a robust stand of plants isolated from other stands by a distance of at least 5 m. Alternatively, the stands had to be separated by a distance of at least 10 m if there were sparse *P. australis* stems between robust stands.

We used a global-positioning system (Garmin GPS 12CX; Olathe, Kansas) to determine the geographic location of each patch by, in most cases, walking its perimeter. If access to a patch was limited, we estimated the size and/or location in the field by using select GPS waypoints and delineating the stand on a 1998 natural color 1”=200’ scale aerial photograph (MrSid, VarGIS, Herndon, VA, USA). Using the GPS data and field sketches, we digitized the 212 patches that we located in 2007 (Fig. 1) using the geographic information system, ArcGIS 9.2 (ESRI; Redlands, California). We used a similar digitization of McCormick and Somes’ (1982) vegetation maps obtained from Maryland Department of the Environment to relate the positions of patches in 1971–72 compared to the current distribution of patches. We also used the digitization of McCormick and Somes’ (1982) vegetation

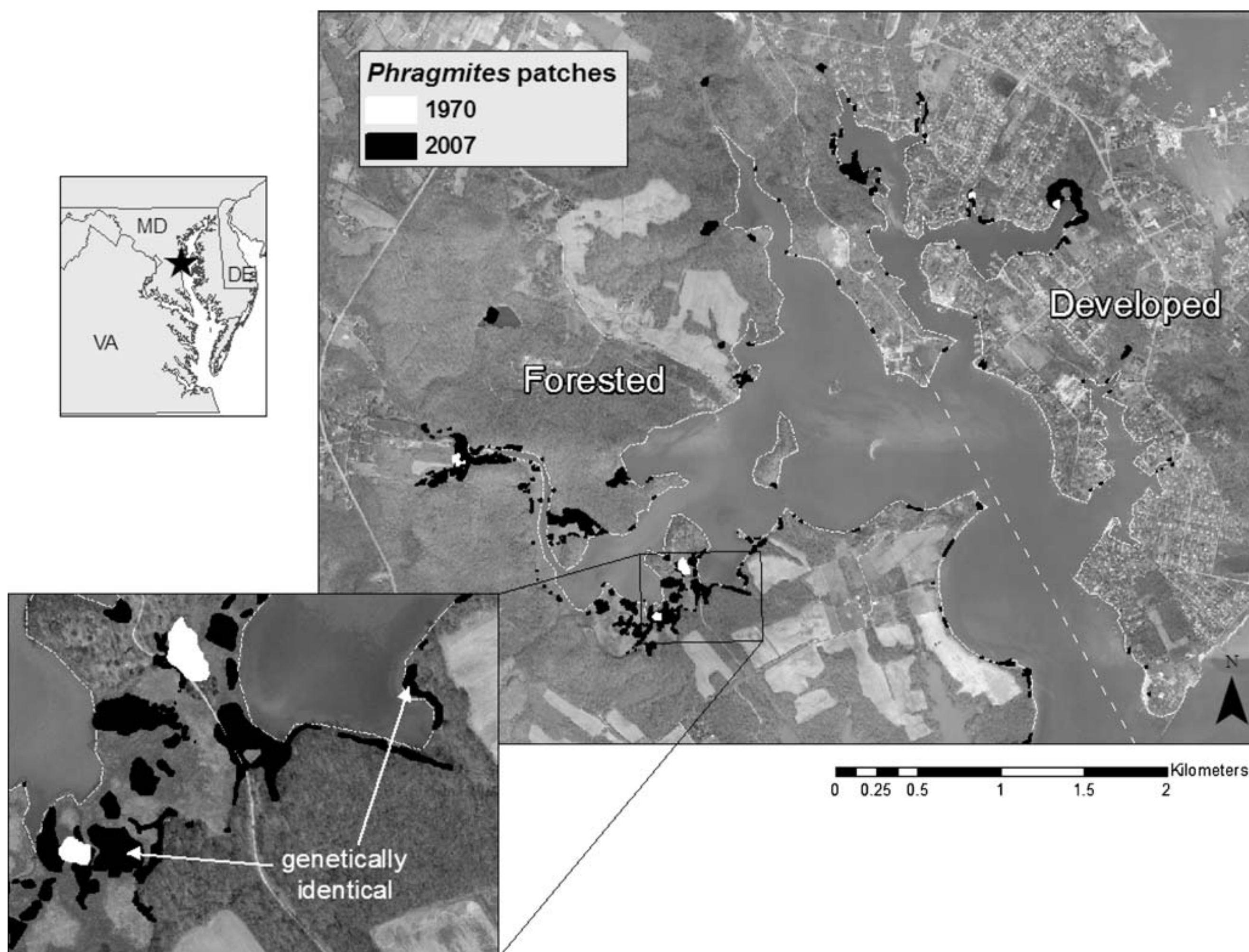

Fig. 1 Map of *Phragmites australis* patches in the Rhode River in 1971–72 (*white*, from McCormick and Somes 1982) and 2007 (*black*). The one pair of identical gene phenotypes is identified by a pair of white arrows in the insert

maps to calculate the total wetland area for comparison with the area covered by *P. australis*.

Genetic Variation Assessment

We collected the freshest leaves from shoots at each of four sampling points approximately equally spread around the perimeter of each of the 212 patches located in 2007. In a few very small patches only one leaf was collected. The leaves were stored in plastic storage bags at 4°C until DNA was extracted. DNA was extracted from approximately 20 mg of fresh tissue using a BioSprint 96 (QIAGEN, Inc.; Valencia, California) adhering to the supplied protocol.

We assessed multilocus gene phenotypes of individual shoots within patches using eight microsatellite markers targeting different regions of the DNA (Saltonstall 2003a; Table 1). Gene phenotypes (i.e., each allele is classified as present or absent) were used because determination of the number of copies of each allele is unreliable in polyploid species (Saltonstall 2003a). Annealing temperatures for each primer pair were determined during trials prior to analysis of samples for maximum yield of amplification product. PCR amplification was performed using a PTC-200 DNA Engine thermal cycler (MJ Research, Inc.; Waltham, Massachusetts) programmed using the following conditions: an initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 50–58°C (See Table 1 for temperatures used for each primer pair) for 30 s, and 72°C for 10 s, with a final polymerization step at 72°C for 2 min. PCR was run as 12.5 µl volume reactions with concentrations as follows: 1.25 µl template DNA (diluted 1:5–1:100 depending on fluorophor and primer pair, see Table 1), 3.2 µl distilled water, 0.75 µl of each primer, 0.3 µl 25 mM $MgCl_2$, and 6.25 µl RedMix Plus (Gene Choice, Inc.; Frederick, MD, USA).

After amplification, PCR product amplified with different fluorophores and with different expected fragment sizes were combined prior to fragment size analysis as follows:

Table 1 Microsatellite primers and PCR conditions used in the current study. Primer names reference Saltonstall (2003a). Alleles give the number of alleles found among the Rhode River samples for each primer locus

Primer Pair	Annealing Temperature (°C)	Fluorophor	DNA Dilution	Alleles
PaGT4	50	FAM	1:100	5
PaGT9	50	HEX	1:50	7
PaGT12	56	FAM	1:100	5
PaGT13	50	HEX	1:50	6
PaGT14	58	FAM	1:100	5
PaGT16	56	NED	1:10	8
PaGT21	58	HEX	1:50	11
PaGT22	50	NED	1:10	8

primers 4+9+16, primers 12+13+22, and primers 14+21. Fragment sizes were determined using GeneMapper v4.0 (Applied Biosystems, Inc.; Foster City, California). Amplified samples were subjected to analysis on an ABI 3100 Automated Capillary DNA Sequencer (Applied Biosystems, Inc.; Foster City, California) using a custom ROX size standard to determine fragment sizes. Fragments for all samples were aligned using a TRFLP peak sorting function for Excel (Rees et al. 2004, http://www.wsc.monash.edu.au/~cwalsh/treeflap.xls) and stutter peaks were removed manually.

When using a finite number of gene loci, it is possible that a sexually produced offspring could show the same gene phenotype as the parent, strictly as a result of chance selection of genes. We calculated the probability of a repeated gene phenotype arising by chance sexually rather than asexually as $P=(\Pi p_i q_i)2^h$, where p_i and q_i are the frequencies of the two alleles at the ith locus and h is the number of heterozygous loci in the genotype (Pollux et al. 2007) modified for gene phenotypes (i.e., X1/X2 in a tetraploid could be 1, 2, or 3 copies of allele X1 and 3, 2, or 1 copies of allele X2). This method uses the number of different alleles present at each locus of the parent's gene phenotype to calculate the probability of a sexually produced seed having a gene phenotype identical to its parent. So, for example, a parent that was homozygous at all eight loci and received pollen from a plant with an identical gene phenotype would produce only seeds that had gene phenotypes identical to itself for the eight loci we used. We used a moderately conservative cutoff of $P<0.001$ to identify repeated gene phenotypes arising through asexual reproduction rather than through seed because inbreeding can greatly increase the probability of repeated gene phenotypes arising through sexual reproduction (Pollux et al. 2007).

Data Analysis

DNA samples with identical multilocus genotypes were assumed to result from asexual reproduction. We compared all multilocus gene phenotypes found within and among patches to identify all repeated phenotypes, which were indicative of patches that had become established from rhizome fragments as well as patches in which all of the shoots were produced by clonal propagation. We compared the genetic relationships between patches using several approaches. First, on the assumption that patches that were closer to each other had a higher probability of being genetically similar, we used Moran's *I* statistic (SPAGeDi v1.2 g, Hardy and Vekemans 1999, 2002) to calculate genetic similarity between patches. We calculated Moran's *I* at distances of 10, 50, 100, 200, 500, 1000, and 5000 m. Second, the genetic similarity of gene phenotypes among patches was compared within patches and to other patches in the Rhode River using *F*- and *R*-statistics. *F*-statistics were used to examine the distribution of genetic variation within and among patches relative to the whole subestuary, regardless of distance separating patches. *F*-statistics were calculated both with and without repeated samples removed (Halkett et al. 2005) using the method of Weir and Cockerham (1984), which is appropriate for polyploid samples. *R*-statistics (Slatkin 1995) were also calculated for comparison. *F*-statistics assume an infinite allele model and *R*-statistics assume a stepwise mutation model. In particular, the two statistics differ in how they are affected by the mutation rate. Substantial mutation rates, as might be expected for microsatellite loci, depress F_{ST} values. In contrast, R_{ST} is unaffected by mutation rates but produces a statistic with much higher variation. The two statistics represent opposing extremes of mutation models, neither of which is likely to strictly match the mutation of microsatellite loci. Rather each is more appropriate in some conditions, so both are reported here as suggested by Balloux and Lugon-Moulin (2002).

Results

Defining and Mapping Patches

The 212 *P. australis* patches identified in the Rhode River subestuary in 2007 (patches shown in black in Fig. 1)

represent a dramatic increase from the 5 patches identified in 1971–72 (patches shown in white on Fig. 1). At the scale of the entire Rhode River subestuary, *P. australis* coverage expanded from 7,294 m^2 in 1971–72 to 183,369 m^2 in 2007 and the number of patches and the area they covered increased dramatically from 1971 to 2007 on both sides of the subestuary (Table 2). While only 10.2% of the total area of intertidal wetlands in the subestuary is located on the developed side (Table 2), by 2007, 69.9% of the wetland area was dominated by patches of *P. australis*. On the forested side of the subestuary, only 22.3% of the 604,568 m^2 was dominated by patches of *P. australis* by 2007. The mean patch area of *P. australis* was similar on both sides of the subestuary in 1971–72 and 2007.

Spread within Patches and Establishment of New Patches

Overall, 55 of 57 patches from which we obtained multiple DNA samples contained multiple gene phenotypes. In 33 patches all four samples had unique gene phenotypes. The number of gene phenotypes per *P. australis* patch did not differ between the subsections of the subestuary dominated by development and forests (Table 2).

Six pairs of patches had identical gene phenotypes. Calculation of the probability of repeated gene phenotypes arising by chance revealed that five of the six pairs of identical gene phenotypes could have reasonably have arisen by seed ($P>0.01$). In these five repeated pairs the high probability of arising by chance resulted from missing data at 3–5 of the 8 loci and the presence of common alleles at the loci for which data were present in both samples being compared. The single remaining case of repeated gene phenotypes had very low probability of arising by chance ($P=0.00002$) with respect to the overall frequency of alleles in the subestuary and so was considered to be a case where a patch could have been established by rhizome.

Genetic Similarity with Distance

Across the Rhode River subestuary, plants in patches that were physically closer together were genetically more similar than those farther apart (Fig. 2). Moran's *I*, a measure of spatial autocorrelation, decreased quickly with distance separating samples, indicating greater genetic similarity of patches closer together compared to two plants chosen at random in the subestuary. However, Moran's *I* was significantly different from 0 even out to 1000 m, indicating that pairs of plants separated by 1000 m or less were significantly more closely related than two plants chosen at random in the subestuary. This was true regardless of whether repeated samples within patches (likely repeated sampling of the same genet) were included in the analysis, so the data presented do not include within-patch repetitions.

Wright's *F*-statistics jackknifed across all eight loci indicated that there was substantial genetic variation among patches ($F_{ST}=0.225\pm0.025$ SE) and lower heterozygosity within patches ($F_{IS}=0.161\pm0.077$), indicative of inbreeding or selfing, and samples within patches were significantly inbred (local pollen exchange) relative to the subestuary as a whole ($F_{IT}=0.351\pm0.072$). Removing repeated gene phenotypes produced a slight decrease in F_{ST}, a corresponding increase in F_{IS} and little change in F_{IT}, indicating that the presence of significant inbreeding within patches and significant genetic differences among patches was not dependent on multiple samplings of single genets. For comparison, *R*-statistics were also calculated without repeated gene phenotypes and jackknifed across all eight loci. R_{IS} and R_{IT} values were similar to F_{IS} and F_{IT} ($R_{IS}=0.222\pm0.182$ SE, $R_{IT}=0.319\pm0.145$ SE), but R_{ST} was lower than F_{ST} ($R_{ST}=0.129\pm0.026$ SE), indicating that interpretation of the distribution of genetic variation changes little when different mutation models are considered.

Discussion

The spread of *P. australis* in the Rhode River subestuary has been substantial since McCormick and Somes (1982) mapped tidal wetland vegetation in the early 1970s, and the non-native haplotype has spread throughout the entire subestuary (Fig. 1). We do not know how quickly colonization of wetlands in the forested versus developed parts of the subestuary occurred but the large amount of shoreline development and the high percentage of total

Table 2 Characteristics of wetlands on the developed and forested sides of the Rhode River subestuary. The total wetland area, number of *P. australis* patches and the wetland area they covered in 1971–72 were calculated based on McCormick and Somes (1982)

		1971–2			2007			
Wetland Area (m^2)		*Phrag.* patches	*Phrag.* Area (m^2)	Patch size ($\log(m^2)\pm$ SE)	*Phrag.* patches	*Phrag.* Area (m^2)	Patch size ($\log(m^2)\pm$ SE)	Phenotypes (mean±SE)
Developed	69,050	2	1,346	2.819±0.089	49	48,238	5.025±0.223	3.0±0.30
Forested	604,568	3	5,947	3.252±0.135	159	135,131	5.045±0.168	3.5±0.11

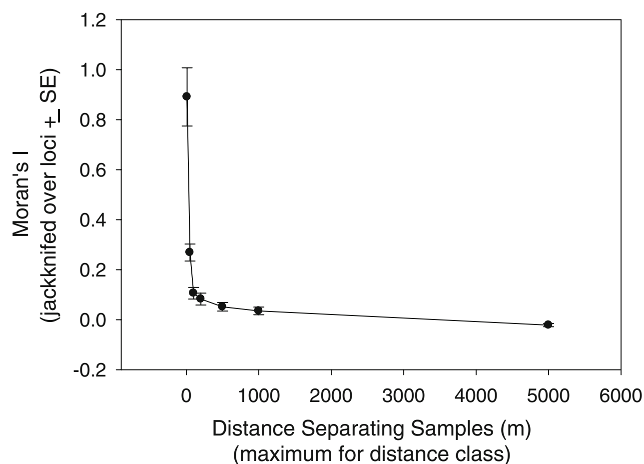

Fig. 2 Genetic similarity (Moran's *I*) as a function of distance separating plant samples from Rhode River *Phragmites* patches. Number of pairs of samples for each point are 10 m: *N*=19, 50 m: *N*=317, 100 m: *N*=549, 200 m: *N*=1,404, 500 m: *N*=4,809, 1,000 m: *N*=8,610, and 5,000 m: *N*=3,6618

wetland area occupied by *P. australis* indicates that establishment from seed has been more common on the side of the estuary dominated by human activities associated with suburban development (Fig. 1). The larger number of patches and the greater total area of *P. australis* on the forested side of the subestuary (Table 2) is interesting because it suggests that seedling establishment can occur in areas with no obvious large-scale disturbance. However, we found that disturbances as small as 30 cm in diameter can be important in *Phragmites* invasion by seed in the Rhode River (*Unpubl. data*). Similar mean patch size on the forested and developed sides of the subestuary (Table 2) indicates that the rate of spread of *P. australis* patches does not differ between the two sides of the subestuary and the higher percentage of the total wetland area occupied by *P. australis* on the developed side (69.9 versus 10.2%) is likely due to the fact that establishment of patches from seeds has been more common on that side of the subestuary. Independent of the metric used to evaluate the spread of the non-native genotype of *P. australis*, it has clearly colonized and spread substantially on both sides of the subestuary. We do not know when the five patches of *P. australis* mapped by McCormick and Somes (Fig. 1) first appeared but they were relatively small and our examination of aerial photographs taken at different times indicated that most of the expansion occurred after 1980. The factor (s) responsible for the relatively recent expansion of *P. australis* in the Rhode River and other subestuaries in the Chesapeake Bay are unknown but the most likely scenario is that the non-native genotype has responded to environmental and/or genetic changes, resulting in the development of patches with multiple genotypes and subsequent spread through the production and dispersal of viable seeds (Kettenring et al. in press). A similar phenomenon was identified for *Spartina alterniflora* in San Francisco Bay where it is an invasive species (Blum et al. 2007).

Despite suggestions that the spread of invasive *P. australis* in the U.S. occurs primarily through vegetative reproduction (e.g., Pellegrin and Hauber 1999, Keller 2000, Saltonstall 2002), we found little evidence that it is an important mechanism for the spread of *P. australis* in the Rhode River subestuary. Nearly all discrete patches were genetically unique, indicating they were established by seed. Furthermore, the expansion of individual patches seems to include both clonal propagation and multiple genotypes recruited from seed, as we found that 96% of patches were composed of multiple genotypes. Other investigators have also reported both monoclonal and polyclonal patches of *P. australis* (Clevering and Lissner 1999, Koppitz 1999, Guo et al. 2003, Alvarez et al. 2005).

The one case where two patches displayed a repeated gene phenotype could also have arisen from self-fertilized or inbred seed, since inbreeding would dramatically increase the probability of repeated genotypes arising through sexual reproduction. Although self-fertilization is thought to be uncommon, Lambert and Casagrande (2007) have found that it can occur in *P. australis*. Even if self-fertilization does not occur, our genetic analysis demonstrates that there is substantial inbreeding among close relatives so, while the probabilities will not be as high as for selfing, they will be substantially higher than those calculated based on random mating. Because the production of seeds with gene phenotypes identical to a parent may be quite high, we must interpret the evidence for asexual spread of *P. australis* (i.e., patches with identical gene phenotypes) with caution.

Regardless of whether spread via rhizomes occurred, it is clear that the production of viable seeds is a major factor in the spread of invasive *P. australis* in the Rhode River subestuary. The relationship between genetic similarity and distance between samples can clarify the scale of gene flow. The high genetic similarity (Moran's *I* values, Fig. 2) at 10 and 50 m and significant F_{IT} values demonstrate that most pollen and/or seed dispersal has been very local, primarily within a patch. This has important implications for understanding factors involving spread and seed production.

Other researchers report that *P. australis* is at least partially self-incompatible both within its native and invasive ranges (Ishii and Kadono 2002, Lambert and Casagrande 2007) and Ishii and Kadono (2002) suggest that outcrossed pollen was important for seed production. Similarly, we have found that outcrossed inflorescences produced a much larger proportion of viable seeds than did selfed inflorescences (*Unpubl. data*). Our results demon-

strate that most pollen and seed dispersal in the Rhode River subestuary has occurred within patches, suggesting that accumulation of genetic variation within patches and availability of outcross pollen may be important for *P. australis* spread.

It is possible, as suggested by Keller (2000), that genetic variation within and among patches could have arisen as accumulated somatic mutations rather than from seed recruitment. Mutation is common at microsatellite loci and it is just this high level of variation that makes them so useful for population genetic studies (Balloux and Lugon-Moulin 2002). However, the distribution of genetic variation seen in this study seems unlikely to have been generated by mutation accumulation for several reasons. First, if establishment of new patches was primarily vegetative, a substantial number of cases of repeated gene phenotypes would be expected even in the face of high levels of somatic mutation. Second, somatic mutation would be expected to produce a high proportion of heterozygotes relative to homozygotes (Balloux et al. 2003), whereas observed heterozygosity in Rhode River *P. australis* was 0.59. Finally, high mutation rates deflate F_{ST} values (Balloux and Lugon-Moulin 2002) relative to R_{ST} values, which are independent of mutation rate, yet F_{ST} values for Rhode River samples (0.225) were relatively high and were higher, rather than lower, than R_{ST} (0.129).

The results of this study provide evidence that seedlings produced by outcrossing become established within as well as beyond the patch of their origin. Samples within patches were, on average, much more genetically similar than samples drawn at random in the subestuary, reflected in the highly significant Moran's I statistic at distances of 100 m or less and by the significant F_{IS} and F_{IT} statistics. However, not all of the genotypes within a patch were closely related. In 30 of the 57 patches from which we had multiple samples, least one genotype was no more similar to the other genotypes in the patch than it was to samples drawn at random from the subestuary (Moran's $I<0.1$). This is unlikely to arise from seed recruitment within a patch. We interpret this as evidence that within patch genetic variability contributes to high viable seed production rather than results from high seed production.

Taken together, the results of this study provide strong support for seed as the primary mechanism by which invasive *P. australis* spreads. While expansion of individual patches is, without question, accomplished substantially through rhizome growth and spread by rhizome fragments has been documented (e.g., Bart and Hartman 2003), increased production of viable seeds coupled with local pollen dispersal and occasional long distance dispersal of seeds (Coops and Van der Velde 1995, Soons 2006) may be in large part responsible for the rapid spread of *P. australis* in the Rhode River. Dominance of *P. australis* spread by seed does not appear to be limited to the Rhode River subestuary. We have found similar dependence on seed for both patch establishment and expansion for more limited studies in nine other Chesapeake Bay subestuaries with land-use that ranged from largely forested to highly developed (*Unpubl. data*).

We found that 1) both the number and areal coverage of patches increased dramatically on both the developed and forested sides of the Rhode River subestuary, 2) increases occurred disproportionately on the developed side of the subestuary but substantial increases also occurred in forested areas, and 3) new patches were founded primarily by seed and some spread within patches was also a result of seed recruitment. Taken together, these results suggest that development in one portion of a subestuary has implications for the entire subestuary and that management of seed production and recruitment will be essential for management of this species.

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