ABSTRACT

Title of Document: **GENETIC CONSEQUENCES OF HABITAT FRAGMENTATION AND RESTORATION.**

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The objective my dissertation was to assess the effects of habitat loss and fragmentation on genetic diversity and landscape connectivity. I focused on *Vallisneria americana* Michx. (Hydrocharitaceae), a submersed aquatic plant species found in the Chesapeake Bay. *Vallisneria americana* has undergone dramatic changes in abundance and distribution throughout its range and has been targeted for restoration, which makes it ideal for examining the effects habitat loss and fragmentation.

I examined the naturally occurring genetic diversity across the Chesapeake Bay and its major tributaries. Sites were genetically diverse, but had a range of genotypic diversities. There were four genetic regions, corresponding with geographic regions in the Bay. *Vallisneria americana* has been the target of restoration, and restoration techniques could be influencing genetic diversity and potentially lowering overall success. I examined various restoration techniques across eight restoration sites, and

found that technique did not greatly influence genetic diversity. However, small population size, significant inbreeding coefficients, and low overlap of allele composition among sites provide cause for concern.

Measures of functional and potential connectivity provide insights into the degree of contemporary gene flow occurring across a landscape. Pollen dispersal distance was measured using indirect paternity analysis, and is spatially restricted to only a few meters. Dispersal at this scale imposes small genetic neighborhoods within sites, evidenced by high seed relatedness within mothers. I used a graph theoretic approach to examine the distribution and potential connectivity of historic and current patches of *V. americana*. There was a high turnover in the distribution of patches, and connectivity varied through time, but even if all habitat were occupied, increases in overall network connectivity would not necessarily be observed.

I developed an individual based model that I used to test the ability of measures of genetic differentiation to detect changes in landscape connectivity. Genetic differentiation measures became significant after two generations, but the magnitude of change in each was small in all cases and extremely small when population sizes are greater than 100 individuals. These results suggest that genetic differentiation measures alone are inadequate to rapidly detect changes in connectivity.

GENETIC CONSEQUENCES OF HABITAT FRAGMENTATION AND RESTORATION.

By

Michael Warren Lloyd

Dissertation submitted to the Faculty of the Graduate School of the
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Preface

This dissertation contains an introductory chapter, five chapters, and a concluding chapter. Each chapter is presented in manuscript format; therefore, background and methods may be repeated, and pronouns usage reflects manuscript authorship. Copyright clearance has been obtained as required. A single reference section containing all literature cited throughout the dissertation is presented at the end.

Dedication

To my parents, Jean and Jim Lloyd, without their steadfast support and love I would not be the person I am.

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Introduction

Habitat Loss, Fragmentation & Resulting Impacts on Landscape Connectivity

Habitat loss and fragmentation are considered to be the most imminent threats to biological diversity worldwide and thus are fundamental issues in conservation biology (Huxel & Hastings 1999; Lawler *et al.* 2002; McKinney 2002; Rouget *et al.* 2003; Wilcove *et al.* 1998). Habitat loss is the process by which habitat is converted to a different type that supports only a fraction of original species (Bender *et al.* 1998; Brooks *et al.* 2002; Sih *et al.* 2000). Fragmentation is a more complex phenomenon that is simultaneously a consequence of habitat loss and a process in and of itself (Fahrig 2003; McGarigal & McComb 1995; Saunders *et al.* 1991). It is a function of the extensiveness of individual patches, the configuration of those patches across a landscape (Neel *et al.* 2004; Pascual-Hortal & Saura 2006; Tischendorf & Fahrig 2000a), and the nature of the land use types between the habitat patches (Ricketts 2001).

Understanding the joint and independent effects of habitat loss and fragmentation remains a major focus of landscape ecology and conservation (e.g., Belisle & Clair 2002; Bender *et al.* 1998; Collingham & Huntley 2000; Fahrig & Jonsen 1998; Fahrig & Merriam 1985; Tischendorf *et al.* 2005; Trzcinski *et al.* 1999). When the specific effects are examined separately, habitat loss has larger impacts including, reduced biodiversity (Brooks *et al.* 2002; Fahrig 2003), population declines in interior species (Bender *et al.* 1998), decreased species distribution and abundance (Fahrig 2002;

McGarigal & McComb 1995), and increased likelihood of population extinction (Fahrig 1997). Genetic consequences of habitat loss include increased rates of inbreeding and genetic drift due to reduced effective population size within habitat patches (Frankham 1995b, 1996). Increased inbreeding is expected to lower probabilities of survival and reproduction (Frankham 1995a; Reed & Frankham 2003), thus increasing the probability of extinction (Saccheri *et al.* 1998; Westemeier *et al.* 1998) in the remaining patches. Genetic drift will reduce allelic richness within patches and increase differentiation among patches (Frankham 1995b, 1996; Keller & Largiader 2003b; Templeton *et al.* 1990; Young *et al.* 1996).

The effects of habitat fragmentation that are independent of habitat loss are due to increased distance and thus decreased connectivity among remaining patches. Although it typically has smaller effects, increased isolation alone has been implicated as a driver of population extinctions (Burkey & Reed 2006), declining population size of interior species (Bender *et al.* 1998; Parker & MacNally 2002), altered social behavior (Cale 2003), reduced population viability (Harrison & Bruna 1999; Patten *et al.* 2005), demographic change (Hovel & Lipcius 2001; Jules 1998), and the spread of invasive species (With 2004). Isolation can exacerbate effects of genetic drift if migration rates are reduced (Frankham 1995a, 2005a; Hartl & Clark 2007; Schwartz *et al.* 2007; Wright 1951).

Neutral landscape models predict a non-linear increase in the number of patches and a change in the distribution of those patches as habitat loss proceeds, with a threshold when habitat occupies 50%-59% of a random landscape depicted by square grid cells (Gardner & O'Neill 1991; With 1997; With & King 1997). Below this

threshold, additional habitat loss primarily further reduces patch size and number, whereas near the threshold habitat loss results in dramatic changes that can affect potential connectivity across the landscape (Turner *et al.* 2001; With & King 1999).

Landscape connectivity was defined by Taylor (1993) as "the degree to which the landscape facilitates or impedes movement among resource patches." This definition was later refined by With *et al*. (1997) as "the functional relationship among habitat patches, owing to the spatial contagion of habitat and the movement responses of organisms to landscape structure." Thus, degree of connectivity in a landscape depends both on how potential habitat patches are structured (Baudry & Merriam 1988; Merriam 1984) and how individual organisms functionally use different patch types and move among them within a landscape (Brooks 2003; McRae 2006; Ricketts 2001; Taylor *et al.* 1993; Taylor *et al.* 2006; Tischendorf & Fahrig 2001).

For species that occur in discrete populations, the point at which separate patches are actually isolated from one another depends on the scale at which a species perceives and interacts with the landscape, including the degree to which the unusable or less-preferred matrix habitat between discrete patches are barriers to movement (Crooks & Sanjayan 2006; Holland *et al.* 2004; Levin 1992; Taylor *et al.* 2006). Ultimately, the level of connectivity among resource patches throughout a landscape facilitates both ecological and evolutionary processes (Baudry & Merriam 1988; Chetkiewicz *et al.* 2006; Fahrig & Merriam 1994; Merriam 1984, 1991; Taylor *et al.* 2006; Wiegand *et al.* 2005).

Structural changes in landscape composition and configuration associated with the fragmentation process have been quantified and monitored using an extensive array of landscape indices (Gustafson & Parker 1994; Hargis *et al.* 1998; Jaeger 2000; McGarigal *et al.* 2002; Saura & Martinez-Millan 2001; Schumaker 1996; Urban & Keitt 2001). Measures include the shape, size, and position of features in a landscape, irrespective of the species of interest (Collinge & Forman 1998; Tischendorf & Fahrig 2000b, 2001). Functional connectivity links actual measures of an organism's movement within and among habitat patches with these structural characteristics (Brooks 2003; Manel *et al.* 2003; Sork & Smouse 2006; Tischendorf & Fahrig 2000a, b, 2001).

These links are often based on habitat suitability, mark-recapture, radio-telemetry, experimental removal-recolonization studies (Bender *et al.* 2003; Tischendorf & Fahrig 2000b) and demographic monitoring (Bowers & Dooley 1999; Bruna & Oli 2005; Dooley & Bowers 1998). Unfortunately, such studies can be so data- and time-intensive that there is little practical application for conservation activities for most species (e.g., Calabrese & Fagan 2004; Urban 2005). Genetic methods are ideally suited to inferring the degree of functional connectivity in a landscape by providing minimally-invasive or non-invasive methods of documenting movement of a large number of individuals (Holderegger & Wagner 2008; Kendall *et al.* 2009; Storfer *et al.* 2007). Additionally, observing physical movement of primarily sessile organisms with limited motile life stages such as plants or highly cryptic organisms is impractical (Ellstrand 1992; Wunsch & Richter 1998). Genetic techniques are especially useful in these situations because they quantify dispersal

events that are difficult to observe directly (Austerlitz *et al.* 2004; Austerlitz *et al.* 2007; Austerlitz & Smouse 2001a, b, 2002; Smouse *et al.* 2001; Smouse & Sork 2004; Sork *et al.* 2002; Sork *et al.* 1999; Sork & Smouse 2006; Sork *et al.* 2005). Furthermore, population genetic parameters may be more sensitive to changes in fragmentation and connectivity than demographic estimates that have large error components (Ims & Andreassen 1999). Both indirect and direct measures of gene flow among resource patches derived from genetic data have been used (Sork *et al.* 1999).

Indirect measures of historic gene flow versus isolation among populations have been based on Wright's fixation index F_{st} under a number of demographic models (e.g. island, stepping-stone, isolation by distance, metapopulation). More recently, coalescent theory has been used to provide estimates of migration among populations (Beerli & Felsenstein 1999, 2001). Methods that directly define the distances over which dispersal events are currently occurring, e.g. pollen (Austerlitz & Smouse 2001a; Smouse *et al.* 2001) and seed (Godoy & Jordano 2001; Grivet *et al.* 2005) movement, provide measures of contemporary functional landscape connectivity (Sork *et al.* 1999; Sork & Smouse 2006). Comparing long-term and current gene glow estimates using both indirect and direct approaches can provide insight into changes in connectivity from historical conditions.

Despite their potential utility, attempts to link indices of landscape structure to ecological and evolutionary processes have not yielded consistent relationships between fragmentation and genetic diversity (Wiegand *et al.* 1999; Wiens *et al.* 1993; Young *et al.* 1996). In particular, despite clear theoretical predictions (e.g. increased

drift, reduction in effective population size, and increased inbreeding) regarding the impacts of fragmentation on genetic diversity, empirical data are often equivocal. There are several potential causes for this lack of consistent connection. It may result in part because relationships between many of the landscape metrics and landscape configuration are not monotonic (Neel *et al.* 2004). Further, there may be non-linear or threshold-like ecological and population responses to changes along the fragmentation gradient.

The lack of consistent effects could also be due to characteristics of Wright's F_{st} and subsequent derivations, which have a number of specific assumptions that are almost always violated in natural systems and complicate the interpretation of genetic differentiation and gene flow among populations (Bossart & Prowell 1998; Neigel 2002; Whitlock 1992; Whitlock & McCauley 1999). In addition, because F_{st} integrates over evolutionary time, it is not possible to separate current from historical processes based on pattern alone. Because of this integration, F_{st} may be slow to reflect a change in migration following a fragmentation event, especially if N_e remains large. Additionally, the alleles that are most likely to be lost through drift are at low frequencies in populations and contribute little to estimates of F_{st}. When connectivity is only reduced rather than eliminated entirely, F_{st} and its analogues may remain close to zero (Neigel 2002). Furthermore, sample sizes may be insufficient to detect differentiation even if it has occurred. Finally, measures of genetic differentiation (e.g. F_{st}, G_{st}, Φ_{st}) can be depressed they are when calculated using data derived from highly diverse marker systems (e.g. microsatellites; Hedrick 2005; Jost 2008; Meirmans 2006). The depression occurs when within-subpopulation

heterozygosity or variance is high relative to among-subpopulation heterozygosity or variance. When a measure of genetic differentiation are calculated from such data, the measure will never approach unity regardless of the underlying patterns of allelic diversity (Hedrick 2005; Jost 2008; Meirmans 2006). Jost (2008) has proposed a measure of genetic differentiation *D* that removes the biases associated with use of heterozygosity for calculating G_{st} and related measures by using allelic diversity among populations, allowing it to freely vary between 0 and 1. As a result, Jost's *D* may provide greater ability to detect recent fragmentation events. When comparing *D* with historically used measures of genetic differentiation (G_{st}) among 34 published studies, Heller and Siegismund (2009) found that *D* was roughly 60 times greater than G_{st}, illustrating that *D* more accurately depicted levels of genetic differentiation.

Another potential explanation for the lack of consistent relationships between fragmentation and Wright's F_{st} is that not all habitat that is perceived as fragmented by humans is actually fragmented from the perspective of a species of interest. These investigators may be trying to quantify something that does not exist. Conversely, when presented with low measures of genetic differentiation investigators may conclude that fragmentation has not occurred when in fact it has. Such results can also be obtained when samples were drawn at too small of a spatial scale. Moreover, even if a landscape is fragmented such that current movement is impeded or precluded, long-lived individuals remaining in a particular location may predate the fragmentation event and therefore provide a genetic signature of connectivity that no longer exists (Young *et al.* 1996). Therefore, simply determining which landscapes are actually fragmented is less than straightforward. These issues can be reduced

through careful study design that matches the sampling scales with potential scales of fragmentation and considering the potential for functional fragmentation given the biology of the organism (Lee-Yaw *et al.* 2009; Zellmer & Knowles 2009).

I used both indirect and direct genetic approaches to quantify effects of habitat loss and to determine if these losses have altered connectivity in the submersed aquatic plant species *Vallisneria americana* Michx. (Hydrocharitaceae) within the Chesapeake Bay in eastern North America (Figure I.1). Submersed aquatic vegetation (SAV) communities in the Bay have been greatly affected by habitat loss and degradation. Pollen composition in sediment cores shows that from the 1700's to 1930, SAV was highly abundant in the Bay and its major tributaries (Brush & Hilgartner 2000; Davis 1985; Orth & Moore 1984). From 1931 through the present day, levels of abundance, distribution, and diversity have fluctuated dramatically as the result of pathogenic infection, hurricanes and tropical storms but have generally declined due to introduction of non-native species, high nutrient and sediment levels, and poor water quality (Davis 1985; Dennison *et al.* 1993; Kemp *et al.* 2005; Orth & Moore 1983, 1984). Extensive modification of the 167,000 km^2 Chesapeake Bay watershed by human population growth and its association agriculture, industrialization, and urbanization (Cooper 1995; Costanza & Greer 1995) have yielded increased industrial and municipal pollution, toxic pesticides, infectious wastes, wetland loss, channel dredging and spoil disposal, power plant effects, overharvesting of fisheries, nutrient runoff, and sediment loads to the Bay that have all affected submersed aquatic vegetation (Boesch *et al.* 2001; Cooper 1995; Costanza & Greer 1995).

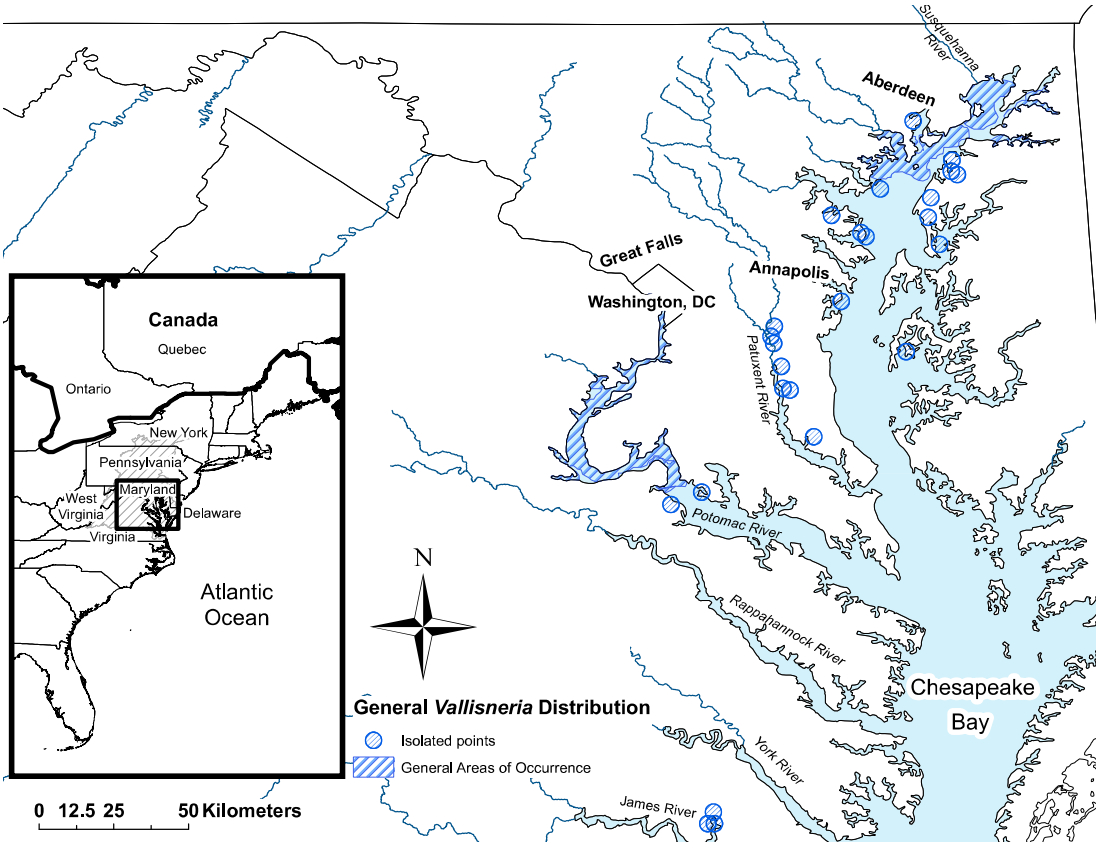

Figure 1.1 General distribution of *Vallisneria americana* in the Chesapeake Bay

Vallisneria americana Michx. is a dioecious, perennial, clonal macrophyte that is native to eastern North American freshwater and oligohaline habitats (Catling *et al.* 1994; Korschgen & Green 1988; Wilder 1974). The distribution of *V. americana* is driven by habitat availability and quality (e.g., water depth, turbidity, temperature, water chemistry, and flow rate) as well as competition among SAV species and grazing by animals (Barko *et al.* 1982; Doering *et al.* 2001; Hunt 1963; Jarvis & Moore 2008; Kemp *et al.* 2004; Korschgen & Green 1988). Vegetative reproduction in *V. americana* is common (Doust & Laporte 1991; Titus & Stephens 1983) and can be more frequent than sexual reproduction (Doust & Laporte 1991). Titus and Stephens (1983) noted within a locality only 24% of ramets sampled had flowered during the growing season. Additionally, sex ratios within a given population can be highly biased to the extreme of having only one sex present in a given area (Doust & Laporte 1991; Lokker *et al.* 1994). Dispersal occurs via pollen, seed, and vegetative tissues. Pollination occurs when pistillate flowers, borne on the water surface, are fertilized by free-floating staminate flowers (Korschgen & Green 1988). Seed dispersal is accomplished when fruits rupture and deposit clusters of seeds, bound in a gelatinous matrix, into the water column (Korschgen & Green 1988). The length of time in the water column is variable, but generally seeds settle quickly upon release (Kaul 1978). Furthermore, fruits and seeds are also moved by waterfowl, either through ingestion or seeds clinging to feathers (Figuerola *et al.* 2003; Higgins *et al.* 2003; Santamaria & Klaassen 2002). Additional long distance dispersal is accomplished when fruits remain attached to the maternal plant as it dislodges from the substrate and floats freely at the end of the season (Korschgen & Green 1988).

In addition to empirically investigating the effects of fragmentation, I also examined the effectiveness of genetic differentiation measures in detecting recent fragmentation events. My goal was to evaluate the ability to detect genetic effects of fragmentation with F_{st} and D over time frames associated with anthropogenic habitat modification (i.e., <200 years) while controlling for population size. The number of generations necessary to make such an evaluation renders the task infeasible in a field setting. Therefore, I developed an individual-based population model to simulate genetic divergence among recently fragmented populations and measured F_{st} and D over time. To isolate fragmentation from habitat loss, population sizes remain relatively constant, only levels of connectivity are explicitly altered. I examined the influence of types and duration of isolation, population size, overlapping generations, and sampling effort in terms of individuals and loci on ability to detect a significant change in F_{st} and D. Additionally, as Wright's F_{st} can be downwardly biased when utilizing highly diverse marker systems, I therefore calculated Jost's D, a measure of 'true' genetic differentiation (Jost 2008), and compared its performance against F_{st}.

Restoration as a means to ameliorate effects of fragmentation

The effects of habitat loss, degradation, and fragmentation can be ameliorated or offset through habitat or population restoration (Huxel & Hastings 1999; Kareiva & Wennergren 1995; Lewis *et al.* 1996; Tilman *et al.* 1997). Recognition of the sharp declines in overall Chesapeake Bay health (biotic and abiotic) has lead to a number of agreements among federal, state, local, and private organizations to protect and restore the Bay. Most recently in 2003, the Chesapeake Bay Foundation set forth a goal requiring that 74,866 ha of SAV cover the bottom of the Bay and its tidal

tributaries by 2010 (Chesapeake Executive Council 2003). However, this is a mere fraction of the 250,000 hectares estimated to have existed historically, and although there have been recent increases in SAV quantity throughout the Bay, current SAV coverage has not exceeded 32,000 ha in recent history (Dennison *et al.* 1993; Orth *et al.* 2008; Stevenson & Confer 1978). The total acreage in a given year remains low, and the sum of SAV acreage occupied across all years from 1984 to 2010 is 76,836 ha (Figure 1.2).

Reaching the stated goal of 74,866 ha of SAV requires expansion of current populations and recolonization of areas denuded of vegetation (Orth *et al.* 2002). Because propagule dispersal distances are thought to be small, unaided colonization is not expected over large distances (Orth *et al.* 2002). As a result, several federal, state, local, and private organizations have attempted to transplant and restore several SAV species throughout the Bay. Restoration goals include increasing the quantity of vegetation across the Bay, reestablishing 'lost' populations, and increasing species diversity and the size of small populations such that they can resist episodes of poor water quality (Orth *et al.* 2002).

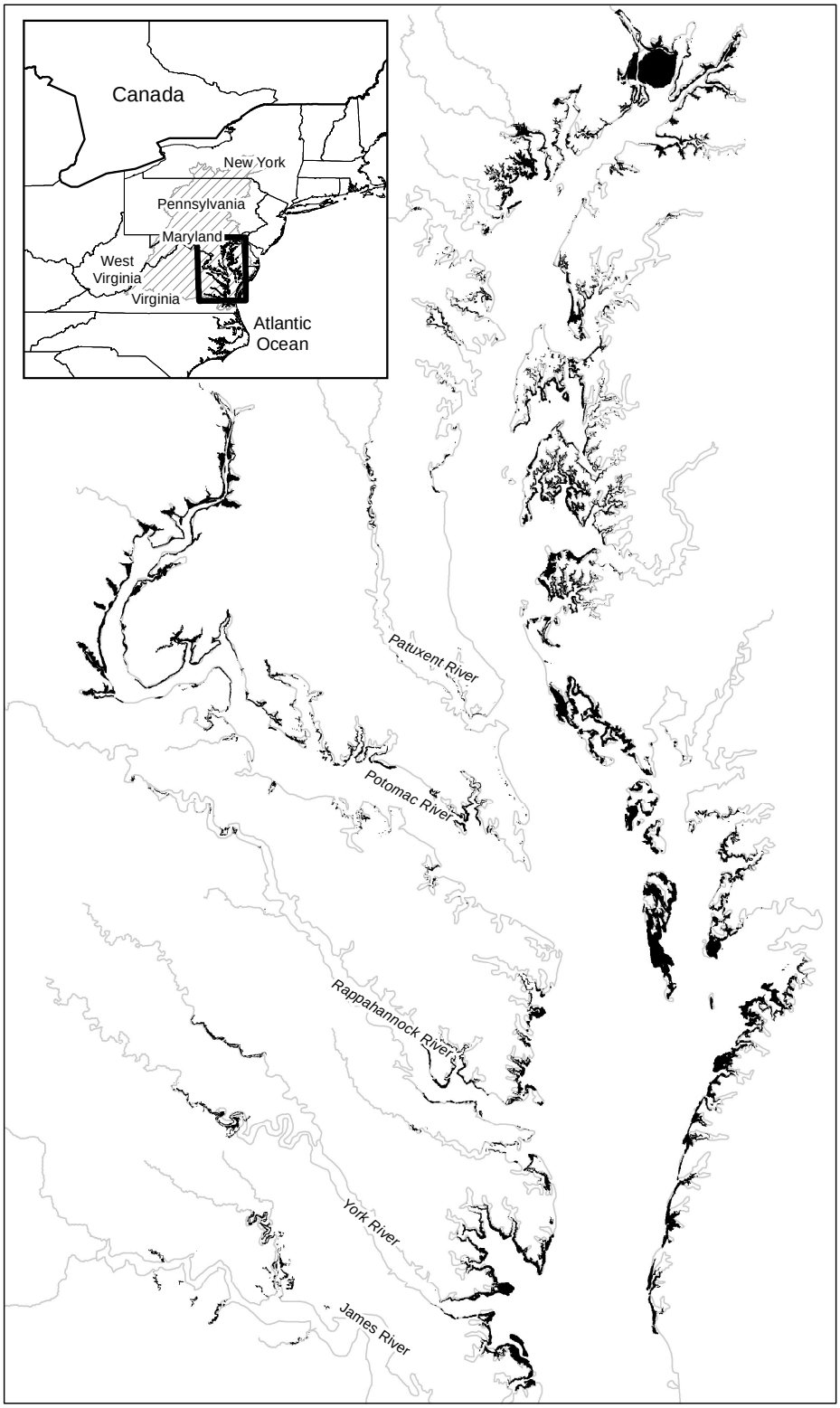

Figure 1.2 Total SAV coverage from 1984 to 2010

The ultimate goal of ecological restoration is the creation of a self-sustaining ecosystem that will be resilient to future perturbation without additional human input (Broadhurst *et al.* 2008; Liu *et al.* 2008; Procaccini & Piazzi 2001; Ramp *et al.* 2006; Rice & Emery 2003). In practice restoration can range from simply creating suitable physical conditions and allowing natural colonization, to supplementing of one or few species within a relatively intact ecosystem, to constructing diverse communities on a denuded sites (Montalvo *et al.* 1997). Spatial scales of efforts range from small local projects (e.g. 1-10 ha) to projects that cover broad geographic scales (e.g. 102-106 ha; Broadhurst *et al.* 2008). Regardless of the scope and scale of a project, populations that have had successful restoration persist in dynamic settings in the short term (Jordan *et al.* 1988) and also retain the capacity to undergo adaptive evolutionary change in the long term (Montalvo *et al.* 1997; Rice & Emery 2003).

Success is a function of adequate environmental conditions (Boesch *et al.* 2001; van Katwijk *et al.* 2009), selection of suitable planting material (Broadhurst *et al.* 2008; McKay *et al.* 2005), and spatial arrangement of restored sites (Tilman *et al.* 1997). The latter two issues can be informed by understanding the distribution of genetic diversity within individuals, among individuals within populations, and among populations and by understanding the effects of these types of diversity on fitness. Understanding differences between historic versus current-day gene flow can inform choices of the most effective spatial distribution of restoration sites to ameliorate loss of connectivity.

Unfortunately, restoration using *V. americana* in the Chesapeake Bay has had limited success. Poor site selection coupled with reduced habitat quality has likely

contributed to the failure of many restoration projects (van Katwijk *et al.* 2009). These are not problems unique to *V. americana*. Most seagrass restoration efforts have resulted in a net loss of habitat, and worldwide success of seagrass transplantation as judged by persistence and bottom coverage is roughly 30% (Fonseca *et al.* 1998). It is also possible that genetic factors are contributing to failures if the genetic diversity of planted individuals is not representative of the overall diversity found within and among natural populations. Little is known about the genetic diversity of *V. americana* in natural populations or restoration stocks that have been, and continue to be utilized, for site restoration in the Bay. My goal was to understand the effect of restoration practices on genetic diversity.

When transplanting individuals or seed, selection of stocks for use is of key importance (Broadhurst *et al.* 2008). Three aspects of genetic diversity can impact plant fitness and diversity among individuals: levels of inbreeding within individuals (Dudash 1990; Gigord *et al.* 1998), levels of diversity among individuals (Hufford & Mazer 2003; Williams 2001; Williams & Davis 1996), and the adaptation of individuals to local environments (Fenster & Galloway 2000; Hufford & Mazer 2003; Montalvo & Ellstrand 2000; Montalvo & Ellstrand 2001). Increased genetic diversity has also been shown to confer resiliency and long-term persistence of populations when presented with an environmental stressor such as grazing (Hughes & Stachowicz 2004), or heat shock (Reusch *et al.* 2005). Finally, transplantation success has been shown to increase with the use of genotypically diverse stock (Procaccini & Piazzi 2001).

Restoration stocks are commonly selected based only on the location and availability of source populations (Ramp *et al.* 2006). Because genetic data are generally unavailable when restoration source material is selected, genetic diversity is often only indirectly factored into restoration planning (Procaccini *et al.* 2007) through following a set of general sampling guidelines and propagation procedures that attempt to mitigate or avoid negative genetic influences (Broadhurst *et al.* 2008; Montalvo *et al.* 1997). When stocks are collected without consideration of the distribution and levels of genetic diversity, limited diversity might be represented in restoration plantings.

When limited numbers of individuals are represented in restoration plantings, reduced effective population sizes will lead to increased rates of genetic drift and inbreeding (Hartl & Clark 2007). Increased inbreeding can decrease reproduction, and overall fitness of individuals (Keller & Waller 2002) leading to decreased success or failure of a restoration project. In small populations, if genetic drift overwhelms natural selection it can lead to fixation of maladapted alleles (Whitlock 2000). Additionally, population bottlenecks resulting from the use of limited genetic stocks can also decrease overall fitness of restored individuals (Hufford & Mazer 2003; Williams 2001; Williams & Davis 1996). Conversely, in simulations, increased genetic diversity within a population increased the ability of that organism to expand into additional ecological niches (Vellend 2006).

Inappropriate use of locally adapted genetic stocks can also have substantial negative impacts on restoration success (Hufford & Mazer 2003; Montalvo *et al.* 1997). Restoration failure can result from stocks being maladapted to local

conditions (McKay *et al.* 2005), leading to reduced survival and growth (Mortlock 2000), genetic 'pollution' of local gene pools (Potts *et al.* 2003), outbreeding depression (Templeton 1997), and resource provisioning at inappropriate times (Jones *et al.* 2001). However, in the face of sweeping environmental changes, as might result from climate change, the 'local is best' policy is considered by some to potentially be detrimental to long-term restoration efforts (Broadhurst *et al.* 2008), and evidence of outbreeding depression is limited (Frankham *et al.* 2011).

The ecological consequence of varying levels of genetic diversity as it relates to restoration success has been examined in several seagrass species. Williams and Davis (1996) noted that genetic diversity of transplanted *Zostera marina* beds was reduced as compared with natural beds in California due to limited stock selection. Furthermore, the decreased genetic diversity contained within the transplanted beds was shown to negatively impact both population growth and individual fitness (Williams 2001). In *Posidonia oceanica*, genetic polymorphism in restoration stock was positively correlated with increases in rhizome length, number of ramets per genet, and survival rate (Procaccini & Piazzi 2001). Increased genotypic diversity of *P. oceanica* within populations was also positively correlated with increased shoot density (Zaviezo *et al.* 2006). A similar trend was noted in *Z. marina*; however, a positive relationship between genotypic diversity and shoot density only existed in winter, potentially aiding in the overwintering of populations when they experienced abiotic and biotic stressors (Hughes & Stachowicz 2009).

Chapter Summaries

In the first chapter, I seek to understand the patterns of genetic diversity in *Vallisneria americana* in the Chesapeake Bay to inform restoration of the species. Measures of genetic diversity, and genetic structure from 26 sampled sites are presented. I also quantify a measure of historic gene flow among genetic regions. The implications of these data on restoration practice are discussed.

In the second chapter, I quantify the effect of restoration practices on genetic diversity. It is critical to understand not only the patterns of genetic diversity within and among natural populations but also within restoration stock when conducting any transplantation project. Measures of genetic diversity from eight restored sites, which were planted using a variety of techniques, are presented and discussed.

In the third chapter, I use population structure and paternity analyses to determine if female plants are being pollinated from genetically structured pollen pools across a range of geographic distances. I conducted an indirect paternity analysis to assess patterns of contemporary pollen dispersal. The consequences of limited gene flow, and small genetic neighborhoods are discussed.

In the fourth chapter, I use a graph theoretic approach to examine the distribution and potential connectivity of submersed aquatic vegetation patches in the Chesapeake Bay that potentially contain *Vallisneria americana*. I compare recently occupied patches with historically occupied patches to examine how potential connectivity has changed in time. Specific focus is given to dispersal distances that are most relevant to *V. americana*, which provides insight into the connectedness of *V. americana* patches across the Bay.

In the fifth chapter, I focus not on *V. americana* specifically, but on the effectiveness of measures of genetic differentiation among populations to rapidly detect changes in functional connectivity. I utilize an individual-based population model to simulate genetic divergence. Specifically looking at if measures of genetic differentiation can detect recent fragmentation among populations that were historically connected. I compare the performance of Jost's *D* against Wright's F_{st}. I also examine the influence of population size, overlapping generations, and sample effort in terms of individuals and loci on the ability of these statistics to detect a significant change in the measure of genetic differentiation.

Chapter 1: The structure of population genetic diversity in *Vallisneria americana* in the Chesapeake Bay: Implications for restoration

Submersed aquatic macrophyte beds provide important ecosystem services, yet their distribution and extent has declined worldwide in aquatic ecosystems. Effective restoration of these habitats will require, among other factors, reintroduction of genetically diverse source material that can withstand short- and long-term environmental fluctuations in environmental conditions. We examined patterns of genetic diversity in *Vallisneria americana* because it is a cosmopolitan freshwater submersed aquatic macrophyte and is commonly used for restoring freshwater habitats. We sampled 26 naturally occurring populations of *V. americana* in the Chesapeake Bay estuary and its tributaries and found that the majority of populations have high genotypic diversity and are not highly inbred. Fourteen of the populations had high allelic and genotypic diversity and could serve as source sites for restoration material. However, substantial geographic structuring of genetic diversity suggests that caution should be used in moving propagules to locations distant from their source. In particular, we suggest that propagules at least be limited within four primary geographic areas that correspond to freshwater tidal and non-tidal, oligohaline, and seasonally mesohaline areas of the Chesapeake Bay.

Introduction

Beds of submersed aquatic vegetation (SAV) provide habitat for fish and aquatic invertebrates (Rozas & Minello 2006; Rozas & Odum 1987, 1988; Wyda *et al.* 2002) and food resources for migratory waterfowl (Korschgen & Green 1988; Krull 1970).

SAV also provides critical ecosystem services in that it improves water quality by stabilizing sediments (Madsen *et al.* 2001; Sand-Jensen 1998) and buffering nutrient levels (Brix & Schierup 1989; Moore 2004; Takamura *et al.* 2003). Unfortunately, the abundance, distribution, and diversity of SAV beds in coastal aquatic habitats have declined world-wide owing to extensive agricultural, industrial, and urban development in coastal zones (Cooper 1995; Orth *et al.* 2006; Procaccini *et al.* 2007; Short & Wyllie-Echeverria 1996). Such is the case in the Chesapeake Bay estuary (Boesch *et al.* 2001; Costanza & Greer 1995; Kemp *et al.* 2005), where current SAV coverage is < 15% of the 250,000 hectares estimated to have existed historically (Dennison *et al.* 1993; Orth *et al.* 2008; Stevenson & Confer 1978).

Programs to restore SAV acreage to the Chesapeake Bay and its tributaries have been implemented to mitigate declines. However, these programs have resulted in minimal increases in SAV extent. Poor water and habitat quality at many restoration sites are likely the primary reasons for disappointing results (van Katwijk *et al.* 2009). Our goal in this paper is to assess the amounts and patterns of genetic diversity in the submersed aquatic plant species *Vallisneria americana* Michx. (Hydrocharitaceae) to begin to investigate the possibility that genetic factors are contributing to low restoration success rates (Frankel 1974; Frankham 1995a; Hughes *et al.* 2008). Genetic diversity can affect population persistence in dynamic environments (Lande & Shannon 1996) and the chances for successful establishment of restored populations (Williams 2001). Unfortunately, assessments of this type of diversity often are not directly included in management and restoration plans because it is hard to quantify without sophisticated equipment and substantial expense. Our intent is to

provide a description of spatial patterns of genetic variation within and among populations of *V. americana* that can contribute to the design of restoration efforts.

Amongst SAV species, *Vallisneria americana* has suffered substantial population size declines in the northern freshwater reaches of the Chesapeake Bay and its tributaries (Kemp *et al.* 1983). *V. americana* is a cosmopolitan, dioecious, perennial macrophyte that is native to eastern North American freshwater and oligohaline habitats (Catling *et al.* 1994; Korschgen & Green 1988). The species reproduces sexually and vegetatively (Wilder 1974) and the relative frequency of the two reproductive modes is unknown. Distribution of *V. americana* is limited to habitats characterized by a maximum water depth of 7m in clear water, substrates ranging from gravel to hard clay, water temperatures between 20 and 40°C, and salinity below 18ppt (Korschgen & Green 1988). It is further limited by turbidity, nutrient content in the water column, water pH, gas exchange, water current, and competition with other plant species and grazing by animals (Barko *et al.* 1982; Doering *et al.* 2001; Hunt 1963; Jarvis & Moore 2008; Kemp *et al.* 2004; Korschgen & Green 1988; Titus & Stephens 1983).

Full restoration of *V. americana* within the Chesapeake Bay will depend on linking both physical and biological factors (Allendorf & Luikart 2007). Previous investigations across a wide range of habitats have examined the abiotic growth requirements and ecology of *V. americana*. These include salinity (Boustany *et al.* 2010; Doering *et al.* 2001; Kreiling *et al.* 2007), light attenuation (Boustany *et al.* 2010; Korschgen *et al.* 1997; Kreiling *et al.* 2007; Titus & Adams 1979), temperature (Titus & Adams 1979), suspended nitrogen (Kreiling *et al.* 2007), germination

requirements (Jarvis & Moore 2008), effects of competition (Titus & Stephens 1983), and sex-ratios and natural fecundity (Doust & Laporte 1991; Titus & Hoover 1991). Here we build on this previous knowledge and quantify the levels and patterns of genetic diversity within and indirect measures of gene flow among naturally occurring sites supporting Vallisneria americana in the Chesapeake Bay.

Given the magnitude of decline in *V. americana* population size and extent in the Bay, we wanted to quantify the levels of genetic diversity and inbreeding overall and within remaining populations (Hufford & Mazer 2003; Williams 2001; Williams & Davis 1996) to know if levels were low enough to cause concern for survival and reproduction (Dudash 1990; Frankham 1995a; Gigord *et al.* 1998; Reed & Frankham 2003; Saccheri *et al.* 1998; Westemeier *et al.* 1998). We also wanted to know what amounts of genetic diversity are available because this diversity can affect probability of persistence of remaining populations, potential for unaided recovery, and selection of source material for propagation and planting. Unfortunately, there is no way to know how much genetic diversity there was prior to population size declines, nor exactly how much is enough to be safe from genetic concerns. We compare current levels of genetic diversity with those in other SAV species to understand if amounts of genetic diversity are substantially lower than expected such that they would cause concern for elevated levels of risk. We also wanted to understand patterns of differentiation because they provide insight into ecological and evolutionary processes that are relevant to restoration. For example, if populations are naturally highly differentiated, moving material among locations could have negative consequences due to outbreeding depression resulting from moving locally adapted

individuals to less suitable locations (Montalvo & Ellstrand 2001). On the other hand, if historically high connectivity among populations of *V. americana* had been reduced or eliminated (Young *et al.* 1996), effective population size within habitat patches would be reduced, and the rate of inbreeding and genetic drift increased relative to historical conditions (Frankham 1995b, 1996). In this circumstance, knowledge of long-term patterns of gene flow can focus restoration efforts on locations that have potential for reestablishing natural movement among anthropogenically isolated sites. In total, the genetic data we present here provide useful guidance for the restoration community actively working with *V. americana* in the Chesapeake Bay.

Methods

Sampling localities and protocol

In 2007, 2008, and 2010, we sampled from 26 naturally occurring sites of *V. americana* present in tidal and non-tidal reaches of Chesapeake Bay tributaries (Table 2.1) to quantify patterns of allelic and genotypic diversity and historic gene flow. Collection sites were identified with the help of managers and scientists working within the Mid-Atlantic region of the U. S. A. Sampling represented the geographical and ecological extent of the species in the Bay (Figure 2.1). Other regions of the Bay are too deep or too saline to support this species. We sampled the Potomac River extensively because plant material from the river has been harvested in the past for use in restoration projects.

From each site, we collected ~30 shoots, each approximately 5-10m apart. Samples were often taken blindly as the water was generally too turbid to see shoots,

but the distances among samples were kept as consistent as possible given the natural variation in densities at sites. Latitude and longitude coordinates were recorded for each sampled shoot using global positioning systems in all but three sites (CBH, CBC, CON). Shoot tissue was placed on ice and frozen at -80°C until DNA extraction and genotyping.

DNA extraction and genotyping

Genomic DNA was isolated and purified using methods described in (Burnett *et al.* 2009). We genotyped 11 microsatellite loci representing tri-nucleotide repeats from each sample using robust primers with specific amplification that were developed for the species (Burnett *et al.* 2009). Polymerase chain reactions (PCR) were performed on an MJ Research PTC-200 Peltier Thermal Cycler using proprietary reagents in the TopTaq DNA Polymerase Kit (QIAGEN). Reaction conditions for all loci followed Burnett *et al*. (2009) with the exception of the locus Vaam_AAG004, for which we added dimethyl sulfoxide and Q-Solution (QIAGEN) to each reaction for optimal specificity. PCR products were separated and measured on an ABI 3730xl DNA Analyzer with GeneScan™ -500 ROX™ or 500 LIZ™ Size Standard (Applied Biosystems) after tagging the PCR product with fluorescent labeled forward primers (Applied Biosystems). Peak data were then analyzed using Genemapper v3.7 (Applied Biosystems) and all allele calls were also visually inspected.

Ambiguity in calls resulting from human or PCR error can result in individuals being misclassified and cascading errors in subsequent analyses. For quality control purposes we reran every ambiguous call up to three times (as necessary). If after

three attempts the sample was still ambiguous, the alleles were coded as missing data. In addition, we confirmed genotype calls by re-extracting DNA from 32 samples, rerunning all PCRs and re-genotyping at all loci. These samples were chosen because together they were present across all eight 96 well plates used in the initial fragment analysis. This confirmatory process was completed several months after the initial analysis of the raw data and scoring was done without looking at the initial scores. We detected no allele scoring differences in any of these samples.

Genotypic diversity

We detected clones within and across sites by identifying identical multilocus genotypes using the program GenClone v2.0 (Arnaud-Haond & Belkhir 2007). Because mutation and scoring errors can lead to individuals originating from the same sexual reproductive event having different genotypes we used Genodive v2.0b17 (Meirmans & Van Tienderen 2004) to quantify pairwise differences in alleles among all individuals. Genodive calculates a distance matrix based on the minimum number of mutation steps that are needed to transform the genotype of one individual into the genotype of the other, summed over all loci. Individuals with distances below a threshold in the distance matrix (threshold = 11) were considered to represent the same genet (Meirmans & Van Tienderen 2004; Rogstad *et al.* 2002). This threshold represents the minimum number of mutation steps that is needed to transform the genotype of one individual into the genotype of another and was chosen because it was it was prior to the point of inflection in the distribution number of clones. Beyond this threshold, genotypes that were different at multiple loci would be identified as one genet, which we considered inappropriate. We compared genets

identified using this method with those that would be identified using complete multilocus matches and found 66 individuals differed due to 3-6 base pair mutation at a single locus and 25 individuals were missing data at one locus but matched exactly at all 9 other loci. Thus, everything we identified as a clone was also identified when exact multilocus matches were required, but we lumped 91 ramets with another genotype that would be identified as unique if missing data or the mutations were coded separately.

We assessed the probability that shoots with identical genotypes were members of the same clone rather than occurring by chance by using P_{gen} (Parks & Werth 1993) to estimate the probability of the occurrence of each genotype based on allele frequencies in each population. We then calculated the probability of sampling a second occurrence of each genotype given the number of genets sampled using P_{sec} (Parks & Werth 1993). These calculations were done using the program GenClone. For each site, the proportion of unique genotypes was calculated as (G-1)/(N-1), where G is the number of unique genotypes and N is the total number of shoots sampled (Arnaud-Haond *et al.* 2007; Pleasants & Wendel 1989). For subsequent analyses, each genet within a population was represented by only one shoot (ramet).

The dispersal of vegetative tissues across long distances has been documented in other submersed aquatics (Fér & Hroudová 2008; Langeland 1996), providing the possibility for sharing of *V. americana* genotypes among sites. To assess the extent of such sharing we pooled all samples, and quantified shared genotypes among sites in Genodive. As with the within-population comparisons, everything we determined to be a clone was an exact multilocus match.

Measures of genetic diversity

For all loci, observed number of alleles (A_n), expected (H_e) and observed (H_o) heterozygosity, proportion of polymorphic loci (P), and private alleles (A_p) within each of the 26 collection sites and across all sites combined were calculated using GDA v1.1 (Lewis & Zaykin 2001). To compare allelic diversity among collection sites and regions, we controlled for varying sample size by conducting a rarefaction analysis using the program HP-Rare v1.0 (Kalinowski 2004, 2005b); rarefied estimates were not used in other analyses. Shannon's information index (I) was calculated using PopGene v1.32 (Yeh *et al.* 1997).

Wright's F_{is} was calculated for the global dataset using the estimator f (Weir & Cockerham 1984) in GDA to test for site-level deviations from Hardy-Weinberg equilibrium. Significance of F_{is} was tested by obtaining confidence limits around each estimate generated by 1000 bootstraps in GDA. Significant departures from Hardy-Weinberg equilibrium can indicate a departure from random breeding.

We examined each site that had more than 2 genotypes for presence of a recent genetic bottleneck using a test for heterozygote excess in the program Bottleneck v 1.2.02 (Cornuet & Luikart 1996). Bottleneck computes heterozygote excess as the difference between expected heterozygosity (H_e) and heterozygosity expected at equilibrium (H_{eq}) for each site from the number of alleles given the sample size (Cornuet & Luikart 1996). Significance of the difference between H_e and H_{eq} was tested using a one-tailed Wilcoxon's sign rank test under a two-phase mutation model which provides results intermediate between an infinite allele model and a stepwise

mutation model that are considered to be most appropriate for microsatellites (Di Rienzo *et al.* 1994).

Population differentiation

We assessed patterns of genetic differentiation in three complementary ways. First we used the program Structurama v1.0 (Huelsenbeck & Andolfatto 2007) to identify theoretical *a posteriori* 'populations' from our collection of sites based on minimal deviations from both Hardy-Weinberg and linkage equilibrium as in Pritchard *et al*. (2000). Structurama differs from the program Structure (Pritchard *et al.* 2000) in that the number of theoretical populations is included as a parameter in the model and a posterior distribution of the probabilities of each number is generated. Prior number of populations and expected number of populations were set as random variables. The sampler was run for 1,000,000 generations and sampled every 25 generations for a total of 40,000 samples. Four heated chains (temperature = 0.1) were used in the analysis. Data were summarized after discarding 10,000 burn-in samples. We chose the mean partition value as the number of theoretical populations (K) containing the highest posterior probability. Because Structurama lacks clearly interpretable visualization of individual assignments we used Structure v2.3.2 (Pritchard *et al.* 2000) to assess distinctiveness of theoretical populations (Berryman 2002) by assigning individuals to the number of populations inferred by Structurama. Structure was run assuming prior admixture, with 1,000,000 steps in the Bayesian sampler, using a burn-in of 50,000 steps. The analysis was run 10 times, and the best run was selected based on the highest likelihood score.

To provide a general overview of site-level differentiation, we calculated global and pairwise estimates of Wright's F_{st}, using Weir and Cockerham's (1984) estimate θ as calculated in GDA. Significance was assessed by generating confidence limits derived from 1000 bootstrap samples. All θ values were normalized to account for the theoretical maximum value and thus allow for future comparison across studies (Hedrick 2005; Meirmans 2006) using the program Genodive (Meirmans & Van Tienderen 2004). There is no significance test for these normalized values (Meirmans 2006). To account for potential limitations of F_{st} in quantifying differentiation (Hedrick 2005; Jost 2008), we also calculated pairwise and global values of Jost's (2008) measure of genetic differentiation, *D*, using Chao *et al.*'s (2008) estimate D_{est_Chao} in SMOGD v 1.2.5 (Crawford 2009). Significance was assessed by generating confidence limits derived from 1000 bootstrap samples in SMOGD.

We tested for relationships between linearized pairwise F_{st} (F_{st} / (1 - F_{st}) (Slatkin 1995) among sites and two different geographic distances using a Mantel test as implemented by the program IBDWS v3.16 (Jensen *et al.* 2005). Significance was assessed using 1,000 randomizations in IBDWS. We used pairwise Euclidean geographic distances calculated from the GPS coordinates collected in the field, and the shortest distance over water among paired sites using Pathmatrix V1.1 (Ray 2005). Euclidian distance is potentially realistic for seed dispersal by waterfowl that can fly over land whereas the weighted geographic distances are more realistic for water-dispersed pollen.

We used principal components analysis (PCA) on the variance-covariance matrix of allele frequencies, using Genodive, to understand the distribution of variance among sampled locations that is a function of variation in allelic composition. PCA provides a different perspective from the Structurama/Structure analyses because it represents the relative degree of genetic similarity among sites in a continuous rather than categorical framework.

Estimates of gene flow among populations

Because coalescent-based methods can provide more accurate and powerful estimates of migration than classical frequentist estimates (Holsinger & Weir 2009; Rosenberg & Nordborg 2002), we quantified migration among population groupings using Migrate-n v3.2.6 (Beerli 2006; Beerli & Felsenstein 1999, 2001). Migrate-n employs a likelihood method of parameter estimation utilizing coalescent theory to estimate asymmetric migration among populations under an equilibrium model that assumes migration has been constant over time (Beerli & Felsenstein 1999). Estimating migration among all sites would require estimating 462 parameters. To estimate a reasonable number of parameters given our data, we limited migration to four groupings based on results from the Structurama/Structure analyses and geographic proximity of sites. The HL locality was difficult to assign to a group in Structure (Figure 2.1) due to assignment probabilities being split between groupings and geographic distance from other sites; it therefore was excluded from this analysis.

Migrate-n was run with the following parameters. Data were treated under a Brownian motion mutational model where mutation rate was calculated as a random variable from the data and missing alleles were discarded. The Bayesian sampler

started from a random genealogy with a full migration model, where both migration rate (M) and population size (θ) were free to vary. The sampler utilized uniform priors for both M and θ. To reduce the size of the tree-space explored by the samples, the priors were constrained based on exploratory analyses between 0 – 4.5 with delta = 0.01 for θ, and 0 – 150 with delta = 30 for M each with 500 bins. Four parallel chains with a swap interval of 1.0 were run with heating values of 10, 7, 4, and 1. One long chain of 80,000 recorded steps was sampled every 20 steps, for a total of 1,600,000 sampled parameters values. Subsequent posterior distributions were summarized after a burn-in of 10,000 steps. The burnin value was selected following examination of exploratory data analyses. Convergence of the run was assessed using effective sample size calculated in migrate-n.

The number of immigrants per generation (Nm) was estimated as $4N\mathrm{m}_j = M_{ij} \times \theta_j$, where θ_j is the effective population size of the recipient population and M_{ij} is the migration rate from population i to population j.

Results

Genetic diversity

We sampled a total of 675 shoots, representing 427 unique genotypes. Within each of 26 locations, we sampled an average of 26.0 shoots (Table 2.1). A median of 68% of sampled shoots within sites represented unique genets, but the proportion of shoots representing multiple genets varied from 0.00 to 1.000 (Table 2.1). Eight of nine sites upstream from and including PL in the Potomac River and site HL in the Mattaponi River were particularly low in genotypic diversity, with genotypic

diversity ranging between 0 and 0.38 of sampled shoots being unique genets (Table 2.1). Site PL was the most extreme, with all 30 samples representing a single genotype. Two exceptions to the trend of low genotypic diversity upstream of PL in the Potomac River were WF and WSP that had clonal diversity values of 0.58 and 0.76, respectively.

Five genotypes were shared among sites within the upper Potomac River (Table 2.2). Two of these genotypes dominated multiple sites, often comprising 53%-100% of sampled shoots. Those two genotypes spanned large geographic distances; one genotype covered approximately 160 river km and the other was present across 132 river km. We found no genotypes shared among other sites within the Chesapeake Bay.

The probability of recovering any given genotype by chance ranged from 5.63×10^{-16} to 5.75×10^{-7} (SD = 3.97×10^{-8}). The probability of finding a second occurrence of each genotype, given the number of genets sampled, ranged from 2.37×10^{-13} to 2.45×10^{-4} (SD = 1.70×10^{-5}). The genotypes that spanned large geographic distances in the Potomac River ranged in the probability of occurrence from 6.5×10^{-11} to 1.5×10^{-7} and in the probability of re-sampling one of those genotypes from 2.75×10^{-8} to 6.57×10^{-5} (Table 2.2). Thus we consider these identical genotypes to be clones that resulted from the same sexual reproduction even.

Table 2.1 Measures of genotypic and genetic diversity in populations of *Vallisneria americana* sampled from the Chesapeake Bay, North America. N = number of sampled shoots; G = unique genets; genotypic diversity = (G-1)/(N-1); *A* = average number of alleles (rarefied allelic diversity not shown); A_p = number of private alleles; *I* = Shannon's information index; *P* = proportion of polymorphic loci; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{is} = correlation of alleles within individuals within populations. TPM = *P* value for Wilcoxon one tail for heterozygosity excess test using the two-phase model. F_{is} and TPM in bold typeface are significant at $P < 0.01$. Population groups were identified using the Structure analysis.

Population Grouping	Sample Locality	Code	N	G	Genotypic Diversity	*A*	A_p	*I*	*P*	H_o	H_e	F_{is}	TPM
Northern Bay	Conford Point	CP	29	26	0.89	5.2	1	1.15	1.0	0.54	0.59	0.089	0.615
	Elk Neck	EN	30	23	0.76	5.5	1	1.22	0.9	0.64	0.60	**-0.057**	0.500
	Fishing Battery	FB	30	26	0.86	4.8	0	1.16	0.9	0.63	0.60	-0.044	0.082
	Sassafras River	SASS	30	29	0.97	5.8	5	1.24	0.9	0.61	0.61	0.004	0.285
Central Bay	Mariner Point	MP	30	24	0.79	4.6	0	1.20	0.9	0.62	0.63	0.003	**0.002**
	Dundee Creek	DC	30	30	1.00	5.5	1	1.12	1.0	0.58	0.61	0.052	0.313
	Chesapeake Bay Hot	CBH	25	16	0.63	5.1	0	1.24	1.0	0.65	0.64	-0.014	0.313
	Chesapeake Bay Cold	CBC	25	18	0.71	5.3	2	1.27	1.0	0.64	0.65	0.014	0.278
	Hawks Cove	HWC	29	27	0.93	5.8	3	1.32	1.0	0.67	0.66	-0.011	0.065
	Shallow Creek	SCN	30	6	0.17	3.1	0	0.92	0.9	0.50	0.57	0.138	**0.014**
	South Ferry Point	SFP	15	5	0.29	3.8	0	1.06	0.9	0.60	0.63	0.055	0.633
Upper Potomac	Upper Potomac 1	TOUR1	15	3	0.14	2.1	0	0.59	0.7	0.57	0.45	**-0.36**	0.055
	Upper Potomac 2	TOUR2	15	2	0.07	1.7	0	0.46	0.7	0.60	0.47	**-0.667**	N/A
	Conococheague Creek	CON	12	2	0.09	1.6	0	0.38	0.5	0.45	0.35	**-0.500**	N/A
	Hancock	HCK	25	8	0.29	3.2	0	0.79	0.7	0.48	0.45	-0.070	0.406
	Williamsport	WSP	22	17	0.76	3.0	0	0.77	0.8	0.45	0.45	0.002	0.125
	Brunswick	BWK	20	6	0.26	2.8	0	0.76	0.8	0.45	0.48	0.057	0.230
	Point of Rocks	POR	33	13	0.38	2.6	0	0.74	0.7	0.49	0.45	-0.099	**0.012**
	Whites Ferry	WF	20	12	0.58	2.9	0	0.75	0.8	0.50	0.44	**-0.151**	0.098
	Pennyfield Lock	PL	30	1	0.00	1.5	0	0.35	0.6	0.50	0.50	N/A	N/A
Lower Potomac	GW Parkway	GWP	30	26	0.86	4.2	0	0.89	1.0	0.39	0.46	**0.160**	0.862
	Piscataway Park	SWP	30	29	0.97	4.2	1	0.89	0.8	0.42	0.46	0.083	0.629
	Gunston Manor	GM	30	17	0.55	4.1	0	0.95	0.9	0.51	0.50	-0.014	0.545
	Leesylvania Park	LSP	30	26	0.86	5.0	0	1.06	1.0	0.42	0.52	**0.193**	0.839
	Aquia Landing	AL	30	30	1.00	5.5	1	1.07	1.0	0.42	0.51	**0.193**	0.862
Mattaponi	Horse Landing	HL	30	5	0.14	2.7	1	0.73	0.8	0.62	0.48	**-0.356**	0.320
		Average	25.96	16.42	0.57	3.9	0.62	0.93	0.85	0.54	0.53	-0.052	N/A
		SD	6.08	10.36	0.34	1.4	1.17	0.28	0.14	0.09	0.09	0.211	N/A

Table 2.2 Number of *V. americana* shoots, and P_{gen} and P_{sec} of each genet (Parks & Werth 1993) that are shared among sites on the main stem of the Potomac River. Sites are ordered from upstream (left) to downstream.

Genotype	Tour 1 n=15	Tour 2 n=15	HCK n=25	WSP n=22	BWK n=20	POR n=33	WF n=20	PL n=30	P_{gen}	P_{se}
1	8	12	12	1	7	2			1.55×10^{-09}	6.61×10^{-07}
2				6	9	15		30	1.54×10^{-07}	6.57×10^{-05}
3			1	3					6.47×10^{-11}	2.76×10^{-08}
4			3	1					1.93×10^{-09}	8.25×10^{-07}
5						1	5		8.85×10^{-10}	3.78×10^{-07}

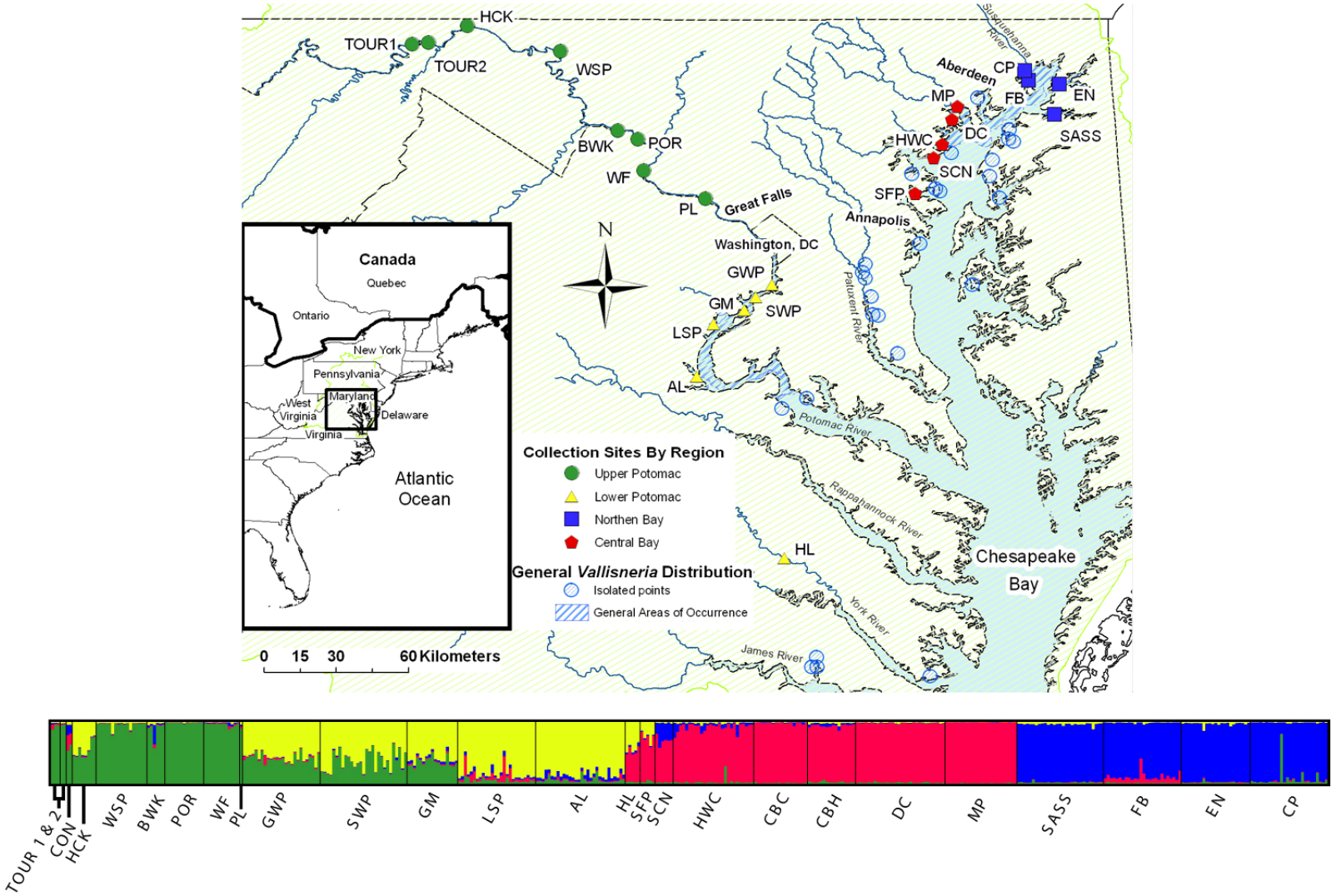

Figure 2.1 Structure results (bottom; colored bars) for *Vallisneria americana* collection sites (top; colored symbols) visited in 2007, 2008, and 2010. Coloring of bars corresponds to coloring of symbols. When K = 4, collection sites from the upper Potomac, lower Potomac, central Bay, and northern Bay form four distinct groupings. PL was excluded from the analysis due to low genotypic diversity. Sites not shown are CON (near WSP), and CBH/CHC (near DC). Dark blue hashed areas represent general and isolated areas where *Vallisneria* occurs in the Bay (Moore *et al.* 2000).

Many loci showed departure from Hardy-Weinberg equilibrium (HWE); however, the degree of deviation was often minimal (Table 2.3). The locus AAGX013 showed significant departure from HWE, and also had a large amount of missing data (31.92%); therefore, it was excluded from subsequent analyses. The amount of missing data in the remaining 10 loci was negligible, averaging 0.84% and ranging from 0.23 - 2.35%.

Table 2.3 Genetic diversity of individual loci averaged over all *V. americana* populations. A = total number of alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{is} = correlation of alleles within individuals within populations. Bold = $P < 0.05$.

Locus	A	H_o	H_e	F_{is}	Percent Missing Data
AAGX071	10	0.681	0.753	**0.095**	0.7
AAGX051	16	0.789	0.865	**0.087**	0.94
AAGX012	6	0.406	0.441	**0.078**	0.23
ATG002	10	0.723	0.771	**0.062**	0.23
AAGX030	5	0.312	0.350	**0.107**	0.23
M49	14	0.607	0.694	**0.124**	0.47
M13	9	0.631	0.807	**0.218**	1.64
AAG002	4	0.547	0.568	0.036	1.17
M16	4	0.082	0.084	0.017	0.47
AAG004	9	0.580	0.688	**0.156**	2.35
Average	8.700	0.536	0.602	0.109	0.843
SD	4.084	0.213	0.244	0.058	0.703
Excluded Locus					
AAGX013*	7	0.152	0.582	**0.740**	31.92

The proportion of polymorphic loci within sites was 0.854 (SD = 0.139). The average number of alleles per locus across all sites combined was 8.70 (SD = 4.08) and within sites was 3.91 (SD = 1.40). When we standardized by number of genets, the number of alleles among sites was similar indicating that genotypic diversity largely controlled allelic diversity. Between one and five private alleles were found

in nine populations. Seven of the sites with private alleles were in the main stem of the Chesapeake Bay (Table 2.1). Sites with private alleles were also relatively high in genotypic diversity (>18 genets). None of the sites with low genotypic diversity in the Potomac River had private alleles.

Observed heterozygosity was high at all sites (avg H_o = 0.535; SD = 0.086). Nine sites departed significantly from Hardy-Weinberg equilibrium (Table 2.1); six sites had more heterozygotes than expected (EN, Tour1, Tour2, CON, WF, and HL) and three had fewer heterozygotes (GWP, AL, LSP; Table 2.1). Shannon's information index was similar among all sites except the HL site, and those sampled in the Potomac River above Great Falls, MD (Table 2.1).

Based on analysis with the program Bottleneck (Cornuet & Luikart 1996), 3 of the 24 sites we could analyze (MP, SCN, and POR) showed evidence that H_e significantly exceeds H_{eq}, which suggests that they have undergone recent genetic bottlenecks (Table 2.1). Of the sites in the lower Potomac with significant F_{is}, two of these sites supported only two genotypes and thus did not have the minimum number of samples to run Bottleneck; the third only met the minimum requirement of three genotypes. Lack of a significant bottleneck for this site could easily have been due to the small sample size.

Population differentiation

Bayesian clustering analysis as implemented by Structurama indicated that there are four genetic subdivisions in the 26 sampled locations of *V. americana* in the Chesapeake Bay (Pr[K = 4 | X] = 0.9993). When Structure was run assuming K = 4 to visualize individual clusters three primary divisions were noted: northern Bay

localities, central Bay localities, and Potomac River localities (Figure 2.1). A further subdivision between the upper and lower Potomac River was identified. Mixed population assignments of individuals provide evidence of similarity among all members of the upper Potomac and several lower Potomac sites (GWP, SWP, GM). The sites LSP and AL had low probability of assignment into the upper Potomac localities (Figure 2.1). The Potomac River sites also have a very small degree of admixture with the central Bay sites, which is most evident in LSP (Figure 2.1). Site HL from the Mattaponi River was difficult to assign, with assignment probabilities being split between the Potomac group and the central Bay group.

Overall, we observed moderate levels of global genetic differentiation among all sites combined (θ = 0.114, 95% CI = 0.081 – 0.152). The PL location was excluded from these analyses because it is not possible to calculate F_{st} or D for a site with only one sample. Within regions identified in Structure, the median pairwise values of θ among sites ranged from ~ 0.020 in the upper and central Bay, to 0.043 among sites in the lower Potomac, to 0.10 in the upper Potomac. The median pairwise θ value of sites from different regions was 0.114 and the range was from 0.013 to 0.32. Thus, the pairwise differences among sites from the upper Potomac (range was from -0.02-0.31) were similar to differences among other sites from different regions. The global D_{est_Chao} (0.124, 95% CI = 0.008 – 0.352) was slightly higher than θ. The median pairwise D_{est_Chao} among regions was 0.07. Within region median values of D_{est_Chao} were lower than those observed with θ (northern Bay = 0.02; central Bay = 0.01; upper Potomac = 0.01; lower Potomac = 0.009), and indicate that differentiation within regions was substantially lower than among regions.

There were significant relationships between genetic distance and both straight-line ($r = 0.39$; $p < 0.001$) and weighted ($r = 0.59$; $p < 0.001$) distances (Figure 2.2) for all sites combined. Relationships with both geographic distances were also significant in the upper (straight-line: $r = 0.41$; $p < 0.001$; weighted: $r = 0.47$; $p < 0.001$) and lower Potomac River (straight-line: $r = 0.69$; $p < 0.001$; weighted: $r = 0.93$; $p < 0.001$) groups. In the northern Chesapeake Bay, neither measure of geographic distance provided a significant correlation. The central Chesapeake Bay tended to have larger genetic distances among sites relative to the northern Chesapeake Bay (distance table not shown); however, the correlation was not significant for either distance measure.

The PCA on the variance-covariance matrix of allele frequencies showed that allelic composition was generally more similar within than among the four geographic regions within the Chesapeake Bay identified in the Structure analysis (Figure 2.3). The first axis explained 27.58% of the variance in allele frequencies and captured differences among the regions. The second axis explained 18.65% of the variance and was driven primarily by two sites with extremely low genotypic diversity (G=2 in CON and G=1 in PL). Both populations were distinct due to chance fixation of some alleles and the fact that given small number of genets present in each site, allele frequencies are by necessity limited to a small range of values, and those values happened to be higher than those in other populations. The alleles that were fixed in these sites were also present in other sites but the resulting large differences in allele frequency placed CON and PL away from all other sites, and compressed the remaining sites into a small portion of Axis 2 (Figure 2.3).

Migration

Effective sample size, a measure of convergence, exceeded 1000 samples for all parameters. The number of migrants per generation ($4Nm$) among the four groups identified using Structure and geographic proximity varied from 7.69 to 29.91 (Figure 2.4). The upper Potomac River population grouping was largely isolated from all other populations. The lower Potomac River population grouping had apparent migrant exchange with both the northern and central population groupings with relatively equal frequency ($4Nm$ = 25.41 to 29.91). The northern Chesapeake Bay received nearly the same number of migrants from ($4Nm$ = 28.14; CI = 23.21 – 32.96) as it contributed to ($4Nm$ = 21.29; CI = 17.06 – 26.24; Figure 2.4) the central Chesapeake Bay. In contrast, the upper Potomac River appeared to share more migrants with the lower Potomac ($4Nm$ = 17.39; CI = 12.44 – 21.62) than the lower Potomac shared with the upper Potomac ($4Nm$ = 9.91; CI = 7.67 – 13.61), but the confidence intervals in these estimates overlapped to a small degree.

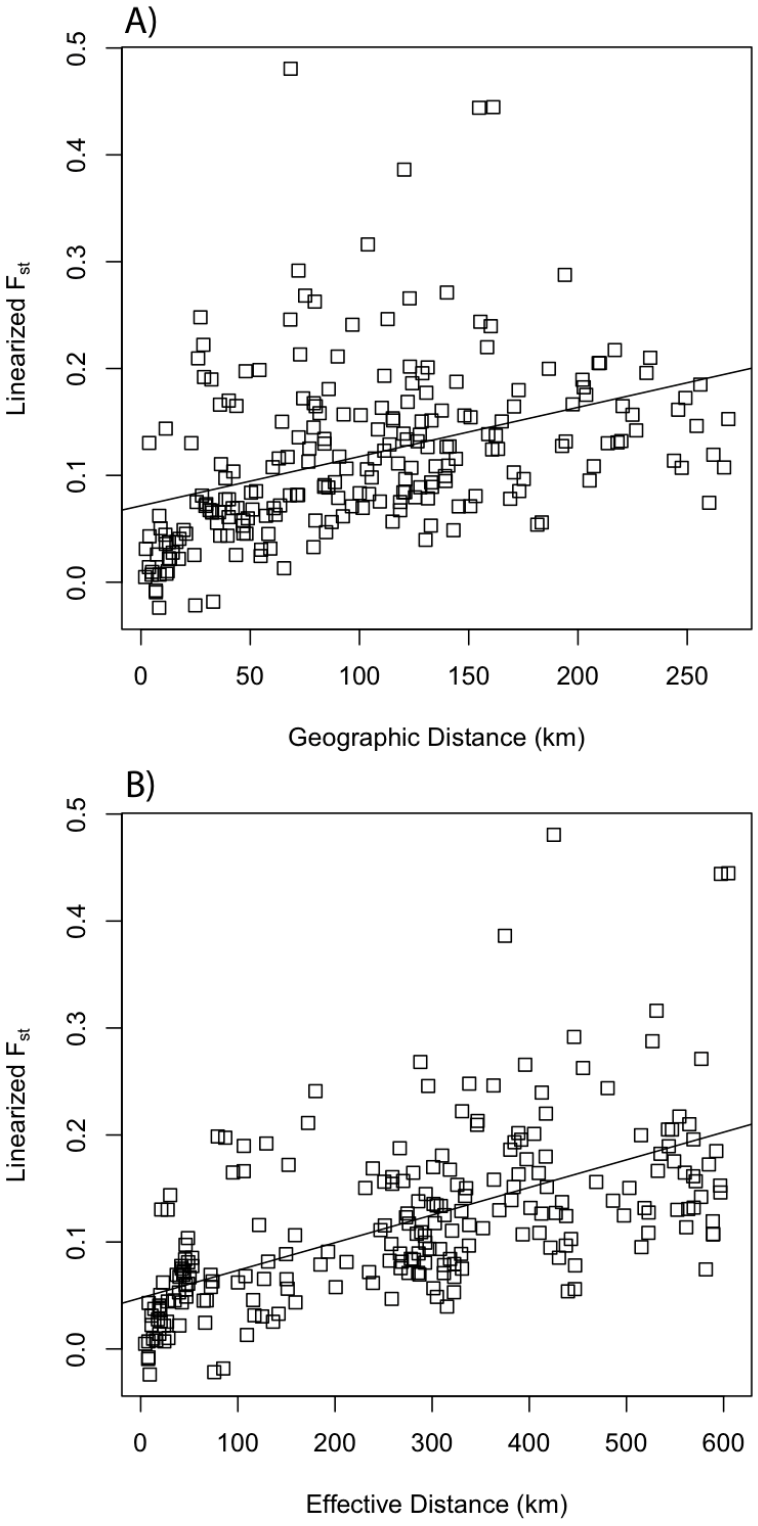

Figure 2.2 Linearized F_{st} (F_{st} / (1 - F_{st}) (Slatkin 1995) genetic distance regressed against A) Euclidean geographic distance and B) the shortest distance over water among collection sites (weighted geographic distance).

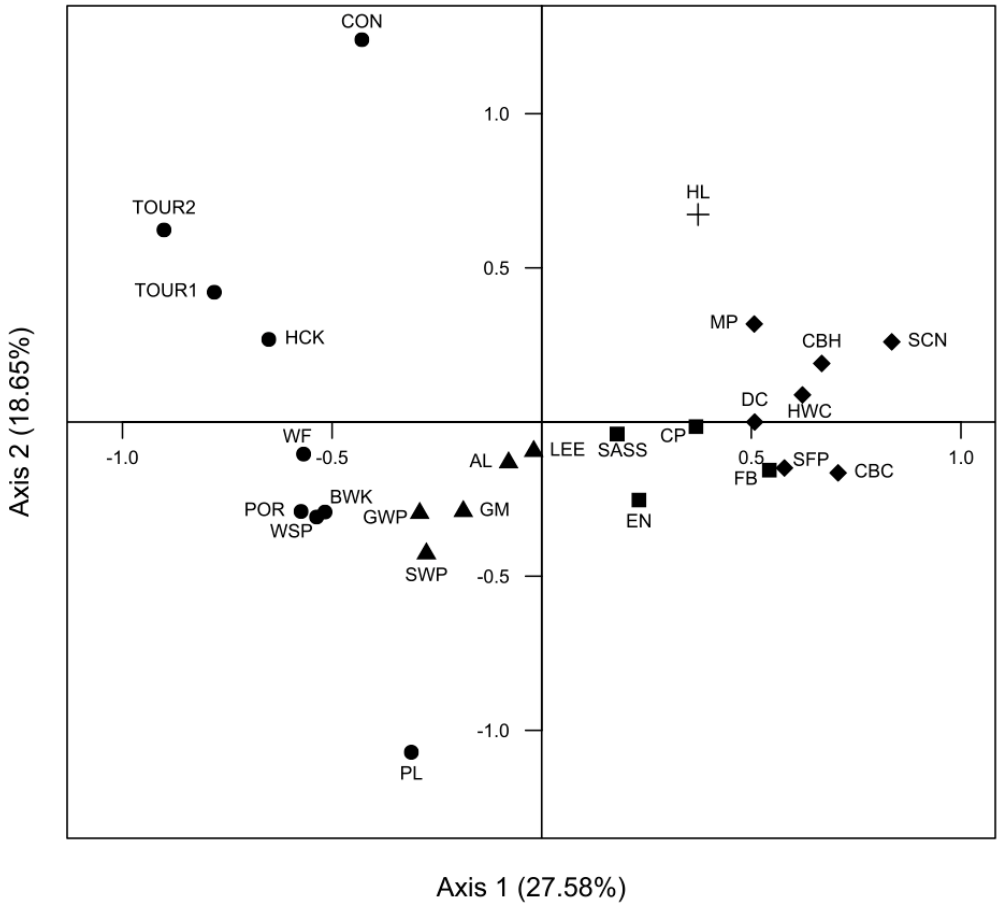

Figure 2.3 Principal components analysis of the covariance matrix of allele frequencies. Axis 1: Eigenvalue = 0.29, percent of variation explained = 27.58; and axis 2: Eigen value = 0.19, percent of variation explained = 18.65. Symbols represent the four genetic regions within the Chesapeake Bay

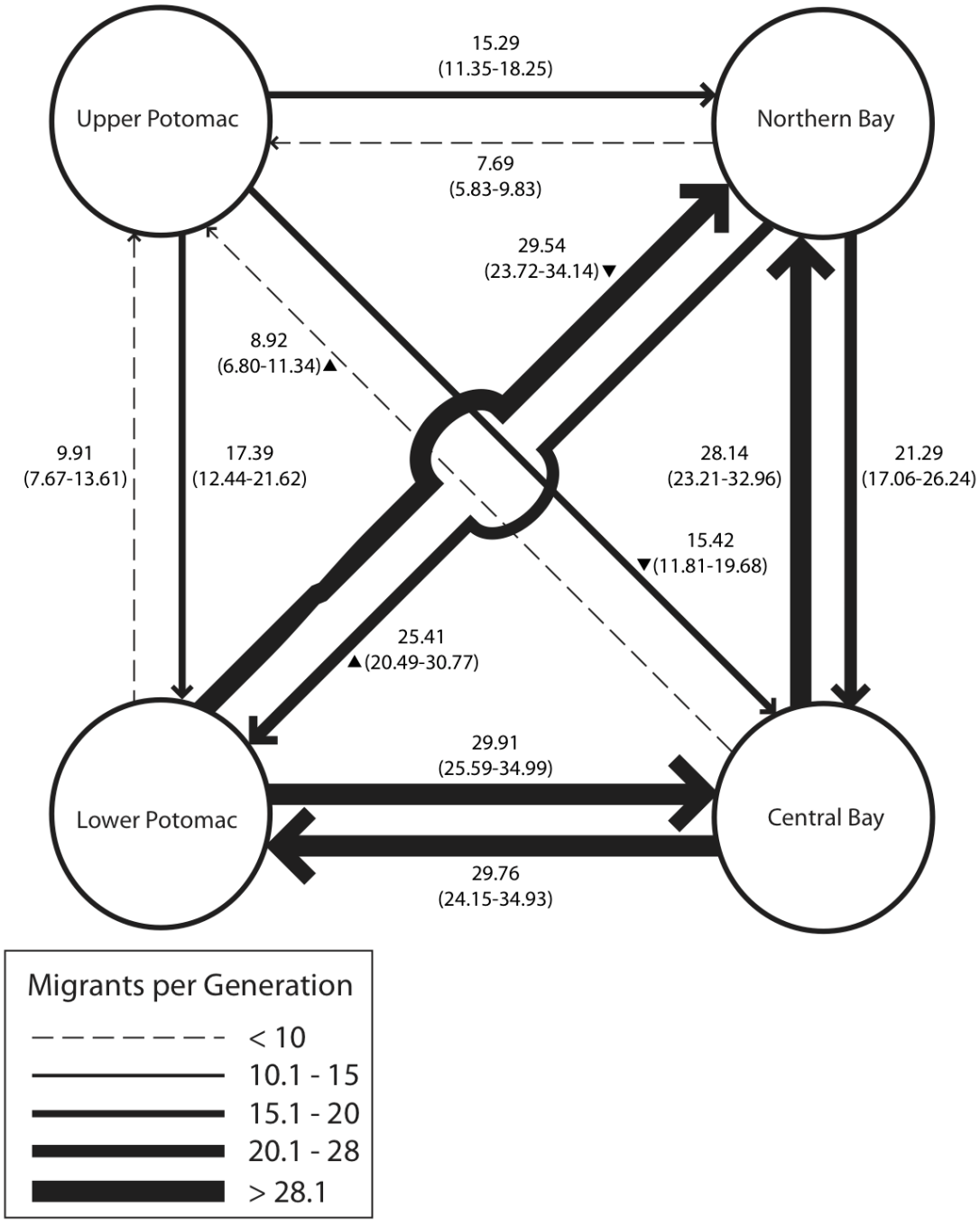

Figure 2.4 Per generation bidirectional migration rates (4*N*m) among the four population grouping recovered from analysis in Migrate-n.

Discussion

Overall, most sites of *Vallisneria americana* in the Chesapeake Bay support a diversity of genotypes and alleles, and most are not highly inbred. This is good news for the future of the species in the Bay because high genetic diversity increases a population's capacity to persist under variable environmental conditions (Frankham 1995a; Procaccini & Piazzi 2001; Reed & Frankham 2003; Williams 2001) and to adapt to novel conditions (Barrett & Schluter 2008; Frankham 2005b; Lavergne & Molofsky 2007). The genotypically diverse sites can also serve as sources of material for restoring *V. americana* to currently unoccupied sites. The geographic structuring of genetic diversity we documented is important to consider if movement of propagules around the Bay is proposed. Despite the relatively positive general outlook, evidence for recent bottlenecks in three sites, signs of inbreeding at three sites, and low genotypic diversity in the upper Potomac River raise concern for long-term effects of the previous population declines.

Genetic diversity

Species level allelic richness in the Chesapeake Bay and its tributaries was on par with what has been found in other SAV species from throughout the world, which ranges from 2 to 18 alleles per locus (Campanella *et al.* 2010; Pollux *et al.* 2007; Reusch *et al.* 1999b, 2000; Rhode & Duffy 2004; van Dijk *et al.* 2009). Our site-level allele richness was also mostly within the typical ranges of values found in these same studies of other SAV species (2.3-10.5 alleles per locus). The three exceptions that had particularly low allelic richness (1.5-1.7 alleles/locus) supported only 1 or 2 unique genotypes each (Table 2.1). Beyond these extreme cases, lower allelic

diversity was associated with lower genotypic diversity, typically with < 30% of sampled shoots in low allelic diversity sites being unique genets.

Evidence of recent bottlenecks based on heterozyote excess in three sites (MP, SCN, and POR) and the significant inbreeding coefficients in three sites in the lower Potomac River (GWP, LSP, AL; Table 2.1) cause some concern. However, widespread inbreeding was not observed despite low levels of genotypic diversity (and therefore effective population size). The dioecious mating system of *V. americana* enforces outcrossing and may explain why inbreeding was not more prevalent. Determining the full implications of apparent bottlenecks and inbreeding requires understanding their fitness consequences, which is beyond the scope of this study.

One of our more striking results is that genotypic diversity ranged from 0-1.0, meaning that sites ranged from being monoclonal to every sampled shoot being distinct. It also means sites range from having no detectable sexual reproduction to no detectable asexual reproduction. Such variation in mating structure across this same spatial scale is not common in aquatic species but has been documented in *Typha minima* Hoppe (Till-Bottraud *et al.* 2010) and in *Posidonia oceanica* Delile (0.1-0.97; Arnaud-Haond *et al.* 2010). The general paradigm that *Vallisneria* populations are maintained primarily by vegetative reproduction (e.g., McFarland & Shafer 2008) is not supported by our data.

The sites with low genotypic diversity relative to other *V. americana* locations in the Bay are those in the upper Potomac River, site HL in the Mattaponi River, and sites SCN and SFP in the central Chesapeake Bay. Variation in levels of genotypic

diversity among sites is interesting because of the advantages typically associated with high genotypic diversity and for the insights into the potential mechanisms that might have caused these sites to have fewer, more extensive clones than other sites in the Bay. Higher genotypic diversity has been correlated with increased resistance to periodic stressors and more resilience after climatic extremes in experimental settings (Hughes & Stachowicz 2004, 2009; Reusch *et al.* 2005) and with increased survival of transplants (Procaccini & Piazzi 2001). Thus, although sites in the upper Potomac River support extensive cover, the few highly successful genotypes may not provide the genetic variation necessary to withstand novel perturbations or adapt to future conditions. It is important to note that the effect of genotypic diversity on the stability of SAV beds is still unclear. At least some field observations indicated higher mortality in more genetically diverse populations of *Posidonia oceanica* (Arnaud-Haond *et al.* 2010). Further, sedimentation rate was a stronger predictor of shoot mortality in *Posidonia oceanica* than were genetic diversity or even demographic parameters (Arnaud-Haond *et al.* 2010).

Clearly, at extreme levels of disturbance that exceed physiological tolerances, no amount of genetic diversity will be sufficient to withstand or overcome perturbations, and environmental factors become more important. Short of such extremes, it is plausible that a limited number of genotypes will be sufficiently resistant to survive perturbations, which would result in less genotypically diverse populations in high disturbance sites. Conversely, low genotypic diversity in more stable sites has been explained as resulting from one genotype becoming dominant. Periodic or fluctuating disturbance could foster more genotypic diversity if survival and fitness of genotypes

differed across conditions (Hammerli & Reusch 2003). The patterns observed in any particular case will depend on the magnitude and frequency of disturbance and the interaction between that disturbance and genotypic or phenotypic abilities to withstand it. Without monitoring over time, it is not possible to know if low genotypic diversity is a signature of past environmental perturbations that have left only tolerant genotypes or the result of stochastic losses.

In addition to having low genotypic diversity, multiple sites along the upper Potomac River shared the same genotype (Table 2.2). The geographic extent of the five shared genotypes is remarkable: two of them extended a distance of 130 and 160 river kilometers, and the remaining three genotypes covered distances of 50 river kilometers. The probability of recovering the specific genotypes by chance if they were not identical by descent given global allele frequencies is astronomically small 10^{-7} to 10^{-11} (Parks & Werth 1993), and the probability of finding a second occurrence of each genotype, given the number of genets sampled, is 10^{-5} to 10^{-8} (Parks & Werth 1993). A typical mutation rate of microsatellite loci (~10^{-3} to 10^{-4} per allele per generation; Thuillet et al. 2002; Vigouroux et al. 2002) does provide the possibility that these genotypes are merely identical in state (Mank & Avise 2003); however, it is highly unlikely that mutation events simultaneously produced identical individuals across such a large geographic range. Although a large proportion of studied angiosperm species exhibit clonality that extends across more than one location (Ellstrand & Roose 1987), extremely large clonal extent is rare. Examples of the larger known clonal extents include a single *Populus tremuloides* Michx. clone that covers an area of roughly 43 ha (Mitton & Grant 1996), and several submersed

aquatic species that are known to have clones that extend > 5 km (Reusch *et al.* 1999a; Ruggiero *et al.* 2002). Most studies of other SAV species indicate that clones are primarily limited to within individual sites (Campanella *et al.* 2010; Titus & Hoover 1991) with extents typically limited to the scale of ~18 m (Becheler *et al.* 2010), to 78 m (Arnaud-Haond *et al.* 2010), to ~250 m (Zipperle *et al.* 2009).

Vegetative expansion of *V. americana* through rhizomes is generally limited to within a few meters of the parent plant (Titus and Hoover 1991). Maximum seasonal lateral growth of *V. americana* from the upper Potomac River genotypes is 60cm under greenhouse conditions (Engelhardt, unpublished data). At this ideal growth rate it would take roughly 260,000 years to grow 130-160km, and even supposing growth occurred from a central location outward, it would take 130,000 years to traverse that distance. It is unlikely that habitat necessary to allow this vegetative growth would have been sufficiently continuous and stable throughout the stretch of the river for such a long period of time. Thus, although lateral vegetative growth within sites could potentially lead to local dominance by one or a few genotypes, it is highly improbable that lateral growth alone is responsible for genotypes extending 50-160 km along the Potomac River.

The question, then, is how did these few genotypes come to extend and dominate over such large areas? Specific mechanisms could include passive stochastic loss and colonization, deterministic processes based on competitive ability, selective advantages due to environmental tolerance of particular genotypes, or a combination of passive and deterministic processes. Passive processes could include initial chance colonization by few genotypes that expanded in place, or stochastic loss of genotypes

within sites followed by repeated recolonization by a small number of genotypes. More deterministic processes include selection in response to abiotic factors or competition. If particular genotypes were resistant to abiotic stressors, they would become dominant as other genotypes were eliminated. Dominance by a few clones could also result if downstream sites were colonized by a small number of competitively superior vegetative propagules from upstream populations, widespread dominance of a limited number of genotypes would result. We offer these mechanisms as possible explanations; our current data are not sufficient to infer mechanism but are more consistent with some possibilities than others, and clearly point to the need for further experiments.

Tubers of *V. americana* are generally negatively buoyant, but they can become positively buoyant if attached to shoot fragments (Titus & Hoover 1991). The extensive clones we observed in the Upper Potomac River could have originated from dislodged shoots and tubers that were carried downstream in floods (Fér & Hroudová 2008). Flooding events sufficiently extreme to cause scouring are common in the Potomac River and removal of individuals from suitable habitat would create opportunities for expansion of chance colonists. It is likely that upstream populations have either had low diversity due to founder events, or that diversity has been lost from small, isolated sites. Once upstream populations have low genotypic diversity, opportunities to gain new diversity would be limited due to unidirectional water flow from headwaters to mouth. Large distances from other major bodies of water yield small chances of recolonization from sources other than nearby low diversity sites (Chen *et al.* 2007). The process could generate a positive feedback loop in that as

particular genotypes become more dominant, they become more likely to be source material for additional colonizations. An additional consequence of low genotypic diversity that may in turn facilitate dominance of a few genotypes is the reduced probability of having both males and females, which limits sexual reproduction. Existing clones could have higher potential to spread and occupy larger areas than they might in populations that also had sexually produced propagules. We have no quantitative data on sex ratios but we have observed fertile fruits at all sites, indicating some sexual reproduction is occurring. However, for the same level of search effort, we found substantially fewer fruits at many of the upper Potomac River sites than we found in other locations throughout the Bay.

Another explanation that we considered to possibly explain widespread dominance was the introduction of competitively superior genotypes into the Potomac River via restoration or other activities, or through natural mechanisms such as ingestion and dispersal of tubers via waterfowl. We know of no restoration activities within any of these regions. Additionally, many of the sites visited were not easily accessible, which would hinder the inadvertent introduction by humans through recreational activities such as boating or through activities such as dumping of aquaria.

It is most likely that the unprecedented size of the large *V. americana* clones in the Potomac River has resulted from a combination of local spread via rhizomes and repeated longer distance dispersal of tubers during storm events. Clearly, much still needs to be learned regarding dispersal of vegetative propagules from parent populations (Titus & Hoover 1991). Regardless of the mechanisms, lower genotypic

and allelic diversity in the upper Potomac River sites compared to other localities in the Bay suggests that they should be considered cautiously as source material for restoration plantings. Sampling shoots from even widespread locations is highly likely to yield the same genotype. If the upper Potomac River were used as a source for restoration, using seed rather than vegetative material would improve chances of representing more genetic diversity and of including both male and females in restoration plantings.

Genetic differentiation and migration

The overall patterns of genetic differentiation among sites in the Bay related strongly to geographic distance (both straight line and weighted and is indicative of equilibrium between genetic drift and gene flow (Hutchison & Templeton 1999). Beyond coarse geographic trends, Structure analysis indicated the Chesapeake Bay can be broken into four genetic regions. These subdivisions roughly correspond to regions of differing salinity. The northern Chesapeake Bay is oligohaline and the central Chesapeake Bay is oligohaline to seasonally mesohaline (Pritchard 1952). Sites in the lower Potomac River are oligohaline and are strongly tidally influenced while the upper Potomac River is entirely freshwater. Such environmental differences can increase isolation among populations (Doebeli & Dieckmann 2003; Keeley 1979; Stanton *et al.* 1997), influence patterns of occurrence and hybridization (Blum *et al.* 2010; Crain *et al.* 2004), and drive adaptation to local conditions (Antonovics 2006; Antonovics & Bradshaw 1970; Clausen *et al.* 1941; Linhart & Grant 1996).

The admixture among the regions implies at least historic gene flow among sites, and results from the full Migrate-n analysis show evidence of some exchange between the two regions within the Potomac River (Figure 2.4). Even with this admixture, the level of substructuring we detected is surprising given the potential for the Bay to represent one large, hydrologically connected unit (e.g., van Dijk *et al.* 2009). The degree of substructuring is greater than has been found in other studies at similar scales (Campanella *et al.* 2010).

The level of differentiation we observed among sites within each region is similar to levels documented from hydrologically connected populations of several *Vallisneria* species (G_{st} = 0.02 – 0.06; Lokker et al. 1994; Chen et al. 2007) and other seagrass populations sampled from similar spatial scales (Campanella *et al.* 2010). When sites are pooled, the degree of genetic differentiation between the north and central Chesapeake Bay (D_{est_Chao} = 0.060) is at the upper range of the levels documented among connected sites. Levels of differentiation among sample sites in different regions are more similar to those found in isolated water bodies: F_{st} = 0.132 - 0.202 and G_{st} = 0.457 (Laushman 1993; Wang *et al.* 2010). Interestingly, the amount of gene flow between the north and central localities estimated by Migrate-n is theoretically enough ($4Nm$ = 21.29 – 28.14) to swamp out genetic differentiation among populations. If successful migration among populations is sufficiently common (e.g., > 1 migrant per generation), genetic subdivision is not likely to occur (Slatkin 1981, 1985, 1987; Wright 1931). Several factors could be influencing the observed patterns of gene flow among the populations. Coalescent-based analyses integrate estimates of migration and effective population size over $4Ne$ generations

(Kingman 1982a, b). A disconnect between current patterns of genetic differentiation and the amount of historic gene flow among populations could exist (Sork *et al.* 1999). In addition, genetic differentiation can occur in presence of substantial gene flow (Morrell *et al.* 2003). In cases where extreme environmental heterogeneity exists among sites, reproductive isolation can develop and be sustained even in the face of genetic exchange among populations (Antonovics 2006; Caisse & Antonovics 1978).

We interpret the inferred regions cautiously because sampling from a continuous population with local mating structure can yield 'populations' using the program Structure (Schwartz and McKelvey 2008). However, most sites we sampled in the northern and Central Bay were from discrete beds that are isolated from other beds by depth and salinity beyond the limits of tolerance for *Vallisneria*. Thus, although they would have been more extensive historically, it is not likely that many of the now isolated beds would ever have been continuous. In contrast, the upper Potomac River is probably best considered one extensive relatively continuous population with a combination of extensive vegetative dispersal and of sexual reproduction among spatially proximal individuals. Within the upper Potomac, F_{st} and Jost's D values (Table 2.1) reflect local mating structure while the extensive distribution of some genotypes (Table 2.2) indicate connectivity over large distances that is not reflected in other statistics calculated including only one representative of each genotype. There are no extensive natural physical barriers along this part of the river, and there is no abrupt environmental change. There are several small dams that cause 1-2km breaks in the distribution of *Vallisneria* by increasing sediment deposition

immediately upstream and causing extensive scouring immediately below. In contrast, differences in F_{st} and Jost's D between the upper and lower Potomac are more similar to those in between other regions, and no genotypes are shared. The major environmental difference between two parts of the river is the tidal influence in the lower reaches of the river that is absent above Great Falls, MD. More intensive sampling between our existing sampling locations is needed to elucidate finer scale patterns of population structure, clonal diversity, and clonal extent, which are necessary to understand spatial mating and dispersal structure.

Implications for restoration

Goals for 'restoration' can range from simply returning vegetation to a site, to full-scale ecological restoration. Ecological restoration is defined as, "an intentional activity that initiates or accelerates the recovery of an ecosystem with respect to its health, integrity and sustainability" (Society for Ecological Restoration International Science & Policy Working Group 2004). This definition requires, the restored ecosystem to be self-sustaining and be sufficiently resilient to endure the normal periodic stress events in the local environment. (http://www.ser.org/content/ ecological_restoration_primer.asp#5). There are three main paradigms for selecting material for revegetation efforts.

1. Select a few particularly well performing genotypes for a particular set of criteria and propagate those genotypes in a manner similar to development of cultivars in agriculture and horticulture. This approach lends itself to efficient commercial production of source material and development of material with resistance to known pests or

pathogens or with characteristics that meet specific needs. Planting one or a few genotypes over broad areas may be successful in the short-term but provides no raw material for evolution to changing abiotic conditions or novel pathogens. Although it is sometime applied in revegetation project, it is generally not considered acceptable in ecological restoration.

2. Select propagules such that amounts and types of genetic diversity in restored populations reflect those found in surrounding natural populations. This approach recognizes the importance of local adaptation and uses local genetic stock. A major goal is to prevent founder events in the restoration process that can occur during collection, cultivation or planting so that future evolutionary potential is maintained. At the same time, propagule sources can be selected based on spatial proximity or habitat similarity (van Katwijk *et al.* 2009) between the source and reference site that are deemed to be sufficiently local. This approach can be problematic if individual sites are genetically depauperate and or inbred, but prevents planting maladapted stock or causing genetic pollution of local populations (McKay *et al.* 2005). However, the presence of local adaptation is not documented for most species and the spatial scale at which such adaptations may occur is likely to be idiosyncratic. Unnecessarily restricting source material for widespread species with little or no local adaptation can severely hamper restoration efforts (Broadhurst *et al.* 2008).

3. Use large numbers of propagules of diverse origin, letting natural selection sort out appropriate genotypes for a particular site (Broadhurst *et al.* 2008). This approach is suggested for relatively common, widespread species that have long-distance dispersal abilities but that are now fragmented and in which individual remnants do not support much remaining diversity or in which inbreeding depression may be causing reduced fitness. Such an approach is also suggested for large-scale regional restoration efforts in which sufficient propagules may not exist within small isolated fragments. Advocates of this approach suggest that the genetic diversity of the source material is as important as or more important than being 'local.' Inappropriate use of genetic stocks in environments to which they are not adapted can substantially impact the success of restored populations (Hufford & Mazer 2003; Montalvo *et al.* 1997). Restoration failure may result when the foreign genetic stock provisions resources at inappropriate times (Jones *et al.* 2001), is maladapted to local conditions (McKay *et al.* 2005), or contributes to outbreeding depression (Montalvo & Ellstrand 2001; Potts *et al.* 2003; Templeton 1997).

Although they provide insight into only the one aspect of genetic diversity, our results inform aspects of each of these potential approaches. We found that levels of genotypic and allelic diversity at most sites are high and can serve as source populations for restoration material. Exceptions include upper Potomac River sites (e.g., HCK, POR, WF), and two sites in the central Bay (SCN, SFP). Low diversity

in sites and presence of shared genotypes among sites in the upper Potomac River also cautions against the use of that region for source material without prior thought and understanding of the potential implications of low diversity collections. On the other hand, the widespread genotypes in the low diversity sites could be candidates for intensive propagation if their dominance was shown to relate to superior competitive ability that confers resistance to environmental stressors affecting the Potomac River. We do not advocate approaches that reduce genetic diversity, but as part of a comprehensive restoration program, having genotypes that can withstand and even flourish under stressful conditions could be beneficial. Our current data only provide a starting point for investigation of such possibilities.

Based on the diversity we observed, we found no compelling evidence for the need for genetic rescue of any population through introduction of genotypes or the need to mix genotypes in restoration plantings (Hedrick & Fredrickson 2010). We have no way of knowing the original levels of genetic diversity in the Bay, but, despite extensive population size declines, there is no evidence of catastrophic losses in that most remaining sites are not genetically depauperate or homogeneous. Confirmation of this assertion requires comparing fitness in apparently bottlenecked populations with populations that have no indication of severe reduction.

The spatial substructuring we detected among sites in the northern and central Bay suggests that caution should be used in moving propagules to locations distant from their source. It is also necessary to more thoroughly understand the population structure within the Potomac River to determine the scales at which there is genetic interaction from dispersal of vegetative propagules, pollen, and seed. Specifically,

we suggest that movement of propagules for restoration activities be limited to within each of the four primary geographic areas that are related to environmental factors, in particular salinity. We find no strong evidence against moving propagules within regions. Our data do not allow us to assess the degree to which the genetic differences we detected indicate adaptation to local environmental conditions. We are just beginning to conduct experiments to determine whether there is evidence for local adaptation within these regions and if there are fitness consequences of crossing individuals from different regions. Until more investigations relating these patterns with fitness are completed, it is prudent to be cautious and carefully select plant material from within one of the genetic regions.

Chapter 2: Does genetic diversity of restored sites differ from natural sites? A comparison of *Vallisneria americana* (Hydrocharitaceae) populations within the Chesapeake Bay

The goal of ecological restoration is to re-establish self-sustaining ecosystems that will resist future perturbation without additional human input. We focus here on the re-establishment of submersed aquatic macrophyte beds in the restoration of the Chesapeake Bay estuary. Degraded environmental conditions are often to blame for poor bed establishment, but genetic factors could also be contributing to low survival. We quantified the effect of restoration practices on genetic diversity in the submersed aquatic plant species *Vallisneria americana* Michx. (Hydrocharitaceae) in the Chesapeake Bay. In 2007, we collected 440 shoots from 8 restored/natural site pairs and 4 restoration stock repositories, and genotyped those individuals at 10 microsatellite loci. Restoration practices do not appear to negatively impact genetic diversity, and basic measures of genetic diversity within restored sites overlap with natural sites. However, small population size of restored sites, significant inbreeding coefficients within 3 sites, and low overlap of allele composition among sites provide cause for concern. These problems are relatively minor, and we propose several corrections that would alleviate them altogether. Managers should be encouraged by our findings as well as the current state of the genetic diversity within *V. americana* restoration efforts.

Introduction

The ultimate goal of ecological restoration is re-establishing self-sustaining ecosystems that will be resilient to future perturbation without additional human input

(Broadhurst *et al.* 2008; Liu *et al.* 2008; Procaccini & Piazzi 2001; Ramp *et al.* 2006; Rice & Emery 2003). In practice, restoration can range from simply creating suitable physical conditions to allow natural colonization; planting pioneer species that will facilitate succession and eventual growth of target species; supplementing one or few species within a relatively intact ecosystem; to constructing diverse communities on denuded sites (Montalvo *et al.* 1997). Spatial scales of efforts range from small, local projects (e.g., <10 ha) to plantings that cover broad geographic areas (e.g. >100 ha; Broadhurst *et al.* 2008). Regardless of the scale of a project, restored populations must persist in dynamic settings in the short term (Jordan *et al.* 1988) and also retain the capacity to undergo adaptive evolutionary change in the long term (Montalvo *et al.* 1997; Rice & Emery 2003) to be considered successful. Genetic diversity of planting materials is a key consideration for restoration success for both time frames.

Unfortunately, genetic diversity is often not explicitly measured or considered in restoration and, owing to logistical constraints, restored populations are frequently founded with a limited number of individuals that may represent only a portion of the genetic diversity present in natural populations. Small numbers of founding individuals can have two main genetic consequences. First, reduction in effective population size (Frankham 1995b, 1996) can directly impact fitness due to increased inbreeding (Dudash 1990; Gigord *et al.* 1998; Keller & Waller 2002). Second, reduced effective population sizes can diminish allelic diversity and thus long-term evolutionary potential through increased rates of genetic drift (Hartl & Clark 2007; Whitlock 2000). Low levels of diversity can also arise from initial selection of few genotypes. Whether it results from initial selection of planting stock or losses over

time, low genetic diversity can limit potential for resilience of populations under environmental stressors such as grazing (Hughes & Stachowicz 2004), heat shock (Reusch *et al.* 2005), or nitrogen loading (Tomas *et al.* 2011). In contrast, increased diversity resulting from restoration techniques can provide short-term benefits that can increase transplantation success (Procaccini & Piazzi 2001). There is also evidence to suggest that genetic diversity affects the structure of communities (Ellers *et al.* 2011; Rowntree *et al.* 2011; Wimp *et al.* 2005) and ecosystem functioning (Tomas *et al.* 2011) and may therefore be important in the provision of ecosystem services.

Such diversity can come from within one or a few local sites or can come from combining individuals from a site from a broader geographic area. Source populations are critical in restoration because selecting individuals from a limited number of sites can lead to the use of individuals adapted to environments that differ from environmental conditions at the restoration sites can negatively affect restoration efforts (Fenster & Galloway 2000; Hufford & Mazer 2003; Montalvo & Ellstrand 2000; Montalvo & Ellstrand 2001).

Thus, genetic diversity is critical to restoration success, and yet restoration practices themselves can negatively affect diversity. These potential consequences led us to quantify the effect of restoration practice on genetic diversity in the submersed aquatic plant species *Vallisneria americana* Michx. (Hydrocharitaceae). Submersed aquatic vegetation (SAV) communities are among the most threatened on earth (Short & Wyllie-Echeverria 1996). SAV declines have been well documented in the Chesapeake Bay (Dennison *et al.* 1993), one of the largest estuaries in the

world. Dramatic reductions in *V. americana* cover and extent in the northern freshwater reaches of the Chesapeake Bay and its tributaries in the 1970s and 1980s (Kemp *et al.* 1983) led to targeted efforts to restore this taxon to denuded areas. These efforts have resulted in low establishment rates that are not unique to *V. americana;* most seagrass species have experienced net loss of habitat even with restoration efforts, and worldwide success of seagrass transplantation as judged by persistence and bottom coverage is roughly 30% (Fonseca *et al.* 1998).

Inappropriate site conditions coupled with continued poor water quality have likely contributed to low establishment rates in many restoration plantings (van Katwijk *et al.* 2009). However, it is also possible that genetic factors are contributing if diversity of planted individuals is lacking or does not represent the genetic diversity found within natural populations. Unfortunately we cannot know the nature of the diversity that was in failed sites in which plants no longer exist. We can only evaluate the diversity in naturally occurring sites and compare them with extant restored populations that vary in age and source.

There is extensive evidence of the ecological consequences of genetic diversity in SAV restoration efforts. For example, Williams and Davis (1996) noted that genetic diversity of transplanted *Zostera marina* L. beds was reduced relative to natural beds. Decreased genetic diversity in transplanted beds was associated with lower population growth and individual fitness (Williams 2001). In *Posidonia oceanica* Delile, genetic polymorphism in restoration stock was positively correlated with rhizome length, number of ramets per genet, and survival rate (Procaccini & Piazzi 2001). Similarly, genotypic diversity of *P. oceanica* within populations was

positively correlated with shoot density (Zaviezo *et al.* 2006). A similar pattern was noted in *Z. marina*; however, a positive relationship between genotypic diversity and shoot density existed only in winter, potentially indicating enhanced tolerance to abiotic and biotic stressors associated with overwintering (Hughes & Stachowicz 2009). Variation in growth rates, production of secondary compounds, and structural characteristics (Tomas *et al.* 2011), may have contributed to the reasons that increased genotypic diversity of *Z. marina* enhanced population recovery and persistence (Hughes & Stachowicz 2004, 2011; Reusch *et al.* 2005) and yet compared with monocultures, and in absence of disturbance, polycultures of *Z. marina* had decreased yield (Hughes & Stachowicz 2011).

We determined the degree to which genetic diversity within restored sites is representative of natural sites. Restored sites within the same tributaries may deviate from paired natural sites when non-local restoration stock or few local genotypes were used to re-establish a population. We compared levels of genotypic diversity and allelic diversity, as well as allelic composition among natural/restored pairs of *V. americana* populations in the Chesapeake Bay and in stock repositories that have been used for restoration activities. Additionally, we compared effective population size estimates of restored versus natural populations. Together, these comparisons allowed us to evaluate the state of natural populations and how restoration practices are affecting genetic diversity in restored populations and nursery stock.

Methods

Sampling locations and protocol

In 2007, we sampled from eight sets of paired natural and restored sites of *Vallisneria americana* located in tidal and non-tidal reaches of Chesapeake Bay tributaries (Figure 3.1). Restoration efforts, including failed attempts, are not documented. We therefore identified restored sites with extant populations and paired natural sites with the help of managers and scientists working within the region. Site pairs were typically located within the same tributary between 165 m and 5 km of each other (Table 3.1). Owing to scarcity of sites, the set in Virginia (HL/TAR) was paired across two tributaries (Figure 3.1). The 8 restored sites differed in age; the oldest site was planted in 1985 and the youngest site was planted only weeks prior to sampling. Restoration efforts varied in techniques and source material (Table 3.2). Rooted plants obtained from areas surrounding the plantings were often used as source material (Table 3.2). From each of the 8 natural locations, we collected up to 30 shoots, each approximately 5-10m apart. Our goal in sampling was to estimate the genotypic and allelic diversity at sites, not to document or compare the spatial distribution of diversity within sites. Therefore, the spatial scale of sampling differed within and among restored and natural sites to account for differences in population size and extent, where the distance between samples depended on the distribution of plants in each site. Latitude and longitude were recorded for each sampled shoot using global positioning system technology.

Table 3.1 Measures of clonal and genetic diversity in populations of *Vallisneria americana* sampled from the Chesapeake Bay. Long. = longitude; Lat. = latitude; Dist. = distance among paired sites (km); N = number of sampled ramets; G = unique genets; genotypic diversity = 1-G/1-N; A = average number of alleles; A_p = number of private alleles; P = proportion polymorphic loci; H_o = observed heterozygosity; H_e = expected heterozygosity; f = correlation of alleles within individuals within populations. Bold = $P < 0.05$.

Sample Location	Code	Long.	Lat.	Dist.	N	G	$\frac{(1-G)}{(1-N)}$	A	A_p	P	H_o	H_e	f
Conford Point[N]	CP	-76.098	39.528		29	26	0.89	5.2	2	1	0.54	0.59	0.089
Conford Point[R]	CPR	-76.100	39.525	0.49	30	17	0.55	4.5	0	0.9	0.64	0.59	**-0.083**
Elk Neck[N]	EN	-75.968	39.480		30	23	0.76	5.5	2	0.9	0.63	0.60	**-0.057**
Elk Neck[R]	ENR	-75.969	39.475	0.63	30	12	0.38	4.2	0	0.9	0.63	0.56	**-0.113**
Fishing Battery[N]	FB	-76.083	39.493		30	26	0.86	4.8	0	0.9	0.63	0.60	-0.044
Fishing Battery[R]	FBR	-76.084	39.492	0.16	30	20	0.66	4.6	1	0.9	0.61	0.58	-0.059
Dundee Creek[N]	DC	-76.363	39.341		30	30	1.00	5.5	1	1	0.58	0.61	0.052
Weir Cove[R]	WC	-76.333	39.314	5.00	15	13	0.86	4.7	2	1	0.49	0.61	**0.199**
Hawks Cove[N]	HWC	-76.404	39.254		29	27	0.93	5.8	2	1	0.67	0.66	-0.014
Long Cove[R]	LOC	-76.408	39.254	0.47	15	13	0.86	4.7	0	1	0.57	0.65	**0.126**
Shallow Creek[N]	SCN	-76.437	39.205		30	6	0.17	3.2	0	0.9	0.52	0.58	0.127
Shallow Creek[R]	SCR	-76.438	39.206	0.20	15	15	1.00	4.4	0	1	0.63	0.61	-0.032
South Ferry Point[N]	SFP	-76.505	39.071		15	5	0.29	3.8	0	0.9	0.60	0.63	0.055
Grachur Camp[R]	GC	-76.525	39.088	2.55	3	2	0.50	2.3	0	0.9	0.55	0.62	0.154
Horse Landing[N]	HL	-76.993	37.706		30	3	0.07	2.3	0	0.6	0.60	0.45	**-0.469**
Tar Bay[R]	TAR	-77.190	37.307	47.76	10	3	0.22	2.4	0	0.6	0.37	0.46	**0.241**
	Avg.				23.19	15.06	0.62	4.24	0.63	0.90	0.58	0.59	0.011
	SD				9.27	9.52	0.32	1.15	0.89	0.13	0.07	0.06	0.166
Restoration Repositories													
Wisconsin Nursery	WISC	N/A	N/A	N/A	5	3	0.50	2.4	0	0.7	0.40	0.47	0.172
Anne Arundel Com. College	AACC	N/A	N/A	N/A	4	1	0.00	1.9	1	0.9	0.90	0.90	N/A
Kollar Nursery	FARM	N/A	N/A	N/A	30	28	0.93	4.4	0	0.9	0.60	0.60	-0.004
USDA Plant Material Center	USDA	N/A	N/A	N/A	30	9	0.28	4.2	2	1	0.67	0.65	-0.031
	Avg.				17.25	10.25	0.43	3.23	0.75	0.88	0.64	0.65	0.034
	SD				14.73	12.31	0.39	1.26	0.96	0.13	0.21	0.18	0.093

[N] Natural sites
[R] Restored sites

Table 3.2 Location and associated details for restoration planting sites. All sites were planted with rooted shoots or tubers, the origin and source of which varied.

Abbreviated Name	Year(s) Planted	Region Whole Plants Obtained	Local Source Material	Type of Whole Plants Used	Repeated Planting
CPR/ENR/FBR	1985-1990	Confluence of the Susquehanna River	Yes	Fresh harvest from natural sites	Yes
WC	1997	Mouth of Dundee Creek (Gunpowder River), AACC, USDA	Yes & No	Repository & fresh harvest from natural sites	No
LOC	2006	Susquehanna Flats, Gunpowder River	No	Grown from seed harvested from natural sites	No
SCR	1999-2001, 2003	AACC, USDA	No	Repository	Yes
GC	2007	Susquehanna Flats	No	Grown from seed harvested from natural sites	No
TAR	1999*, 2004, 2006, 2008**	Repository stocked from the Potomac River	No	Repository stocked with seed and entire plants harvested from natural sites	Yes

*Plant material removed by herbivory.

**Post-sampling

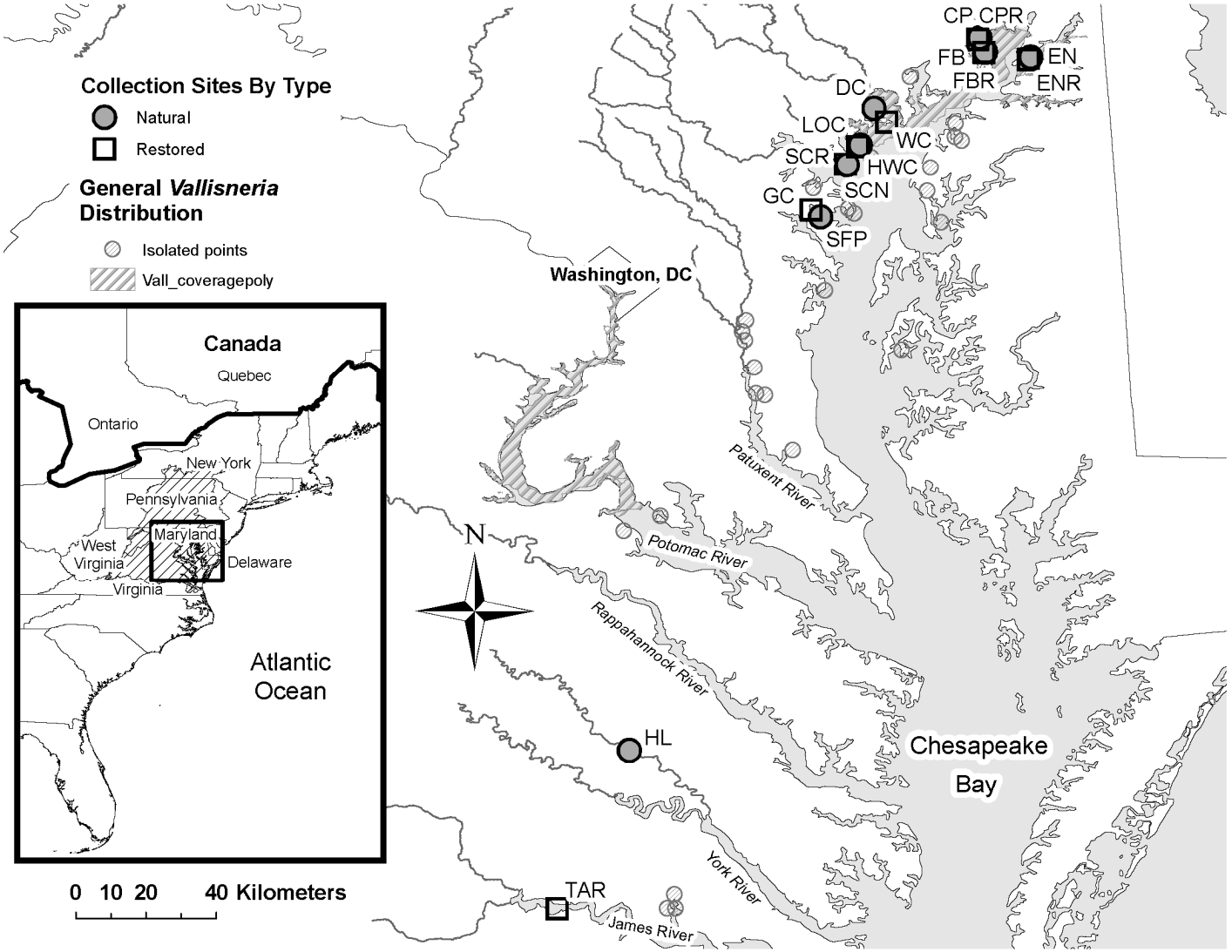

Figure 3.1 Paired natural restored collection sites sampled in 2007.

Sampling within restored sites was limited by the size of the plantings and if the planting had expanded. In the older restoration sites without herbivore exclosures (i.e., CPR, FBR, ENR, SCR), we sampled at 5-10 m increments as these sites had expanded to areas ~150 m^2 and contained > 500 stems. The WC site, although open, was of similar size to an enclosed site. Sites with herbivore enclosures (i.e., LOC, GC, TAR) were typically small ~1.5 m x 3 m in area and had < 100 stems. We collected fewer shoots from these smaller restored sites to limit impacts to the new plantings. Although only a small amount of tissue is needed for genotyping, poor visibility prevented seeing plants to sample and simply accessing enclosed restored sites caused extensive dislodging of plants and we chose to minimize our access time. In these circumstances, we made a concerted effort to collect representative samples while not causing unnecessary damage. Despite the smaller sample sizes, samples from restored sites likely represent a larger proportion of the total number of shoots at a site than do samples from natural populations. Genotypic diversity of dense or extensive natural sites may be comparatively underestimated.

In addition to sites located within the estuary, three local restoration stock repositories were sampled: a propagation facility at Anne Arundel Community College in Maryland, the USDA Native Plant Materials Center in Beltsville, Maryland, and a nursery facility in Baltimore County, Maryland. We also sampled from a nursery in Wisconsin to compare local nursery stock with nursery stock that is shipped throughout the U.S.A. Sample sizes from repositories were limited by the amount of material provided by each center. For example, tissue is cultured in jars at the AACC, and to avoid contamination of jars due to sampling we were provided four

jars from the repository. Field-collected shoot tissue was placed on ice within 1 hour of collection, and immediately transported to the University of Maryland College Park, where the material was frozen at -80°C until DNA extraction.

DNA extraction and genotyping

Genomic DNA was isolated and purified using methods outlined in Burnett et al. (2009). We genotyped 10 microsatellite loci from each shoot using robust primers with specific amplification that we developed for the species (Burnett *et al.* 2009). Polymerase chain reactions (PCR) were performed as in Burnett et al. (2009), with the exception of the locus Vaam_AAG004, for which we added dimethyl sulfoxide and Q-Solution (QIAGEN) to reactions to optimize specificity. PCR products were separated, measured, and peaks analyzed using identical methods and quality control procedures, which included repeated analyses to ensure high reproducibility of PCR reactions, as detailed in Lloyd et al. (2011). Our final dataset contained 0.5% missing data.

Genotypic diversity

We detected clones within and across sites by identifying identical multilocus genotypes using the program GenClone v2.0 (Arnaud-Haond & Belkhir 2007). Because mutation and scoring errors can lead to assigning different genotypes to individuals that actually represent clones, we used GenClone to identify cases in which there was only a one-allele difference among genotypes. We examined these cases by hand to confirm scoring, and, when warranted, modified clonal assignments, which resulted in changing assignment of 16 genotypes. Within sites, the proportion

of unique genotypes was calculated as (G-1)/(N-1), where G is the number of unique genotypes and N is the total number of shoots sampled (Arnaud-Haond *et al.* 2007; Pleasants & Wendel 1989). Each genet was represented by only one shoot in subsequent analyses. We also identified clones that were shared across sites by repeating the GenClone analysis after pooling all samples.

Measures of genetic diversity

We used GDA v1.1 (Lewis & Zaykin 2001) to calculate proportion of polymorphic loci (*P*), observed number of alleles (*A*), private alleles (A_p), unbiased expected heterozygosity (H_e), and observed heterozygosity (H_o) within sampling locations. We used rarefaction on pairs of restored and natural sites to account for different sample sizes (HP-Rare v1.0; Kalinowski 2004, 2005b). We tested for differences in genetic measures among all natural, restored and stock sites using Kruskal-Wallis tests in R v2.12.1 (R Development Core Team 2010). Differences in all genetic measures among the paired sites were examined using Mann-Whitney tests in R. Simple linear regression was used to test the relationship between genotypic diversity and all basic statistics (*P*, *A*, A_p, H_e, and H_o) in R to test for the influence of genotypic diversity on the basic statistics. Finally, the relationship between restoration practices (i.e., age of sites, source of plants, type of plants, # plantings per site; Table 3.2) and measures of genetic diversity were assessed using Kruskal-Wallis tests, Mann-Whitney tests or simple linear regression in R (R Development Core Team 2010). We accounted for multiple comparisons in tests that determined differences among individual site pairs. The format of the response variable determined the type of test used.

Departure from Hardy-Weinberg equilibrium

Wright's F_{is} was calculated for each site using the estimator *f* (Weir & Cockerham 1984) in GDA to test for population level deviations from Hardy-Weinberg equilibrium. *f* is the correlation of genes within individuals relative to each site (Weir & Cockerham 1984). We used confidence limits around each estimate generated by 1000 bootstraps in GDA to assess significance of *f*, which indicates departure from random mating.

We examined each site and the three repositories that had two or more genetically distinct individuals for presence of a recent genetic bottleneck using a test for heterozygote excess in the program Bottleneck v1.2.02 (Cornuet & Luikart 1996). Bottleneck computes heterozygote excess as the difference between expected heterozygosity (H_e) and heterozygosity expected at equilibrium (H_{eq}) for each site from the number of alleles given the sample size (Cornuet & Luikart 1996). We tested significance of the difference between H_e and H_{eq} using a one-tailed Wilcoxon signed-rank test under a two-phase mutation model. This model provides results intermediate between an infinite allele model and a stepwise mutation model and is considered to be most appropriate for microsatellites (Di Rienzo *et al.* 1994).

Shared allelic identity among natural and restored pairs

We used principal components analysis (PCA) to assess similarity of allelic composition among genets sampled from natural versus restored sites. We implemented one individual-based PCA using the variance-covariance matrix for all unique genets sampled in Genodive v2.0b17 (Meirmans & Van Tienderen 2004). We then calculated percent overlap in the distribution of PCA scores of individuals in

individual natural-restored population pairs along the first and second PCA axes. For restoration stock repositories, we compared the composition to all sampled natural diversity in the Chesapeake Bay. The degree of overlap at the site level provides insight into how well allelic diversity in restored sites represents local natural diversity. The degree of deviation from 100% overlap along either axis indicates the degree to which allelic composition differs among population pairs within the context of the total diversity in the sampled sites (Figure 3.2).

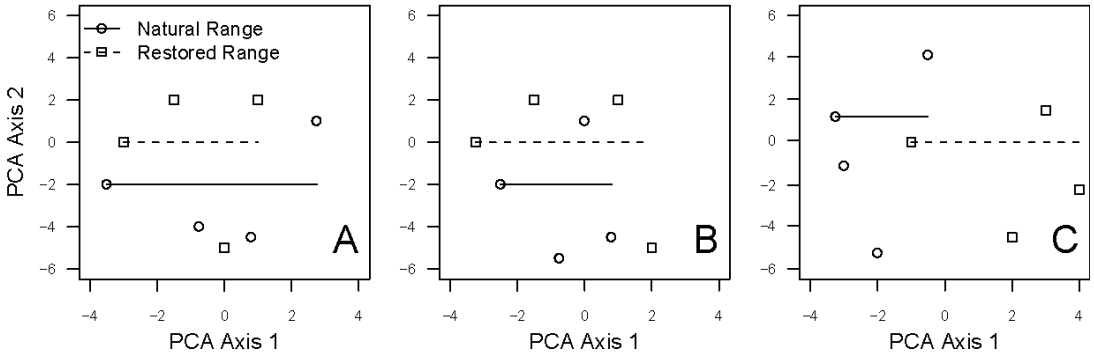

Figure 3.2 Caricature of PCA arrangements showing: A) allelic composition within natural sites larger (100% overlap) than within restored sites (< 100% overlap), B) allelic composition within restored sites greater (100% overlap) than within natural sites (< 100% overlap), C) asymmetric allelic composition among natural and restored sites (both < 100% overlap). Round and square symbols represent 8 theoretical genets from one natural / restored site pair (4 genet per site). Lines show the range of values along one of two PCA axes. Each axis is examined separately.

Effective population size

We estimated effective population size (N_e) using LDNe v1.31 (Waples & Do 2008) with $P_{crit} = 0.05$. LDNe utilizes the Burrows method to calculate linkage disequilibrium, which is subsequently used to calculate N_e from a single population sample (Waples 2006).

Results

Genotypic diversity

We sampled 223 shoots from 8 natural and 148 shoots from 8 restored sites within the Chesapeake Bay (Table 3.1). On average, we sampled fewer individuals from each restored site ($\bar{x}$ = 18 shoots) than from natural sites ($\bar{x}$ = 28 shoots) due to the small size of many restoration plantings. A total of 69 shoots were sampled from the stock repositories, with an average of 17 shoots per repository.

Of the shoots sampled within the Chesapeake Bay, 241 were unique genets, and 41 of the shoots sampled from stock repositories were unique. Genotypic diversity as measured by (G-1)/(N-1) ranged from 7% to 100% with an average of 62% (Table 3.1). The percentage of unique genets did not differ between natural versus restored sites ($\bar{x}$ = 62% for both; W = 45; p = 0.41). However, within paired comparisons genotypic diversity was greater in natural sites than restored sites five out of the eight times (Table 3.1). In general, sites with the highest genotypic diversity were located in the northern Chesapeake Bay. The lowest genotypic diversity sites were the HL/TAR, SCN and SFP sites (Table 3.1). Samples taken from stock repositories typically supported fewer genotypes (43% unique genets), but they were not significantly less than either natural or restored sites (H = 0.31; d.f. = 2; p = 0.86). The Wisconsin stock repository supported the highest genotypic diversity with 93% of sampled shoots belonging to different genets, and the 4 Anne Arundel Community College samples represented the same genet.

Specific genotypes were shared among paired sites within two sets. In the EN/ENR set, one genotype was sampled 4 times in EN and 11 times in ENR; in the FB/FBR set, one genotype was sampled 1 time in FB and 9 times in FBR.

Measures of genetic diversity

The proportion of polymorphic loci (*P*) within genets sampled among sample locations averaged across populations was $\bar{x} = 0.90$ (SD = 0.12). On average, genets at natural and restored sites did not differ in polymorphic loci ($\bar{x}$ natural $P = 0.90$, SD = 0.13; $\bar{x}$ restored $P = 0.90$, SD = 0.13; W = 40.5; $p = 0.68$). Within restoration stock repositories, the average proportion of polymorphic loci for sampled genets was $P = 0.88$ (SD = 0.13), which was not different from either natural or restored sites (H = 0.21; d.f. = 2; $p = 0.90$). There was no difference in the proportion of polymorphic loci between any of the 8 pairwise sets.

The average number of alleles per locus (*A*) across all sampled genets and loci was 8.10 (SD = 3.25). The average number of alleles per locus within individual sites was 4.24 (SD = 1.15). Genotypic diversity and uncorrected allelic diversity were strongly correlated ($y = -15.4231 + 7.3077x$; $R^2 = 0.77$; $p < 0.001$). Natural and restored sites supported genets with similar numbers of alleles per locus (natural $A = 4.51$, SD = 1.26; restored $A = 3.98$, SD = 1.02) before (W = 54; $p = 0.09$) and after (W = 48; $p = 0.27$) using rarefaction. Restoration stock repositories supported fewer alleles than either natural or restored sites, $\bar{x} = 3.22$ (SD = 1.26; H = 9.84; d.f. = 2; $p = 0.007$), and the difference remained following rarefaction (H = 5.94; d.f. = 2; $p = 0.05$). Following correction for multiple comparisons and rarefaction, allelic richness did not differ among any of the paired sites.

Eight sampled sites supported at least one of 13 private alleles. Each sample type (natural, restored, stock repository) supported at least one rare allele. Relative frequency of the private alleles in all but one of the sites varied from 0.02 to 0.07 (Table 3.3). Allele 150 at the AAGX030 locus had a frequency of 0.50 because it was present in a heterozygous state in the single genotype of the AACC stock repository sample.

Table 3.3 Private allele frequency for 13 alleles found across 8 sampled populations

Type	Code	Locus	Allele	Frequency
Natural	CP	AAGX030	165	0.038
	CP	M16	184	0.019
	EN	AAG004	400	0.043
	EN	AAG004	403	0.043
	DC	M49	195	0.033
	HWC	M49	198	0.019
	HWC	AAGX051	199	0.019
Restored	FBR	AAGX051	202	0.026
	WC	M13	286	0.038
	WC	M16	196	0.077
Stock	AACC	AAGX030	150	0.500
	USDA	AAGX071	248	0.056
	USDA	AAGX051	204	0.056

Average observed heterozygosity of genets within all sample sites was 0.58 (SD = 0.07), and did not differ between all natural and restored sites combined ($\bar{x}$ natural H_o = 0.59, SD = 0.05; $\bar{x}$ restored H_o = 0.56, SD = 0.09; W = 45; $p > 0.41$). There was also no difference in observed heterozygosity between any of the 8 pairwise sets of natural-restored sites. Average observed heterozygosity of genets sampled from restoration stock repositories (H_o = 0.64 SD = 0.21) did not differ from natural or restored sites (H = 3.60; d.f. = 2; p = 0.16).

Departure from Hardy-Weinberg equilibrium

Four loci departed significantly from Hardy-Weinberg equilibrium (Table 3.4). Three restored sites showed heterozygote deficit (Table 3.1): WC (f = 0.20; 0.04 – 0.26), LOC (f = 0.13; 0.04 – 0.21), and TAR (f = 0.24; 0.03 – 0.44). The AACC sample had only 1 unique genotype; therefore, f could not be estimated. Four sites showed signs of heterozygote excess: EN (f = -0.06; -0.13 – -0.03), HL (f = -0.47; -1.00 – -0.47), CPR (f = -0.08; -0.22 – -0.01), and ENR (f = -0.11; -0.27 – -0.04).

Based on analysis with the program Bottleneck (Cornuet & Luikart 1996), 4 of the 18 sites we could analyze (LOC, p = 0.007; HL, p = 0.008; FARM, p = 0.001; USDA, p = 0.007) showed evidence that H_e (expected heterozygosity) significantly exceeds H_{eq} (heterozygosity expected at equilibrium) indicating potential of a recent bottleneck.

Table 3.4 Genetic diversity of individual loci over all samples. A = total number of alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{is} = correlation of alleles within individuals within all samples. Bold = $P < 0.05$.

Locus	A	H_o	H_e	Percent Missing Data	F_{is}
AAGX071	10	0.74	0.78	0.355	0.055
AAGX051	13	0.80	0.87	2.482	**0.073**
AAGX012	6	0.61	0.62	0.000	0.024
ATG002	8	0.73	0.77	0.000	0.047
AAGX030	5	0.58	0.56	0.000	-0.043
M49	12	0.63	0.74	0.000	**0.138**
M13	10	0.67	0.80	1.773	**0.156**
AAG002	4	0.54	0.54	0.000	-0.007
M16	4	0.11	0.12	0.000	**0.116**
AAG004	9	0.59	0.63	1.064	0.065
Average	8.1	0.60	0.64	0.567	0.064
SD	3.25	0.19	0.21	0.903	0.063

Restoration practices related to genetic diversity

Of all restoration practices and genetic diversity measures, only the correlation between age of restoration sites and inbreeding coefficient was significant ($y = 0.21 – 0.013x$; $R^2 = 0.67$; $p = 0.008$; Figure 3.3).

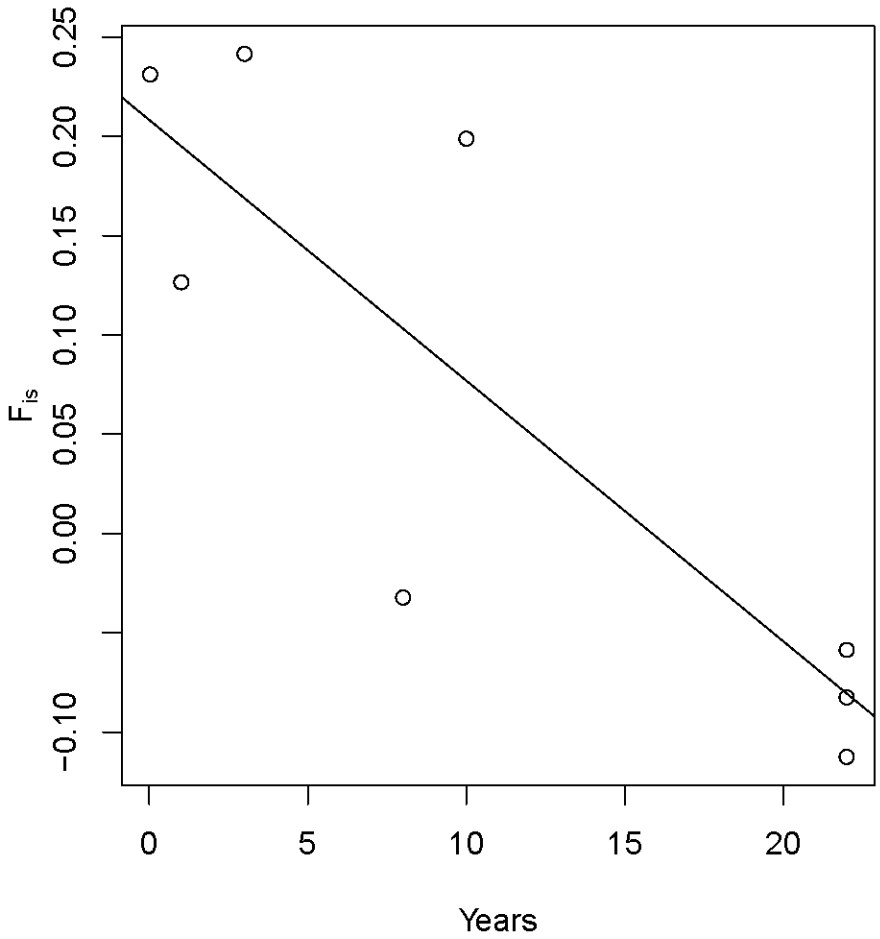

Figure 3.3 Inbreeding coefficient (F_{is}) of restoration sites against age of restoration site in years.

Shared allelic identity among natural and restored pairs

The first axis explained only 10.14% of the variation in allele frequencies, and the second axis explained only 6.31% of the variance. The small amount of variance explained by these axes indicates that variance in frequencies of different alleles cannot be easily collapsed into a smaller number of axes. Although the total variance

is low, the amount represented on each of the first two axes captures the largest concentration of variation due to allele composition. Percent overlap within individual pairs of natural/restored sites along the first and second PCA axes provides a measure of similarity in allelic composition between genets planted within a restored site and genets growing in natural sites. We observed three different patterns of overlap among restored and natural sites (Figure 3.2). A) The range of allelic composition within natural sites was larger than the range within restored sites (Figure 3.2A; Table 3.5). B) The range in allelic composition within restored sites was greater than the range within natural sites (Figure 3.2B; Table 3.5). C) Overlap of allelic composition among natural and restored sites was asymmetric, where both natural and restored sites had allelic composition that fell outside the range of the other site (Figure 3.2C; Table 3.5).

When compared to all sites sampled from natural locations within the Chesapeake Bay, genets from the WISC stock repository were limited to a small portion of multivariate space (4.6 % along axis 1, and 21.78 % along axis 2). Genets from the USDA stock repository occupied 55% of the first axis and 64% of PCA axis 2. The single AACC genet fell within the range of allelic diversity sampled from the Chesapeake Bay, but many genets sampled from the FARM stock repository fell outside of the multivariate space occupied by genets we sampled Bay-wide.

Effective population size

Effective population size (N_e) in 8 sites (3 natural, 3 restored, and 2 stock repositories) ranged between 1.9 and 41.0 genets (Table 3.6). In the remaining 12

sites, estimates of N_e were indistinguishable from infinity, which occurs when linkage disequilibrium is less than sampling error (Waples 1991).

Table 3.5 Percent overlap of paired natural-restored sites on the first and second PCA axes. Scenario type corresponds to diagrams in Figure 3.2; each axis was examined separately. Percent overlap for stock repositories was calculated based on all populations. Presence of only one genet within AACC precluded inclusion of this repository in this analysis.

	PCA Axis 1 Restored Overlap with Natural	PCA Axis 1 Natural Overlap with Restored	Scenario Type	PCA Axis 2 Restored Overlap with Natural	PCA Axis 2 Natural Overlap with Restored	Scenario Type
CP/CPR	100.00	70.71	A	93.36	99.93	C
EN/ENR	100.00	73.41	A	16.58	26.24	C
FB/FBR	81.67	100.00	B	100.00	79.05	A
DC/WC	96.68	85.86	C	67.93	90.84	C
HWC/LOC	92.41	88.32	C	67.37	100.00	B
SCN/SCR	95.27	82.27	C	45.74	100.00	B
SFP/GC	98.38	79.16	C	92.99	71.44	C
HL/TAR	100.00	50.84	A	7.48	100.00	B
WISC	100.00	4.60		100.00	21.78	
FARM	100.00	55.31		64.47	50.45	
USDA	100.00	55.31		100.00	53.59	

Table 3.6 Mean effective population size estimates and upper and lower 95% confidence intervals of sample sites. CP/CPR and FB/FBR are paired sites. FARM and USDA are repositories.

Abbreviated Name	N_e	Lower 95%	Upper 95%
CP	34.50	18.20	109.60
CPR	29.70	12.70	595.90
FB	41.00	20.40	183.50
FBR	27.80	13.80	108.20
LOC	15.10	6.60	69.00
SCN	1.90	1.00	13.10
FARM	11.00	11.00	17.20
USDA	10.70	10.70	197.90

Disussion

Our results suggest that natural populations of *V. americana* in the Chesapeake Bay are genetically diverse (see also Lloyd et al. 2011) and that restoration practices are generally successful in re-establishing populations that are as genetically diverse as natural populations, especially when calibrated for the number of individuals sampled. All basic measures of genetic diversity (i.e., number of alleles, heterozygosity, proportion of polymorphic loci, number of genets) within restored sites mirror the levels of genetic diversity contained within their natural paired sites. N_e overlaps substantially among two paired natural / restored sites in which it could be measured. The detection of shared genotypes at two of the eight-paired sites shows that managers are at least in some cases either actively using local genets from adjacent sites, or that the restored sites have become integrated with their natural counterparts through vegetative expansion. The local nature of planting stock is also confirmed by substantial overlap in allele composition between paired natural and restored sample sites that indicates that genetic material mostly does not originate from sources foreign to surrounding natural genets. Although the outlook is generally very positive, we found evidence for use of non-local stock in three sites and also identified signs that some restored sites might be planted with genetic stock from multiple populations based on differences in allele composition and departure from Hardy-Weinburg equilibrium. We also found reduced effective population size at eight sites that cause concern for future loss of diversity.

Genotypic diversity

Although genotypic diversity of *V. americana* in restored sites did not differ statistically from natural sites, five of the eight restored sites supported fewer genotypes than the paired natural sites (Table 1). Reduced genotypic diversity in *Zostera marina* has been associated with decreased shoot density (Ehlers *et al.* 2008), decreased resistance to heat shock (Reusch *et al.* 2005), and decreased resistance to grazing (Hughes & Stachowicz 2004). It has been associated with increased selfing (Reusch 2001), which can have subsequent effects on genetic diversity beyond initially low diversity. Prolonged periods of mating among close relatives within submersed aquatics can lead to substantial declines in reproductive fitness (Ruckelshaus 1995); however, the effect of inbreeding on fitness varies greatly among species (Hedrick & Kalinowski 2000). Because *V. americana* is dioecious, there is no risk of increased selfing. Rather, lower genotypic diversity decreases chances that both male and female plants will be present at a site, thereby reducing the potential for sexual reproduction. For example, the 6 genotypes documented from the SCN population are male, the SFP population is skewed towards female genets (4 female, 1 male; Engelhardt *pers. obs.*) and the sample of 30 ramets from HL supported 1 known male and 1 female genotype. The low number of genets decreases opportunities for recombination at those sites and increased inbreeding will ensue as full and half sib offspring from a small number of parents reproduce (Hedrick & Kalinowski 2000).

Genotypic diversity affects basic measures of genetic diversity such as allelic diversity, heterozygosity, and Wright's fixation indices (Kalinowski 2005a; Pruett &

Winker 2008). Consequently, the number of genotypes present in a population will necessarily affect analyses that rely on such basic genetic statistics. Our genetic diversity results could reflect low genotypic diversity in source populations. Moreover, the low genotypic diversity we observed in three natural sites (i.e., SCN, SFP, and HL) presents a larger problem. If these sites were subsequently used for restoration stock material the resulting restored site would also have low genotypic diversity potentially creating the negative consequences that were discussed in the paragraph above.

Non-Random Mating

We detected departure from random mating based on significant heterozygote deficits in three (WC, LOC, TAR) of the eight restored sites (Table 1). Although the number of restored populations showing significant heterozygote deficit may be relatively small, they represent a large proportion of populations when compared to a Bay-wide sample, where only three of 27 natural populations showed significant heterozygote deficit (Lloyd *et al.* 2011). The deficits in the restored populations could be the result of true inbreeding or of mixing individuals from different gene pools during planting, in essence a restoration-induced Wahlund effect. The three sites with significant positive *f* values were planted with individuals from multiple donor populations (LOC and TAR) or from multiple repositories (WC; Table 2). Planting materials in the three sites were either plants germinated from seed exclusively or in addition to freshly harvested tissue.

Use of planting material derived from seeds germinated from a limited number of fruits, or sampled from one or a few clones could lead to planting a large number

of full and half siblings. Subsequent mating among those individuals would increase the degree of inbreeding relative to source populations. If fruits are collected from different but spatially aggregated maternal genotypes, the same father or small set of fathers may have sired the seeds, which would also yield many full and half siblings. In fact, we saw that inbreeding coefficients of restoration sites significantly declined with the age of restored sites (Figure 3), which supports the hypothesis that apparent inbreeding is due to mixing gene pools. The declines in the inbreeding coefficient with time could be the result of the establishment of Hardy-Weinberg equilibrium as individuals from the different gene pools mate and generate the expected number of heterozygotes. The majority of populations were not heterozygote deficient; thus, minimizing inbreeding is a relatively minor management concern. However, the issue could be completely avoided by increasing the spatial extent of sampling from within natural source populations thereby avoiding the use of many individuals from any single clone. Additionally, planting restoration sites in proximity to natural sites would facilitate gene flow among the sites and increase mixture among non-related individuals.

Genets sampled from restoration stock repositories were not out of Hardy-Weinberg equilibrium, which suggests that managers have been avoiding increased mating among close relatives. Alternatively, the original material grown in repositories may have be representative of a source population that were in Hardy-Weinberg equilibrium and those genotypes have been maintained following initial cultivation. There is evidence of population bottlenecks within both the FARM and USDA sites. This is a situation that gives cause for concern if the lack of genotypic

diversity and apparent population bottlenecks are not due to sampling error but rather due to a true lack of diversity in FARM and USDA stocks. Periodically adding new genetic material from the wild to repositories is essential for alleviating the issues of low genotypic diversity and the effect of bottlenecks.

Effective population size

Guidelines for effective population sizes necessary to maintain genetic diversity range from $N_e \geq 50$ to prevent greater than 1% loss of heterozygosity per generation (Franklin 1980; Soule 1980), to $N_e \geq 500$ to prevent loss of alleles through genetic drift (Soule 1980), to upwards of N_e 1000-5000 (Lynch & Lande 1998) to maintain long-term evolutionary potential. Although there is still debate about which if any of these effective population sizes are necessary or sufficient for maintaining genetic diversity, the mean estimates of N_e in the 8 sites we could measure (N_e = 1.90 – 41) were well below all of the commonly accepted suggestions, and the upper limit of the 95% confidence interval exceeded 500 in only one site. Large deviations between N_e and census size are known for a number of marine organisms (Palstra & Ruzzante 2008) and can result from sampling across genetic neighborhoods in continuous populations (Neel *et al.* In Review). The small sizes we observed are potentially of concern, thus their cause needs to be better understood.

Reduced effective population size can rapidly increase rates of loss of genetic diversity (Ellstrand & Elam 1993), leading in general to increased inbreeding, decreased fitness, and decreased survivorship (Hedrick & Kalinowski 2000; Newman & Pilson 1997). Low population size or planting densities may suffer from decreased population growth rates (i.e., Allee effect), which can play a substantial role in the

outcome of restoration plantings (Deredec & Courchamp 2007). Evidence of population bottlenecks within one restored site (LOC) reinforces that either planting sizes may be too small or initial plantings contained too few individuals to support long-term fitness and survivorship. We know from plant invasion literature that number of propagules and invasion events are critical determinants of persistence and expansion (e.g., propagule pressure hypothesis; Richardson & Pysek 2006; Zayed *et al.* 2007). Thus, the number of individuals planted and the number of planting events should impact the overall success of a restoration effort as well. In animal restoration programs, increased reintroduction size and frequency are known to correlate with restoration success (Griffith *et al.* 1989; Hopper & Roush 1993). We recognize the practical constraints associated with restoration plantings. However, plantings in larger areas should be encouraged or, at the very least, a large number of individuals should be planted within each site through time. Collecting individuals from large areas within donor patches would also be beneficial.

Overlap of allelic composition

The large degree of overlap in allele composition among natural, restored, and stock repositories implies that managers are typically matching the allelic composition of adjacent natural sites. However, interpretation of these results is hampered by the low explanatory power of the first two PCA axes (Figure 2). The limited variation explained is indicative of a high degree of shared alleles among individuals across sites. Regardless, each of the three scenarios of allele composition overlap we note (Figure 2; Table 5) highlights a different type of genetic risk. When individuals for restoration represent a limited genetic pool, the allelic composition of

the resulting restored site will represent a subset of the natural allelic composition, which can negatively impact both immediate plant growth and long-term individual plant fitness (Williams 2001; Williams & Davis 1996). The departures were minimal in this direction, which is a positive result and indicates low potential for genetic diversity being limited due to poor stock selection.

Conversely, genetic diversity of restored and restoration stock genets either did not overlap or extended beyond observed natural variation in three cases: SCR, ENR, and FARM. This potentially indicates mixing of sources or potential sample bias that is introduced when sampling a greater proportion of the population in sparse restoration sites. The FARM site in particular had greater range along PCA axis 2 than did any other site we sampled. This could be problematic if individuals from this repository were used for planting within the Chesapeake Bay. When allelic composition of restored sites does not overlap with natural diversity, populations can experience outbreeding depression (Fenster & Dudash 1994; Montalvo & Ellstrand 2001) or exhibit decreased fitness as the result of being maladapted to local conditions (Fenster & Galloway 2000; Linhart & Grant 1996; Montalvo & Ellstrand 2000). We observed negative inbreeding coefficients within both the EN and ENR sites and also within CPR, which indicates an excess of heterozygous individuals at these sites. An excess of heterozygous individuals can result from the recombination of genotypes from populations with different allelic composition. Given the geographic proximity of EN and CP to their restored counterparts, it is possible that the recombination of diverse gene pools is driving the observed negative inbreeding coefficients.

Further investigation is required to determine if either scenario (greater diversity or lack of overlap) affects fitness of restored or stock populations, but it does emphasize the need to avoid planting too few individuals or genotypes with limited or highly varied genetic diversity. An effort to avoid exchanging materials among regions should also be actively adopted to maintain similar patterns of allelic composition. We detected genetic isolation between the Northern and Central Bay, with the division line roughly between DC and FB (Lloyd *et al.* 2011), suggesting that movement of genetic materials across large geographic distances is limited. However, evidence of outbreeding depression is limited (Frankham *et al.* 2011), fitness recovery following hybridization is possible (Erickson & Fenster 2006).

Conclusions

The issues relating to small population size, increased inbreeding, and a lack overlap in allelic composition are not ubiquitous across *Vallisneria americana* restoration sites and stock repositories. With a few minor changes to propagation and planting protocols, as well as propagule collection techniques, we expect that the genetic diversity of restored populations will directly mirror naturally occurring genetic diversity within the Chesapeake Bay. However, simply mirroring naturally occurring genetic diversity may not be enough. The relationships between genetic diversity of *V. americana* and the resulting ecological functioning and ecosystem services are ripe for increased investigation. Such understanding will provide insight into the role of genetic diversity in returning seagrass beds to their prior ecological prominence in the Chesapeake Bay.

Chapter 3: Pollen dispersal distance of *Vallisneria americana* Michx. (Hydrocharitaceae)

Dispersal within and among habitat patches is a key process that influences both ecological and evolutionary dynamics of plant populations. Fragmentation and habitat loss have the potential to reduce pollination effectiveness. Using an indirect paternity method we examined pollen dispersal and seed paternity of the water-pollinated plant *Vallisneria americana*, which has been fragmented and reduced in size from historic coverage. Using the KinDist method on samples of 19 – 39 mothers from 3 sites across 2 years, we found that correlated paternity, within- and among-sibling relatedness, and neighborhood size all indicated pollen dispersal that is limited to 0.80 to 20.63 m. Limited pollen dispersal establishes genetic neighborhoods, which unless overcome seed and propagule dispersal, will lead to genetic differentiation among neighborhoods. Unless loss and fragmentation drive populations to extreme ratios of females to males, local pollen dispersal is likely to be relatively unaffected by habitat loss and fragmentation because the typical spatial scale of patch isolation already exceeds pollen dispersal distances.

Introduction

Dispersal within and among habitat patches is an important ecological process that influences the evolutionary dynamics of plant populations (Austerlitz & Garnier-Gere 2003; Austerlitz *et al.* 2000). Gene flow within and among populations determines the spatial distribution of alleles and individuals of a species (Broquet &

Petit 2009; Slatkin 1985). In plants genes are transferred via pollen, seed, and vegetative (e.g., tuber dispersal) movement. Understanding the scale at which each of these act brings insight into potential genetic connectivity and structure of populations (Ashley 2010; Manel *et al.* 2003; Storfer *et al.* 2010). The degree to which sites are connected by gene flow versus isolated largely depends on the scale at which a species perceives and interacts with the landscape (Crooks & Sanjayan 2006; Holland *et al.* 2004; Levin 1992; Taylor *et al.* 2006).

Any alteration of the landscape, via processes such as habitat loss and fragmentation, has the potential to disrupt genetic connectivity and isolate populations (Ellstrand & Elam 1993; Luque *et al.* 2012; Young *et al.* 1996). Increased isolation in conjunction with a reduction in population size can both increase the likelihood of mating among close relatives and decrease the genetic diversity of a population (Frankham 1995b, 1996), both of which are known to reduce plant fitness (Frankham 2005a). The magnitude of the effects of isolation are largely dependent on the mating system of the species. With predominantly outcrossing, or dioecious plant species being more susceptible to the effects of inbreeding depression (Barrett & Charlesworth 1991; Charlesworth & Charlesworth 1987). Furthermore, species that are self-incompatible may suffer Allee effects as population size declines and isolation increases if they lack suitable mates (Gascoigne *et al.* 2009).

The distance that pollen moves is also a determinant of the degree of genetic isolation within and among sites. The number and quality of pollen grains dispersing among sites is expected to decline with increasing distance, and fragmentation negatively affects the number of pollinator visits, quality of pollen, and seed set in

insect pollinated species at scales of 100 m – 1000 m (Jennersten 1988; Steffan-Dewenter & Tscharntke 1999; Wolf & Harrison 2001). In wind pollinated tree species the scale of dispersal (250 m to over 3 km) often surpasses the distance among isolated patches of habitat and thus maintains connectivity in discontinuous habitat (Ashley 2010). Water-born pollen, like wind, can occur in three-dimensions; however, it is expected to be locally limited as compared to wind pollination (Laushman 1993; Les 1988), and is influenced by the prevailing current at a site. Two-dimensional water pollination (surface only) has the potential to be more effective than wind pollination, but is still limited to a water-body (Cox 1988; Laushman 1993). A number of submersed aquatic plants rely on either true hydrophily (sub-surface) or epihydrophilous (water-surface) dispersal for pollination. However, reduced patch density and increased isolation can decrease seed set in such species (Reusch 2003; van Tussenbroek *et al.* 2010). Worldwide SAV species have experienced habitat loss and fragmentation (Short & Wyllie-Echeverria 1996). Submersed aquatic vegetation (SAV) communities in the Chesapeake Bay have been greatly affected habitat fragmentation and loss, due to anthropogenically-influenced eutrophication and increased sedimentation (Dennison *et al.* 1993; Orth & Moore 1983).

Declines in the epihydrophilous species *Vallisneria americana* Michx. (Hydrocharitaceae) have been especially pronounced (Kemp *et al.* 1983; Moore *et al.* 2010). In any one year, total potential habitat ranges from 9494 ha to 15612 ha and patches are on average between 6.2 ha and 12.9 ha in size (Lloyd et al., In Prep). Relative to total recently occupied habitat the number of *V. americana* patches

occupied decreased, and many of the patches that are present have been broken into discrete remnants (Lloyd et al., In Prep). Distribution of this dioecious, perennial, clonal plant (Catling *et al.* 1994; Korschgen & Green 1988; Wilder 1974) is driven by habitat characteristics (primarily water depth and salinity) and water quality (e.g., turbidity, temperature, chemical composition), by competition among SAV species, by herbivory (Korschgen & Green 1988), and by the ability of species to disperse within and among patches. Determining how the distribution and isolation of discrete patches may impact water-born pollination requires an assessment of the dispersal distance of pollen within sites. Understanding the nature of genetic connectivity within and among sites is necessary when a restoration program is being conducted, and determining the scale over which pollen is distributed highlights the potential of inbreeding and local adaptation to impact restoration activity (Weeks *et al.* 2011). *Vallisneria americana* has multi-seeded fruits, and multiple fathers can sire seed in a single fruit. Multiple sirings has the consequence of potentially reducing genetic relatedness among offspring (Ritland 1989), and increasing genetic diversity of seeds within a single fruit. By measuring offspring in conjunction with maternal tissue, we are able to determine the number of potential sires contributing to each fruit, and the inbreeding coefficient within the next generation of seed.

Water surface pollination in *Vallisneria americana* occurrs when pistillate flowers, borne on the water surface, are fertilized by free-floating staminate flowers that are moved by currents, winds, and tides (Korschgen & Green 1988). Measuring the distance over which pollen is dispersed provides a measure of genetic connectivity that can be extrapolated across patches. Indirect molecular methods

provide the most effective means of measuring pollen dispersal distance. These methods rely on the combination of population structure and paternity analyses to determine if female plants are 'sampling' from genetically structured pollen pools across a range of geographic distances (Austerlitz *et al.* 2004; Robledo-Arnuncio *et al.* 2006; Smouse *et al.* 2001). Indirect genetic measures of pollen dispersal distance have been used extensively in wind-pollinated trees (Ashley 2010; Smouse & Sork 2004), and have been adapted to other species (e.g., Fenart *et al.* 2007). We use the KinDist method (Robledo-Arnuncio *et al.* 2006) to estimate the distance over which pollen is dispersed in the hydrophilous species *V. americana*. We measured pollen dispersal and seed paternity across two years among open water and shoreline sites. The combination of paternity data with pollen dispersal distances provides a foundation for understanding the degree of patch isolation, and the impacts that isolation of patches can play on the genetic connectivity of *V. americana* populations.

Methods

Sampling locations and protocol

In October 2008 and 2009, we sampled plant material from three sites supporting *Vallisneria americana* within the Chesapeake Bay (Figure 3.1). Sites were selected to represent different conditions found within the Bay and its tributaries that have potential to affect pollen movement: open water (OB) and shoreline (EN, MP; Figure 3.1). The KinDist method requires sampling a minimum of 20 seeds from each of 20 females across a range of spatial distances within populations (Robledo-Arnuncio *et al.* 2006). Each year we collected 40 mothers within each site at a minimum distance

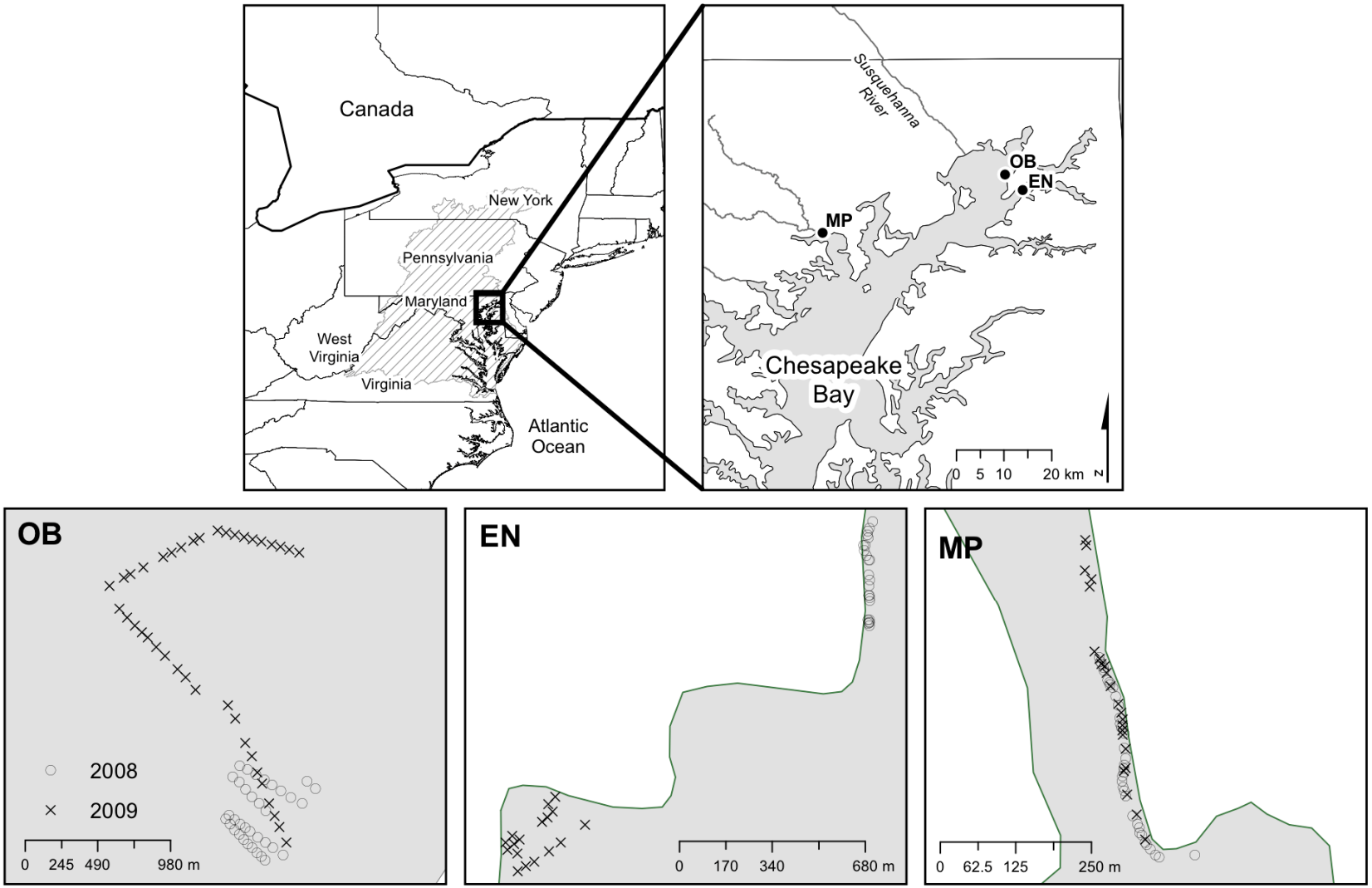

Figure 3.1 Overview of *Vallisneria americana* sites sampled in 2008 and 2009, and the distribution of samples analyzed. Note the difference in scale among the three sites.

of 10 – 15m in an attempt to capture the critical distance over which pollen is no longer transferred (Figure 3.1). We used a boat to sample the OB (2008, 2009) and EN (2009) sites, which resulted in lag distances of 40 – 100 m among samples. The boat pilot in 2009 moved at a higher rate of speed, which resulted in different lag distances in that year (50 – 120 m) compared to 2008 (30 – 80 m) among samples in the OB site. Lag distances at MP were dictated by where fruits were found in both years and accessibility. As *V. americana* is clonal, we were concerned that we sampled identical maternal genotypes at these scales due to clonality; however, different ramets have the potential to sample different pollen pools. Using the program GenClone v2.0 (Arnaud-Haond & Belkhir 2007) we checked for multilocus genotype matches among mothers.

For each sample, we collected both a single fruit and the peduncle attached to the fruit. *Vallisneria americana* fruits have an average of 150-200 seed (B. West, personal communication), and fruits ripen in late summer to early fall (Catling *et al.* 1994). We waited until fruits from each site appeared to contain mature seed prior to sampling. Latitude and longitude coordinates were recorded for each sampled fruit using global positioning system technology. Harvested fruits were immediately placed into individually labeled WhirlPack bags and transported to University of Maryland College Park. Peduncles were separated at the time of collection, placed in separate labeled containers on ice, and transported to University of Maryland College Park and stored at -80°C until extraction. Twenty randomly chosen seeds from each fruit were subsequently placed into individual wells in 96 well plates and stored at -80°C until extraction.

DNA extraction, and genotyping

DNA from maternal tissue and 20 seeds per mother were extracted using a modified Chelex extraction protocol (Walsh *et al.* 1991). Prior to extraction, the seed coat was removed and subsequently embryonic tissue was placed in strip-tubes was macerated with a sterilized fire-sealed glass pipette tip prior to lysis. Using methods outlined in Burnett et al. (2009) we genotyped in five robust microsatellite loci (AAG_X012, M13, AAG_X051, M49, ATG002). Five loci were shown to provide adequate power to utilize the KinDist method (Robledo-Arnuncio *et al.* 2006), and we used the program Cervus 3.0 (Kalinowski *et al.* 2007) to assessed the probability of excluding a candidate parent when the other parent is known when using these five loci for each year.

Polymerase chain reactions (PCR) were performed as in Burnett et al. (2009). PCR products were separated and measured on an ABI 3730xl DNA Analyzer with GeneScan™ - 500 LIZ™ Size Standard (Applied Biosystems, Carlsbad, California, USA) after tagging the PCR product with fluorescent labeled forward primers (Applied Biosystems). Peak data were analyzed using Genemapper v3.7 (Applied Biosystems) and all allele calls were also visually inspected for quality control.

Measures of genetic diversity

Number of alleles (A), expected (H_e) and observed (H_o) heterozygosity were separately calculated for mothers and offspring with the program GDA v1.1 (Lewis & Zaykin 2001). Wright's F_{is} was calculated using the estimator f (Weir & Cockerham 1984) in GDA to test for deviations from Hardy-Weinberg equilibrium for the mothers and offspring. Significance of F_{is} was tested by obtaining confidence limits

around each estimate generated by 1000 bootstraps in GDA. Significant departures from Hardy-Weinberg equilibrium can indicate a departure from random breeding. We tested for differences in genetic measures, among mothers and offspring and sites across years using Kruskal-Wallis tests in R v2.12.1 (R Development Core Team 2010), and we accounted for multiple comparisons in tests using Tukey's multiple test correction.

Paternity analysis and seed relatedness

For each site and year, we used PolDisp v1.0c (Robledo-Arnuncio *et al.* 2007) to calculate correlated within-sibship paternity. We calculated the number of pollen donors contributing to a mother (i.e., neighborhood size) as the inverse of the correlated paternity (Ritland 1989). Using the program Coancestry v1.0 (Wang 2011), we calculated the average within- and among-sibship pairwise relatedness with the Wang (2002) estimator, and the proportion of within- and among-sibship seed pairs that that were ½ sibs or full sibs. The Wang estimator was selected based on the results of Monte-Carlo simulations that determined which relatedness estimator best fits the microsatellite markers we used in this study (West et al., In Prep). Relatedness does not account explicitly for parentage, and a high proportion of seeds with relatedness values above full sib indicates that few fathers have contributed pollen to that fruit. We tested for differences in paternity and relatedness measures among mothers and offspring and sites across years using between offspring and mothers using Kruskal-Wallis tests in R v2.12.1 (R Development Core Team 2010), and we accounted for multiple comparisons in tests using Tukey's multiple test correction.

Average pollen dispersal distance and pollen dispersal functions

The program PolDisp v1.0c was used to calculate the average pollen dispersal distance (δ) and variance associated with the measure with the KinDist approach. KinDist calculates δ based on a normalized measures of correlated paternity among mothers, which factors out the unknown male density parameter (Austerlitz & Smouse 2001a; Robledo-Arnuncio *et al.* 2006; Smouse *et al.* 2001). To estimate δ, a probability function is estimated based on the expected decline in correlated paternity with increasing geographic distance among maternal pairs. The probability function describes the probability of a pollen grain dispersing a given distance from a source plant. The average pollen dispersal distance is then calculated as the first moment of that probability distribution. We explored the behavior of the dispersal parameter using a normal, exponential, exponential-power, geometric, and 2-dimensional student's *t* distributions; however, the estimated value of δ is relatively insensitive to the particular function selected (Robledo-Arnuncio *et al.* 2006). Using the estimated PDF parameters, we calculated the probability of pollen dispersal at distances of 5, 10, and 30 m for all functions in all sites for both years.

Results

Fruits appeared ripe when sampled; however, upon processing many contained either immature or rotten seeds. We attempted to amplify all loci for all individuals; however, the immature and old seeds did not consistently amplify and resulted in a reduction in the number of maternal samples analyzed relative to the number collected (N = 19-39; Table 3.1). The number of seeds analyzed from each site

ranged between 274 – 780, with an average of 14 – 20 offspring analyzed per mother (Table 3.1). There was 2.5% missing data in the final total dataset.

Genetic diversity

We detected a total of 51 alleles in 2008 and 53 alleles in 2009 with an average of 10.2 and 10.6 alleles per locus in 2008 and 2009 respectively. There was no difference in the number of alleles, observed heterozygosity, and expected heterozygosity among mothers and offspring or among sites across years (Kruskal-Wallis tests $p > 0.05$; Table 3.1). There were departures from Hardy-Weinberg equilibrium (Table 3.1). In the offspring, two sites had fewer heterozygotes than expected (2008 MP, 2009 EN), and in the mothers, two sites had more heterozygotes than expected (2009 EN, 2009 MP), and the differences among mothers and offspring within sites were significant (Kruskal-Wallis test $p = 0.0121$; Table 3.1).

Parentage and relatedness

The combined probability of excluding an unknown father using the genotype data (Jamieson & Taylor 1997) was 99.987% for 2008 and 99.985% for 2009, suggesting that these loci were suitable for subsequent analyses. There was one maternal genotype shared among three ramets in the 2008 MP sample, and two other genotypes were shared by two maternal ramets each in the 2009 MP sample. All other genotypes at all sites in both years were unique.

Within-sibship correlated paternity averaged over all sites was 0.28 (SD = 0.12) in 2008 and 0.30 (SD = 0.14) in 2009 (Table 3.2) and ranged from 0.008 – 0.97 depending on the site and year. The average number of potential pollen donors (i.e.,

neighborhood size) was 7.08 (SD = 4.77) in 2008 and 8.46 (SD = 5.16) in 2009. The largest estimated neighborhood size was 113 fathers in the 2009 EN sample. The average within-sibship relatedness was 0.37 (SD = 0.49) for 2008 and 0.36 (SD = 0.49) for 2009 indicating on average seeds were more than half siblings. The proportion of offspring that were estimated to be either ½ or full siblings was higher within maternal pairs than among maternal pairs (Kruskal-Wallis test $p < 0.001$; Table 3.2). There were no significant differences among years averaged across sites for any statistic (Kruskal-Wallis test $p > 0.05$); however, there were differences among individual sites from 2008 to 2009 (Kruskal-Wallis test $p < 0.0001$; Table 3.2).

Average pollen dispersal distance and pollen dispersal functions

An underlying assumption to the KinDist approach is that the magnitude of correlated paternity among mothers declines with increased geographic distance. Among sib-ship correlated paternity is normalized so that the average over all offspring pairs will be zero; therefore, as is typically seen (Robledo-Arnuncio *et al.* 2006, 2007), correlations were both positive and negative at short geographic distances (Figure 3.2). Negative values indicate paternal relatedness between pairs of mothers that are less than the average. Correlated paternity decreased slightly in 2008 and 2009 in the EN and MP sites, but did not in OB for either year (Figure 3.2). The declines observed were on par with what has been posited as acceptable for estimating dispersal distances (see data in Robledo-Arnuncio *et al.* 2007).

Table 3.1 Measures of genetic diversity in sites of *Vallisneria americana* sampled from the Chesapeake Bay in 2008 and 2009 for both mothers and offspring. N_m = number of mothers analyzed; N_o = number of offspring analyzed; *A* = average number of alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; and *f* = inbreeding coefficient. Bold = $P < 0.05$. Different letters indicate significant differences between *f* values (Kruskall-Wallis $p = 0.0121$).

Site	Year	N_m	N_o	Maternal *A*	Offspring *A*	Maternal *Ho*	Offspring *Ho*	Maternal *He*	Offspring *He*	Maternal *f*	Offspring *f*
OB	2008	35	682	6.4	8.8	0.77	0.72	0.74	0.74	-0.04^{BC}	0.03^{AB}
	2009	39	780	7.6	9.2	0.76	0.73	0.74	0.74	-0.02^{BC}	0.01^{AB}
EN	2008	24	380	6.0	7.6	0.68	0.71	0.68	0.71	0.00^{AB}	0.00^{ABC}
	2009	19	274	4.6	7.6	0.70	0.63	0.62	0.67	**-0.14^{C}**	**0.07^{A}**
MP	2008	36	619	6.0	7.6	0.68	0.71	0.68	0.74	0.03^{AB}	**0.03^{AB}**
	2009	23	418	5.4	8.8	0.70	0.71	0.62	0.74	**-0.13^{C}**	0.04^{AB}
	2008	AVG		6.13	8.00	0.71	0.72	0.70	0.73	0.00	0.02
		SD		0.23	0.69	0.05	0.00	0.03	0.02	0.04	0.02
	2009	AVG		5.87	8.53	0.72	0.69	0.66	0.72	-0.10	0.04
		SD		1.55	0.83	0.03	0.06	0.07	0.04	0.06	0.03

Table 3.2 Measures of shared paternity and relatedness in sites of *Vallisneria americana* sampled from the Chesapeake Bay in 2008 and 2009. Mean correlated paternity; average neighborhood size = 1/correlated paternity; Mean *r* = Wang (2002) relatedness estimator; and the Mean proportion of within- and among-maternal siblings that greater than 0.25 and 0.5 relatedness. Different letters indicate significant differences between *f* values (Kruskall-Wallis p < 0. 0001). Standard error in parentheses.

Site	Year	Mean Correlated Paternity	Mean Neighborhood Size	Mean Within Mother *r*	Mean Among Mother *r*	Mean Proportion Half Sib Within Mothers	Mean Proportion Half Sib Among Mothers	Mean Proportion Full Sibs Within Mothers	Mean Proportion Full Sibs Among Mothers
OB	2008	0.14 (0.09)^{D}	12.56 (12.47)^{A}	0.31 (0.08)^{A}	0.04 (0.26)^{A}	0.60 (0.12)	0.22 (0.06)	0.23 (0.10)	0.04 (0.02)
	2009	0.23 (0.18)^{C}	11.06 (16.40)^{B}	0.33 (0.11)^{B}	0.03 (0.25)^{B}	0.62 (0.14)	0.2 (0.06)	0.27 (0.16)	0.03 (0.02)
EN	2008	0.32 (0.17)^{B}	4.75 (5)^{CD}	0.39 (0.13)^{C}	0.09 (0.26)^{C}	0.65 (0.15)	0.33 (0.19)	0.37 (0.19)	0.10 (0.13)
	2009	0.22 (0.21)^{CD}	11.81 (26.24)^{BC}	0.34 (0.10)^{B}	0.14 (0.34)^{D}	0.71 (0.18)	0.40 (0.07)	0.32 (0.11)	0.14 (0.04)
MP	2008	0.37 (0.22)^{B}	3.92 (2.76)^{D}	0.40 (0.10)^{C}	0.05 (0.26)^{E}	0.73 (0.13)	0.24 (0.06)	0.37 (0.16)	0.05 (0.02)
	2009	0.47 (0.18)^{A}	2.51 (1.13)^{E}	0.41 (0.10)^{D}	0.02 (0.26)^{F}	0.75 (0.14)	0.23 (0.09)	0.41 (0.16)	0.05 (0.04)

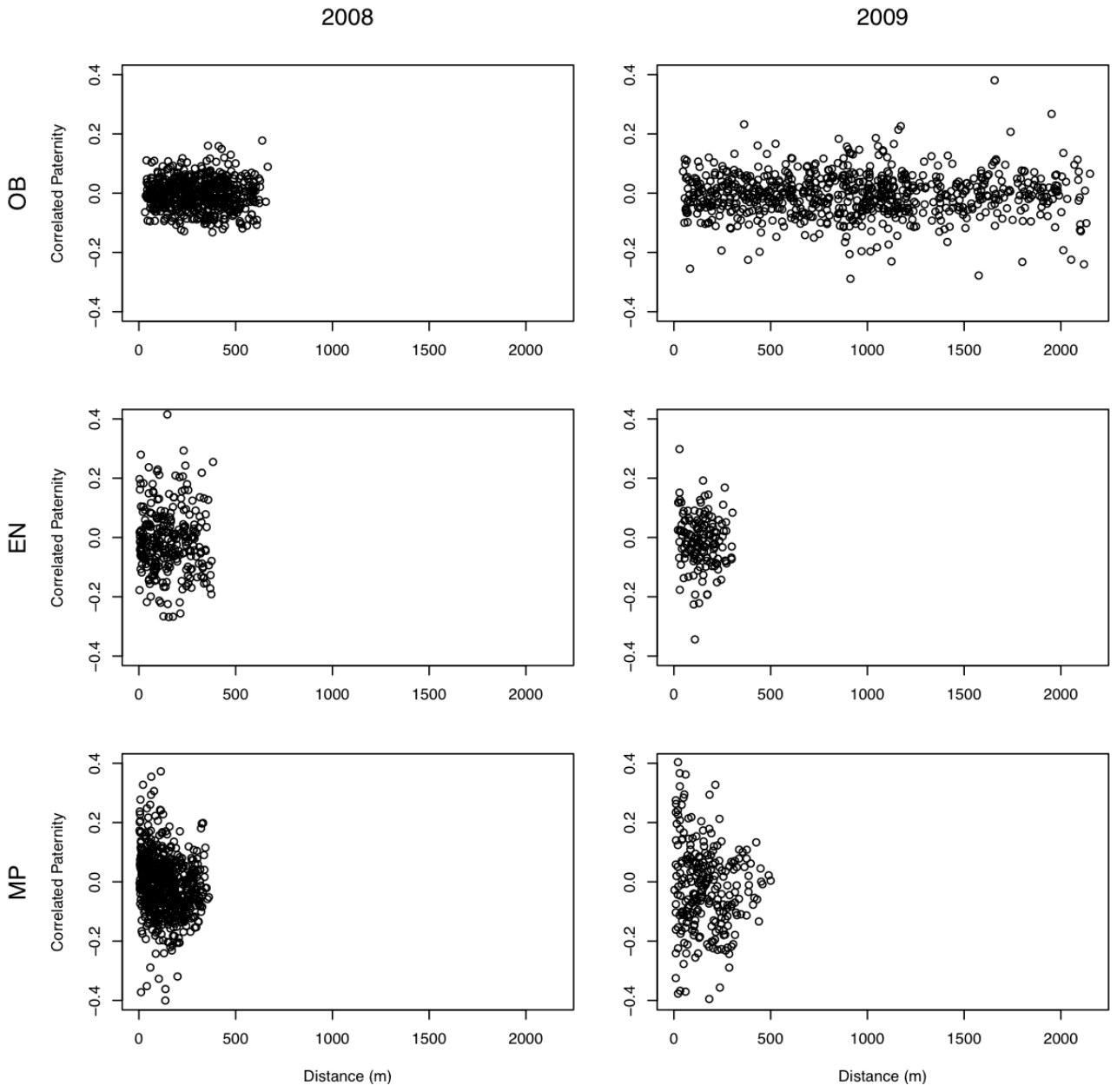

Figure 3.2 The relationship between correlated paternity among maternal pairs and geographic distance (m) among paired mother for all sites and years. Correlated paternity is expected to decline with increasing geographic distance among maternal pairs. The x-axis is set to the largest distance sampled (2009 OB) for comparison among sites.

Of the five probability density functions (PDF), only the one-parameter normal and exponential distributions provided informative results (Table 3.3). The two-parameter functions (exponential-power, geometric, and 2-dimensional student's *t*) provided parameters that estimated δ to include infinity. Estimates of infinity indicate a poor model fit and were therefore not used. Estimates of δ as calculated with a normal or exponential distributions were similar; however, the exponential distribution increased the distribution tail length (Figures 3.3 & 3.4). As a result of the longer tail, the exponential distributions had an increased probability of dispersing 5, 10, and 30 m; however, they was still very small (5 m dispersal probability range = $1 \times 10^{-3} - 1 \times 10^{-5}$; 30 m dispersal probability range = $1 \times 10^{-4} - 1 \times 10^{-30}$; Table 3.3). Across years within a site, there were minimal differences in estimates of δ and pollen dispersal functions. The largest difference in δ was seen in the EN site; however, this difference confounded by different lag distances between mothers. The estimates of δ are similar between the 2009 EN sample and the 2008 and 2009 OB samples.

Table 3.3 Best fit models for all sites and years as calculated in PolDisp. *a* = estimated model paramerter (see Austerlitz *et al.* 2004; for model details); δ = average pollen dispersal distance; σ^2 = variance; residual = least-square residual; and 5, 10, 30 m = probability of dispersing given the estimated model parameter.

	OB		EN		MP	
Normal	2008	2009	2008	2009	2008	2009
a	0	23.28	1.48	17.47	3.91	0.91
δ	0	20.63	1.31	15.48	3.47	0.80
σ^2	0	16.46	1.05	12.35	2.77	0.64
Residual	69.37	71.15	28.56	22.00	42.17	21.11
5 m	NA	5.6E-04	1.6E-06	9.6E-04	4.1E-03	3.0E-14
10 m	NA	4.9E-04	2.2E-21	7.5E-04	3.0E-05	1.4E-53
30 m	NA	1.1E-04	5.2E-180	5.5E-05	5.6E-28	0
Exponential						
a	0.63	9.77	0.80	9.48	2.17	0.45
δ	1.25	19.55	1.61	18.96	4.34	0.89
σ^2	1.08	16.93	1.39	16.42	3.76	0.77
Residual	74.66	71.32	28.55	20.73	41.82	20.76
5 m	1.4E-04	1.0E-03	4.8E-04	1.0E-03	3.4E-03	1.2E-05
10 m	5.1E-08	6.0E-04	9.3E-07	6.2E-04	3.4E-04	1.8E-10
30 m	8.4E-22	7.7E-05	1.3E-17	7.5E-05	3.3E-08	8.8E-30

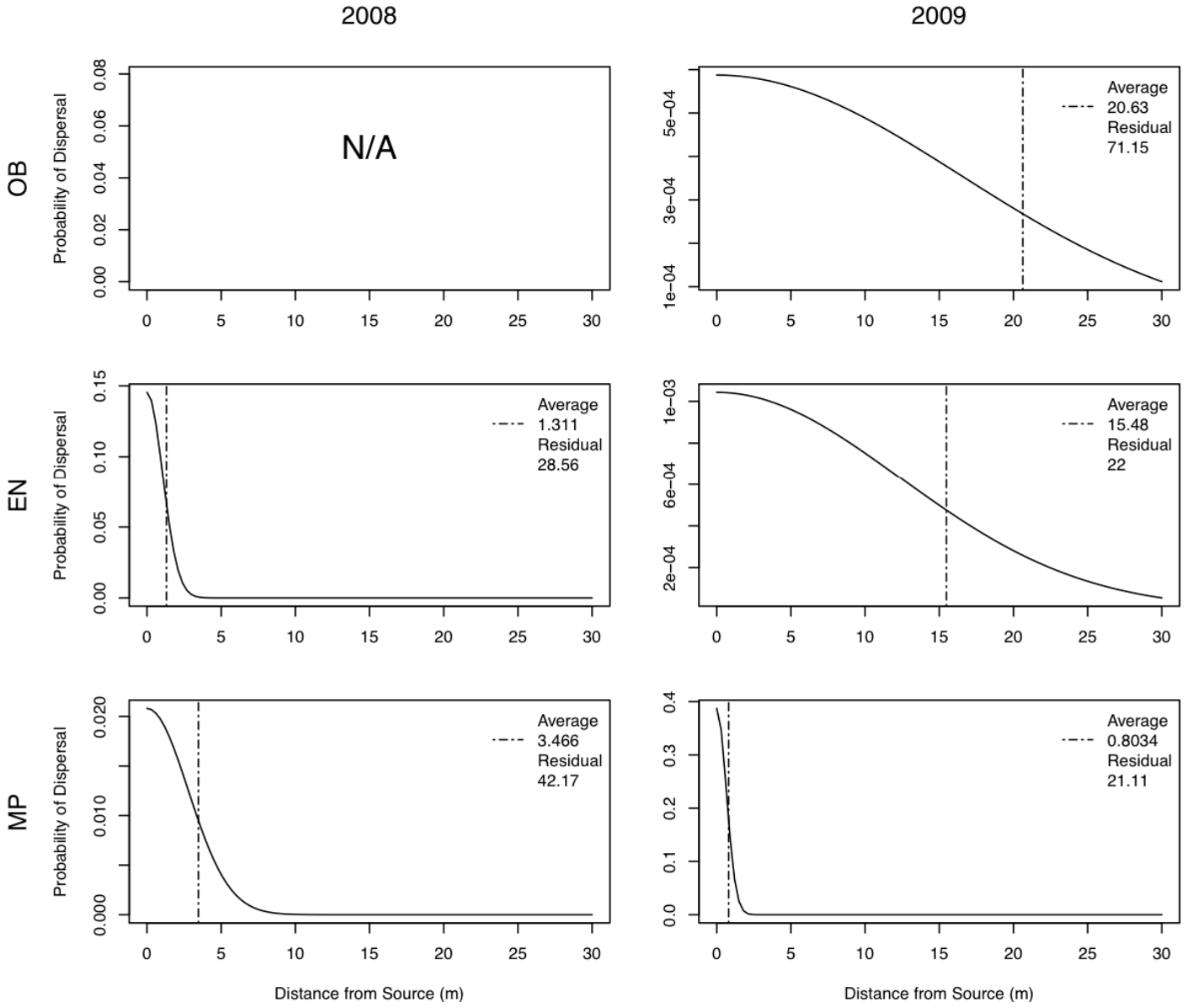

Figure 3.3 Probability of a pollen grain dispersing a given distance from a pollen source (m) for all sites and years. The normal probability function was fit. The 2008 OB site was unable to fit a model. The average pollen dispersal distance (dashed lines) and least-square residual for each model are presented.

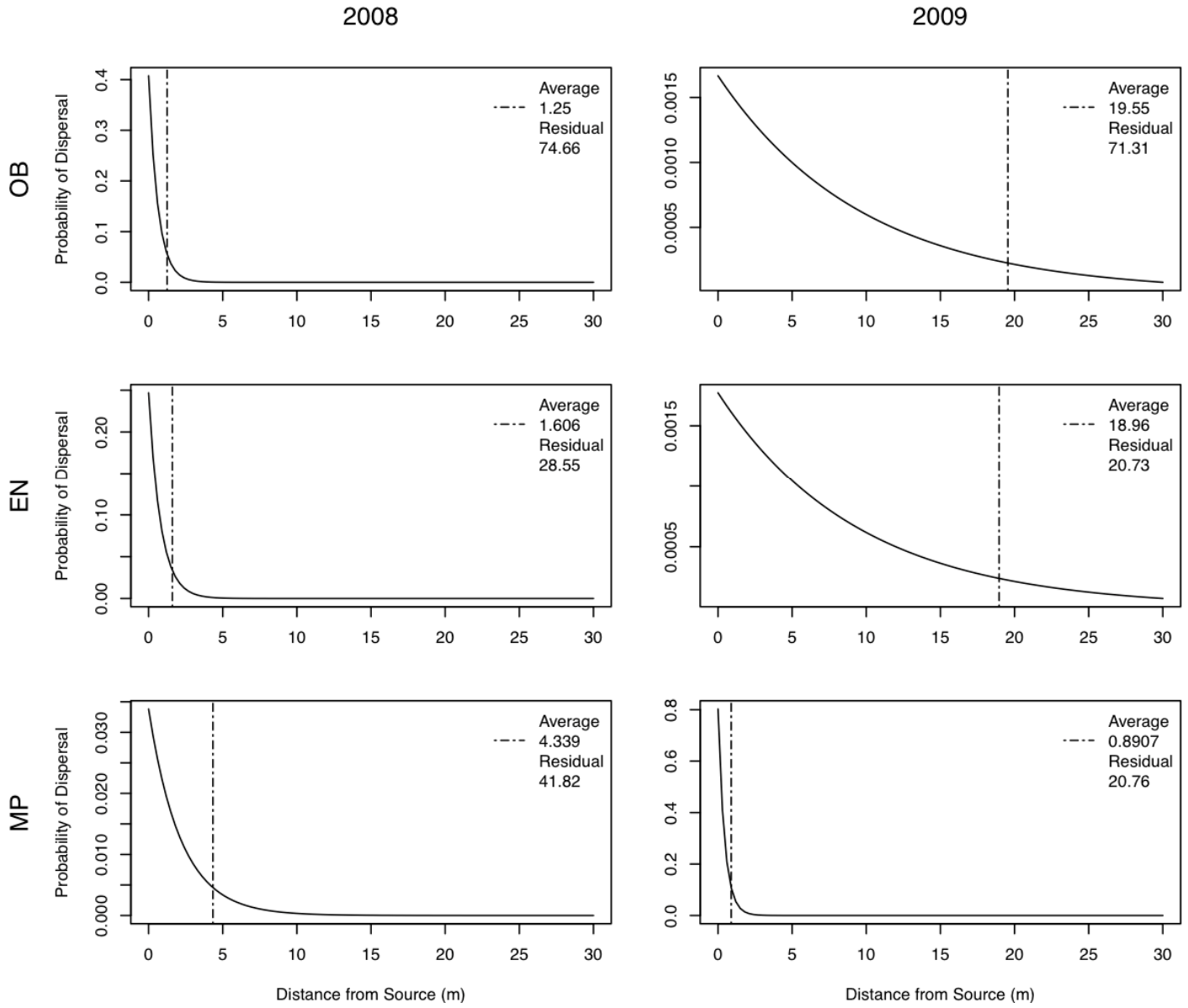

Figure 3.4 Probability of a pollen grain dispersing a given distance from a pollen source (m) for all sites and years. The exponential probability function was fit. The 2008 OB site was unable to fit a model. The average dispersal distance (dashed lines) and least-square residual for each model are presented.

Discussion

Our results indicate that the scale of pollen dispersal for *Vallisneria americana* is on the order of 1 – 20 m. The different sample types, open water versus shoreline, did not greatly impact pollen dispersal, and the differences among years were negligible. Experimentally derived pollen dispersal distances in *Zostera marina* L. (Zosteraceae), a monoecious species with water-dispersed pollen, were shown to be < 15 m (Harwell & Orth 2002; Ruckelshaus 1995). These distances are on the lower end of within site dispersal distances observed in wind-pollinated trees (15 m – 7.6 km), in insect pollinated trees (21 m – 88.6 km) and below what is seen in shrubs and herbaceous plants (113 m – 5 km; see studies in Ashley 2010). In insect pollinated plants, isolation negatively affects seed number and flower visits only when sites are beyond the effective dispersal distance of the pollen vector (Jennersten 1988; Steffan-Dewenter & Tscharntke 1999; Wolf & Harrison 2001). Habitat loss and fragmentation can affect pollen dispersal in *V. americana* is if males are lost within a site as the patch contracts and becomes isolated. Skewed sex ratios, as might result by chance during isolation, lead to decreased reproductive success within a site (Shelton 2008). The probability of a pollen grain encountering a flower upon emergence is also dependent upon female density as much as geographic proximity (Cox 1988). Lower female density would increase the time a pollen grain spends on the surface of the water, which then increases potential dispersal distance. However, decreased population densities lead to decreased outcrossing, pollen limitation, and reduced seed set (Groom 1998; Murawski & Hamrick 1991; Reusch 2003; Van Treuren *et al.* 1993; van Tussenbroek *et al.* 2010). Reduction in population density

may also cause a decrease in population growth rates (Stephens *et al.* 1999). We sampled dense patches of *V. amerciana* with aerially mapped crown covers of 75-95% (Orth *et al.* 2009, 2010a) and females were locally abundant. As *V. americana* is dioecious it would be of interest to examine the role density plays on pollen dispersal, as there is likely a threshold ratio of males to females below which pollination cannot occur.

Our results are potentially influenced by the lack of decline in correlated paternity with distance, which has several possible explanations. The first is that our markers did not provide enough resolution to provide accurate estimates of paternal pollen structure. Our markers were variable, highly heterozygous, and provided a high probability of exclusion. In power analyses preformed by Austerlitz and Smouse (2002), and Robledo-Arnuncio et al. (2006) five loci with between five and ten alleles per locus were sufficient to provide high exclusion probability, minimal bias and mean square error when estimating δ (Austerlitz & Smouse 2002; Robledo-Arnuncio *et al.* 2006). The five loci used in the present investigation had an average of ten alleles per locus, which suggests that we had sufficient power for estimating unbiased estimates of δ.

Even with a sufficient marker system the sample lag distances can influence estimates of dispersal distance. We could have sampled at scales either too large, or sample at too small a distance to detect a decline in correlated paternity. If long distance dispersal exists relative to the population density the overall genetic structure of the pollen pool would be low. In such a case, if we sampled at a scale that was within mixed pollen pools, we would be unable to detect a decline in correlated

paternity and estimates of pollen dispersal would be under estimated (Robledo-Arnuncio *et al.* 2006, 2007). Additionally, fathers are not restricted to a single point in space, which violates assumptions of the KinDist model. A single male clonally spread across a population can eliminate the genetic structure of pollen pools. In such an extreme case, there would be no way to determine the distance any single pollen grain is transferred because regardless of the lag distance between mothers, the proximity to the same paternal genotype would not change.

Spatially restricted pollen flow would also result in no decline in among-sibship correlated paternity. If this were the case, we potentially sampled at distances too great and all mothers we sampled would have been pollinated from unique pollen pools. In such a scenario the number of fathers contributing to a fruit can be high, but there would be no correlation among fathers contributing to mothers. In our case, the neighborhood size shows that on average 7 fathers contribute to each seed, but can range as high as 113 fathers; furthermore, we observed higher within mother seed relatedness compared to among mother seed relatedness. The combination of these data supports that pollen dispersal is occurring over 1 – 20 m.

The number of pollen donors at local distances determines fine scale genetic structure (Koenig & Ashley 2003; Pluess *et al.* 2009). The short pollination distance establishes an unequal contribution of pollen from local (1 – 20 m) fathers, which can generate genetic neighborhoods within continuous patches of a species via isolation-by-distance (Wright 1946). Increased local pollination over consecutive generations, in conjunction with limited seed dispersal can lead to closely related individuals mating with one another (Turner *et al.* 1982). We did observe slightly elevated

inbreeding coefficients in two pools of offspring. The maternal genotypes were highly heterozygous showing that selection against homozygous individuals could exist, and be acting on seeds either prior to or following germination. Limited gene flow will also reduce genetic diversity within the genetic neighborhoods relative to the total population (Maruyama 1972), but genetic diversity will increase near the center of the population or species range (Wilkins & Wakeley 2002). In a continuous population effective population sizes (N_e) remain below global N_e and close to Wright's neighborhood size within local genetic neighborhoods (Neel *et al.* In Review), and genetic divergence among neighborhoods will increase as gene flow distance is decreased (Wilkins & Wakeley 2002).

It is important to remember that pollen dispersal is not acting alone. We know from a Bay-wide population genetic surveys of *V. americana*, that there is genetic connectivity within geographic regions (Lloyd *et al.* 2011). Within the northern and central Bay regions, the genetic differentiation was low among sites (D_{est_Chao} = 0.01 – 0.02) compared to among regions (D_{est_Chao} = 0.124). Genetic connectivity among sites suggests that even if local genetic neighborhoods are being established by limited pollen dispersal, the movement of seed and propagules within and among sites overcomes limited pollen dispersal prior to genetic differentiation. While it is possible that there are long distance pollen dispersal events occurring, the probability of such an event is too low to account for the degree of genetic connectivity observed among patches throughout the Chesapeake Bay. Even with low genetic differentiation within regions it is highly unlikely that patches are connected over distances of greater than 5km (Lloyd et al., In Prep). There is a genetic break that is

present between regions of the Bay that has been separated by a roughly 5 km gap in patches that has only recently (since 2000) begun to be occupied (Lloyd et al., In Prep.; Lloyd *et al.* 2011). It is likely that dispersal is occurring in a stepping stone pattern and patches are connected by gene flow when they are within the maximum dispersal distance. When patches that serve as bridges among regions are lost, genetic connectivity is also lost. The establishment of genetic neighborhoods via limited pollen dispersal only becomes important if patches are too distant from one another, if environmental factors prevent migration of seed among local sites, or if fragmentation isolates previously connected patches. If any of these isolates patches to the point that seed and propagules do not disperse among local genetic neighborhoods, genetic differentiation will occur and the genetic diversity of the new neighborhood will be limited to what is present at the time of isolation.

Summary

Pollen dispersal in *Vallisneria americana* is spatially restricted to only a few meters. Unless habitat loss and fragmentation drive population male densities low, pollen dispersal will likely remain intact. The scale of pollen dispersal has the potential to establish genetic neighborhoods embedded within larger populations, which influences local genetic structuring. A broad scale genetic survey of *V. americana* (Lloyd *et al.* 2011) shows that seed and propagule movement are likely driving dispersal among patches. If populations are fragmented and isolated from seed and vegetative movement, these local genetic neighborhoods can begin to differentiate.

Chapter 4: Potential landscape connectivity of *Vallisneria americana* in the Chesapeake Bay provides guidance for conservation and restoration prioritization.

We used graph theoretic approaches to examine the distribution and potential connectivity of submersed aquatic vegetation patches in the Chesapeake Bay that potentially contain *Vallisneria americana*. We examined critical distances from complete patch isolation to connection of all patches in coverages that represent the sum of all potential *V. Americana* between 1984 and 2010 and in coverages from individual years within that timeframe for which complete survey data were available. We found that if all sites that have been occupied in the recent past were occupied in a single year, the total amount of SAV coverage would be sufficient to exceed the 2010 restoration goal. Additionally, there was a high turnover in the distribution of patches. If the high turnover is due to lack of persistence in marginal habitat, reductions in turbidity could increase the growth of *V. americana* such that persistence of colonized sites is improved and direct restoration is less necessary. Connectivity varied through time, but even if all habitat were occupied, increases in overall network connectivity would not necessarily be observed. Finally, most of the thresholds in connectivity are beyond reasonable dispersal distances for *V. americana* and we recommend that restoration efforts focus on bridging gaps between patches that are less than 4 km apart.

Introduction

By the 1970's submersed aquatic vegetation (SAV) in the Chesapeake Bay of eastern North America was drastically reduced to a small fraction of its historic abundance and extent due to eutrophication and increased sedimentation (Dennison *et al.* 1993; Orth & Moore 1983). The degree of habitat loss is of a sufficient magnitude to raise concern that increased isolation of the remaining habitat patches could substantially reduce connectivity (Gardner *et al.* 1987; Helm *et al.* 2006; Jaeger 2000; Keller & Largiader 2003a; Prugh *et al.* 2008; Thrush *et al.* 2008). Baywide SAV coverage has increased since the 1980's (e.g., Orth *et al.* 2010a) due to improvements in water quality (Carter *et al.* 1994; Rybicki & Carter 2002; Rybicki & Landwehr 2007), and extensive restoration efforts (Moore *et al.* 2010). Despite these efforts to increase acreage to return the keystone functions performed by these species, annual acreages have remained at ~30,000 ha since the early 1990's (Orth *et al.* 2010a). As of early 2012 SAV abundance still fell far below target levels (U.S. Environmental Protection Agency 2010) and was a fraction of the estimated ~250,000 ha known historically (Dennison *et al.* 1993; Orth *et al.* 2008; Stevenson & Confer 1978). The acreage of SAV has been well documented but to date there has been no analysis of connectivity of the recovering habitat. To begin to fill this gap, we used graph theoretic approaches to quantify extent and patterns of potential connectivity in one of the dominant SAV species, *Vallisneria americana* Michx. (Hydrocharitaceae; American water celery). Lack of distribution data prior to major declines precludes comparison with baseline levels of abundance or connectivity, and instead we

compare the observed patch distribution with probable dispersal distances and with genetic data that contain a record of long-term movement in the Bay.

Much of the theoretical understanding of fragmentation (i.e., loss of connectivity) is based on effects of converting extensive, relatively continuous habitats to smaller and increasingly isolated patches. As with many natural habitats, sites supporting particular SAV species in the Chesapeake Bay were likely always patchily distributed due to species-specific limitations imposed by physiological tolerances to light (i.e., depth) and to salinity. Even in suitable habitat, SAV patches are known to be somewhat ephemeral (e.g., Rybicki & Carter 2002), becoming extirpated and later reappearing, indicating the potential that SAV species exhibit metapopulation (*sensu* Levins 1969) or source-sink (*sensu* Pulliam 1988) dynamics. In these situations, patch isolation may be less serious than it is for species that rely on large tracts of connected habitat. Still, the severity of habitat lost has almost certainly increased distances among remaining patches, such that changes in dispersal among patches of suitable and occupied habitat could affect overall network persistence (Hanski 1998) making it critical to understand connectivity.

Although amount of habitat is typically the most important factor in maintaining species (Fahrig 1997, 2003), ecological and evolutionary processes are driven by interactions between total habitat area, extent and size of continuous habitat patches, and connectivity among discrete patches (Baudry & Merriam 1988; Chetkiewicz *et al.* 2006; Fahrig & Merriam 1994; Merriam 1984, 1991; Taylor *et al.* 2006; Wiegand *et al.* 2005). Connectivity is facilitated by both relatively continuous large patches and among discrete patches that lie sufficiently close to one another that propagules

can move through the intervening matrix (Ferrari *et al.* 2007; Saura *et al.* 2011; Saura & Rubio 2010). For any amount of habitat, its spatial distribution will strongly affect movement that can confer resistance and resilience to perturbations and determine the amount of habitat available to an organism. As patches are lost and decrease in size, distances among remaining patches can increase and dispersal can be reduced or eliminated. Thus, preserving and restoring acreage of sites that contribute to connectivity can have greater ecological benefits than will simply adding habitat area alone. Graph theoretic measures are superior for assessing potential connectivity because they are efficient at identifying the dispersal distances required for any observed patch distribution to remain connected through both intra-and interpatch movement (Calabrese & Fagan 2004; Galpern *et al.* 2011; Pascual-Hortal & Saura 2006; Saura *et al.* 2011; Zetterberg *et al.* 2010). By identifying graph structures that develop from habitat patches within defined distances we document the location and extensiveness of networks of *V. americana* in the Chesapeake Bay of eastern North America that are potentially connected by ecological processes and highlight the distances at which changes in connectivity of these networks exhibit threshold like behavior (Bunn *et al.* 2000; Urban & Keitt 2001). Such thresholds represent the dispersal distance at which a landscape changes from being connected to disconnected for organisms with dispersal distances less than the distance at which the threshold occurs.

The degree to which potential connectivity translates to functional connectivity depends on the size of and distances among patches (Baudry & Merriam 1988; Ferrari *et al.* 2007; Merriam 1984) and how individual organisms perceive, use and

move among those patches (Brooks 2003; McRae 2006; Ricketts 2001; Taylor *et al.* 1993; Taylor *et al.* 2006; Tischendorf & Fahrig 2001). In absence of complete information on usage and movement, the scale of thresholds in patch isolation can be compared with what is known or inferred about dispersal capabilities of individual species, (e.g., Calabrese & Fagan 2004; Urban 2005). If scales of isolation coincide with distances that are likely to be important for dispersal, actions can be targeted to ameliorate risk or more detailed behavioral, demographic research can be initiated at the appropriate spatial scales and locations to assess effects on movement.

We focused on *V. americana* because this meadow forming species is one of the dominant and more persistently occurring members of SAV communities in freshwater and oligohaline tributaries of the Bay. These areas have suffered the largest SAV declines (Kemp *et al.* 1983; Moore *et al.* 2010), and as a result this species has been a target of restoration efforts for over 20 years. Additionally, genetic data for *V. americana* (Lloyd et al. 2011) provides insight into levels of long-term gene flow among sites.

We estimated dispersal distances for the species using literature on SAV dispersal and from data on genetic differentiation among *V. americana* populations in the Bay. *Vallisneria americana* disperses via seed and vegetative propagules and gene flow also occurs via pollen dispersal. Pollination occurs when pistillate flowers, borne on the water surface, are fertilized by free-floating staminate flowers (Korschgen & Green 1988). Once released to the water column, pollen remains viable for only a few days (McFarland & Shafer 2008), and individual female flowers remain receptive for approximately 24 hours (West *et al.* Submitted) indicating little

potential for movement beyond short distances. Unpublished data indicate that on average pollen moves <20m and in some populations movement is <3m (Chapter 3). Pollen movement of <15m has been documented for *Zostera marina* (Harwell & Orth 2002).

Seed dispersal occurs by three mechanisms. First, fruits can rupture while they are attached to the mother plant, releasing clusters of seeds bound in a gelatinous matrix into the water column (Korschgen & Green 1988). Once freed, seeds generally settle quickly within 10's of meters from the mother plant (Kaul 1978). In other cases, fruits become detached from the mother plant or entire reproductive ramets become dislodged and float freely. Movement distances for seeds dispersed in this way are not known but floating reproductive shoots are commonly seen in the fall and can be carried appreciable distances by currents. *Zostera marina* is known to disperse seed in this manner between 5 m and 10 km (Harwell & Orth 2002; Orth *et al.* 2012), and shoots are known to remain buoyant for up to two weeks and retain seeds for three weeks in laboratory conditions (Harwell & Orth 2002). Fragments of reproductive *Z. marina* shoots with viable seeds have been found washed up on shore up to ~34 km from established patches, but successful reestablishment is considered more likely after dispersal of 1-10 km (Harwell & Orth 2002; Orth *et al.* 2012). Vegetative dispersal of *V. americana* can also be accomplished if dislodged ramets become reestablished in a new location through rerooting or deposition of tubers (underground, overwintering organs) that later resprout (Korschgen & Green 1988). Potential dispersal distances of floating plant material vary between riverine environments with directional flow, tidal streams with predominantly downstream but

semi-diurnally alternating flow direction, and open bay environments subject to tidal flow, currents, and wind fetch. Finally, dispersal of fruits and seeds by waterfowl, either through ingestion or through adhesion to feathers (Figuerola *et al.* 2003; Higgins *et al.* 2003; Santamaria & Klaassen 2002), could potentially connect populations from distant reaches of the Bay and beyond. The relative frequency of dispersal by these different mechanisms is unknown.

Three primary genetic regions have been documented in the Chesapeake Bay (Lloyd *et al.* 2011) including the Potomac River, the northern Bay, and the central Bay (Figure 3.1). The Potomac River is further differentiated into non-tidal and tidal regions, only the latter of which is included within the VIMS SAV survey. Genetic differentiation patterns suggest that long-term gene flow has been lower among regions than among patches within these regions. Only one population from a southern tributary of the Bay was sampled and it was classified into the Potomac region, but showed admixture between the Potomac and central regions (Figure 3.1). Preliminary evidence that crosses among individuals from the same region are more successful than are crosses among individuals from different regions (West *et al.* Submitted) indicates potential that differentiation among the regions is evolutionarily significant.

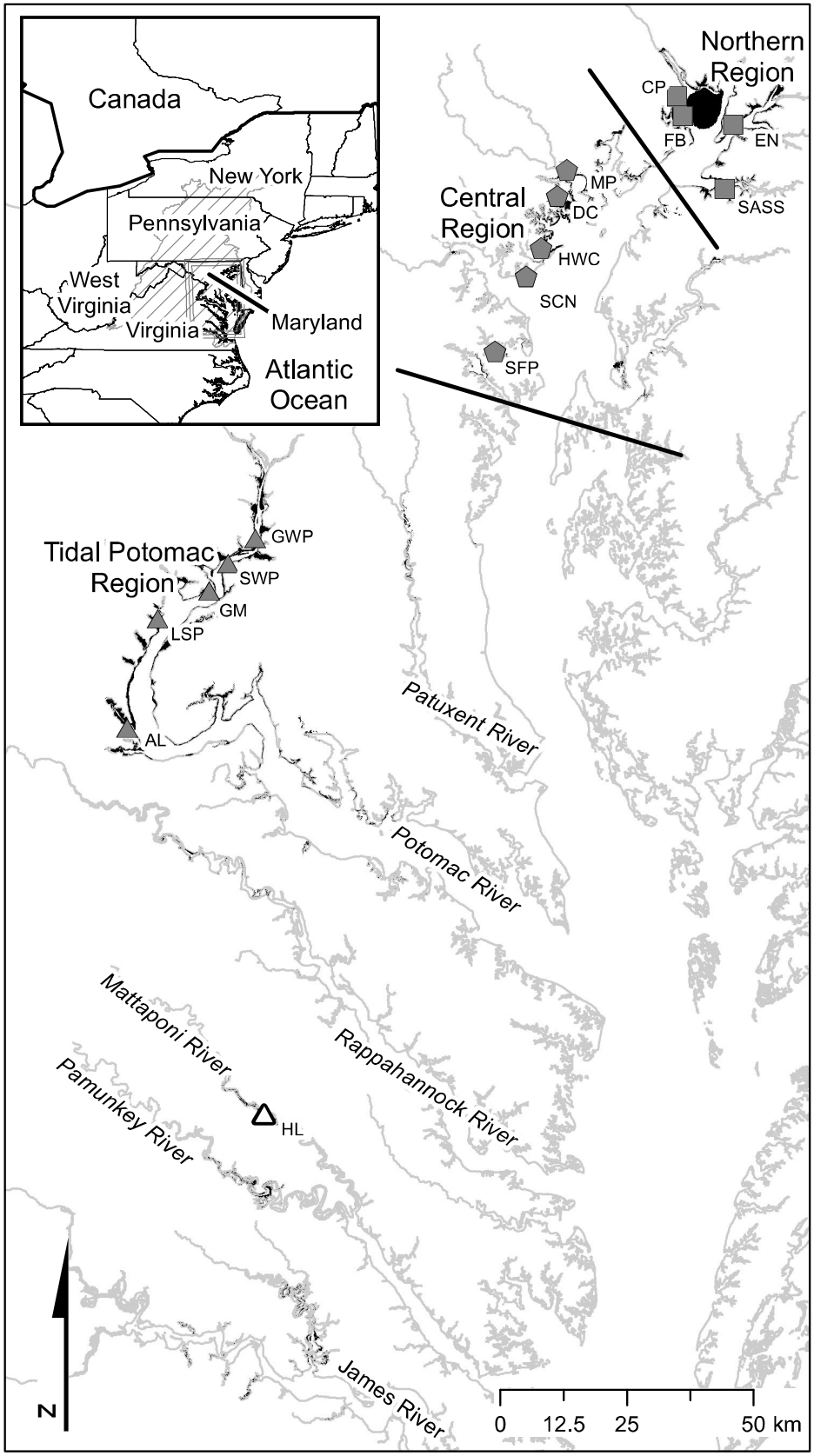

Figure 4.1 Distribution of potential patches of *Vallisneria americana* within the Chesapeake Bay for the composite coverage along with sites sampled for genetic diversity coded for three genetic regions [Northern Bay, Central Bay, Potomac River] found within the Chesapeake (see Lloyd *et al.* 2011). The site on the Mattaponi River presented difficulty in assignment, but was most similar to sites in the Potomac River.

We examined critical distances from complete patch isolation to connection of all patches in coverages that represent the sum of all potential *V. Americana* between 1984 and 2010 and in coverages from individual years within that timeframe for which complete survey data were available. We more closely examined critical distances ≤10km that we considered to have high potential to be important for *V. americana* based on inferences from the dispersal mechanisms and genetic information described above. For each critical distance, we quantified network extensiveness as number of components, the landscape coincidence probability (LCP), integral index of connectivity (IIC), and equivalent connectivity (EC) (Saura *et al.* 2011; Saura & Pascual-Hortal 2007). We identified distances at which there were large changes in connectivity as measured by these metrics. Examining the summary composite coverage gives insight into connectivity of all recently occupied SAV habitat that meets the habitat requirements for *V. americana*. Quantifying the change in occupied area and connectivity within years in which surveys of SAV in the Bay were complete allowed us to investigate how the scale and nature of networks changed across time and how individual years differed from the composite coverage. These changes in patch distribution through time give insight into the population dynamics of the species and its potential for persistence.

Methods

We created coverages with patches that had potential to contain *V. americana* by intersecting coverages of all mapped SAV and clipping by appropriate depth and salinity limits as described below. We obtained coverage data for the distribution of SAV in tidal regions of the Chesapeake Bay from 1984 to 2010 from the Virginia

Institute of Marine Science (VIMS; http://web.vims.edu/bio/sav/gis_data.html). VIMS has mapped SAV using aerial photography each year from 1971-2010, except for 1988 using methodology described in annual reports (e.g., Orth *et al.* 2010a). Due to lack of water quality data for earlier years, we used SAV coverage data from only 1984-2010. We converted polygon coverages to raster format with a cell size of 30 m.

We obtained bathymetry data for the southeastern U.S. Atlantic coast at a scale of 3 arc-seconds from the National Geophysical Data Center coastal relief model (www.ngdc.noaa.gov) and clipped it to include only the tidal portion of the Chesapeake Bay. The bathymetry data were reprojected from GCS North American 1983 to the NAD 1983 UTM Zone 18N projection, creating a raster with 84 m cells that was resampled to 30 m to facilitate clipping the SAV raster.

We obtained salinity data from all available monitoring stations (range 104 – 430 stations per year) from the Chesapeake Bay Programs water quality database (www.chesapeakebay.net/data_waterquality.aspx) for all years between 1984 and 2010 that had SAV coverage data. These geo-referenced monitoring stations record monthly water quality data from throughout the Chesapeake Bay and its major tributaries. We calculated the maximum yearly salinity at each station and used these values to interpolate a continuous salinity surface with a 30 m cell size across the Bay for each year using kriging with default settings within ArcMap v10.0 (ESRI 2011). We also explored using average salinity; however, the resulting coverages overestimated the extent of *V. americana* compared to observed occurrences (Moore *et al.* 2000) and it is likely that maximum salinity is a more important limiting factor

than average salinity. Although kriging does not take into account directionality of water flow or currents, it provided a reasonable continuous surface representation of salinity between the discrete monitoring stations.

The bathymetry and salinity data were used to clip the SAV layers to sites meeting known requirements for growth of *V. americana*. We used the single depth limit≥ −5m that represents the lower limit of prevalent growth of *V. americana* in low turbidity environments (Dutton & Juday 1944; Sheldon & Boylen 1977; Titus 1983). Temporal and spatial scales of water quality data were not sufficient to model the complex relationships between SAV growth, depth, and turbidity (Hudon *et al.* 2000). However, because we limited our potential *V.* a*mericana* habitat to areas occupied by SAV, turbidity limitations on SAV growth were *de facto* incorporated. Our depth limit encompassed the approximate extent of mapped SAV and *V. americana* is known to be one of the most tolerant of SAV species to low light conditions (Batiuk *et al.* 2000).

Each annual SAV raster was clipped independently using salinity data from that survey year. We explored using four salinity levels: ≤8, 10, 12, and 15 ppt. Competitive ability and growth of *V. americana* declines when salinity is >8 ppt (Boustany *et al.* 2010; Cho & Poirrier 2005; Doering *et al.* 2001) and growth is minimal at ≥15 ppt (Boustany *et al.* 2010; Doering *et al.* 2001; French & Moore 2003; Twilley & Barko 1990). Predicted habitat based on salinity values ≥12 included many sites in which *V. americana* has never been documented. Coverages resulting from 8 ppt versus 10 ppt were nearly identical and yielded distributions that coincided with many confirmed *V. americana* occurrences (Moore *et al.* 2000). We

used the salinity limit of 10 ppt to provide a generous but realistic distribution of *V. americana*. The resulting coverages represent patches of SAV in which *V. americana* is likely to occur given environmental limits of the species, but only a subset have been confirmed through ground truthing. Thus, we likely over-estimated the extent of the species, but did so in the same manner for all data sets and we consider this best-case scenario of the distribution and abundance of *V. americana* a reasonable basis for assessing potential connectivity.

To determine the full extent of recent potential *V. americana* within the Bay, we combined the separately clipped annual raster data from 1984–2010 into one composite raster. Using information provided in the VIMS annual reports we determined which flight lines within USGS quadrangles were either partially flown or not flown each year. Each cell was coded with the number of times it was included in the survey, the number of times it supported SAV, and the number of times the quadrangle containing that cell was surveyed. We then calculated the average and maximum within-patch cell age and percentage of time each quadrangle was surveyed to provide insight into the persistence of patches through time. Additionally, we individually examined nine years for which all flight lines were flown yielding complete survey data (1998, 2000, 2002, 2004, 2006, 2007, 2008, 2009, 2010) to assess changes through time and to compare extent and connectivity within years to what had been occupied at any time between 1984 and 2010.

Landscape analyses

For all 10 data sets (1 composite and 9 individual years) we calculated the area of each patch and of all patches combined with Fragstats v3.4 (McGarigal *et al.* 2002).

We measured potential connectivity using standard graph theoretic statistics based on the number of patches (*nodes*) that lay within a range of critical threshold distances and thus were connected into *components* by *edges*. Input files of discrete patches were created with the REGIONGROUP tool using an eight-neighbor rule in ArcMap v10 (ESRI 2011). The SAMPLE tool was used to extract text file representations of all rasters and we used GenGraph (Urban 2003) to create node files. We used the landscape genetics toolbox "Cost-Distance Matrix" tool (Etherington 2011) in ArcMap 10 to calculate effective pairwise distances among patches allowing only dispersal across water because pollen dispersal and most seed dispersal is limited to the water column (Harwell & Orth 2002; Kendrick *et al.* 2012) and even waterfowl which could disperse across land tend to follow waterways during localized and long distance flight (Hochbaum 1955). Resistance layers were constructed for each year assigning a dispersal cost of 1 to water, a cost of 0 to occupied habitat, and no-data (i.e., no dispersal) to land. Calculating effective distance in this way provides measures that are analogous to edge-to-edge distances across water; however, graph edges are shown as straight lines from patch centroids for graphical convenience.

Node and effective distance files for the composite and annual coverages were submitted to the program Conefor Sensinode v2.6 (Saura & Torné 2009) to evaluate networks at critical distance thresholds in 100 m increments ranging from 100 m to the distance at which all nodes were connected by edges into a single component. We examined distances ≤10 km more closely as this distance represents the upper limit at which we considered likely for dispersal. At each critical distance we calculated the number of components, the landscape coincidence probability (*LCP*), integral index

of connectivity (*IIC*), and equivalent connectivity *EC(IIC)* (Saura & Pascual-Hortal 2007). Both *LCP* and *IIC* are fractions with total landscape area in the denominator, which results in minute values; therefore we used the numerators of both statistics (*LCPnum* and *IICnum*). *LCPnum* is the summation across components of the squared sum of habitat area belonging to each component (Saura & Pascual-Hortal 2007). The maximum *LCPnum* value is obtained when all patches are connected at which point it is equal to total patch area squared. *IICnum* defined as $\sum_{i=1}^{n}\sum_{j=1}^{n}\left(\frac{a_i \cdot a_j}{1+nl_{ij}}\right)$ where a_i and a_j are the areas of patches *i* and *j* and nl_{ij} is the shortest number of edges require to link patches *i* and *j* (Saura & Pascual-Hortal 2007). As *IICnum* integrates interpatch connectivity with habitat area it is considered a habitat availability index (Pascual-Hortal & Saura 2006). *IICnum* is maximal for one large contiguous patch and declines as as patch size declines and patches become numerous and more distant from one another.

To facilitate comparisons across landscapes we used the *IICnum* values to calculate equivalent connectivity (*EC*). *EC(IIC)* (the square root of *IICnum*) is interpreted as the size of a single habitat patch that would provide the same *IIC* value as the actual habitat pattern (Saura *et al.* 2011). We compared proportional differences in *EC(IIC)* (*dEC(IIC)*) as a function of proportional differences in habitat area (*dA*) to provide insights on the degree to which differences in area yield changes in connectivity. We calculated these values in two ways.

First, to examine changes in connectivity through time, we compared each sequential pair of years by calculating dEC(IIC) as $\left(\frac{EC(IIC)_{year_1} - EC(IIC)_{year_0}}{EC(IIC)_{year_0}}\right)$ and

then dA as $\left(\frac{Area_{year_1} - Area_{year_0}}{Area_{year_0}} \right)$ (Saura *et al.* 2011). Second, to examine if current levels of habitat occupancy have connectivity consequences relative to the maximal composite coverage, we compared the maximal composite coverage compared to each year with complete survey coverage. This comparison was accomplished by calculating *dEC(IIC)* as $\left(\frac{EC(IIC)_{year} - EC(IIC)_{composite}}{EC(IIC)_{composite}} \right)$ and then calculating the proportion of change *dA* in the same manner (Saura *et al.* 2011). Comparison between changes in EC and area provide a straightforward assessment of the impact changes in habitat amount alter overall connectivity (Saura *et al.* 2011). When *dEC(IIC) > dA*, the additional habitat area is making substantial contributions of connectivity (Saura *et al.* 2011). Conversely, when *dEC(IIC) < dA* the additional habitat represents isolated patches that make only a modest contribution to increased habitat connectivity (Saura *et al.* 2011). Finally, when *dEC(IIC) = dA* the additional habitat area is adjacent to or contiguous with the original habitat area and corresponds to a neutral area gain in connectivity (Saura *et al.* 2011).

Results

The total amount of SAV acreage occupied between 1984 and 2010 was 76,836 ha, an amount much greater than is found in individual years. Of the total composite SAV acreage between 1984 and 2010, we estimated the total area of potential *V. americana* based on salinity and depth in the composite coverage was 27,264.4 ha. This acreage was distributed across 2644 patches that ranged in size from 0.09 ha to 4838.7 ha (median = 0.27 ha; Table 4.1).

Table 4.1 Landscape characteristics of potential *Vallisneria americana* habitat: number of patches, area, average patch area, critical distance required to yield 7 main components, maximum critical distance required to connect all patches across the 9 years with complete flight line coverage and the composite coverage.

Year	Number of Patches	Total area (ha)	Avg. Patch Size (ha)	Distance to 7 components (km)	Max. Critical Distance (km)
1998	1240	7038	5.68	14.2	182.9
2000	1310	9779	7.47	*13.2	185.4
2002	1033	7794	7.55	22.6	202.9
2004	1548	12305	7.95	16.2	169.4
2006	1781	11027	6.19	12.5	192.8
2007	2061	13346	6.48	12.4	199.4
2008	2195	15130	6.89	11.9	216.0
2009	2160	15379	7.12	12.0	194.6
2010	2237	14329	6.41	12.6	194.8
Composite	2644	27264	10.31	24.5	157.8

* In the year 2000 there were only 6 main components due to lack of patches in the Mattaponi.

Based on the number of times a 30m cell occurred in annual surveys, the average patch age was 2.07 years, and the maximum age averaged was 13.5 years. Each individual cell within a patch could have been observed up to 26 times (seen for cells in 2 patches), but on average the maximum individual cell age was 2.97 years (range – 1-26). Patch age is dependent upon the number of times each quadrangle was surveyed. The quadrangles, in which patches potentially containing *Vallisneria americana* were found, were surveyed 75.3% of the time (range 19-100%).

The vast majority of patches (n=1436) were found in only a single year. These single patches were located in quadrangles that were surveyed an average of 78.1% of the time. An additional 837 patches were present in 2 to 5 years, and the quadrangles in which these patches were located were surveyed 69.9% of the time. Only 37 patches were documented to have been in place for over 20 years, and all of these had expanded greatly during that time. Patches existing for more than 20 years were

found in quadrangles that were surveyed an average of 98.95% of the time. These oldest patches were mainly located in the northern Chesapeake Bay and Potomac River; however, 3 small patches (0.9, 2.7 and 5.4 ha) have existed in the Gunpowder River (adjacent to the Bush River) and 10 other larger patches have existed for 15-19 years in the central region.

In the composite coverage, we identified three critical distances at which there were rapid changes in connectivity as measured by *LCPnum*, and *IICnum* (Figure 4.2). The first threshold at 24.5 km represents connection of patches into seven main components (Figures 4.2 and 4.3) that corresponded to one network that included patches in the northern and central Bay regions and networks within of the following six rivers: Patuxent, Potomac, Rappahannock, Pamunkey, Mattaponi, and James (Figure 4.3). A threshold at 84.8 km corresponded to connection of the north-central component with the Patuxent and Potomac Rivers into one component. The third increase in the metrics came when that component and components in the tributaries in the southern part of the Bay all joined into one component at a critical distance of 157.8 km (Table 4.1; Figures 4.2 and 4.3).

At the 10 km critical distance, components in each of the six rivers remain intact but the component in the north-central Bay broke apart forming one isolated component on the northeastern shore and the several smaller components on the central western shore (Figure 4.3). At critical distances <9 km additional components formed along the western and eastern shores, within the combined northern and central regions, but the two regions as identified with genetic data did not become disconnected from each other until critical distances were ~4.5 km (Figure 4.3).

Patches in the lower Potomac River began to form separate components below 8 km, and patches in the James River did the same below 9 km (Figure 4.3).

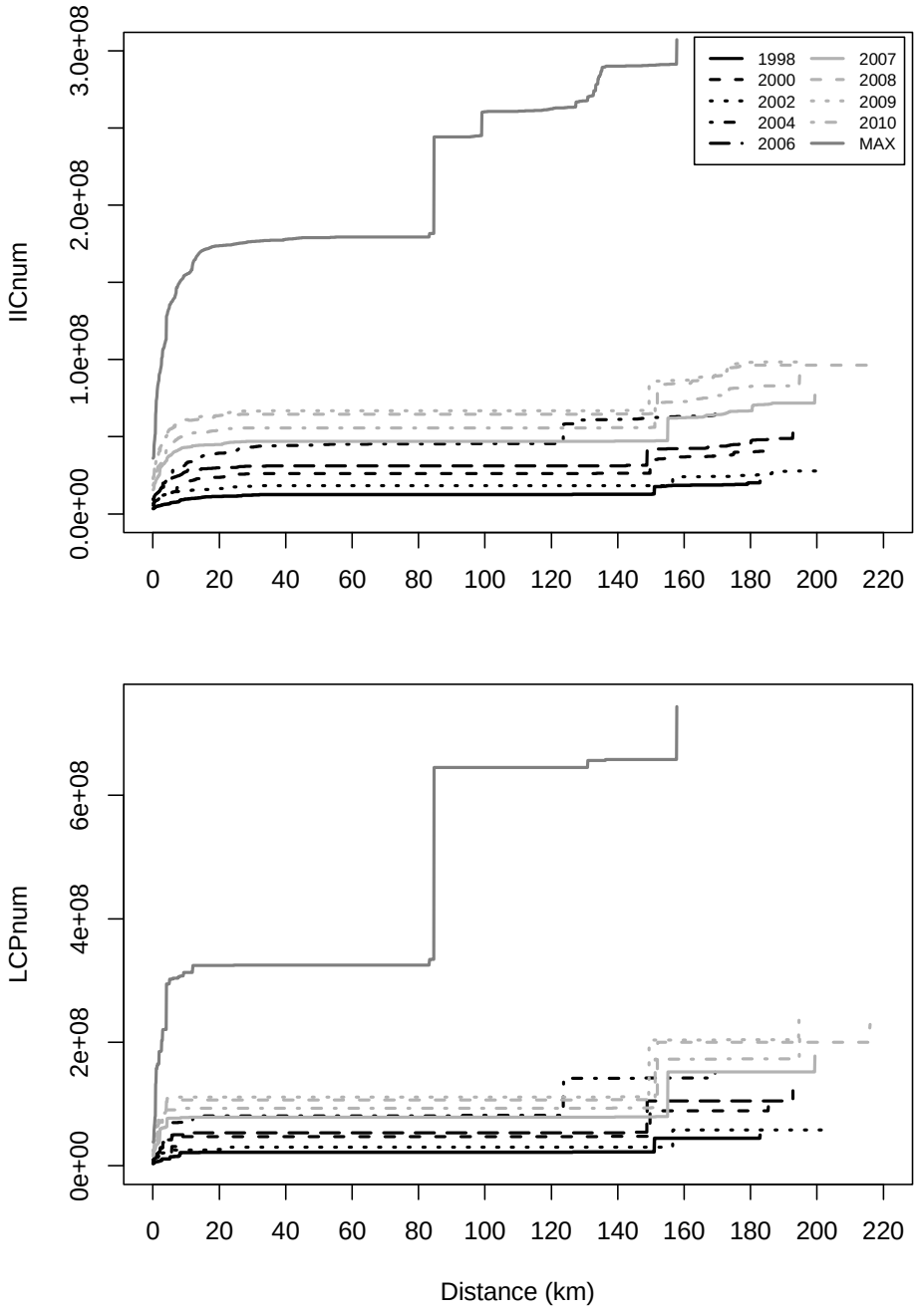

Figure 4.2 Landscape coincidence numerator (LCPnum) and integral index of connectivity numerator (IICnum) for each critical distance (km) and coverage.

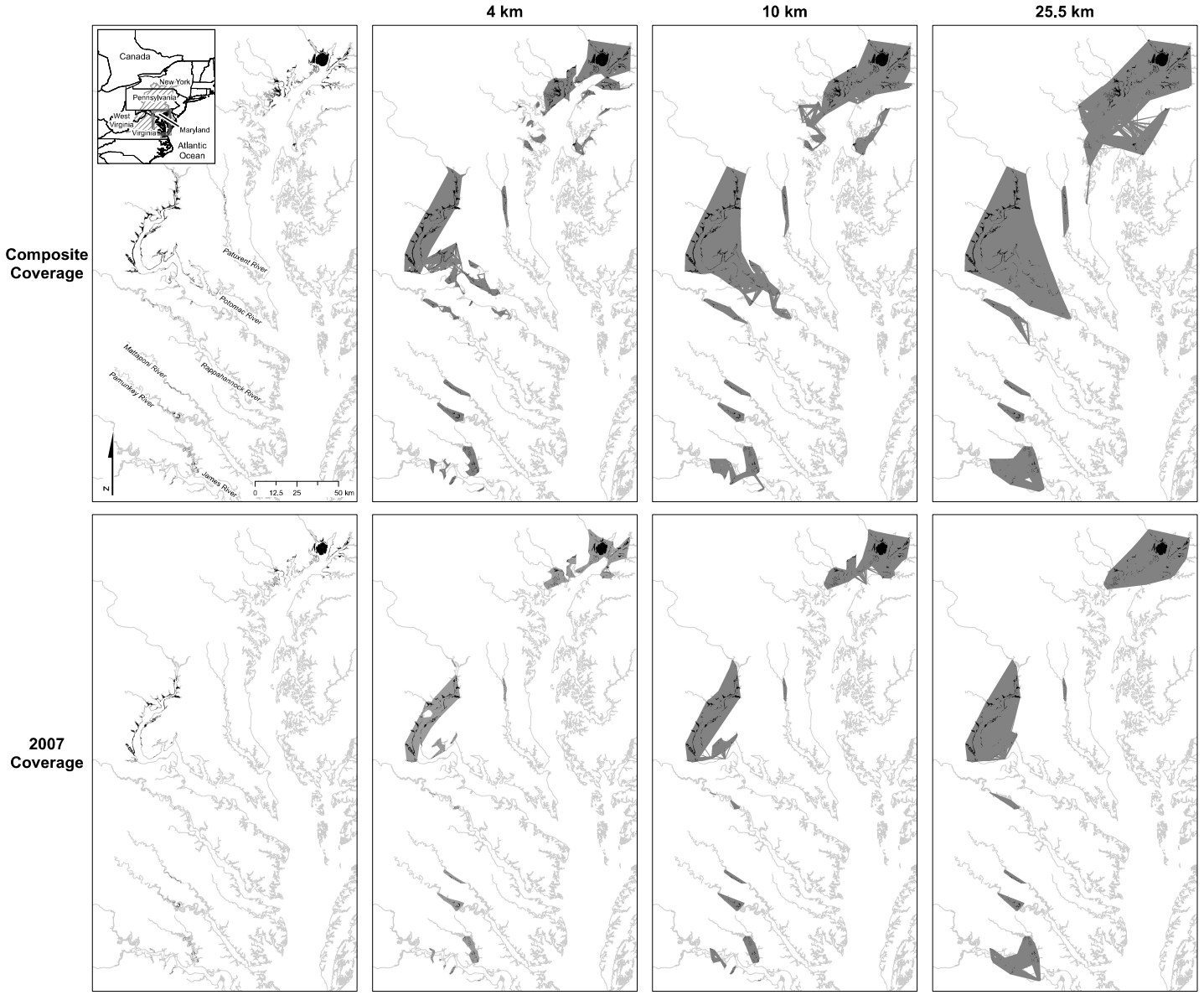

Figure 4.3 Distribution of potential patches of *Vallisneria americana* within the Chesapeake Bay for the composite and 2007 coverages. The 2007 coverage was selected as a representative of the years with complete survey coverage. Connectivity among patches (edges) at critical distances of 4.0, 10.0 and 25.5 km are presented.

The years with complete survey data (1998, 2000, 2002, 2004, 2006, 2007, 2008, 2009, 2010) each represented between 26% and 56% of the total area recently occupied potential *V. americana* (Table 4.1). With some annual fluctuations, the number of patches generally increased from 1240 in 1998 to 2237 in 2010 and area increased from 7038 ha to 15379 in 2009 with a decline to 14329 ha in 2010 (Table 4.1). Patch connectivity as measured by *LCPnum* varied up to 20% from year to year (Figure 4.4); however, the broad patterns of connectivity mirrored the composite coverage. Thresholds in *LCPnum* and *IICnum* similar to those observed in the composite coverage were observed for each of the individual years; however, the distances at which these thresholds were reached differed (Figure 4.2). The rank order of both *LCPnum* and *IICnum* values is directly related to the amount habitat present in each coverage indicating that habitat area drives these metrics.

Patches were united into a single component at critical distances ranging from 182.9 km to 292.9 km, exceeding the distances required in the composite coverage by 15.9%-85.6%. The same seven main components seen in the composite coverage were formed at the first threshold in the individual years except 2000 at distances between 11.9 and 22.6 km (Table 4.1). In the year 2000, no SAV patches were mapped on the Mattaponi River, and therefore only 6 major components were formed and this occurred at a distance of 13.2 km. Components formed at shorter distances within individual years relative to the composite coverage due to absence of patches located along the central western shore of the Bay (Figure 4.3). The lack of these patches, however, increased the distance required to connect the north-central region with the Patuxent and Potomac Rivers. For example, in all years except 2004,

between 149 km and 156.7 km was required to link the north-central component with the Patuxent and Potomac Rivers (Figure 4.2). In 2004, the critical distance required to for this component only 123.8 km (Figure 4.2) due to presence of several patches along the northeastern shore of the Bay that were not documented in the other individual years.

At the 10 km critical distance, there were between 8 and 14 components within individual years compared to the 12 components in the composite coverage (Table 4.2). Below 10 km, *LCPnum* and *IICnum* values at a given critical distance fluctuated across years, but the rank order of these values was directly related with habitat area (Figure 4.5). At these shorter critical distances the exact distribution of components varied from year to year as a function of patch presence or absence and patch size; there were, however, general patterns across all years. In general, the middle portions of the Potomac River remained internally well connected until below 2 km, but the patches in lower reach of the Potomac disconnected from the middle reach below 7.5 km. The northern and central regions of the Bay broke into separate components in a similar way to the composite coverage and two closest populations in the two regions became isolated from each other at distances of 5-7 km. These populations are separated by a peninsula of land between the Bush River and Romney Creek (Figure 4.4).

Table 4.2 Number of components at 10 km within each major region or river drainage.

Year	North - Central	Patuxent	Potomac	Rappah-annock	Pamunkey (York)	Mattaponi (York)	James	Components at 10km
1998	1	1	2	1	1	1	2	9
2000	1	1	1	1	1	NA	4	9
2002	3	1	2	1	1	1	4	13
2004	4	1	3	1	1	1	3	14
2006	1	1	2	1	1	1	3	10
2007	1	1	1	2	1	1	4	11
2008	1	1	1	1	1	1	3	9
2009	1	1	1	1	1	1	3	9
2010	1	1	1	1	1	1	2	8
Composite	*5	1	1	2	1	1	1	12

*2 of the 5 components extend south beyond what is observed in individual years with complete coverage

Figure 4.4 Connectivity among patches (edges) at a critical distance of 4.0 km for all years with complete survey data and the composite coverage.

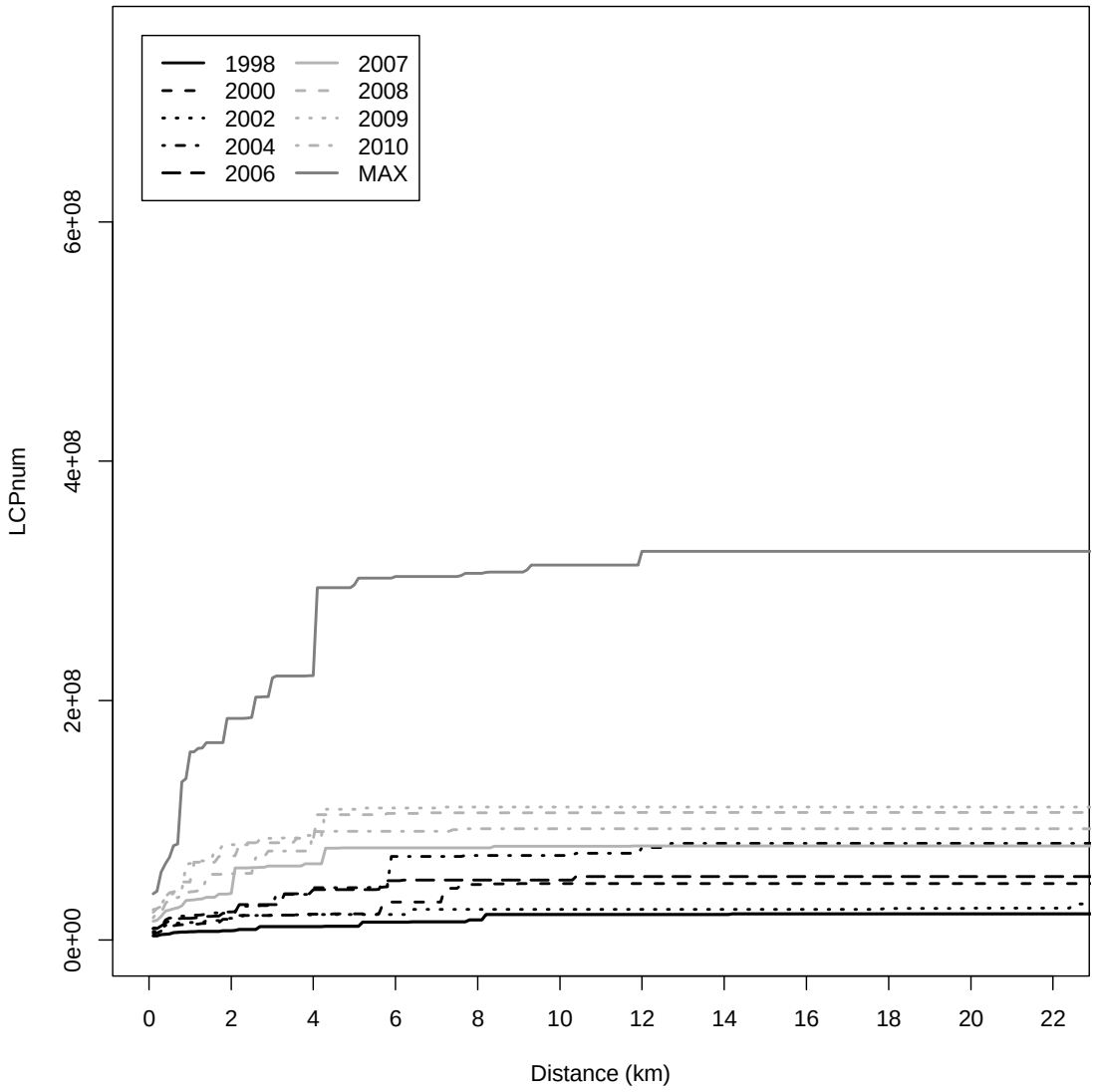

Figure 4.5 Zoomed view of landscape coincidence numerator for each critical distance (km) and coverage.

The values of *dEC(IIC)* across sequential years in relation to *dA* varied slightly across critical distances and pairs of years (Figure 4.6). Increased area tended to conferr increased connectivity as *dEC(IIC)* was greater generally than *dA* for pairs of years. Below 10 km, moving from 1998-2000 fluctuated around no change in connectivity relative to area until ~8km at which point the additional area in 2000 corresponded to an increase in connectivity. Across the same critical distances, the shift in area between 2002-2004, and 2009-2010 did not contribute to additional connectivity. 2008-2009 *dEC(IIC)* relative to *dA* fluctuated around zero change in connectivity across all distances. The change in area from 2004-2006 intially provided additional connectivity, at distances <~12 km provided no additional benefit to connectivity.

When comparing each year with complete survey coverage to the composite coverage, which represents total possible connectivity, the values of *dEC(IIC)* in relation to *dA* varied widely across critical distances and years (Figure 4.7). Below ~1.5 km the composite coverage had greater connectivity for all years as *dEC(IIC)* was always larger than *dA*. Between 1.5 and ~8 km, the additional area in the composite coverage did not increase overall connectivity relative to the 1998, 2000, and 2004 coverage as *dEC(IIC)* was always less than *dA*. At ~8km 2000, and 2004 the difference between *dEC(IIC)* and *dA* became positive. The value of *dEC(IIC)* - *dA* 2006 fluctuated around 0 until ~8 km when it became positive. At 26.9 The value of *dEC(IIC)* - *dA* km 1998 became positive, along with all other years. At a distance of 84.7 km there was an abrupt change in all years, which corresponded to the dramatic increase in connectivity in the composite coverage at that distance. Beyond this distance all values remained negative indicating that *dEC(IIC)* was always less

than *dA* and the additional area did not result in greater connectivity, except for 2004, which dipped negative from 123.7 to 163.4km and 2002, which was negative from 186.6 km to 192.4 km.

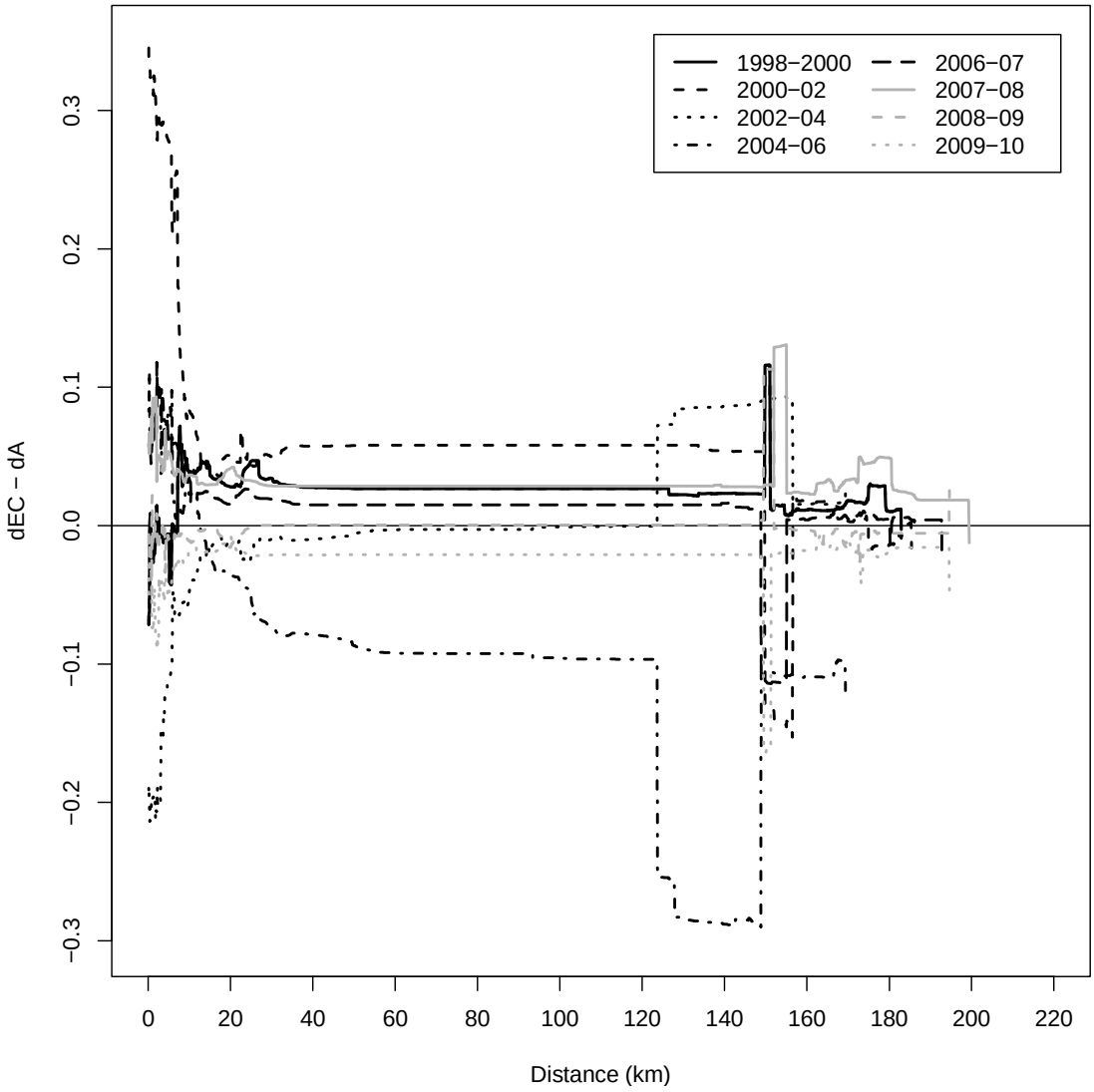

Figure 4.6 Difference in dEC(IIC) and dA for each pair of subsequent years. When dEC(IIC) > dA, the additional habitat area contributes additional connectivity (Saura *et al.* 2011). Conversely, when dEC(IIC) < dA the additional habitat represents isolated patches and makes only a modest contribution to increased habitat connectivity (Saura *et al.* 2011). Finally, when dEC(IIC) = dA the additional habitat area is adjacent to, or overlapping the original habitat area and corresponds to a neutral area gain in connectivity (Saura *et al.* 2011).

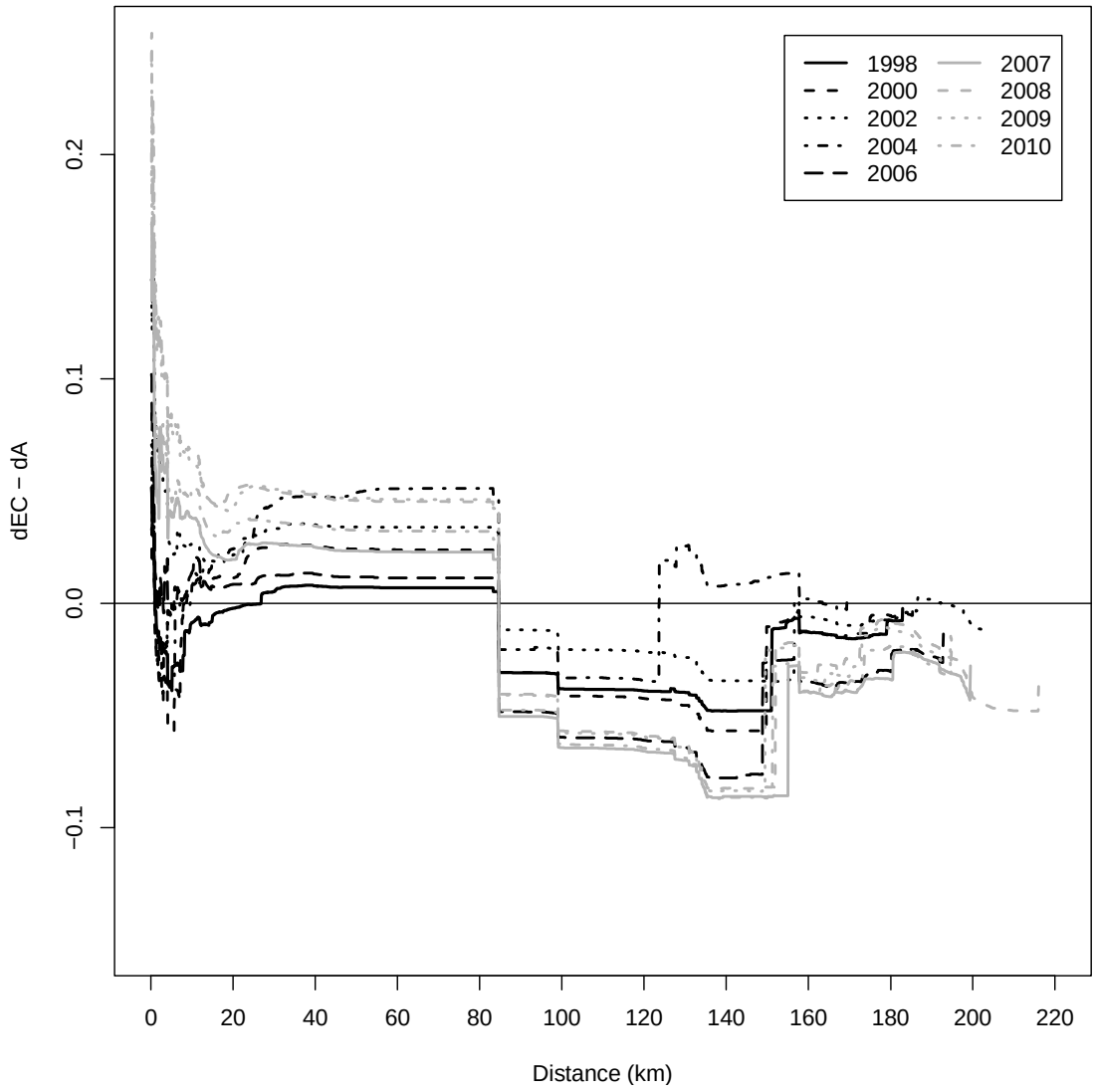

Figure 4.7 Difference in dEC(IIC) and dA for each year compared with the most recent maximal connectivity observed in the composite coverage across all critical distances. When dEC(IIC) > dA, the additional habitat area in the composite coverage has generally been connected to previously existing habitat areas (Saura *et al.* 2011). Conversely, when dEC(IIC) < dA the additional habitat represents isolated patches and makes only a modest contribution to increased habitat connectivity (Saura *et al.* 2011). Finally, when dEC(IIC) = dA the additional habitat area is adjacent to, or overlapping the original habitat area and corresponds to a neutral area gain in connectivity (Saura *et al.* 2011).

Discussion

Our analysis of patch distributions and connectivity yielded three striking results. First was the discrepancy between the amount of SAV in any one-year and the total recently occupied acreage. A second, and related finding is that the vast majority of patches are ephemeral, being observed in only one or two years. The third is the affect this patch turnover has on connectivity metrics from year to year.

Although the overall amount of total SAV in the Chesapeake increased slightly between 1984 and 1993, it has since fluctuated around 30,000 ha (Orth *et al.* 2010a). Lack of further increase has been attributed to continuing poor water quality creating degraded habitat conditions. However, the sum of SAV acreage occupied between 1984 and 2010 was 76,836 ha. Thus, even under the compromised environmental conditions in the Bay, much more acreage SAV has been supported than is occupied at any one time. The inter-annual variation could result from conditions that fluctuate from suitable to unsuitable (e.g., salinity, temperature, turbidity; Carter *et al.* 1994; Rybicki & Landwehr 2007), or the ephemeral locations might have marginal conditions that can be tolerated for short time periods but that do not facilitate persistence. Even within the subset of 27,264 ha considered to potentially be *V. americana* based on salinity and depth, acreage within individual years represented only 26%-56% of acreage in all years combined.

The discrepancy between the patch number and extent within years relative to the possible extent based occupancy between 1984 and 2010 has implications for long-term persistence of the species in the Bay. The average patch age in the composite coverage of 2 years shows that the majority of patches are short-lived and

locations that are occupied in a given year are a shifting mosaic within the larger area of suitable habitat. This pattern indicates that the network is potentially functioning as a metapopulation as classically defined (Levins 1969); however, given that a few patches are always present and many patches are present only one or a few years, source-sink dynamics are also potentially at play (Pulliam 1988). It is possible that small patches remain but are not detectable in aerial photographs and thus patches are more persistent than it appears. On the other hand, some of the sites we consider to be *V. americana* could have been invasive species such as *Hydrilla* and even less optimistic than we assert.

We expected turnover because substantial yearly changes in the local and regional distribution of submersed aquatic species are common (Cristofor *et al.* 2003; Demars & Harper 2005; Lirman *et al.* 2008), and are a function of the dynamic nature of aquatic environments (O'hare *et al.* 2012; Orth *et al.* 2010b; Santos *et al.* 2011; van der Nat *et al.* 2003). Still, the degree to which patches were extirpated was surprising. In addition to outright patch turnover, annual changes include increasing or decreasing size of patches that persist, coalescing of patches, colonization of new areas that do persist, and formation of multiple patches from a single patch. Effective dispersal to colonize or recolonize sites requires the movement of seed or propagules and will become less likely if distance among occupied sites and other suitable sites increases beyond a reasonable dispersal distance.

Although baywide connectivity provides a benchmark for distribution of *V. americana*, it is unreasonable to expect that pollen, seed, or propagules would commonly disperse across the distances required to connect all patches (157.8 km to

216.0 km). In fact, only dispersal of seed by waterfowl has a reasonable potential to span those distances and there is no evidence regarding how commonly such dispersal occurs. At critical distances most relevant to dispersal based on SAV in general and a similar species *Zostera marina*, (< 10 km for seed and < 15 m for pollen; Harwell & Orth 2002; Kendrick *et al.* 2012; Orth *et al.* 2012), we found a high degree of connectivity within river drainages and among patches in the northern and central regions, with the majority of the seven main components either being fully connected or broken into between 1 and 5 components (Table 4.2; Figure 4.3).

The component associated with the Potomac River is consistent with the tidal portion of one of the three primary genetic regions identified by Lloyd et al. (2011). Patches in this tributary were connected at critical distances <7.5 km, and all but several of the more downstream patches were connected until the critical distance was <2.0 km. The two genetic sampling sites associated with the more isolated downstream components show evidence of recent bottlenecks, which could indicate recent colonization or reduction in size associated with this greater isolation. The possibility of recent recolonization is supported by documented dramatic changes in distribution and abundance of *V. americana* in the Potomac River between 1985-2001 as the result of competition with *Hydrilla verticillata*, water clarity, water temperature, and nitrogen levels (Carter *et al.* 1994; Rybicki & Carter 2002; Rybicki & Landwehr 2007).

In the northern and central regions, relationships between potential connectivity based on patch distributions and long-term connectivity indicated by the genetic record are more complex and difficult to interpret. In particular, the amount of

genetic differentiation between the two regions found by Lloyd et al. (2011) is substantial enough to represent long-term lack of gene flow that quite possibly predates declines over the last 100 years. However, this genetic differentiation occurs in an area in which the closest patches are isolated by only 4.5 km in the composite coverage and by 5-7 km in individual years (Figures 4.3 and 4.4). Further, we expect the amount of SAV even in the composite coverage to be less than existed prior to historic declines and thus historic isolation distances could have easily been even shorter. This result would suggest that common dispersal distances are closer to 4.5 km than the 10 km we estimated based on the literature on dispersal. Yet, other patches within each of the central and northern bay regions that are more distant from each other maintain genetic similarities. There are several potential explanations for the discrepancy, none of which can be ruled out or supported with the currently available data.

A peninsula that lies between Bush River and Romney Creek separates the most proximal populations at the boundary between the northern and central regions (Figure 4.5). This peninsula may act as a barrier that keeps the main components of the central and northern Bay disconnected from one another. Additional sampling of *V. americana* patches between the sampled locations at Mariner Point (MP) and Fishing Battery (FB) would be required to better resolve their genetic affinities and verify the nature of the genetic differentiation (e.g., if there is a step cline that indicates a barrier to gene flow or strong selective pressure). A second possibility is that the central Bay represents relictual populations that persisted through the major decline of SAV in the Bay, and the northern Bay populations represent more recent

recolonization from populations in the non-tidal regions of the Susquehanna River. However, within the central Chesapeake Bay region, there are 10 patches that have existed for 15-19 surveys, and 3 small patches that have existed for over 20 surveys. Whereas, there were at least 30 patches in the northern region existed for over 20 surveyed years. Additional evidence against this scenario, is that genetic samples in both the northern and central bay are diverse in genotypes and alleles and no northern Bay populations show evidence of a recent bottleneck (Lloyd et al 2011). This set of characteristics would not be expected for recently colonized sites unless propagule pressure was high and the source of propagules was exceedingly diverse. Thorough sampling of patches of different ages throughout the central and northern Bay regions and in non-tidal portions of the Susquehanna River would provide insight into the sources and timing of patch colonization.

The two southernmost sampling locations in the central Bay (SFP and SCN; Figure 4.1) also show evidence that is consistent with patch isolation affecting genetic diversity. Although they had the highest probability of belonging to the central Bay region, these sites showed affinities to other regions (the Potomac for SFP and the northern for SCN (Lloyd *et al.* 2011). The difficulty in placing these sites within the central Bay grouping indicates either lower levels of gene flow than occurred among other central populations or additional gene flow from other regions. The admixture between regions is consistent with the greater connection distances required to link these sites to other members of the central region. SFP is not connected to the rest of the central region until the critical distance is ~9 km in the composite coverage and the site is absent from the individual years with complete data, which reinforces that 9

km is beyond the distance of regular gene flow. The nearest Potomac region location is over 100 km away, a distance over which dispersal is not likely to occur. Additionally, the affinity of SCN to other northern populations would require gene flow from populations that do not become connected until critical distances reach 24.5 km. Both SFP and SCN had low genotype diversity and SCN showed evidence of a recent bottleneck indicating they might represent recent colonization. Cell age data corroborated this possibility; the first year these locations were mapped was 1996 and 2002 respectively.

Our empirical evaluation also highlights the nature and utility of a range of graph theory metrics. It is well known that multiple metrics are required to fully understand different aspects of connectivity (Laita *et al.* 2011). Of the metrics available, those that incorporate habitat area (or other patch weight) with a measure of graph extensiveness are the most comprehensive and informative (Galpern *et al.* 2011). However, we find that using such metrics to examine patterns of connectivity through time is less than straightforward precisely because they integrate both patch area and isolation. Of the metrics we examined both *LCPnum* and *IICnum*, are a complex interaction between habitat area, patch number, and patch distribution. The rank order of these metrics at each critical distance was directly related to the amount of habitat present in each coverage. As such, using these metrics alone did not provide insights into changes in connectivity independent of area. Equivalent connectivity overcomes this challenge by standardizing the units to area (or other chosen patch weighting e.g., population size, habitat quality). Furthermore, change in equivalent

connectivity [*dEC(IIC)*] allows for a direct comparison among datasets with different amounts of habitat.

Changes in area between sequential years did not necessarily strongly affect connectivity. In pairs in which area increased (e.g., 1998-2000, 2002-2004), versus decreased (e.g., 2000-2002, 2009-2010) there was not a consistent directional effect on connectivity. Rather, the arrangement of habitat following addition or subtraction of habitat area drives the metric values. In 2004 there were habitat patches along the lower central eastern and western shores of the Bay that were not observed in any other year with complete survey coverage. Between 2004 and 2006, *dEC(IIC)* relative to *dA* was strongly negative indicating that loss habitat along lower central eastern and western shores impacted overall connectivity, but only beyond 12 km critical distances. Beyond this distance the differences in patch distribution becomes significant, because their presences provides a bridge between the northern/central region and the Potomac/Patuxent. Whereas, below 12 km, connectivity is not affected within regions. In cases where habitat increased, but was similarly distributed (e.g., from 2006 to 2007), there were only small additional gains to connectivity, as connectivity at shorter distances within regions and at longer distances among regions, was largely unaffected by the fluctuations in patch area. Examining the relationship between *dEC(IIC)* and *dA* provides an assessment of connectivity through time that is difficult to obtain with other connectivity metrics.

Additionally, having all possible habitat occupied does not necessarily confer connectivity benefits above and beyond the benefits of additional area. Below 1.5 km the additional area in the composite coverage always increased connectivity (Figure

4.7) indicating the additional patches were located within this distance of patches within years and provided stepping stones connections between otherwise separate components. Between 1.5 and ~8 km, the added area in the composite coverage had little effect on connectivity in those years where habitat amounts were relatively low (1998, and 2000), and when habitat was more evenly distributed across the landscape (2004). In years where a larger amount of habitat existed, and was compactly distributed (e.g., 2002, 2007-2010), the additional area in the composite coverage served to either link separate components at these larger distances. It is not until beyond 84.8 km that additional benefits to connectivity gained in the composite coverage are negated by the fact that the majority of the landscape in composite coverage is within a few components.

The central dogma of submersed aquatic restoration in the Chesapeake Bay has held that environmental factors are limiting SAV to abundances below restoration goals. If the high turnover is due to lack of persistence in marginal habitat, reductions in turbidity and nutrients could increase the growth of *V. americana* (Rybicki & Carter 2002) such that persistence of colonized sites is improved and direct restoration is less necessary. We have shown that if all sites that have been occupied in the recent past were occupied in a single year, the total amount of SAV coverage would be sufficient to exceed the 2010 restoration goal by 1969.2 ha. Areas suitable for *V. americana* represent ~33% of this acreage and if it were all occupied the occupancy would be up to ~360% of what we find in individual years. Simply this increased acreage would greatly enhance probabilities of persistence and ecosystem service benefits. However, to facilitate movement and increase network connectivity

we recommend that restoration efforts focus on bridging gaps between patches that are less than 4 km apart. Most of the thresholds in connectivity are beyond reasonable dispersal distances for *V. americana* and genetic evidence indicates Baywide no long-term connections between more distant regions of the Bay.

Chapter 5: The Power of Wright's F_{st} and Jost's D to Detect Recent Fragmentation Events

Habitat loss and fragmentation are imminent threats to biological diversity worldwide and thus are fundamental issues in conservation biology. Increased isolation alone has been implicated as a driver of negative impacts in populations associated with fragmented landscapes. Genetic monitoring and the use of measures of genetic divergence have been proposed as means to detect changes in landscape connectivity. Our goal was to evaluate the sensitivity of Wright's F_{st} and Jost's D to recent fragmentation events across a range of population sizes and sampling regimes. We constructed an individual-based model, which used a factorial design to compare effects of varying population size, presence or absence of overlapping generations, and presence or absence of population sub-structuring. Increases in population size, overlapping generations, and population sub-structuring each reduced θ and D_{est_Chao}. The signal of fragmentation was detected within two generations for both θ and D_{est_Chao}. However, the magnitude of the change in each was small in all cases, and when N_e was >100 individuals it was extremely small. Multi-generational sampling and population estimates are required to differentiate the signal of background divergence from changes in θ and D_{est_Chao} associated with fragmentation. Finally, the window during which rapid change in θ and D_{est_Chao} between generations occurs can be small, and if missed would lead to inconclusive results. For these reasons, use of F_{st} or D for detecting and monitoring changes in connectivity is likely to prove difficult in real-world scenarios. We advocate use of genetic monitoring only in conjunction with estimates of actual movement among patches.

Introduction

Habitat loss and fragmentation are considered to be among the most imminent threats to biological diversity worldwide and thus are fundamental issues in conservation biology (Lawler *et al.* 2002; McKinney 2002; Rouget *et al.* 2003; Wilcove *et al.* 1998). Fragmentation is a complex phenomenon that is simultaneously a consequence of habitat loss and a process in and of itself (Fahrig 2003; McGarigal & McComb 1995; Saunders *et al.* 1991). It is a function of the extensiveness of individual patches, distances among those patches (Neel *et al.* 2004; Pascual-Hortal & Saura 2006; Tischendorf & Fahrig 2000a), the nature of the landscape between the patches, and how individual species are affected by each of those aspects (Ricketts 2001). Understanding the joint and independent effects of loss and configuration of the remaining habitat has long been a major focus of landscape ecology and conservation (e.g., Belisle & Clair 2002; Bender *et al.* 1998; Fahrig & Jonsen 1998; Fahrig & Merriam 1985; Trzcinski *et al.* 1999).

Although the two phenomena are intertwined, when they are examined separately habitat loss has repeatedly been shown to have larger detrimental effects than fragmentation alone (Bender *et al.* 2003; Brooks *et al.* 2002; Fahrig 1997, 2002, 2003; McGarigal & McComb 1995). Still, increased isolation has been implicated as a driver of population extinctions (Burkey & Reed 2006), declining population size of interior species (Bender *et al.* 1998; Parker & MacNally 2002), altered social behavior (Cale 2003), reduced population viability (Harrison & Bruna 1999; Patten *et al.* 2005), demographic change in general (Hovel & Lipcius 2001; Jules 1998; Kennedy *et al.* 2010), and spread of invasive species (With 2004). Reduced

migration under lower levels of connectivity will have genetic consequences of reduced effective population size (N_e) and increased rates of inbreeding and genetic drift within newly isolated habitat patches that will affect short- and long-term potential for survival (Frankham 1995a, 1996; Saccheri *et al.* 1998; Westemeier *et al.* 1998).

Changes in landscape composition and configuration associated with the fragmentation process have been quantified and monitored using an extensive array of landscape indices (Gustafson & Parker 1994; Hargis *et al.* 1998; Jaeger 2000; McGarigal *et al.* 2002; Saura & Martinez-Millan 2001; Schumaker 1996; Urban & Keitt 2001). Assessing the consequences of these structural changes for populations and processes fundamentally requires linking these structural attributes of landscape pattern with potential or actual movement of individuals among patches (Collingham & Huntley 2000; Taylor *et al.* 2006; Tischendorf & Fahrig 2000a, b; Urban & Keitt 2001). Movement is often documented using habitat suitability, mark-recapture, radio-telemetry, experimental removal-recolonization studies (Bender *et al.* 2003; Tischendorf & Fahrig 2000b) and demographic monitoring (Bowers & Dooley 1999; Bruna & Oli 2005; Dooley & Bowers 1998). Unfortunately, such studies can be so data- and time-intensive that there may be little practical application for conservation of most species (e.g., Calabrese & Fagan 2004; Urban 2005). Observing physical movement of cryptic or primarily sessile organisms in which mobility is limited to particular life stages is especially challenging (Ellstrand 1992; Wunsch & Richter 1998).

Genetic monitoring, has been proposed as a minimally invasive, relatively cost-effective solution for providing such understanding (Schwartz *et al.* 2007) by quantifying genetic effects of changes in landscape structure in patches of remnant habitat or documenting movement of individuals (Kendall *et al.* 2009). Population genetic parameters may be more sensitive for detecting changes in fragmentation and connectivity than traditional demographic estimates that have large error components (Ims & Andreassen 1999). Thus, although in many cases conservation biologists are concerned about genetic diversity for its own sake, here we are interested in the potential for using genetic changes that result from fragmentation to quantify changes in the ecological process of movement.

Direct genetic methods have been developed to detect actual dispersal events (Robledo-Arnuncio *et al.* 2006; Smouse *et al.* 2001; Sork *et al.* 1999), for example pollen and seed dispersal in plants (Coates & Atkins 2001; Coates *et al.* 2003; Diniz-Filho & De Campos Telles 2002; Dyer & Nason 2004). However, still the most commonly used approach to document fragmentation is to use indirect methods to quantify the amount of divergence in populations in putatively fragmented habitat [e.g., Wright's F_{st} (1951) and its analogues (Schwartz *et al.* 2007)]. Even with development of potentially more powerful methods (Kingman 1982a, b; Pearse & Crandall 2004; Slatkin 1991), many investigators continue to use indirect measures to assess functional connectivity among populations (Hall *et al.* 1996; Hanfling *et al.* 2004; Krauss *et al.* 2004; Li *et al.* 2008; Matern *et al.* 2009; Meldgaard *et al.* 2003; Meyer *et al.* 2009; Wallace 2002; Young *et al.* 1999). Recent simulation studies have indicated that N_e estimators based on linkage disequilibrium (England *et al.* 2010)

may be more sensitive at detecting recent isolation in discrete populations and Mantel's r may be more sensitive in continuous populations as long as populations support <500 individuals, generations are not overlapping, and samples of individuals and loci are moderately large (>100 individuals and 30 loci; Landguth *et al.* 2010).

Despite its fundamental importance and strong theoretical foundations, detecting fragmentation effects in the wild has not been as straightforward as one might expect. Attempts to link indices of landscape structure to ecological and evolutionary processes have not yielded consistent relationships and many empirical investigations of fragmentation fail to detect definitive effects (Wiegand *et al.* 1999; Wiens *et al.* 1993; Young *et al.* 1996). In particular, empirical data are often equivocal relative to predictions of the impacts of fragmentation on genetic divergence. There are several potential causes of the lack of consistent connection including: non-monotonic relationships between many landscape metrics and landscape configuration (Neel *et al.* 2004) or non-linear or threshold-like population responses along the fragmentation gradient. Additionally, as mentioned above, the point at which discrete patches are actually fragmented depends on the scale at which a species perceives and interacts with the landscape (Crooks & Sanjayan 2006; Holland *et al.* 2004; Levin 1992). For species in patchy habitats, connectivity ultimately depends on the degree to which land cover types between discrete patches are barriers, versus filters, versus easily traversable, which is lacking for most species. Because not all habitat that is perceived as fragmented by humans is actually fragmented from the perspective of a species of interest, some investigations may be trying to quantify effects of fragmentation where it actually does not exist. Moreover, even if movement through

a landscape is impeded or precluded, long-lived individuals pre-date the fragmentation event and provide a genetic signature of connectivity that no longer exists (Young *et al.* 1996). These issues can be addressed through careful study design in which temporal and spatial sampling scales match potential scales of fragmentation based on the biology of the focal organism.

Of greater concern is the potential that characteristics of F_{st}-related values might make them insufficient for detecting habitat fragmentation on time scales that are relevant for conservation management. Because F_{st} integrates over evolutionary time it is difficult to separate current from historical processes based on a single estimate of pattern alone and it may be slow to reflect changes in migration following a fragmentation event, especially if N_e remains large. Additionally, the alleles that are most likely to be lost through drift are at low frequencies in populations and these alleles contribute little to F_{st} values. Slow response may also arise from the fact that, when connectivity is only reduced rather than eliminated entirely, estimates of F_{st} may remain close to zero (Neigel 2002). Finally, measures of genetic divergence (e.g., F_{st}, G_{st}, Φ_{st}) can be depressed when within-subpopulation heterozygosity or variance is high relative to among-subpopulation heterozygosity or variance which is common in highly diverse marker systems (e.g., microsatellites; Gerlach *et al.* 2010; Hedrick 2005; Jost 2008; Meirmans 2006; Meirmans & Hedrick 2011). F_{st} related measures calculated from such data will never approach unity regardless of the underlying patterns of allelic diversity and do not behave monotonically. Jost (2008) proposed a measure of genetic divergence based on allelic diversity (D) that removes these biases and varies between 0 and 1 regardless of within-population

heterozygosity. Heller and Siegismund (2009) found that values of Jost's *D* in 34 published studies were roughly 60 times greater than G_{st}. The increased magnitude and potential range of values may provide greater ability to detect recent fragmentation events. Additionally, *D* could be more sensitive because it is calculated based on number of alleles, which will be lost at a much higher rate than heterozygosity (Allendorf 1986).

Because we were interested in effects of fragmentation independent of habitat loss, we evaluated the ability to detect genetic effects of fragmentation with F_{st} and *D* over time frames associated with anthropogenic habitat modification (i.e., <200 generations) while controlling for population size. We chose to exclude G'_{st} because it is built on the same underlying assumptions as F_{st} and can be misleading when mutation rates are high (Jost 2009; Ryman & Leimar 2009). The number of generations necessary to make such an evaluation renders the task infeasible in a field setting. Therefore, we developed an individual-based population model to simulate genetic divergence among recently fragmented populations and measured F_{st} and *D* over time. Potential for detecting change in these metrics will vary based on the amount and nature of migration among populations; therefore, we simulated two severe cases of fragmentation. In the first, migration among a set of historically panmictic populations was abruptly and completely stopped. In the second, limited gene flow among populations was allowed and subsequently ceased. The first scenario provides the most ideal situation for detecting change from a base condition of a population at panmixia to complete isolation. The second provides a more realistic starting condition in which there is a pre-existing level of divergence among

populations onto which anthropogenic fragmentation is imposed. We complement a recent investigation of the effect of dispersal distance among individuals on the time required to detect an abrupt barrier to gene flow (Landguth *et al.* 2010) by examining multiple discrete populations and by quantifying the influence of population size, overlapping generations, and sampling effort in terms of individuals and loci on ability to detect a significant change in F_{st} and Jost's D.

Methods

Using a model we wrote in Perl script, we generated six homogenous panmictic populations of equal size at the start of each run. Panmixia among populations was created by allowing mating at random among individuals in all populations. The model allows variation in distances among individual population pairs but for the purposes of this evaluation all populations were equally isolated at an arbitrary distance of 1 km. Census size maxima (N_{max}) within populations were set to 25, 75, 100, 500, 1000, and 3000 individuals (N_e was subsequently calculated) which encompasses the size ranges of populations of most plant species listed under the U.S. Endangered Species Act (Neel unpublished data) and 71% of minimum viable population estimates for plant species world wide (Traill *et al.* 2007). Initial size of each population was set to 75% of the size limit for each run and the size cap was reached within one or two generations due to the population growth parameters discussed below.

At initiation, individuals were assigned two alleles at each of 20 unlinked microsatellite loci. Allele size ranged between 5 and 50 repeat units. Alleles for each locus could take on any value within the given range, and were drawn from a normal

distribution with parameters μ = mean of the size range of the locus and $\sigma^2 = 5$. Drawing initial allele frequencies from a normal distribution allows for accurate simulation of the stepwise mutational model of microsatellite evolution throughout a simulation (Whittaker *et al.* 2003). These starting conditions yielded between 7 and 40 alleles per locus at the start of each simulation depending on the population size. The initial proportion of heterozygous individuals was arbitrarily set to 0.50 for all loci, although varying the initial proportion of heterozygous individuals between 0.1 and 0.9 did not influence final results (data not shown). Mutations occurred every 0.004 gamete transfer events (Whittaker *et al.* 2003). By using a stepwise mutational model of microsatellite evolution, changes follows a normal distribution ($\mu = 0$; $\sigma^2 = 3$) in allelic state were smaller changes are more likely than larger changes, and the direction of mutation tended toward the mean size range of each locus (Whittaker *et al.* 2003).

Individuals were simulated to be hermaphroditic, annual plants that were self-compatible, but that did not self-fertilize more than what would be expected at random, and therefore the amount of selfing depended upon population size. All individuals had an equal probability of mating each generation. Individuals from within a population had an equal probability of being a father for all individuals within that population. The proportion of individuals contributing seed to the next generation varied around a normal distribution with the parameters μ = 50% total population size and $\sigma^2 = 1$. The number of seeds produced per female was drawn from a normal distribution with parameters $\mu = 35$ and $\sigma^2 = 5$ to provide stochastic variation around a likely number of seeds per plant. Each seed had a randomly

selected father. When a seed bank was included in the model, those seeds not germinating entered the seed bank; otherwise, seeds that did not germinate immediately were removed. Germination potential of seeds in the seed bank decreased over time for five generations following a negative function (germination rate $t_2 = 6\%$, $t_3 = 4\%$, $t_4 = 2\%$, $t_5 = 1\%$, $t_6 = 0.5\%$). As the size of each population approached the population size limit, the number of viable seeds produced was reduced to reflect density dependence (Silander & Pacala 1985).

Each cap size was run under four conditions that independently varied presence or absence of a seed bank (i.e., non-overlapping versus overlapping generations) and presence or absence of preexisting population structure prior to population isolation. To simulate absence of population structure, panmictic populations (i.e., those without prior substructure) were immediately isolated to yield an abrupt fragmentation event with the highest likelihood of being detected. In a second more realistic scenario, we simulated preexisting population structure by limited seed and pollen migration as described below for 500 generations prior to stopping all migration.

At least 85% of pollen grains remained within a population and 15% had some probability of moving. If part of the 15% of pollen grains did not disperse, they remained within the source population. Probability of dispersal from a population followed a Laplace distribution ($\mu = 0.4$, $b = 0$), sites were set at an arbitrary distance of 1 km apart. The Laplace distribution is a commonly used dispersal kernel for plants that reflects a range of common dispersal syndromes (Bullock & Moy 2004; Bullock *et al.* 2003; Bullock *et al.* 2006; Neubert & Caswell 2000). Seeds produced

from matings within populations could either stay within the population in which they were generated or they could disperse. Probability of dispersal followed the same dispersal kernel described above. After the dispersal step, seeds had a 10% chance of germinating the year after they were produced and their ultimate fate depended on whether or not generations overlapped. Although the specific values for seed production, seed germination, and pollen and seed dispersal were arbitrary, they were within the range of values that have been documented for plant species (Fox *et al.* 2006; Kahmen & Poschlod 2008; Kalamees & Zobel 1997; Kelly 1989; Schiller *et al.* 2000; Weekley *et al.* 2007; Zammit & Zedler 1990).

Simulations with preexisting population structure ran under the above conditions for 500 generations prior to complete isolation, those that began from panmixia were immediately isolated. Following isolation in both simulation types, the model proceeded for 200 additional generations with no migration among the 6 populations. We conducted 200 independent simulations for each of the four conditions for each of the six population size caps, yielding 24 model configurations. The resulting 4,800 independent simulations were run on The Lattice Project, a Grid computing system (Bazinet & Cummings 2008; Bazinet *et al.* 2007; Myers *et al.* 2008; Myers & Cummings 2003).

During simulations, individual populations were allowed to go extinct and to be recolonized with migrants from other populations (when migration was allowed) or from the seed bank (when overlapping generations were present). This process was stochastic and resulted from the lack of individual replacement at smaller population sizes. At small population sizes, individual populations would frequently go extinct.

When all populations went extinct, the simulation was restarted. However, extinction of all six populations occurred in only ~1/100 cases. We determined the total number of alleles, observed (H_o) and expected (H_e) heterozygosity at each generation.

In simulations without overlapping generations, we calculated the inbreeding N_e at each generation as $Ne = \frac{N(\bar{k})-1}{\bar{k}-1+\frac{V_k}{\bar{k}}}$ where $\bar{k}$ is the mean number of progeny and V_k is the variance in the number of progeny at each generation (Kimura & Crow 1963). In simulations with overlapping generations, N_e was calculated as $N_e = T(N_b)$ where T is generation time defined as the average age of parents including dormancy (Nunney 2002) calculated following Vitalis *et al*. (2004) and N_b is the effective number of breeders in a given year (Waples 2002). Effective population size for each population, and for each run was calculated as the harmonic mean across all generations and then averaged across simulation runs.

Population divergence was quantified using Weir and Cockerham's (1984) unbiased estimate θ and using Jost's D (Jost 2008) using the estimator D_{est_Chao} following Chao et al. (2008). We estimated θ and D_{est_Chao} from the total number of individuals using all 20 loci at each generation to provide the census or "true" estimate of θ and D_{est_Chao} for comparison with the subsamples of individuals and loci discussed below.

We used a permutation test to assess whether each estimated θ was significantly different from 0, assuming individuals were members of a global population and then randomly reallocated to populations while maintaining sample sizes at the realized values, and recalculating θ (Excoffier *et al.* 1992). The actual value for each run was

compared with the distribution of 2000 such randomizations to obtain a p-value. Significance of D_{est_Chao} was assessed using the bootstrap method described by Chao et al. (2008). The number of generations after population isolation at which θ and D_{est_Chao} became significantly different from values at the last time-step with gene flow was tested using a one-way Dunnet multiple mean comparison test in SAS (SAS Institute Inc., Cary, NC). The magnitude and rate of change between consecutive generations was calculated for the first 24 generations following fragmentation for all simulations. To determine the power to detect differences we calculated the proportion of metric values from each run, at each generation that were significantly different from 0.

We sampled factorial combinations of 10, 15, and 20 loci, and 20, 30, and 50 individuals (as allowed by total maximum population sizes) at every generation over the course of each simulation run. To evaluate the effect of sample size on potential to detect fragmentation, we compared estimates of θ and D_{est_Chao} calculated for all factorial combinations of individuals and loci to the corresponding census value using a Tukey multiple comparison test in SAS v9.1 (SAS Institute Inc., Cary, NC). In addition, we tested estimates of θ and D_{est_Chao} from all factorial combinations for significant departure from 0 using the methods described above.

Results

All individuals and loci

As expected, the number of alleles, H_o and H_e tended to be higher through time in larger populations (Figure 5.1). Model runs with overlapping and non-overlapping

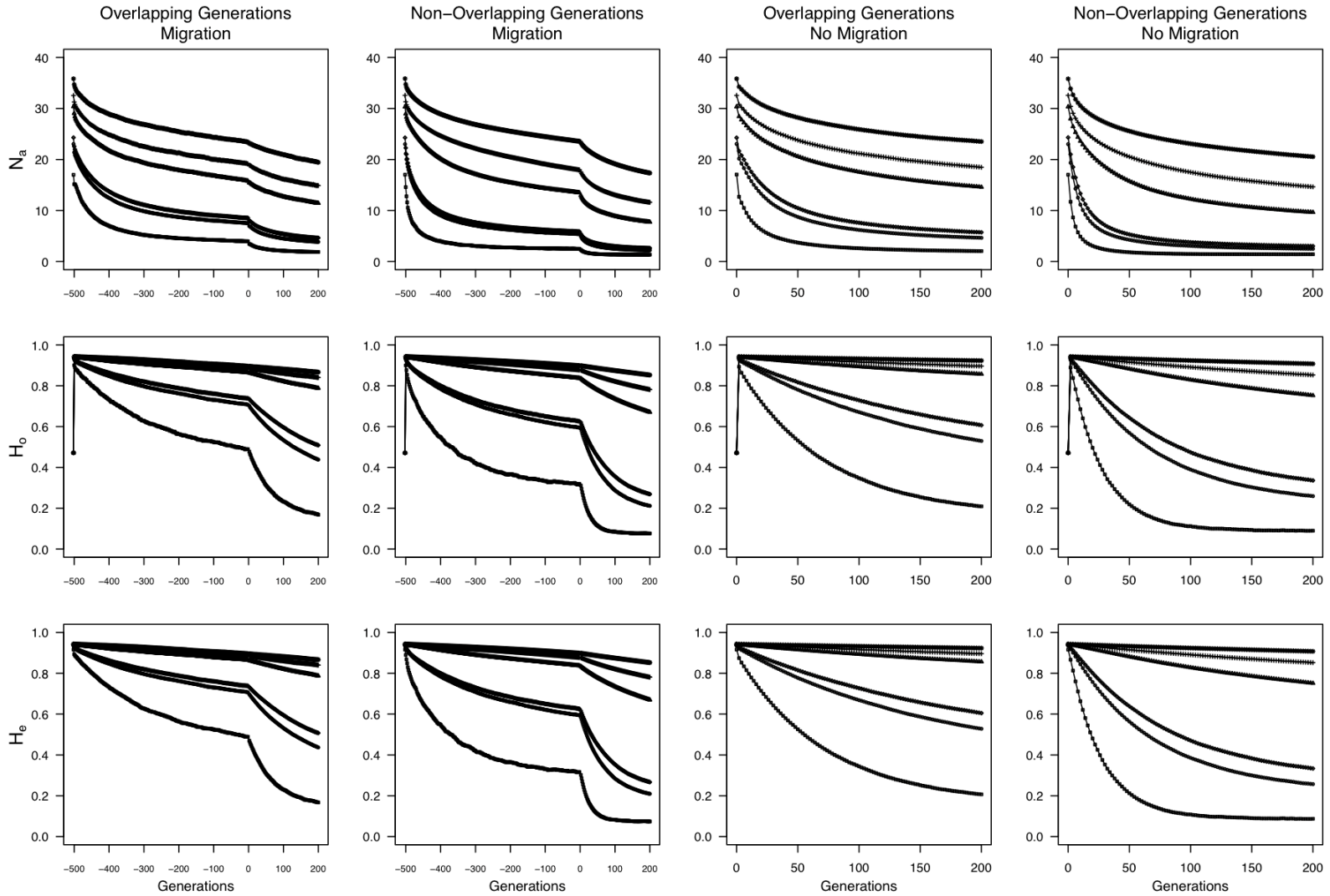

Figure 5.1 Values of N_a, H_o, and H_e for 20 loci and all individuals across all simulation conditions. Lines from top to bottom represent the $N_{\max}$'s of 3000, 1000, 500, 100, 75, and 25 individuals.

generations yielded similar average allelic diversity for any given N_{max} (2 – 36 alleles per locus). However, model runs with overlapping generations tended to yield higher average H_o and H_e through time than did runs with non-overlapping generations, and differences were more pronounced at smaller population cap sizes (Figure 5.1).

In the absence of overlapping generations, the harmonic mean values of N_e estimates for each of the six subpopulations based on all individuals averaged over all runs were 13, 40, 52, 265, 531, 1601 individuals which was roughly half the actual N_{max} values of 25, 75, 100, 500, 1000, and 3000, respectively. With overlapping generations, the harmonic mean of N_e estimates for each subpopulation averaged over all runs was roughly twice the N_{max}: 43, 143, 193, 975, 1994, 5994 individuals, respectively.

As expected from theory, behavior of θ and D_{est_Chao} at a given time point depended on three factors: the maximum population size, presence or absence of overlapping generations, and presence or absence of population sub-structuring prior to fragmentation. Smaller maximum population sizes predictably yielded larger θ and D_{est_Chao} values for any given time step (Figure 5.2 & 5.3). For a given maximum population size θ and D_{est_Chao} were lower in simulations with overlapping generations than those without (Figure 5.2 & 5.3). In simulations with population sub-structuring prior to fragmentation, θ values followed similar trajectories to those in which isolation occurred immediately after a period of panmixia (Figure 5.2). D_{est_Chao} values after isolation were lower when prior population sub-structuring was included (Figure 5.3).

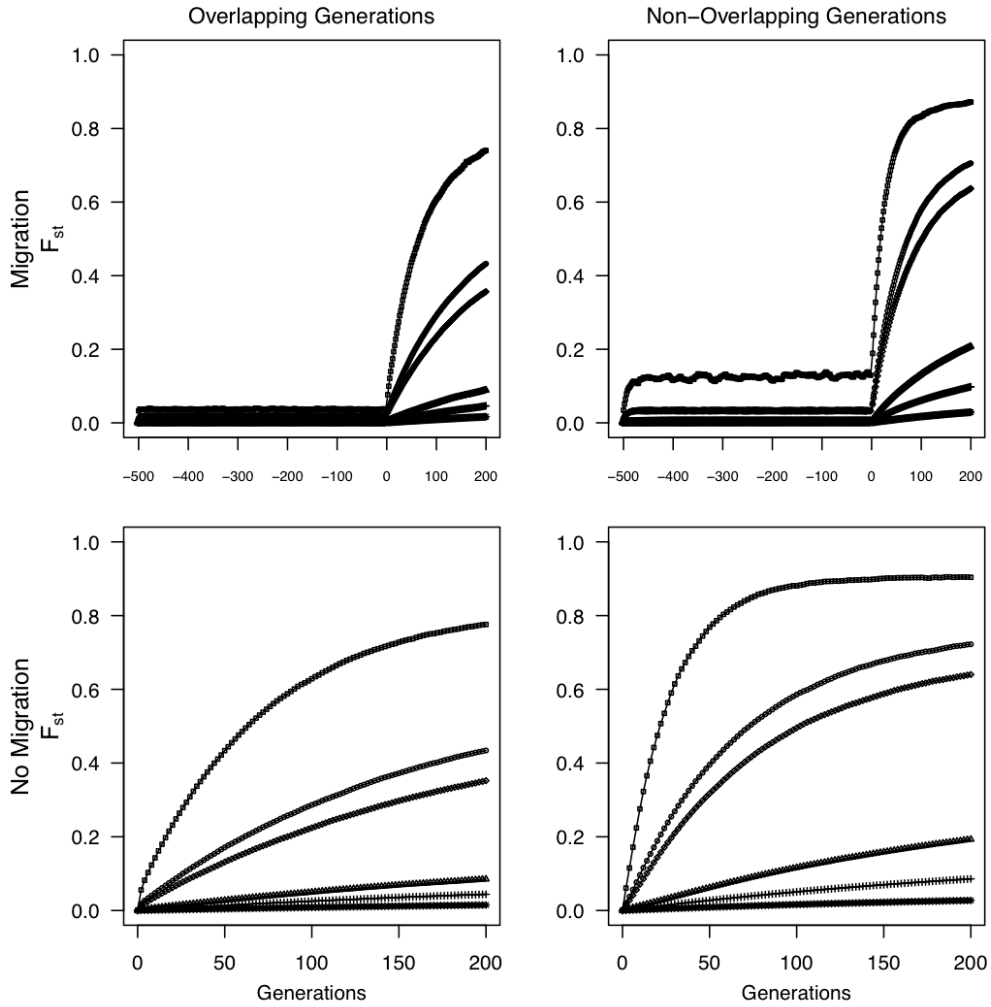

Figure 5.2 Change in average θ calculated from all individuals through time for all N_{max} sizes. Negative generations indicate generations with migration prior to the fragmentation event. Lines from bottom to top represent the N_{max}'s of 3000, 1000, 500, 100, 75, and 25 individuals.

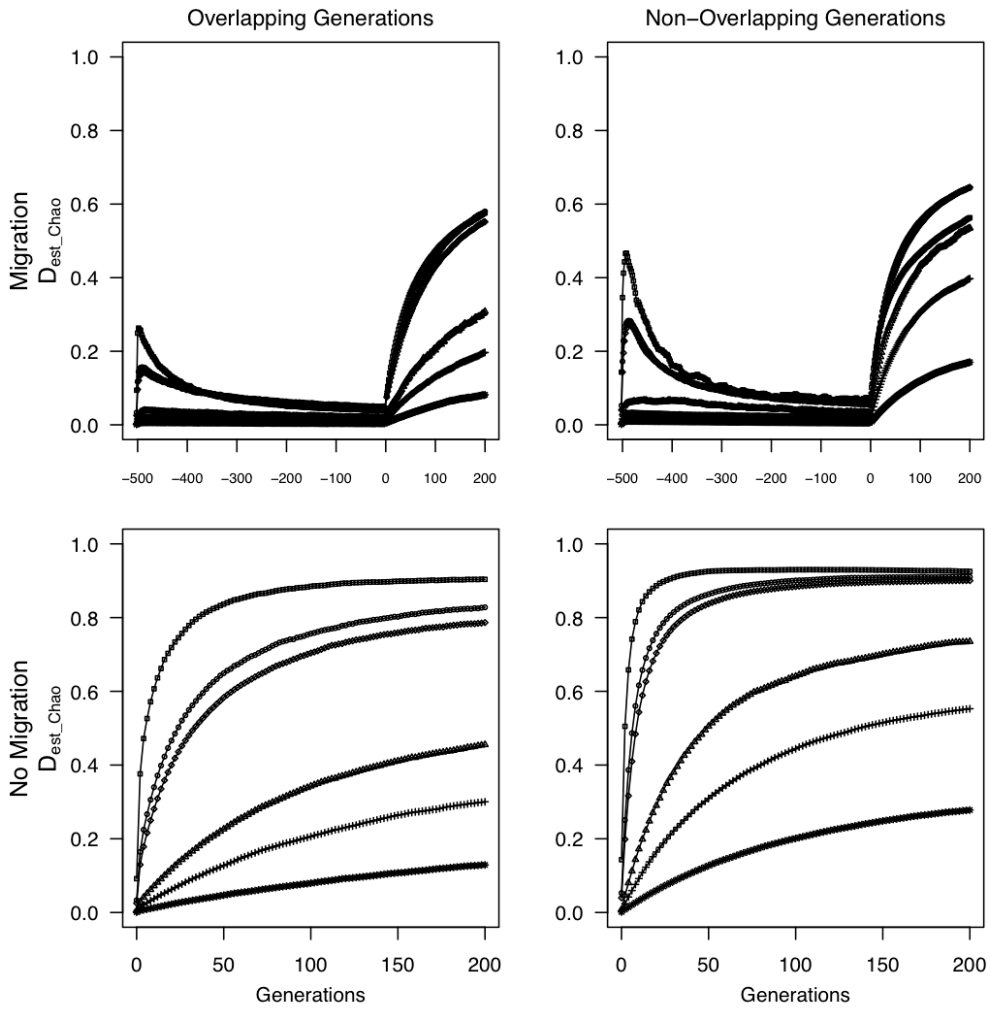

Figure 5.3 Change in average *D* calculated from all individuals through time for all N_{max} sizes. Negative generations indicate generations with migration prior to the fragmentation event. Lines from bottom to top represent the N_{max}'s of 3000, 1000, 500, 100, 75, and 25 individuals.

Across all simulations, values of D_{est_Chao} were generally larger than θ under the same conditions. One exception was that when population sub-structuring preceded fragmentation the magnitude of D_{est_Chao} was initially lower than θ for $N_{max} = 25$, and after 200 generations of isolation. We found two additional anomalies: a small peak in D_{est_Chao} existed at the start of simulations that included migration when maximum population sizes were ≤100 individuals (Figure 5.3), and $N_{max} = 25$ with non-overlapping generations and population sub-structuring, had a shallower rate of increase than the $N_{max} = 75$ and $N_{max} = 100$ under the same conditions.

An asymptote in θ and D_{est_Chao} values is expected as mutation-drift equilibrium is reached (Hedrick 2005; Jost 2008). For θ, this asymptote was not reached during the 200 generations in any case when generations overlapped (i.e., with or without migration; Figure 5.2). For simulations with non-overlapping generations and limited migration prior to fragmentation, θ and D values reached equilibrium after 60 generations when N_{max} was 25 individuals. By the 200th generation, when N_{max} was 75 or 100 individuals, θ had reached an asymoptote (Figure 5.2). D_{est_Chao} was just approaching equilibrium at these N_{max} values by the 200th generation (Figure 5.3). When prior population sub-structuring was included (with or without overlapping generations) D_{est_Chao} did not reach equilibrium at any population cap size. For population sizes >500 individuals, there was no asymptote in θ or D_{est_Chao} values within time scales that would affect monitoring of anthropogenic effects, regardless of the simulation conditions.

When calculated using all loci and individuals, it took two generations after cessation of gene flow for θ to become significantly different from zero in runs

starting from panmixia and from the final time step with migration in the runs with pre-existing structure (Table 5.1). For the four combinations of pre-existing structure versus panmixia and overlapping versus non-overlapping generations, the magnitude of θ, when it became significant following the fragmentation event, was between 3.68×10^{-4} and 0.060 (Table 5.1). Regardless of the simulated conditions, the absolute magnitude of change in θ between generations was exceedingly small ($< 1.0 \times 10^{-3}$) for population cap sizes >500 (Table 5.1).

Estimates of D_{est_Chao} also took only two generations following the fragmentation event to become significantly different from zero. The magnitude of D_{est_Chao} at two generations post-fragmentation was between 1.17 and 12 times the analogous θ values in all cases except when $N_{max} = 25$ and prior structure was present without overlapping generations (Table 5.1). In this single scenario the magnitude of D_{est_Chao} was half that of θ.

The magnitude of change in θ and D_{est_Chao} between generations in the scenario with highest likelihood of detection (i.e., no overlap in generations and isolation occurred from panmixia) decreased sharply following the initial ten generations after isolation (Figure 5.4). In the worst-case scenario for detecting change (overlap in generations and isolation from population sub-structure), the decline in magnitude was less pronounced across generations; however, the average change between generations never exceeded 0.042 for either θ or D_{est_Chao} (Figure 5.4). The magnitude of change in both parameters across generations was a function of the maximum population size and time since isolation. At all time points, the magnitude of change dramatically decreased as N_{max} increased, and as the asymptote was

approached and passed for all N_{max} values (Figure 5.4D). Results for the two remaining cases, 1) generations overlapped and isolation occurred from panmixia and 2) generations did not overlap and prior population structure was included were intermediate to the presented cases (data not shown).

The rate of change in θ and D_{est_Chao} between generations was consistent with the magnitude of change in those same parameters and was consistent across all maximum population sizes. The rate of change was lowest in the worst-case scenario (overlap in generations and isolation from population sub-structure) with an average of 23.8% change in θ and 23.1% in D_{est_Chao} from generation 2-4. In contrast, in the best case scenario the rate of change in D_{est_Chao} from generation 2-4 was 83.6% for θ and 62.0%, respectively. In this best case, near the asymptote (generation 30), the rate of change decreased to ~ 0.2% and beyond the asymptote to ~ 0.01%. In comparison to θ, D_{est_Chao} had either a slightly slower or equivalent initial rate of change; but because, the magnitude change over the same time period was substantially greater for D_{est_Chao} than θ it could be easier to detect a change.

Estimates from samples

Values of θ and D_{est_Chao} calculated from samples taken at each time point were statistically indistinguishable from the census estimate at all time points sampled, across all simulation conditions (Tukey multiple comparison test not shown). Thus, the samples are unbiased and accurate estimates of the census values.

Table 5.1 Difference in mean θ and D values between the final migration step and 2 generations following cessation of migration for 200 runs under each set of simulation conditions. We provide results for two generations because this was the point at which there was a significant difference from the last time step with migration. All differences were significant at $P > 0.05$.

Overlapping Generations With Migration			Non-Overlapping Generations With Migration		
N_{max}	Magnitude of Difference in θ	Magnitude of Difference in D	N_{max}	Magnitude of Difference in θ	Magnitude of Difference in D
25	0.04005	0.04220	25	0.05930	0.03330
75	0.01370	0.03064	75	0.02019	0.03094
100	0.01038	0.02696	100	0.01751	0.02939
500	0.00215	0.01004	500	0.00366	0.02010
1000	0.00105	0.00567	1000	0.00158	0.01067
3000	0.00037	0.00221	3000	0.00039	0.00324
From Panmixia			From Panmixia		
*N*max	Magnitude of Difference in θ	Magnitude of Difference in D	N_{max}	Magnitude of Difference in θ	Magnitude of Difference in D
25	0.05592	0.28437	25	0.06080	0.36174
75	0.01828	0.13102	75	0.02003	0.19777
100	0.01377	0.10443	100	0.01484	0.15884
500	0.00272	0.02368	500	0.00295	0.03858
1000	0.00136	0.01184	1000	0.00147	0.01981
3000	0.00045	0.00394	3000	0.00049	0.00672

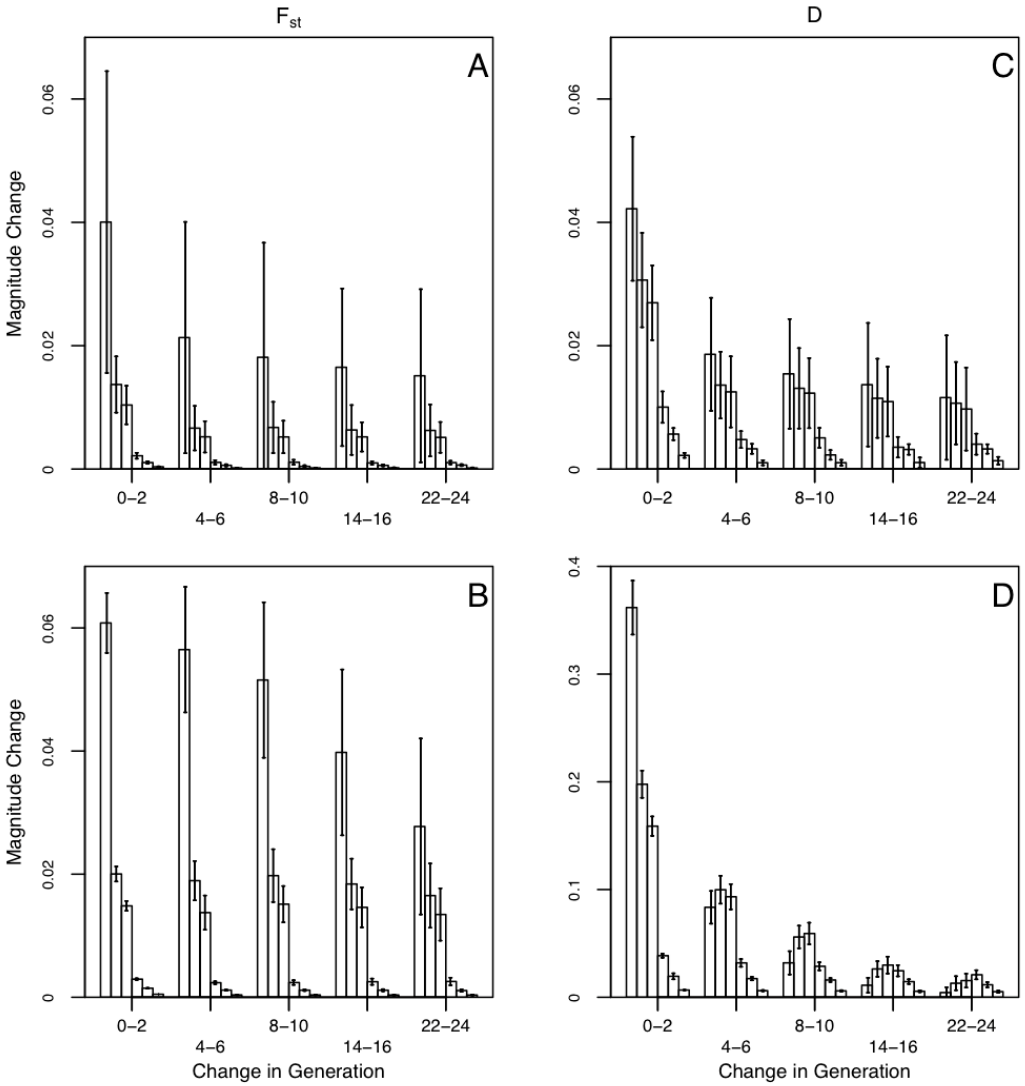

Figure 5.4 Magnitude of change between consecutive sets of two-generations over the first 24 generations following termination of migration. Bars from left to right are N_{max}'s = 25, 75, 500 and 3000 with standard error. Note the different scale in figure 4D. Figure 4A & 4C overlapping generations within migration. Figure 4B & 4D non-overlapping generations from panmixia.

In addition to being related to maximum population size, the ability to detect a significant difference between each sampled θ and panmixia or the last generation with migration was a function of the number of individuals sampled and number of loci sampled. All samples drawn from simulations that had population sub-structuring prior to complete isolation had θ and Jost's D estimates that were significantly different from zero by generation 2. When starting from panmixia, θ values were significantly different from 0 and from the last time point with migration in 100% of replicates at generation 2 only when N_{max}<500 (Figure 5.5). When sampling 20 individuals and 10 loci with overlapping generations and isolation occurred from panmixia, 52 generations were required before 100% of samples were significantly different from 0 at $N_{\max}$ = 3000. In the same conditions 12 generations were required when $N_{\max}$=1000 and 8 generations were required when N_{ma} =500 (Figure 5.5). When generations did not overlap the time required to obtain 100% significant replicates was reduced (Figure 5.5). The time required to detect a θ value greater than zero decreased with either larger numbers of individuals or numbers of loci (Figure 5.6). The addition of 10 sampled loci provided an equivalent gain, to that provided by addition of 10-20 sampled individuals (Table 5.2).

All sampled $D_{\mathrm{est\_Chao}}$ values for all simulation conditions were significantly different from zero at two generations post fragmentation and from the last time step with migration for all maximum population sizes. Furthermore, there was no significant difference among the factorial combinations of the number of sampled individuals and sampled loci across all simulation conditions (not shown).

Table 5.2 Percentage of 200 replicate runs that yielded significant θ values 2 generations after the cessation of migration for all factorial combinations of sampled individuals and loci. For overlapping generations and non-overlapping generations where fragmentation occurs at panmixia

N_{max}	Number Sampled Individuals	Number Sampled Loci			Number Sampled Individuals	Number Sampled Loci		
		20	30	50		20	30	50
500	10	64.5	87	100	10	68.5	95.5	99.5
	15	78	98.5	100	15	81.5	98.5	100
	20	85.5	97.5	100	20	92	99	100
1000	10	28.5	44	82	10	33.5	60	85.5
	15	39.5	56.5	95	15	36.5	65.5	95.5
	20	45	74	95	20	45	77.5	97
3000	10	6.5	16.5	25	10	9	16.5	22.5
	15	11.5	18	29	15	10	19.5	35
	20	16	20	34	20	15	20	37

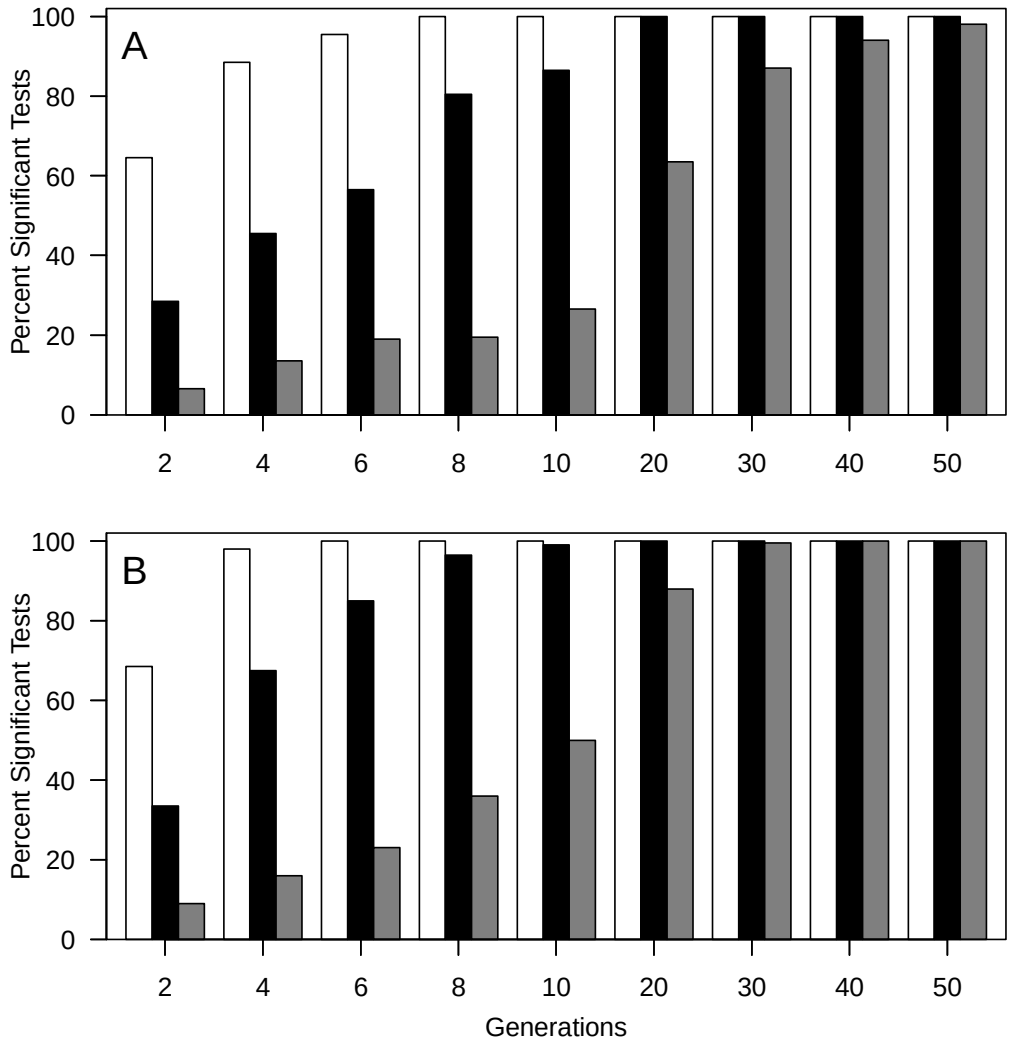

Figure 5.5 Percentage of 200 replicate runs that yielded significant θ values beginning at two generations after the cessation of migration from panmixia for 20 sampled individuals and 10 sampled loci in populations with 5A overlapping generations and 5B non-overlapping generations. Open bars N_{max} = 500, closed bars N_{max} = 1000, gray bars N_{max} = 3000.

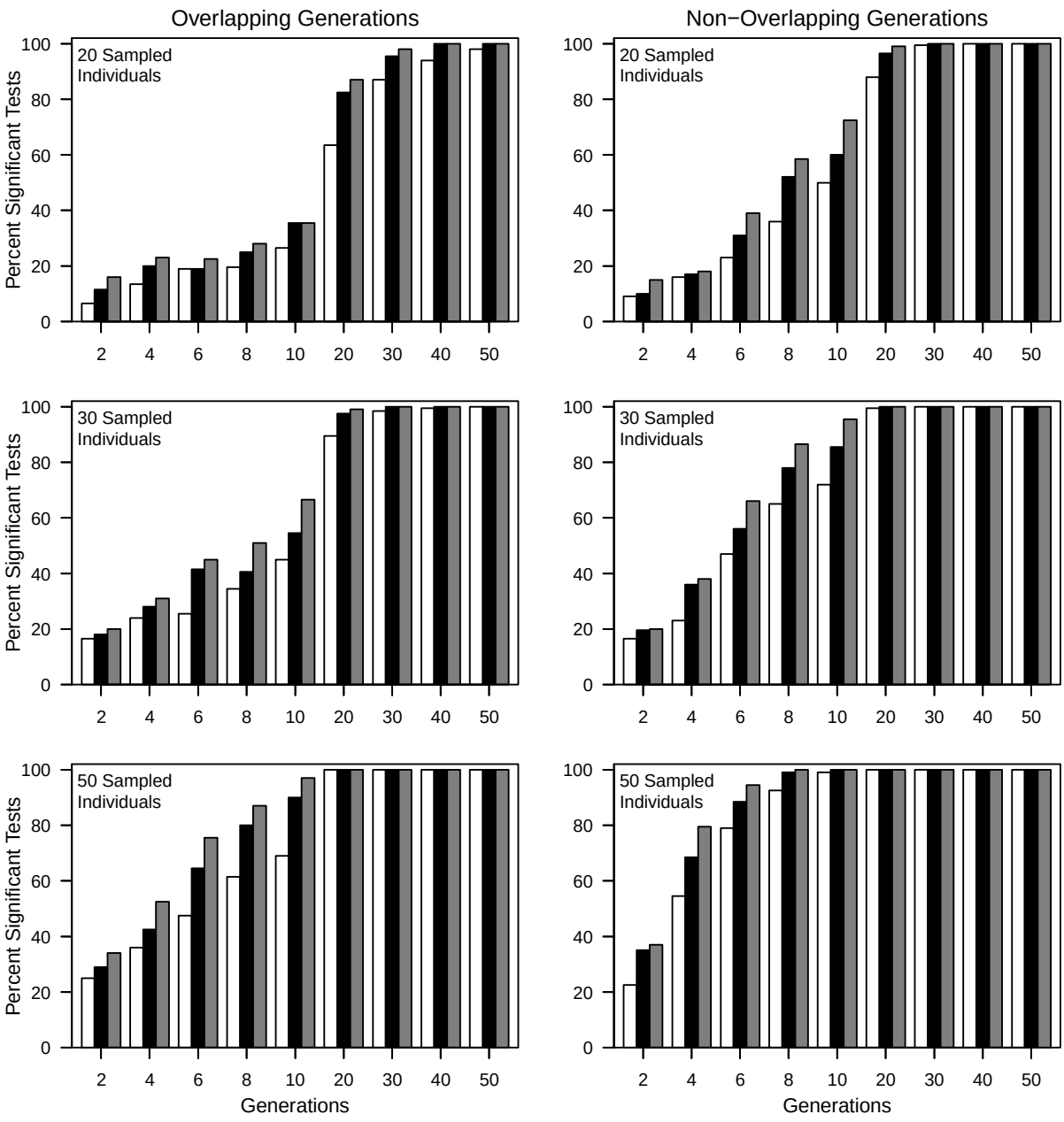

Figure 5.6 Effect of number of individuals (20, 30 and 50), number of loci (10, 15, 20), and overlapping versus non-overlapping generations on the percentage of the 200 replicate runs that yielded significant θ values 2 to 50 generations after cessation of migration for N_{max} = 3000. Closed bars 10 loci, open bars 15 loci, grey bars 20 loci.

Discussion

Ideally, detecting changes in connectivity will provide early warning that biologically relevant habitat fragmentation has occurred so management action can be taken before the consequences become irreversible (Procaccini *et al.* 2007; Schwartz *et al.* 2007). The potential utility of indirect genetic methods for this purpose relies on a substantial and significant increase in genetic divergence following the end of migration relative to preexisting structure, as well the ability to detect that change under realistic field conditions. We documented changes in θ and D_{est_Chao} of sufficient magnitude (> 0.05) under several combinations of population size and life history in our models to meet the first criterion. However, because the conditions under which such changes are likely to be detected are fairly restrictive and because the values that would indicate fragmentation can be obtained with natural subdivision, we suggest that θ and D_{est_Chao} alone are problematic for detecting changes in landscape connectivity in time frames that will inform management. In general though, D_{est_Chao} was far superior to θ, for early detection.

Estimates of θ and D_{est_Chao} in populations with < 500 individuals were significantly different from 0 and from pre-fragmentation values within 2 generations of isolation. A similar study examined a continuous population of 1000 individuals divided in half by a barrier to gene flow (Landguth *et al.* 2010). Depending on dispersal distances, it took >100 generations to detect a barrier using θ, while only 1-15 generations were required for detection with Mantel's *r* based on approaching equilibrium (Landguth *et al.* 2010). However, Landguth et al. (2010) did not report the magnitude of change in the metrics or effective population sizes, limiting direct

comparison to our results. Although we found significant differences, the magnitude of increased genetic divergence was often so small that detection in the field would be difficult.

Magnitudes were largely dependent on the demography of the populations under investigation and to a lesser extent on sample size. In the best-case scenario for detecting change (N_{max} = 25 with no overlap in generations and isolation occurred directly from panmixia), the magnitude of θ two generations after isolation compared with the last generation with connectivity could increase by 0.056 to 0.066 resulting in θ values ranging from 0.06 to 0.12. In populations with >500 individuals, the change in θ from prior to fragmentation to the second generation post-fragmentation was ≤0.002, which would be exceedingly difficult to recognize as biologically significant. The magnitude of change in Jost's *D* was larger under these conditions; with the change between the two generations ranging from 0.34 to 0.39. In the most difficult circumstances for detecting change (when a seed bank was present and population sub-structuring was established prior to isolation) increases between generations in neither θ nor D_{est_Chao} exceeded 0.042 (± 0.01), which reflects final values from 0.03 to 0.075. Changes of this magnitude are well within the range of sampling error in real populations (Avise 2004; Hamrick & Godt 1996; Whitlock & McCauley 1999), and indicate that the detection of change in θ or D_{est_Chao} over timeframes of 2-3 generations could be difficult at best. Further, such detection presumes having samples that represent conditions prior to fragmentation for comparison. It is more likely that assessments of connectivity will happen only after

changes in habitat amount and configuration have occurred because most often species are not studied prior to becoming of conservation concern.

Without having pre-fragmentation data, it is not possible to attribute causes to significant values of θ to changes, because the same values of θ can be obtained in very different ways. For example, when N_{max} >500 individuals and there is no migration θ values were identical to cases with limited migration when N_{max} <100 individuals (Table 5.1). Without having precise population size estimates, it would not be possible to determine whether a given θ or D_{est_Chao} value was due to small population size with a low level of migration or due to lack of migration among larger populations, and it would be impossible to determine if a shift in connectivity had been reduced from a single estimate (Chiucchi & Gibbs 2010). As such, there would be no way to distinguish between genetic divergence as the result of historic isolation and recent population fragmentation and fragmentation from anthropogenic activities could be improperly implicated for naturally occurring population sub-structuring.

Several approaches can possibly overcome lack of pre-fragmentation data. One potential approach is to compare multiple populations in heterogeneous habitat matrix in which there is strong contrast in gene flow among the matrix landscape types (i.e. barrier to gene flow; Balkenhol *et al.* 2009; Cushman & Landguth 2010; Jaquiery *et al.* 2011).

Alternatively, the change in D_{est_Chao} and θ across generations after fragmentation when N_{max} <100 individuals indicates that samples at multiple time points after isolation could allow detection fragmentation and thus provide a solution to the lack of pre-fragmentation data (Figures 5.2 & 5.3). On average, the rates of

change across generations far exceeded change seen in absence of fragmentation or in populations with substructuring due to limited migration prior to fragmentation (Figures 5.2 & 5.3). Thus sampling at multiple time points after landscape change or sampling from multiple demographic cohorts representing different generations and quantifying the amount of change in divergence between generations could provide evidence of fragmentation.

However, rates of change that we observed across generations may not be sufficient to detect signatures of fragmentation in field conditions. Likelihood of detection depends on time since isolation in addition to population size and whether or not generations overlap. Near or after the point of inflection where the mutation-drift equilibrium is reached, one would detect highly differentiated populations, but there would be little change between generations. The window of time after fragmentation during which it is possible to detect appreciable increases in divergence between 2 consecutive generations when $N_{\max}$ is below 100 individuals ranges from 8-10 generations. Beyond 10 generations the rate of change between two consecutive generations is dramatically reduced and is indistinguishable from that seen in populations prior to fragmentation even though the absolute values of θ or D_{est_Chao} could be higher. If the sampling time frame misses the window when rapid magnitude change in genetic divergence is occurring or if an initial estimate of genetic subdivision among sites is by chance high, the resulting time series would be inconclusive regarding any contemporary change in genetic connectivity. Complicating matters further; when a barrier to gene flow is removed the signature of restricted gene flow (e.g. high θ) can persist for 15-300 generations depending on the

dispersal distances (Landguth *et al.* 2010). A legacy of historical isolation within currently connected populations would result in misidentifying such populations as not connected by gene flow.

In larger populations, divergence continues increasing for at least the 200 generations we modeled thus providing a longer temporal window for detecting changes across generations; however the rate of divergence is extremely low throughout the 200-generation sampling period. Additionally, with these maximum population sizes, the divergence rates were highly variable, making change detection more difficult (Figure 5.4). This yields a frustrating conundrum in that N_{max} sizes that are most likely to have detectable change are also those for which the number of generations across which change will be detectable which variance is highest. Further, for all but annual species with no seedbank, the number of years required to sample across generations could be too large to provide reasonable recommendations in timeframes that are responsive to management concerns. If generations are 5-10 years, the 10-30 years necessary for the signal of fragmentation to be clear does not yield an early warning. Conversely, the timeframe is not appropriate for documenting that management actions have successfully reestablished connectivity and thus would not support adaptive management approaches (Walters 1986) that require regular and rapid assessment of the effects of management treatments.

Thus, Wright's F_{st} and Jost's D can only detect fragmentation when populations are monitored for multiple generations either before and after a fragmentation event or across multiple generations post-isolation. Despite the fact that genetic monitoring by definition requires a multi-year approach to be effective (Schwartz *et al.* 2007),

few published studies of fragmentation have included such temporal sampling (Barrett *et al.* 2005; Hoffman & Blouin 2004; Morris *et al.* 2002; Nussey *et al.* 2005; Poulsen *et al.* 2006; Thornhill *et al.* 2006), and even these generally do not extend more than a few generations. A few studies have sampled across generations by either comparing seedlings and adults (Young & Merriam 1994) or across strata in a soil seed bank (Baskauf & Snapp 1998). Most genetic evaluations of fragmentation have been based on one sampling time, so it is not possible to assess the cause of the observed patterns. Comparing estimates of gene flow using multiple analytical approaches that reflect different time frames has been suggested as a way to compare long-term and short-term levels of differentiation from a single sample (Chiucchi & Gibbs 2010).

Should the issues surrounding sampling at the correct time and for a sufficient length of time be overcome, there is the potential that error in estimates of θ or D_{est_Chao} associated with samples could prevent detection of changes in genetic divergence. However, our results show that even relatively few sampled individuals (20) or loci (10) provided unbiased estimates. When using D_{est_Chao}, sampling 20 sampled individuals per population at 10 microsatellite loci was sufficient to detect the small changes associated with cessation of migration. Detection using θ required greater numbers of loci or individuals when population sizes exceed 500 individuals, and addition of 10-20 individuals provides gains equivalent to addition of 10 loci. Given that it is often not cost effective or feasible to obtain both additional individuals and loci, it is encouraging that both options can improve estimates. It is important to note that our recommendations apply only to the use of genetic data to

detect a shift in genetic connectivity and are not generalizable to all types of genetic estimates. For example, a minimum of 60 individuals, sampled at least 5 years apart, and genotyped at 15 loci are required to provide a reliable and unbiased estimate of trends in effective population size (Tallmon *et al.* 2010). Thus, if there were multiple goals for genetic monitoring, the sample sizes indicated here could be inadequate.

In general we found that D_{est_Chao} (Jost 2008) represented genetic divergence more rapidly than did θacross all simulation conditions. This is not that surprising given that D_{est_Chao} avoids biases related to high sample heterozygosity (Hedrick 2005; Jost 2008; Meirmans 2006) because it is calculated directly from allele frequencies. Although there has been disagreement surrounding the appropriateness of use of D_{est_Chao} to the exclusion of heterozygosity-based measures (Ryman & Leimar 2009; Whitlock 2011), *D* has been shown to behave appropriately across a wide range of allele diversities, heterozygosities, and mutation rates (Gerlach *et al.* 2010; Jost 2008, 2009). We found that Jost's *D* was significantly greater than zero for all simulation conditions, including large effective population sizes, and usually was larger than Wright's F_{st}. During the initial 70 generations, when $N_{cap} \leq 100$, there was a peak in D_{est_Chao}, which resulted from drift overwhelming migration, or from the initial increase in the number of individuals as the population cap size is reached. D_{est_Chao} had a slower initial rate of change compared to θ; however, the magnitude change over the same time period was substantially greater for D_{est_Chao} (10^{-1}) than θ (10^{-2}). The initial magnitude of θ is much reduced relative to D_{est_Chao}, such that even a minute change in the magnitude of θ would bring about large relative changes. Values of D_{est_Chao} estimated from all factorial combination of sampled

individuals and loci were statistically indistinguishable from the census values indicating that the minimal number of individuals and loci need to be used to obtain a valid estimate of D_{est_Chao}.

Estimates of θ exceeded D_{est_Chao} when N_{max} was small (e.g., $N_{max} = 25$) and migration was present. The combination of small population size and migration lead to fixation of common alleles in several populations. The pattern of fixation is what subsequently resulted in depression of θ relative to D_{est_Chao}. Because θ is based on a ratio of partitioned of variance that includes heterozygosity, identical alleles that are fixed within multiple populations to the exclusion of others do not contribute to heterozygosity. When such a fixed allele is shared across two or more populations to the exclusion of others, θ is unable to account for the shared alleles and is therefore artificially high (Table 5.3). The magnitude of the decrease in θ will be a function of the number of fixed alleles, but in all such cases θ is misrepresenting the underlying pattern of differentiation, and is consequently over estimating the degree of genetic differentiation relative to D_{est_Chao}.

To conclude, we find that use of F_{st}-related statistics or D for detecting and monitoring changes in connectivity is problematic in real world scenarios. Although we were able to detect significant changes in θ and D_{est_Chao}, the magnitude of those changes was often small (< 0.03), especially as population sizes increased above 100 individuals. Even in cases when the magnitude of change was large (> 0.1), errors associated with measuring θ and D_{est_Chao} would decrease the likelihood of detecting change. Sampling across multiple generations and estimations of population size are also required to differentiate the signal of background differentiation from changes in

θ and D_{est_Chao} associated with the loss of genetic connectivity. This multi-generation sampling must occur within the window during which rapid change is occurring to either θ or D_{est_Chao} to yield conclusive results. At the same time the number of years required for a sufficient number of generations for detection of a change to even be possible may preclude utility. For these reasons, we caution against using indirect techniques alone for detection of fragmentation events, and advocate their use only in conjunction with estimates of actual movement among patches such that one could compare current movement with the genetic signature of past movement to determine that there has been a change.

Table 5.3 Example cases of allelic composition drawn from N_{max} = 25, which included population sub-structuring; values calculated for θ and D_{est_Chao} from these sample data. The fixation of common alleles removes all heterozygosity and results in inflated estimates of genetic differentiation when using F_{st} as opposed to D.

F_{st}	0	1	1	1	1
D	0	0.5	0.6	0.8	1
Pop 1	A/A	A/A	A/A	A/A	A/A
	A/A	A/A	A/A	A/A	A/A
	A/A	A/A	A/A	A/A	A/A
Pop 2	A/A	A/A	A/A	A/A	B/B
	A/A	A/A	A/A	A/A	B/B
	A/A	A/A	A/A	A/A	B/B
Pop 3	A/A	B/B	B/B	B/B	C/C
	A/A	B/B	B/B	B/B	C/C
	A/A	B/B	B/B	B/B	C/C
Pop 4	A/A	A/A	B/B	C/C	D/D
	A/A	A/A	B/B	C/C	D/D
	A/A	A/A	B/B	C/C	D/D

Conclusions

When Captain John Smith first explored the Chesapeake Bay and its tributaries in 1608, he encountered a land modified only by Native American settlement and agriculture (Cooper 1995). The subsequent substantial modification of the Chesapeake Bay watershed, in conjunction with abiotic and biotic stressors led to declines in abundance and distribution of submersed aquatic plants found in the Bay. As species become targeted for restoration, the impact that such wide scale declines have had on the genetic diversity of SAV species in the Bay becomes of key importance. My dissertation examined the impact of fragmentation and habitat loss on *Vallisneria americana* a species which has undergone substantial declines in the freshwater tidal reaches of the Chesapeake Bay.

The overall genotypic and allelic diversity of most sites were high enough to severe as restoration material, and the majority of sights did not show signs of population bottlenecks as might be expected with a decline in population size, or isolation. Furthermore, restoration techniques have not been impacting genetic diversity of restored sites. These data are a strong foundation for future work to examine the link between the genetic diversity data presented in this dissertation, and plant growth characteristics. Such a link would provide a wealth of data for the management and restoration of the species.

The synthesis of my work provides several additional issues that are, for the moment, unexplored. Pollen dispersal in *V. americana* is limited to within sites, but genetic data suggests that there is connectivity within regions among sites. The

mechanism of what is connecting these populations is unknown, and requires additional investigation. First, the true scale of pollen dispersal needs to be elucidated. My pollen results were hampered by the lack of decline in correlated paternity with distance. Additional intensive sampling within one site is required to confirm that pollen flow is truly limited. Exhaustively sampling ramets within a site, in combination with a few mothers, would provide the genotypes of potential fathers in addition to the maternal genotypes. This would conclusively show the scale at which pollen is moving.

Genes of *Vallisneria americana* are not only dispersed via pollen. The movement of seed and of propagules within and among sites is required to fully understand the degree of functional connectivity across a landscape. Measuring seed dispersal is still a difficult challenge made harder by the fact that *V. americana* is a submersed aquatic. Directly trapping seed following dispersed, or tracking seed with molecular methods (e.g., parentage, assignment tests) require sampling widely enough to find the source of a migrant (Cain *et al.* 2000). The dispersal of propagules among sites provides an equal challenge to detecting the movement of seed among sites; however, the wide-scale movement of clonal individuals in the Potomac river may provide insight that can be extrapolated more broadly through the Bay.

Upper Potomac sites vary from being dominated by a single clonal individual to being made up of mostly unique individuals that have resulted from separate sexual reproduction events. The processes that generated this variation can provide insight into how individuals are dispersing among sites. Sampling at intervals down the length of the Potomac River will show the distribution of extensive clones.

Combining genetic data from such a sampling with fitness measures from the sampled plants will provide information on if extensive clones are dominant because they are competitively superior or if they were left by chance after population bottlenecks.

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