Genetic Diversity and Spatial Structure of *Spartina alterniflora* at Four Spatial Scales

by

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Abstract

Spartina alterniflora, salt marsh cordgrass, is the dominant plant in coastal wetlands along the North American Atlantic coast. Ecological disturbances in salt marshes, such as coverage by wrack, disease, and eat-outs, affects *Spartina* marshes from the Gulf of Mexico to New England and may reduce the diversity of *S. alterniflora* clones within a population or alter other genetic characteristics of a population by eliminating some genotypes. Nine polymorphic microsatellite loci were used to quantify the genetic characteristics (e.g., allelic richness, diversity, polyploidy, fixation index) of the *S. alterniflora* populations at five salt marshes, as well as, to measure the spatial structure (size and shape of clones) of a single population in Upper Phillips Creek marsh (UPC), a marsh that experienced dieback. Over 250 individual plant samples were collected at three spatial scales for these experiments. Clones were found at all three spatial scales. However, at UPC marsh, over 53 unique genotypes were found corresponding to a high clonal diversity index of 0.944. All other marshes had indices above 0.9, except for Indiantown marsh, which had a low diversity index of 0.378. Although spatially separated by as much as 1, 15, 20, and 35 km, the five marshes were genetically connected as indicated by percent similarity calculations based on genetic similarity and geographic location. The high clonal diversity found and the large number of multilocus genotypes indicated that sexual reproduction and seedling recruitment are underappreciated processes that may contribute to marsh resilience and resistance to disturbance and climate change at the VCR LTER.

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Glossary of Terms

Allele – a form or specific variation of the gene

Allele Count -the number of times an allele is present within the population (GenoDive manual)

Allele Frequency - the sum of the allele counts divided by the sums of all allele counts (GenoDive manual)

Clonality - a form of plant growth that produces genetically identical individuals that are all capable of independent reproduction and growth (Vallejo-Marín et al. 2010)

Electropherogram - a graphical representation of the fluorescent dye intensity (referred to as peak height which is plotted on the y-axis of the MegaBACE output) and the time that it takes the fragment to travel the length of the capillary column (Figure 4)

Evenness - a measure of how the genotypes are distributed throughout the population in which a value of one indicates that all genotypes have an equal frequency throughout the population (GenoDive manual)

Gene – stretch of DNA that determines a certain trait

Genet – ramets produced by the same genotype (a clone)

Genetic distance - the number of mutations required to convert one of the sample pairs to the other (using Nei's diversity index)

Genotypic Richness - the proportion of different genets (genotypes) in the population calculated as $R = (G-1)/(N-1)$, where G is the number of genets and N is the sample size (Olivia et al. 2014)

Initial Seedling Recruitment (ISR) - species that reproduce sexually only in initially disturbed areas **(**Eriksson 1989)

Inbreeding coefficient (G_{is}) – a fixation index that compares the observed heterozygosity to the expected heterozygosity on a scale of -1 to 1, a positive number correlates to a deviation from Hardy-Weinberg Equilibrium and a lower observed heterozygosity than expected or inbreeding, while a negative number suggests outbreeding is occurring.

Microsatellites – tandem repeats of one to six nucleotides that vary in length between five and forty repeats depending on the species, a molecular technique which allows identification of plant genotypes, heterozygosity, and clonal diversity (Selkoe and Toonen 2006)

Multilocus Genotype (MLG) – the genotype is determined by using many different loci, in this study 9 primers were used

Nei's Diversity Index – Simpson's diversity index adjusted for clonal growth

Ramet – the individual plant sample

Recruitment at Windows of Opportunities (RWO) – species utilize sexual reproduction and seedling recruitment at optimal times during ideal natural conditions, when there are 'windows of opportunities' (Eriksson 1997)

Repeated Seedling Recruitment (RSR) – species that utilize sexual reproduction and seedling recruitment continually (Eriksson 1989)

Singleton - any sample that does not match the genotypes of the other samples (Douhovnikoff and Hazelton 2014)

Stepwise Mutation Model (SMM) – calculates genetic distance by assuming that alleles that differ only a few repeats in length are thought to be of more recent common ancestry than alleles that differ a lot of repeats in length (GenoDive manual)

Introduction

Salt marshes are critical habitat along mid-latitude coasts (Gedan et al. 2009). They provide valuable ecosystem services (Costanza et al. 1997, Levin et al. 2001); they prevent shoreline erosion and attenuate storm surge (King and Lester 1995, Moeller et al. 1996), reduce nitrogen inputs to coastal water (Valiela and Teal 1979), store carbon (Chmura et al. 2003), provide critical habitat for fish, shellfish, birds (Boesch and Turner 1984), and mammals, and offer opportunities for recreation (Costanza et al. 1997). It is these ecosystem services that attract human populations to live near salt marshes. Proximity to human populations has led to hydrodynamic alteration, use for waste disposal, over harvesting of fish and shellfish, invasion of exotic plants and animals, and conversion to residential and industrial sites and ports (Gedan et al. 2009). Accelerating sea-level rise that is occurring as a consequence of climate change is an additional threat to these critical terrestrial-marine transition zones and the services they provide.

Ecological Disturbance: Dieback

Anthropogenic impacts are not the only disturbances experienced by salt marshes. There also are many natural processes that can cause ecological disturbance in the marsh, such as high salinity, coverage by wrack, change in the tidal regime (Hartman 1988), fire (Turner 1987), disease (Kaur et al. 2010; Daleo et al. 2013), and eat-outs, which are extreme cases of goose herbivory in which large numbers of plants are uprooted and consumed (Adams 1963; Miller et al. 2005). Based on the intensity of these disturbances, open patches of vegetation can be created. More severe disturbances have the potential to kill both aboveground and belowground vegetation creating bare patches that can persist for a long time (Hartman 1988).

In some cases, the cause of plant death is not clear, as in the case of fire or herbivory, and these unknown bare patches can be so persistent and extensive that the events have been referred

to as salt marsh dieback. These events are thought to occur when the physiological or ecological limits of the marsh plants are exceeded. Other names given to this phenomenon are brown marsh, marsh balding, salt marsh dieback, and sudden wetland dieback. The geographic extent of brown marsh is broad. It has been noted along the USA Atlantic and Gulf Coasts (Alber et al. 2008, Osgood and Silliman 2009) and the frequency and intensity of dieback appears to be on the rise (Alber et al. 2008). The marsh plant most frequently affected is a salt marsh cordgrass, *Spartina alterniflora*, although other marsh plants may be affected. The cause of dieback is not clear but there is evidence that some combination of factors associated with drought (Mendelsshon and McKee 1988, Hughes et al. 2012), and pathogens or herbivory (Elmer et al. 2012, Silliman et al. 2005) may be involved. Dieback is a concern because *S. alterniflora* habitat is critical habitat for shellfish, fish, and birds, protects upland areas from storm surge, and stabilizes sediments.

Environmental disturbances in the marshes may become more pronounced as climate change continues to impact environmental conditions and systems. Rising carbon dioxide levels have the potential to drive shifts in temperature, circulation, nutrient input, and productivity effecting ecosystem function (Doney et al. 2012). Warming has been shown to decrease the diversity of salt marsh plant communities via loss of foundation species, thus affecting the function of the salt marsh ecosystem (Gedan and Bertness 2010). These shifts have been shown to alter biodiversity within a system. Climate change coupled with anthropogenic deterioration of marine systems will impact salt marshes due to multiple-stressors leading to the estimated deterioration of 50% of salt marshes worldwide (Jackson 2010).

As the intensity of environmental disturbances increases and multiple-stressors become more apparent, a genetically diverse population of *S. alterniflora* will have a greater likelihood of

survival (Travis et al. 2002). It is important to understand how *S. alterniflora* is reproducing and colonizing new areas in order to better understand disturbances in the marsh.

Understanding S. alterniflora reproductive systems

  Spartina alterniflora is a rhizomatous plant that reproduces asexually primarily by clonal expansion (Shumway 1995) and sexually via seeds (Edwards et al. 2005). Clonal growth may allow for individual persistence in well-established communities, rapid colonization of environments, and growth in stressful environments where seedling establishment is not favored (Pennings and Bertness 2001). After initial colonization by propagules or seedlings, populations of *S. alterniflora* develop in circular patches due to clonal growth. This circular growth is only disrupted when either environmental conditions change or competition with other plants prevent further expansion (Proffitt et. al 2003).

  It is important to understand the meaning of plant clonality. Clonality is a form of plant growth that produces genetically identical individuals that are all capable of independent reproduction and growth (Vallejo-Marín et al. 2010). These new individuals formed by clonal propagation are considered ramets and all ramets produced by the same genotype are referred to as a genet. The number of ramets in a population, however, does not reflect the number of genets. This means that some populations can be composed of a single clone; while in other populations, each ramet could represent a unique genotype or individual (Vallejo-Marín et al. 2010).

  Additionally, the spatial arrangement of ramets can have an impact on mating opportunities. *S. alterniflora* typically has a clonal architecture that is characterized by rapid spread and greater separation between ramets, known as a "guerrilla" strategy (Castillo et al. 2010). This architecture creates a greater intermingling of ramets from different genets.

S. alterniflora has typically been understood as a clonal plant (Shumway 1995). However, the reproduction strategies of S. alterniflora are not fully understood in terms of the fitness of the population. Growth via seedling recruitment promotes genetic and clonal diversity, which helps maintain the potential for outcrossing and a greater survival during environmental disturbance (Travis et al. 2002). Thus, there appears to be trade-offs between reproducing asexually and sexually.

Eriksson (1989) described clonal species' reproduction as a continuum. At one end of the spectrum are those species that utilize "initial seedling recruitment" (ISR). These species only reproduce sexually in disturbed areas. The other end of the spectrum represents those species that utilize seedling recruitment continually, "repeated seedling recruitment" (RSR) (Eriksson 1989). *S. alterniflora* has been described as an ISR species, but Travis et al. (2004) found an outcrossing rate of about 90 percent in Louisiana marshes, suggesting that *S. alterniflora* may be more characteristic of a new group of clonal species termed RWO, "recruitment at windows of opportunity" (Eriksson 1997, Travis et al. 2004). This "window of opportunity" indicates that *S. alterniflora* only utilizes seedling recruitment when it is readily available, for example during ideal natural conditions – limited competition, low stress, and room for seed settlement.

Dieback and other disturbances could create a 'window of opportunity,' where substrate becomes bare and seedling recruitment is favored. Researchers working on the Eastern Shore of Virginia noticed a dieback at Upper Phillips Creek marsh (UPC) in the summer of 2004 (Marsh 2007). The areas affected in this dieback were all monocultures of *S. alterniflora*. UPC marsh is one of the only marshes on Virginia's Eastern Shore that has experienced dieback. Understanding the spatial structure of the clones within the UPC population and this population's diversity in the context of nearby marshes that have not experienced dieback may provide an

opportunity to learn more about the effect of salt-marsh dieback on the genetic diversity of *S. alterniflora* clones and aid in the understanding of *S. alterniflora* reproduction and colonization.

Genetic Analysis: Microsatellites

Quantification of *S. alterniflora* spatial structure depends upon the ability to identify individual cordgrass genotypes using a molecular approach such as allozymes, mitochondrial and nuclear DNA, or microsatellites. Microsatellites produce a more precise and statistically powerful way of comparing populations and individuals because the results come from many loci, specific locations on the gene.

Microsatellites are tandem repeats of one to six nucleotides that vary in length between five and forty repeats depending on the species (Selkoe and Toonen 2006). The DNA surrounding the microsatellite locus is termed the flanking region. Plant microsatellites are rich in adenosine (A) and thymidine (T). For example, a plant four-repeat microsatellite might be ATATATAT and the flanking region to which the primer attaches could be TTACCCTCATCCGAGTCAAAA, a flanking region for primer SPAR 01 used in this investigation. The flanking regions change only slowly across individuals of a species, thus a particular microsatellite can be identified by DNA of the flanking regions. Unlike the flanking regions, the microsatellite sequences mutate frequently during DNA replication, thus altering the length and number of repeats within the sequence. (Selkoe and Toonen 2006). Nine microsatellite primers for *S. alterniflora* are readily available and were used in this study.

The advantages of microsatellite markers are that they can be used to identify plant genotypes, heterozygosity, allelic richness (number of different types of a single gene), and population diversity and divergence (e.g., speciation) (Selkoe and Toonen 2006), so that ecological questions about clonal identity and the genetic relatedness of individuals within and

between population can be addressed using a single molecular technique. They allow questions such as, "What are the genetic relationships of individuals within and among marshes?", or "Which individuals are clones within a marsh?" to be addressed (Selkoe and Toonen 2006). The differences in the length and number of repeats in the microsatellites can be easily identified via gel electrophoresis making identification of individual plants of a species possible. The high mutation rates and abundance of the microsatellites in plants allows for identification of individuals and assessment of population diversity in a statistically powerful way. (Selkoe and Toonen 2006).

Research Questions

This thesis will address two questions: (Q1) What is the spatial structure of *S. alterniflora* genotypes in Upper Phillips Creek (UPC) marsh?; and (Q2) what is the genetic relatedness of this population to populations in nearby marshes? UPC marsh is one of the only marshes on Virginia's Eastern Shore that has experienced dieback. Understanding the spatial structure of the clones within the UPC marsh population and this population's diversity in the context of nearby marshes may provide an opportunity to learn more about the reproductive mechanisms of *S. alterniflora* and the effects of disturbance.

Methods

Research Setting

Samples were collected along the Eastern Shore of Virginia at Upper Phillips Creek marsh (UPC) and in four other nearby marshes (Lower Phillips Creek (LPC), Indiantown (ITM), Oyster Harbor (OHM), and Cushman's Landing (CLM) marshes; Figure 1). Upper Phillips Creek (UPC) marsh is on the Brownsville Plantation, located near the town of Nassawadox, Virginia. This marsh is located within the Nassawadox, Virginia, 7.5 minute quadrangle at

approximately latitude 37° 27' 50" N and longitude 75° 50' 04.99" W. The marsh is classified as

a valley marsh and is typical of 67% of the marshes along the Virginia portion of the eastern side

of the Delmarva Peninsula (Oertel and Woo 1994). UPC marsh was sampled at three spatial

scales (20 cm, 1 m, and 5 m) in order to determine spatial structure within a marsh (Q1).
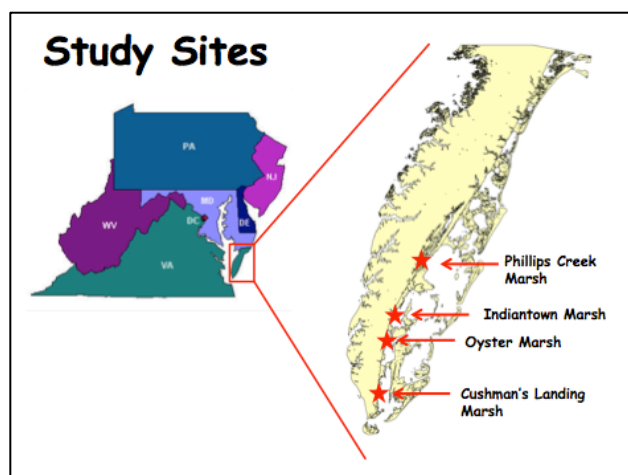
Figure 1. The five marshes sampled are located on the Eastern Shore of Virginia and are sites where the Virginia Coast Reserve Long-Term Ecology Research program monitors marsh grass production annually. The sites sampled were Upper Phillips Creek (UPC), Lower Phillips Creek (LPC), Indiantown (ITM), Oyster Harbor (OHM), and Cushman's Landing (CLM).

The four other nearby marshes, sampled for this study, are Virginia Coast Reserve Long-

Term Ecological Research monitoring sites for marsh grass production (Figure 1). These four

marshes are representative of the other mainland geomorphic marsh types found in this region

(Oertel and Woo 1994). They are located approximately 1, 15, 20, and 35 km (LPC, ITM, OHM,

and CLM; respectively) from UPC marsh and were selected to be at increasing distances from

UPC to determine if the distance between populations was correlated with the genetic relatedness

of the cordgrass populations of the lower Delmarva Peninsula (Q2).

It's important to note that the five marshes that are included in this study are all mainland

marshes. However, these marshes differ in their configuration and have different sedimentation

rates (Oertel and Woo, 1994), which could independently affect dispersal of reproductive

structures (seeds and rhizomes) and growth of genetically different *S. alterniflora* in the

geomorphic settings. Because greater genetic diversity may indicate differences in plant susceptibility to dieback or other types of disturbance, sampling marsh grass populations from the five different geomorphic marsh types increases the potential for capturing the greatest possible range of genetic diversity for mainland marshes at the VCR. Therefore, sampling from different marsh types does not create a confounding variable.

Plant Sampling Schemes

All five marshes were sampled in June 2013 to allow for diversity comparisons (Q2) and to establish a general spatial scale (> or < 10 m) for determination of within-marsh spatial structure (Q1). In June 2013, ten individual stems of short-form *S. alterniflora* were collected 10m apart along a transect that was parallel to the tidal creek (i.e., at similar elevation and hydroperiod) (Appendix I). Each plant stem was clipped, wrapped in a paper towel, and placed in individual zip-top bags. The samples were kept cool during transportation back to Charlottesville. In the lab, plants were refrigerated until DNA extraction was performed – within a week after sampling. The apex of each sample was used for extraction using Qiagen's DNeasy Mini Plant Kits (Qiagen, Inc., Valencia, CA, USA) (Appendix II). All 50 DNA samples were preserved for further genetic analysis (see below).

In June 2014, a different sampling approach was be used to address Q1 in UPC marsh. The sampling design was a nested approach (Figure 2). A 1000 x 1000-cm grid made of nylon string was constructed over an area of short-form *S. alterniflora*. Individual plant stems were sampled along the grid every 100 cm where the strings intersected. Within the large grid, two additional 100 x 100-cm grids were constructed at random. Individual plant stems were sampled every 20 cm where the strings intersected. Additionally, a 50 m transect was constructed off the corner node, perpendicular to the plot. Samples were taken every 5 m along this transect (Figure

2). This approach yielded 204 samples in total. Individual stems were clipped, wrapped in a

paper towel, placed in zip-top bags, kept cool, transported back to Charlottesville, extracted, and

preserved for further genetic analysis in the same way as described above.
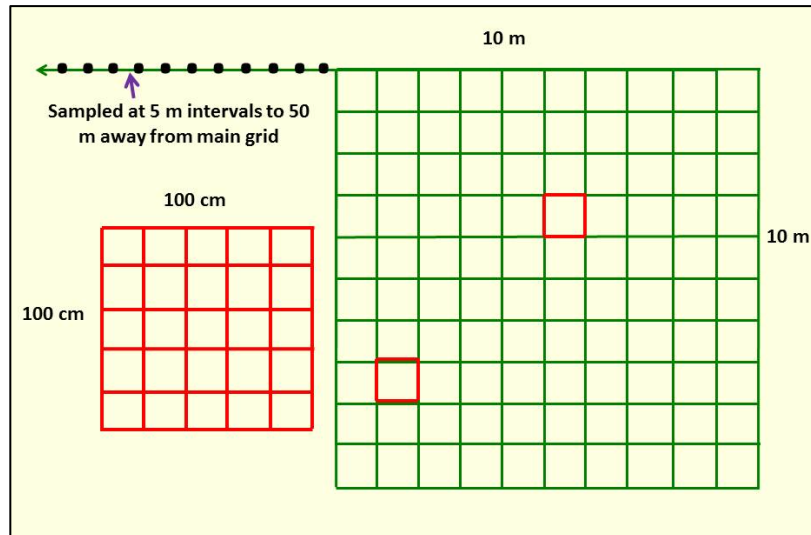
Figure 2. Schematic of the 10 x 10-m sampling grid used to examine the spatial distribution of clones at Upper Phillips Creek marsh in June 2014. At each node of the grid, a single plant stem was clipped, including along the perimeter of the grid. Within the large grid, two additional 100 x 100-cm grids were clipped. An additional, 11 samples were collected along a 50 m transect perpendicular to the grid.

Microsatellite genotyping

Nine microsatellite primers for *S. alterniflora* were readily available and thus easily

accessible (Blum et al. 2007) (Appendix III). For each primer pair, the forward primer was

fluorescently tagged with HEX, NED, and FAM. All Polymerase Chain Reactions (PCR) were

generated on a MJ Research PTC 200 thermocycler (Bio-Rad Laboratories, Inc.,) in order to

amplify the DNA microsatellite regions of interest. Approximately 1 µl of DNA (consisting of

10-50 ηg of genomic DNA) was used as a template in a 15 µl PCR. Each PCR also contained 7.5

µl of TypeIT (Qiagen, Inc., Valencia, CA, USA), 0.06 µl of the forward primer (with flouro-tag),

0.06 µl of the reverse primer, and 6.02 µl of biograde molecular water. Thus, creating a 14 µl

master mix for each reaction. PCR products were generated using a heated lid at 105°C, an initial

denaturing stage at 95°C for 5 min, and 30 thermal cycles of 95°C for 30 s, 60°C for 90 s, 72°C

for 30 s, a final extension stage at 60°C for 30 min, and a cool down stage at 20°C for 30 s

(Appendix II). Following initial PCR's, PCR products were visualized on a 1.5% agarose gel,

where bands of approximate expected size according to locus primer design signified successful

amplification (Figure 3). The PCR procedure produces a mixture of short, fluorescently-tagged

fragments that differ in the number of base pairs or fragment length. The fluorescent tags are

used to visually distinguish PCR products containing the targeted microsatellite regions of

interest from unintentionally amplified DNA.
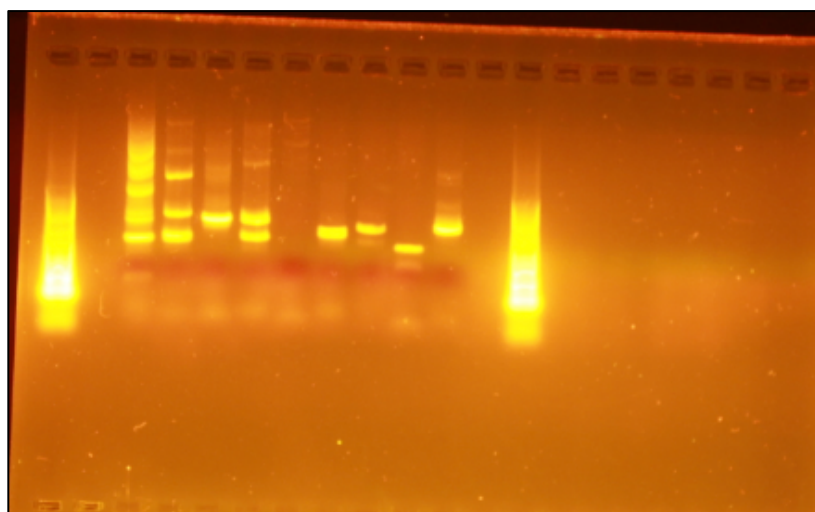
Figure 3. Photograph of an electrophoretic gel showing PCR product for primers 1 through 9.
Gels were prepared to determine if DNA was amplified during PCR and to determine if the
primers produced multiple, clear bands as compared to a ladder (DNA of a known number of
base pairs). Ladders are in lanes 1 and 11.

After confirmation of 8-10 successful PCR products via gel electrophoresis, PCR

products were analyzed by capillary electrophoresis on a MegaBACE 1000 (GE Biosciences,

Pittsburgh, Pennsylvania, USA) with ET 400-Rox (GE Biosciences) internal size standard in

each sample, as per manufacturer's instructions, and microsatellite genotyping (Amersham

Biosciences 2003) (Appendix II). MegaBACE output (Figure 4) was scored using a standard

approach (see below) utilizing the software Fragment Profiler, version 1.2 (Amersham

Biosciences). The first 50 samples collected from the five marshes in 2013 were analyzed using

the in-house MegaBACE and scored on Fragment Profiler.
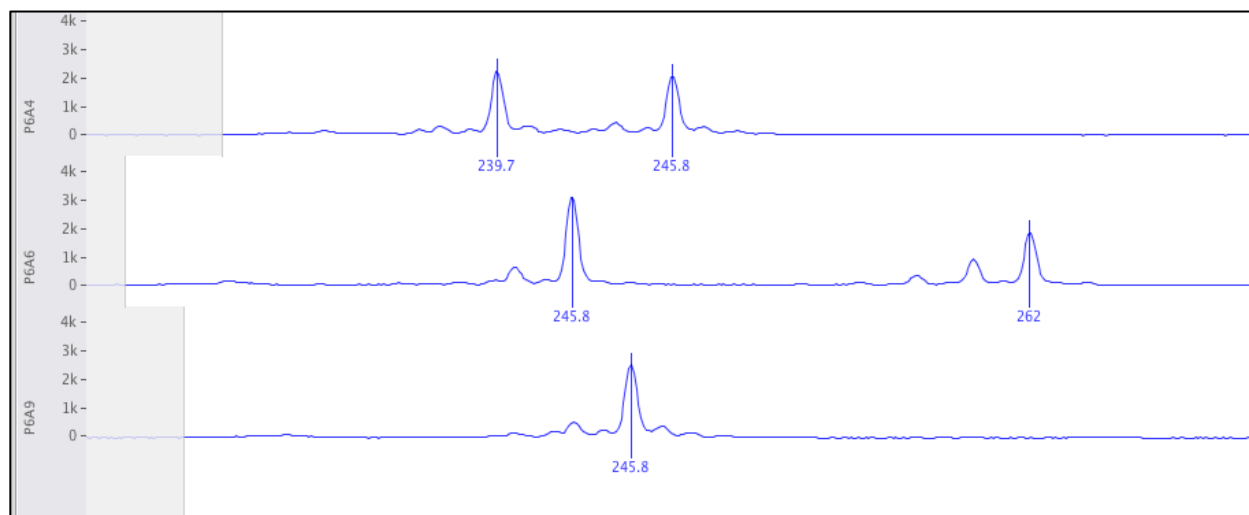
Figure 4. MegaBACE output as visualized in Geneious software, version 7.1. Peaks represent allelic amplification height on the y-axis and the length of the fragment relative to the standard in units of base pairs on the x-axis. Two peaks (top and middle) represent a heterozygote, while one peak represents a homozygote (bottom). It should be noted that Fragment Profiler had a similar interface with similar scale (used in Q2), and that both the MegaBACE and Fragment Profiler gave identical results for samples analyzed using both machines.

The samples from UPC (204 samples collected in 2014) were sent to Georgia Genomics

Facility (University of Georgia, Athens, GA) for analysis. PCR was performed in house, then 1

µl of PCR product and 39 µl of biograde molecular water were sent to Georgia (1:40 µl dilution).

The Georgia facility runs samples using a 3730xl DNA Analyzer (Applied Biosystems) with a

ROX-500 standard. To test accuracy between in-house and out-of-house results, multiple plates

were run at both facilities. Results from Georgia were analyzed and scored utilizing the software

Geneious, version 7.1 (Biomatters Limited) (Figure 4). Due to variability in genomic DNA

concentrations per sample, some samples had to be resent to Georgia. When samples were run

twice, a 1:20 µl dilution was used instead of the 1:40 µl to insure enough DNA was available for

use at Georgia.

During capillary electrophoresis, the mix of fragments produced by PCR is loaded into capillary tubes that contain a gel solution that serves as a sieving matrix. An electrical voltage is applied to the gel so that one end is positively charged and the other is negatively charged. Because DNA has a slight negative charge, the fragments move in the gel. The different length fragments move at different rates and so separate from one another based on size, with smaller fragments traveling faster through the gel. When each fluorescently-tagged fragment reaches the end of the capillary tube, the tag is excited by a laser beam and the results visualized in a plot called an electropherogram.

An electropherogram is a graphical representation of the fluorescent dye intensity (referred to as peak height which is plotted on the y-axis of the output) and the time that it takes the fragment to travel the length of the capillary column (Figure 4). The travel time through the capillary column is proportional to the length of the fragment (i.e., number of base pairs in the fragment) and is plotted on the x-axis of the electropherogram. All is relative to standards of known base number that are run at the same time as the samples being analyzed. Thus, each peak on the electropherogram represents an allele; a sample with a single peak has two identical alleles for that microsatellite or is homozygous, while a sample with two peaks has two different alleles and is heterozygous (Figure 4).

Identification of Alleles: Scoring Output from Electropherograms

Identification of alleles is done by eye and referred to as scoring. Scoring involves examination of the electropherograms to identify peaks representing alleles. *A priori*, a minimum peak height of 200 was established to avoid artifacts associated with a "noisy" baseline (Figure 4). Samples were scored as heterozygous if the electropherogram had two peaks of similar height, within a range of 2000 from peak to peak. In some cases, three alleles were identified for

a sample indicating that the individual was a triploid. Triploidy and tetraploidy (four alleles) is common within the genus *Spartina*; however, when triploids were found, the samples were rerun in order to confirm the plant's status as triploid.

To allow comparison between samples analyzed at UVa in 2013 and those analyzed at the University of Georgia's genomic facility the following year, samples run in 2014 were sent to both locations in order to test the consistency between both facilities. Although the electropherograms were created using Fragment Profiler software by the UVa instrument and with Geneious software by the UGA instrument, both software interfaces produce identical types of electropherograms and intercomparison between the two machines gave identical results.

Even with careful DNA extraction, repeated PCR, and reanalysis by capillary electrophoresis, some sample-primer combinations had no identifiable alleles. Out of 1,836 sample-primer combinations for the 2014 data, there were 244 missing combinations, roughly 13% of the combinations. For the 2013 data, there were 450 sample-primer combinations and 38 missing combinations, about 8% of the combinations. For any given sample, typically only one of the nine primers used was missing allele information (Table 1 and 2). Because some of the statistical tests used to examine population genetics require that there are no missing values in a dataset, missing alleles can be either assigned or the samples can be removed from the analysis (GenoDive manual, version 7.1).

Table 1. Total alleles per primer, missing sample data, and number of triploids for Upper Phillips Creek Marsh (2014 data). Of the 1,836 sample-primer combinations filled in using approach 1, there were 244 missing combinations, about 13%.Filling in the rest via approach 2, alleles for only two samples could not be assigned, and so, these samples were dropped, leaving 202 samples for statistical analyses.

Primer	Total alleles	Triploids	Missing sample-primer combinations	Approach 1	Approach 2
1	12	0	73	16	55
2	8	2	35	11	23
3	5	6	13	12	0
4	9	0	18	11	6
5	10	4	26	8	17
6	8	1	14	5	9
7	14	0	41	7	33
8	13	0	16	5	11
9	14	0	8	3	4
Total	93	13	244	78	158

Table 2. Total alleles per primer, missing sample data, and number of triploids for the collective five marshes (2013 data). Of the 450 sample-primer combinations, there were 38 missing combinations, about 8%. Alleles for all samples were assigned; thus, all 50 samples were available for statistical analyses.

Primer	Total alleles	Triploids	Missing sample-primer combinations	Replaced
1	16	0	11	11
2	13	0	0	0
3	6	0	3	3
4	21	0	4	4
5	17	4	3	3
6	16	0	0	0
7	14	0	6	6
8	14	1	2	2
9	19	0	9	9
Total	136	5	38	38

The statistical package used, GenoDive, recommends assigning random alleles drawn from the pool of alleles present in the population, so that the alleles present at the highest frequency are more likely to be picked than alleles present at lower frequencies (GenoDive manual, version 7.1). This avoids throwing out samples for which only one of nine primers did not yield useful data. Instead of a random approach, for the 2014 data (Q1), I choose a more

conservative approach in which nearest geographical neighbors were examined and alleles were assigned based on that examination (Table 1).

Two approaches were used to examine the nearest neighbor. The first approach (approach 1) was to identify the eight sample-primer combinations surrounding the sample with the missing allele, for a specific primer. If four of the eight combinations surrounding the missing value had the same allele, then that allele was assigned to the missing sample for that specific primer. This approach filled-in data for 78 sample-primer combinations and left only 166 without a full set of alleles for all nine primers. The 166 remaining sample-primer combinations without an allele were compared to the surrounding nearest-neighbors across all nine primers (approach 2). If alleles for seven of the nine primers matched between two samples, then the sample with a missing allele was assumed to be from the same clone (i.e., the same genotype) and the missing allele was assigned to create the closest genotype. This approach favors asexual reproduction and clonal growth, as well as reducing the probability of overestimating population diversity. After this procedure, only two samples did not have a complete set of alleles for all nine primers; these two samples were dropped from statistical analyses requiring no missing values. Thus, 202 samples were available in order to answer Q1.

The missing sample-primer combinations from 2013 had to filled in with a different approach (Table 2). Since nearest neighbor was difficult to identify due to the sampling scheme, a more random approach was needed. Each marsh was treated as a separate population when filling in missing values, thus still utilizing a conservative approach. Within a given marsh, the highest allele frequency for each specific primer was used to fill in the remaining samples. All 38 missing sample-primer combinations were filled in for the 2013 data, thus all 50 samples were available to answer Q2.

Determination of Population Genetics Statistics

GenoDive, version 2.0b23, was used to perform the statistical and genetic analyses. GenoDive was chosen for its ability to perform population genetic analyses and generate a genetic distance matrix (aiding in further spatial genetic analysis) for clonal populations. The first population statistics determined were the allele count and allele frequencies for each primer and each marsh. The allele count represents the number of times an allele is present within the population and the allele frequency represents the sum of the allele counts divided by the sums of all allele counts (GenoDive manual, version 7.1).

To perform any heterozygosity-based genetic analyses, the genotypes (clones) had to be identified and assigned based on allele identification as described above. Clones are assigned within GenoDive using an algorithm that first calculates genetic distance and then applies a user-defined threshold distance (see below). The threshold is the level of genetic similarity necessary for samples to be considered a clone. The algorithm uses a stepwise mutation model (SMM) to calculate genetic distance. A SMM assumes "that alleles that differ only a few repeats in length are thought to be of more recent common ancestry than alleles that differ a lot of repeats in length" (GenoDive manual, version 7.1). The genetic distance is reported by the software as the number of single stepwise mutations necessary to convert one genotype to another. Genetic similarity was calculated as genetic distance between a pair divided by the maximum genetic distance within the population. This number was multiplied by 100 to give percent similarity.

A threshold distance of nine base pairs was chosen for each of the populations examined (UPC, LPC, ITM, OHM, and CLM). This means that samples could be as many as nine base pairs different in size and still be considered genetically identical (members of the same clone), while samples that differ by 10 base pairs are different individuals or clones. The need for a

threshold arises as a result of errors occurring during extraction, PCR, scoring, or somatic

mutations (not associated with DNA replication during meiosis). A nine-base pair threshold was

chosen for the UPC data (Q1) because it was the inflection point where rate of clone decrease

slowed dramatically (Figure 5), indicating that threshold no longer affected the number of

clones. A threshold of zero was chosen for the collective five marsh data (Q2) because the

number of clones was unaffected by the threshold selected.
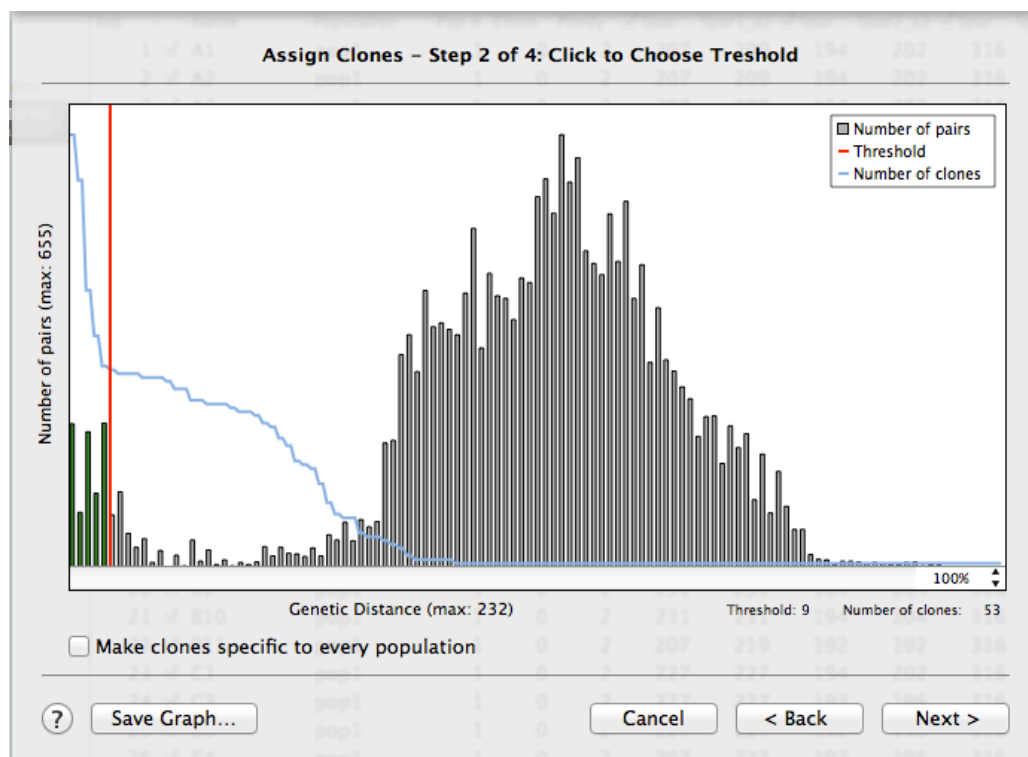
Figure 5. GenoDive interface printout showing how differences in the number of base pairs between samples in pair-wise comparisons affect the number of clones identified. A threshold value of nine base pairs was selected to assign clones at UPC (Q1). This means that in a pair-wise comparison, samples with as many as nine different base pairs were considered to be 100% similar.

Clone assignment also tested for the clonal population structure by determining clonal

diversity. Nei's diversity index (Simpson's diversity adjusted for clonal growth) was used in

order to calculate genetic distance and other diversity indices. Several other measures of

diversity also were determined including the number of genotypes, effective number of

genotypes (genotypes reproducing sexually), evenness, and genotypic richness. Evenness is a measure of how the genotypes are distributed throughout the population in which a value of one indicates that all genotypes have an equal frequency throughout the population (GenoDive manual). Genotypic richness is the proportion of different genets (genotypes) in the population and was calculated R = (G-1)/(N-1), where G is the number of genets and N is the sample size (Olivia et al. 2014).

GenoDive can calculate heterozygosity-based statistics in order to examine genetic diversity within a population or among populations. This genetic diversity function provides information regarding observed and expected heterozygosity, which helps better understand the fixation indices. The fixation index measures how populations differ genetically and the extent to which they differ, values typically range from 0 (no differentiation) to 1 (distinct populations) (Norrgard and Schultz 2008). Among the fixation indices, an inbreeding coefficient (G_{is}) was calculated in order to determine the departure from the Hardy-Weinberg equilibrium (HWE) within a population. The inbreeding coefficient compares the observed heterozygosity to the expected heterozygosity on a scale of -1 to 1. A positive number correlates to a deviation from HWE and a lower observed heterozygosity than expected or inbreeding, while a negative number suggests outbreeding is occurring.

To better understand the relatedness between clones, a dendrogram was created for both sampling schemes – UPC (Q1) and the five marshes collectively (Q2). A dendrogram is a tree diagram demonstrating percent similarity. Dendrograms were created using percent similarity calculated from Nei's diversity index. SPSS (version 21) software was used to cluster the data.

Determination of UPC Spatial Structure (Q1)

Geostatistical tools can be used to assess spatial structure and quantitatively determine spatial variation based on the amount of autocorrelation between two samples as a function of the distance between them (see review by Legendre 1993). Geostatistical analyses are commonly used in soil science (Goovaerts 1999) but have also been used in ecological studies (see review by Rossi et al. 1992).

The spatial structure of the UPC marsh *S. alterniflora* population was characterized by geostatistical analysis using the genetic similarity generated by the genetic distance matrix (Nei's) and genetic distance was plotted as a function of the geographic distance between pairs of samples to create a semivariogram.

Results

Q1 – What is the spatial structure of *S. alterniflora* genotypes in Upper Phillips Creek (UPC) marsh?

Population Genetics Statistics

Of the nine-microsatellite markers across the 202 samples from UPC, 93 unique alleles were identified (Table 1). There were an average number of 10 alleles per loci (Table 3), of which 6 were considered effective based on their frequencies. Overall, there were 53 unique multilocus genotypes in UPC, of which 16 were considered effective within the population. The most frequent genotype or largest clone consisted of 28 ramets or 14% of the total ramets (or samples).

Table 3. Clonal diversity measures for 202 samples at Upper Phillips Creek Marsh, 2014 data. Statistics were computed using GenoDive. See glossary (p. vii for explanation of terms)

Population Statistic	Value	Population Statistic	Value
N, number of samples amplified	202	Number of single unique MLGs (singletons)	28
A, average number of alleles per loci	10	Singletons % of samples amplified	14
A_E, effective number of alleles	6	Singletons % of genotypes	53
Number of unique multilocus genotypes (MLGs)	53	Genotypic richness	0.26
Number of effective unique MLGs	16	Expected heterozygosity/ Observed heterozygosity (range 0 to 1; 1= all heterozygotes)	0.944/ 0.562
Most frequent genotype (number of ramets)	28	Evenness	0.313
Most frequent genotype (% of samples)	14	Inbreeding Coefficient (G_{is}) (range -1 to 1 but decreases with increasing allele numbers above 2; positive numbers suggest inbreeding)	0.292
Simpson's diversity index (range 0 to 1, 1 = genetically distinct)	0.944		

When analyzing the number of genotypes, it is important to highlight the number of singletons within the population. Singletons are any samples that do not match the genotypes of other samples within the population, thus could disproportionately affect clonal diversity measures. (Douhovnikoff and Hazelton 2014). At UPC, 28 singletons were identified, 14% of the samples identified and 53% of the number of genotypes identified (Table 3).

Genetic and clonal diversity statistics were calculated for UPC marsh. A relatively low genotypic richness of 0.26 was found within the population, which puts the population at risk for extinction or loss of the genotypes at UPC from the regional population. The genotypes were not distributed evenly throughout the population, corresponding to an evenness value of 0.313. The expected heterozygosity was 0.944 suggesting a very heterozygous population; however, the observed heterozygosity was 0.562, thus deviating from Hardy-Weinberg Equilibrium. There

was a positive inbreeding coefficient of 0.292 due to the lower observed heterozygosity. The Simpson's diversity index was high at 0.944 indicating high clonal diversity.

53 unique genotypes cluster from a range of 95.7% similarity between samples B6 and B7 to 0% similarity for sample pair C9 and E1 (Figure 6). It is important to note that similarity is compared based on nine microsatellite primers, not the whole genome so that samples that are 100% dissimilar do not share any alleles for only the nine microsatellites examined and the remainder of the genome may be fully similar.

Spatial Structure

A genotype map (Figure 7) was constructed based on genetic distance determined as a corrected Nei's diversity index. Genetic distance is the number of mutations required to convert one of the sample pairs to the other. Of 202 samples analyzed, the figure depicts the 53 genotypes that were identified. 50 genotypes were found within the 10 x 10-m sampling grid. Each color symbol indicates a clone and the open symbols are singletons. Figure 7 allows a visual representation of the spatial structure of UPC at three different spatial scales – 0.2 m, 1.0 m, and 5 m.

A spatial autocorrelation was examined as the function of the genetic distance and geographic distance (Figure 8). These results demonstrate that even though plants can be physically close together, they can be genetically very different, while genetically similar plants can be physically far apart.
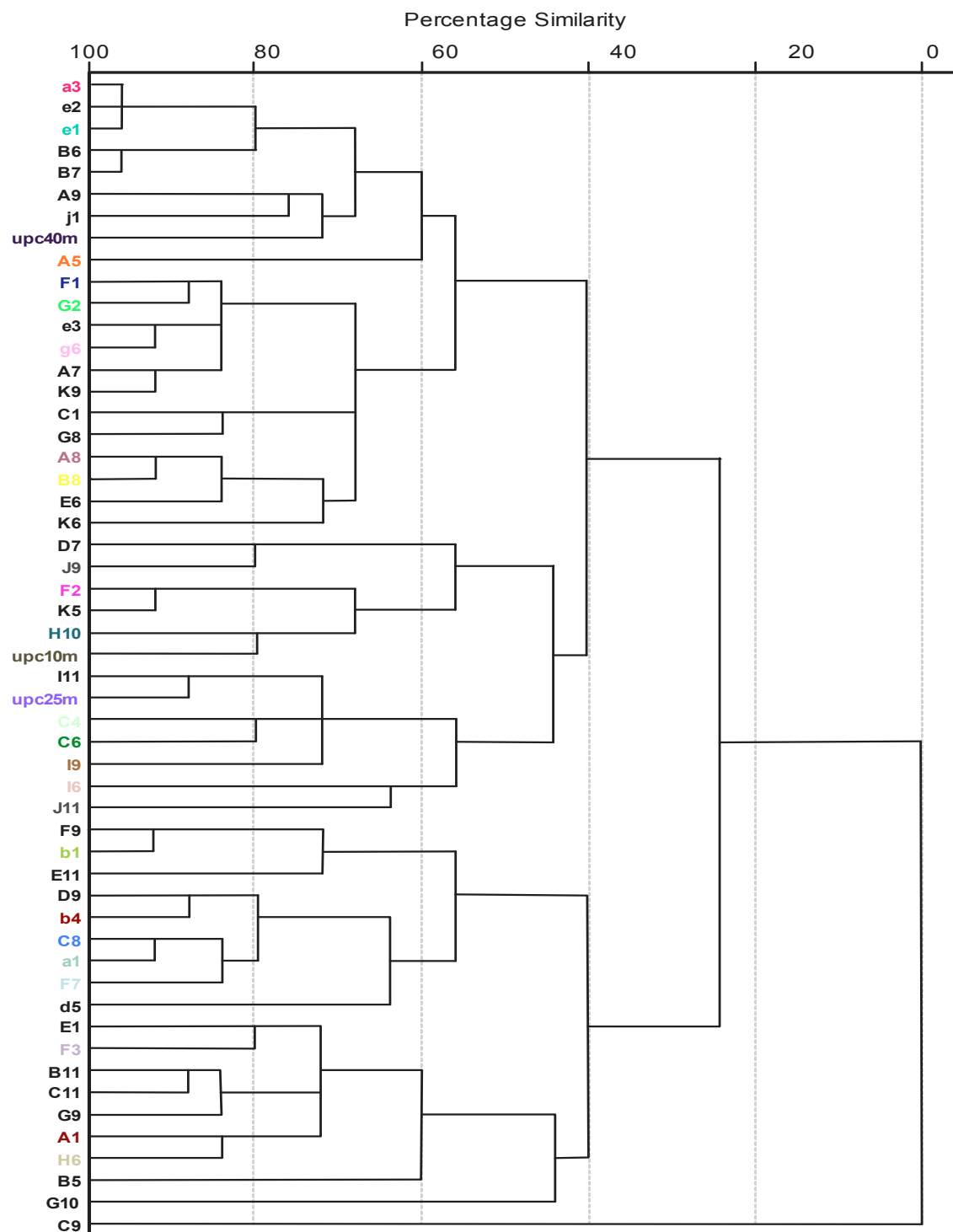
Figure 6: Cluster analysis of 202 samples collected at Upper Phillips Creek marsh in 2014 at three spatial scales. The dendrogram shows the percent similarity of 53 unique genotypes based on Nei's diversity index. Genotype colors along the y-axis are code to match those in the genotype map in Figure 7. Cluster analysis was done in SPSS.
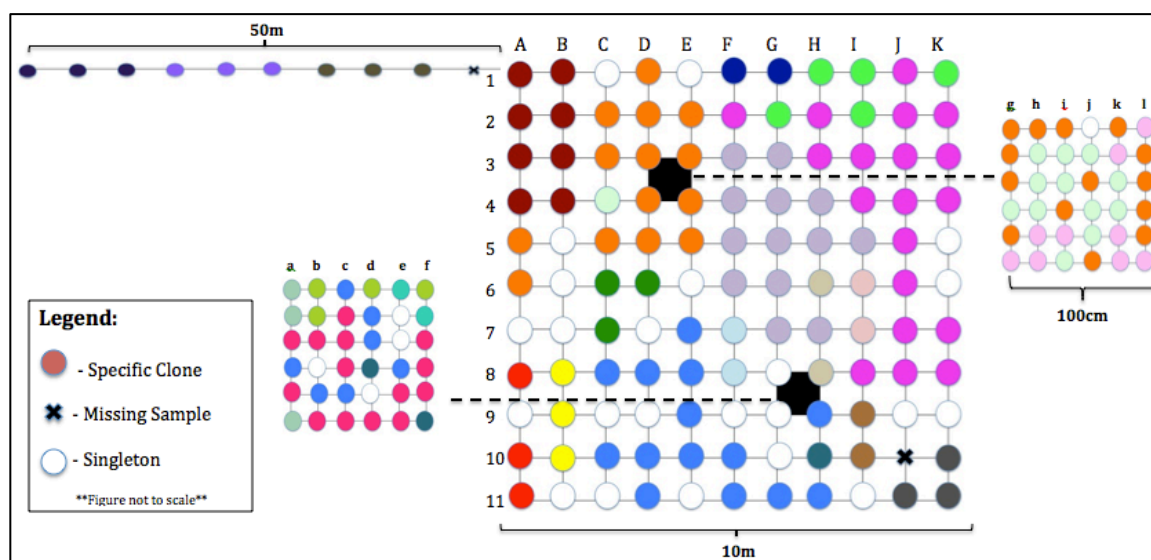
Figure 7: A visual representation of the 53 genotypes collected in 2014 at Upper Phillips Creek marsh. Same-color symbols indicate samples that were from the same genotype while white symbols indicate genotypes that were unique and **x** indicate missing samples.
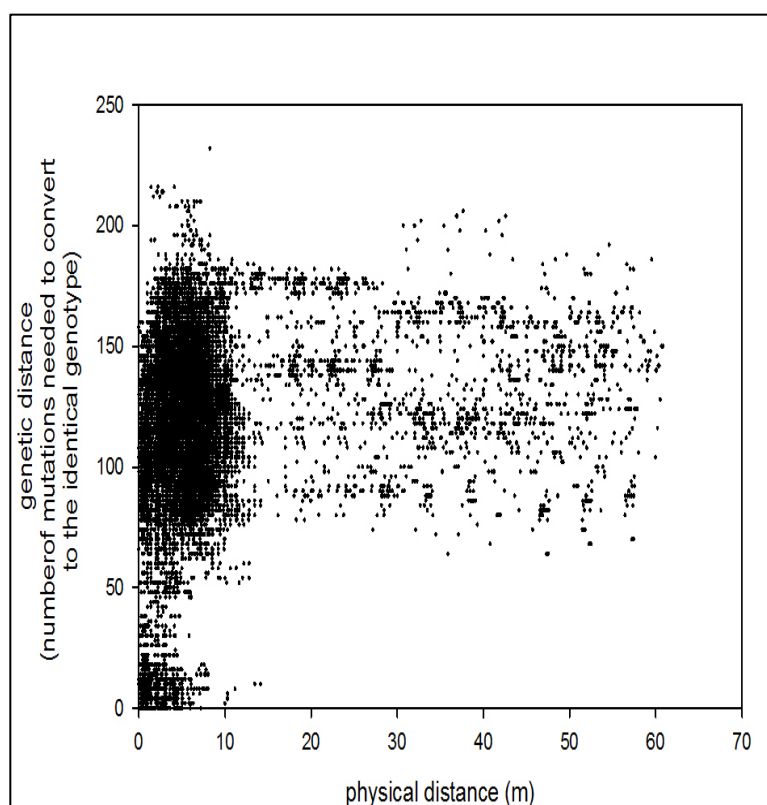
Figure 8: Semivariogram comparing the genetic distance and the geographic distance of samples at Upper Phillips Creek marsh.

Q2 – Genetic Relatedness of 5 marshes, 2013 data

Of the nine-microsatellite markers across the 50 samples from all five marshes (UPC

ITM, OHM, CLM, and LPC), 136 unique alleles were identified (Table 2). Each of the five

marshes had 10 samples analyzed (Table 4). In UPC (analyzed the first time in 2013), CLM, and

LPC, there were an average of 7 alleles per loci, of which 5 were considered effective. In UPC

and CLM, 8 unique multilocus genotypes (MLGs) were identified. In LPC, 10 unique MLGS

were identified and all 10 were found to be effective. In ITM, there was an average of 4 alleles

per loci and in OHM, there was an average of 8 alleles per loci. ITM had 3 unique MLGS, 2

effective; and OHM had 10 MLGS, which were all effective. ITM was the most clonal marsh

with only 3 unique genotypes, with the most frequent genotype consisting of 8 ramets (Table 4).

Table 4. Clonal diversity measures for 50 samples from the five marshes sampled in 2013.
Statistics were computed using GenoDive.

| Marsh	N	A	A_E	Number of (MLGs)	Number of effective MLGs	Most frequent genotype (number of ramets)	Most frequent genotype (% of samples)	Simpson's diversity index
UPC	10	7	5	8	6	3	30	0.933
ITM	10	4	3	3	2	8	80	0.378
OHM	10	8	5	10	10	1	10	1
CLM	10	7	5	8	7	2	20	0.956
LPC	10	7	5	10	10	1	10	1

The Simpson's diversity index was calculated for each marsh. UPC had a high diversity

index of 0.933, which was very similar to the 2014 sampling results (Table 4). OHM and LPC

had an index of 1, indicating that all samples had unique MLGs. ITM had the lowest Simpson's

diversity index of 0.378, indicating low clonal diversity.

To compare similarity between the genotypes from the different marshes, a dendrogram

representing percent similarity between each sample was constructed (Figure 9). All 50 samples

were compared to one another in order to determine if each marsh was a distinct population from another. Thirty-nine unique genotypes cluster from a range of 98.5% similarity between samples OHM28 and OHM29 to 0% similarity for sample pair OHM30 and CLM31. These results show that genotype similarity can be greater between individuals from different marshes than between samples collected within the same marsh. For example CLM31 clusters more closely with the large clone at ITM (55% similarity) than with CLM32 (22.9% similarity), even though CLM31 and CLM32 were samples taken 10 m apart (Figure 9).

Percent Similarity
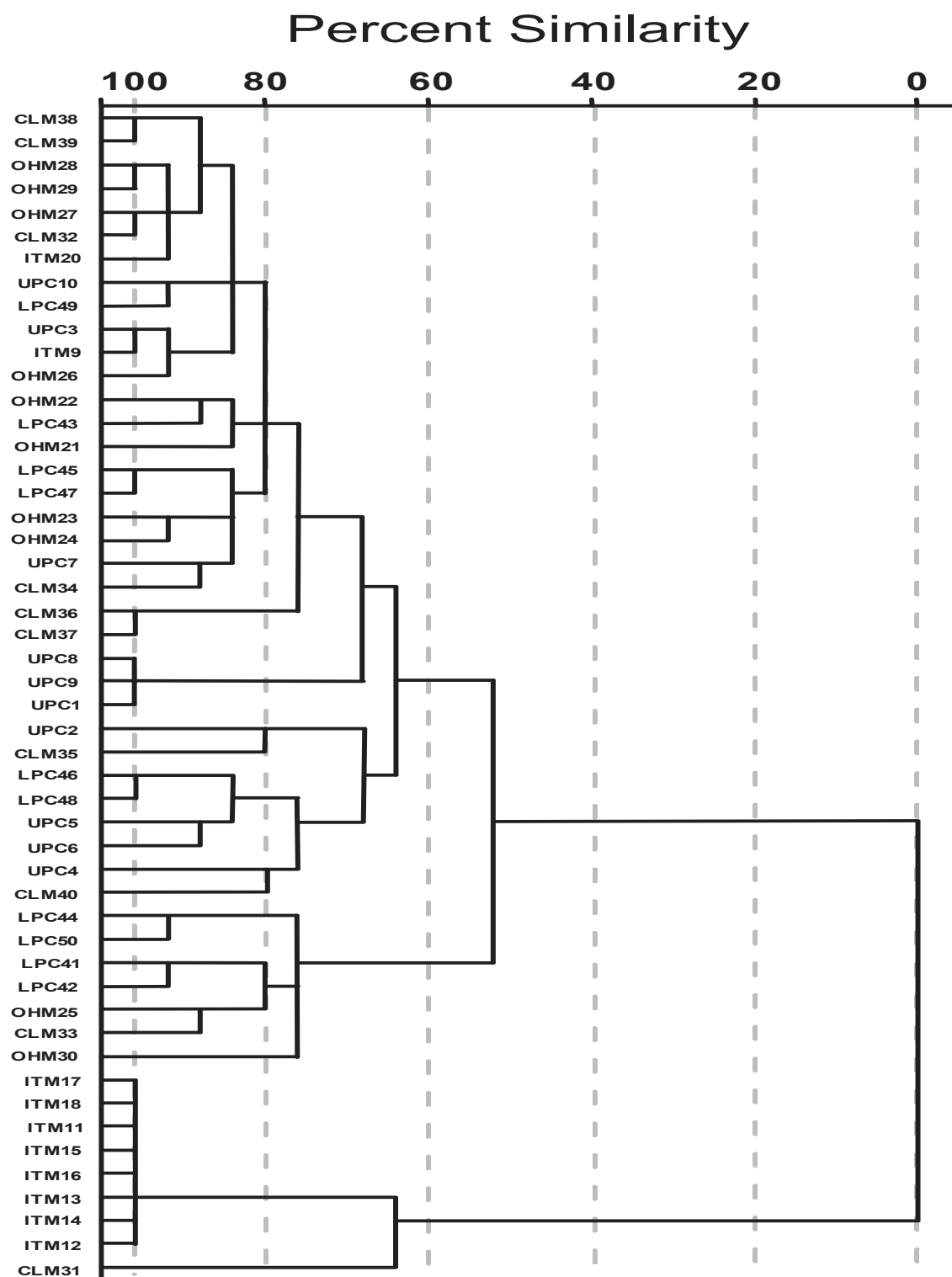
Figure 9: Cluster analysis of samples collected at five marshes in 2013. Samples were collected along an elevation contour at 10 m intervals. The dendrogram shows the percent similarity of the 39 unique genotypes from the 50 samples based on Nei's diversity index. Cluster analysis was done in SPSS.

Discussion

The absence of a viable seed bank has lead marsh ecologists to assume that *Spartina alterniflora*, a clonal plant, colonizes open patches of the marsh by clonal growth and that populations consist of a few very large clones (Hartman 1998, Shumway 1995). Limited diversity and large clones were expected in this study; however, I found evidence of a high degree of sexual reproduction and seedling establishment in four out of the five marshes along the Eastern Shore. High clonal diversity indices and many genotypes indicated that seeds are more important in the growth of *S. alterniflora* in these salt marshes than previously understood.

Q1 - What is the spatial structure of *S. alterniflora* genotypes in Upper Phillips Creek (UPC) marsh?

The genetic spatial-structure of *S. alterniflora* plants in Upper Phillips Creek (UPC) marsh was mapped and visually represented to draw conclusions regarding colonization strategies within the marsh. The clonal map (Figure 7) suggested that sexual reproduction and seedling establishment of *S. alterniflora* was occurring in this marsh. The map shows that there were a small number of large clones and a high number of individual genotypes (called singletons in the literature), thus leading to many multilocus genotypes (MLGs, see glossary on p. vii) in UPC marsh. This spatial structure correlated with the high clonal diversity index (Table 4), indicating that sexual reproduction is important to the cordgrass population within UPC marsh. Further, it was found that plants that are physically close in space can be genetically different (Figure 8). These data counter the idea of extensive clonal expansion (Shumway 1995).

Several plant colonization strategies have been proposed for *S. alterniflora* as alternatives to clonal expansion including "initial seedling recruitment" (ISR), "recruitment at windows of opportunity" (RWO), and "repeated seedling recruitment" (RSR) (Travis et al. 2004).

Characteristics of ISR species include a small seed bank, seedling recruitment in areas where

disturbance has occurred, and reduced clonal diversity as the population ages (Travis et al. 2004,

Erikkson 1989). Although *S. alterniflora* has a small seed bank, Travis et al. (2004) proposed

that *S. alterniflora* might be more characteristic of a RWO species. Species exhibiting RWO

characteristics have high levels of diversity like a species exhibiting repeated seedling

recruitment (RSR species); however, the recruitment is more sporadic, and thus potentially

correlated with small-scale disturbances (Travis et al. 2004). The high clonal diversity index

coupled with the many MLGs at UPC marsh may suggest that UPC marsh has experienced or is

experiencing small-scale disturbances that create a 'window of opportunity' allowing seedling

recruitment establishment. While the data presented in this thesis does not clearly support any of

these alternative colonization strategies, disturbances have occurred in UPC marsh that may

create windows of opportunity for seedling establishment such as drought (Porter et al. 2014),

wrack deposition (personal observation), salt-marsh dieback (Marsh et al. submitted), and eat-

outs (J. Haywood, personal communication).

   The population statistics of *S. alterniflora* in UPC marsh also provided evidence that the

marsh most likely has been experiencing a disturbance or is still recovering from one. A low,

positive inbreeding coefficient was found suggesting that there is a low level of inbreeding

depression within UPC marsh. Inbreeding depression decreases the overall fitness of the

population reducing the ability of a population to respond to environmental change. One

explanation of inbreeding depression could be from "biparental inbreeding" or mating between

close relatives (Nuortila et al. 2002). Because there is no *S. alterniflora* seed bank and the seeds

are viable for only about two weeks after maturation, there is little opportunity for seedling

recruitment from other nearby populations. Thus, if the *S. alterniflora* is in fact a RWO species,

seedling recruitment is most probable from within the immediate UPC marsh population resulting in a situation that provides ample opportunity for ramets to reproduce with their close relatives, giving way to an inbreeding depression. This low level of inbreeding depression could further predispose the marsh to disturbance thereby further favoring seedling recruitment. In order to confirm that the type of inbreeding in the marsh is "biparental," additional parent-genetic analysis would have to be performed.

The presence of triploids additionally supports the idea of disturbance within UPC marsh playing an important role in population genetic structure. Members of the genus *Spartina* have a basic chromosome number of $\chi = 10$; *S. alterniflora* is considered a hexaploid with 60-62 chromosomes or has 6 sets of the same 10 chromosomes (Ainouche et al. 2009). Although a hexaploid, the microsatellites of *S. alterniflora* behave as diploid markers (Blum et al. 2004). Thus, indication of a triploid would indicate a deviation from the typical ploidy. A total of 13 different triploids were found within UPC. Some plants experience changes in ploidy as a response to abiotic stress or are more frequent in extreme environments (Madlung 2013). Plants with changes in ploidy have been hypothesized as conferring a greater ability to adapt to environmental stress. Liu and Adams (2007) examined the expression of a gene in cotton (*Gossypium hirsutum)* under different abiotic stress treatments. They (Liu and Adams 2007) found that each stress treatment altered the gene expression of the genes depending on their ploidy. The indication of triploids could correspond with disturbance-induced stress at UPC marsh.

Thus, the question becomes whether or not UPC marsh is experiencing seedling recruitment due to the documented dieback in 2004 or if UPC is experiencing some type of ongoing disturbance. Additional experimentation would be required to establish a link between

disturbance and the genetic population structure of the UPC marsh *S. alterniflora* population. However, it is clear that sexual reproduction is more prevalent within the marsh than previously thought. In order to better understand the role of sexual reproduction in this UPC marsh population, sampling grids similar to those used in my experiments but distributed throughout UPC marsh would be required. If similar high clonal diversity was found throughout the marsh, then UPC marsh may be experiencing some type of disturbance, thereby opening up the opportunity for sexual reproduction to dominate. Alternatively, experimental disturbances could be created to examine the impact on clonal diversity.

Although the indication of the high level of clonal diversity in UPC marsh differs from the general hypothesis of dominant clonal growth in salt marshes (Shumway 1995), other investigators have found evidence of the importance of sexual reproduction for *S. alterniflora.* For example, Richards et al. (2004) examined the connection between genotypes and marsh zones in Sapelo Island, GA salt marshes. They hypothesized that large clones would span across strong environmental and elevation gradients; however, they found high clonal diversity values of 0.96 and 0.99, higher than those at UPC marsh, indicating the presence of a high degree of sexual reproduction and seedling recruitment. Richards et al. (2004) draws attention to the potential "underestimated" importance of sexual reproduction, a conclusion that the results at UPC marsh also support.

Although the broad implications of Richards et al.'s (2004) work and mine highlight the critical role of sexual reproduction and the population genetics of a clonal plant, there are important differences. Richards et al. (2004) sampled across what they describe as a 'severe' environmental gradient from creek bank to high marsh zones and used allozyme genetic markers (DNA coding for proteins), concluding that large clones are limited to distinct zones along the

gradients. They (Richards et al. 2004) speculate that sexual reproduction may be important in coping with the differing conditions along the gradients. Strong gradients, like those examined by Richards et al. (2004), likely provide strong selection pressures that might be expected to increase genetic diversity of allozyme genetic markers across the gradients by selecting for genotypes adapted to the differing conditions. In contrast, the part of UPC marsh that was sampled was a homogenous environment where there was no apparent environmental gradient; yet, the clonal diversity index was nearly as high at UPC marsh as was found by Richards et al. (2004) where the selection could increase the potential for genetic variation.

In spite of the clear observation that sexual reproduction is critical to the genetic structure of the cordgrass population at UPC marsh, clonal growth appeared to be an ongoing and viable process as well. Clones were found at all three spatial scales at which samples were collected (5m, 1m, and 0.2m). The variation in scales illustrated the high degree of intermingling among ramets from different genets, which is typical of guerilla clonal architecture of *S. alterniflora* described by Castillo et al. (2010) (Figure 7). This highly intermingled spatial arrangement of ramets further promotes opportunities for sexual reproduction in the marsh due to the high degree of diversity among ramets in close physical proximity to one another. However, this architecture could also be driving the inbreeding present in the marsh as well. Although different genotypes, the ramets could be relatives thereby leading to an inbreeding depression.

When the clonal diversity index, inbreeding coefficient, and clonal spatial structure are examined holistically, it is apparent that sexual reproduction is an important colonization strategy in UPC marsh, but seedling recruitment into UPC marsh from other populations may be insufficient to overcome inbreeding depression. Alternatively, the UPC marsh population may be genetically so similar to other populations within the region that seedling recruitment from those

populations is insufficient to overcome inbreeding depression. Thus, it is important to understand the genetic relatedness of the UPC marsh population to populations in nearby marshes.

Q2 - What is the genetic relatedness of UPC marsh to populations in nearby marshes?

Michael Blum and colleagues were among the first to develop microsatellite primers for *Spartina alterniflora* (Blum et al. 2004). Their motivation for developing the primers was to examine the evolutionary history of *S. alterniflora* over a large geographic scale to provide insight into the mechanisms underlying the success of non-native *Spartina*. They (Blum et al. 2007) found evidence of low gene flow and isolation-by-distance of native *S. alterniflora*. The paper attributed many of the genetic differences of *S. alterniflora* to an interaction of factors, such as biogeographical provinces, physical barriers inhibiting migration, and response to specific environmental changes or disturbance (Blum et al. 2007). Their study lends to the discussion of the large geographic differences of *S. alterniflora,* but does not address the local genetic differences of *S. alterniflora.*

Given the unexpected results of high clonal diversity at UPC marsh, important questions arise regarding whether or not UPC marsh is an unusual situation or if UPC marsh is genetically related to other marshes within the VCR LTER. UPC marsh is hydrologically isolated from ITM, OHM, and CLM, thus it was hypothesized that there would be limited gene flow between these marshes. UPC and LPC were expected to be the most genetically similar due to their geographical location, 1km apart. These four marshes were sampled to better understand the genetic relatedness of marshes within the VCR LTER.

Although analyzed as five distinct populations, the results indicate that the marshes are genetically connected. It was hypothesized that cluster analysis of the individual ramets would show five distinct groups, one cluster for each marsh if the marshes were genetically isolated

(Figure 9). However, the 50 samples were very intermingled and were not clustered in any recognizable pattern; samples from UPC marsh were as closely related to samples from other marshes as they were to samples from UPC marsh. This mixture of samples from different marshes within clusters seems to suggest that the five marshes are genetically connected, sharing very similar alleles. The cluster analysis results mirror the autocorrelation results from UPC marsh (Figure 8), that plants can be physically close in space but genetically different and vice a versa, in this case in regards to marsh distance and connectivity.

The results of the genetic analysis at ITM are intriguing. The largest clone at ITM was 0% similar to its other samples (across 9 primers) at ITM and had a very low clonal diversity index (Table 5). Taken together, these two findings could suggest elimination of many clones through competition, leaving large clones that are very genetically different, explaining why the clones are not clustering together. Another possibility is that ITM is experiencing a low frequency of disturbance, and therefore experiencing fewer 'windows of opportunities' than the other four marshes.

The limited sampling (10 samples) within each marsh makes it risky to do more than speculate and begs more questions rather than providing insight into the mechanisms underlying the genetic structure of *Spartina* marshes within the region. For example, because UPC marsh was sampled at three different scales (in 2014) and clonal growth was found at each scale, would finer sampling resolution increase the number of alleles that were found at UPC marsh and at the four other marshes sampled in 2013? Would these four marshes have a spatial structure similar to UPC marsh in that ramets from different genets are intermingled? The results based on the Simpson's diversity index suggest that LPC, CLM, and OHM would all have similar spatial structures as UPC marsh. However, ITM seems to differ from the other five marshes, with a

greater clonal presence. If the ITM genetic structure is different, what combination of factors are responsible for those differences?

Link to Ecological Theory

The role of disturbance may be an influential mechanism in UPC marsh. The genetic connectivity of the VCR LTER marshes sampled and the high clonal diversity at UPC marsh suggests that sexual reproduction and seedling recruitment are prevalent in all of these marshes. Disturbance may be opening 'windows of opportunity,' which then favor colonization and establishment by seeds rather than through clonal extension.

The idea that disturbance can open up opportunities for other processes is a prominent theory in the field of ecology. It is especially important in dynamic systems, such as salt marshes (Brinson et al. 1995). Connell (1978) was the first to clearly articulate the potential for disturbance to play a critical role in the biodiversity of ecosystems based on his studies of biodiversity in tropical rainforests and coral reefs. Connell divides his 'intermediate disturbance hypothesis' (IDH) into two categories based on whether or not the system is typically in equilibrium or in disequilibrium. Due to the dynamic nature of salt marshes, they are seldom in equilibrium (Morris et al. 2002). When a system is in disequilibrium, Connell (1978) suggests that diversity is the highest when disturbances are intermediate in frequency and intensity. In this instance, as the interval between disturbances increases, diversity will also increase in response to elimination or reduction in the populations of potential competitors making resources relatively more available to new populations. When disturbance is too frequent, diversity will decline as the number of species resilient to more frequent or intense disturbances becomes fewer.

The IDH was developed to explain multi-species diversity in ecosystems and communities. More recently, Peleg et al. (2008) applied the concept to a single plant species and found that higher levels of microsatellite diversity was associated with intermediate levels of water stress than when plants were exposed to either constantly low or high levels of moisture. Here I suggest that IDH may have application to intra-population (i.e., clonal) diversity of *S. alterniflora* and could be one explanation of the high degree of clonal diversity in UPC marsh. As disturbances occur in the marsh, space is opened which allows for seedling recruitment and establishment, increasing genetic diversity of the population. As the disturbances become less frequent, expansion of the most competitive clones through vegetative growth would be favored, increasing the size of clones and further decreasing genetic diversity as less competitive clones are lost from the population. Thus, a genetically diverse population might be expected in marshes with intermediate levels of disturbance and highly clonal marshes may be representative of infrequent disturbances.

The timing of disturbances in *S. alterniflora* marshes may also be critical to population diversity. Disturbances occurring when viable seeds are present would maximize the potential for seed germination and establishment, and so, population diversity. Because *S. alterniflora's* seeds remain viable for only approximately one month post maturation, which means there is a very limited seed bank, and because seeds mature at different times even on the same plant (Mooring et al. 1971), the optimum timing for disturbances to promote seedling germination and establishment should be in August and September at the VCR (personal observation of flowering and seed production). At other times of the year, disturbance would favor clonal expansion by vegetative growth and therefore, potentially lower diversity. Examination of the types of disturbances and their frequency throughout the year could offer insight into what role

disturbance might play in facilitating either sexual (via seeds) or asexual (via vegetative) growth of *S. alterniflora* populations.

The five marshes studied for this project, while separate populations, were found to be genetically connected. This connectivity could result from among population pollination because *S. alterniflora* is wind-pollenated and the flowers are highly fertile (c.a. approximately 21-fold greater more fertile than *Spartina foliosa* (Anttila et al. 1998)). Alternatively, storm tides or wrack deposits might be responsible for dispersal of seeds among marshes, while at the same time being a source of disturbance favoring seedling recruitment from nearby marshes.

One of the perplexing aspects of the genetic analyses of the UPC marsh population is that this clonal population exhibits high genetic diversity in species that also shows evidence of inbreeding (Table 4). In a species that is able to self-pollinate, like *S. alterniflora,* in combination with the low number of effective alleles found in this population, genetic diversity should be low. The IDH is a way to explain these seemingly contradictory results. Disturbances may affect the genes within a population by altering the reproduction strategies, as well as providing selective pressure on certain genes. Clearly, more research needs to be done to better understand the influence of disturbance on the genetic structure of *S. alterniflora* populations and the implications of genetic structure on salt marsh responses to changing climate.

Restoration and Climate Change Implications

The indication of high clonal diversity and the high degree of sexual reproduction in UPC marsh of *S. alterniflora* provides useful information to support salt marsh restoration efforts. Restorations typically favor a limited number of genotypes that are handpicked for fast growth with little consideration of genetic diversity because it is cheaper and more easily accomplished than working with a variety of genotypes (Travis et al. 2004). For example, Travis et al. (2010)

found that restoration sites in Louisiana were less diverse than a nearby reference marsh. While it is a common restoration practice, use of a limited number of genotypes will establish a population that is susceptible to inbreeding depression and has limited resilience to major environmental disturbances.

If intermediate levels of disturbance have the potential to promote seedling recruitment thus increasing clonal diversity, restoration with a genetically diverse base population could promote more rapid restoration of salt marsh structure and functioning. Travis et al. (2002) suggests collecting seeds from at least three different sites to use in restoration of a salt marsh. This way a genetically diverse population is established, setting up the population with the ability to be resilient to disturbance and resistant to inbreeding. My results are consistent with those of Travis et al. (2010); their study and mine demonstrate that sexual reproduction is an important reproduction strategy for *S. alterniflora.*

The ability to sexually reproduce will become even more important as climate change rates accelerate. Climate change has the ability to increase the intensity of environmental disturbances. A genetically diverse population of *S. alterniflora* will have a greater likelihood of survival (Travis et al. 2002). It is also important to understand the frequency and intensity at which disturbances occur and are predicted to occur in the future. I have hypothesized that disturbance promotes seedling recruitment thereby promoting sexual reproduction, yet there may be a tipping point in which the magnitude of the disturbance is too great for the marsh, and seedling recruitment is no longer favored. This tipping point may be the point at which clonal growth becomes favored again rather than seedling recruitment. Furthermore, some disturbances may not create this 'window of opportunity,' but rather prevent it. Additional studies need to be

conducted to better understand this critical point between clonal growth and seedling recruitment.

Conclusion

The spatial and genetic structure of *Spartina alterniflora* clones in Upper Phillips Creek marsh is very complex. In a 10 m x 10 m area of Upper Phillips Creek marsh, 53 unique multilocus genotypes of *Spartina alterniflora* were found by molecular genetic analysis of nine microsatellites. Sixteen of the 53 genotypes were identified as contributing to the allelic richness of the population. The most frequent genotype or largest clone consisted of 28 plant stems or 14% of the 202 samples collected. 93 unique alleles were identified and there were an average number of 10 alleles per loci. The spatial pattern of the clones and the number of unique genotypes suggests that there is a high degree of sexual reproduction occurring in this marsh. The high degree of sexual reproduction (seeds) and the genetic relatedness of five geographically widely-spaced populations in the marshes in the VCR LTER illustrate that *S. alterniflora* clonal expansion by vegetative growth is not the predominant driver of population structure at the regional or individual marsh scale.

Although *S. alterniflora* displays several characteristics of an "initial seedling recruitment" species, the spatial structure of UPC marsh populations and evidence of seedling recruitment suggests that *S. alterniflora* is most likely a species that recruits at 'windows of opportunity' (RWO) species. Given the highly dynamic nature of salt marshes at the VCR LTER, disturbance may provide a window of opportunity that favors a sexual (seed-based reproduction) strategy over an asexual (clonal expansion via vegetative growth) strategy.

The high genetic diversity of clones yet low number of effective alleles and relatively high inbreeding coefficient (G_{is}) observed at UPC marsh can be explained by the Intermediate

Disturbance Hypothesis (IDH). While IDH has been used to explain multi-species diversity, it may also be useful to understand diversity patterns within a population. Disturbances can affect species intraspecifically, impacting diversity and reproductive strategies. If this is the case, then disturbances may be vital in marsh systems in order to maintain the structural integrity of the vegetation.

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Appendices

Appendix I. GPS coordinates for each *Spartina alterniflora* stem collected in June 2013. Ten plant stems were collected at each of the five marshes at Virginia Coast Reserve Long-Term Ecological Research sites. These samples were used to determine the genetic relatedness of the Upper Phillips Creek population to populations in nearby marshes (Q2).

Marsh	Plant	GPS (N)	GPS (W)
Upper Phillips Creek	1	37.45957	75.83501
	2	37.45952	75.83489
	3	37.45946	75.83485
	4	37.45938	75.83487
	5	37.45935	75.83489
	6	37.45939	75.83497
	7	37.45944	75.83504
	8	37.45951	75.83499
	9	37.45956	75.83495
	10	37.45962	75.83495
Indiantown	11	37.35117	75.90057
	12	37.3511	75.90058
	13	37.35103	75.90055
	14	37.35103	75.90047
	15	37.3511	75.90049
	16	37.35115	75.90045
	17	37.35118	75.9005
	18	37.3512	75.90056
	19	37.35112	75.90063
	20	37.35114	75.90054
Oyster Harbor	21	37.28786	75.92876
	22	37.28785	75.92866
	23	37.28782	75.9286
	24	37.28778	75.92865
	25	37.28773	75.92863
	26	37.28775	75.92879
	27	37.2877	75.92886
	28	37.28763	75.92899
	29	37.28764	75.92905
	30	37.28778	75.92844
Cushman's Landing	31	37.18211	75.94212
	32	37.18215	75.94215
	33	37.18222	75.94214
	34	37.18228	75.94207
	35	37.18227	75.942

	36	37.18222	75.94195
	37	37.18217	75.94194
	38	37.18211	75.94193
	39	37.18206	75.94188
	40	37.18202	75.9418
Lower Phillips Creek	41	37.36068	75.93994
	42	37.45367	75.83395
	43	37.45366	75.83405
	44	37.45372	75.83401
	45	37.45372	75.83406
	46	37.45375	75.83406
	47	37.45378	75.83409
	48	37.45389	75.83414
	49	37.45375	75.83428
	50	37.45388	75.83425

Appendix II. A detailed list of procedures for DNA extraction, PCR, and MegaBACE as performed in house for all samples. (Adapted from the Zieman Lab Protocols)

DNA extraction Using Qiagen kits (DNeasy Mini Plant Kit)

- Use Wet Sample – grind sand + 600 ul AP1 buffer in bowl, place in tube
- Add 4 ul Rnase A (vortex before using) to tube and close lids tightly, vortex the tube well.
- Incubate in dry-block for 10 min at 65 degrees C. Mix tubes 2-3 times during incubation by inverting tubes. (this step lyses cells)
- Add 130ul AP2 buffer, mix well by vortexing, then put tubes on ice for 5 min (this step precipitates detergent, proteins, and polysaccharides)
- Centrifuge at max speed for 5 min (this step separates the precipitates from the lysate solution)
- Pour lysate into lilac topper (QIAshredder Mini spin column) found in a 2ml collection tube (topper + tube is a single unit supplied by kit) and centrifuge at 13,200 rpm for 2 minutes (this step "shreds" DNA and removes most precipitates and cell debris).
- Transfer 400ul of the flow through fraction from previous step into a new microcentrifuge tube (our own) without disturbing the cell-debris pellet. Discard the remaining flow through and the tube.
- (Do this with manual pipette). Add 1.5 volume of AP3 Buffer (600 ul if you were able to get exactly 400ul from the last step) to the cleared lysate and mix by pipetting. Once fully mixed (the oil-slick look needs to be totally gone), pipet 600 ul of the mixture into the white topper (DNeasy Mini spin column) found in a 2 ml collection tube (topper + tube is a single unit supplied by kit). Centrifuge for 1 minute at >8,000 rpm and discard the flow through, reusing the collection tube in next step.(this step collects and concentrates DNA onto filter in white toppper)
- Pipet the remaining mixture from the microcentrifuge tube into the white topper returned to the same 2 ml collection tube. Centrifuge for 1 minute at >8,000 rpm and discard the flow through.
- Place the white topper into a new 2 ml collection tube (no top, part of kit), add 500 ul buffer AW, and centrifuge for 1 min at >8,000 rpm. Discard flow through and reuse collection tube in next step (this step washes DNA)
- Add 500 ul AW to the white topper and centrifuge for 2 minutes at high speed to dry membrane. Discard flow through and collection tube. (when removing tube from centrifuge, DO NOT splash white topper with flow through. If this happens recentrifuge. Need membrane to be ethanol free).
- Transfer the white topper to a microcentrifuge tube (labeled 1st elution tube) and pipet 75 ul AE buffer slowly onto membrane. Incubate for 5 minutes, then centrifuge for 1 min at >8,000 rpm. (this step elutes (pulls out of membrane and puts back into solution) DNA)
- Take white topper and place in new microcentrifuge tube (labeled 2nd elution). Pipet 75 ul AE buffer slowly onto membrane. Incubate for 5 minutes, then centrifuge for 1 min at >8000rpm
- Test both elutions on a DNA gel (refer to Running a gel for DNA quantification section for instruction) Decide which elution will be the experimental elution from which you make the working stock and which will be stored in the sample bank. (One with cleanest bands become the experimental elution)
- Make aliquots of stock for qualification, ~5-10ng/ul. in microcentrifuge tubes. Use filter-tips in original extraction tubes to avoid any contamination. Pipet 31 ul DNA from the experimental elution (30 ul is enough sample to make a feeder plate if it turns out you don't need to dilute the DNA) and put in working stock tubes. Use 1 ul DNA for a second DNA gel in order to determine the concentration of DNA (dilution factor (amount water added) depends on outcome of gel-keep record of these dilutions)
- Store Tubes in freezer.

Do a PCR with TypeIT (Use the 8 samples)

15ul reaction per sample.

Mix for	1 reaction	9 reactions (8, plus 1 extra)
TypeIT	7.5ul	67.5ul
Primer-F (1/4th of M13 primer)	0.06ul	0.54ul
Primer-M13Fam, Ned, or Vic	0.24ul	2.16ul
Primer-Rpig	0.24ul	2.16ul
H20 (top up to 14ul)	5.96ul	53.64ul

 --REMOVE PRIMER-F, add the .06ul to H20

Add 14 ul of master-mix to each of the 8 tubes in a strip-cap.
Or if testing in a plate use the width of the plate. You can test 12 different primers at once.

Template (test DNA)	1.0ul	-

Cycling program:

TYPEIT PCR Program (heated lid 105C)			
Step	Temp (degree C)	Time	Action
1	95	5:00	Initial Denaturing
2	95	:30	Denaturing
3	60	1:30	Annealing
4	72	:30	Extension
5	go to 2	30 times	Cycling
6	60	30:00	Final Extension
7	20	:30	Cool Down
8	End		

MegaBACE Prep and Run: ZNA Machine Notes

- At start:
    - Charge the multichannel 10, multichannel 1200, and the single 100 pipettes
    - Defrost the PCR dilution plate(s) (green) , vortex and centrifuge them
- Prepare: 6 matrix tubes, 6 buffer tubes, new buffer plate (blue), and sample plate (natural color) (old buffer plate should already be made)
    - Matrix tubes
        - Get 6 tubes per run from Meg's fridge-in crisper drawer
        - Spin at full speed (13.2 rpm) for ~1 min
    - Buffer plate
        - Use a trough (take from the bottom of stack, not the top) and the 1200 multichannel pipette
        - Get a plate from the box labeled "clean buffer plates"
        - Distribute 100uL of 1x LPA buffer solution into EACH well
        - Put the plate in a plastic bag and centrifuge up to 400-600 rpm and stop
        - Set near machine still in bag until ready to put into MegaBACE
    - Buffer tubes
        - Use clear centrifuge tubes and the remaining buffer in trough (throw away any extra buffer in trough after making plate(s) and tubes for the day)
        - Make 6 per run
        - Use a disposable pipette to fill to stripe ring on tube
        - Close with the blue/brown buffer tops and centrifuge (full speed, 1 min)
    - Sample plate
        - Rox
            - Get from Meg's fridge - labeled as "MegaBACE ET200-R"
            - Vortex the Rox well
            - Add 880 uL of bio-grade water to 15uL of Rox in a labeled colored microcentrifuge tube, vortex
        - Plate
            - Use the natural/clear colored plates
            - Label on the front side of plate: the primers on the left, run name in the center, and amount of Rox mix and amount of PCR product on the right
            - Unseal the defrosted diluted PCR plate (should have already vortexed and centrifuged)
            - Add 8 uL of Rox mixture to EACH well of the sample plate - use the single 100 pipet to distribute this
            - Add 1.5uL of diluted PCR product to the sample plate - use multichannel pipet
            - Put a clear plastic sheet on the sample plate and briefly centrifuge (up to 300 rpm and stop - confirm all liquid is in the bottom of the well, re-centrifuge as needed)
            - Place plate in thermocycler if going to run the program 'Eric': PREMEGA; or turn on the heat block with plate insert in place to 120° if you will do the premega step manually
- Create Plate files (you must do this before running programs in the ICM)

- Import file to ICM
- Starting the run
    - In Instrument Control Manager, on Instrument Control tab
        - Start 'Matrix Fill and Prerun'
        - Follow the program prompts displayed on the machine screen
        - If it has been more than 15 min since you ran 'Matrix Fill and Prerun' before running 'Inject Samples' you will be prompted to run 'Prerun Only'
    - Have ready: old buffer plate (in machine or right in front of it), matrix tubes, buffer tubes, new buffer plate and sample plate
    - Start PREMEGA with sample plate in the thermocycler after the "load new buffer plate" step or run it manually - 1 min at 95 on heat block, 2-3 min on ice block
    - Start 'Inject Samples and Run' - have nanopure tank and sample plate ready
        - Follow the program prompts
    - After the run has started:
        - Check run time (should be 70 min - may need to add 5 min by clicking on the run time and then adding 5)
        - Check the current monitor - under 'Options' (all wells should be between 9-12)

Appendix III: Forward and reverse sequences of the nine microsatellite primers used in this study that were known to amplify *Spartina alterniflora* DNA. The calculated expected heterozygosity of the each primer is also shown. All information is from Blum et al. (2007).

Spar1)   Forward: TTACCCTCATCCGAGTCAAAA
      Reverse: GGTGGCGGTGTGGTTCAC
      Repeat Motif: $(CT)_{13}$
      Expected Heterozygosity: 0.76

Spar2)   Forward: GAAGGACGAGTCTCATTTGG
      Reverse: GGCTGCCCCTGTTTCACG
      Repeat Motif: $(CT)_{14}$
      Expected Heterozygosity: 0.66

Spar3)   Forward: CTCAGCTTCCTCCAGAGTGC
      Reverse: TTGAAGAGACGTGGGAATACG
      Repeat Motif: $(CTT)_{6}$
      Expected Heterozygosity: 0.45

Spar4)   Forward: GCCTTCTCGGTCCTTCAG
      Reverse: TGGGTTGTCGCAGTTATTGG
      Repeat Motif = $(AAG)_{15}$
      Expected Heterozygosity: 0.52

Spar5)   Forward: AGGTAACACCGAACGAGTC
      Reverse: CCTACGACATCACCGATA
      Repeat Motif = $(AG)_{18}$
      Expected Heterozygosity: 0.70

Spar6)   Forward: CGGTTGTTTTTGATTGTC
      Reverse: GGTTCTTGGGGAGTTGATTTC
      Repeat Motif = $(CT)_{18}$
      Expected Heterozygosity: 0.61

Spar7)   Forward: TTTCATTTCTGCCGCTTTTAC
      Reverse: GTCGCCCCCTAATCTTTCTC
      Repeat Motif = $(AG)_{11}$
      Expected Heterozygosity: 0.55

Spar8)   Forward: CTAAGGTCCCAAACGACGAC
      Reverse: GCGACGAGCGAGGATTTAC
      Repeat Motif = $(AG)_{14}$
      Expected Heterozygosity: 0.57

Spar9)   Forward: GTGGCCTAGCCTATCGACCT
      Reverse: TGAATGGAAAGGGGAAATGA
      Repeat Motif = $(CT)_{12}$
      Expected Heterozygosity: 0.68