Genetic diversity in *Spartina patens* in remnant patches in the New Jersey Meadowlands

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Habitat fragmentation is a factor that influences virtually all plant and animal communities. For plants, it typically reduces the size, and increases spatial isolation of populations and causes a decrease in genetic variation. *Spartina patens*, a clonal and salt-tolerant grass, is commonly used in local high marsh ecosystem restoration. When an ecosystem is restored, as is the case for many urban salt marshes, the genetic profiles of plants propagules employed are often ignored. This investigation was conducted not only to identify the genetic profiles of *S. patens* in the Hackensack Meadowlands New Jersey for restoration, but also to understand the influence of patch size in distribution of genetic diversity of populations.

To address these questions, ISSR (inter-simple sequence repeats) analysis was utilized to establish molecular genotype signatures for clones of *S. patens* that have been used for restoration. Approximately 83% of polymorphic
bands were obtained using fourteen primer combinations. The number of polymorphic bands among the six populations/patches ranged from 15 to 52. Shannon’s index also indicated that larger patches had higher genetic variation than small patches had. Analysis of the resulting patterns suggests that the Hawk Property Large, River Bend Small, and Fish Creek Large populations are genetically closely related. The River Bend Large population is related to Fish Small, and Hawk Property Small. This indicates that geographical distance is not related to genetic variation among populations. Based on the analysis of molecular variance (AMOVA), 58% of the genetic variation accounts for the differences among populations. It indicates that the divergence among populations is higher than the variation within population. In order to enhance the conservation of habitat of S. patens, the samples should be collected all over the mash. In this way, it may ensure the survival of the species when they were transplanted to a new habitat due to more genetic variation.
Acknowledgements and/or Dedication

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# Table of Contents

Abstract ...................................................................................................................................................... ii

Acknowledgements and/ or Dedication ........................................................................................................ iv

Table of Contents ........................................................................................................................................ v

Lists of Tables ........................................................................................................................................... vi

List of Illustrations .................................................................................................................................... vii

Introduction .................................................................................................................................................. 1

The study organism: *Spartina patens* ........................................................................................................ 6

Molecular markers in plant ecological research ....................................................................................... 8

Material and Methods ............................................................................................................................... 14

Description of sampling site ..................................................................................................................... 14

Collection of genetic material ................................................................................................................... 15

ISSR procedure ......................................................................................................................................... 16

Data analysis ............................................................................................................................................... 17

Results ....................................................................................................................................................... 19

ISSR polymorphism .................................................................................................................................. 19

Discussion .................................................................................................................................................. 21

Genetic structure of populations ............................................................................................................... 25

Implications for conservation ................................................................................................................... 26

Conclusion ................................................................................................................................................ 27

Reference .................................................................................................................................................. 28

Curriculum Vitae ..................................................................................................................................... 46
Lists of Tables

Table 1. List of sample locations of *Spartina patens*................................. 42

Table 2. Sequences and their annealing temperatures of selected primers. ............................................................................................................. 43

Table 3. Genetic diversities in *Spartina* populations, measured as the number of polymorphic ISSR marker per patch. ................................. 44

Table 4: Analysis of Molecular Variance (AMOVA) for six populations of *Spartina patens* in the Meadowlands, New Jersey............................ 45
List of Illustrations

Figure 1: Phylogenetic tree of the genus *Spartina*. ........................................... 37

Figure 2. The principle of ISSRs. ........................................................................ 38

Figure 3: Map of Locations of *S. patens* sample collection sites in
Hackensack Meadowlands, New Jersey.............................................................. 39

Figure 4. Dendrogram of six populations of *S. patens* in Hackensack
Meadowlands, New Jersey.................................................................................. 40

Figure 5. Result of Principal Coordinates Analysis (PCA) of six
populations of *Spartina patens* in Hackensack Meadowlands, New
Jersey..................................................................................................................... 41
Introduction

Global biodiversity and ecosystem structure and functions are affected by invasion of exotic species and human disturbances. This becomes a significant issue for native species conservation and native habitat preservation to maintain the biodiversity. For example, human activities may lead to the invasion success of non-native species and result in habitat degradation or fragmentation for native species. When local communities are degraded, damaged or destroyed through human activities, restoration plays an important role to maintain existing habitat and biodiversity. Recent studies have shown the importance of using local ecotypes in restoration efforts. Hufford and Mazer (2003) showed that genotypic differentiation in plant materials used in restoration can be crucial for the success. Mayr (1963) documented that large genetic variation within local populations may increase a species’ ability to adapt to changing environmental conditions.

In order to achieve success with restoration efforts, three basic criteria must be met. First, protecting sufficient habitat must be done for the continued persistence of a species (Gilpin and Soule, 1986). Studies show that habitat fragmentation not only affects species richness, but also changes the composition of ecosystems and communities, and that it can alter the structure of populations (Bierregaard et al., 1992). The impacts of habitat fragmentation include increased mortality rates (Jules, 1998; Dixon et al., 2007), reduced
abundance (Flather and Bevers, 2002; Dixon et al., 2007), changed movement
patterns (Brooker and Brooker, 2002; Dixon et al., 2007), disturbed social
structures of populations (Ims and Andreassen, 1999; Cale, 2003; Dixon et al.,
2007), adversely affected environmental conditions (Laurance and Williamson
2001), and reduced population viability (Harrison and Bruna 1999; Davies et al.,
2001; Dixon et al. 2007).

Second, demographic information must be collected because life history
traits and initial conditions will influence survival, reproduction, long-term
population vigor, and short and long term genetic process (Lande, 1988). Finally,
once these fundamental criteria for population survival are met, genetic variation
needs to be considered as an issue in restoration and conservation (Fenster and
Dudash, 1994). Thus, the maintenance of genetic diversity is a crucial purpose in
the management of populations of endangered species conservation (Frankham
et al., 2002).

Genetic diversity allows a population to mount a successful evolutionary
response to unpredictable environmental challenges such as changing weather,
disturbance events, variations in resource availability, and population sizes of
competitors (Falk et al., 2006). Hoffmann and Willi (2008) showed that the level
of genetic variation influences the ability of species to respond to threats and
environmental changes. When a population has a high level of genetic variation,
it is better able to adapt to new environmental challenges. On the other hand, low
genetic variation in a population will limit a species’ ability to respond to the
changes in the short term and to persist in the long term (Amos and Harwood, 1998).

Genetic diversity is affected by several factors such as mating patterns, migration, natural selection, and genetic drift. Mating patterns are important because nonrandom mating can occur when a population interbreeds. Nonrandom mating includes inbreeding and outbreeding. Inbreeding, which can occur via selfing or biparental fertilization, decreases genetic diversity because the gametes are closely related and genetically very similar. Inbreeding by self fertilization is prevented in some plants through different mechanisms such as physiological, chemical or genetic barriers. Biparental inbreeding occurs in small populations when pollen or seeds are restricted spatially in their dispersal pattern (Ellstrand and Elam, 1993). Outbreeding tends to increase genetic diversity.

Genetic drift is the change in relative allele frequency, which can either increase or decrease by chance over time. When genetic drift occurs in small populations, it may decrease the genetic diversity of population or cause extinction through lost alleles in a population resulting in only one allele present at a particular locus. Natural selection is a process that pressures populations to become better adapted to their environment. Those individuals in a population suited to the new environment will produce more offspring through natural selection.
Genetic migration is a movement of genetic diversity between distinct populations of a species. It influences many important ecological and evolutionary properties of populations such as the adaptive ability in environmental changes. It also helps a species reduce genetic diversity and genetic drift. Population size and the movement of genes from one population to another population (gene flow) are factors that determine the possibility of adaptation of a species. Plants use pollen dispersal, seed dispersal and vegetative propagules to exchange genes between populations. Normally, gene flow can be calculated by the equation

$$N_m = \frac{(1-F_{st})}{4F_{st}}$$

in which $N_m$ is the number of number of migrants per generation and $F_{st}$ is the amount of the total genetic variation explained by the difference between samples. This equation is for diploids. For haploids, the equation for calculating gene flow is

$$N_m = \frac{(1-F_{st})}{2F_{st}}$$

(Cockerham and Weir, 1993; Xu, 2005). $G_{st}$ values are identical to $F_{st}$ values when a locus have more than two alleles (Nei, 1973). In addition, effective population size is another critical factor for maintaining genetic variation. Several studies show that small population size may lead to genetic erosion and increasing genetic divergence among populations by random genetic drift. Additionally, small population size may increase levels of inbreeding and reduce gene flow (Peterson, 2008).
Genetic divergence among subpopulations can be assessed by measuring the proportion of total genetic variation that exists among subpopulations. There are many genetic divergence criterion such as $G_{st}$, $F_{st}$, $\Phi_{st}$ (phi-st) and $R_{st}$ that concentrate on estimating genetic divergence among populations. Wright’s inbreeding coefficient ($F_{st}$) and Nei’s coefficient of gene variation ($G_{st}$) are common statistical methods to detect differences among populations. Both indicate the degree of heterozygosity within and among populations based on differences in allele frequencies (Wright, 1969; Nei, 1975; Chai, 1976; Wright, 1978). $F_{st}$ is the proportion of the total genetic variance included in a subpopulation relative to the total genetic variance of a species. Nei(1973) defined $G_{st}$ as the proportion of interpopulation genetic diversity to total genetic diversity. $\Phi_{st}$ is also an indicator that shows the proportion of the variance among populations relatives to the total variance. It is an analog to $F_{st}$ when the data are haploid or binary (Excoffier et al., 1992).

$G_{st}$ can be utilized for one or many loci and would not be influenced significantly by mutation rates. It also can be analyzed without the specific number of populations. Then, it is relatively responsive to changes in allele frequencies over time. Values of $F_{st}$ and $G_{st}$ are different from 0 to 1 (Nei, 1975; Hedrick 1985; Crow, 1986; Hartl and Clark, 1997). A high $F_{st}$ suggests that the differentiation is greater among rather than within populations. When $G_{st}$ in species is low to near 0, it means that the majority of variation occurs within populations. This shows that individuals within populations are likely to be
generically different, but each population has the same complement of alleles in
similar frequencies. On the other hand, when \( G_{st} \) is high, it means that individuals
within a population are relatively similar but populations are drastically different.

The study organism: *Spartina patens*

*Spartina* (Poaceae) is a genus of grasses of C4 cordgrass distributed over
the tropic, subtropics and temperate zones. It forms a monophyletic lineage in
the subfamily Chloridoideae, all species of which are erect perennial grasses with
strongly articulated leaves and ciliate ligules. There are thirteen species
represented in the genus *Spartina*: *Spartina alterniflora, Spartina anglica,
Spartina bakeri, Spartina cynosuroides, Spartina densiflora, Spartina foliosa,
Spartina gracilis, Spartina maritima, Spartina patens, Spartina pectinata, Spartina
spartinae, Spartina × townsendii, Spartina × caespitosa* (Baumel et al., 2002).

*Spartina patens* is a rhizomatous, perennial, salt-tolerant grass that forms
dense turf or sod with fine, matted and decumbent stems. *S. patens* can be
found from the U.S. Atlantic coast to Canada, the Caribbean and Central
America. It can dominate the upper salt marsh zone and colonize sand dunes,
swale grasslands, sand flats and coastal scrublands (Silander, 1984). *S. patens*
acts as a buffer against shoreline erosion and its dense root system stabilizes the
marsh from saline, brackish, and freshwater tidal flows (Hamer et al., 1988).
S. patens has been used in marsh planting efforts in different coastal areas along with other species due to it not only regulates abiotic stresses but also support populations of amphipods and crustacean for predatory fish and crabs. When S. patens was removed, abiotic stress increased, the abundance of detritivores reduced (Gendan and Bertness, 2010). S. alterniflora dominated in a low salt marsh are moving landward and replacing S. patens, Distichilis spicata, and Juncus gerardii mainly occupied in high marsh zone on northern New England coastline by the influence of global warming and subsequent sea-level rise (Donnelly and Bertness, 2001).

Although plants may produce both via sexual reproduction by seed and through asexual reproduction by rhizome, vegetative reproduction is the major mode of reproduction in this species. Rhizomes from a single plant spread out and from circular clones, forming extensive patches or meadows. Studies reveal that S. patens may have about 97% of the productivity of U.S. Gulf coast marsh (Pezeshki and Delaune, 1991; White and Simmons, 1998; Lonard et al., 2010). It is an important species for coastal conservation and reclamation project based on the character of phalanx growth and high productivity. Marchant (1968) demonstrated that the chromosome numbers of Spartina species are either tetraploid (4n=40), hexaploid (6n = 60- 62), or dodecaploid (12n = 122, 124)(Fig 1). S. patens is tetraploid and closely related to S. bakeri and S. gracilis, S. arundinacea, and S. pectinata.
Prior suggestions that plants propagating through clonal or asexual reproduction have low genotypic diversity (Ellastrand and Roose, 1987) have been disproven. Silander (1979), for example, showed that environmental factors associated with habitat differentiation correspond to genetic divergence among populations of *S. patens* in North Carolina based on common environment experiments. Silander’s research also supported the idea that the genetic diversity of populations in different habitats like dune, swale and salt marsh is associated with competitive ability. Further, some studies have demonstrated that clonal plants have moderate to high levels of intra- and inter- population diversity (Ellstrand and Roose, 1987; Hamrick and Godt, 1990). Although several studies suggest that clonal plants do not necessarily have lower levels of genetic diversity, the genetic diversity of *S. patens* in Hackensack Meadowlands is not clear yet.

Molecular markers in plant ecological research

Molecular markers have proven to be useful tools in evaluating genetic diversity, phylogenetic relationships, genetic fidelity, disease resistance, and early determination of sex in plants before they enter the reproductive stage (Sharma et al., 2008). The choice of molecular markers for investigating the structure and dynamics of genomes at the level of populations and of individuals is based on their precision, ease and speed of assay, and cost per specimen. There are several techniques to assess the genetic diversity of plant populations
such as allozyme electrophoresis, and polymerase chain reaction-based (PCR) markers such as restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), microsatellites and inter simple sequence repeats (ISSR). Each technique has its own advantages and disadvantages.

Allozymes are allelic variants of enzymes encoded by structural genes. Enzymes are proteins that consist of amino acids, which are electrically charged or neutrally charged. The net electric charge of an amino acid depends on the conformation of the protein. When a DNA mutation causes the amino acid sequence to change, the net electric charge of the protein may be altered and the overall conformation of the molecule can become modified. Since these changes will affect the protein migration rate in an electric field, the allelic variation can be detected by gel electrophoresis. Allozyme analysis is comparatively simple because allozymes can be analyzed with enzyme staining reagents without DNA extraction or the use of primers or probes. Its main weakness is the relatively low abundance and low level of polymorphism in allozymes. Furthermore, allozymes are enzymes that might be tissue specific and analyses will be thus affected when using different plant parts like root or leaf tissue (Kumaret al., 2009).

Restriction fragment length polymorphisms (RFLP) assesses to DNA fragments, usually within the range of 2-10 kb, that are the result of digestion of genomic DNA with restriction enzymes. These DNA fragments are separated by agarose gel electrophoresis and are detected by Southern blot hybridization to a
labeled DNA probe. Labeled probes are sequences contained with a radioactive isotope or with alternative non-radioactive stains such as fluorescein. The advantage of RFLPs lies in detecting co-dominant alleles and having high reproducibility under it high genomic abundance and random distribution. It is also relatively easy to score due to large size difference between fragments and no sequence information required. The disadvantages of RFLP are the requirement of laborious and technically demanding methodological procedures and high cost such as high quantity and quality of DNA and development of specific probe libraries for the species (Kumar et al., 2009).

Random amplified polymorphic DNA (RAPD) uses DNA fragments amplified by the polymerase chain reaction (PCR) using short synthesis primers of random sequence length from 10 -15 bp (Williams et. al, 1990). These oligonucleotides serve as both forward and reverse primers. Amplified fragments are separated by agarose gel electrophoresis, and polymorphisms are detected by the presence or absence of bands resulting from the amplifications. The benefits of RAPD are quickness and easy to assay because prior information of sequences is not necessary for primer construction. Besides, RAPD markers have a high genomic abundance and are randomly distributed throughout the genome. The limitation of the RAPD technique is its low reproducibility due to the sensitivity of primers that can affect the reaction under different conditions.

AFLPs are DNA fragments obtained after digestion of genomic DNA with restriction endonucleases, followed by ligation of oligonucleotide adapters to the
digestion products and selective amplification by the PCR. Polymorphisms are detected from differences in the length of the amplified fragments by polyacrylamide gel electrophoresis or by capillary electrophoresis. The strengths of AFLPs are their high genomic abundance, considerable reproducibility, and no sequence information needed. The disadvantages of AFLPs involve in possible nonindependence of fragments, problems of homology assignment of fragments, asymmetry in the probability of losing and gaining fragments and problems in distinguishing heterozygote from homozygote bands (Koopman, 2005).

Microsatellites or simple sequence repeat (SSR) loci are found throughout the nuclear genomes of eukaryotes and prokaryotes. They have also been identified in plant chloroplasts. Microsatellites range from one to six nucleotides in length and are classified as mono-, di-, tri-, tetra-, penta- and hexanucleotide repeats. They are tandemly repeated (usually 5-20 times) in the genome with a minimum repeat length of 12 base-pairs (Tóth et al., 2000). Although microsatellites are specific and highly polymorphous, they require knowledge of the genomic sequence to design specific primers. Besides, they may not be readily available for all species. The reason is that flanking sequences of interested species must be known to design 5’-anchors for PCR primers (Godwin et al., 1997). Furthermore, mutations in the primer annealing sites of microsatellite may cause the occurrence of null alleles, which may lead to errors in genotype scoring. This error will affect the detected genetic diversity of species (Semagn et al., 2006).
Inter simple sequence repeats (ISSRs) utilize DNA polymerase chain reaction (PCR) and microsatellite primers designed for di- or tri-nucleotide repeats which are anchored to one to three random nucleotides at the 3′ end or 5′ end such as (CA)$_4$ (Gupta et al., 1994, Fig. 2). The PCR products are separated by gel electrophoresis and scored as bands that represent the presence or absence of fragments of a particular size. Since PCR is used in the analytical procedure, ISSR has advantages over other markers. For example, there is no requirement of prior knowledge of target sequences for designing ISSR primers when compared with microsatellite markers or SSR markers (Li and Ge 2001). ISSR markers are more reproducible than RAPD markers since ISSR primers are designed to anneal to a microsatellite sequence and the length of primer is longer than RAPD primers (Sharma et al., 2008).

Although ISSR analysis has its weaknesses such as the possible non-homology of similar size fragments and reproducibility problems, several studies still proved ISSR as a useful tool for analyzing genetic diversity such as rice (Zietkiewicz et al., 1994; Goodwin et al., 1997; Parsons et al., 1997), corn (Kantety et al., 1995), and the Poaceae family in general (Akagi et al., 1996b). Several studies show that ISSRs can provide a higher reproducibility of bands in analyzing population genetics than RADPs (Nagaoka and Ogihara, 1997; Wolfe and Liston, 1998; Wolfe et al. 1998; Camacho and Listion, 2001). This is the reason that ISSR is chosen to use in this study.
Goals of thesis

Due to the invasion of non-native species (in particular a non-native genotype of *Phragmites australis*) and human activities in the New Jersey Meadowlands, high marsh patches previously dominated by *Spartina patens* are now fragmented and vary in size and the pattern of genetic diversity within and among remnant *S. patens* patches has not been known previously. In order to develop an appropriate conservation management strategy for preserving *S. patens*, it is necessary to clarify the genetic structure of the remnant *S. patens* patches in the Meadowlands. The first question addresses whether the current small patches are remnants of formerly larger patches that contain genetically close-related individuals or whether each small patch contain distinct individuals. Then, if remnant patches are heterogeneous, the following question is that whether the size of a remnant patch has any influence on the genetic diversity of *S. patens* within it. As the patches differed in current size, and plant material of these patches might differ in their suitability for restoration, it is important to understand the population genetic structure of these patches to establish a better management for *S. patens* conservation. The goal of this thesis was to collect and analyze samples to answer these questions.
Material and Methods

Description of sampling site

The New Jersey Meadowlands, also called the Hackensack Meadowlands, are the wetland ecosystems of the Hackensack River in northeast New Jersey. It is also the largest brackish water complex in the New York / New Jersey Harbor Estuary. The Meadowlands are about 16 kilometers long north-to-south and cover an area of about 8300 hectares, including residential, commercial, industrial development areas, landfills, roads and railways, natural uplands, and wetlands (Kiviat and McDonald, 2004). The vegetation of natural uplands and wetlands mainly consist of various marsh plant communities such as *Phragmites australis* (common reed) and *Spartina* marshes and hardwood swamp. The salinity of marshes in tidal area ranges from nearly fresh to very brackish (0 to 16 parts per thousand).

The Hackensack Meadowlands is an important habitat to fish and wildlife. In addition, it is a main area in the Atlantic Flyway route for supporting migratory bird species. It also provides habitat for over 275 plant species, 50 species of fish and shellfish, 25 species of reptiles and amphibians and 24 species of mammals (U.S. Fish and Wildlife Service, 2003). The common reed (*Phragmites australis*) is a common invasive plant in the Meadowlands that has also spread throughout North American wetlands. It has become the dominant plant species in the Meadowlands, crowding out native plant species, and has been reported to have
low value to local fish and wildlife. *Phragmites australis* has been found to reduce biodiversity in wetland ecosystems (Marks et al., 1994; Chambers, 1999; Myerson et al., 2000; Wilcox et al., 2003).

Due to the invasion of *Phragmites australis* in the Meadowlands, only a few portions of brackish marsh are still dominated by the low marsh cordgrass, *Spartina alterniflora*, and high marsh cordgrass, *Spartina patens*. Data have suggested that wildlife prefer to inhabit *Spartina* marshes rather than marshes dominated by invasive *Phragmites*. (Benoit et al., 1999; Able et al., 2003; Raichel et al., 2003). For example, short-eared owls and northern harriers forage in *Spartina* high marsh areas. Therefore, preserving the *Spartina* marsh is important for providing habitat to the wild life in the Hackensack Meadowlands. Fish Creek, Hawk Property and River Bend were selected for this study because they are a natural site in the Meadowlands, which is dominated by *S. patens* high marsh (Fig. 3).

Collection of genetic material

*Spartina patens* materials were sampled from paired small patch (<200 m²) and large patch (>3000 m²) in three locations in the Hackensack Meadowlands northern New Jersey (Table 1). Genomic DNA was extracted from fresh leaves and stems using Qiagen DNeasy Plant Mini kits (Qiagen, Valencia, California, USA) according to the manufacturer’s instructions. 100mg fresh leaves and stems were ground to a fine powder in a mortar with liquid nitrogen.
DNA was eluted with 100 ul AE buffer. The concentration of DNA was estimated by absorbance at 260 nm. Isolated DNA was stored at -20°C freezer until ISSR analysis was performed.

ISSR procedure

Fourteen microsatellite-based primers (out of 60 tested) from University of British Columbia (UBC), Vancouver, Canada (Ayres and Strong, 2001), were selected for PCR amplification (Table 2). Amplifications for ISSR analysis were conducted as follows: each PCR volume of 12.5 μl contained 6.25μl Promega GoTaq Green master mix (Promega, Madison, Wisconsin, USA), 1μl 20 μM primer, 1μl (40 ng) genomic DNA template and 4.25μl nuclease-free water. Amplifications were performed with a thermal cycler (PTC-100, MJ Research, Inc.) using the following procedure (after Ayres and Strong, 2001): denaturing at 94°C for 1.5 minutes, followed by 40 cycles of denaturing (15 seconds at 94 °C), annealing for 30 seconds at the appropriate primer melting temperature, elongation (2 minutes at 72 °C) and a final elongation step of 4 minutes at 72 °C. Final products were stored at 4 °C. Negative controls without DNA template were run for each experiment. PCR products were separated by electrophoresis on horizontal 1% (w/v) agarose gels with ethidium bromide in TAE buffer (90 Volt, 100mA) and visualized under ultraviolet light. Images were taken with a Kodak image system1D 3.5.4 with three to six second exposures and a Canon image
system with Sony image software. The molecular weight of each band was estimated using a 100 bp DNA ladder (Promega, Madison, Wisconsin, USA).

Data analysis

Spartina patens PCR products were analyzed by scoring the presence (1) /absence (0) of polymorphic bands. Ambiguous bands were not included in the analysis. Genetic diversity of populations was analyzed by Popgene (version 1.32) (Yeh et al., 1997). The parameters for estimating genetic diversity include the percentage of polymorphic loci, the number of polymorphic loci, and Shannon’s information index (Lewontin, 1972). Generally, calculation of observed heterozygosity (Ho) is included in studies of genetic diversity. Nevertheless, ISSR markers are dominant markers, and S.patens violates assumptions of Hardy-Weinberg due to it is polyploid and asexual reproducing, thus, observed heterozygosity (Ho) and the allele frequencies cannot be determined.

Yeh (1997) proved that the equation of the Shannon information Index is

\[ I = \sum p_i \log_2 p_i \]

where \( p_i \) is the frequency of the presence or absence of a band. As a result, Shannon’s information index is able to represent the level of heterozygosity of the population. Shannon’s information index has been used in other studies when dominant markers such as RADPs or ISSRs are utilized in clonal species (Li and Ge, 2001). A dendrogram of the genetic relationship of each patch was prepared using Treecon for Windows (version 1.3b) (Van de Peer and De Wachter, 1994).
Genetic differentiation within populations and among populations was estimated by using Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992). AMOVA is a method to calculate population differentiation directly from molecular data and to test hypotheses about genetic variation within the populations and among the populations and generates a Φ statistic (an F-statistic analog). Variance estimates were based on 1000 permutations. AMOVA was conducted using GenAlEx V6.3 (Peakall and Smouse, 2006).
Results

ISSR polymorphism

Fourteen primers produced clear and reproducible PCR amplicons. In total, the percentage of polymorphic bands (PPB) for this species was 83.84%. The genetic diversity within the populations ranged from PPB values of 15.15% (population RBS) to 52.53% (population HPL) (Table 3). For each of the three locations, number of polymorphic bands was less for small patches than for large patches (Table 3): FC small (27) < FC large (37), population RB small (15) < RB large (37), and population HP small (31) < HP large (52). This shows that genetic variation was related to the patch size and genetic variation was reduced in small populations. The Shannon’s index was calculated to find genetic variation within populations. It ranged from 0.0081 (RB small site) to 0.251 (HP large) (Table 3). All measures indicated that there were varying amounts of genetic diversity among populations.

The dendrogram (Fig. 4) indicated all populations are distinct populations in Hackensack Meadowlands. We can see distinct populations from the dendrogram but we cannot tell their genetic relationship to each other. The inter-population relationships in unknown based on this data. Principal Coordinates Analysis (PCA) (Fig 5) showed that the populations grouped into three distinct clusters. FCS, RBL and RBS were located close to each other in the ordination space. FCL and HPL were closed to each other and HPL form a separate group.
The PCA results indicated that there were no relationship between genetic
closeness and geographic distance as neighboring small patches and large
patches within a given location are typically were not genetically close.

The AMOVA analysis for populations showed significant differentiation (P
<0.001), with 42% of the differentiation attributed to within populations and 58%
attributed to among populations (Table 4). The phi PT estimates were high
(0.573) and highly significant (P < 0.001). These outcomes indicated that there
was a larger amount of variation among populations than within populations. The
coefficient of genetic variation among populations ($G_{st}$) was 0.575 which showed
57.5% genetic variation among S. patens populations. Gene flow ($N_m$) was
estimated as 0.369 illustrated as 0.369 individuals per generation. This
demonstrates a large amount of differentiation among populations with low gene
flow between them.
Discussion

ISSR markers have been established to study cultivar identification and genomic variation in plants (Wolfe and Liston, 1998). Many studies have supported ISSR markers as a powerful molecular tool, by virtue of its high reproducibility and efficiency (Nagaoka and Ogihara, 1997; Devarumath et al., 2002; Galvan et al., 2003). They also are used to estimate the genetic variability among of natural populations and related species (Ayres and Strong, 2001, Camacho and Liston, 2001; Deshpande et al., 2001; Wolfe and Randle, 2001).

In this study, fourteen ISSR primers were selected that provided 99 clear, reproducible amplicons, 83 of which were polymorphic. This leads us to conclude that ISSR markers are useful in analyzing genetic diversity of *Spartina patens* populations due to the level of polymorphism. In this study, ISSR markers show that large and small populations/patches are different. Large populations/patches had higher genetic diversity than small populations/patches. It expected that the genetic diversity between large patches and neighboring small patches would be very similar due to asexual reproduction and close geographic distance. However, the results show this not to be the case. Geographic distance and fragmentation may affect the genetic diversity in *Spartina* populations.

Relatively high seedling recruitment or increased somatic mutation might explain why the genetic diversity in populations in large patches is higher than in small patches (Hamrick and Godt, 1990, Lewis and Crawford, 1995; Maguire and
Sedgley, 1997; Ranker, 1994; Zawko et al., 2001). Hartl and Clark (1997) proposed that small populations possess lower levels of genetic variation than large populations after the influence of genetic drift or inbreeding. Given the recent history of S. patens in the Meadowlands, shrinking population size and fragmentation have probably been significant factors in shaping the residual patterns of genetic diversity. This may explain why the large patches have more standing genetic variation than smaller neighboring patches.

Alleles or genotypes that are non-randomly distributed in space represent ‘genetic structure’. In the Meadowlands, 58% of the genetic variation in S. patens is accounted for by population/patch differences, with the remaining 42% accounted for by variation within patches. Interestingly, the three ‘locations’ in the marsh account for virtually none of the variation, and the pairwise distance analysis and the Principle Coordinates Analysis indicate that genetic affinity is not necessarily closer for the small and large fragmented patches at a single location than it is for patches in different locations. However, a positive correlation was demonstrated between genetic distance and geographic distance when gene flow and genetic drift of populations reached equilibrium (Wright, 1942; Hutchison and Templeton, 1999). A possible explanation may be that gene flow was not a significant factor for genetic diversity among the small and large fragmented patches of a single location.

The high $G_{st}$ value (0.575) and the low $N_{m}$ value (0.369) both indicate rapid genetic differentiation among the six populations/fragments. Loveless and
Hamrick (1984) indicated that genetic diversity of plant populations is largely influenced by mating system, genetic drift, evolutionary history, and life history. Generally speaking, outcrossing species have a higher degree of genetic diversity than selfing and clonal plants (Rossetto et al., 1995). *S. patens* is a cross-fertilized plant (Lonard et al., 2010) and although *S. patens* uses vegetative reproduction as its major spreading mode, sexual reproduction does occur in *S. patens*. This may increase the possibility of genetic recombination and subsequently increase the genetic diversity. This may also explain why populations of *S. patens* in the Meadowlands had high genetic variation. However, comparing with the mean $G_{st}$ value presented by Nybom and Bartish (2000), self-fertilization appears to have occurred in populations of *S. patens* in Meadowlands ($G_{st}$ = 0.58). Nybom and Bartish (2000) compile different mean $G_{st}$ values for selfing plants ($G_{st}$ = 0.59), mixed mating plants ($G_{st}$ = 0.19), and outcrossing plants ($G_{st}$ = 0.23).

Reproductive systems are correlated with genetic diversity. This may explain why *S. patens* uses asexual reproduction and displays self-fertilization. Reproduction may occur via selfing and asexual reproduction when the environmental conditions decrease opportunities for a union of gametes produced by different individuals. Under the certain circumstances, selfing and asexual reproduction are beneficial due to an individual passing on copies of its genome for every copy passed on by an outcrossing individual. This “cost of outcrossing” can provide fitness advantage to help a population resist
environmental changes and survive. In addition, these two reproductive systems could be considered as a reproductive assurance mechanism or a means of fixing co-adapted genes under pollination limitation (Barringer, 2007). Furthermore, Barringer (2007) observed that annual, herbaceous perennial and woody perennial polyploid plants generally have higher rates of self-fertilization than diploid plants. This provides evidence that self-fertilization appears to have occurred in populations of *S. patens* in Meadowlands due to the polyploid nature of *S. patens*.

Gene flow is an important factor maintaining genetic diversity of populations in that pollen and seed dispersal and population size contribute to gene flow. Slatkin (1987) reported that gene flow could prevent decline of genetic variation within populations and decrease inter-population differentiation. Soltis and Soltis (1990a) observed that Nm represented the level of gene flow among populations. If Nm is larger than one, the high level of gene flow among population is indicated. Compared with the values of mixed-mating (Nm = 0.727) and outcrossed animal-pollinated species (Nm = 1.154), gene flow per generation of *S. patens* is low (Nm = 0.369).

The low Nm of *S. patens* indicated limited gene flow among populations. Wright (1965) suggested that small populations have distinct genetic differentiation from genetic drift when Nm values are lower than 1.0. A possible explanation could be that genetic drift could happen in the small populations and random loss and fixation of alleles could occur in populations over time. Isolation
or fragmentation also could affect gene flow among populations, because when populations are isolated, the chance of inbreeding may increase and reduce the genetic diversity. As a result, whether the population size of *S. patens* is disturbed via human activities or invasion of non-native species, genetic drift may occur and lead to a decrease in genetic variation and differentiation. Silander and Antonovics (1979) discovered that pollen dispersal in *S. patens* is a factor affecting gene flow due to the short distance of pollen dispersal.

Genetic structure of populations

PCA showed that the populations/patches are distinct of *S. patens*. The results suggest that the species is not monoclonal in the Meadowlands. There are multiple genets that exist in the different patches. *S. patens* utilizes asexual reproduction as the major reproduction. Asexual reproduction is advantageous resulting in the ability to forge for resources, supporting the establishment of offspring or reducing the mortality risk of a genet. But, it also has disadvantages, including like reduction of genetic diversity, and easily transplanting diseases through rhizomes. Many clonal plants have the capability for sexual reproduction and display a range of genetic variability (Ellstrand and Roose, 1987; Widén et al.,1994). This could explain why multiple genets exist in the different patches because genetic combination and seed recruitment could take place when sexual reproduction occurs.
Implications for conservation

Falk and Holsinger (1991) describe the goal of conservation as maintaining the genetic diversity and evolutionary processes in natural populations in order to prevent extinction. The level of genetic variation directly influences the ability of a population to respond via selection and adaptation (Huenneke, 1991). Thus we can expect that a species would not respond well under environmental changes if it has a narrow genetic diversity. Therefore, information about the genetic diversity of the species can make conservation efforts more efficient.

The common reed (*Phragmites australis*), a common invasive plant, has become the dominant plant species in the Meadowlands. In order to maintain the habitat of *S. patens*, it is important to understand the genetic structure of local populations of *S. patens*. Before choosing the source of population for recovery plan for *S. patens*, the preparation should include information of the genetic composition of each population. Due to the value of *S. patens* in the salt marsh, broad conservation at the Meadowlands would be enhanced by returning the site to dominance of *S. patens*. Based on the results of this investigation, the samples for transplanting should be collected from all over the marsh and predominately from more genetically diverse larger patches. This would allow for the largest possible expansion of genetic coverage and ensure the survival of the species.
Conclusion

Fragmentation can influence the genetic structure of a population by isolation or by reduction of the population size. ISSRs are useful molecular markers, with which to analyze the genetic diversity within a species, by virtue of a high level of polymorphism. In this study, the genetic variation among patches is larger than that within patches. In addition, the internal genetic variation of large patches is larger than that within the small patches. To maintain the dominance of *S. patens* in the Meadowlands habitat, it is important to understand the genetic structure of local populations of *S. patens*. It can be an indicator for long-term *S. patens* population sustainability.
Reference


Figure 1: Phylogenetic tree of the genus *Spartina* (from Ainouche, 2004).
Figure 2. The principle of ISSRs. Three illustrations represent ISSR-PCR with a single primer (AG)$_n$, unanchored (a), 3'-'anchored(b) and 5'-'anchored (c) targeting a (TC)$_n$ repeat to amplify PCR product flanked by two inversely oriented (TC)$_n$ sequences. (a) Unanchored(AG)$_n$ primer can anneal any (TC)$_n$ repeat region on the template DNA; (b) (AG)$_n$ primer anchored 2 nucleotides (NN) at the 3' end can anneal at specific region on the template DNA and make clear bands (c) (AG)$_n$ primer anchored with 2 nucleotides (NN) at the 5' end anneals at specific regions and amplifies part of the repeat region producing larger size PCR product. (from Semagn et al., 2006 ).
Figure 3: Map of Locations of *S. patens* sample collection sites in Hackensack Meadowlands, New Jersey. The black spots represent the location of *Spartina* large patches. The yellow spots represent the location of *Spartina* small patches (from Holzapfel and Kirby 2011).
Figure 4. Dendrogram of six populations of *S. patens* in Hackensack Meadowlands, New Jersey. Dendrogram generated using UPGMA (unweighted paired group method of cluster analysis using arithmetic average) analysis shows genetic dissimilarity in populations. The numbers above the branches indicate the bootstrap value among the samples. Cut off point is fifty.
Figure 5. Result of Principal Coordinates Analysis (PCA) of six populations of *Spartina patens* in Hackensack Meadowlands, New Jersey. PCA of six populations of *Spartina patens* sampled from Meadowlands in New Jersey based on ISSR fragments.
Table 1. List of sample locations of *Spartina patens*. Large denotes large patches (>3000 m²). Small denotes small patches (<200 m)

<table>
<thead>
<tr>
<th>Location</th>
<th>Sample collected site</th>
<th>Sample size (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish Creek</td>
<td>Fish Creek Large (FCL)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Fish Creek Small (FCS)</td>
<td>10</td>
</tr>
<tr>
<td>Hawk Property</td>
<td>Hawk Property Large (HPL)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Hawk Property Small (HPS)</td>
<td>10</td>
</tr>
<tr>
<td>River Bend</td>
<td>River Bend Large (RBL)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>River Bend Small (RBS)</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 2. Sequences and their annealing temperatures of selected primers (Ayres and Strong, 2001).

<table>
<thead>
<tr>
<th>ISSR primers</th>
<th>Sequences</th>
<th>annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>849</td>
<td>GTG TGT GTG TGT GTG TYA</td>
<td>50.5</td>
</tr>
<tr>
<td>842</td>
<td>GAGAGAGAGAGAGAGAYG</td>
<td>47.2</td>
</tr>
<tr>
<td>854</td>
<td>TCT CTC TCT CTC TCT CRG</td>
<td>48</td>
</tr>
<tr>
<td>850</td>
<td>GTG TGT GTG TGT GTG TYC</td>
<td>53</td>
</tr>
<tr>
<td>840</td>
<td>GAGAGAGAGAGAGAGAYT</td>
<td>45.8</td>
</tr>
<tr>
<td>856</td>
<td>ACA CAC ACA CAC ACA CYA</td>
<td>49.8</td>
</tr>
<tr>
<td>857</td>
<td>ACACACACACACACACACYG</td>
<td>63.3</td>
</tr>
<tr>
<td>855</td>
<td>ACACACACACACACACYT</td>
<td>49.8</td>
</tr>
<tr>
<td>869</td>
<td>GTTGTTGTTGTTGTTGTT</td>
<td>55.9</td>
</tr>
<tr>
<td>812</td>
<td>GAGAGAGAGAGAGAGAA</td>
<td>44.3</td>
</tr>
<tr>
<td>835</td>
<td>AGAGAGAGAGAGAGAGYC</td>
<td>45.6</td>
</tr>
<tr>
<td>844</td>
<td>CTCTCTCTCTCTCTCTCRC</td>
<td>46.5</td>
</tr>
<tr>
<td>878</td>
<td>GGATGGATGGATGGAT</td>
<td>66.5</td>
</tr>
<tr>
<td>873</td>
<td>GAC AGA CAG ACA GAC A</td>
<td>45</td>
</tr>
</tbody>
</table>

Single letter abbreviations for mixed-base position: Y = (C, T), R = (A, G)
Table 3. Genetic diversities in *Spartina* populations, measured as the number of polymorphic ISSR marker per patch.

<table>
<thead>
<tr>
<th>Population ID</th>
<th>Number of polymorphic loci</th>
<th>PPB(%)</th>
<th>$I$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCL</td>
<td>37</td>
<td>37.37</td>
<td>0.169</td>
</tr>
<tr>
<td>FCS</td>
<td>27</td>
<td>27.27</td>
<td>0.126</td>
</tr>
<tr>
<td>RBL</td>
<td>37</td>
<td>37.37</td>
<td>0.185</td>
</tr>
<tr>
<td>RBS</td>
<td>15</td>
<td>15.15</td>
<td>0.081</td>
</tr>
<tr>
<td>HPL</td>
<td>52</td>
<td>52.53</td>
<td>0.251</td>
</tr>
<tr>
<td>HPS</td>
<td>31</td>
<td>31.31</td>
<td>0.152</td>
</tr>
<tr>
<td>Total</td>
<td>83</td>
<td>83.84</td>
<td>0.370</td>
</tr>
</tbody>
</table>

PPB: percentage of polymorphic bands; $I$: Shannon's information index; FCL: Fish Creek large patch; FCS: Fish Creek small patch; RBL: River Bend large patch; RBS: River Bend small patch; HPL: Hawk Property large patch; HPS: Hawk Property small patch.
Table 4: Analysis of Molecular Variance (AMOVA) for six populations of *Spartina patens* in the Meadowlands, New Jersey. (levels of significance are based on 1000 iteration steps, d.f.: degree of freedom; S.S.: sum of square; Est. Var.: estimate variation)

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Est. Var.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among Regions</td>
<td>2</td>
<td>158.133</td>
<td>79.067</td>
<td>0.000</td>
<td>0%</td>
</tr>
<tr>
<td>Among Pops</td>
<td>3</td>
<td>248.850</td>
<td>82.950</td>
<td>7.734</td>
<td>58%</td>
</tr>
<tr>
<td>Within Pops</td>
<td>54</td>
<td>302.900</td>
<td>5.609</td>
<td>5.609</td>
<td>42%</td>
</tr>
</tbody>
</table>

![Image of Table 4]

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Table 4 provides a detailed analysis of molecular variance among and within populations of *Spartina patens* in the Meadowlands, New Jersey. The table summarizes the results of an AMOVA analysis, which assesses genetic diversity and structure across different regions and populations. The table includes the following columns: Source (Among Regions, Among Pops, Within Pops), df (degrees of freedom), SS (sum of squares), MS (mean square), Est. Var. (estimate of variation), and % (percentage of total variance explained by each component). The significance levels are based on 1000 iteration steps, and the calculations account for degrees of freedom (d.f.) and sum of square (S.S.).
Curriculum Vitae

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