Genetic diversity of historic and range-expansion populations of *Avicennia germinans* (Black Mangrove) along a latitudinal gradient of the Gulf Coast of Florida, Louisiana, and Texas, USA.

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Abstract

Avicennia germinans (black mangrove) is a species of mangrove occurring along the Gulf Coast of the continental U.S., and is an important tropical, intertidal foundation species that provides many ecosystem services such a storm energy attenuation, nursery habitat for many juvenile marine species, water filtration, and carbon sequestration. With recent climate shift resulting in warmer winter temperatures and sea level rise along tropical-temperate ecotones along the Gulf Coast, mangrove populations are exhibiting northward and inland migration, potentially foreshadowing large-scale ecosystem shifts in this region. Biotic drivers, such as levels of genetic diversity and inbreeding within a species and among its populations, are equally important in the assessment of possible future range shifts of mangroves in the Gulf. The genetic diversity of a species and its populations plays a large role in its fitness, resilience, and predisposition for successful establishment into new territory, and is thus an important parameter when assessing future mangrove range-expansion into novel environmental conditions

Little is known about the reproductive patterns of *A. germinans*, and previous studies have found confounding and contradictory data on the mating systems and the overall trends in genetic diversity in populations of this species, suggesting population genetics in mangrove species are highly influenced by local conditions. This study used 12 microsatellite loci to examine the genetic diversity among seven populations of *A. germinans* sampled along the coast of the Gulf of Mexico, and compared the genetic diversity and inbreeding coefficients of two new, range-expansion populations (Dog Island, FL and Leeville, LA) to the other historic, within-range populations (Port Aransas, TX; Port Isabel, TX; Cedar Key, FL; Emerson Point Preserve, FL, Site 2, FL). The research addressed whether the populations sampled were within Hardy-Weinberg Equilibrium, supporting at least moderate levels of outbreeding and genetic diversity, or whether the populations appeared highly inbred. Further, this study explored whether populations exhibited a latitudinal gradient of decreasing genetic diversity with increasing latitude, and how heterozygosity and inbreeding within the two new, range-expansion populations compared to the other historic, within-range populations. Eight microsatellite loci were then used to compare genetic characteristics of the parents and progeny of the two new, range-expansion populations (Leeville, LA and Dog Island, FL)

to a historic, within-range population (Port Aransas, TX) to illuminate how stressful novel conditions may affect the reproduction and resulting genetic diversity of range expansion populations.

Overall, the results affirm previous findings that populations of *A. germinans* may support moderate to high levels of inbreeding within populations but that the genetic variation among populations can still be high, suggesting local conditions exert great influence over mangrove population genetics. None of the populations were within Hardy-Weinberg Equilibrium, and expressed heterozygote deficiency. Overall observed heterozygosity among populations was low (H_o = 0.04 – 0.28), and inbreeding coefficients were moderate to high (F_{IS} = 0.20 – 0.70). There was no latitudinal gradient of decreasing genetic diversity with increasing latitude, however northern populations did tend to exhibit greater inbreeding coefficients than the more southern populations. Genetic differentiation among populations was moderate (F_{ST} = 0.39; p-value < 0.001) and pairwise fixation indices (F_{ST}) ranged from -0.033 to 0.66, displaying a wide range of differentiation among populations.

High inbreeding, extremely low observed heterozygosity, and very high genetic differentiation from all other populations sampled revealed that the new, range-expansion population Dog Island, FL may be experiencing founder effects. Dog Island, FL is the only population in which the inbreeding coefficient increased from parent to progeny, and AMOVA data suggest significant genetic differentiation between the parent and progeny (F_{ST} = 0.48; p-value < 0.001), alluding to possible genetic drift. Leeville, LA – a more developed and less isolated new, range-expansion population – appears bereft of founder effects. The observed heterozygosity and inbreeding coefficients overall and between parent and progeny are comparable to other historic, within-range populations, suggesting that over time, long distance dispersal events of propagules may mitigate and counteract founder effects.

Genetic linkage groups seemingly follow the predominant west-to-east flow of the Gulf Stream and Loop currents within the Gulf Basin: the Texas and Louisiana populations form a cluster, Dog Island, FL forms a cluster, and the three historic Florida sites form a cluster. The irregularity and complexity of smaller loops, eddies, and gyres

within the Gulf basin, compounded by the possibility of long-distance dispersal events of propagules, may lend to why some populations may be more isolated and genetically distinct from others along the same coastline and some populations appear most closely linked to populations from across the basin.

In conclusion, these data support previous research by maintaining that *A. germinans* can support moderate to high levels of inbreeding and in fragmented or isolated populations, there may be high levels of bi-parental inbreeding, geitonogamy, and self-fertilization, but genetic variation among populations can still be high. In new or isolated range-expansion populations, inbreeding, isolation from diverse pollen donors or incoming migrants, and natural selection favoring adaptations to local conditions, may drive great genetic differentiation over short timescales. Despite theories asserting that small and fragmented colonizing populations have poor genetic diversity and thus reduced fitness, it is possible for colonizing populations to maintain genetic diversity through genetic variation within and among individuals, and to share high genetic connectivity with other populations through long-distance dispersal events, mitigating the consequences of the founder effect over time. Such populations may also offer interesting case studies in the development of local adaptations in range-expansion populations that allow for species resilience against future environmental change.

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Glossary of Genetics Terms

Allele – a particular form, or variation, of a given gene

Allelic diversity – The average number of alleles per locus (location on gene) for a population

Effective population size (N_e) – The size of an ideal population (under Hardy-Weinberg conditions) that will lose genetic variation through drift at the same rate as an actual population.

Fixation Index - F_{ST} – A measure of the degree of inbreeding within a subpopulation relative to the total population. Reflects the probability that two alleles drawn at random from within a subpopulation are identical by descent. This term is the most common measurement used to describe the genetic differentiation between subpopulations, and how genetically similar two populations are to one another. Calculated as:

$$F_{ST} = \frac{H_T - H_S}{H_T}$$

Where H_T is the expected heterozygosity of the total population and H_S is the heterozygosity that would be expected if the subpopulation is in Hardy-Weinberg Equilibrium (HWE). Analogs of F_{ST} include G'_{ST}, G''_{ST}, Jost's D, and Rho_{ST}.

Founder effect – The changes in allele frequencies, relative to the source population, that are often evident in populations which have been founded by a small number of individuals.

Genetic bottleneck – A severe, temporary reduction in the size of a population that also reduces the number of alleles in a population

Genetic Drift – A process that changes the allele frequencies within a population from one generation to the next because of random sampling of gametes.

Gene flow – The transfer of genetic material from one population involving successful dispersal and subsequent reproduction

Genetic Structure – Genetic structure of a population is characterized by the number of subpopulations within it, the frequencies of different genetic variants (alleles) in each subpopulation, and the degree of genetic isolation of the subpopulations.

*(Definition taken from: Chakraborty R. 1993. Analysis of Genetic Structure of Populations: Meaning, Methods, and Implications. In: Majumder P.P. (eds) Human Population Genetics. Springer, Boston, MA.)

Hardy-Weinberg equilibrium – A predictable ratio of genotype frequencies in a sexually reproducing population of infinite size with random mating and no selection

Homozygous – An individual that has only one type of allele at a particular locus (ex: aa)

Heterozygous – An individual that has more than one type of allele at a particular locus (ex: Aa)

Inbreeding coefficient (F_{IS}) – the probability that a diploid individual has two alleles at a particular locus that recently descended from a single common ancestor. Measures the degree of inbreeding within individuals relative to the rest of the subpopulation. Calculated as:

$$F_{IS} = \frac{H_S - H_I}{H_S}$$

Where H_I is the observed heterozygosity in a subpopulation at the time of investigation (individual heterozygosity), and H_S is the heterozygosity that would be expected if the subpopulation was in HWE. When $F_{IS} = 0$, there is no inbreeding; when $F_{IS} = 1$, all individuals within a population are homozygous and there may be complete inbreeding.

Linkage Disequilibrium – Occurs when the alleles at two or more loci co-occur more often than is expected based on their frequencies.

Locus – The location of a particular gene or region of DNA on a chromosome

Microsatellite – A stretch of DNA consisting of short tandem repeats of up to 5 base pairs

Nuclear DNA – The complement of DNA that is arranged in chromosomes and located in the nucleus of a cell; used in microsatellite analysis

Polymerase chain reaction (PCR) – A procedure that denatures DNA to amplify specific segments of DNA

Population genetic structure – Refers to the way in which a population can be divided into subpopulations or local populations based on different allelic frequencies or genetic characteristics.

Private Alleles – Alleles found in only one subpopulation, or population, relative to all populations sampled.

* Unless otherwise indicated, all definitions provided in the Glossary are from Freeland et al. 2011

Introduction

Mangrove species are foundational species that wield great influence on the wetland ecosystem dynamics and ecological communities they inhabit. They play major roles in improving water quality through runoff filtration, storing carbon, protecting coastlines from erosion and storm energy, and supporting vital fish and invertebrate populations (Tomlinson 1986; Twilley 1998; Hogarth 1999; Nagelkerken et al. 2008; Cerón-Souza et al. 2012; Osland et al. 2013; Yando et al. 2016; Thomas et al. 2017). Additional unique distinguishing attributes of mangrove species include the ability to generate diverse microhabitats that support a vast array of vertebrates and invertebrates, the ability to grow in very saline and anoxic environments, and alter the geomorphology and geochemistry of the substrate toward more favorable conditions for other marine species, and their high tolerance for various environmental stressors (FWS 1999). Such attributes make mangrove forests some of the most productive and biologically diverse habitats in the world (FWS 1999), as well as resilient and potentially very large carbon sinks (Doughty et al. 2016; FWS 1999).

Mangroves are cryptoviviparous, halophilic woody trees and shrubs that establish along tropical coastal intertidal zones, defined by evolutionary convergence and ecological role rather than by taxonomy (Tomlinson 1986; Hogarth 1999). Typically found in highly anoxic habitats subject to frequent inundation, mangrove species have developed specific biological structures and pathways to cope with these challenges, including establishing aerial roots, pneumatophores, and various salt-excreting and aeration pathways. These adaptions not only generate spatially complex ecosystems but also alter the physical and geochemical characteristics of the environment within their immediate vicinity. Therefore, mangrove species are considered important foundational species in coastal marine environments – resilient species that play a vital role in physically stressful environments by creating more suitable habitats, modulating ecosystem dynamics, and facilitating the development of entire ecological communities (Osland et al. 2013).

Reflecting their robust nature, mangrove species seedlings, or propagules, are specially designed for long periods of marine travel and for enduring a range of salinity

and temperature thresholds. Some species' propagules remain viable for up to one year after detaching from the parent tree (Rabinowitz 1978; Hogarth 1999). This combined strategy of cryptovivipary and resilient, floating propagules promotes not only the widespread dispersal of mangroves, but also enables seedlings to travel to novel regions and establish themselves quickly once they encounter the appropriate substrate (Odum and McIvor 1990; Hogarth 1999; Alleman and Hester 2014).

Only three major groups of true mangroves exist in the continental United States: *Rhizophora mangle* (red mangrove), *Avicennia germinans* (black mangrove), and *Laguncularia racemosa* (white mangrove), mostly found along the coast of Florida, southern Louisiana, and Texas (Tomlinson 1986; Hogarth 1999; Deltares 2014). While salt marsh ecosystems have historically dominated most of northern Florida and southern Louisiana, recent data project the possibility of further northward and inland mangrove migration due to sea level rise, warmer winter temperatures, and reduced freezing events, suggesting possible salt marsh displacement and ecosystem shifts at the Gulf Coast's ecotone boundaries (Ross et al. 2009; Saintilan et al. 2009; McKee et al. 2012; Osland et al. 2013).

Though salt marsh and mangrove species occupy similar geomorphic settings, facilitate similar ecological dynamics, and provide similar ecosystem services, their environmental stress limits differ (Friess et al. 2011; Osland et al. 2013). Mangrove species have broader temperature and inundation ranges, enabling them to generally out-compete salt marsh species in more tropical settings. Marshes historically dominate the temperate, boreal, and arctic coastlines (Adam 1990; Pennings and Bertness 2001), whereas mangrove seedlings are highly susceptible to winter freeze events (Stuart et al. 2007; Pickens and Hester 2011; Osland et al. 2013; Madrid et al. 2014) and thus typically cannot successfully establish above their latitudinal thresholds (Osland et al. 2013). Near tropical to temperate transition zones, however, warmer winter temperatures, less extreme freezing events, and increased tidal encroachment may potentially enable poleward mangrove migration and niche competition, possibly resulting in salt marsh displacement (Osland et al. 2013; Madrid et al. 2014).

Ecosystem shifts in such areas would likely affect the botanic, vertebrate, and invertebrate species abundances and distributions, geomorphology, hydrology, and

carbon storage potential of the affected coastlines. Thus, for conservation and environmental planning purposes, it is important to accurately predict where these ecosystem shifts may occur. Various studies focused on dispersal ranges and temperature thresholds have examined the abiotic limitations on possible mangrove migration (Osland et al. 2013; Madrid et al. 2014; Peterson and Bell 2012; 2015), and a few studies have examined possible biotic limitations by exploring differences in genetic diversity and physiology within populations and individuals subjected to novel stressful conditions (Salas-Levia et al. 2009; Nettel-Hernanz et al. 2013; Proffitt and Travis 2014; Madrid et al. 2014). However, we have yet to specifically examine the possible differences in genetic diversity between historic mangrove populations and range-expansion populations as a source of physiological differences that allow for successful mangrove migration and establishment into novel territories.

For this thesis research, I use the interesting case study of present-day transition zones along the northern rim of the Gulf Coast to directly examine patterns in genetic diversity within and among populations of *Avicennia germinans* (black mangrove) at various sites in northern Florida, southern Texas, and southern Louisiana. I chose *A. germinans* since it is the most successful northward migrating mangrove species (personal observation, Kent 2017). Since differences in genetic diversity are directly or indirectly influenced by species/population breeding patterns (Freeland et al. 2011), I used microsatellite molecular markers to quantify genetic diversity and measure inbreeding within and among Gulf Coast *A. germinans* populations. I specifically focused on identifying differences between historic populations and range-expansion populations, and whether there is high inbreeding between parent and progeny.

Such data have many important practical applications for conservation and restoration, including the facilitation of more accurate predictions of whether a species or population is likely to survive novel conditions. This, in turn, promotes more efficient management to maximize evolutionary potential and address important ecological questions about future ecosystem dynamics (Freeland et al. 2011).

Populations and species with low genetic diversity are less successful at adapting to changing environmental conditions, and usually exhibit reduced fitness and increased susceptibility to disease and environmental strain. Populations demonstrating high

genetic diversity, however, are often more resilient against environmental stressors, and are more likely to survive novel conditions (Freeland et al. 2011). Identifying such populations will help focus conservation efforts, funds, and man-power, in order to yield the most likely outcome for success in preserving healthy populations (Freeland et al. 2011).

Mating systems and pollen dispersal patterns in plants play a key role in establishing spatio-temporal patterns of genetic diversity, which directly influence reproductive ability, population size, degree of inbreeding, genetic variation, genetic structure, and speciation (Nettel-Hernanz et al. 2013). Literature on levels of genetic diversity in mangrove populations suggest disputing inferences as to whether mangroves are highly selfing and inbred, or whether levels of inbreeding and outcrossing can vary greatly both geospatially and temporally.

Proffitt and Travis (2014) attempted to broach the subject of genetic diversity by assessing whether population reproduction and outcrossing rates in the red mangrove (*Rhizophora mangle*) were affected by cold stress (measured through latitude), anthropogenic stress (measured through human population density), and natural disturbance (measured through years since a major hurricane). A grand-scale survey of 104,211 trees and 102 forest stands used the frequency of occurrence of heterozygous trees (exhibiting normal and albino propagules) and the deviation from the 3:1 Mendelian ratio of heterozygous trees to homozygous tress (exhibiting only normal propagules) as a proxy for mutation rates and outcrossing rates. The resulting data found the number of reproducing trees within a stand varied by site and increased with latitude, and that outcrossing increased under conditions of cold and anthropogenic stress. Cold stress appeared to increase reproductive output (number of reproducing trees) within populations, increase outcrossing, and suppress mutation (presence of albino propagules). Anthropogenic stress, however, elicited the opposite response. Stands in close proximity to human populations exhibited greater presence of mutants (albino propagules), decreased stand reproduction, and increased outcrossing. The study concluded the potential for colonization of northern Florida salt marshes by mangroves is enhanced by increased reproductive and outcrossing rates, which should enhance genetic variation,

thereby promoting adaptation to novel environmental conditions (Proffitt and Travis 2014).

Though early literature proposes mangrove species may self-pollinate, are predominantly inbred, and consequently generally exhibit very low levels of genetic diversity, more recent surveys of mangrove populations support the findings of Proffitt and Travis (2014) that the breeding patterns and resulting genetic diversity may vary greatly depending on local environmental conditions. Salas-Leiva et al. (2009) studied natural and re-forested areas of *A. germinans* in Colombia using microsatellites (genetic markers) and found that inbreeding coefficients were greater in re-forested areas compared to natural, undisturbed areas, and that all populations sampled deviated from the Hardy-Weinberg Equilibrium. Younger trees in a region where a new highway was constructed expressed higher rates of inbreeding than their older counterparts. On another transect with no recent construction, young and old trees appeared to have similar inbreeding coefficients, alluding to a negative effect of stress on breeding and resulting genetic diversity (Salas-Leiva et al. 2009).

Nettel-Hernanz et al. (2013) conducted an analysis of mating systems in *A. germinans* populations along the Chiapas, Mexico coastline using microsatellite analysis of parent and progeny DNA. Results found inbreeding coefficients were close to zero and that none of the studied populations significantly deviated from the Hardy-Weinberg Equilibrium. Detected levels of bi-parental inbreeding were low, and the proportion of siblings sharing the same "father" tree was also low. The study concluded that *A. germinans* populations in this area are predominantly outcrossing but can support moderate levels of self-fertilization. Nettel-Hernanz et al. (2013) claim these findings are consistent with previous predictions that *Avicennia* mating patterns consist of a mixture of outbreeding and selfing, where outcrossing events are more predominant but random, with little effect on population substructure and biparental inbreeding.

Based on data from such studies, one can surmise that mangrove populations may often be inbred, but that genetic variation among populations can still be high (suggesting separate populations are isolated from one another). Further, genetic variation within a population varies from one population to another, suggesting local conditions exercise great influence over patterns of population genetics in mangroves.

To quantify genetic diversity and measure inbreeding within and among *A. germinans* populations along the Gulf Coast, I focused on identifying differences between historic populations and range-expansion populations by sampling leaf tissue from mature trees at seven sites along the Gulf Coast (Figure 2.1; Table 2.1). To compare the inbreeding coefficient between parent and progeny, and within the population in historic versus range-expansion populations, I sampled leaf and corresponding propagule tissue from two range-expansion populations and one historic population along the Gulf Coast (Figure 3.1; Table 3.1). Nuclear DNA was isolated and extracted from the leaf and propagule tissue (following the protocol outlined in Chapter 1). Genetic markers for *A. germinans*, identified from existing literature, were used to target loci of interest, and then amplified using polymerase chain reaction techniques (PCR). PCR products were sent to Georgia Genomics Facility (University of Georgia, Georgia, USA) to be sequenced on a capillary-based 3730xl DNA Analyzer (Applied Biosystems). The results were analyzed using Geneious V7.1.5 (Biomatters Ltd.).

I conducted a comparative population genetic structure analysis of *A.germinans* along the Gulf Coast, and between historic and leading-edge populations, to examine the following questions and test the following hypotheses:

(1) Do populations of *A. germinans* along the Gulf coasts of Texas, Louisiana, and Florida appear highly inbred, or do they appear to adhere to Hardy-Weinberg levels of interbreeding? How much genetic diversity exists within and among these populations of *A. germinans*? Is there a latitudinal pattern or trend in the degree of genetic diversity of *A. germinans* populations sampled along the Gulf Coast?

Hypotheses: The populations sampled will exhibit at least intermediate levels of outbreeding (inbreeding coefficient of 0.4 or lower), thus allowing for propagules with the genetic plasticity necessary to succeed in new environments (as exemplified by successful range-expansion of mangroves into northern Florida and Louisiana). I further expect populations at higher latitudes, closer to the boundaries of typical mangrove latitudinal ranges, to exhibit decreased genetic

diversity compared to more southern populations, due to selection and a bottleneck of pollen contributors (founder effect).

(2) When comparing the genetic diversity and inbreeding coefficients in mature individuals of *A. germinans* to their respective progeny, do the progeny reflect similar or significantly different levels of genetic diversity and inbreeding? And, if they are different, does this appear to result in significant genetic differentiation between the parent and progeny subpopulations of each population? How do the differences in observed heterozygosity and inbreeding coefficients between the parent and progeny subpopulations in the new, range-expansion populations compare to differences between the parent and progeny subpopulations in the historic, within-range population? Is there significant genetic differentiation among the populations? Does the level of differentiation among all populations differ between the "parent" and "progeny" subpopulations? Is there significant genetic differentiation among regions, when the two new, range-expansion populations are compared to the historic population? What implications does this assessment have for the genetic diversity and resilience of future range-expansion populations of mangroves?

Hypotheses: There will be a difference in the genetic diversity and inbreeding coefficients between the parent subpopulation and the progeny subpopulation in the two new, range-expansion populations. I expect this difference to reflect less genetic diversity and greater inbreeding coefficients in the progeny compared to the parents due to isolation from pollen donors, resulting in a genetic bottleneck or a founder effect. I posit that this will result in significant genetic differentiation among the parent and progeny within the Dog Island, FL, and Leeville, LA, populations. However, I also postulate that the parent subpopulations of the two new, range-expansion populations (Dog Island, FL and Leeville, LA) will exhibit greater genetic diversity than the historic, within-range population (Port Aransas, TX), thus exhibiting the genetic diversity needed for the individuals in the range-

expansion populations to succeed in novel environmental conditions. As the historic, within-range population (Port Aransas, TX) is less fragmented and well within the species' distribution range, thus having ample access to pollen donors and being spared the stress of novel environmental conditions, I do not anticipate significant genetic distinction between parent and progeny in this population. Due to differing forces of selection, and possibly genetic drift, acting on the range-expansion populations compared to the historic, within-range population, I surmise there will be significant genetic differentiation among the three populations, and that these genetic distinctions will be reflected in both the parent and progeny subpopulations of each population.

The unique characteristics of mangrove species that allow them to disperse to and successfully colonize novel regions, as well as modulate and facilitate the dynamics and development of the ecosystems in which they exist, mark mangroves as particularly interesting in terms of future climate and ecosystem shifts. Tropical coastal wetland regions are particularly sensitive to the effects of climate shift and sea level rise, casting uncertainty about the future of the ecosystems in these areas, particular along the Gulf Coast, where transitions are already being observed. Such data, in conjunction with previous research on abiotic drivers of mangrove migration and possible salt marsh displacement, will foster more accurate ecosystem shift predictions and help inform more effective conservation and restoration initiatives.

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Chapter 1

Methods in tissue storage and the extraction of nuclear DNA from leaf and propagule tissue from *Rhizophora mangle* and *Avicennia germinans*

Abstract

The isolation of pure, high-quality DNA is vital for molecular study. However, DNA isolation from higher plants with recalcitrant tissue is usually difficult due to high concentrations of secondary metabolites and structural tissue, which usually results in low, often contaminated, yields of DNA. Several pathways for a more efficient and cost-effective means of extracting high quality and quantity DNA from recalcitrant tissue have been identified but tend to be time-consuming, expensive, use dangerous and/or toxic chemicals such as liquid nitrogen and phenols, and may require fresh or very young tissue samples in order to yield sufficient results. Plant DNA extraction kits, such as those provided by QIAGEN (QIAGEN Pty Ltd, Valencia, California, USA), are straightforward, simple, contain fewer harmful chemicals, and are less time-consuming. However, when dealing with recalcitrant plant tissue, the kit-based protocols tend to generate questionably poor DNA quality and very low DNA yields per reaction solution volume. Thus, there is a need for an efficient, inexpensive, non-hazardous protocol for storing plant tissue and isolating and extracting genomic DNA that will produce products of sufficient yield and purity for polymerase chain reaction (PCR) and further molecular study. Mangrove species have highly recalcitrant tissue, thus making these species promising candidates for refining the protocol for nuclear DNA extraction from higher plants with high concentrations of secondary metabolites and structural tissue.

Tissue samples from young and mature leaves, as well as tissue samples from propagules (seedlings) of *Avicennia germinans* (black mangrove) and *Rhizophora mangle* (red mangrove) collected from various locations along the Gulf Coast, USA, were used to demonstrate how modified tissue storage and preparation, in conjunction with a modified protocol for the QIAGEN DNeasy Plant Mini Kit (QIAGEN Pty Ltd, Valencia, California, USA), can result in viable DNA yields of sufficient quality for PCR and further molecular work. The DNA concentrations were verified as between 5 ng/μL – 30 ng/μL with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, California, USA) and randomly chosen DNA samples were amplified for loci of interest using the Type-it® Microsatellite PCR Kit (QIAGEN, QIAGEN Pty Ltd, Valencia, California, USA). Polymerase chain reaction (PCR) conditions were set to the manufacturer's optimized cycling conditions and customized with additional steps to include the optimum

annealing temperatures for the various microsatellite primers used, as suggested by their source literature. The PCR products were imaged with 1.5 % agarose gel electrophoresis, providing evidence that the protocols outlined will yield sufficient DNA quality and quantity for PCR amplification and further molecular work.

1. Introduction

The isolation of pure, intact, and high-quality DNA is vital for molecular study, however DNA isolation from higher plants, particularly plants with recalcitrant tissue, is usually difficult due to excessive contamination by high concentrations of secondary metabolites that vary from species to species, and, in some species, from population to population or individual to individual, influenced by local environmental conditions (Sangwan et al. 1998; Sahu et al. 2012). Several pathways for a more efficient and cost-effective means of extracting higher quality and higher quantity DNA from recalcitrant tissue containing high concentrations of secondary metabolites have been identified but tend to be time-consuming and expensive, and use dangerous and/or toxic chemicals (Porebski et al. 1997; Sahu et al. 2012).

The cetyl trimethylammonium bromide protocol (CTAB) developed by Cullings (1992) is complicated, lengthy, and uses harmful chemicals such as phenols, chloroform, and liquid nitrogen. The CTAB method has been adjusted and modified throughout the years, however recent modifications, such as by Thangjam et al. (2003), take over 3-4 hours, also use harmful chemicals such as chloroform and phenol, and yield about 50 – 60ng DNA per 25µl reaction mixture. Another recent modification proposed by Sahu et al. (2012) eliminates the need for liquid nitrogen and phenols, yet still employs chloroform and requires that the tissue be frozen in order to be ground.
Alternative attempts to eliminate the need for hazardous chemicals and expensive kits generally resulted in labor-intensive, low purity yields with limited shelf life and poor amplification (Dilworth and Frey 2000; Ikeda et al. 2001; Sahu et al. 2012). Additionally, these protocols require fresh tissue samples for genomic DNA isolation, which may be difficult to procure, if research sites are remote, or if transport of the tissue to a lab is necessary (Sahu et al. 2012). Plant DNA extraction kits, such as those provided by

QIAGEN (QIAGEN Pty Ltd, Valencia, California, USA), are simple, contain fewer harmful chemicals, and take less time and handling. However, when dealing with plant tissue laden with structural tissue and high concentrations of secondary metabolites, the kit-based protocols tend to generate questionably poor DNA quality and very low DNA yields per reaction solution volume.

Young leaves that still have relatively low concentrations of secondary metabolites, polyphenols, tannins, and structural tissue are ideal for DNA extraction, and many existing protocols use young leaves and therein have the best success (Moreira and Oliveira 2011; Sahu et al. 2012). Young leaves may not always be available, however, so there is a need for an easy extraction method that yields viable DNA from both young leaves as well as mature leaves. If the samples are to be collected from distant locations and stored and transported prior to extraction, then the drying and storage of the plant tissue in order to prevent tissue degradation is also very important. Thus, there is a need for an inexpensive, non-hazardous, efficient protocol for preserving and storing plant tissue and isolating and extracting genomic DNA that will produce products of sufficient yield and purity for further molecular study. Mangrove species have highly recalcitrant tissue and are becoming the focus of various population genetics studies due to their great ecological value; this makes these species promising candidates for refining the protocol for nuclear DNA extraction from higher plants with high concentrations of secondary metabolites and structural tissue for the purpose of using molecular ecology techniques in conservation.

Mangroves are specially adapted halophilic woody trees and shrubs that establish along tropical, coastal intertidal zones, and are typically found in highly stressful and anoxic environmental conditions. In order to cope with their taxing living conditions, mangrove species' tissue is highly recalcitrant, containing high concentrations of polysaccharides, polyphenols, and other secondary metabolites such as tannins, alkaloids, and flavonoids. These compounds, when oxidized, bind with DNA and make it resistant to restriction enzymes, making it notoriously difficult to isolate and extract viable DNA, and making the DNA highly susceptible to degradation (Kathiresan and Bingham 2001; Bandaranayake 2002; Sahu et al. 2012; Rawat et al. 2016).

The biochemical composition of the living tissue can vary considerably among mangrove species. Depending on differing environmental conditions, individuals among and within populations of the same mangrove species can also exhibit considerable variations in the biochemical composition of leaf and propagule (seedling) tissue. This makes the mangrove an excellent test subject for a standardized storage and extraction protocol that will hopefully be translatable to other plants species with recalcitrant tissue. *Rhizophora mangle* (red mangrove) and *Avicennia germinans* (black mangrove) are the two intertidal species of the three main mangrove species found on continental North America and will be the species used for this study.

This paper presents a standardized protocol for the drying and storage of both young and mature leaves for *R. mangle* and *A. germinans*, as well as the drying and storage of propagule (seedling) cotyledon tissue from *R. mangle* and *A. germinans*. These protocols eliminate the need for storing leaf and propagule tissue frozen, and eliminate the need to dehydrate the tissue further prior to extraction. This paper will also outline simple adjustments to the protocol of an existing plant extraction kit, the QIAGEN DNeasy Plant Mini Kit (QIAGEN Pty Ltd, Valencia, California, USA), in order to quickly and easily generate high yields of DNA that are viable for polymerase chain reaction (PCR) and further molecular study from mangrove leaf and propagule tissue.

2. Materials and Methods

Fresh leaf and propagule samples were collected from *R. mangle* and *A. germinans* populations from various locations along the Gulf Coast of Florida, Texas, and Louisiana, USA. Using a modified protocol of the QIAGEN DNeasy Plant Mini Kit (QIAGEN Pty Ltd, Valencia, California, USA), genomic DNA was isolated from the dried, mature leaves and propagules from various individuals of *R. mangle* and *A. germinans* and verified with 1 % agarose gel electrophoresis. The DNA concentrations were verified with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, California, USA). Randomly chosen DNA samples were diluted down to ~5 ng/μL and amplified for loci of interest using the Type-it® Microsatellite PCR Kit (QIAGEN, QIAGEN Pty Ltd, Valencia,

California, USA). PCR conditions were set to the manufacturer's optimized cycling conditions and customized with additional steps to include the optimum annealing temperatures for the various microsatellite primers used, as suggested by literature (Appendix: Table 1 and Table 2). The various samples of DNA isolated from *R. mangle* leaf tissue were amplified with primers RM38 and RM41 (Rosero-Galindo et al. 2002), and the various samples of DNA isolated from propagule tissue were amplified with primers RM19, RM21, and RM36 (Rosero-Galindo et al. 2002). The various samples of DNA collected from A. *germinans* leaf tissue were amplified with primer Agerm1-22 (Mori et al. 2010), and the various samples of DNA collected from *A. germinans* propagule tissue were amplified with primer Agerm1-18 (Mori et al. 2010). The PCR products were imaged with 1.5 % agarose gel electrophoresis (Figure 1.1 and Figure 1.2).

2.1 Tissue Drying and Storage Protocols

2.1.1 Leaf Drying, Storage, and Preparation for DNA Extraction

Gently place up to three leaves, either young or mature, in an empty cotton filter-paper tea bag. Take special care not to rip or tear the leaves, as this will release secondary metabolites and begin to brown and degrade the tissue. For small, young leaves, add 10 – 15 mL silica gel beads (roughly 1 tablespoon) into the tea bag with the leaves. For large, mature leaves, add 15 – 25 mL (roughly 2 tablespoons) of silica gel beads into the tea bag and fold shut or seal. Store sealed tea bags in a larger, sealed plastic bag or container bedded with silica gel beads. Keep the container in a cool, dry place out of direct sunlight. The dehydrating effect of the tea bag in addition to the silica usually eliminates the need for freezing the samples during storage or transport and effectively dries the tissue, until it is crisp but usually still green. If tissue turns brown, but is dry, it is usually still viable for the following DNA isolation and extraction protocol. Samples should be checked regularly to ensure tissue is drying.

2.1.2 Propagule Drying, Storage, and Preparation for DNA Extraction

2.1.3 *Rhizophora Mangle*

When working with the long, cigar-shaped propagules of *R. mangle*, there are two ways in which the tissue can be effectively dehydrated, stored and transported. Upon collection, place the propagules in a cool, dry storage container (plastic bag or plastic container) taking care not to snap the propagule or tear the green surface tissue. Add 10 – 15 mL silica gel beads per propagule, kept in the same container to aid the complete removal of moisture, and store in a cool, dry place out of direct sunlight. When storing for the long term, freeze the container with silica beads and propagules until use. Check the silica regularly and replace if saturated to ensure thorough drying.

(*Note*: Proceed to the following steps only once a freezer storage space is available, or directly prior to DNA extraction, as cutting or thawing mangrove tissue will exponentially increase tissue degradation.)

Since the majority of the propagule volume is a core made up of xylem and phloem sieve cells, devoid of DNA, it becomes apparent that only the green, epidermal tissue of the propagule is useful for isolation of DNA. In order to harvest this DNA-viable tissue, use a clean knife or vegetable peeler to gently shave off the epidermal tissue in thin strips. Once all the green epidermal tissue is peeled from the spongy xylem and phloem core, store the strips in a cotton filter paper tea bag with 15 – 25 mL of silica gel beads. Seal the tea bag and store in a plastic container or plastic bag bedded with silica gel beads. Seal container or bag and store in a cool, dry place. For the most efficient preservation of shaved tissue, freeze as soon as possible, as the tissue is now more vulnerable to degradation from oxidation and secondary metabolites. Only thaw sufficient tissue for each immediate extraction process, as thawing the tissue will cause cellular lysing and progressive tissue degradation. Check the silica regularly and replace if saturated to ensure thorough drying.

(*Note*: Thick shavings may not entirely dehydrate; wet shavings can still be used for the extraction protocol below.)

2.1.4 *Avicennia germinans*

When working with the lima-bean shaped propagules of *A. germinans*, place a single propagule in a cotton filter paper tea bag without damaging the protective outer layer of the pericarp. (If the propagule has already dropped from the parent tree, it will have shed its pericarp and the cotyledons will be exposed.) Place 10 – 15 mL of silica gel beads in the tea bag with the propagule and seal. Store tea bags in a sealed container or plastic bag bedded with silica gel beads, and keep in a cool, dry place or freeze until use. Check the silica regularly, and replace if silica becomes saturated to ensure thorough drying. If the propagules are very large and fleshy, they may need several renewals of silica gel bedding. Taking extra precaution to store propagules in a freezer when possible may be wise.

When preparing the *A. germinans* propagules for DNA extraction, gently peel apart the propagule's protective pericarp to expose the green cotyledons within. Use the green cotyledon tissue for DNA extraction. Once the desired amount of green tissue has been harvested, carefully fold the protective outer layer of the propagule and pericarp back around the cotyledon tissue if possible, and return the propagule to the tea bag with silica and store in a freezer. This will help preserve the remaining tissue for subsequent extractions. Any propagule that has been opened should be frozen to prevent further tissue degradation caused by tissue damage.

(*Note*: *A.germinans* propagules may not thoroughly dehydrate, especially if the propagule is large and fleshy. These storage methods will help preserve the cotyledon tissue, even if it does not entirely dehydrate. Wet or dried cotyledon tissue can be used for the following extraction protocol.)

2.2 DNA Extraction and Quantification: Adjusted QIAGEN DNeasy Plant Mini Kit

2.2.1 DNA Isolation and Extraction

This protocol employs the QIAGEN DNeasy Plant Mini Kit (QIAGEN Pty Ltd, Valencia, California, USA) and offers a few adjustments to standardize and optimize the process of isolating and extracting DNA from dried young and mature mangrove leaf tissue, as well as propagule tissue.

When extracting DNA from dried young or mature leaf tissue from *R. mangle* and *A. germinans*, place 0.026g – 0.040g (optimum dry weight range) in a ceramic mortar and pestle. When extracting DNA from *R. mangle* propagules, place 0.024g – 0.18g shaved epidermal tissue in a ceramic mortar and pestle. For extracting DNA from *A. germinans* propagules, place 0.16g – 0.30g (optimum dry weight range) or 0.20g – 0.23g (optimum wet weight range) of cotyledon tissue in a ceramic mortar and pestle. Immediately add 1,000 µL of warmed AP1 extraction buffer to the mortar to slow tissue oxidation and DNA degradation.

Add a pinch of sand to the mortar and pestle to facilitate the grinding of the tissue into the AP1 buffer. Add AP1 buffer in increments of 200 µL as needed until the mixture is a homogenous liquid. (Make note of how much AP1 buffer is needed, as every 1,000 µL of AP1 used should be matched with 4 µL RNase-A, in the following step.)

(*Note*: Use filter tips throughout this protocol to avoid DNA contamination. Discard all remaining tubes and liquids into the appropriate chemical waste disposal receptacle.)

Carefully pour the homogenous solution from the mortar and pestle into a micro-centrifuge tube (not included in kit). Vortex the RNase-A (provided by kit) and add 4 µL for every 1,000 µL of AP1 buffer used to the micro-centrifuge tube. Vortex the mixture thoroughly.

(*Note*: If close to 2,000 µL of AP1 is needed to make the mangrove tissue and buffer solution a homogenous liquid, it is best to pour the mixture into two separate micro-centrifuge tubes –1,000 µL of mixture in each – before proceeding with the rest of the protocol in order to maintain proper reagent to solution ratios.)

Incubate the micro-centrifuge tube on a dry block for 10 minutes at 65 °C, mixing the content of the micro-centrifuge tube 3 - 4 times during the incubation by inverting or vortexing the tubes every 3 minutes.

Following the dry block incubation, add 130 µL of P3 buffer (formerly called AP2 buffer) to each micro-centrifuge tube and vortex thoroughly. Incubate the tubes on an ice block or in a tub of ice for 5 minutes.

Centrifuge the tubes for 7 minutes at 13, 200 rpm so that a hard pellet of organic debris is formed at the bottom of the tubes, leaving a clear supernatant liquid.

Being careful not to disturb the hard pellet, pipet 600 µL of the supernatant into the lilac QIAshredder Mini spin column placed in a 2.0 mL collection tube (included in kit, lilac / purple tubes) and centrifuge for 2 minutes at 13, 200 rpm. This step may need to be repeated if clear supernatant is left over from the previous step.

(***Note for next step*: At minimum, 400 µL of flow-through fraction (supernatant) from the lilac tubes in the previous step is needed for transfer into new micro-centrifuge tubes (not included in kit) for this next step; 600 µL of flow-through fraction (supernatant) is optimal. Pipet no more than 600 µL of supernatant from the lilac tubes into each new micro-centrifuge tube in the next step, as excess will cause over-flow, leaking, and possible contamination. If more than 600 µL is acquired from the previous step with the lilac micro-centrifuge tubes, pipet the excess amount into a second, separate micro-centrifuge tube (not included in kit) and follow the directions for the next step with two microcentrifuge tubes for that same sample. Make sure to record the supernatant volume in each individual micro-centrifuge tube for the next step.)

Once all of the supernatant from the previous step is run through the lilac QIAshredder Mini spin column placed in a 2.0 mL collection tube, transfer 400 - 600 µL of the flow-through fraction (supernatant) from the 2.0 mL collection tube into a new micro-centrifuge tube (not included in kit). When pipetting, take care not to disturb the cell-debris pellet at the bottom of each lilac 2.0 mL collection tube (if one is present).

Add a volume of AW1 buffer (formerly called AP3 buffer) equal to 1.5 times the volume of the supernatant within each micro-centrifuge tube to the supernatant in each micro-centrifuge tube and carefully mix by pipetting until the solution is clear. (*Note*: AW1 volume added = volume in each micro-centrifuge tube x 1.5)

Pipet 600 µL of the mixture in the micro-centrifuge tube into a clear DNeasy Mini spin column placed in a 2 mL collection tube (included in kit, clear/white tubes).

Centrifuge the DNeasy Mini spin column placed in a 2 mL collection tube for 1 minute at 8,000 rpm and discard the flow-through (liquid in the bottom of the 2 mL collection tube) into a chemical waste jug. Repeat the previous step until all of the mixture from the micro-centrifuge tubes has passed through a corresponding DNeasy Mini spin column.

Remove the DNeasy Mini spin column (top attachment of tube) and place it in a new 2 mL collection tube (included in kit).

Add 500 µL of AW2 buffer (formerly called AW buffer) to the DNeasy Mini spin column placed in a 2 mL collection tube and centrifuge for 1 minute at 8,000 rpm. Discard the flow-through (liquid in the 2 mL collection tube) and reuse the DNeasy Mini spin column and collection tube.

Add 500 µL AW2 buffer to the DNeasy Mini spin column and centrifuge for 2 minutes at 13, 200 rpm to dry the membrane.

Keep the DNeasy Mini spin column and discard the flow-through (liquid in the 2 mL collection tube) and the collection tube into an appropriate chemical waste disposal receptacle.

Transfer the DNeasy Mini spin column to a new 2 mL micro-centrifuge tube that will hold the ultimate DNA elution (not included in kit).

Slowly pipet 50 µL of AE buffer onto the mesh membrane of the DNeasy Mini spin column and incubate at room temperature for 7 minutes. Centrifuge the DNeasy Mini spin column for 1 minute at 8,000 rpm to yield Elution #1.

Transfer the DNeasy Mini spin column to a new micro-centrifuge tube (not included in kit) and repeat the previous step to yield Elution #2.

Discard the DNeasy Mini spin column and store final DNA elution tubes in a freezer.

(*Note*: Depending on the number of samples, up to 24 samples, this procedure averages between 2 and 4 hours total.)

3. Results and Discussion

The leaf tissues of both species, which were stored from a few months up to a year, were either completely dried or were in various stages of becoming dried and brittle, all still green. Propagules remained green and, when frozen, also remained wet. Propagules not frozen began to dry and harden over time as well, making it easier to powder the tissue and mix homogenous solutions. This study found unfrozen, thoroughly dried propagule and leaf tissue the most amenable to genomic DNA isolation and extraction. Frozen tissue yielded no significant decrease in DNA yield or quality, but prolonged the isolation and extraction process. Tissue frozen and thawed multiple times may also expedite tissue degradation over time. Thus, when possible, thoroughly drying the tissue is the best approach.

Although the biochemistry and recalcitrance of mangrove tissue can be highly variable between species and among populations of the same species, these methods—when paired with the recommended tissue storage methods—consistently yielded between 5 and 30 ng/µl of DNA, sufficient DNA concentrations for PCR and further molecular ecological work. The DNA was verified with 1 % agarose gel electrophoresis, and the concentrations were verified at 5 ng/µL or above with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, California, USA).

In Figure 1.1 and Figure 1.2, PCR products from amplified regions of genomic DNA from *R. mangle* leaf tissue, *R. mangle* propagule tissue, *A. germinans* leaf tissue, and *A. germinans* propagule tissue exhibit clearly defined bands in the agarose gel, denoting products of similar length, clarity, and concentration. These samples have demonstrated adequacy for further molecular ecology work, as these methods of DNA isolation, extraction, and amplification have been used for sample processing, sequencing, and fragment analysis for further genetic study of mangrove populations in Chapters 2 and 3 of this thesis.

In conclusion, these methods in mangrove leaf and propagule tissue storage, DNA isolation from mangrove leaf and propagule tissue, and customized PCR and thermocycling programs offer a rapid, simple, and efficient protocol for mangrove tissue storage and processing. They eliminate the need of freezing samples for storage during transport, and in some cases eliminate the need for freezing samples for storage at all. Additionally, these methods also alleviate the need for large, expensive dehydrators. The use of pre-existing DNA extraction kits with single-usage, disposable components largely reduces the probability of cross-contamination. The QIAGEN DNeasy Plant Mini Kit (QIAGEN Pty Ltd, Valencia, California, USA) also reduces the amount of harmful or toxic chemicals needed to process the tissue, and streamlines the process to a series of simple steps, allowing for simultaneous processing of multiple samples. These protocols have great potential for further development toward being transferable to other higher plants with highly recalcitrant tissues.

a.)

b.)

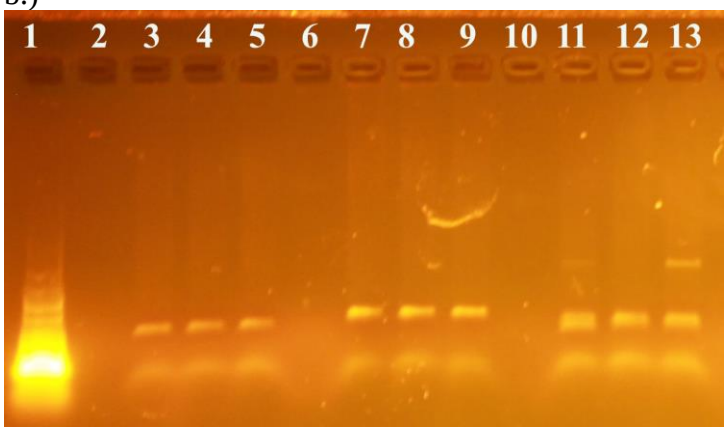
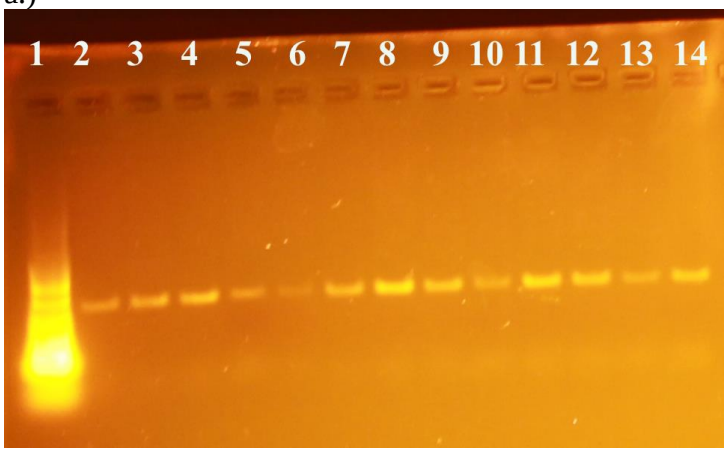


Figure 1.1. PCR products resolved on 1.5% agarose gel. The first lane in each gel is loaded with hyperladder (25bp, Bioline, Taunton, MA, USA). Empty lanes are left as a gap to distinguish different microsatellite primers used on samples. a.) PCR products of various leaf samples of *R. mangle* genomic DNA amplified with microsatellite primers RM38 and RM41, respectively (Rosero-Galindo et al. 2002).
b.) PCR products of various samples of *R. mangle* genomic DNA extracted from propagule tissue and amplified with microsatellite primers RM19, RM21, and RM36, respectively (Rosero-Galindo et al. 2002).

a.)



b.)

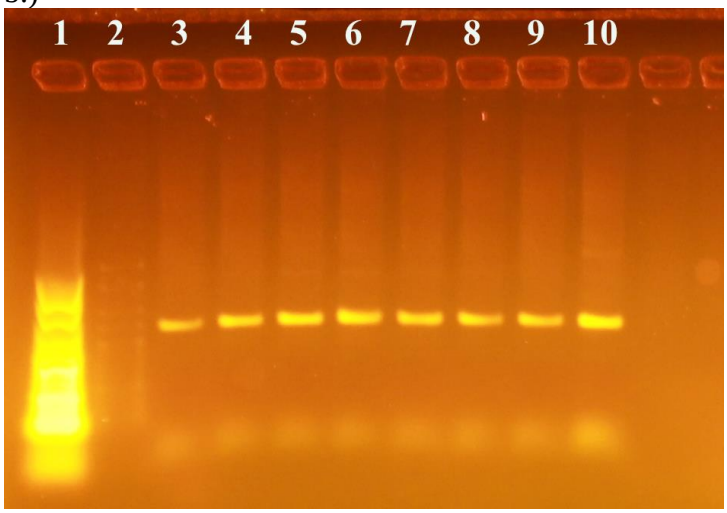


Figure 1.2. PCR products resolved on 1.5% agarose gel. The first lane in each gel is loaded with hyperladder (25bp, Bioline, Taunton, MA, USA). Empty lanes are left as a gap to distinguish different microsatellite primers used on samples.
a.) PCR products of various samples of *A. germinans* genomic DNA extracted from leaf tissue, amplified with microsatellite primer Agerm1-22 (Mori et al. 2010).
b.) PCR products of various samples of *A. germinans* genomic DNA extracted from propagules, amplified with microsatellite primer Agerm1-18 (Mori et al. 2010).

Appendix / Supplemental Material

Figure 1. *R. mangle* leaves and shaved *R. mangle* propagules prior to extraction.

Figure 2. *A. germinans* leaves and propagules prior to DNA extraction.

Table 1. Modified PCR thermocycler program for Type-it® Microsatellite Multiplex PCR Kit (QIAGEN) used for *A. germinans* samples.

Step	Temperature (°C)	Time	Action
1	95	5:00	Initial Denaturing
2	95	0:30	Denaturing
3	65.5*	1:30	Annealing
4	72	0:30	Extension
5	95	0:30	Denaturing
6	63.4*	1:30	Annealing
7	72	0:30	Extension
8	95	0:30	Denaturing
9	60**	1:30	Annealing
10	72	0:30	Extension
11	95	0:30	Denaturing
12	59.6*	1:30	Annealing
13	72	0:30	Extension
14	95	0:30	Denaturing
15	56.7*	1:30	Annealing
16	72	0:30	Extension
17	95	0:30	Denaturing
18	55*	1:30	Annealing
19	72	0:30	Extension
20	95	0:30	Denaturing
21	50*	1:30	Annealing
22	72	0:30	Extension
23	go to 20	23 times	Cycling
24	60	1:30	Final Extension
25	4	END	Chill

* Specific annealing temperature of a primer as outlined by literature
** Optimum annealing temperature per QIAGEN

Table 2. Modified PCR thermocycler program for Type-it® Microsatellite Multiplex PCR Kit (QIAGEN) used for *R. mangle* samples.

Step	Temperature (°C)	Time	Action
1	95	5:00	Initial Denaturing
2	95	0:30	Denaturing
3	54*	1:30	Annealing
4	72	0:30	Extension
5	95	0:30	Denaturing
6	52.0*	1:30	Annealing
7	72	0:30	Extension
8	95	0:30	Denaturing
9	51.0*	1:30	Annealing
10	72	0:30	Extension
11	95	0:30	Denaturing
12	50.0*	1:30	Annealing
13	72	0:30	Extension
14	go to 11	23 times	Cycling
15	60	1:30	Final Extension
15	4	END	Chill

* Specific annealing temperature of a primer as outlined by literature

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Chapter 2

An assessment of latitudinal patterns in genetic diversity and inbreeding coefficients for *Avicennia germinans* (*Black Mangrove*) and a comparative analysis of its historic and range-expansion populations along the Gulf Coast of Florida, Louisiana, and Texas, USA.

Abstract

Within the last 10 – 15 years, new, range-expansion populations of *Avicennia germinans* have established themselves along the northern Gulf Coast, representing a northward migration of these mangrove populations as winter temperatures along the tropical-temperate ecotone grow milder. The genetic diversity of mangrove populations along the Gulf Coast – and thus their population fitness, resilience, and predisposition for successful establishment into novel territory – remain in question, especially for new range-expansion populations, which tend to be relatively small and fragmented. Previous research suggests high inbreeding and decreased genetic diversity in new, range expansion populations, placing such populations at great risk for decreased fitness due to inbreeding depression and founder effects. Thus, genetic diversity is an important parameter to consider when assessing future mangrove range expansion, and future mangrove conservation and management This study used 12 polymorphic microsatellite loci to assess the regional variation in genetic diversity within and among the populations of *A. germinans* along the Gulf Coast of Florida, Louisiana, and Texas. The study aimed to determine whether there was a latitudinal gradient of genetic diversity in the populations sampled, and how the genetic diversity of two new, range-expansion populations (Dog Island, FL, and Leeville, LA) compared to the genetic diversity represented in the other five historic, within-range populations sampled along the Florida and Texas Gulf coasts.

The results of the study indicated all populations expressed heterozygote deficiency and revealed moderate to high levels of inbreeding within populations (F_{IS} = 0.20 – 0.70). Observed heterozygosity was very low (H_o = 0.04 – 0.28), and there was no discernable gradient of decreasing genetic diversity with increasing latitude. The youngest and most northern range-expansion population sampled, Dog Island, FL, exhibited the lowest observed heterozygosity and the highest inbreeding coefficient, whereas the older, more developed range-expansion population exhibited heterozygosity and inbreeding coefficients comparable to the historic populations, suggesting a possible temporal effect on the severity of founder effects enacting on young, range-expansion populations possibly due to long-distance dispersal events. Despite low observed heterozygosity and moderate to high inbreeding, AMOVAs suggest populations do

exhibit significant genetic distinction from one another (F_{ST} = 0.39; p-value < 0.001). This data supports prior research that mangrove populations can sustain moderate to high levels of inbreeding and low levels of heterozygosity, and still maintain high genetic variation among populations likely due to the influence exerted by local environmental conditions.

Introduction

Most of northern Florida and southern Louisiana are historically dominated by salt marsh ecosystems. Recent data, however, project the possibility of northward and inland mangrove migration into salt marsh systems due to sea level rise, warmer winter temperatures, and reduced freezing events. This, in turn, suggests possible salt marsh displacement and ecosystem shifts in ecotones along the Gulf Coast (Ross et al. 2009; Saintilan et al. 2009; McKee et al. 2012; Osland et al. 2013).

Mangrove and salt marsh habitats support vital coastal and offshore fish and invertebrate populations and are important nurseries for juvenile life stages of many marine species. Mangrove and salt marsh species are considered foundational species, meaning they exert great influence on wetland ecosystem dynamics and the ecological communities they inhabit (Tomlinson 1986; Twilley 1998; Hogarth 1999; Cerón-Souza et al. 2012; Osland et al. 2013; Yando et al. 2016; Thomas et al. 2017). For example, the presence of mangroves or marshes affects the geomorphology and geochemistry of the environment, improving offshore water quality through runoff filtration, sequestering carbon in plant tissue and in the organic horizon of the sediment, and buffering coastlines from erosion and storm energy (Hogarth 1999; Nagelkerken et al. 2008Osland et al. 2013; Peterson and Bell 2012; 2015; Yando et al. 2016). Though salt marsh and mangrove species can occupy similar geomorphic settings, they have differentiated environmental stress limits. These respective stress limits generally set strict latitudinal constraints on mangrove-salt marsh zonation (Friess et al. 2011; Osland et al. 2013).

Mangrove species have broader temperature and inundation ranges than salt marsh species, and as a whole tend to out-compete them in more tropical settings. But mangrove seedlings are highly susceptible to winter freeze events (Stuart et al. 2007; Pickens and Hester 2011; Osland et al. 2013; Madrid et al. 2014) and usually cannot successfully establish north of their tropical latitudinal thresholds (Osland et al. 2013). Marshes, in turn, historically dominate the temperate, boreal, and arctic coastlines (Adam 1990; Pennings and Bertness 2001). Near tropical to temperate transition zones, however, warmer winter temperatures, less extreme freezing events, and increased tidal encroachment will potentially enable poleward mangrove migration and niche competition, which may result in salt marsh displacement (Osland et al. 2013; Madrid et al. 2014). A study by Peterson and Bell (2015) even found that the presence of salt marsh grasses facilitates mangrove propagule entrapment, promoting inland propagule transportation and improving chances of successful mangrove establishment.

Ecosystem shifts in these areas will affect botanic, vertebrate, and invertebrate species abundances and distributions, as well as coastal geomorphology, hydrology, and carbon storage potential (Osland et al. 2013; Doughty et al. 2015; Yando et al. 2016). Thus, for conservation and environmental planning purposes, it is important to accurately predict where these ecosystem shifts may occur. Various studies have examined the abiotic limitations on possible mangrove migration by evaluating dispersal ranges, dispersal mechanisms, and temperature thresholds (Bialozyt et al. 2006; Nettel and Dodd 2007; Stuart et al. 2007; Alleman and Hester 2011; Pickens and Hester 2011; McKee et al. 2012; Osland et al. 2013; Cavanaugh et al. 2014; Madrid et al. 2014; Peterson and Bell 2012; 2015). Few studies have examined possible biotic limitations of mangrove migration, investigating differences in reproductive strategies, physiological and phenotypic differences among populations, and the genetic diversity within and among populations in response to novel stressful conditions (Arnaud-Haond et al. 2006; Cerón-Souza et al. 2012; Nettel-Hernanz et al. 2013; Madrid et al. 2014; Proffitt and Travis 2014; Sandoval-Castro et al. 2014; Dangremond and Feller 2016; Hodel et al. 2016; Millán-Aguilar et al. 2016; Salas-Leiva et al. 2009). Literature on levels of genetic diversity in mangrove populations draw contradictory conclusions as to whether mangroves are highly selfing and overall inbred, or whether local environmental

conditions govern the levels of inbreeding and outcrossing, thus enabling the species to vary greatly both geospatially and temporally.

Proffitt and Travis (2014) surveyed population reproduction and outcrossing rates in *Rhizophora mangle* (red mangrove) in Florida in relation to cold stress (measured through latitude), anthropogenic stress (measured through human population density), and natural disturbance (measured through years since a major hurricane). They used the frequency of occurrence of heterozygous trees (exhibiting normal and albino propagules) and the deviation from the 3:1 Mendelian ratio of heterozygous trees to homozygous tress (exhibiting only normal propagules) as a proxy for mutation rates and outcrossing rates. The resulting data found the number of reproducing trees within a stand varied by site and increased with latitude, and that outcrossing increased under conditions of cold and anthropogenic stress. Cold stress appeared to increase reproductive output (number of reproducing trees) within populations, increase outcrossing, and suppress mutation (presence of albino propagules). Anthropogenic stress, however, elicited the opposite response. Stands in close proximity with human populations exhibited a greater rate of mutation, decreased stand reproduction, and increased outcrossing. The study concludes that the potential for colonization of northern Florida salt marshes by mangroves is enhanced by increased reproductive and outcrossing rates, which should also enhance genetic variation and promote adaptation to novel environmental conditions (Proffitt and Travis 2014). Another study by Dangremond and Feller (2016) observed that individuals of *R. mangle* in range-expansion populations in eastern Florida became reproductively active at younger ages than individuals of southern populations, further supporting the findings by Proffitt and Travis (2014).

Salas-Leiva et al. (2009) studied natural and re-forested areas of *A. germinans* (black mangrove) in Colombia using microsatellite genetic markers, and found that all populations sampled deviated from the Hardy-Weinberg Equilibrium, and that inbreeding coefficients increased in re-forested areas. Younger trees in a region where a new highway had been built expressed higher rates of inbreeding than their older counterparts. On another transect with no such construction activity, young and old trees appeared to have similar inbreeding coefficients, alluding to the negative effect of stress on breeding and resulting genetic diversity (Salas-Leiva et al. 2009).

Nettel-Hernanz et al. (2013) conducted an analysis of mating systems in *A. germinans* populations along the Chiapas, Mexico coastline using microsatellite analysis of parent and progeny DNA. Results found that inbreeding coefficients were close to zero, and none of the studied populations significantly deviated from the Hardy-Weinberg Equilibrium. Detected levels of bi-parental inbreeding were low, and the proportion of siblings sharing the same "father" tree was also low. The study concluded that *A. germinans* populations in this area were predominantly outcrossing but could support moderate levels of self-fertilization. Nettel-Hernanz et al. (2013) claim these findings are consistent with previous predictions (Clarke and Myerscough 1991; Arnaud-Haond et al. 2006; Giang et al. 2003; Cerón-Souza et al. 2005) that *Avicennia* mating patterns consist of a mixture of outbreeding and selfing, with outcrossing events being more predominant but random, and exerting little effect on population substructure and bi-parental inbreeding.

In a study using microsatellite loci to examine genetic diversity among 15 populations of *A. germinans* along the eastern and western coasts of Florida, Hodel et al. (2016) observed greater heterozygosity than expected, with H_o ranging from 0.175 to 0.838 and H_e ranging from 0.243 to 0.553. The overall average H_o across all 15 sites was 0.67. The lowest levels of heterozygosity were found on the southwestern tip of Florida, in Flamingo ($H_o = 0.175$). The highest levels were found along the southern Florida Keys ($H_o = 0.71 – 0.77$), New Port Richey ($H_o = 0.75$), and West Palm Beach ($H_o = 0.775$). The northernmost sites, however, on the east and west coasts of Florida, near Seahorse Key and Cape Canaveral respectively, exhibited reduced heterozygosity ($H_o = 0.65$; $H_o = 0.613$). Hodel et al. (2016) also found significant gene flow between populations of *A. germinans*. However, the gene flow from the Gulf to the Atlantic is roughly equivalent to gene flow in the opposite direction, and thus shows no clear directional pattern. This suggests the primary directional ocean currents in this region do not exert a major influence on genetic flow and population connectivity in *A. germinans*. But for *R. mangle*, gene flow from the Gulf to the Atlantic appears to follow the pattern of directional currents in the Gulf Stream, indicating that, in the case of *R. mangle*, ocean currents do affect gene flow in this region (Hodel et al. 2016).

Based on the data from these studies, one can surmise that mangroves appear to support moderate levels of inbreeding, yet the range of genetic variation among populations is high, suggesting separate populations are generally isolated from one another. This, in turn, indicates that local conditions exert great influence over patterns of population genetics in mangroves.

Researchers have yet to use molecular techniques to specifically examine differences in genetic diversity in northern and southern mangrove populations compared to range-expansion populations in northern Florida and southern Louisiana. Such data would offer many important practical applications for modeling future mangrove migration, identifying populations to prioritize for conservation, and managing overall coastal conservation and restoration.

In this study, I used 12 species-specific polymorphic microsatellite loci to assess the regional variation in diversity within and among populations of *A. germinans* along the Gulf Coast. I set out to answer the following questions: (1) Do populations of *A. germinans* sampled appear highly inbred, or do they appear to adhere to Hardy-Weinberg levels of interbreeding? (2) How much genetic diversity exists within and among populations of *A. germinans*? (3) Is there a latitudinal pattern or trend in the degree of genetic diversity of *A. germinans* populations sampled along the Gulf Coast?

I hypothesized that the populations sampled will exhibit at least intermediate levels of outbreeding (inbreeding coefficient of 0.4 or lower), thus allowing for propagules with the genetic plasticity necessary to succeed in new environments (as exemplified by successful range-expansion of mangroves into northern Florida and Louisiana). I further expect populations at higher latitudes, closer to the boundaries of typical mangrove latitudinal ranges, to exhibit decreased genetic diversity compared to more southern populations, due to selection and a bottleneck of pollen contributors (i.e. founder effect).

Methods

Sample Collection and Preparation

Leaf samples from mature trees of *A. germinans* were collected from a total of seven locations (populations) along the west coast of Florida, southern coast of Louisiana, and southern coast of Texas (Figure 2.1). Two of these locations – Dog Island, Florida and Leeville, Louisiana – were identified as new range-expansion populations, estimated to have established within the last 10 years (Table 2.1). Individuals within the range-expansion population were more sparsely distributed along the coastline than more developed, historic mangles, which are often very dense. Individuals from the new, range expansion populations also appeared largely homogeneous in height, usually standing roughly four to five feet tall, with some apparently younger generations only standing two to three feet tall. While southern Florida populations have individuals standing as high as 30 feet, more stressed, northern-latitude populations can exhibit much smaller statures, even in mature mangles. The main distinguishing feature of the new, range expansion populations was the sparse distribution of individuals.

In each of the seven populations, leaf samples were obtained from 10 - 15 individuals of *A. germinans*. To minimize the chance of collecting parents and their progeny within the same location, only trees located along the outer edge of the mangle (stand of mangroves, mangrove forest) and at least 10m apart were sampled. In the two range-expansion populations, where the mangles were fairly young, small, and densely packed, only trees 3m or more apart were sampled.

The leaves were stored in labeled bags with silica gel as outlined in Chapter 1 until DNA extraction in a lab at the University of Virginia. The genomic DNA was isolated and extracted using a modified protocol for the DNeasy™ Plant Mini Kit (QIAGEN Pty Ltd, Valencia, California, USA) as outlined in Chapter 1. Extracted DNA was quantified on a Qubit® 2.0 Fluorometer (Invitrogen). DNA was diluted to a working stock concentration of ~5ng μL^{-1}.

Table 2.1. Classification, name, abbreviation, and GPS coordinates of the seven sites of *A. germinans* populations sampled for leaf tissue.

Leading Edge Populations	Dog Island, Apalachicola, FL (DI)	29°48'48.1"N 84°35'05.3"W
	Leeville, LA (Leeville)	29°14'49.2"N 90°12'37.5"W
Historic Range Populations	Emerson Point Preserve, FL (EPP)	27°31’56.8”N 82°37’46.3”W
	Port Aransas, TX (PA)	27°52'04.7"N 97°05'15.8"W
	Port Isabel, TX (PI)	26°00'59.8"N 97°16'25.6"W
	Cedar Key, FL (CK)	29°08'09.4"N 83°01'48.0"W
	"Site 2", Smallwood Dr., Ochopee, FL (Site2)	25°49’17.15”N 81°21’33.86”W

*10 individuals were sampled at CK, EPP, and Site 2; 15 individuals were sampled at DI, Leeville, PA, and PI

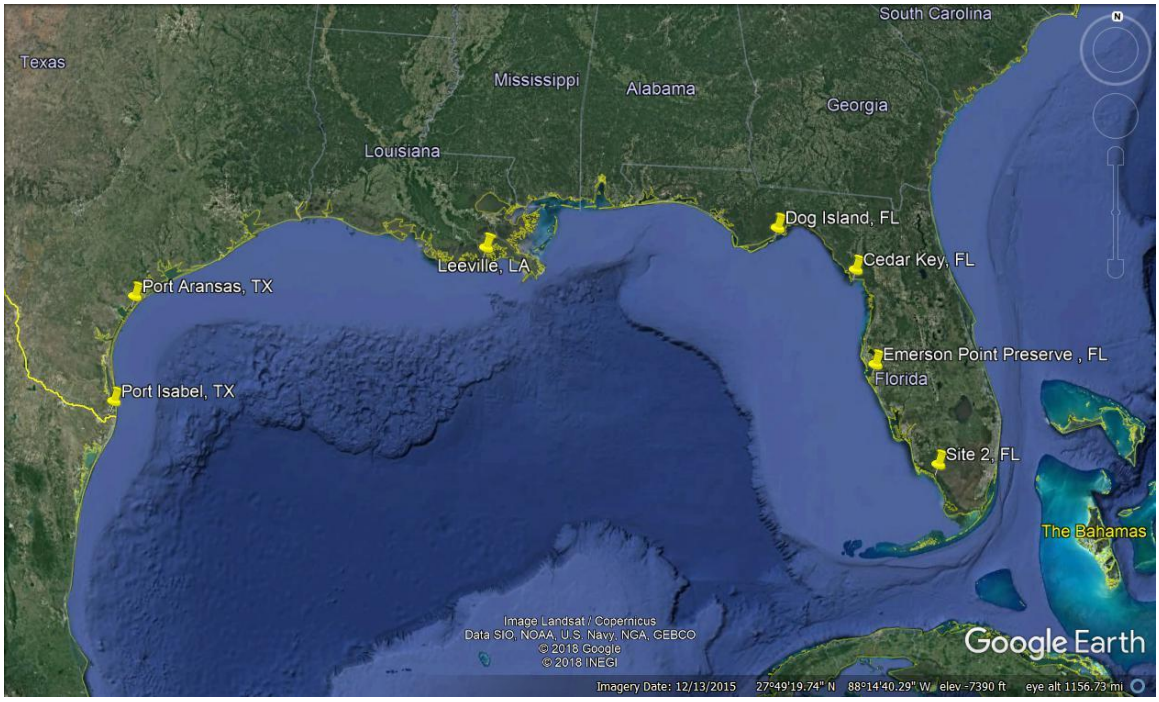


Figure 2.1. Map of the 7 locations along the Gulf Coast sampled for *Avicennia germinans*. From left to right, the sites are as follows: Port Isabel, TX; Port Aransas, TX; Leeville, LA; Dog Island, FL; Cedar Key, FL; Emerson Point Preserve, FL; “Site 2,” FL. Leaves from 15 individuals were sampled at each location.

Microsatellite Amplification and Analysis

Polymerase chain reaction (PCR) and fluorescently labeled primers identified by Nettel and Dodd (2005), Cerón-Souza et al. (2006), and Mori et al. (2010) were used to amplify 12 microsatellite loci of interest for *A. germinans* (Appendix: Table 1). Twelve loci were

considered sufficient, since previous studies have detected phylogeographic structure, genetic diversity, and genetic connectivity with similar or fewer microsatellite markers (Salas-Leiva 2009; Pil et al. 2011; Hodel et al. 2016).

(*Note:* Using a Bonferroni corrected α-value ($\alpha < 0.004$; $p < 0.004$), the chosen loci were examined for linkage disequilibrium. Linkage disequilibrium was detected between two loci targeted by the primers Agerm1-01 and Agerm1-12 (Mori et al. 2010) in the populations Leeville, LA, Port Aransas, TX, and Port Isabel, TX, and all loci also indicated significant deviation from Hardy-Weinberg Equilibrium ($p < 0.05$), exhibiting heterozygote deficiency. As these are published primers used in various other genetics studies of *A. germinans*, the lean toward heterozygote deficiency is likely a product of the high levels of inbreeding within the populations rather than a factor of the chosen loci. Published loci are assumed to be neutral.)

PCR was conducted in three multiplex panels using a Type-it® Microsatellite Multiplex PCR Kit (QIAGEN). When the PCR products were too similar in fragment length, and the products overlapped when pooled together and visualized for analysis, PCR was conducted in simplexes using the Type-it® Microsatellite Multiplex PCR Kit (QIAGEN). Following standard M13 protocols (Shuelke 2000; Boutin-Ganache et al. 2001) and using four fluorescent labels (FAM, NED, VIC), the PCR recipe (14 μL reactions per well) is as follows: 7.5 μL of Type-it® PCR Multiplex Master Mix, 0.06 μL of 10 μM microsatellite primer-forward with M13 tail attached, 0.24 μL of 10 μM microsatellite primer-reverse, 0.24 μL of 10 μM primer-M13 tag (FAM/NED/VIC), 5.96 μL H_2O, and 1 μL of diluted working stock DNA.

PCR conditions were set to a modified version of the manufacturer's (QIAGEN) optimized cycling conditions to include the optimum annealing temperature for the Type-it® Microsatellite Multiplex reagent and the suggested optimum annealing temperatures of the various microsatellite primers used. PCR protocol was optimized using a touch-down approach from 65.5°C to 50°C to better target individual primer annealing temperatures (Appendix: Table 3).

PCR products were sequenced on a capillary-based 3730xl DNA Analyzer (Applied Biosystems) with an internal ET-ROX 500 size standard at the Georgia Genomics Facility (University of Georgia, Georgia, USA). Fragment lengths for each locus were determined using Geneious V7.1.5 (Biomatters Ltd.).

Allele frequencies and genetic diversity statistics

For each population the number of different alleles (N_a), effective number of alleles (N_e), number of private alleles (N_p), percent polymorphism (%P), observed heterozygosity (H_o), expected heterozygosity (H_e), and the inbreeding coefficient (F_{IS}) were calculated in GENALEX version 6.5 and GenePop 4.2 (Peakall and Smouse 2006; 2012). Loci were tested for linkage disequilibrium using a Bonferroni corrected α-value and p-value, and populations were tested for deviations from Hardy-Weinberg Equilibrium by measuring heterozygote excess and heterozygote deficit in GENEPOP web version 4.2 (Raymond and Rousset 1995; Rousset 2008).

Genetic differentiation: Analysis of molecular variance, pairwise F-statistics comparisons, and PCA

An analysis of molecular variance (AMOVA) was performed to assess overall genetic differentiation among populations under the assumptions of the Infinite Allele Model in GENODIVE version 2.0 (Miermans and Tienderen 2004). Standard deviations for AMOVA F-statistics were calculated by jackknife resampling over loci, and permutation tests (1,000 and 10,000 permutations – latter not reported) were used to assess significance. Confidence intervals of 95% of F-statistics were obtained through bootstrapping over loci.

In order to assess the significance of genetic differentiation between northernmost, range-expansion populations and historic, within-range populations, another AMOVA was performed in GENODIVE (Miermans and Tienderen 2004) by grouping new populations (DI and Leeville) into a “northern” region and historic range

populations (CK, EPP, PA, PI, Site 2) into a "historic" region. Populations were nested within regions for the analysis. Standard deviations for AMOVA F-statistics were calculated by jackknife resampling over loci, and permutation tests (10,000 permutations) were used to assess significance. Confidence intervals of 95% of F-statistics were obtained through bootstrapping over loci.

Fixation indices were calculated to measure population differentiation and genetic distance. Fixation indices F_{ST} (Weir and Cockerham 1984), Rh_o (Ronfort et al. 1998), Nei's D (Nei 1972), and Jost's D (Jost 2008; 2009) were calculated for all possible pairwise population combinations using GENODIVE. As these terms are analogous (Freeland et al. 2011), and the results and patterns are maintained across the pairwise combinations, only F_{ST} and Jost's D are reported. Principal component analysis (PCA) without the assumption of hierarchical genetic structure was performed in GENODIVE using a covariance matrix based on individual allele frequencies to determine whether geographically proximal samples exhibit similar allele frequencies. The two axes that accounted for the most variance within the data were graphed to visually depict the variations among population clusters.

Population Structure

To determine the most likely number of population clusters (K) based on the genetic data collected, population assignment using a Bayesian approach was performed in the genetic software program STRUCTURE (Pritchard et al. 2000). Admixture was specified in the model, and model parameters were set to K = 1 – 20, with an initial burn-in period of 10,000 iterations followed by 10,000 Markov Chain Monte Carlo (MCMC) repetitions, as suggested by Evanno et al. (2005). To account for the variation of the likelihood for each K, each data set, K = n, was run 20 times and averaged. The average for each K = n was used to calculate the most likely number of population clusters using the ad hoc quantity, ΔK (Evanno et al. 2005). To check the agreement of the results using parameters suggested by Evanno et al. (2005) with longer burn-in periods and MCMC iterations, the analysis was repeated by setting K = 1 – 15, with 50,000 burn-in iterations followed by 250,000 MCMC repetitions. Once the most likely population clusters were

identified, they were visualized using the bar plot feature in STRUCTURE (Pritchard et al. 2000).

(*Note:* Missing data may bias the results of PCA and STRUCTURE analysis tests by artificially grouping together samples with missing data at the same loci (Miermans and Van Tienderen 2004). Thus, separate tests were run with and without filling in missing values by using randomly drawn alleles from relative allele frequencies for each population, as recommended by Miermans and Van Tienderen (2004), to assure the results were not significantly different. The results were not found to yield significant differences, so the original datasets without missing data filled in were reported for this study.)

Results

Allele frequencies and genetic diversity statistics

Using 12 microsatellite primers to target 12 loci, the number of different alleles (N_a), effective number of alleles (N_e), number of private alleles (N_p), percent polymorphism of loci (%P), observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient (F_{IS}), were calculated based on the 90 individual samples collected at seven sampling locations (see Figure 2.1 and Table 2.1 for sampling sites; see Appendix: Table 1 for loci information). Though GENODIVE detected 66 different alleles in total across the 12 loci (see Appendix: Table 2), the average number of different alleles per population detected by GenAlEx is 2.4 (Table 2.2). The highest number of different alleles (N_a) occurs in Site 2, FL, at 3.2, and the lowest number of different alleles occurs in Dog Island, FL, and Port Aransas, TX, at 1.8. The effective number of alleles (N_e), however, is consistently lower across all sites, averaging to an overall 1.5. The highest values for number of private alleles occur in Emerson Point Preserve, FL, and Site 2, Florida (0.58 and 0.67, respectively); the lowest values for number of private alleles

occur in Port Aransas, TX, and Dog Island, FL (0 and 0.08, respectively; Table 2.2). The percent of polymorphic loci across all populations ranges from 41.6% in Port Aransas, TX, to 100% in Site 2, FL.

Overall, the observed heterozygosity was lower than the expected heterozygosity, ranging from 0.04 – 0.28 and 0.14 – 0.43, respectively (Table 2.2). The average observed heterozygosity across all sites was 0.16, with the greatest heterozygosity occurring in Site 2, FL (0.28) and the lowest occurring in Dog Island, FL (0.04). Port Aransas, TX, Port Isabel, TX, and Leeville, LA, all exhibited similar observed heterozygosity (0.11, 0.11, 0.12), as did Cedar Key, FL, Emerson Point Preserve, FL, and Site 2, FL (0.22, 0.23, 0.28). The average expected heterozygosity across all sites was 0.27, and ranged from 0.43 (Site 2, FL) to 0.14 (Dog Island, FL). Cedar Key, FL and Site 2, FL, had similar expected heterozygosity (0.42, 0.43). Dog Island, FL, Port Aransas, TX, and Port Isabel, TX, also had similar values of expected heterozygosity (0.14, 0.17, 0.17). The greatest difference between observed heterozygosity and expected heterozygosity occurs in Cedar Key, FL (0.22 vs. 0.42).

The inbreeding coefficient (F_{IS}) ranges from high (0.70 in Dog Island, FL) to relatively low (0.20 in Emerson Point Preserve, FL). The average inbreeding coefficient among populations is relatively moderate (0.42). All populations indicated significant deviation from Hardy-Weinberg Equilibrium ($p < 0.05$), exhibiting great heterozygote deficiency. Using the Bonferroni corrected α-value, $p < 0.004$, linkage disequilibrium was detected between two loci targeted by the primers Agerm1-01 and Agerm1-12 (Mori et al. 2010) in the populations Leeville, LA, Port Aransas, TX, and Port Isabel, TX.

Table 2.2. Summary of the genetic statistics for all populations of *Avicennia germinans*. Number of individuals (N), number of different alleles (N_a), effective number of alleles (N_e), number of private alleles (N_p), percent polymorphism (%P), observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient (F_{IS}) are reported for each population. Standard error is included for H_o, H_e, and F_{IS}. The total number of samples used, and the averages for each statistic, is included at the bottom of the table. All populations exhibit significant deviation from Hardy-Weinberg Equilibrium ($p < 0.05$).

Population	N	Na	Ne	%P	Ho	He	Fis
PI	15	2.080	1.230	58.33%	0.11 ± 0.055	0.17 ± 0.061	0.45 ± 0.126
PA	15	1.830	1.260	41.67%	0.11 ± 0.053	0.17 ± 0.067	0.34 ± 0.127
Leeville	15	2.330	1.480	66.67%	0.12 ± 0.053	0.23 ± 0.076	0.43 ± 0.118
DI	15	1.830	1.210	58.33%	0.04 ± 0.022	0.14 ± 0.046	0.70 ± 0.120
CK	10	3.000	1.910	91.67%	0.22 ± 0.063	0.42 ± 0.054	0.52 ± 0.122
EPP	10	2.500	1.560	66.67%	0.23 ± 0.07	0.30 ± 0.075	0.20 ± 0.115
Site 2	10	3.250	2.030	100.00%	0.28 ± 0.072	0.43 ± 0.069	0.28 ± 0.125
Averages	**Total = 90**	**2.403**	**1.526**	**69.05%**	**0.16**	**0.266**	**0.419**

Genetic Differentiation: Analysis of molecular variance (AMOVA), Pairwise comparisons of Fixation Indices, and PCA

The AMOVA analyzing differentiation among all 7 populations revealed that the greatest amount of variance occurred among populations (39%), followed by within individuals (37%), and that there is significant genetic differentiation among populations ($F_{ST} = 0.387 \pm 0.068$; p-value < 0.001; Table 2.3).

The AMOVA analysis of the genetic differentiation between new, "northern" leading-edge populations (DI, Leeville) and "historic" populations (CK, EPP, PA, PI, Site 2), nested into respective "northern" and "historic" regions, found that most of the variance within the data set is attributed to among populations within regions (39%) followed by within individuals (38%). There is no significant genetic differentiation between the "northern" and "historic" regions ($F_{CT} = -0.001 \pm 0.057$, p-value = 0.475; Table 2.4).

Table 2.3. Analysis of Molecular Variance (AMOVA) results treating every sampling location as a population. Standard deviation is reported for F-statistic values.

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	37.1	F_it	0.629	0.064	--
Among Individual	Population	24.1	F_is	0.394	0.079	< 0.001
Among Population	--	38.7	F_st	0.387	0.068	< 0.001

Table 2.4. Analysis of Molecular Variance (AMOVA) results per grouping new populations (DI, Leeville) into a "northern" region and grouping historic range populations (CK, EPP, PA, PI, Site 2) into a "historic" region. Standard deviation is reported for F-statistic values.

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	37.3	F_it	0.627	0.065	--
Among Individual	Population	24.2	F_is	0.394	0.085	< 0.001
Among Population	region	38.6	F_sc	0.386	0.078	< 0.001
Among region	--	-0.1	F_ct	-0.001	0.057	0.475

The results and patterns of the pairwise population differentiation comparisons were largely consistent across all differentiation statistics, F_{ST}, F'_{ST}, Rh_o, Jost's D, and Nei's D (data not shown). As these statistics are analogous (Freeland et al. 2011), only F_{ST} and Jost's D – two of the most commonly used indices to describe genetic differentiation among populations – will be discussed in detail.

The Fixation Index, F_{ST}, ranges from -0.033 – 0.66, and Jost's D ranges from 0 – 0.59 across all population combinations (Figure 2.2). The "new" populations, Dog Island, FL, and Leeville, LA, exhibited a pairwise F_{ST} differentiation range of 0.45 – 0.66 and 0.05 – 0.56, respectively. Dog Island exhibited high genetic differentiation when paired with any other population, and its values were generally higher than most other population combinations overall. The highest pairwise values for Dog Island occurred when paired with Port Aransas and Port Isabel, TX (0.66 and 0.65, respectively). Dog Island's lowest pairwise differentiation values were when paired with Emerson Point Preserve, FL (0.45). Leeville's highest pairwise differentiation values occurred when paired with Dog Island (0.56), and the lowest values occurred when paired with Port Aransas and Port Isabel (0.05). The Texas sites, Port Aransas and Port Isabel, have a

negative pairwise differentiation F_{ST} value (-0.033) when paired with each other. The Florida sites – Cedar Key, Emerson Point Preserve, and Site 2 – also have relatively low pairwise differentiation values when paired together, ranging from 0.17 – 0.26. Site 2, FL, has relatively high pairwise differentiation values when paired with Dog Island, FL, Leeville, LA, Port Aransas, TX, and Port Isabel, TX (0.50, 0.52, 0.56, and 0.57, respectively).

These trends correspond with the trends seen in the Jost's D distance pairwise combinations. Leeville, LA, Port Aransas, TX, and Port Isabel, TX, have the lowest values when paired together (all 0), and Dog Island, FL, tends to have relatively high values when compared with any other population. Contrary to the trend of Dog Island, FL, generally being the most highly differentiated population in the F_{ST} pairwise combinations, the highest Jost's D values appear when Site 2, FL, is paired with Leeville, LA, Port Aransas, TX, and Port Isabel, TX (0.59, 0.57, 0.58, respectively; Figure 2.2).

x ≤ 0	Very little differentiation
0 < x < 0.05	Little differentiation
0.05 - 0.09	Moderate differentiation
0.1 - 0.25	Great differentiation
0.25 < x	Very great differentiation

Pairwise Fst

Population	Type	PI	PA	Leeville	DI	CK	EPP	Site2
Historic	**PI**	0						
Historic	**PA**	-0.033	0					
New	**Leeville**	0.049	0.05	0				
New	**DI**	0.648	0.656	0.56	0			
Historic	**CK**	0.396	0.397	0.348	0.487	0		
Historic	**EPP**	0.523	0.533	0.504	0.446	0.25	0	
Historic	**Site 2**	0.567	0.563	0.523	0.505	0.256	0.17	0

Pairwise Jost's D

Population	Type	PI	PA	Leeville	DI	CK	EPP	Site2
Historic	**PI**	0						
Historic	**PA**	0	0					
New	**Leeville**	0	0	0				
New	**DI**	0.397	0.408	0.342	0			
Historic	**CK**	0.285	0.286	0.282	0.363	0		
Historic	**EPP**	0.331	0.339	0.383	0.301	0.204	0	
Historic	**Site 2**	0.582	0.573	0.589	0.392	0.289	0.163	0

Figure 2.2. Pairwise comparisons of all population combinations using F_{ST} and Jost's D. Populations are organized west-to-east along the Gulf Coast.

The principal component analysis (PCA) indicates that the first and second axes of the PCA are responsible for 52.2% and 24.8% of the total variance, respectively (Figure 2.3). The populations clustered into two, perhaps three, population clusters. Dog Island, FL, clustered separately from all other sites, save for an outlier clustered with Cedar Key, FL and Emerson Point Preserve, FL. The Florida sites (Cedar Key, Emerson Point Preserve, Site 2) predominantly clustered together. The Texas and Louisiana sites – Port Aransas, Port Isabel, and Leeville – clustered together, and appear to share some proximity with Cedar Key, FL.

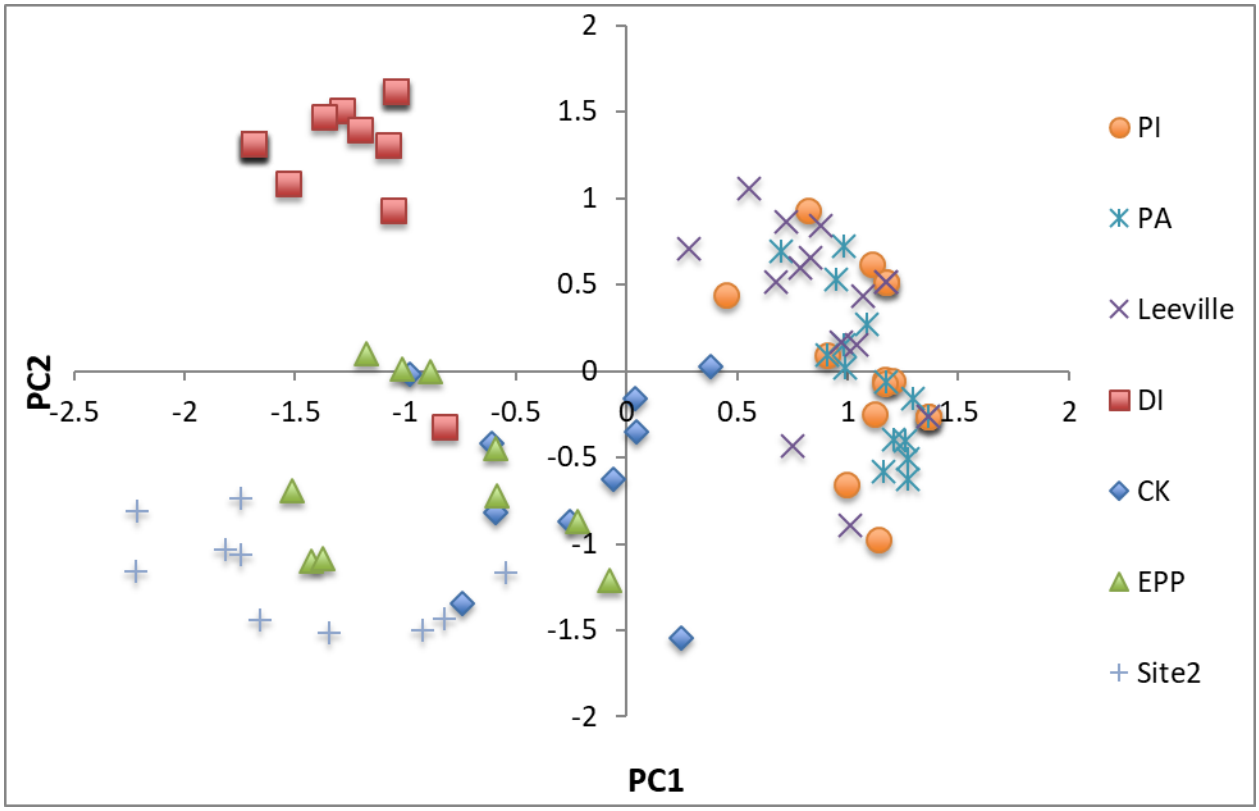


Figure 2.3. Results from the principal component analysis (PCA) plotted for the two principal component axes containing the greatest amount of variance: PC 1 (52.2% variance) and PC 2 (24.8% variance). Populations are listed in order of west to east along the Gulf Coast.

Population Structure

Population structure based on the genetic data showed the greatest statistical support for K = 2 (ΔK = 85) and K = 4 (ΔK = 16.67) when model parameters were set to K = 1 – 20, with an initial burn-in period of 10,000 iterations followed by 10,000 Markov Chain Monte Carlo (MCMC) repetitions, as suggested by Evanno et al. (2005; Figure 4). When the STRUCTURE model parameters were set to K = 1 – 15, with an initial burn-in period of 50,000 iterations followed by 250,000 Markov Chain Monte Carlo repetitions, the greatest statistical support shifted to K = 2 (ΔK = 447) followed by K = 3 (ΔK = 190.94; Figure 5).

For K = 2, the STRUCTURE bar plot depicts Cedar Key, FL, Dog Island, FL, Emerson Point Preserve, FL, and Site 2, FL clustered together, and Leeville, LA, Port Aransas, TX, and Port Isabel, TX clustered together (Figure 6a).

For K = 3, Cedar Key, FL, Emerson Point Preserve, FL, and Site 2, FL, clustered together. Dog Island clustered on its own, and Leeville, LA, clustered with Port Aransas, TX, and Port Isabel, TX (Figure 6b).

For K = 4, Cedar Key, FL, clustered on its own, as did Dog Island, FL. Emerson Point Preserve, FL, clustered with Site 2, FL. Leeville, LA, clustered with Port Aransas, TX, and Port Isabel, TX (Figure 6c).

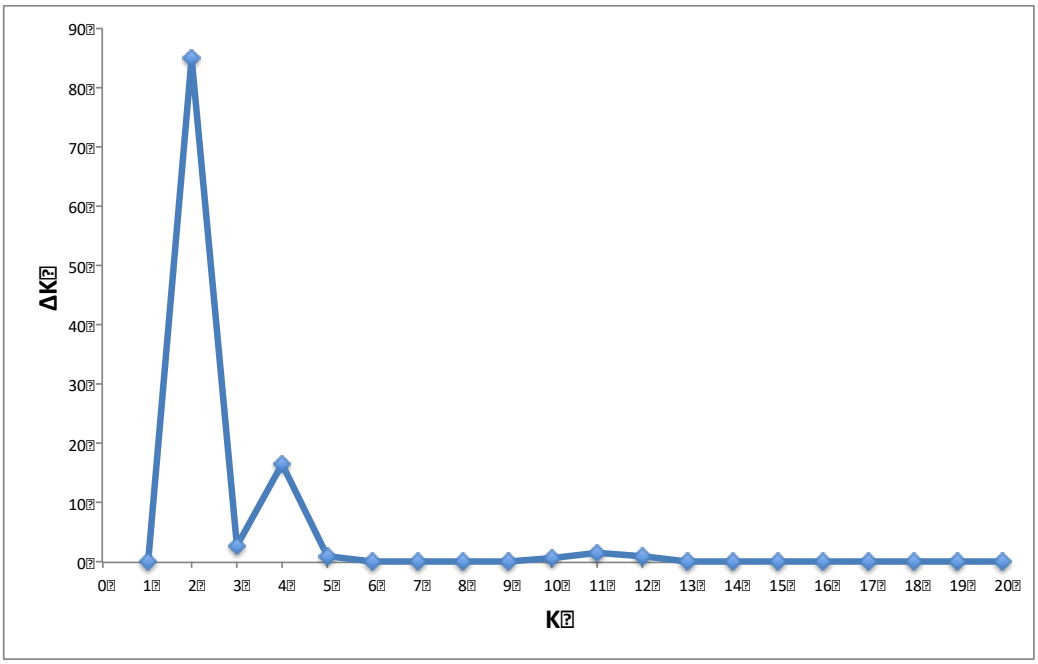


Figure 2.4. ΔK plot of STRUCTURE results with a burn-in period of 10,000 iterations and 10,000 MCMC repetitions, with 20 runs for each K as K = 1 – 20, as suggested by Evanno et al. (2005). The 7 sampled populations grouped into 2, possibly 4, distinct clusters.

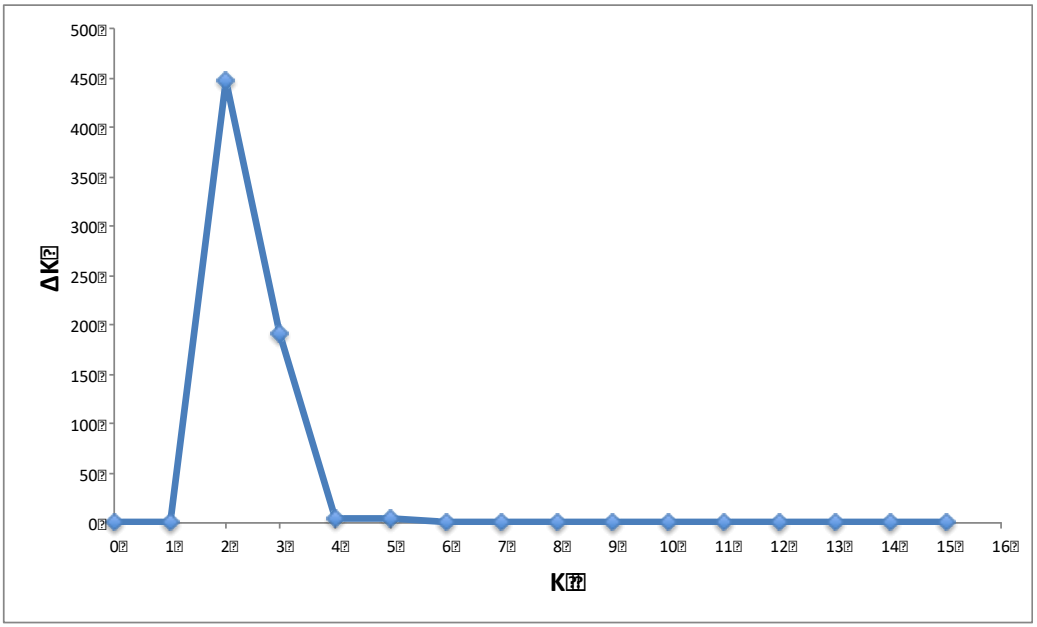
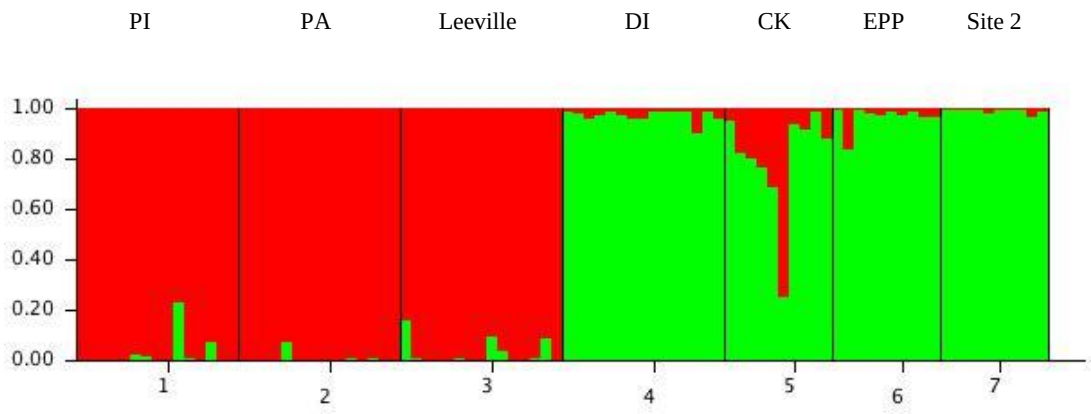
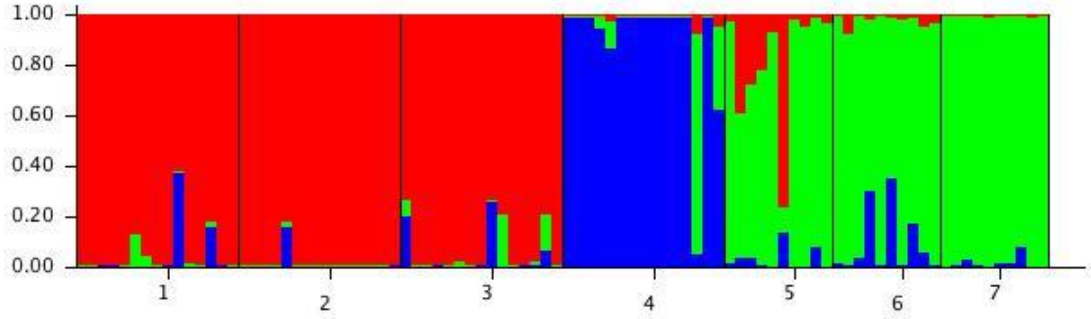


Figure 2.5. ΔK plot of STRUCTURE results with a burn-in period of 50,000 iterations and 250,000 MCMC repetitions, with 20 runs of each K as K = 1 – 15. The 7 sampled populations grouped into 2, possibly 3, distinct clusters.

a)



b)



c)

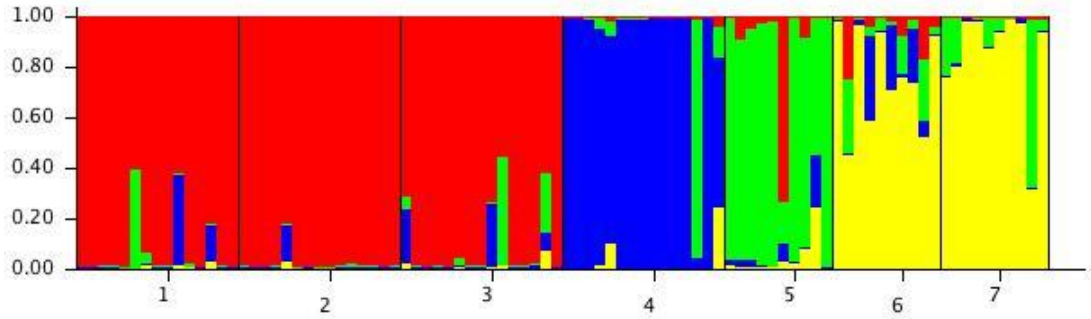


Figure 2.6. Results for STRUCTURE bar plot of the 7 sampling locations for *Avicennia germinans* using (a) K = 2 (b) K = 3 (c) K = 4 cluster assignments. Populations are listed along the x-axis, and individual genotypes are represented as vertical bars of color within each population. Subgroups and individual membership to a cluster is represented by color. Admixture was specified in the model, therefore membership to more than one cluster may occur. (Populations are listed in order of west-to-east orientation around the Gulf Coast as follows: 1 = PI, 2 = PA, 3 = Leeville, 4 = DI, 5 = CK, 6 = EPP, 7 = Site 2.)

Discussion

In this study, I set out to examine the genetic diversity among seven populations of *Avicennia germinans* (black mangrove) sampled along the coast of the Gulf of Mexico, encompassing Florida, Louisiana, and Texas. Genetic diversity is an important component in a population's resiliency to novel stressful conditions, and thus becomes an important parameter when considering ecological conservation initiatives. *A. germinans* is a key foundational species of mangroves in tropical intertidal ecosystems along the Gulf, and plays a large role in coastal geomorphology, coastal hydrology, carbon storage, water filtration, and marine biodiversity (Viosca 1928; Davis 1940; Zedler and Kercher 2005; Barbier et al. 2011; Osland et al. 2013).

With climate change and sea level rise beginning to drive ecosystem shifts in temperate-tropical ecotones, such as seen along the Gulf of Mexico, the fate of many tropical species is uncertain (Osland et al. 2013; Profitt and Travis 2014; Sandoval-Castro et al. 2014; Peterson and Bell 2012; 2015). Biotic drivers, such as levels of genetic diversity within a species and among its populations, are equally important in the assessment of possible future range shifts of tropical species in the Gulf. Understanding both abiotic and biotic thresholds of key species will vastly improve our understanding of future ecosystem shifts and better inform conservation efforts.

As little is known about the reproductive patterns of *A. germinans*, and previous studies have found confounding and contradictory data on the mating systems and the overall trends in genetic diversity in populations of this species, I set out to assess the levels of genetic diversity of populations of *A. germinans* along the Gulf Coast, a hotspot for future coastal ecosystem changes (Osland et al. 2013; Profitt and Travis 2014; Sandoval-Castro et al. 2014; Peterson and Bell 2012; 2015). The aim of the study was to determine whether populations were within Hardy-Weinberg Equilibrium conditions, supporting at least moderate levels of outbreeding and genetic diversity, or whether the populations were highly inbred. Further, this study explored whether a latitudinal pattern or trend in the degree of genetic diversity exists for *A. germinans* populations sampled along the Gulf Coast.

I hypothesized that the populations sampled would exhibit at least intermediate levels of outbreeding (inbreeding coefficient of 0.4 or lower), and at least moderate levels of heterozygosity, allowing for the genetic plasticity necessary to succeed in new environments (as exemplified by successful range-expansion of mangroves into northern Florida and southern Louisiana). I further expected a decrease in genetic diversity in populations of *A. germinans*, as latitude increases toward the northern limit of the species' range, due to selection and a bottleneck of pollen contributors (founder effect). Overall, the results affirm previous findings that populations of *A. germinans* may support moderate to high levels of inbreeding within populations, but that the genetic variation among populations can still be high (Parani et al. 1997; Hogarth 1999; Salas-Leiva et al. 2009; Cerón-Souza et al. 2012; Nettel-Hernanz et al. 2013). This suggests that the sampled populations may be largely isolated from one another and that local conditions exert great influence over patterns of population genetics in *A. germinans*.

Upon examination of the genetic characteristics of each population (Table 2.2), the results fall somewhere in the middle of previous studies. None of the sampled populations were within Hardy-Weinberg Equilibrium, and expressed heterozygote deficiency and very low levels of observed heterozygosity with moderate to high inbreeding coefficients. Sandoval-Castro et al.'s study (2014) assessing levels of genetic diversity in *A. germinans* populations along the Pacific and Atlantic coasts of Mexico found low levels of observed heterozygosity as well, ranging from 0.01 – 0.48, with many populations falling between H_o = 0.01 – 0.36, similar to the ranges of H_o in this study. Sandoval-Castro et al. (2014) also found that in nearly all sampled populations, observed heterozygosity was lower than expected heterozygosity, implying heterozygote deficiency in these populations, too. These findings are supported by numerous other studies of populations of *A. germinans* in more southern locations (Salas-Leiva et al. 2009; Nettel-Hernanz et al. 2013; Millán-Aguilar et al. 2016). The populations that Sandoval-Castro et al. (2014) sampled from the Gulf Coast occur along the southwestern rim, the same side of the Gulf as the Port Aransas and Port Isabel, TX, sites, which exhibited the low H_o values of 0.11.

Conversely, results from a recent study published by Hodel et al. (2016) on the genetic diversity of populations of *A. germinans* along the east and west coasts of Florida, found observed heterozygosity values ranged from 0.60 – 0.83, and were consistently greater than expected heterozygosity (H_e) for each population, indicating heterozygote excess. As heterozygosity is frequently used as a measure of genetic diversity, the study by Hodel et al. (2016) suggests that most populations of *A. germinans* in this region are relatively quite diverse. The data from this thesis, however, suggest otherwise, finding instead H_o consistently lower than H_e, supporting aforementioned conclusions from Sandoval-Castro et al. (2014) positing that many mangrove populations in this region exhibit heterozygote deficiency. Observed heterozygosity values in this study ranged from 0.28 (Site 2, FL) to 0.04 (Dog Island, FL), and overall observed heterozygosity was quite low (average $H_o = 0.16$; Table 2.2). Hodel et. al's (2016) findings could result from the inclusion of populations along the east coast of Florida and the Florida Keys, regions more accessible to a greater variety of potential donor populations from the Caribbean and other more distant populations, permitting greater potential for the introduction of new genotypes.

The sites collected in Florida – Cedar Key, FL; Emerson Point Preserve, FL; and Site 2, FL – exhibited the highest H_o values (0.22, 0.23, 0.28, respectively) of the seven populations sampled in this study. These data, in conjunction with the data collected by Hodel et al. (2016) and Sandoval-Castro et al. (2016), allude to a possible decrease in heterozygosity in *A. germinans* populations from east to west across the Gulf of Mexico.

My results support the assertion by Hodel et al. (2016) that there is no apparent latitudinal gradient of decreasing genetic diversity among populations of *A. germinans* with increasing latitude but suggests there is greater genetic diversity along the eastern Gulf coasts (Florida) than the western coasts (Texas). The observed heterozygosity (H_o) values in the historic, northern populations sampled – Cedar Key, FL, and Port Aransas, TX – were 0.22 and 0.11, respectively. The H_o values in the southern, historic sites – Emerson Point Preserve, FL; Port Isabel, TX; and Site 2, FL – were 0.23, 0.11, and 0.28, respectively (Table 2.2). Thus, we do not see a clear decrease in heterozygosity and, presumably, genetic diversity, with increasing latitude.

My data, however, also hint at notable founder effects in the two northern range-expansion populations - Dog Island, FL and Leeville, LA - as the observed heterozygosity for these two populations were some of the lowest values of H_o = 0.04 and 0.12, respectively (Table 2.2). These two northern range-expansion sites, especially Dog Island, the most northern and most fragmented population of *A. germinans*, seem to support the theory of a decrease in genetic diversity with increasing latitude. Yet perhaps the decrease in heterozygosity is also heavily influenced by how young the established mangrove stands are, and how geographically isolated and fragmented a population is (McCauley 1991; Hewitt 2000; Dutech et al. 2003; Bialozyt et al. 2006; Petit et al. 2003; Sandoval-Castro et al. 2014).

Sandoval-Castro et al. (2014) found a linear relationship between decreasing heterozygosity and increasing latitude in the populations of *A. germinans* along the Pacific coast of Mexico, but further found that this pattern was not maintained in the Atlantic coast populations. They noted that the northern populations of *A. germinans* along the Pacific coast tended to be sparse and patchy in distribution.

Similarly, the Dog Island, FL population is located on a small barrier island, distant from other populations, and is very small and sparse. In contrast, the Leeville population along the Louisiana coast is less fragmented and is proximal to other small, patchy coastal mangrove stands. Both populations have only established roughly within the last 10 years (Osland et al. 2013). Recent colonization or population fragmentation may intensify bi-parental inbreeding and genetic drift, resulting in recently colonized populations usually harboring decreased levels of genetic diversity (McCauley 1991; Hewitt 2000; Dutech et al. 2003; Bialozyt et al. 2006; Petit et al. 2003; Sandoval-Castro et al. 2014). In patchy distributions, the propagule dispersal of *A. germinans* may also remain very local, leading to greater bi-parental inbreeding, increasing population genetic differentiation (Kalisz et al. 2001; Yu et al. 2010; Cerón-Souza et al. 2012), though frequent occurrences of long-distance dispersal (LDD) events may result in exceptions to this pattern.

In assessing whether latitudinal gradients correlate with genetic diversity in *A. germinans*, I also looked at the dominant trends in mangrove reproduction using the

inbreeding coefficient. *Avicennia germinans* has a perfect flower, and though the protandry reported for *A. germinans* supports evidence that this is mainly an outcrossing species (as this makes within-flower pollination unlikely), there is no direct evidence of self-incompatibility (Rathcke et al. 1996). In isolated individuals, and isolated or low-density populations, geitonogamy (pollination of different flowers on the same plant) could be a very advantageous breeding strategy.

Other *Avicennia* species from the Indo-West Pacific region show this type of reproduction strategy. Sandoval-Castro et al.'s (2014) interesting data, showing a greater genetic structure (more genetic subpopulations within a metapopulation) in the assumed outcrossing *A. germinans* species compared to the mixed-mating *R. mangle* mangrove species, further supports the possibility of mixed-mating in *A. germinans* (Loveless and Hamrick 1984; Aluri 1990; Clarke and Myerscough 1991; Hamrick 2004; Sandoval-Castro et al. 2014).

Data from Nettel-Hernanz et al.'s (2013) study of mating systems of *A. germinans* populations along the Mexican coastline also support the possibility of a mixture of outcrossing, self-fertilization, and bi-parental inbreeding, which may increase genetic differentiation among populations as well as affect the spatial genetic structure of populations, especially in isolated or fragmented populations (Kalisz et al. 2001; Yu et al. 2010; Nettel-Hernanz et al. 2013; Sandoval-Castro et al. 2014). This could explain why molecular genetics studies on *Avicennia* in India, comparing the amount of genetic diversity within and among populations of the same species, found that genetic variation among populations is relatively high, while the amount of genetic variation within populations varies from one population to another (Parani et al. 1997), and may be very low despite the great genetic variation among populations.

My data appear to support differentiation due to isolation, fragmentation, and possible genetic drift. The sampled populations express moderate to high inbreeding coefficients, yet maintain high genetic variation among populations.

The inbreeding coefficients (F_{IS}) for the seven populations sampled ranged from $F_{IS} = 0.20$ in Emerson Point Preserve, FL, to $F_{IS} = 0.70$ in Dog Island, FL; the average inbreeding coefficient among populations was $F_{IS} = 0.42$ (Table 2.2). Most populations

ranged between F_{IS} = 0.2 – 0.45, implying that most of the sampled populations are predominantly outbreeding, yet can sustain moderate levels of inbreeding.

Here, the data reveal what resembles a possible gradient of increasing inbreeding coefficients with increasing latitude. The northern sites, with increasing latitude, are as follows: Port Aransas, TX; Cedar Key, FL; Leeville, LA; and Dog Island, FL. Their respective F_{IS} values are 0.34, 0.52, 0.43, and 0.70 (Table 1). The southern sites, with decreasing latitude, are as follows: Emerson Point Preserve, FL; Port Isabel, TX; and Site 2, FL. Their respective F_{IS} values are 0.20, 0.45, and 0.28.

Dog Island, FL, the population with the lowest reported observed heterozygosity (H_o = 0.04), also has the highest inbreeding coefficient (F_{IS} = 0.70) (Table 2.2). The Dog Island, FL population also exhibits some of the highest pairwise F_{ST} (Fixation Index, genetic differentiation) values when paired with all other populations (Figure 2.2). This further supports probable founder effects enacting on the population, and possible genetic drift and isolation due to the young and fragmented nature of this particular population.

Consistent with moderate to high inbreeding coefficients, the analysis of molecular variance (AMOVA) attributes most of the genetic variance in these populations to among populations (38.7%), followed by within individuals (37%) when analyzing all populations separately (Table 2.3). Only 24% of the variance is attributed to among individuals within populations. The AMOVA calculation of the inbreeding coefficient among individuals within populations is F_{IS} = 0.39 (close to the average reported in Table 2.2: F_{IS} = 0.419).

The genetic differentiation among populations, however, is highly significant (F_{ST} = 0.38; p-value = 0.001), denoting what would usually be considered a very pronounced genetic differentiation (Freeland et al. 2011). Nevertheless, in the case of this study, and a few others observing F_{ST} values among populations of *A. germinans* around the world (Sandoval-Castro et al. 2014; Cerón-Souza et al. 2012), this value implies relatively moderate differentiation (Table 2.3).

These trends in variance are nearly identical to the AMOVA testing populations in the new, “northern” region (e.g., Leeville, LA and Dog Island, FL) against the

populations in the "historic region" (all other populations), finding the greatest amount of variance among populations within a region (38.6%), followed by within individuals (37%; Table 2.4). Again, only 24% of the variance was attributed to among individuals within populations.

The genetic differentiation between the "northern" and "historic" regions, however, was highly insignificant, indicating no notable differences between regions (F_{CT} = -0.001; p-value = 0.475), confounding whether founder effects or genetic drift are significantly distinguishing the new, "northern" leading-edge populations from the "historic" range populations (Table 2.4).

Pairwise combination tests using the Fixation Index, F_{ST}, and genetic distance, Jost's D, further illuminate the lack of directional gene flow among populations as observed by Hodel at al. (2016) and Sandoval-Castro et al. (2016). These pairwise combination tests also support that populations can be highly differentiated despite geographical proximity, and shed light on which populations appear more or less genetically similar.

In the pairwise combination tests of all seven populations, the Fixation Index, F_{ST}, ranged from -0.033 – 0.66 (Figure 2.2). The "northern" populations – Dog Island, FL and Leeville, LA – exhibited a pairwise F_{ST} differentiation range of 0.45 – 0.66 and 0.05 – 0.56, respectively, when paired with other populations. Dog Island exhibited high genetic differentiation when paired with any other population, and its values were generally higher than most other population combinations overall, implying great genetic distance from all other populations sampled. The highest pairwise values for Dog Island occurred when paired with Port Aransas and Port Isabel, TX (F_{ST} = 0.66 and 0.65, respectively). Dog Island's lowest pairwise differentiation values were when paired with a more southern Florida site, Emerson Point Preserve, FL (F_{ST} = 0.45), despite having greater proximity to Cedar Key, FL and Leeville, LA (F_{ST} = 0.49 and F_{ST} = 0.56, respectively).

Likewise, Leeville's highest pairwise differentiation values occurred when paired with Dog Island (F_{ST} = 0.56), despite Dog Island, FL being its most proximal population. Surprisingly, Leeville's lowest pairwise F_{ST} values occurred when paired with Port Aransas and Port Isabel, TX (F_{ST} = 0.05), implying high genetic similarity to these distant

Texas populations (~ 427 to 482 miles). The Texas sites, Port Aransas and Port Isabel, have a negative pairwise differentiation value (F_{ST} = -0.033), indicating extreme genetic similarity. The Florida sites – Cedar Key, Emerson Point Preserve, and Site 2 – also have relatively low pairwise differentiation values, ranging from F_{ST} = 0.17 – 0.26. Site 2, FL, has relatively high pairwise differentiation values when paired with Dog Island, FL; Leeville, LA; Port Aransas, TX; and Port Isabel, TX (F_{ST} = 0.50, 0.52, 0.56, and 0.57, respectively), signifying great differentiation among the south-eastern populations and the northern and western populations.

These trends are similar to the trends seen in the Jost's D distance pairwise combinations. In the pairwise combination tests of all seven populations, the genetic distance, Jost's D, ranged from 0 – 0.59 (Figure 2.2). Leeville, LA; Port Aransas, TX; and Port Isabel, TX have the lowest values when paired together (all 0), representing close genetic proximity. Dog Island, FL, in turn, tends to have relatively high values when compared with any other population, denoting greater genetic distance.

Contrary to the trend of Dog Island, FL generally being the most highly differentiated population, as seen in the F_{ST} pairwise combinations, the highest Jost's D values appear when Site 2, FL is paired with Leeville, LA; Port Aransas, TX; and Port Isabel, TX (Jost's D = 0.59, 0.57, 0.58, respectively; Figure 2.2). The next highest values occur when Dog Island, FL is paired with other populations.

The principal component analysis (PCA; Figure 2.3), ΔK calculation plots (Figure 2.4 and 2.5), and STRUCTURE bar plots (Figure 2.6) further illuminate distinct clusters of genetically similar groupings. These data show that, based on the genetic information alone, the seven sampled populations cluster into two to perhaps three or four populations, with Leeville, LA; Port Aransas, TX; and Port Isabel, TX always clustering into a linkage group. Dog Island, FL clusters with the Florida sites when ΔK = 2, but for ΔK = 3 or ΔK = 4, Dog Island, FL clusters alone in a separate linkage group from the other Florida sites and from the Texas-Louisiana sites. The PCA appears to most support the ΔK bar plot of K = 3, with Dog Island, FL acting as one linkage group, the Texas-Louisiana sites acting as another linkage group, and the other three Florida sites as a third linkage group, with some overlap between the Florida and Texas-Louisiana populations.

These data further foster the conclusion of a lack of discernable latitudinal gradients in genetic diversity of *A. germinans* along the Gulf Coast, and that even proximal populations may exhibit high levels of genetic differentiation, as suggested by Hodel et al. (2016). Contrary to the findings by Hodel et al. (2016), however, there does appear to be a discernable pattern of west-to-east gene flow among the populations along the Gulf Coast.

These genetic linkage groups as depicted by the STRUCTURE population cluster assignments (Figure 2.6) and PCA (Figure 2.3) may be attributed to the patterns and interactions of the Loop Current, the Mexican Current, and the Gulf Stream Current's effect on the long-distance dispersal of *A. germinans'* buoyant propagules. The genetic clusters appear to follow the predominant west-to-east pattern of the Gulf Stream current, but the irregularity and complexity of smaller loops, eddies, and gyres within the Gulf Stream current and the Gulf basin may lend to why some populations may be more isolated and genetically distinct from others along the same coastline. As previously hypothesized by various studies, populations of *A. germinans* appear to be highly influenced by local conditions, and thus heterozygosity and genetic diversity can be highly variable from population to population and location to location (Parani et al. 1997; Hogarth 1999; Arnaud-Haond et al. 2006; Salas-Leiva et al. 2009; Cerón-Souza et al. 2012; Nettel-Hernanz et al. 2013). Usual patterns of decreasing heterozygosity, resulting from isolation by distance or decreasing genetic diversity with increasing proximity to a species' usual distribution range, may be disrupted by frequent long-distance dispersal events (Kalisz et al. 2001; Yu et al. 2010; Cerón-Souza et al. 2012) or confounded by untraditional long-distance dispersal patterns, as propagules may be re-distributed randomly according to currents and weather phenomena affecting water transport.

The geomorphology of the oceanic basin, and the gyres and looping meanders of the various dominant currents influencing water transport in the Gulf of Mexico may provide an interesting area for further research related to genetic linkage groups and trends in genetic diversity of *A. germinans* populations along the Gulf's coasts. Mangrove propagules may be "trapped" in several areas of the basin, where currents circulate close to the shore, encouraging redistribution of propagules to similar places

with each long-distance dispersal event, thus resulting over time in higher levels of inbreeding in some populations and close genetic linkages among others despite geographic distance.

Further research into these relationships may also elucidate why the Dog Island, FL population is so genetically distinct from the others, despite a low number of private alleles and its position within the basin near possible neighboring donor populations on both the east and west. Hodel et al. (2016) posit a similar situation to account for a population in Flamingo, FL that appears highly differentiated despite very low genetic diversity – i.e., ocean currents preventing gene flow may be to blame. Dog Island's distinction, however, may also be attributed to its high levels of inbreeding and its isolation by distance, causing genetic drift from other populations within the Gulf.

Per my findings, I support the conclusions of Hodel et al. (2016) and Sandoval-Castro et al. (2014) that there is not a clear latitudinal gradient of genetic diversity along the Gulf Coast, however, contrary to their findings, there may be a perceptible pattern of west-to-east gene flow among Gulf Coast populations of *A. germinans*. Also contrary to Hodel et al.'s findings (2016), and in support of other, earlier studies (Salas-Leiva et al. 2009; Nettel-Hernanz et al. 2013; Sandoval-Castro et al. 2014; Millán-Aguilar et al. 2016), I found all sampled populations of *A. germinans* out of Hardy-Weinberg Equilibrium, expressing heterozygote deficiency. I also found that observed heterozygosity was low, and in fact, consistently lower than expected heterozygosity.

Overall, the populations appeared to express moderate to high levels of inbreeding and low levels of genetic diversity, but genetic differentiation among populations was still high. Despite greater inbreeding coefficients and lower observed heterozygosity in the two new, northernmost range-expansion populations on Dog Island, FL and Leeville, LA – indicating possible founder effects enacting on these populations – the AMOVA indicated there was no significant genetic distinction between these two new, range-expansion populations and the historic populations. It may be necessary to conduct a more fine-scaled genetic survey of these populations over a greater period of time to uncover how selection and a potential genetic bottleneck will affect the genetic resilience of these range-expansion populations in the future.

Understanding patterns of genetic diversity among populations of a species, and tracking genetic diversity in relation to various biotic and abiotic factors, allows us to better distinguish resilient populations from fragile populations. This, in turn, will help identify which populations will make suitable donor populations for conservation and restoration efforts. To deter the deleterious effects on fitness that stem from inbreeding depression, efforts should be taken to diversify genotypes in re-forestation efforts and to discourage the re-introduction of high inbreeding into populations for rehabilitation efforts (Freeland et al. 2011; Salas-Leiva et al. 2009). Sites with particularly low heterozygosity and high inbreeding should be carefully monitored, as such sites are most susceptible to adverse effects from natural and anthropogenic disturbances, and more likely to be altered by novel environmental conditions. Alternatively, such populations may also offer interesting case studies in the development of local adaptations in range-expansion populations that allow for species resilience against future environmental change (Arnaud-Haond et al. 2006) and should be carefully monitored for conservation purposes.

Molecular genetics studies on *Avicennia* in India have been used to assess and compare the amount of genetic diversity within and among populations of the same species and found that between populations genetic variation is relatively high, suggesting that the separate populations are largely isolated from one another, and there is little mutual gene flow among them. The amount of genetic variation within populations varies from one population to another, suggesting within-population variation is largely dictated by local circumstances (Parani et al. 1997). Research by Arnaud-Haond et al. (2006) corroborates this evidence. Their data found populations of *Avicennia* species in Vietnam, the northern Philippines, and Australia expressed a high level of genetic structure and inbreeding, showing that populations may be functioning as independent evolutionary units rather than subpopulations connected by gene flow, and that peripheral populations thus may be likely to develop local adaptations that will allow for resilience against future environmental changes and novel conditions. More data on such cases will help us better understand probabilities for mangrove species adaptation, range-expansion, and ecosystem shifts in the face of climate change.

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Appendix

Table 1. Microsatellite primers chosen for this study of *Avicennia germinans*.

Literature	Primer Name	Repeat Type	Expected Heterozygosity	Annealing Temperature (° C)
Cerón-Souza et al., 2006	Agerm_CTT_001 (DQ240228)	$(CTT)_8$	0.571	55
Cerón-Souza et al., 2006	Agerm_GT_002 (DQ240226)	$(TG)_{12}$-A-$(TG)_3$	0.651	55
Nettel et al., 2005	AgT31 (AY741800)	$(CA)_{12}(TA)_2GA(CATA)_9$	0.24	60
Nettel et al., 2005	AgT7 (AY741799)	$(CAT)_2(AT)_3(GTAT)_5$	0.33	50
Mori et al., 2010	Agerm1-01 (HM470003)	$(AC)_{15}$	0.51014	53.4
Mori et al., 2010	Agerm1-02 (HM470004)	$(CA)_{11}$	0.75	53.4
Mori et al., 2010	Agerm1-12 (HM470014)	$(AC)_{15}$	0.83382	53.4
Mori et al., 2010	Agerm1-14 (HM470016)	$(CA)_8(AT)_6$	0.72657	59.6
Mori et al., 2010	Agerm1-16 (HM470018)	$(TG)_9$	0.28	53.4
Mori et al., 2010	Agerm1-18 (HM470020)	$(AG)_{16}$	0.62368	64.5
Mori et al., 2010	Agerm1-21 (HM470023)	$(CA)_8$	0.61469	61.8
Mori et al., 2010	Agerm1-22 (HM470024)	$(TTTCTT)_4$	0.04	63.4

Table 2. Literature cited, primer name, and allele information per this study as assessed by GENODIVE (based on 90 individuals and 12 loci).

Literature	Primer Name	# Alleles (in this study)
Cerón-Souza et al., 2006	Agerm_CTT_001 (DQ240228)	3
Cerón-Souza et al., 2006	Agerm_GT_002 (DQ240226)	5
Nettel et al., 2005	AgT31 (AY741800)	4
Nettel et al., 2005	AgT7 (AY741799)	9
Mori et al., 2010	Agerm1-01 (HM470003)	8
Mori et al., 2010	Agerm1-02 (HM470004)	7
Mori et al., 2010	Agerm1-12 (HM470014)	8
Mori et al., 2010	Agerm1-14 (HM470016)	7
Mori et al., 2010	Agerm1-16 (HM470018)	4
Mori et al., 2010	Agerm1-18 (HM470020)	4
Mori et al., 2010	Agerm1-21 (HM470023)	4
Mori et al., 2010	Agerm1-22 (HM470024)	3
		Total: 66

Table 3. Modified PCR thermocycler program for Type-it® Microsatellite Multiplex PCR Kit (QIAGEN).

Step	Temperature (°C)	Time	Action
1	95	5:00	Initial Denaturing
2	95	0:30	Denaturing
3	65.5*	1:30	Annealing
4	72	0:30	Extension
5	95	0:30	Denaturing
6	63.4*	1:30	Annealing
7	72	0:30	Extension
8	95	0:30	Denaturing
9	60**	1:30	Annealing
10	72	0:30	Extension
11	95	0:30	Denaturing
12	59.6*	1:30	Annealing
13	72	0:30	Extension
14	95	0:30	Denaturing
15	56.7*	1:30	Annealing
16	72	0:30	Extension
17	95	0:30	Denaturing
18	55*	1:30	Annealing
19	72	0:30	Extension
20	95	0:30	Denaturing
21	50*	1:30	Annealing
22	72	0:30	Extension
23	go to 20	23 times	Cycling
24	60	1:30	Final Extension
25	4	END	Chill

* Specific annealing temperature of a primer as outlined by literature
** Optimum annealing temperature per QIAGEN

Chapter 3

Comparative analysis of genetic diversity and inbreeding in parent and progeny of a historic and two range-expansion populations of *Avicennia germinans* along the Gulf Coast of Florida, Louisiana, and Texas, USA.

Abstract

Avicennia germinans (black mangrove), an important foundational mangrove species found along the tropical, coastal intertidal zones of the Gulf Coast of Florida, Texas, and Louisiana. Recent *A. germinans* range expansion into more northern latitudes are likely due to increasing winter temperatures and sea level rise, and have important implications for large-scale ecosystem shifts along the tropical-temperate coastal ecotones in the Gulf. To better understand the probability of successful northward mangrove migration, it is imperative to also understand biotic factors that influence mangrove fitness, resilience, and ability to adapt to novel conditions, such as the reproductive patterns and mating strategies that result in the genetic diversity within a population. Reproductive patterns and mating systems are poorly understood in *A. germinans*, especially in range-expansion populations, which may be highly fragmented and isolated, and susceptible to a genetic bottleneck due to the founder effect, leading to inbreeding depression and reduced fitness. Information on genetic diversity and inbreeding between parent and progeny of such populations would be valuable for shedding insight on the effect of stressful, novel conditions on the genetic diversity and resilience of new, young populations.

The purpose of this study was to examine the degree of genetic diversity and inbreeding between parents and progeny within two new, range-expansion populations and one historic, within-range mangrove population. Eight microsatellite loci were amplified from DNA isolated from leaf tissue from adult individuals and propagule cotyledon tissue from respective progeny. Results found that the parent and progeny in the two new range-expansion populations –Dog Island, Florida and Leeville, Louisiana – expressed lower observed heterozygosity and higher inbreeding coefficients than the parent and progeny in the historic, within-range population, Port Aransas, Texas. Leeville, however, was comparable to Port Aransas, perhaps due to its less-fragmented, more developed nature. Observed heterozygosity increased from parent to progeny in all populations, and the inbreeding coefficient decreased from parent to progeny in Port Aransas and Leeville, but increased from parent to progeny in Dog Island. Dog Island exhibited significant genetic differentiation between parents and progeny (F_{ST} = 0.47; p-value < 0.001) and great genetic distance from the other two populations (F_{ST} = 0.49 –

0.55), indicating possible genetic drift due to isolation from pollen donors and incoming migrants. In future research, more range-expansion populations of varying age, level of fragmentation, and degree of isolation should be examined in comparison to a variety of historic, within-range populations to further illuminate patterns in early mangrove population colonization and development.

Introduction:

Mangroves are halophilic woody plants that establish along the intertidal zones of tropical and subtropical coasts. Many mangrove species are considered important foundational species as well as ecosystem engineers, providing a plethora of ecosystem services that improve the health of surrounding environments, boost flora and fauna biodiversity, and protect coastlines from erosion (Tomlinson 1986; Twilley 1998; Hogarth 1999; Nagelkerken et al. 2008; Cerón-Souza et al. 2012; Osland et al. 2013; Thomas et al. 2017). Over the last few decades, *Avicennia germinans*, the black mangrove, has expanded northward from its historic northern latitudinal extent along the coast of the Gulf of Mexico, establishing in northern Florida and southern Louisiana. This northward, and also inland, migration into traditionally temperate, salt marsh-dominated territory is generally attributed to sea level rise, warmer winter temperatures, and fewer freeze events along the Gulf of Mexico's northern coasts, in conjunction with *A. germinans'* ability to withstand freeze events and high soil salinity (Rogers et al. 2005; Osland et al. 2013; Saintilan et al. 2014; Madrid et al. 2014; Cavanaugh et al. 2014).

Mangrove dominance in the northern latitudes may increase carbon storage, coastal sediment stabilization, biodiversity, and storm energy attenuation (Osland et al. 2013; Chmura 2003; Krauss et al. 2009; Dangremond and Feller 2016). This northward migration may also, however, displace salt marshes and disrupt salt marsh food webs, resulting in adverse consequences for the current flora and fauna abundance and distribution, and potentially damaging traditional fisheries in these areas (Dangremond and Feller 2016; Glick and Clough 2006).

Therefore, the ability to predict mangrove expansion and range shifts is vital in foreseeing and understanding possible future coastal ecosystem dynamics and the

potential repercussions of such changes. While several studies have modeled probable abiotic drivers and suppressors of mangrove migration, such as temperature thresholds and freezing events, only a few studies have examined possible biotic limitations, such as differences in reproductive strategies and the genetic diversity and physiology within and among populations in response to novel stressful conditions.

Current literature on the mating systems and resulting levels of genetic diversity in mangrove populations draw contradictory conclusions for whether mangrove populations are consistently highly inbred, either from self-pollination or genetic bottlenecks, or whether mangrove populations exhibit genetic diversity consistent with high levels of outcrossing. In a study using microsatellite loci to examine genetic diversity among 15 populations of *A. germinans* and *R. mangle* along the east and west coasts of Florida, Hodel et al. (2016) observed greater average heterozygosity among *A. germinans* populations than expected. H_o ranged from 0.175 to 0.838 and H_e ranged from 0.243 to 0.553, with an overall 0.67 average H_o across all 15 sites and a 0.46 overall average for H_e. Additionally, the study found no apparent pattern of increasing or decreasing levels of genetic diversity among populations of *A. germinans* in relation to increasing latitude or proximity to northern range-edge locations. While Hodel et al. (2016) found significant gene flow between populations of *A. germinans*, they identified no discernable directional pattern of gene flow.

Nettel-Hernanz et al. (2013) analyzed mating systems in *A. germinans* populations along the Chiapas, Mexico coastline using microsatellite analysis of parent and progeny DNA. The resulting analysis determined inbreeding coefficients were close to zero and that none of the studied populations significantly deviated from the Hardy-Weinberg Equilibrium. Detected levels of bi-parental inbreeding were low, and the proportion of siblings sharing the same "father" tree was also low. The study concluded that *A. germinans* populations in this area were predominantly outcrossed but could support moderate levels of self-fertilization. Nettel-Hernanz et al. (2013) claim these findings are consistent with previous hypotheses that *Avicennia* mating patterns consist of a mixture of outbreeding and selfing, with outcrossing events being more predominant but random enough to exert little effect on population substructure and biparental inbreeding.

Several other studies, however, suggest a possible discernable difference in the genetic diversity and inbreeding coefficients of stressed mangrove populations compared to "comfortable" populations. Compared to individuals in more southern populations, individuals in northern, range-edge *R. mangle* populations begin to reproduce at younger ages, exhibit an increased rate of flowering, and produce larger propagules (Dangremond and Feller 2016). Based on their garden experiment, which determined that the effect of cold stress was not the sole driver of precocious reproduction in more northern *R. mangle* populations, Dangremond and Feller (2016) concluded that genetics must be the basis for the different reproductive strategies.

Proffitt and Travis (2014) established that the maternal line influences reproduction in *R. mangle* offspring along the Gulf Coast of Florida. This further supports the existence of early-reproducing genotypes, and indicates that these genotypes may dominate the northern mangrove ranges. Dangremond and Feller (2016) speculate that this genotypic dominance may result from the colonization of northern populations by early-reproducing genotypes through the founder effect. Plants at the leading-edge of environmental thresholds tend to be more solitary and may be forced to self-pollinate.

Thus, precocious reproduction may result from inbreeding within a small group of genotypes, which pass on the early-reproduction gene to their progeny. Alternatively, early-reproducing genotypes may simply spread their genes more quickly via increased reproductive output, thus out-competing other genotypes. Local adaptation may also influence this process if early-reproducing individuals exhibit increased fitness compared to later-reproducing individuals, as likely occurs in regions with more frequent freeze events.

In support of attributing the differences in reproductive strategy to a founder effect or forced inbreeding, Salas-Leiva et al. (2009) found that younger mangroves in a reforested area in Colombia, recently disturbed by highway construction, exhibited increased inbreeding compared to their older counterparts within the same population. This increase in inbreeding in young, stressed individuals is believed to result in higher inbreeding coefficients in subsequent maturing reforested areas when compared to preserved, undisturbed mangrove forests.

Notwithstanding the studies described above, there is a paucity of literature specifically examining the genetic diversity of mature mangrove trees compared to their progeny, to include case studies of young, northward migrating populations in northern Florida and southern Louisiana. Such information would be valuable in further illuminating the reproductive strategies and the genetic diversity of the novel range-expansion populations, and shedding insight on the resilience of the new populations and the probability of further northward advancement.

Because of the vast array of biodiversity that mangroves support, and the invaluable ecosystem services they provide – e.g., coastal storm buffering, sediment stabilization and protection from coastal erosion, carbon sequestration, fishery support, and water filtration - it is imperative that conservation efforts have data to help identify the best source mangrove populations for restoration and reforestation (Tomlinson 1986; Twilley 1998; Hogarth 1999; Cerón-Souza et al. 2012; Osland et al. 2013; Thomas et al. 2017). Additionally, since mangrove migration threatens the displacement of salt marshes and a potential grand-scale shift in coastal flora and fauna, we need to understand both abiotic and biotic drivers to accurately model where such shifts may occur.

The purpose of this study is to examine the genetic diversity of progeny compared to parents within two new range-expansion mangrove populations and one historic, within-range mangrove population.

This research attempted to answer the following questions: 1.) When comparing the genetic diversity and inbreeding coefficients in mature individuals of *A. germinans* to their respective progeny, do the progeny reflect similar or significantly different levels of genetic diversity and inbreeding? And, if they are different, does this appear to result in significant genetic differentiation between the parent and progeny subpopulations of each population? 2.) How do the changes in observed heterozygosity and inbreeding coefficients between the parent and progeny subpopulations in the new, range-expansion populations compare to changes between the parent and progeny subpopulations in the historic, within-range population? 3.) Is there significant genetic differentiation among all the populations? Does the level of differentiation among all populations change between the "parent" and "progeny" subpopulations? Is there significant genetic differentiation

among regions, when the two new, range-expansion populations are compared to the historic population?

I hypothesized that there would be a difference in the genetic diversity and inbreeding coefficients between the parent subpopulation and the progeny subpopulation in the two new, range-expansion populations. I expect this difference to reflect less genetic diversity (lower observed heterozygosity) and greater inbreeding coefficients in the progeny subpopulations compared to the parent subpopulations due to the founder effect. I posit that this would result in significant genetic differentiation among the parent and progeny subpopulations within the Dog Island, FL, and Leeville, LA, populations. However, I also postulate that overall, the two new, range-expansion populations (Dog Island, FL and Leeville, LA) will exhibit greater genetic diversity compared to the historic, within-range population (Port Aransas, TX), which may allow the individuals in the leading-edge populations to succeed in novel environmental conditions. As the historic, within-range population (Port Aransas, TX) is less fragmented and well within the species' distribution range, thus having ample access to pollen donors and being spared the stress of novel environmental conditions, I do not anticipate significant genetic distinction between parent and progeny in this population. Due to differing forces of selection acting on the range-expansion populations compared to the within-range population, I surmise that there will be significant genetic differentiation among the three populations, and that these genetic distinctions will be reflected in both the parent and progeny subpopulations of each population.

Methods

Sample Collection and Preparation

Leaf samples from the mature trees of *A. germinans* were collected from a total of three locations: Leeville, LA; Dog Island, FL; and Port Aransas, TX (Figure 3.1). These locations will hereafter be termed populations. Dog Island, Florida and Leeville, Louisiana were identified as new range-expansion populations, and appear to have been

established within the last 10 years (Table 3.1). Individuals within the range-expansion populations were more sparsely distributed along the coastline than more developed, historic mangles, which are often very dense. Individuals from the new, range expansion populations also appeared largely homogeneous in height, usually standing roughly four to five feet tall, with some apparently younger generations only standing around two to three feet tall. The individuals of the historic population found at Port Aransas are also very small in stature (~ five feet tall), however are very densely packed along the coastline. The main distinguishing feature of the new, range expansion populations was the sparse distribution of individuals.

In each of the three populations, leaf samples from 15 trees were collected for the "parent" subpopulation. Of those 15 trees, 8 trees were randomly chosen for propagule sampling, for the "progeny" subpopulation. Fifteen propagules were collected per parent tree. Only propagules still attached to the parent tree were sampled to ensure a parent-progeny relationship.

To minimize the chance of collecting leaf tissue from parents and their offspring within the same location, samples were obtained only from trees located along the outer edge of the mangle, with access to water, and at least 10m apart. In the two range-expansion populations, where the mangles were fairly underdeveloped and covered relatively small stretches of the coastline, samples were obtained from trees 3m or more apart.

The leaves and propagules were stored in labeled bags with silica gel as outlined in Chapter 1 until DNA extraction in a lab at the University of Virginia. The genomic DNA was isolated and extracted from leaf and propagule tissue using a modified protocol for the DNeasy™ Plant Mini Kit (QIAGEN Pty Ltd, Valencia, California, USA) as outlined in Chapter 1. Extracted DNA was quantified on a Qubit® 2.0 Fluorometer (Invitrogen) and then diluted to a working stock concentration of ~5ng μL^{-1}.

Table 3.1. Classification of sites of *A. germinans* populations sampled for leaf and propagule tissue.

Leading Edge Populations	Dog Island, Apalachicola, FL (DI)	29°48'48.1"N 84°35'05.3"W
	Leeville, LA (Leeville)	29°14'49.2"N 90°12'37.5"W
Historic Range Population	Port Aransas, TX (PA)	27°52'04.7"N 97°05'15.8"W

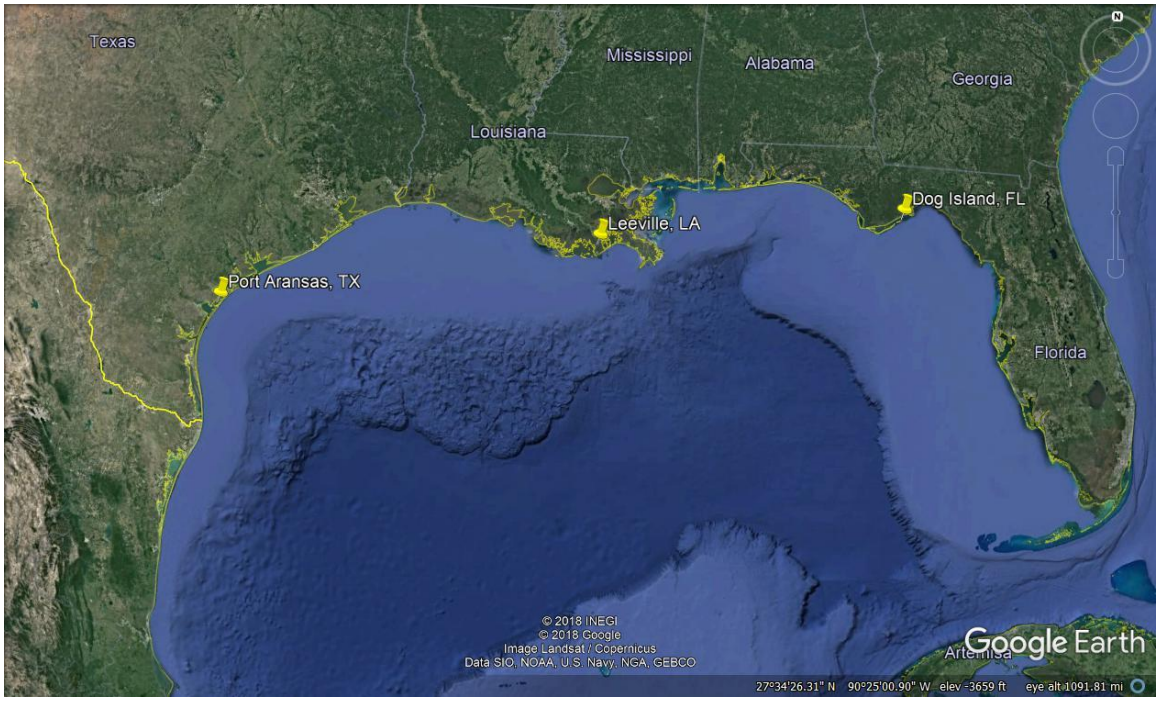


Figure 3.1. Map of the three locations along the Gulf Coast sampled for *Avicennia germinans*. From left to right, the sites are as follows: Port Aransas, TX; Leeville, LA; and Dog Island, FL.

Microsatellite Amplification and Analysis

Polymerase chain reaction (PCR) and fluorescently labeled primers identified by Nettel and Dodd (2005), Cerón-Souza et al. (2006), and Mori et al. (2010) were used to amplify 8 microsatellite loci of interest for *A. germinans* (see Appendix: Table 1).

Eight loci were considered sufficient, since previous studies have detected phylogeographic structure, genetic diversity, and genetic connectivity with similar or fewer microsatellite markers (Salas-Leiva 2009; Pil et al. 2011; Hodel et al. 2016).

PCR was conducted in three multiplex panels using a Type-it® Microsatellite Multiplex PCR Kit (QIAGEN). If the fragment lengths of the PCR products were too similar, and the products overlapped when pooled together and visualized for analysis, PCR was conducted in simplexes using the Type-it® Microsatellite Multiplex PCR Kit (QIAGEN). Following standard M13 protocols (Shuelke 2000; Boutin-Ganache et al. 2001) and using three fluorescent labels (FAM, NED, VIC), the PCR recipe (14 μL reactions per well) was prepared as follows: 7.5 μL of Type-it® PCR Multiplex Master Mix, 0.06 μL of 10 μM microsatellite primer-forward with M13 tail attached, 0.24 μL of 10 μM microsatellite primer-reverse, 0.24 μL of 10 μM primer-M13 tag (FAM or NED or VIC), 5.96 μL H_2O, and 1 μL of diluted working stock DNA.

PCR conditions were set to a modified version of the manufacturer's (QIAGEN) optimized cycling conditions to include the optimum annealing temperature for the Type-it® Microsatellite Multiplex reagent and the suggested optimum annealing temperatures of the various microsatellite primers used. PCR protocol was optimized using a touch-down approach from 65.5°C to 50°C to better target individual primer annealing temperatures (See Appendix: Table 1).

PCR products were sequenced on a capillary-based 3730xl DNA Analyzer (Applied Biosystems) with an internal ET-ROX 500 size standard at the Georgia Genomics Facility (University of Georgia, Georgia, USA). Fragment lengths for each locus were determined using Geneious V7.1.5 (Biomatters Ltd.).

Allele frequencies and genetic diversity statistics for "parent" and "progeny" subpopulations

For each sampling location (i.e. population), the number of different alleles (N_a), effective number of alleles (N_e), number of private alleles (N_p), percent polymorphism of loci (%P), observed heterozygosity (H_o), expected heterozygosity (H_e), and the

inbreeding coefficient (F_{IS}) were calculated in GENALEX version 6.5 (Peakall and Smouse 2006; 2012). Populations were tested for deviations from Hardy-Weinberg Equilibrium by measuring heterozygote excess and heterozygote deficit in GENEPOP web version 4.2 (Raymond and Rousset 1995; Rousset 2008). In order to compare these diversity parameters between the "parent" and "progeny" of each population, these parameters were calculated separately for each population's "parent" trees (leaf tissue) and "progeny" (propagule tissue), treating the "parent" and "progeny" as subpopulations of each population. In order to increase the "parent" sample size, the DNA from all 15 mature trees collected per population, including the 8 randomly chosen individuals for propagule collection, was used for the "parent" subpopulation.

Analysis of molecular variance and *F*-statistics

Genetic differentiation among "progeny" subpopulations

An analysis of molecular variance (AMOVA) was performed to assess overall genetic differentiation among the "progeny" subpopulations of each location under the assumptions of the Infinite Allele Model in GENODIVE (Miermans and Tienderen 2004). Standard deviations for AMOVA F-statistics were calculated by jackknife resampling over loci, and permutation tests (1,000 permutations) were used to assess significance. Confidence intervals of 95% of F-statistics were obtained through bootstrapping over loci.

Pairwise F-statistics were calculated in GENODIVE to measure genetic differentiation among the Florida, Louisiana, and Texas "progeny" subpopulations, and to assess the level of differentiation between the new leading-edge populations and the historic population (Miermans and Tienderen 2004; Peakall and Smouse 2006). Fixation indices F_{ST} (Weir and Cockerham 1984), F'_{ST}, G'ST (Nei 1978), Rh_o (Ronfort et al. 1998), Nei's D (Nei 1972), and Jost's D (Jost 2008; 2009) were calculated for all "progeny" subpopulation pairwise combinations. As these terms are analogous (Freeland et al. 2011), and the results and patterns are maintained across the pairwise combination indices, only F_{ST} and Jost's D are reported and discussed. Additionally, AMOVAs were

run on each pairwise combination to further elucidate genetic similarities or dissimilarities.

Genetic differentiation between "parent" and "progeny" subpopulations

In order to assess the genetic differentiation between parents and progeny in each population, AMOVAs under the assumptions of the Infinite Allele Model were conducted in GENODIVE (Miermans and Tienderen 2004) for the "parent" and "progeny" subpopulations of each of the three sampling locations.

Genetic differentiation between "progeny" subpopulations based on regions

An AMOVA under the assumptions of the Infinite Allele Model was run in GENODIVE (Miermans and Tienderen 2004) to test the genetic differentiation between the new leading-edge populations and the historic population by nesting Dog Island, FL and Leeville, LA "progeny" subpopulations into a "new" region, and the Port Aransas, TX "progeny" subpopulation into a "historic" region.

Overall genetic differentiation among populations and regions

To test the overall genetic differentiation among the three sampling locations, the "parent" and "progeny" subpopulations of each population were lumped together for the following AMOVA analyses, conducted in GENODIVE under the assumptions of the Infinite Allele Model (Miermans and Tienderen 2004). The first AMOVA tested the genetic differentiation among all three populations. A second AMOVA nested the Dog Island, FL and Leeville, LA populations into a "new" region, and the Port Aransas, TX, population into a "historic" region, to assess whether significant differentiation among the regions existed when all samples were pooled together. Two subsequent AMOVAs were run to independently test genetic differentiation between Dog Island, FL and Port Aransas, TX, and Leeville, LA and Port Aransas, TX.

Standard deviations for all AMOVA F-statistics were calculated by jackknife resampling over loci, and permutation tests (10,000 permutations) were used to assess significance. Confidence intervals of 95% of F-statistics were obtained through bootstrapping over loci (GENODIVE; Miermans and Tienderen 2004)

Population Structure

To determine the most likely number of population clusters (K) based on the genetic data collected from the "progeny" subpopulations of Leeville, LA, Dog Island, FL, and Port Aransas, TX, population assignment using a Bayesian approach was performed in the genetic software program STRUCTURE (Pritchard et al. 2000). Admixture was specified in the model, and model parameters were set to K = 1 – 10, with an initial burn-in period of 50,000 iterations followed by 250,000 Markov Chain Monte Carlo (MCMC) repetitions. To account for the variation of the likelihood for each K, each data set, K = n, was run 20 times and averaged. The average for each K = n was used to calculate the most likely number of population clusters using the ad hoc quantity, ΔK (Evanno et al. 2005). Once the most likely population clusters were identified, they were visualized using the bar plot feature in STRUCTURE (Pritchard et al. 2000).

Results

Allele frequencies and genetic diversity statistics among "progeny" and among "parent" subpopulations

Using 8 microsatellite primers to target 8 loci, the number of different alleles (N_a), effective number of alleles (N_e), number of private alleles (N_p), percent polymorphism of loci (%P), observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient (F_{IS}) were calculated based on the 45 individual "parent" trees sampled at the three sampling locations (see Appendix: Table 1, for primer and loci information). Though GENODIVE detected 23 different alleles in total across the 8 loci, GENALEX reports on average only two different alleles among parent subpopulations. The effective number

of alleles (N_e) per population is consistently lower across all sites, averaging to an overall 1.36. The average number of private alleles among parent subpopulations is 0.292, with the highest value occurring in Leeville, LA (0.375) and the lowest occurring in Port Aransas, TX (0).

Overall, the observed heterozygosity was lower than the expected heterozygosity, ranging from 0.043 – 0.101 and 0.15 – 0.18, respectively (Table 3.2). The average observed heterozygosity across all sites was 0.08, with the greatest heterozygosity occurring in Port Aransas, TX. Dog Island, FL exhibited a low observed heterozygosity at 0.043. The average expected heterozygosity across all sites was 0.19, and ranged from 0.15 (Dog Island, FL) to 0.25 (Leeville, LA). The greatest difference between observed heterozygosity and expected heterozygosity occurred in Leeville, LA (0.094 vs. 0.25).

The inbreeding coefficient (F_{IS}) ranges from high (0.70 in Dog Island, FL) to moderate (0.39 in Port Aransas, TX). The average inbreeding coefficient among populations is moderately high (0.55). All populations indicated significant deviation from Hardy-Weinberg Equilibrium ($p < 0.05$), exhibiting great heterozygote deficiency. The number of different alleles (N_a), effective number of alleles (N_e), number of private alleles (N_p), percent polymorphism of loci (%P), observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient (F_{IS}) were also calculated based on the 360 individual progeny samples (3 populations x 8 parents x 15 propagules) collected at the three sampling locations (see Appendix: Table 1, for primer and loci information). Though GENODIVE detected 33 different alleles in total across the 8 loci, GENALEX reports an average of 2.5 different alleles per progeny subpopulation. The effective number of alleles (N_e) among subpopulations averages to 1.38. Overall, the number of private alleles (N_p) among populations is relatively substantially higher than in the "parent" subpopulations, with an average of 0.708. Dog Island, FL exhibits the highest values for private alleles and Port Aransas, TX the lowest (0.875 and 0.5, respectively).

The observed heterozygosity among populations was lower than the expected heterozygosity, ranging from 0.052 – 0.12 and 0.23 – 0.12, respectively (Table 3.2). The average observed heterozygosity across all sites was 0.096, with the greatest heterozygosity occurring in Port Aransas, TX, and Leeville, LA, at 0.12. Dog Island, FL exhibited a low observed heterozygosity at 0.052. The average expected heterozygosity

across all sites was 0.18, and ranged from 0.12 (Dog Island, FL) to 0.23 (Leeville, LA). The greatest difference between observed heterozygosity and expected heterozygosity occurred in Dog Island, FL (0.052 vs. 0.12).

The inbreeding coefficient (F_{IS}) ranges from high (0.75 in Dog Island, FL) to low (0.31 in Port Aransas, TX). The average inbreeding coefficient among subpopulations is moderate (0.48). All subpopulations indicated significant deviation from Hardy-Weinberg Equilibrium ($p < 0.05$), exhibiting great heterozygote deficiency.

Table 3.2. Summary of the genetic statistics for "parent" subpopulations (top) and "progeny" subpopulations (bottom) of *Avicennia germinans*. Number of individuals (N), number of alleles (N_a), effective number of alleles (N_e), number of private alleles (N_p), percent polymorphism (%P), observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient (F_{IS}) are reported for each population. Standard error is included for H_o, H_e, and F_{IS}. The total number of samples used, and the averages for each statistic, is included at the bottom of the table. All populations exhibit significant deviation from Hardy-Weinberg Equilibrium ($p < 0.05$).

				Parent				
Population	**N**	**Na**	**Ne**	**Np**	**%P**	**Ho**	**He**	**Fis**
DI	15	1.750	1.237	0.5	62.50%	0.043 ± 0.029	0.15 ± 0.063	0.70 ± 0.167
Leeville	15	2.375	1.553	0.375	75.00%	0.094 ± 0.044	0.25 ± 0.086	0.55 ± 0.141
PA	15	1.875	1.306	0	50.00%	0.101 ± 0.054	0.18 ± 0.074	0.39 ± 0.173
Average:	**Total: 45**	**2**	**1.365**	**0.292**	**62.50%**	**0.080**	**0.195**	**0.547**
				Progeny				
Population	**N**	**Na**	**Ne**	**Np**	**%P**	**Ho**	**He**	**Fis**
DI	120	2.375	1.184	0.875	75.00%	0.052 ± 0.039	0.12 ± 0.055	0.75 ± 0.103
Leeville	120	2.750	1.454	0.750	75.00%	0.12 ± 0.057	0.23 ± 0.085	0.38 ± 0.117
PA	120	2.375	1.511	0.500	75.00%	0.12 ± 0.066	0.19 ± 0.091	0.31 ± 0.130
Average:	**Total: 360**	**2.500**	**1.383**	**0.708**	**75.00%**	**0.096**	**0.18**	**0.48**

Genetic Differentiation: Analysis of molecular variance (AMOVA) among "progeny" subpopulations

The AMOVA revealed that the greatest amount of variance occurred among populations (42%) followed by within individuals (32%), and that there was significant genetic differentiation among populations ($F_{ST} = 0.42 \pm 0.126$; p-value = 0.001; Table 3.3).

Table 3.3. Analysis of Molecular Variance (AMOVA) results per treating the propagules sampled at every sampling location as a population. Standard deviation is reported for F-statistic values. (1,000 permutations)

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	32.2	F_it	0.678	0.079	--
Among Individual	Population	25.4	F_is	0.441	0.085	0.001
Among Population	--	42.4	F_st	0.424	0.126	0.001

Pairwise AMOVAs and pairwise comparisons of F-statistics among "progeny" subpopulations

AMOVA results for each pair of "progeny" subpopulations indicate that there is significant differentiation among all population pairs. When Leeville, LA is paired with Dog Island, FL, the greatest amount of variation occurs among populations (~50%), and there is a significant F_{ST} value of 0.5 (p-value < 0.001; Table 3.4). In contrast, when Leeville, LA is paired with Port Aransas, TX, the greatest amount of variation is attributed to within individuals (52.7%), and very little among populations (9%). There is significant differentiation among the populations, but the differentiation is very low (F_{ST} = 0.09; p-value < 0.001; Table 3.5). The AMOVA between Dog Island, FL and Port Aransas, TX reveals similar differentiation values as between Dog Island, FL and Leeville, LA. Among population variance is responsible for 55.2% of the variance, and there is large, significant differentiation (F_{ST} = 0.55; p-value < 0.001; Table 3.6).

Table 3.4. AMOVA results for genetic differentiation among progeny subpopulations from Leeville, LA, and Dog Island, FL.

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	28.5	F_it	0.715	0.077	--
Among Individual	Population	21.7	F_is	0.432	0.061	< 0.001
Among Population	--	49.8	F_st	0.498	0.137	< 0.001

Table 3.5. AMOVA results for genetic differentiation among progeny subpopulations from Leeville, LA, and Port Aransas, TX.

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	52.7	F_it	0.473	0.178	--
Among Individual	Population	38.3	F_is	0.421	0.156	< 0.001
Among Population	--	9	F_st	0.09	0.055	< 0.001

Table 3.6. AMOVA results for genetic differentiation among progeny subpopulations from Dog Island, FL, and Port Aransas, TX.

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	25.8	F_it	0.742	0.074	--
Among Individual	Population	19	F_is	0.424	0.04	< 0.001
Among Population	--	55.2	F_st	0.552	0.119	< 0.001

The trends exhibited in the paired progeny subpopulation AMOVAs are upheld by the pairwise comparisons of differentiation indices, F_{ST} and Jost's D. Due to all three subpopulations contributing to the total heterozygosity (H_T), we now see even higher values of F_{ST} among progeny subpopulations (Figure 3.2). Dog Island, FL exhibits the highest pairwise differentiation values overall. Dog Island, FL and Port Aransas, TX are most differentiated (F_{ST} = 0.712) followed by Dog Island, FL and Leeville, LA (F_{ST} = 0.636). Leeville, LA and Port Aransas, TX have the lowest pairwise differentiation value (F_{ST} = 0.11). Jost's D mirrors these trends; Leeville, LA is least genetically distant from Port Aransas, TX, and Dog Island, FL is most genetically distant from Port Aransas, TX.

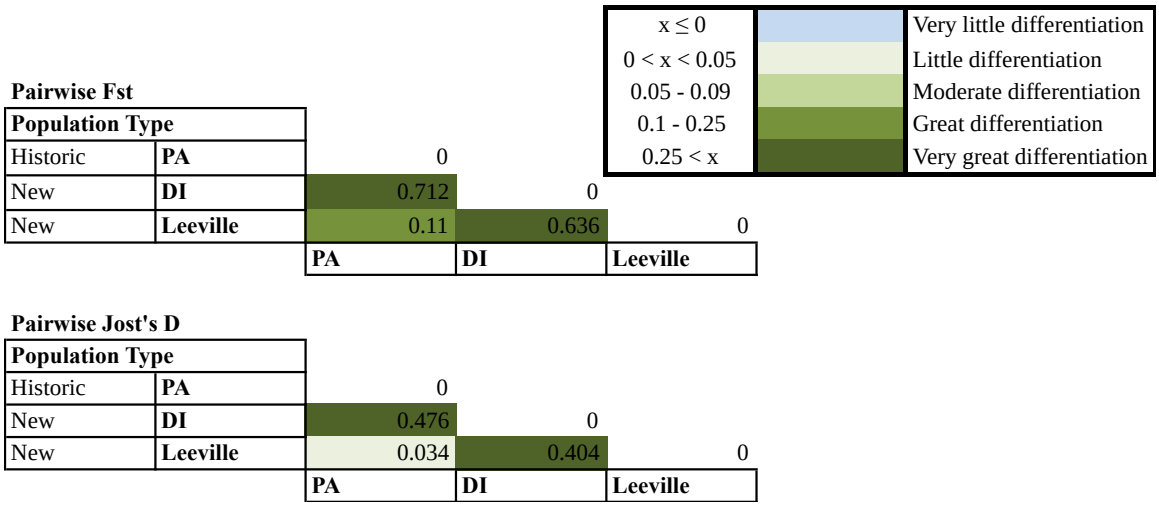


Figure 3.2. Visualization of pairwise comparisons among the "progeny" subpopulations using F_{ST} and Jost's D.

Genetic differentiation between "parent" and "progeny" subpopulations

The AMOVA testing the molecular variance between "parent" and "progeny" subpopulations of Leeville, LA (Table 3.7) shows that nearly equal variance is attributed to within individuals (50%) and among individuals within a population (49%). There is moderately high, significant inbreeding among individuals within a population (F_{IS} = 0.5; p-value = 0), and no significant differentiation among populations (F_{ST} = 0.002; p-value = 0.353).

The AMOVA testing molecular variance between "parent" and "progeny" subpopulations of Dog Island, FL (Table 3.8), attributes most of the variation to among populations (47.5%), followed by nearly equal variance for within individuals (24%) and among individuals within a population (28.5%). There is significantly high inbreeding among individuals within a population (F_{IS} = 0.54, p-value < 0.001) and significant differentiation among populations (F_{ST} = 0.47; p-value < 0.001).

The AMOVA testing molecular variance between "parent" and "progeny" subpopulations of Port Aransas, TX (Table 3.9) attributes most of the variance to within

individuals (60%), followed by among individuals within populations (37%). There is significant, moderate inbreeding among individuals within populations (F_{IS} = 0.38; p-value < 0.001), and nearly significant genetic differentiation among populations (F_{ST} = 0.025; p-value = 0.055).

Table 3.7. Analysis of Molecular Variance (AMOVA) results per testing the "parent" subpopulation of Leeville, LA, against the "progeny" subpopulation of Leeville, LA. Standard deviation is reported for F-statistic values.

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	50.1	F_it	0.499	0.194	--
Among Individual	Population	49.7	F_is	0.498	0.195	< 0.001
Among Population	--	0.2	F_st	0.002	0.01	0.353

Table 3.8. Analysis of Molecular Variance (AMOVA) results per testing the "parent population" of Dog Island, FL against the "progeny population" of Dog Island, FL. Standard deviation is reported for F-statistic values.

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	24	F_it	0.76	0.214	--
Among Individual	Population	28.5	F_is	0.543	0.181	< 0.001
Among Population	--	47.5	F_st	0.475	0.443	< 0.001

Table 3.9. Analysis of Molecular Variance (AMOVA) results per testing the "parent population" of Port Aransas, TX, against the "progeny population" of Port Aransas, TX. Standard deviation is reported for F-statistic values.

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	60.4	F_it	0.396	0.116	--
Among Individual	Population	37	F_is	0.38	0.122	< 0.001
Among Population	--	2.5	F_st	0.025	0.03	0.055

Genetic differentiation between "progeny" subpopulations based on regions

The greatest amount of variance stems from among populations within a region (62%), followed by within individuals (34%; Table 3.10). There is moderate inbreeding among individuals within a population (F_{IS} = 0.44; p-value < 0.001), and moderately high differentiation among populations within a region (F_{SC} = 0.5; p-value < 0.001). However,

there is no significant differentiation among the "new" and "historic" regions (F_{CT} = -0.23; p-value = 0.67).

Table 3.10. Analysis of Molecular Variance (AMOVA) results per sorting "progeny" subpopulations from Leeville, LA, and Dog Island, FL, into a "new" region, and Port Aransas, TX, into a "historic" region.

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	34	F_it	0.66	0.074	--
Among Individual	Population	27.1	F_is	0.443	0.08	< 0.001
Among Population	region	61.9	F_sc	0.503	0.147	< 0.001
Among region	--	-23	F_ct	-0.23	0.157	0.668

Overall genetic differentiation among populations and regions

The AMOVA testing the genetic variance among all three populations as a whole (pooling parent and progeny subpopulations together) attributes most of the variance to among populations (42%), with similar variances for within individuals (30%) and among individuals within populations (28%). The individuals within populations are moderately inbred (F_{IS} = 0.48; p-value < 0.001), but there is significant, moderate differentiation among populations (F_{ST} = 0.42; p-value < 0.001; Table 3.11).

When running an AMOVA on these three populations divided into a "new" region (Leeville, LA and Dog Island, FL) and a "historic" region (Port Aransas, TX), most of the variance is attributed to among populations within a region (~60%). There is significant inbreeding among individuals within a population (F_{IS} = 0.47; p-value < 0.001) and significant differentiation among populations within regions (F_{SC} = 0.49; p-value < 0.001). However, there is no significant differentiation among regions (F_{CT} = -0.21; p-value = 0.67; Table 3.12).

Using the AMOVA to test the "new" populations of Dog Island, FL, and Leeville, LA independently against Port Aransas, TX finds that there is much greater genetic differentiation among Dog Island, FL and Port Aransas, TX than there is among Leeville, LA, and Port Aransas, TX (Table 3.13; Table 3.14). Dog Island, FL is quite differentiated

from Port Aransas, TX (F_{ST} = 0.55; p-value < 0.001), and Leeville, LA is only slightly differentiated from Port Aransas, TX (F_{ST} = 0.086; p-value < 0.001).

Table 3.11. Analysis of Molecular Variance (AMOVA) results among the three populations: Leeville, LA, Dog Island, FL, and Port Aransas, TX.

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	29.8	F_it	0.702	0.074	--
Among Individual	Population	28	F_is	0.484	0.086	< 0.001
Among Population	--	42.1	F_st	0.421	0.104	< 0.001

Table 3.12. Analysis of Molecular Variance (AMOVA) results among the three populations divided into a "new" region (Leeville, LA; Dog Island, FL) and a "historic" region (Port Aransas, TX).

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	32.4	F_it	0.676	0.071	--
Among Individual	Population	29.2	F_is	0.474	0.07	< 0.001
Among Population	region	59.6	F_sc	0.492	0.127	< 0.001
Among region	--	-21.1	F_ct	-0.211	0.146	0.668

Table 3.13. Analysis of Molecular Variance (AMOVA) results for Dog Island, FL (parents + progeny) vs. Port Aransas, TX (parents + progeny).

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	24	F_it	0.76	0.067	--
Among Individual	Population	20.6	F_is	0.462	0.034	< 0.001
Among Population	--	55.4	F_st	0.554	0.099	<0.001

Table 3.14. Analysis of Molecular Variance (AMOVA) results for Leeville, LA (parents + progeny) vs. Port Aransas, TX (parents + progeny).

Source of Variation	Nested in	%var	F-stat	F-value	Std.Dev.	P-value
Within Individual	--	51.5	F_it	0.485	0.177	--
Among Individual	Population	39.9	F_is	0.437	0.151	< 0.001
Among Population	--	8.6	F_st	0.086	0.061	<0.001

Population Structure among "progeny" populations

Population structure, based on the genetic data of the three "progeny" subpopulations, showed the greatest statistical support for K = 2 (ΔK = 3,981.58) when model parameters were set to K = 1 – 10, with an initial burn-in period of 50,000 iterations followed by 250,000 Markov Chain Monte Carlo (MCMC) repetitions (Evanno et al. 2005; Figure 3.3).

For K = 2, Port Aransas, TX clustered together with Leeville, LA (Figure 3.4a).

For K = 3, representing the number of populations sampled in the field, Port Aransas, TX and Leeville, LA still largely clustered together with some overlap from Dog Island, FL into Leeville, LA. Dog Island, FL appears to be divided into two genetic linkage groups, the second (blue) slightly overlapping into Port Aransas, TX (Figure 3.4b).

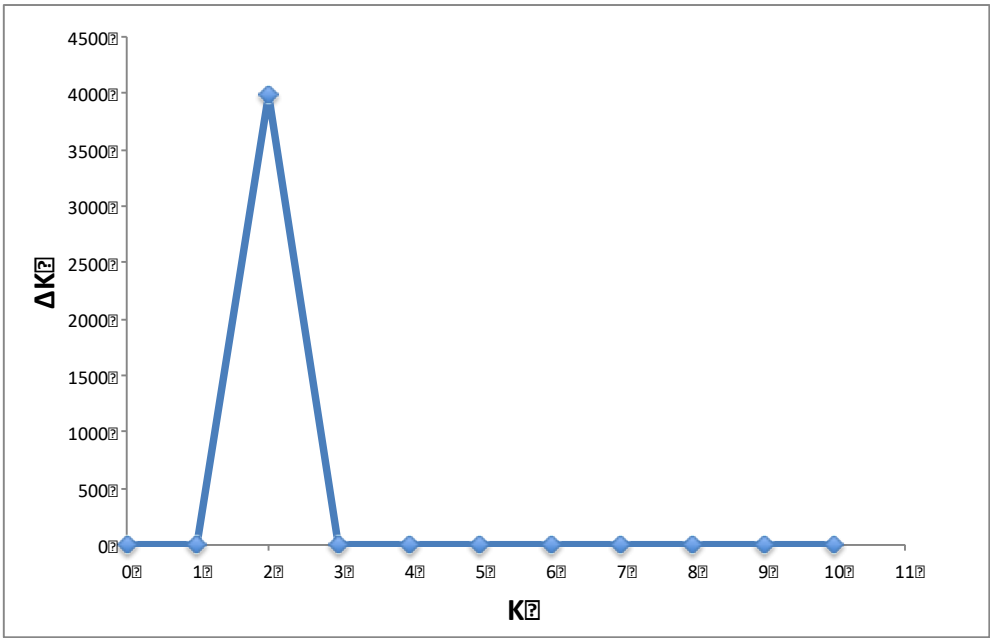
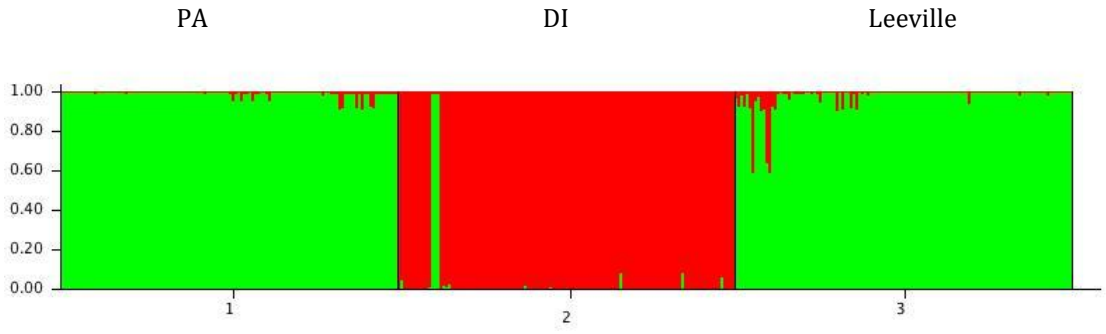


Figure 3.3. ΔK plot of STRUCTURE results with a burn-in period of 50,000 iterations and 250,000 MCMC repetitions, with 20 runs of each K as K = 1 – 10 (Evanno et al. 2005). The 3 sampled populations grouped into 2 distinct clusters.

a.)



b.)

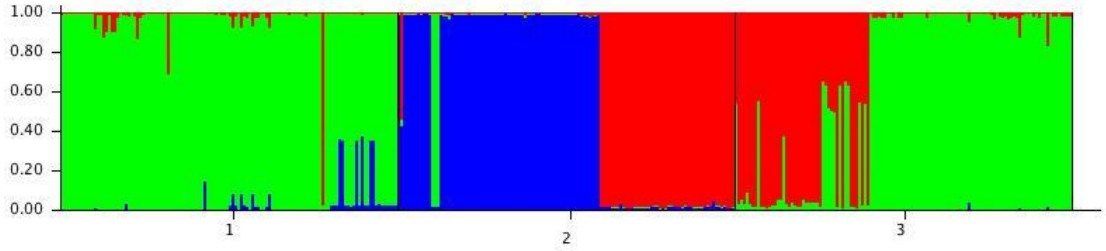


Figure 3.4. Results for STRUCTURE bar plot of the three sampling locations for *Avicennia germinans* using (a) K = 2 and (b) K = 3 cluster assignments. Populations are listed along the x-axis, and individual genotypes are represented as vertical bars of color within each population. Subgroups and individual membership to a cluster is represented by color. Admixture was specified in the model, therefore membership to more than one cluster may occur. (Population names and numbers: 1 = PA, 2 = DI, 3 = Leeville.)

Discussion

Using eight species-specific polymorphic microsatellite loci, this study assessed the genetic diversity of the parent trees compared to their progeny, and explored how the levels of genetic diversity and inbreeding between parents and progeny compared among the populations sampled.

I hypothesized that overall, the two new, range-expansion populations (Dog Island, FL and Leeville, LA) would exhibit greater genetic diversity compared to the historic, within-range population (Port Aransas, TX), which may allow the individuals in the range-expansion populations to succeed in novel environmental conditions. I further postulated that the genetic diversity and inbreeding coefficients would differ between the parent subpopulation and the progeny subpopulation in the two new, range-expansion populations. I expected this difference to reflect a decrease in genetic diversity and an increase in inbreeding coefficients due to the less developed and more isolated nature of the range-expansion populations, possibly resulting in reduced pollen donors and a genetic bottleneck. I posited that this would result in significant genetic differentiation among the parent and progeny subpopulations within the Dog Island, FL and Leeville, LA populations.

Port Aransas, TX, the historic, within-range population, is less fragmented and well within the species' distribution range. This likely means that this population has more ample access to pollen donors, which would spare it from much of the stress that the other two populations experience from novel environmental conditions. I did not, therefore, anticipate a significant genetic distinction between parent and progeny in this population, and expected the levels of observed heterozygosity and inbreeding coefficients to be fairly similar between parent and progeny subpopulations. Due to differing selection forces acting on the range-expansion populations compared to the within-range population, I surmised that there would be significant genetic differentiation among the three populations, and that both the parent and progeny subpopulations of each population would reflect these genetic distinctions.

The parent subpopulation of the within-range population, Port Aransas, TX, exhibits a higher observed heterozygosity (H_o = 0.10) and a lower inbreeding coefficient (F_{IS} = 0.39) than either of the two range-expansion populations, refuting my hypothesis that the parents of the range-expansion populations would harbor greater genetic diversity to sustain the genetic plasticity necessary to adapt to novel conditions (Table 3.2). All populations tested out of Hardy-Weinberg Equilibrium, expressing heterozygote deficiency. This is mirrored by the very low levels of heterozygosity found in both the parent and the progeny subpopulations, ranging from H_o = 0.043 in Dog Island, FL to H_o = 0.10 in Port Aransas, TX and the high inbreeding coefficients, ranging from F_{IS} = 0.70 in Dog Island, FL to F_{IS} = 0.39 in Port Aransas, TX (Table 3.2). In all populations, observed heterozygosity was lower than the expected heterozygosity, with the largest differences between the two values in Leeville, LA and Dog Island, FL (Table 3.2). These values for observed heterozygosity are very low compared to other studies on *A. germinans* (Salas-Leiva et al. 2009; Cerón-Souza et al. 2012; Nettel-Hernanz et al. 2013; Hodel et al. 2016; Millán-Aguilar et al. 2016). Similarly low ranges, however, have been seen in *A. germinans* populations along the Mexican coast (Sandoval-Castro et al. 2016).

Another unexpected finding is the increase in observed heterozygosity, albeit small, from parent to progeny in every population (Table 3.2). Observed heterozygosity increased from H_o = 0.10 to H_o = 0.12 in Port Aransas, TX; H_o = 0.043 to H_o = 0.052 in Dog Island, FL; and H_o = 0.094 to H_o = 0.12 in Leeville, LA. Inbreeding coefficients in Port Aransas, TX and Leeville, LA also decreased from parent to progeny (F_{IS} = 0.39 to F_{IS} = 0.31, and F_{IS} = 0.55 to F_{IS} = 0.38, respectively). In Dog Island, FL, however, there is an increase in the inbreeding coefficient (F_{IS} = 0.70 to F_{IS} = 0.75), denoting further inbreeding in the progeny subpopulation than in the parent subpopulation. If this trend continues, the population is at risk for a reduction in fitness, and truncated genetic plasticity and adaptability due to inbreeding depression (Salas-Leiva et al. 2009; Freeland et al. 2011). The number of different alleles present (N_a) and the number of private alleles (N_p) increase in each population from parent to progeny, suggesting greater genetic distance among the populations in the progeny subpopulations than in the parent subpopulations.

An analysis of the molecular variance (AMOVA) of the progeny subpopulations of each population (Table 3.3) indicates the greatest variance exists among populations (42.4%), followed by within individuals (32.3%). Significant genetic differentiation exists among the populations (F_{ST} = 0.42; p-value < 0.001). These progeny subpopulation results are similar to those of the AMOVA testing for genetic differentiation among the populations as a whole (parent + progeny). The AMOVA also attributes the greatest variance to among populations (42.1%), followed by within individuals (29.8%). There is also a nearly identical value for genetic differentiation among populations as a whole (F_{ST} = 42.1; p-value < 0.001), with 42.1% of the variance also attributed to among populations (Table 3.11), implying the progeny subpopulations behave similarly enough that the pooling of parent and progeny subpopulations does not profoundly alter the levels of differentiation among the three populations.

This inference is corroborated by the analyses of molecular variance (AMOVAs), assessing whether significant genetic differentiation exists between the parent and progeny subpopulations of each population (Tables 3.7, 3.8, and 3.9). The AMOVAs reveal no significant difference between parents and progeny, except in Dog Island, FL where the parent and progeny subpopulations are significantly distinguished (F_{ST} = 0.48; p-value < 0.001). So even though the AMOVAs report the greatest variance occurring within and among individuals for the analysis between the parent and progeny subpopulations in both Leeville, LA and Port Aransas, TX (50% and 60%, respectively), the greatest amount of reported variance is among populations in Dog Island, FL (47.5%). These data suggest interesting genetic shifts due to local adaptations may already be occurring within a single generation of *A. germinans*, resulting in significant genetic differentiation between parent and progeny. Arnaud-Haond et al. (2006) found edge populations of *Avicennia marina* in Vietnam, the northern Philippines, and Australia had significantly reduced gene diversity and higher genetic structure (greater number of subdivisions or subpopulations within a population) compared to core, within-range populations, suggesting inbreeding or selfing may be advantageous in range-expansion habitats due to reproductive assurance and local adaptations. Arnaud-Haond et al. (2006) proposed that the high level of genetic structure and inbreeding suggests that populations of this *Avicennia* species are acting as independent evolutionary units, rather than as units

of a larger population connected through gene flow, likely resulting in the development of local adaptations and successful establishment in novel conditions.

Further review of the differentiation among the progeny populations through pairwise AMOVAs conducted for each population combination suggests which populations may be more or less genetically distinguished from the others (Tables 3.4, 3.5, 3.6). There is significant differentiation among all of the progeny subpopulations, with the highest F_{ST} (fixation index) values occurring between the Dog Island, FL and Port Aransas, TX populations ($F_{ST} = 0.55$; p-value < 0.001; Table 3.6), and between the Dog Island, FL and Leeville, LA populations ($F_{ST} = 0.5$, p-value = 0; Table 3.4).

Most of the variance among the three pairwise AMOVAs is attributed to among populations. The exception to this is in the AMOVA analyzing Leeville, LA and Port Aransas, TX, where most of the variance is credited to within individuals, followed by among individuals within populations (Table 3.5). Furthermore, a very low but significant value of genetic differentiation is reported for Leeville, LA and Port Aransas, TX ($F_{ST} = 0.09$; p-value < 0.001).

A similar situation occurred in a study by Salas-Leiva et al. (2009), where inter-population genetic differentiation in natural and reforested areas of *A. germinans* along the Salamanca Island Parkway (Colombian Caribbean) was as low as $F_{ST} = 0.02$, yet still significant. When all three populations are included in the pairwise population calculations for the fixation index (F_{ST}) and the genetic distance parameter (Jost's D) in Figure 3.2, the values for genetic differentiation are higher but agree with the patterns of genetic similarity and dissimilarity among populations produced in the AMOVAs.

The genetic proximity between Leeville, LA and Port Aransas, TX may explain why the AMOVA assessing differentiation among "regions" reports no genetic differentiation among the range-expansion "region" and the within-range "region" (Table 3.10). Neither the AMOVA sorting the progeny subpopulations into "new" (Leeville, LA and Dog Island, FL) and "old" (Port Aransas, TX) regions, nor the AMOVA sorting populations as a whole into "new" and "old" regions, indicate significant differentiation among regions (Table 3.10; Table 3.12). In both AMOVAs, the greatest variance is credited to among populations within regions, followed by within individuals.

In both cases, the genetic differentiation is very low and highly insignificant, refuting my hypothesis that the new, range-expansion populations are significantly genetically distinguished from the within-range, historic population. Separate AMOVAs run on population combinations, however, further confound this assertion, finding a significant genetic differentiation between Dog Island, FL and Port Aransas, FL (F_{ST} = 0.55; p-value < 0.001; Table 3.13), and between Leeville, LA and Port Aransas, TX (F_{ST} = 0.086; p-value < 0.001; Table 3.14). This differentiation, however, is also very low, similar to the AMOVA testing the progeny subpopulations of Leeville, LA and Port Aransas, TX. In the AMOVAs testing the populations and progeny subpopulations nested within "new" and "historic" regions (Tables 3.10 and 3.12), 60% of the variance stemmed from among populations within regions, followed by within individuals. The uncharacteristically great genetic distance of the Dog Island, FL population from the other two populations may be the main driver of the differentiation among populations within regions. Alternatively, the lack of significant genetic differentiation among regions may be chiefly influenced by the high genetic similarity between Leeville, LA and Port Aransas, TX, and lack of more populations for a more balanced comparison.

The ΔK and STRUCTURE cluster assignments suggest both theories may be correct (Figure 3.3; Figure 3.4). Based on the genetic data, the greatest statistical support occurred for K = 2 (ΔK = 3, 981.58), in which the STRUCTURE bar plot depicts Port Aransas, TX forming a linkage group with Leeville, LA. In K = 3, representing the actual number of populations sampled, the STRUCTURE bar plot reveals a third, unidentified linkage group making up almost half of the Dog Island, FL cluster, and spilling somewhat into the Leeville, LA cluster. This unidentified linkage group may represent Dog Island's donor population, and appears to distribute at least some of its genotypic influence to Leeville, LA as well. No overlap exists between this unidentified group and Port Aransas, TX. Perhaps this peripheral linkage group is the foundation of Dog Island's seemingly particular genetic linkage group, distinguishing Dog Island, FL from several other populations of *A. germinans* along the Gulf Coast (Chapter 2).

In conclusion, I found confounding evidence of maintained genetic diversity between parent and progeny, and possible founder effects in the new, leading-edge populations of *A.germinans* compared to a historic population along the Gulf Coast.

Contrary to my expectations, the historic, within-range population Port Aransas, TX had higher observed heterozygosity in both its parent and progeny subpopulations, compared to the range-expansion populations in Leeville, LA and Dog Island, FL. As predicted, however, the two new populations did exhibit higher inbreeding coefficients compared to the within-range population. Interestingly, data for Port Aransas, TX and Leeville, LA progeny subpopulations revealed an increase in observed heterozygosity and a decrease in inbreeding coefficients compared to the parent subpopulations. This is perhaps due to sufficient pollen donor populations interacting with these populations, or a recent influx of migrants to this population, introducing new, mixed genotypes. Dog Island, FL, the youngest and northernmost population sampled (Osland et al. 2013), did express an increase in the inbreeding coefficient from parent to progeny subpopulations, and had very low observed heterozygosity values compared to the other two populations. This population, remarkably, also expressed a slight increase in observed heterozygosity from the parent to the progeny subpopulations. Further study is necessary to verify this increase in observed heterozygosity and discern to which mechanisms this unexpected increase may be attributed.

A study by Millán-Aguilar et al. (2016) assessing changes in genetic diversity and inbreeding among adults and saplings of perturbed and preserved sites of *A. germinans* in northwestern Mexico found an increase in observed heterozygosity and a decrease in the inbreeding coefficient from the adult subpopulation to the sapling subpopulation in preserved populations of *A. germinans*, similar to my results for Port Aransas, TX and Leeville, LA. In perturbed populations, there was a decrease in observed heterozygosity and an increase in the inbreeding coefficient among adult and sapling trees, similar to my data for Dog Island, FL. This suggests that this population is more stressed compared to the other two populations and may have limited access to pollen donors (Millán-Aguilar et al. 2016). Dog Island, FL was the only population to demonstrate significant, genetic distinction between the parent and progeny subpopulations, likely attributed to genetic selection due to the circumstance of being an isolated, fragmented, small, and young colonizing population. As discussed before, bi-parental inbreeding, isolation from diverse pollen donors or incoming migrants, and natural selection favoring adaptations to local

environmental conditions may be leading to genetic drift and swift genetic differentiation in this population (Slatkin 1987; Arnaud-Haond et al. 2006).

Nonetheless, AMOVAs show no genetic distinction between regions of "new" leading-edge populations and the "historic" within-range populations, despite the presence of significant genetic distinction among populations within those regions. Though the Leeville, LA population conveys significant genetic distinction from Port Aransas, TX, the distinction is very small. The PCA and STRUCTURE bar plots further uncover how genetically linked these two populations may be, perhaps resulting in the skewing of results in the AMOVA detecting variance among "new" and "historic" regions.

These conclusions support previous findings by Nettel-Hernanz et al. (2013) that *A. germinans* may be predominantly outcrossing but can support high to moderate levels of inbreeding and, in fragmented, disturbed populations, there may be high levels of bi-parental inbreeding, geitonogamy, and self-fertilization (Cerón-Souza et al. 2012; Nettel-Hernanz et al. 2013). The observed protandry in *A. germinans* makes self-fertilization unlikely, but little is known about the proportion of bi-parental inbreeding, and there is no direct evidence of self-incompatibility in *A. germinans* (Tomlinson 1986; Rathcke et al. 1996; Cerón-Souza et al. 2012). Other *Avicennia* species from the Indo-Pacific region show this pattern. In isolated or low-density populations, geitonogamy could be a very advantageous breeding system (Clarke and Myerscough 1991; Aluri 1990). Previous studies also suggest that pollen and seed dispersal in patchy distributions of *A. germinans* are very local, leading to greater bi-parental inbreeding and increasing genetic differentiation, possibly by driving genetic drift (Slatkin 1987; Kalisz et al. 2001; Yu et al. 2010).

It is important to understand these biotic drivers toward increased or decreased genetic diversity, as the reduction in fitness due to inbreeding depression may play a large role in the future success of colonizing populations of *A. germinans.* It will also enhance our understanding of, and modeling capabilities for, the resulting ecosystem shifts that may occur along the Gulf Coast in the future.

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Appendix

Table 1. List of primers chosen for this study on *Avicennia germinans*.

Literature	Primer Name	Repeat Type	Expected Heterozygosity	Annealing Temperature (° C)
Cerón-Souza et al., 2006	Agerm_CTT_001 (DQ240228)	$(CTT)_8$	0.571	55
Nettel et al., 2005	AgT31 (AY741800)	$(CA)_{12}(TA)_2GA(CATA)_9$	0.24	60
Nettel et al., 2005	AgT7 (AY741799)	$(CAT)_2(AT)_3(GTAT)_5$	0.33	50
Mori et al., 2010	Agerm1-12 (HM470014)	$(AC)_{15}$	0.83382	53.4
Mori et al., 2010	Agerm1-14 (HM470016)	$(CA)_8(AT)_6$	0.72657	59.6
Mori et al., 2010	Agerm1-18 (HM470020)	$(AG)_{16}$	0.62368	64.5
Mori et al., 2010	Agerm1-21 (HM470023)	$(CA)_8$	0.61469	61.8
Mori et al., 2010	Agerm1-22 (HM470024)	$(TTTCTT)_4$	0.04	63.4

Table 2. Literature cited, primer name, and allele information per this study as assessed by GENODIVE (based on 405 individuals and 8 loci).

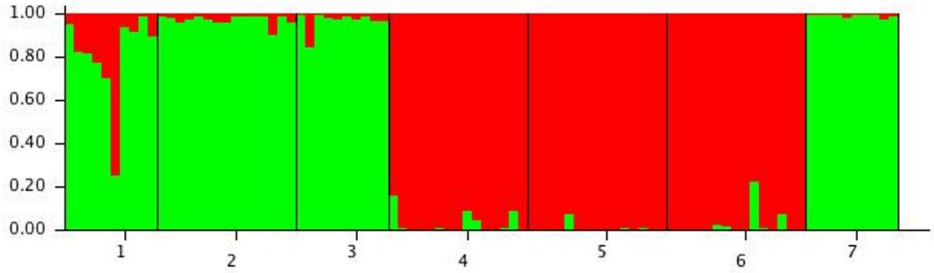
Literature	Primer Name	# Alleles in this study
Cerón-Souza et al., 2006	Agerm_CTT_001 (DQ240228)	2
Nettel et al., 2005	AgT31 (AY741800)	2
Nettel et al., 2005	AgT7 (AY741799)	5
Mori et al., 2010	Agerm1-12 (HM470014)	7
Mori et al., 2010	Agerm1-14 (HM470016)	7
Mori et al., 2010	Agerm1-18 (HM470020)	4
Mori et al., 2010	Agerm1-21 (HM470023)	3
Mori et al., 2010	Agerm1-22 (HM470024)	5
		Total: 35

Table 3. Modified PCR thermocycler program for Type-it® Microsatellite Multiplex PCR Kit (QIAGEN).

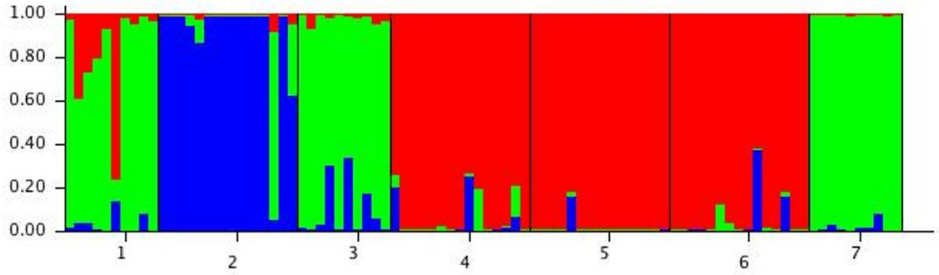
Step	Temperature (°C)	Time	Action
1	95	5:00	Initial Denaturing
2	95	0:30	Denaturing
3	65.5*	1:30	Annealing
4	72	0:30	Extension
5	95	0:30	Denaturing
6	63.4*	1:30	Annealing
7	72	0:30	Extension
8	95	0:30	Denaturing
9	60**	1:30	Annealing
10	72	0:30	Extension
11	95	0:30	Denaturing
12	59.6*	1:30	Annealing
13	72	0:30	Extension
14	95	0:30	Denaturing
15	56.7*	1:30	Annealing
16	72	0:30	Extension
17	95	0:30	Denaturing
18	55*	1:30	Annealing
19	72	0:30	Extension
20	95	0:30	Denaturing
21	50*	1:30	Annealing
22	72	0:30	Extension
23	go to 20	23 times	Cycling
24	60	1:30	Final Extension
25	4	END	Chill

* Specific annealing temperature of a primer as outlined by literature
** Optimum annealing temperature per QIAGEN

a)



b)



c)

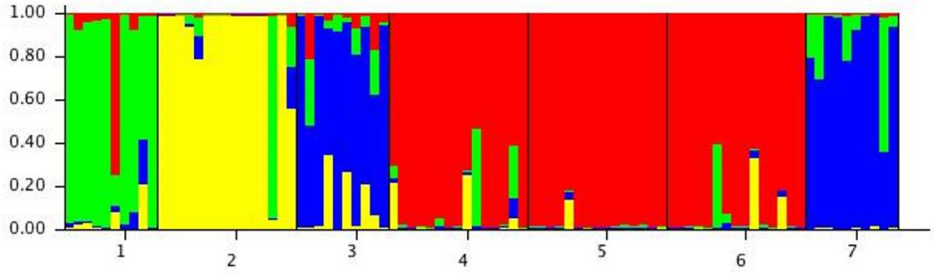


Figure 2.6. Results for STRUCTURE bar plot of the 7 sampling locations for *Avicennia germinans* using (a) K = 2 (b) K = 3 (c) K = 4 cluster assignments. Populations are listed along the x-axis, and individual genotypes are represented as vertical bars of color within each population. Subgroups and individual membership to a cluster is represented by color. Admixture was specified in the model, therefore membership to more than one cluster may occur. (Population names and numbers: 1 = CK, 2 = DI, 3 = EPP, 4 = Leeville, 5 = PA, 6 = PI, 7 = Site 2.)

*Original Figure 2.6 with populations listed in alphabetical order before re-running STRUCTURE data by reorganizing populations by west-to-east orientation along the Gulf Coast.

Summary and Prospects for Future Work

In this thesis, polymorphic microsatellite markers were used to observe the genetic diversity and inbreeding coefficients of seven populations of *Avicennia germinans* (black mangrove) along the Gulf Coast of Florida, Louisiana, and Texas. The analyses included an overall assessment of genetic diversity within and among populations, and compared trends in heterozygosity and inbreeding coefficients among two new, range-expansion populations to the five historic, within-range populations. To gauge the presence and degree of possible founder effects enacting on the two new, range-expansion populations, genetic characteristics between the parent and progeny subpopulations of the two range-expansion populations were compared to a historic, within-range population.

Data indicated that all populations were expressing heterozygote deficiency, and exhibited relatively low levels of observed heterozygosity with moderate to high inbreeding coefficients. There was, however, significant genetic differentiation among the seven populations, supporting assertions by previous studies that populations of *Avicennia germinans* may exhibit moderate levels of inbreeding within populations, but that the genetic variation among populations can still be high, suggesting that local conditions exert great influence over patterns of population genetics in *A. germinans*. There was no significant genetic differentiation among "regions," however, when the two new, range-expansion populations were ground into a "new" region, and the five historic, within-range populations were grouped into an "old" region, there was no distinct separation of leading-edge populations from within-range populations. There was also no discernable latitudinal gradient of heterozygosity or inbreeding coefficients among populations of *A. germinans* along the Gulf Coast.

The youngest, most northern range-expansion population sampled, Dog Island, FL, expressed the greatest genetic distance from other populations, and exhibited the lowest values of observed heterozygosity and the highest values for inbreeding coefficients. In the analysis of the genetic similarities among the parent and progeny subpopulations of the two new, range-expansion populations (Leeville, LA and Dog Island, FL) and the historic, within-range population (Port Aransas, TX), only the Dog

Island population expressed significant distinction between parent and progeny. In all three populations, observed heterozygosity increased from parent to progeny subpopulations, accompanied by a decrease in the inbreeding coefficient, except for in Dog Island, where the inbreeding coefficient increased from parents to progeny. This, in conjunction with the Dog Island, FL population's extreme genetic distance and distinction from all other populations sampled along the Gulf Coast, suggests this population may be highly fragmented and isolated, and is experiencing selection due to a genetic bottleneck, risking inbreeding depression due to the founder effect. The other new, range-expansion population, Leeville, LA, however, has much more moderate levels of heterozygosity and inbreeding, implying that though this population is relatively young and on the leading-edge of the *A. germinans* threshold in the Gulf, it appears connected enough to other populations and potential pollen donors to mitigate the consequences of the founder effect. This theory is supported by the apparent high genetic connectivity among the Leeville, LA population and the two Texas populations, as these three populations consistently cluster together, while Dog Island, FL is the only population of the seven to consistently form its own linkage group.

Implications of such data are particularly interesting when assessing effects of novel environmental stressors due to range-expansion on the genetic diversity – and thus genetic resiliency – of *A. germinans* in the Gulf Coast. It shows the potential of populations of *A. germinans* to successfully colonize and expand further north given the right abiotic and biotic conditions, supporting that favorable physical conditions alone cannot accurately predict potential mangrove expansion in the future and that the biotic limitations must also be taken into account. Further research should include larger sample sizes of both mature "parent" trees and their respective progeny, to better track how observed heterozygosity and inbreeding change between generations, and if there appears to be a correlation with various stressors. Furthermore, more new colonizing populations of *A. germinans* along the northern rim of the Gulf should be carefully monitored and assessed for inbreeding depression, as these fragmented and highly inbred populations may be very susceptible to natural phenomena and thus may not accurately represent enduring mangrove expansion.

Managers may use information from such genetic analyses to properly identify optimum source populations for re-forestation efforts to maximize success of restoring genetically diverse and resilient mangles, and to better inform models of potential coastal ecosystem changes with sea level rise, reduced frequency of winter freeze events, and northward mangrove migration. A better understanding of biotic factors that influence the resiliency of *A. germinans* populations, in addition to the abiotic drivers that dictate mangrove migration, will improve our understanding of potential long-term ecosystem shifts and the cascading environmental effects such shifts may have on the valuable ecosystem services that the Gulf provides.