The genetic structure of Avicennia germinans populations in the northern Gulf of Mexico.

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A Dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Environmental Sciences

University of Virginia
November, 2019

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Abstract

Around the world mangroves occupy around 152,000 km^2 of tropical and subtropical coastline. Mangroves provide critical habitat for estuarine, nearshore, and terrestrial biota and supply crucial services such as shoreline establishment, protection from coastal erosion, coastal livelihood support and atmospheric carbon sequestration. The past and present-day distribution and abundance of mangroves in the Atlantic Eastern Pacific has been established through a combination of habitat suitability, climatic condition, and genetic selection. Mangrove species have been observed to be expanding poleward and landward across the Gulf of Mexico, where mangroves face the unusual situation of being both excellent at long-distance dispersal and constrained in any northward movement by available habitat. Though mangrove populations provide many crucial services, little is known about mangrove population genetic structure in the northern Gulf of Mexico.

The broad goals of this dissertation were to better understand global mangrove expansion at the cost of salt marsh habitat through literature review and to better understand *Avicennia germinans* phylogeography in the northern Gulf of Mexico by use of a modern next generation sequencing technique known as double digest RAD-sequencing. From the literature review, human activities both past and present were found to have significantly altered both salt marsh and mangrove habitat. Currently salt marsh habitat across the world is experiencing large habitat loss and fragmentation, while mangrove populations are expanding both poleward and landward. The current loss and fragmentation of salt marsh habitat along with the superiority of the mangrove species dispersal capability suggests that salt marshes across the globe are ripe for mangrove species encroachment and conversion. For mangrove phylogeography in the northern Gulf of Mexico, the genetic structure and the kinship of *Avicennia germinans* populations

showed highly related populations with low genetic diversity and a high number of individuals with a similar genetic makeup. Taken together, the results of this study help to suggest future directions for mangrove conservation, mangrove genetics research, and the shift of the mangrove salt marsh ecotone in the GOM. Understanding the dynamics of these highly variable genetic relationships and the processes of genetic exchange in mangrove populations will be essential to predicting future impacts of climate change and anthropogenic forcing.

Acknowledgements

First, I would like to thank my first advisor Jay Ziemen, who believed in me and supported me through the being of my time here at the University of Virginia. His friendly ear and open door gave me help and guidance I needed to make a start in a brand-new field for me. I would also like to thank my current advisor Manuel Lerdau, who took over in Jay's stead after his passing. Without his ability to accept me as his student I would not have been able to continue to my Ph.D. candidacy. I would also like to thank my committee members: Karen McGlathery, Matt Reinbach, Martin Wu, and Alan Bergland for their input and support. This work would not have been possible without the hours of statistical and coding help that I received not only from members of my committee, but also from member of the Bergland lab group and Catherine Debban.

Special thanks to the Kenneth Heck lab group at the Dauphin Island Sea lab, for their help in obtaining samples from Horn Island, Mississippi. Thank you as well to the Apalachicola National Estuarine Research Reserve, who helped me to identify sites in the reserve for sampling and on multiple occasion took me and members of my lab group out to Dog Island for sampling. I would also like to thank Meg Miller, Kelcy Kent, and Gina Digiantonio, who gave me support in the lab as well as guidance and friendship throughout all of these years and hardships.

Thank you especially to the great team of doctors and nurses at the UVA cancer center. The care that I received there gave me the hope and strength to carry on in the face of my breast cancer diagnosis. Truly the only reason I am alive to finish this degree is thanks to their quick action and diligent care. I would specifically like to thank my infusion nurse Dennis for his smiling face every week and his love of the movie White Christmas.

Lastly, I would like to thank my family and friends for their love, support, and bravery as I have faced the hardest years of my life. My friends in the department and in the community did so much for me during my treatments with kind words, support, and food. They were my family here, while mine was so far away. To my parents, who taught me to persevere through adversity and to never give up. They have given me the tools, knowledge, and love to make it even through my darkest of days. Thank you to my sister, for being my cheerleader and groaning partner as we both suffered through graduate school together. I would also like to thank my fiancé, Anthony Jennings, who went to every doctor's visit, every infusion, and every defense. He has been my constant support and my rock through everything these past years. I can never thank him enough for listening to me complain every day for the last 8 years.

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Chapter 1: Salt marsh decline and mangrove expansion in the face of anthropogenic forcing

Abstract

In subtropical regions, shifts in vegetation patterns are currently occurring at the boundary where salt marsh and mangrove species coexist. Mangrove species have expanded their range poleward and replaced salt marsh species in some areas at a rapid rate; at the same time, salt marshes are experiencing large die-off events and landscape level habitat loss. The shift poleward of mangrove vegetation and loss of salt marsh habitat could have lasting effects on ecosystem services provided by coastal habitats. The combination of the mangrove's endogenous abilities and anthropogenically caused exogenous forces suggest that mangroves are in an excellent position to continue expanding their range over the coming years. In this review, evidence for and against mangrove poleward and landward expansion and discuss how differences in the basic biology of mangroves vs. saltmarsh species affects mangrove expansion is presented. This review allows for discussion of the roles dispersal limitation and fragmentation play in contributing to the salt marsh to mangrove ecotone shift and for identification of questions that need to be resolved in order to better understand the future role of anthropogenic forcing on the salt marsh mangrove ecotone.

Introduction

For millennia, human actions have shifted the distributions and abundances of plants, animals, and entire ecosystems. From burning trees and shrubs to maintain prairies, to hunting top predators and shifting herbivores compositions, to adding rocks onto sandy beaches to promote oyster beds, humans have manipulated the natural world to promote the ecosystems they desire and retard others. Recently, another type of anthropogenic ecosystem modification has

become more common, indirect modification through altering large-scale parameters such as global temperature and ocean chemistry. While most ecologists have focused on high latitude ecosystems because these are the areas predicted to suffer the largest climatic changes, some of the most striking changes already measurable are in coastal subtropical systems, where mangrove ecosystems are expanding to higher latitudes, with immense impacts on community, ecosystem, and biogeochemical properties.

In the subtropical regions, shifts in vegetation patterns are occurring at the boundaries of where salt marshes and mangroves species coexist. The ecotone occurs as a dynamic stable state between salt marsh grasses and forbs and mangrove woody vegetation. The vegetation composition is controlled by climate and disturbance events. Recently this ecotone has been receiving more attention as mangrove species have expanded their range poleward and replaced salt marsh species in some areas at a rapid rate, as well as salt marshes experiencing large die-off events and landscape level habitat loss. This shift poleward of mangrove vegetation and loss of salt marsh habitat could have lasting effects on ecosystem services provided by coastal habitats such as coastal protection, erosion prevention, nutrient cycling, fisheries maintenance, and rates of carbon sequestration: all of which also influence local and regional economies (Armitage et al. 2015, Doughty et al. 2016, Saintilan et al. 2014).

Contributing to this shift are many anthropogenic forces such as climate change and coastal development. Climate change influences coastal environments in three major ways: sea level rise (SLR), temperature/precipitation shifts, and ocean acidification. To keep pace with SLR and occupy the same coastal areas, salt marshes and mangroves must increase soil elevation at equivalent or accelerated rates compared to rates of SLR. Soil elevation is maintained through a variety of feedbacks. Salt marshes and mangroves have extensive above-ground biomass from

plant shoots and aerial roots that slow water velocities, lower wave height, reduce erosional processes, and enhance soil deposition, These, in turn, increase soil elevation. Below-ground, salt marshes and mangroves have extensive root systems, that exist in a dynamic state of growth, death, or decay.

Within the last century climate change has caused a global average surface temperature increase of 0.74°C (McKee et al. 2012). More significantly for the mangrove-marsh ecotone, minimum winter temperatures globally have been increasing at twice this rate (Saintilan et al. 2014). Mangrove latitudinal limit has been shown to be regulated by minimum winter temperatures and the number of freeze events during the winter months, thus increases in the former and decreases in the latter will cause mangroves to shift their distribution poleward into areas occupied by salt marshes (Osland et al. 2013, Saintilan et al. 2014). Along with changes in temperature, changes in rainfall regimes around the world may affect the mangrove salt marsh ecotone shift, with increases in precipitation promoting mangroves and decreases likely degrading mangrove ecosystems.

Decreases in precipitation regimes can alter freshwater inputs, decreasing the amount of sediment inputs, affecting the ability of mangroves and salt marshes to respond to SLR, and increasing salinity. These changes lead to decreased seedling survival, productivity, growth rates, and they can ultimately result in mangrove and salt marsh die-back and/or collapse (Hughes et al. 2012, Osland et al. 2015, Ward et al. 2016). Increases in precipitation cause coastal habitat to see an increase freshwater inputs that, in turn, increase sediment inputs. The increase in sediment inputs raises surface elevations and enhances resiliency of the coastal habitat to SLR and can result in an enhancement of mangrove landward expansion into salt marshes (Ward et al. 2016). For example, in Moreton Bay, southeast Queensland, Australia, a 32

year study found that periods of increased precipitation enhanced mangrove expansion into salt marshes, while drier periods resulted in diminished mangrove expansion (Eslami-Andargoli et al. 2009).

As noted earlier, coastal land use, as well as climate, has strong impacts on mangrove systems. Coastal development affects coastal wetlands through habitat loss and degradation. Globally, it is estimated that between a one third and one half of vegetated intertidal habitats have been lost due to anthropogenic activities such as land reclamation, coastal development, and agriculture/aquaculture (Perkins et al. 2015). Many countries have lost more than 50% of their intertidal habitat (Table 1). As populations along the coast increase intertidal habitat is predicted to see further losses. For example, China plans to reclaim 563,450 ha by 2020(Perkins et al. 2015). Along with future reclamation projects, predictions of SLR will drive coastal communities to construct erosion/flooding defense structures which cause intertidal habitats to experience coastal squeeze (Perkins et al. 2015). Coastal squeeze occurs when SLR drives coastal wetlands to move inland, but coastal defense structures prevent inland movement leading to wetland fragmentation, vegetation loss, and negatively affect biogeochemical cycling, all of which significantly impact ecosystem function. Another result of coastal urbanization is coastal watershed development. Coastal watershed development increases the amount of impervious surface, which alters the amount of sediment and freshwater received by intertidal habitats and increases nutrient loading. These alterations can cause decreased soil salinities, alter plant assemblages, and affect biogeochemical cycles leading to fragmentation and ecosystem collapse (Noe et al. 2014, O'Meara and Thompson 2015).

Location	Estimated Habitat Loss	Time Period	Reference
Galveston, Texas, USA	10,000 ha, 10%	1930s-1980s, 2001-2011	White & Tremblay 1995, Entwistle 2017
Louisiana, USA	441,334 ha	1978-2000	Barras et al. 2004
Southern California, USA	3,767 ha	1930s-1980s	Zedler 1996
Weeney Bay, Australia	100%	1950-1994	Saintilan & Williams 2000
Hunter River Estuary, Australia	67%	1954-1994	Saintilan & Williams 2000
Tasmania	971 ha	1952-2006	Prahalad et al. 2014
China (all 11 coastal Provinces)	4,150,000 ha, 3,860,000 ha	1950-1991, 1991-2014	Sun et al. 2015

Table 1. Coastal wetland habitat loss estimates for areas around the world

One large consequence of these types of anthropogenic forcing is habitat fragmentation, which results in population fragmentation. Habitat fragmentation can be caused by climate change from temperature/precipitation changes, SLR especially in combination with coastal development (coastal squeeze), changes in pH that can affect biogeochemical cycling causing die-back, pools, and holes, and physical fragmentation from human erosion/flooding defense structures. Increased fragmentation decreases salt marsh connectivity, which in turn decreases the ability of salt marsh vegetation to recolonize areas or colonize new areas. In studies of salt marsh restoration, it has been shown that the most important factor for successful restoration is the distance of the restoration site to an established marsh. This suggests that for marsh restoration or marsh establishment connectivity been marshes is crucial.

Mangroves colonization ability is not as highly influenced by fragmentation as salt marsh vegetation. This difference results from the fact that, unlike salt marsh vegetation that produce dormant seeds, mangroves produce propagules through vivipary or cryptovivipary (Hogarth 1999). Mangrove propagules are mostly retained within the parent population, but unlike salt marsh seeds, they have a larger capacity for long distance dispersal, thus overcoming the distances between fragmented populations. This greater dispersal capability comes from the fact that propagules can float for longer than seeds and they can also survive as free-floating dispersing agents for much longer than seeds. This capability for long distance dispersal in

concert with increases in winter temperatures and decreased freeze event has allowed for mangrove poleward expansion into salt marshes.

This combination of mangrove's endogenous abilities and anthropogenically caused exogenous forces suggest that mangroves are in an excellent position to expand their ranges over the coming years. In this review we (i) discuss the evidence for and against mangrove poleward and landward expansion and (ii) discuss how differences in the basic biology of mangroves vs. saltmarsh species affects mangrove expansion. This review allows us to discuss the roles dispersal limitation and fragmentation play in contributing to the salt marsh to mangrove ecotone shift and to identify questions that need to be resolved in order to better understand the future role of anthropogenic forcing on the salt marsh mangrove ecotone.

(i) Evidence of Mangrove Range Extension

Mangroves have been observed to be expanding poleward and landward into salt marshes across the world with examples coming from every continent except Europe and Antarctica (Saintilan et al. 2014, Ward et al. 2016). Asia accounts for 42% of the global mangrove areal extent and a large portion of the world's mangrove taxonomic diversity, with 51 species near the equator and 11 species at the range limit in southern Japan. Currently, mangrove populations across Asia are experiencing large anthropogenic pressure from agriculture and aquaculture along with what the IPCC (2014) projects to be an observed warming of <2°C to 6°C across Asia by 2100 (Ward et al. 2016). These factors could have significant effects on both biodiversity and mangrove habitat range. Mangroves in Asia reach their range limits at the coast near the city of Fuding in Fujian Province China (27°20' N) (Jia et al. 2014), the Tanshui estuary in Tiawan (25°10' N), and on the coast near the city of Kiire in Kagoshima Prefecture Japan (31°22.5' N) (Spalding 2010). Mangrove expansion at range limits in Asia has been obscured by

anthropogenic influences such as plantings of native and non-native species north of the historic range and large mangrove losses from aquaculture and agricultural pressures. In Kagoshima Prefecture, Japan, and Zheihang, China, plantings of Kandelia obovate have been successful and have extended the historical range limit of this species, suggesting that climatic conditions outside of the historic range may now be suitable for mangrove growth and establishment (Ward et al. 2016). Many mangrove reserves in China have seen an expansion in mangrove populations at the expense of salt marsh habitat including Zhanjiang Mangrove National Nature Reserve and the Zhuhai Qi'ao Provincial Nature Reserve (Saintilan et al. 2014). On Iriomote Island in Japan, Nypa fructicans populations at their range limit have more than doubled over a 15 year period with 27 individuals observed in 1978 and 65 individuals observed in 1993 (Sugai et al. 2016).

In South Africa mangrove area has increased by 40% since the 1970s (Saintilan et al. 2014). In 1962 mangroves were observed at the estuaries of Mtata River (31°57' S), Mngazana River (31°42' S), and isolated clumps at the estuaries of the Mbashe (31°42' S) and Nxaxo (32°35' S). By 1982 mangrove formed extensive stands in estuaries of the Kobonqaba (32°36'S), Nqabara (32°30'S). Xora (32°05'S), and Bulungula (32°08'S) Rivers. The southernmost stand of mangroves was observed in 1969 in the Kwelera River (32°54'S) and later was observed in the Great Kei River (32°40'S) (Saintilan et al. 2014). Since then mangroves have been planted in the Nahoon estuary in 1969 and the Tyolomnqa estuary in the 1990s (Hoppe-Speer et al. 2015, Whitfield et al. 2016). In the Nahoon estuary three species have been planted (A. marina, B. gymnorrhiza, and R. mucronata) and have expanded by a rate of 0.06 ha y-1 over a 33 year period from 1978-2011 (Hoppe-Speer et al. 2015). Even though planting has obscured observations of mangrove expansion B. gymnorrhiza has natural expanded into salt

marshes in the Kariega estuary, which is more than 150 km south of the previously known populations in the Nahoon estuary (Whitfield et al. 2016).

In South America mangroves grow on both sides of the continent. On the Atlantic coast mangroves reach their range limit in Laguna, Brazil (28°30'S) with 2 mangrove species, Avicennia schaueriana and Laguncularia racemosa (Quisthoudt et al. 2012, Ximenes et al. 2016, Soares et al. 2012). The third naturally occurring species in Brazil (R. mangle) reaches its range limit in Florianopolis (27°49'S) (Quisthoudt et al. 2012). The southern limit of mangroves in Brazil has been relatively stable since the late 1980s with some recent evidence of Laguncularia racemosa recruitment further south (Saintilan et al. 2014). In this area the range limit for mangroves has not only been limited by temperature and freeze events, but also by the Falkland current which runs south to north along the South American coast until it meets the Brazil current around Laguna, Brazil. Southern Brazil has recently experienced increases in both annual mean air temperature and annual minimum temperature of surface waters, decreases in the occurrence of major frost events, and decreases in the influence of the Falkland current, suggesting that mangroves will in the future have both better conditions and more available habitat for expansion south of Laguna, Brazil (Soares et al. 2012).

On the Pacific coast of South America, the southern limit of mangroves was described by Clüsener & Breckle (1987) as the mouth of the River Thumbes (3°35'S), with a few individuals of Rhizophora further south at 3°44'S and a small stand of Avicennia even further south at the mouth of the Piura River (5°30'S). In Saintilan et al. (2014) the site at the mouth of the Piura River was found to have extensive mangrove cover of 9.5 km of shoreline and 38 ha in the north arm and 9 ha in the south arm of the San Pedro estuary. This population was found to be absent from aerial photograph of the area taken in the 1970s. Another monospecific stand of A.

germinans has also been observed in Virrila, Peru (5°50'S), which has also had observed increases since the 1970s (Ward et al. 2016).

Mangrove expansion has been observed continuously in southeastern Australia and New Zealand since the 1950s. In New Zealand since the 1940s, mangrove expansion has been observed across 29 locations, with an average increase in area of 165%. In southeastern Australia mangrove expansion has also been observed with 30% of salt marsh losses in the region being lost to mangrove habitat. R. stylosa and B. gymnorrhiza have been a large factor in this mangrove expansion. R. stylosa has expanded 100 km southward from the Corindi estuary to South West Rocks Creek (30°53'16" S) and has been recorded to be expanding within 16 estuaries in southeastern Australia. B. gymnorrhiza has observed to be expanding and colonizing new estuaries such as Sandon, Wooli Rivers, and Moonee Creek (Saintilan et al. 2014). Concurrent with poleward expansion is a lateral range expansion that has been observed at the expense of salt marsh habitat. In southeastern Australia landward expansion of mangroves has been observed over a 70 year period from 1943-2013. Most of this expansion has come at the expense of Sarcocornia-Sporobolus salt marsh as A. marina establishes in new areas (Ward et al. 2016).

Similarly, North American mangroves have expanded both poleward and landward. In the southeastern United States northern mangrove expansion is driven by a decrease in the number of winter freeze events with mangrove establishment observed along the coast of the Gulf of Mexico and the Atlantic coast of Florida. On the Atlantic coast of Florida, Williams et al. (2014) identified the most northern populations of A. germinans, R. mangle, and L. racemosa as 30°7'N, 29°56'N, and 29°44'N respectively. A. germinans was identified 27 km north of its' previous northern limit in 2007, R. mangle was 29 km north of its' previous northern limit in

2006, and L. racemosa was 67 km north of historic observations. The area that mangroves occupy in this region (north of 29.75°N) has increased by a total of 1,700 ha over 27 year from 1984 to 2011 (Cavanaugh et al. 2014). In the Gulf of Mexico, before the heavy frost event in 1983 R. mangle and L. racemose were documented as far north as Cedar Key, with large populations found south of Tarpon Springs; and A. germinans was documented as scattered individuals north of Cedar Key, with large populations found at Cedar Key and to its' south (Odum et al. 1982). The low temperature events in the 1980s caused 98% mangrove mortality at Cedar Key, but a study of aerial photography from 1995 to 1999 found that the extent of mangrove cover expanded in the area at rates of 300 m2 per year to over 844 m2 per year over the five year period. The study predicted that in 25-30 years mangroves would completely cover areas of Cedar Key (Stevens et al. 2006). In 2012, aerial photographs of Cedar Key were examined, and the areas of previous mangrove growth were completely covered 12 years before previously predicted (Saintilan et al. 2014).

Landward expansion of mangroves has occurred throughout southeastern United States. On the Atlantic coast of Florida at 28.3°N mangrove extent increased by 1,039 ha between 2003 and 2010, with a concurrent loss of 651 ha of salt marsh (Doughty et al. 2016). Also, on the Atlantic coast, Anastasia Island, FL (29°73'N), during a period from 1995-2013 mangrove extent increased by 23% increasing from 90.7 ha to 118.4 ha with a concurrent 103% decrease in salt marsh extent, from 295.94 ha to 145.68 ha (Rodriguez et al. 2016). On the Gulf coast of Florida mangrove encroachment has been observed more than 1km into Cladium-Eleocharis marshlands in the Everglades, as well as increases in mangrove extent in the Ten Thousand Islands National Wildlife Refuge of 35% since 1927 and on islands around Cedar Key (Saintilan et al. 2014). Texas has had similar increases at the expense of salt marshes with a 131% increase in mangrove

cover on Harbor Island over a 74 year period from 1930 to 2004 (Montagna et al. 2011) and a 74% increase or growth of 16.1 km2 in observed mangrove extent for the entire coast of Texas observed between 1990 and 2010, with a concurrent loss of salt marsh area of 77.8 km2 or a 24% net loss (Armitage et al. 2015). In Louisiana mangrove area has increased from 356 ha in 1978 to 670 ha in 2011 and is higher in 2014 than any time during the period of 1893-2014 all at the expense of previously salt marsh dominated habitat (Osland et al. 2017).

(ii) Differences in the Dispersal Biology of Mangroves vs. Saltmarsh Species

Across the globe salt marsh habitat has been lost at alarming rates with approximately 48% of the coastal wetland area lost since 1980 (Torio & Chmura 2015, Temmerman et al. 2012). The two main contributing factors to this loss are sea level rise, anthropogenic structure that impede sediment delivery to coastal systems (dams and reservoirs), and coastal development (agriculture, aquaculture, coastal defense structures, etc.). If sediment accretion rates in salt marshes are unable to keep up with sea level rise, inundation stress increases. The increase in inundation stress leads to vegetation die-off, shallow pool formation and bear tidal mudflat formation, in turn causing salt marshes to become fragmented and decreasing connectivity (Schepers et al. 2017, Torio & Chmura 2015, Temmerman et al. 2012, Kirwan & Megonigal 2013, Tian et al. 2017). Examples of saltmarsh fragmentation can be found along coasts around the world (Tian et al. 2017, Xu et al. 2016, Lee et al. 2006, Day et al. 2000, Saintilan 2009) and has been shown to have significant effects on fisheries (Roth et al. 2008, Hitch et al. 2011, Lowe & Peterson 2014) and wildlife (Major et al. 2014, Statham et al. 2016).

The consistent superiority of mangrove propagules over the seeds of salt marsh species gives mangroves a distinct advantage as these habitats become more fragmented and long-distance dispersal more important for re-colonizing areas of vegetation die-off, shallow pools,

and bear tidal flats along with colonizing newly opened areas. The dominant reproduction strategy for salt marsh macrophytes is vegetative propagation, but the most important strategy of reproduction for colonization or re-colonization is sexual reproduction and subsequent dispersal. Seeds from sexual reproduction for salt marsh species disperse through hydrochory, traveling by near coast currents with a viability of several hours to several months (Table 2). This hydrochorous dispersal has been shown to be both infrequent, occurring only during storms or high tides, and near ranging with few seeds escaping the parent marsh (Rand, 2000; Erfanzadeh et al., 2010). Instead of vegetative reproduction, mangroves reproduce sexual through vivipary or cryptovivipary, where the propagule germinates on the parent tree before the propagule drops and disperses via ocean currents.

Mangrove species, much like salt marsh species, have mostly localized dispersal, but mangroves have a greater capacity for long distance dispersal. In studies of mangrove propagule movement with varying species the dominant dispersal direction was observed to be landward, but with seaward movement of propagules being observed in large enough numbers as to make it significant (Peterson & Bell 2015, Van der Stocken et al. 2015, De Ryck et al. 2012). This seaward movement of mangrove propagules along with the long distance dispersal capacity of propagules gives mangroves an increased ability over salt marsh species for re-colonization and colonization (Table 2). The longest float time for a mangrove species was observed in Rhizophora mangle of 365 days in a study by Rabinowitz (1987) were at the end of the float period the propagules were found to be viable when planted. The longest dispersal by distance was observed for the species Ceriops tagal in a study by Van der Stocken et al. (2015) where an individual was observed to travel 320m. None of the studies on float time or dispersal distance had one hundred percent propagule recovery thus suggesting that a small number of propagules

moved outside of the recovery zone, which could mean that propagules could have moved beyond observed distances. This movement outside of known recovery zones is supported by the genetic evidence.

Genetic evidence for long distance dispersal in mangroves comes from various species that span different oceans around the world. In the Atlantic, the genetic structure of two different species Avicennia germinans and Rhizophora mangle have shown continual but rare dispersal across the Atlantic. For Avicennia germinans, a study by Nettel and Dodd (2007) and a study by Dodd et al. (2002) both calculated genetic distance values between African and South American populations that would suggest low but continual migration. Takayama et al. (2013) found strong evidential support in Rhizophora mangle for propagules dispersal across the Atlantic finding no genetic differentiation between populations in the Eastern and Western Atlantic. In the Indian Western Pacific region, genetic structure shows wide spread dispersal of propagules within three regions, with these regions being separated by ocean current and geographic barriers. These regions are slightly different between genus and even between species within the same genus, which these studies propose maybe due to differences in dispersal capability of the different species (Guo et al. 2018, Guo et al. 2017, Lo et al. 2014, Tomizawa et al. 2017, Wee et al. 2015). This genetic evidence supports the idea that different species of mangrove representing multiple genus have long distance dispersal capabilities far exceeding even salt marsh species, thus making mangroves better able to recolonize areas of previous coastal wetland growth and colonize new areas.

Ecosystem	Genus	Species	Floatation Time	Distance	Reference(s)
Saltmarsh	*Aster*				
		tripolium	7–15 days		Huiskes et al. (1995)
Saltmarsh	*Atriplex*				
		portulacoides	45 days		Koutstaal et al. (1987)
Saltmarsh	*Elymus*				
		athericus	10% floating after 30 days		Bockelmann et al. (2003)
Saltmarsh	*Salicornia*				
		europaea	1.5–2 h (50% residence time) (max. 24 h)		Koutstaal et al. (1987); Huiskes et al. (1995)
Saltmarsh	*Spartina*				
		alterniflora	25 days		Elsey-Quirk et al. (2009)
Saltmarsh	*Spergularia*				
		media	1–1.5 h (50% residence time) (max. 7 h)		Koutstaal et al. (1987)
Mangrove	*Acanthus*				
		ilicifolius	<11 days		Ye et al. (2004)
Mangrove	*Avicennia*				
		marina	25 days	10-50 km	Clarke (1993)
			>80% at 15 days		Clarke et al. (2001)
		bicolor/germinans	82 days		Rabinowitz (1987)
		germinans	110 days		
				19.1 m	Sousa et al. (2007)
Mangrove	*Bruguiera*				
		exaristata	>50% at 15 days		Clarke et al. (2001)
		gymnorrhiza	20 to 60% at 15 days		Clarke et al. (2001)
		parviflora	0 to 20% at 15 days		Clarke et al. (2001)
		sexangula	12–63 days (10% floating after 63 days)		Allen & Krauss (2006)
Mangrove	*Ceriops*				
		decandra	>50% at 15 days		Clarke et al. (2001)
		tagal	>80% at 15 days		Clarke et al. (2001)
				320 m, 146 m	Van der Stocken et al. (2015), Ryck et al. (2012)
Mangrove	*Heritiera*				
		littoralis	1 year (max.)		Davis (1940)
			104 days (max.)		Friess (2012)
			150 days (15 parts per thousand salinity)		Ye et al. (2004)
Mangrove	*Laguncularia*				
		racemosa	55 days		Rabinowitz (1987)
				85.3 m	Sousa et al. (2007)
Mangrove	*Lumnitzera*				
		racemosa	<20 days (15 parts per thousand salinity)		Ye et al. (2004)
Mangrove	*Rhizophora*				
		apiculate	15 days average (max. 89 days)		Drexler (2001)
		harrisonii	104 days		Rabinowitz (1987)
		mangle	365 days		Hogarth (1999)
				8 m	Sousa et al. (2007)
		mucronate	150 days		Drexler (2001)
				50 m, 60 m	Van der Stocken et al. (2015), De Ryck et al. (2012)
		stylosa	75 days (max.)		Friess (2012)
			>70% at 15 days		Clarke et al. (2001)
Mangrove	*Xylocarpus*				
		granatum	60 days (max.)		Friess (2012)
		mangle	>80% floating after 63 days (estimated up to 302 days)		Allen & Krauss (2006)
		mucronata	53 days average (max. 150 days)		Drexler (2001)

Table 2. Dispersal characteristics of select mangroves and salt marsh species

(iii) Conclusions

Human activities both past and present have significantly altered coastal ecosystems. These alterations have led to salt marsh loss and fragmentation causing many salt marshes to become patchy with large areas of mudflat and/or shallow open water. At the same time as humans have directly caused this habitat loss, mangrove populations are expanding their range across the globe because of minimum winter temperatures increases, freeze event decreases, and changes in precipitation regimes. Mangroves are uniquely adapted to take advantage of new areas open to colonization due to their ability to produce propagules through vivipary or cryptovivipary that disperse long distances. The loss and fragmentation of salt marsh along with the superiority of the mangrove dispersal capability suggests that salt marshes across the globe are ripe for mangrove encroachment and conversion. Future research is required to support that these factors work in concert to increase the conversion of salt marsh habitat to mangroves. It is imperative for policy makers to understand mangrove expansion at the expense of salt marsh habitat as the location and rate of conversion will have significant effects on ecosystem services and biodiversity.

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Chapter 2: ddRAD-seq based phylogeographic study of Avicennia germinans along the northern Gulf of Mexico

Abstract

Mangrove species across the world are expanding their range poleward as global climate change continues to impact temperature, precipitation, and sea level rise. The Gulf of Mexico is no exception with *Avicennia germinans* experiencing both expansion poleward (as found at Horn Island, MS) and landward (as found at Ten Thousand Islands region of Florida). *A. germinans* in the Gulf of Mexico faces the unusual situation of being both excellent at long distance dispersal and constrained in northward movement by available habitat. To better understand current *A. germinans* population dynamics and future population dynamics in the face of climate change, ddRAD-seq, a denovo SNP discovery and genotyping method, was utilized. For this method, leaf samples for DNA extraction were collected from 50 individuals across 14 populations in the Gulf of Mexico. Expected heterozygosity was utilized to quantify genetic diversity, STRUCTURE and NeighborNet were utilized to access population structure, and identity by state was utilized to determine kinship. Low genetic diversity was observed across all populations with an average of 0.13. In terms of inbreeding, only one population with a negative inbreeding coefficient was observed, and it was in Texas at Boca Chica. The STRUCTURE analysis for K=3 revealed a large proportion of individuals that had similar cluster distributions, which translated into most populations having similar proportions of the three clusters as well. Only two populations showed any real significant discrepancies from this pattern. NeighborNet showed three populations differentiating themselves from the central cluster e, but only one of these populations matched those that showed discrepancies in the STRUCTURE analysis. For

the identity state analysis, most individuals from all the populations had a zero IBS with only a select few individuals having IBS above zero, suggesting that individuals across all populations show similar or identical DNA segments at the loci. The genetic structure and kinship analyses provide a picture of highly related populations in the Gulf of Mexico with individuals that share a close genetic makeup. Given these relationships, conservationist looking to help mangrove environments effected by coastal development and experiencing habitat loss and degradation could replant with mangroves taken from almost any location in the northern GOM without significantly effecting the mangrove population genetics.

Introduction

During the twentieth century, species range shifts both poleward in latitude and upward in elevation have occurred across many geographic regions and in many different species (Walther et al. 2002). Range shifts have been observed poleward in arctic shrubs (Sturm et al. 2001), butterflies (Parmesan et al. 1999, Parmesan et al. 1996), and birds (Thomas and Lennon 1999), as well as upward in alpine plant species (Grabherr et al. 1994). Mangroves populations are no exception with range expansion poleward occurring across the globe, but in the Gulf of Mexico (GOM) mangroves face the unusual situation of being both excellent at long-distance dispersal and also constrained in any northward movement by available habitat. Additionally, they are sensitive to many of the impacts of climate change, such as changes in temperature and precipitation, enhanced CO2, and sea level rise (Saintilan et al. 2014).

Mangroves are a group of tropical woody trees and shrubs, which occupy 152,000 km2 of intertidal shore between the latitudes of 25°N and 30°S. They are not a monophyletic group and consist of 16 different families (110 species) that have evolved convergently to show adaptions

to tropical saline coastal ecosystems. They provide critical habitat for estuarine, nearshore, and terrestrial biota and supply crucial services such as shoreline establishment, protection from coastal erosion, coastal livelihood support, commercial fish nursery grounds, recreation, and atmospheric carbon sequestration (Takayama et al. 2013, Saintilan et al. 2014, Cavanaugh et al. 2014).

In the northern GOM, the salt marsh mangrove ecotone occurs as a dynamic stable state between salt marsh grasses and forbs and mangrove woody vegetation. The vegetation composition is controlled by climate and disturbance events. Recently this ecotone has been receiving more attention as mangrove species have expanded their range poleward and replaced salt marsh species in some areas at a rapid rate, as salt marshes have experienced large die-off events and landscape level habitat loss. This shift poleward of mangrove vegetation and loss of salt marsh habitat could have lasting effects on ecosystem services provided by coastal habitats such as coastal protection, erosion prevention, nutrient cycling, fisheries maintenance, and rates of carbon sequestration: all of these also influence local and regional economies (Armitage et al. 2015, Doughty et al. 2016, Saintilan et al. 2014), thus making better understanding mangrove population genetic diversity and structure in this highly dynamic system vital.

Within the last decade genetics technologies have improved to help better understand populations dynamics such as genetic diversity and genetic structure, which has enhanced the ability to make important conservation decisions. The genetic structure of mangroves can be attributed to propagule dispersal and the geographic and temperature barriers to mangrove movement and establishment, but the pattern of gene flow is still poorly understood (Takayama et al. 2013). For mangroves in the Atlantic Eastern Pacific (AEP), little is known about population genetics, but for the predominant species of mangrove in the Atlantic, Avicennia

germinans, a limited number of studies have probed at genetic diversity and phylogeny. In the Atlantic, high population genetic variation has been observed in studies of A. germinans, which has been attributed to limited propagule exchange between mangrove populations (Ceron-Souza et al. 2012, Dodd et al. 2002, Salas-Leiva et al. 2009).

In A. germinans, propagules are released from the parent tree once fully mature and are buoyant for a duration of approximately 110 days. Most propagules do not disperse far from the parent tree, but a small number of propagules do disperse long distances, carried by tides and coastal currents (Sousa et al. 2007). Long distance dispersal events, although few in number, occur often enough to maintain genetic similarity even over long distances such as the Atlantic Ocean. In a study by Nettel and Dodd in 2007, A. germinans populations across the AEP populations in South America and Africa were shown to have had enough genetic exchange as to maintain some genetic similarity between populations.

This study seeks to set a foundation for future genetics research on mangroves in the northern GOM to help coastal planners and conservationist make decisions about mangrove conservation in the face of future mangrove population expansion using one of the newest genetic sequencing techniques, ddRAD-seq. ddRAD-seq is a denovo single-nucleotide polymorphism (SNP) discovery and genotyping method that deploys restriction enzymes across the genomes of many individuals, cutting at certain predefined sequences to generate a large selection of fragments to interrogate further with statistical techniques.

Methods

Fourteen sites were sampled from across the GOM (Table 1). Sites in Florida, Louisiana, and Texas were sampled in 2015, except for the Chandeleur Island and Horn Island sites, which were sampled in 2014. At each site, three young leaves were collected from fifty trees of A. germinans except for the Horn Island site where only six individuals were sampled. Horn Island is a small newly establishing population that at the time of sampling had experienced a freeze event the prior winter that caused a die back event, leaving only a few individuals with viable growth for sampling. The distance between each tree sampled was at least 2 m to account for direct progeny. The leaves sampled were placed in desiccant and stored until the time of extraction. DNA was extracted from the leaf samples using a Qiagen Kit, specifically the DNeasy Mini Plant. A modified version of the protocol that comes with the Qiagen Kit was used. The modified protocol was developed using preliminary samples taken following the previously stated protocol and manipulating the amounts of buffer and the amount of time allowed for incubation to obtain the highest DNA concentration. DNA concentration was obtained using a Qubit and the standard Qubit protocol that comes with the instrument. For the full study, the extracted DNA from the modified Qiagen kit protocol was stored frozen until library construction.

Library construction was performed following the Double Digest RAD sequencing (DDRAD-seq) protocol of Peterson et al. (2012) using the enzymes Sbf1 and EcoR1. The constructed library was frozen and sent to the Georgia Genomic facility and sequenced on the Illuminia Hiseq at 500 cycles. Stacks v4.8 was used to process DDRAD-seq reads after data quality was checked using FastQC v0.11.8, this established that the best trim length would be 70bp. DDRAD-seq reads were cleaned, demultiplexed, and trimmed to 70bp using process

radtags in Stacks. Several iterations of the dataset were run using the Stacks pipeline of sstacks, cstacks, ustacks, and populations varying one parameter, while keeping all other parameters at the defaults of cstacks parameter m=3, cstacks parameter M=2, and ustacks parameter n=0 just as documented in the procedural paper by Paris et al. 2017. The m parameter was varied from 1 to 6, the M parameter was varied from 0 to 8, and n was varied from 0 to 10. The number of assembled loci, polymorphic loci, and the number of SNPs were observed for each run and accessed to determine the best set of parameters for a finally run through the entire protocol. From these outputs the best set of parameters was determined to be m=2, M=2, and n=0. The output from the final run of the Stacks protocol was exported for analysis along with a basic statistical output for the number of private alleles in per population (P), expected heterozygosity (He), observed heterozygosity (Ho), nucleotide diversity (Π), and inbreeding coefficient (FIS).

The population genetic structure was analyzed using two different methods: a model based method using STRUCTURE and a distance based method using Nei's Da. The model based method using Bayesian clustering analysis was conducted in STRUCTURE v2.3.4 to examine population structure with the number of clusters (K) being estimated using the methods from Falush et al. 2003, where K is estimated without an a priori population assignment of individuals under the admixture model for correlated allele frequencies with 100,000 Markov chain Monte Carlo steps and a burn-in of 10,000. Independent runs were performed 20 times for each value of K from 1 to 16. The meaningful value of K was then determined to be K=3 based on the ΔK method from Evanno et al. 2005. In STRUCTURE the expected heterozygosity and genetic diversity within each cluster was calculate for K=3 along with the Fst value of each cluster.

The distance based method for genetic structure used pairwise genetic distance that was calculated using the Nei's Da method in Populations v1.2.32 (Langella 1999). Based on this pairwise genetic distance, a phylogenetic network using the NeighborNet method (Bryant and Moulton 2004) was constructed using the R package phangorn v2.5.3 (Schliep 2017). To better understand the genetic structure analysis outputs, an identity by state (IBS) analysis was run using the R package SNPRelate (Zheng et al. 2012) to look at kinship.

Results

Even after DNA extraction with the modified protocol, the DNA concentration and quality was low, so many individuals would be expected to be removed during the Stacks pipeline. After sequences with low reads were removed during the first step of the Stacks pipeline, process_radtags, 183 individuals were removed, so only 393 individuals were included in the population map for the remaining steps in the pipeline. The genetic diversity parameters of individual populations are shown in Table 2. The mean statistics of expected heterozygosity and inbreeding coefficient for all populations were 0.13 and 0.01, respectively. Low genetic diversity was observed across all populations with the highest expected heterozygosity being observed for Grand Isle. The only population with a negative inbreeding coefficient was the population at Boca Chica. The population with the highest inbreeding coefficient was Port Fourchon.

In the STRUCTURE analysis, the log-likelihood of the data, LnP(K), increased to K=3 then leveled off before decreasing (Fig. 1a). The ΔK values showed a large peak at K=3 and two smaller peaks at K=13 and K=15 (Fig. 1b). STRUCTURE analysis for K=3 revealed a large proportion of individuals had similar distributions of the three clusters, which translated into

most populations having similar proportions of the three clusters as well (Fig. 2). The only population to show any real significant discrepancies from this pattern was the population at Emerson Point Preserve and Boca Chica.

The results of the NeighborNet analysis is shown in Figure 3. This analysis does not line up completely with the STRUCTURE analysis. In both analyses most populations are clustered together; but unlike STRUCTURE, NeighborNet shows SD, BC, and CS differentiated themselves from the central cluster unlike BC and EPP for STRUCTURE. The Neighbornet analysis also does not show clustering of populations by regions of the GOM with populations from TX, LA, and FL falling throughout the net.

The IBS analysis needs to have no missing values at any locus to run correctly so the data set had to be severely edited. The results of the editing were that only 20 loci had no missing data values. This is a very low number of loci for an IBS analysis but helps as a preliminary analysis to set the ground work for future research with large population sizes and a better DNA extraction and library construction protocols. In this analysis, most individuals from all the populations had a zero IBS with only a select few individuals having IBS above zero (Fig. 4).

Discussion

For all populations in the GOM, genetic diversity was low but was in line with some previous estimates of genetic diversity for populations in the western Atlantic. Dodd et al. (2002) and Nettel & Dodd (2007) found the average He for the Western Atlantic was 0.167 and 0.122 respectively. Each of these studies only had one site in the northern GOM, both of which were located in Florida having He of 0.137 and 0.09. These values fall in line with the average He for this study of 0.13. However, in a study by Ceron-Souza et al. (2015), an average of 0.507

for He was found for populations from the western Atlantic of which only 2 sites came from the northern GOM, a site in Texas with a He of 0.522 and a site in Florida with a He of 0.430. Another study by Hodel et al. (2016) looking at A. germinans on either side of the state of Florida found an average He of 0.456 for all sites. The two studies mentioned with low genetic diversity both used ALFP, the two studies with high genetic diversity values both used microsatellite markers, and the current study used DDRAD. This may account for the discrepancy in the values for similar regions of the western Atlantic; more research in the GOM needs to be performed to determine the true cause for these discrepancies.

At the leading edge of range expansion, populations are expected to experience increased population differentiation and a decrease in genetic diversity. This increase in differentiation and decrease in genetic diversity has a significant impact on leading edge population stability and the ability for future expansion (Excoffier et al. 2009). Range edge expansion has been observed in A. germinans along the Gulf Coast of the United States. The leading edge populations in this study were Dog Island, Florida and Horn Island, Mississippi, with both populations having been established within ten years of the date of sampling. The average genetic diversity for Dog Island was above average having the second highest He in the study. However, Horn Island had the second lowest He of all the populations following the expectations of a leading edge population.

Neither population followed the expectation of increased differentiation when looking at the STRUCTURE or NeighborNet analyses. In the STRUCTURE analysis, neither of this populations showed portions of the three clusters that highly varied from the majority of other populations. In the NeighborNet analysis, Horn Island showed some separation from populations in the center of the net, but no more than Cardinal Sound, Smallwood Drive, and

Port Isabel, whereas Dog Island fell out in the center of the net. Dog Island follows none of the expectations of a leading edge population, suggesting that, even though it is at the range edge of the species and is a newly established population, it is experiencing the same levels of gene flow as populations from the more central parts of the range. Horn Island though having low genetic diversity has similar genetic structure to the rest of the populations in the GOM. This suggests that the population on Dog Island is likely to thrive and expand in the coming years, whereas the Horn Island population may support limited diversity for expansion.

For all populations in the GOM, the STRUCTURE analysis showed that most populations had similar proportions of the 3 clusters, with only Emerson Point Preserve and Boca Chica having differing portions. Across all populations many of the individuals also had similar portions of the 3 clusters, which was supported by the IBS analysis. In the IBS a large proportion of individuals had an IBS of zero suggesting that these individuals are genetically identical individuals (Fig. 4). These results are in line with a previous study by Hodel et al. (2016), which found that for A. germinans in Florida that the cluster proportions from STRUCTURE were similar for most populations. Those populations that did not have proportions were mostly found in the Florida Keys, which was not included in the current study (Hodel et al. 2016).

The NeighborNet analysis revealed no distinct genetic differentiation by region with most populations clustering together, but with differentiation of Boca Chica similar to that from the STRUCTURE analysis. Unlike the STRUCTURE analysis, the NeighborNet analysis also showed population differentiation of Cardinal Sound and Smallwood Drive from the other populations with the strongest differentiation found in the Smallwood Drive population. All three of the previously mentioned populations are at the tip of the range sampled in this study,

suggesting that the northern most populations are experiencing different genetic influences from populations closer to center of the species range. This is not entirely unexpected as the populations included in this study are at the end of the species range with the center of the range being in central America and therefore may be behaving as would be expected from leading edge populations with high differentiation from those in the central range. To confirm this, a study would need to consider not just the populations in the current study but also populations from the center of the species range.

The shift poleward of mangrove vegetation at the expense of salt marsh habitat could have lasting effects on ecosystem services provided by coastal habitats such as coastal protection, erosion prevention, nutrient cycling, fisheries maintenance, and rates of carbon sequestration: all of which also influence local and regional economies (Armitage et al. 2015, Doughty et al. 2016, Saintilan et al. 2014). Such leading edge population expansion has already been observed in the northern GOM at Cedar Key in Florida, where a study of aerial photography from 1995 to 1999 found that the extent of mangrove cover expanded at rates of 300 m2 per year to over 844 m2 per year (Stevens et al. 2006). Further in 2012, aerial photographs of Cedar Key showed that areas of previous mangrove growth were completely covered 12 years before previously predicted (Saintilan et al. 2014). This study supports the idea that leading edge populations in Mississippi and Florida could experience similar stable expansion as observed at Cedar Key as leading edge populations included in the current study showed similar genetic structure and diversity to those of the long established populations across the northern GOM.

As far as the rest of the populations in the northern GOM, the genetic structure and kinship analyses provide a picture of highly related populations with individuals with a close genetic makeup. Given these relationships, conservationist looking to help mangrove environments effected by coastal development experiencing habitat loss and degradation could be able to replant with mangroves taken from almost any location in the northern GOM without significantly effecting the mangrove population genetics. Globally, between a one third and one half of vegetated intertidal habitats have been estimated to have been lost due to anthropogenic activities such as land reclamation, coastal development, and agriculture/aquaculture. Many countries have lost more than 50% of their intertidal habitat. As populations along the coast increase, intertidal habitat is predicted to see further losses (Perkins et al. 2015). The GOM is no exception with some of the most dramatic wetland losses in the US occurring in this region. The GOM was observed to have net loss of 257,150 acres between 2004 and 2009. Losses in the GOM have been attributed to sea level rise, land subsidence, and hurricane frequency and intensity, which are further exacerbated by human activities such as conversion of wetlands to other land uses, alteration to the hydrologic regime, canal and channel dredging, and fluid extraction associated with maritime commerce and energy production (Watson et al. 2015).

This preliminary study looked to use the new genetic technique of ddRAD-seq to access the population genetics in the northern GOM. The ddRAD-seq techniques requires a higher DNA concentration than previous techniques along with higher quality. These numbers were hard to achieve using the DNeasy Mini Plant Kit even with modifications to the protocol. This led to many samples not meeting these standards going into the library construction phase of the study, which in turn led to a number of individuals not making it through process_radtags in the Stacks pipeline. To better access population genetics in *A. germinans* in the future, different

DNA extraction techniques should be tested to achieve both higher DNA concentrations and quality along with testing other sets of enzymes for library construction to achieve the best cuts for better genotyping and SNP recovery.

Even with these limitations, the results of this study can help to suggest future directions for mangrove conservation and research on mangrove population genetics and the mangrove salt marsh ecotone shift in the GOM. Understanding the dynamics of these highly variable genetic relationships and the processes of genetic exchange in mangrove populations will be essential to predicting future impacts of climate change and anthropogenic forcing.

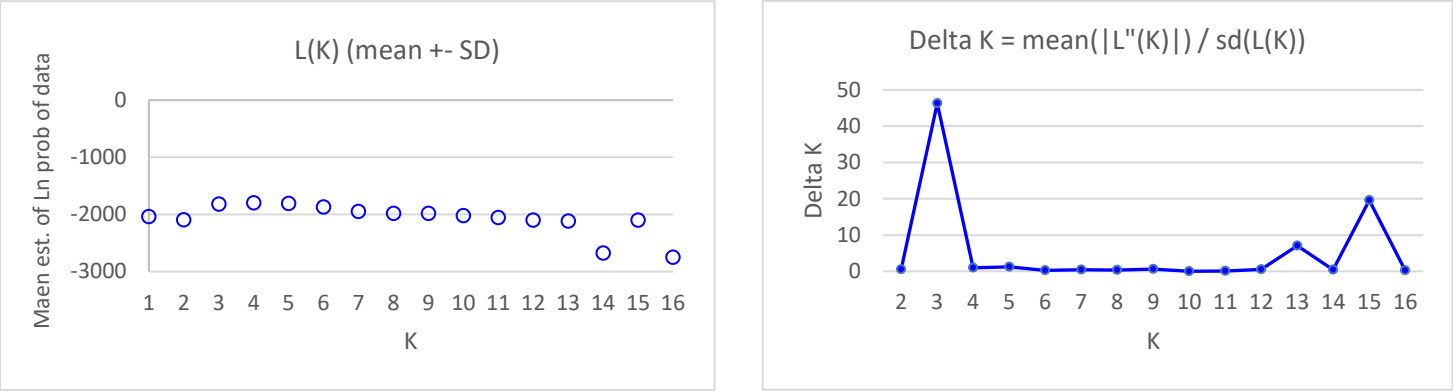

Figure 1. Plot of mean posterior probability (LnP(D)) values per clusters (K), based on 20 iterations per K, from STRUCTURE analyses (a), and delta K analysis of LnP(D) to estimate the genetic structure of the 14 populations of *Avicennia germinans* (b).

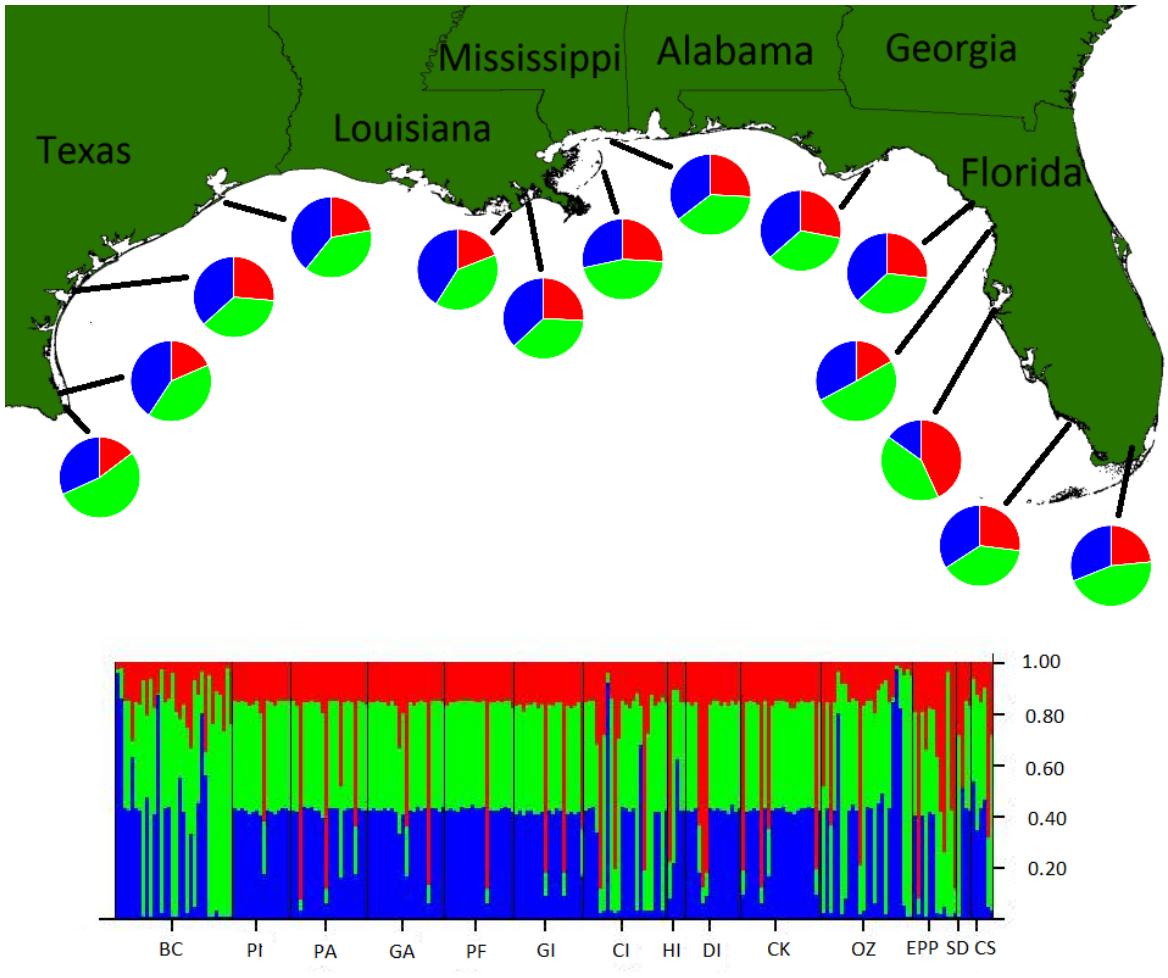

Figure 2. Map of the proportion of each of the 14 *A. geminans* populations assigned to three clusters identified in the program STRUCTURE. The pie charts showing the frequency of each cluster in the population (a). The bar plot showing the frequency of each cluster in the individuals for each population (b).

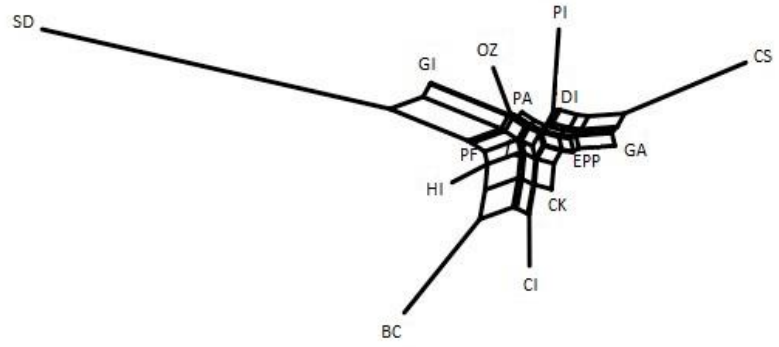

Figure 3. The NeighborNet tree of 14 *A. germinans* populations using Nei's Da method.

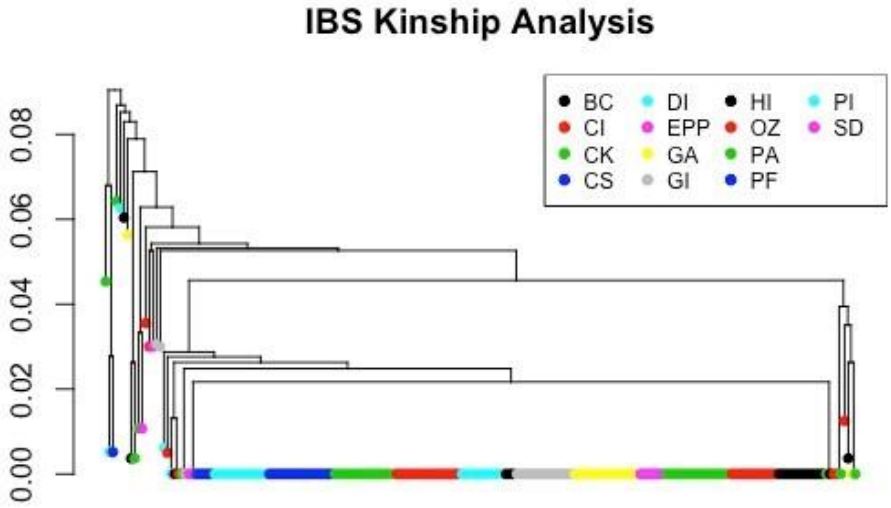

Figure 4. The Identity by state analysis of 14 *A. germinans* populations with a legend identifying the color assignment of each of the 14 populations

Pop ID	Population Name	County/State	Lat/Long
CS	Card Sound Road	Miami-Dade/FL	25°19'33.7"N 80°24'12.4"W
SD	Smallwood Drive	Collier/FL	25°49'4.0"N 81°21'51.2"W
EPP	Emerson Point Preserve	Manatee/FL	27°31'56.8"N 82°37'46.3"W
OZ	Ozello	Citrus/FL	28°52'04.1"N 82°39'55.6"W
CK	Cedar Key	Levy/FL	29°08'09.4"N 83°01'48.0"W
DI	Dog Island	Franklin/FL	29°48'48.1"N 84°35'05.3"W
HI	Horn Island	Jackson/MS	30°14'22.1"N 88°40'17.7"W
CI	Chandeluer Islands	St. Bernard/LA	29°55'08.5"N 88°49'21.0"W
GI	Grand Isle	Lafourche/LA	29°15'51.9"N 89°57'10.6"W
PF	Port Fourchon	Lafourche/LA	29°06'21.3"N 90°11'15.2"W
GA	Galveston	Galveston/TX	29°20'00.1"N 94°44'39.8"W
PA	Port Aransas	Aransas/TX	27°52'04.7"N 97°05'15.8"W
PI	Port Isable	Cameron/TX	26°00'59.8"N 97°16'25.6"W
BC	Boca Chica	Cameron/TX	25°59'53.9"N 97°09'31.8"W

Table 1. Sampling locations, their codes used in figures, and GPS coordinates

Pop ID	N	A_p	H_o	H_e	Π	F_{is}
CS	7	81	0.16	0.09	0.16	0.003
SD	5	67	0.11	0.06	0.11	0.003
EPP	19	135	0.15	0.09	0.15	0.007
OZ	42	563	0.22	0.14	0.23	0.015
CK	40	392	0.20	0.12	0.21	0.011
DI	42	639	0.29	0.17	0.30	0.011
HI	6	74	0.14	0.07	0.14	0.004
CI	29	492	0.23	0.14	0.23	0.006
GI	42	1001	0.32	0.19	0.33	0.015
PF	37	551	0.21	0.14	0.23	0.027
GA	37	328	0.19	0.12	0.19	0.012
PA	38	822	0.26	0.16	0.27	0.020
PI	32	606	0.26	0.16	0.27	0.017
BC	37	799	0.28	0.16	0.27	-0.012
MEAN	30	468	0.22	0.13	0.22	0.010

Table 2. Number of individuals included (N), number of private alleles (A_p), observed Heterozygosity (H_o), expected Heterozygosity (H_e), mean value of nucleotide diversity (Π), and inbreeding coefficient (F_{is}) with yellow representing the highest values and blue representing the lowest values

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Chapter 3: The application of the ddRAD-seq technique for *Avicennia germinans*

Abstract

Phylogeography is a field that uses elements of taxonomy, geography, and population genetics to answer questions about population dynamics, population genetic traits, and distributional history. Recently the field of phylogeography has been undergoing rapid expansion as RAD-seq has become a widely adopted method for genotyping and SNP discovery. Herein, the methods for a phylogeographic study of *Avicennia germinans* using a newly developed RAD-seq method known as DDRAD-seq are detailed. DDRAD-seq improves upon the previous RAD-seq technique by replacing the fragment shearing step with a second restriction digest and including a second index. The DNA quality from DNA extraction proved to be low, even after improvements to the extraction protocol. This led to downstream complications with many samples not meeting standards for library construction. In this study ddRAD-seq provided a preliminary snapshot of *A. germinans* population history in the GOM. This snapshot suggests that most populations of *A. germinans* in the GOM have similar genetic structure and highly related individuals. Understanding these relationships can help determine mangrove regulations and develop future conservation efforts. In order to better understand these relationships, future studies should seek to improved DNA extraction techniques to provide datasets on which in-depth relatedness analyses can be performed to improve understanding of the genetic structure within and between populations.

Introduction

Phylogeography is a field that was coined by Avise et al. (1987) as the connection between population genetics and systematics. Currently, studies use elements of taxonomy, geography, and population genetics to answer questions about population dynamics, local population genetic traits, and distributional history. Phylogeography is currently undergoing rapid expansion from advanced sequencing technologies (Kobayashi et al. 2018). Prior to 2006, studies used one of two techniques developed in the 1990s: either microsatellite markers or amplified fragment length polymorphisms (AFLPs) (Bellows et al. 1999). The term microsatellite was defined by Litt and Luty in 1989, but microsatellites were not widely used until the invention of PCR in the 1990s (Bellow et al. 1999). The microsatellite marker technique assesses polymorphism via markers that are both highly polymorphic and codominant (Evanno et al. 2005). AFLPs generate hundreds of polymorphic bands that are used to assess population genetic relationships (Evanno et al. 2005). Problems exist with both methods. Microsatellites are expensive to develop, time consuming to use, and difficult to work with. In contrast, AFLPs are easier to develop but are frequently dominant, which means that a DNA band is either present or absent. The inconsistency of the method used to describe population structure has led to confusion, as the differences between the properties of the two markers leads to different levels of population structuring (Evanno et al. 2005).

In 2006, Miller et al. published a new genetic sequencing technique to combat these and other issues. The new high-throughput technique was developed based on restriction site-associated DNA sequencing (RADseq). The advantage of RADseq over microsatellite markers and ALFPs is that marker discovery and genotyping is relatively low-cost and can be done for thousands of genetic markers at once (Kobayashi et al. 2018). The following is a detailed

description of the methods for a phylogeographic study on *Avicennia germinans* (black mangrove) using a newly developed RADseq method known as double digest RADseq (DDRAD-seq) (Peterson et al. 2012).

Methods

- Sampling Scheme

Samples were collected from sites across the Northern Gulf of Mexico (GOM) in the United States. The study area for *Avicennia germinans* was determined by using the US Fish and Wildlife Service National Wetlands Inventory (NWI) and online herbarium databases (the University of Florida Herbarium online database, the Shirley C. Tucker Herbarium at Louisiana State University online database, and the Billie L. Turner Plant Resources Center at the University of Texas at Austin). The northern most population of *A. germinans* in Florida was Cedar Key, FL with a few individuals being found in St. Joseph Bay. To confirm that St. Joseph Bay did not have enough individuals to constitute a population, the National Estuarine Research Reserve Nature Center in Eastpoint, FL was consulted. The National Estuarine Research Reserve provided knowledge of a population of *A. germinans* at Dog Island that at the time of the study was undocumented. The Mississippi mangrove population at Horn Island (HI) is a preserve, so Kenneth Heck at the Dauphin Island Sea Lab helped obtain samples. In total the number of sites was 14 with six in FL, one in MS, three in LA, and four in TX to cover the widest range of sites across the northern GOM (Table 1).

Mangrove species, including *A. germinans*, produce propagules that typically drop directly under the parent tree and do not get exported from the area; to account for this when sampling, a distance of five meters was used between individuals to avoid sampling both parent and offspring (Ceron-Souza et al. 2012). An exception was made for HI, as it was a newly established population with very few individuals and as newly established populations are less

likely to have individuals of a mature age and size that are from propagules dropping directly under the parent. The distance allowed for HI was reduced to two meters between individuals. This decrease in sampling distance did not have any apparent on results effect as HI did not have the lowest genetic diversity or the highest level of inbreeding and had similar values as populations in South Florida (Table 4).

Three leaves were obtained from each individual for possible replication. The leaves were stored in tea bags and placed in silica gel beads to quickly dry the samples. Multiple techniques for leaf transport were test, but the selected technique consistently gave the highest DNA quantity results. DNA quantity was tested using the Thermo Fisher Scientific Qubit 2.0, which performs a nucleic acid quantification assay by using a dye that when bound to DNA fluoresces.

The number of individuals samples from each was 50 based on the lack of a reference genome and the lack of studies on genetic diversity in populations of *A. germinans* in the northern GOM (Kess et al. 2018). In systems with no reference genome, sequencing errors may cause difficulties in genotyping. This may, in turn, can cause erroneous base pair calls and problems with heterozygosity, making highly heterozygous populations harder to distinguish from true heterozygous alleles (Xu et al. 2014). To account for these possible errors and the demographic history, intrinsic life-history traits, and overall population characteristics of *A. germinans*, the sampling scheme included more individuals than the minimum required for a population genomic study using next generation technology, which is 2-5 individuals (Nazareno et al. 2017).

- DNA Extraction

The Qiagen DNeasy Mini Plant Kit was used to extract DNA from leaf samples. The standard protocol for the kit was modified to obtain the highest DNA quantity and quality. The kit protocol is a multi-step process that uses manual grinding and heat to break down the cellular

tissue to allow the DNA from the nucleus to be brought into solution. The DNA is then isolated from other cellular material and cleaned. The last step in the process is to move the clean DNA into a holding tube for storage. After initial tests of the DNA extraction protocol, problems arose that caused low DNA quality and quantity output. When the amount of buffer specified in the kit manual was added to the sample, the buffer was absorbed by the leaf tissue. Upon consultation with the manufacturer, the amount of the initial buffer was increased from 400 µL to 1,000 µL along with a five-minute increase of the final incubation time. The extracted DNA was stored frozen until library construction ("DNeasy Plant Handbook" 2015).

- Library Construction

Library construction for DNA sequencing was performed using the DDRAD-seq protocol from Peterson et al. (2012). DDRAD-seq improves upon the previous RAD-seq technique by replacing the fragment shearing step with a second restriction digest and including a second index. This allows for improved size selection accuracy and combinatorial indexing ("Illumina" n.d.). In the current study, the enzymes used for the restriction digests were Sbf1 and EcoR1. Samples were also multiplexed, which is when barcode sequences are added to each DNA fragment so that reads can be identified and sorted during data analysis. Multiplexing also allows for multiple populations to be included on a single plate, which helps account for plate errors in sequencing ("Illumina" n.d.). A total of 14 plates were made during the DDRAD-seq library construction protocol. DNA sequencing was performed at the Georgia Genomics facility using the Illumina Hiseq at 500 cycles. The output from the Illumina Hiseq came back as 30 files containing raw sequence reads, two for each of the 14 plates plus undetermined sequences. The two files for a single plate were concatenated together for a total of 15 files.

- STACKS

The programs FastQC v0.11.8 and STACKS v4.8 were used for quality control and to process the raw Illumina Hiseq reads for downstream statistical analyses following the quality control protocol from Paris et al. (2017). FastQC is a tool used to get a quick overview of the quality of the raw Illumina Hiseq reads. It outputs a modular set of analyses that includes per base sequence quality and per sequence quality scores ("Babraham Bioinformatics" n.d.). FastQC was run for each plate raw output from the Illumina Hiseq, so in total 15 FastQC outputs were generated. The FastQC analyses determined 70bp to be the most appropriate trim length based on the drop in quality scores for base pairs after 70bp. The 70bp trim length was used in the cleaning step of the STACKS pipeline. During the STACKS pipeline, the Illumina Hiseq raw reads are cleaned, demultiplexed, and trimmed by process_radtags. Following process_radtags, ustacks creates matching stacks of short-read sequences by taking the outputs from process_radtags and aligning them. The maximum likelihood framework by Hohenlohe et al. (2010) is used to compare the matching stacks, create a set of putative loci, and to distinguish single nucleotide polymorphisms (SNPs). After ustacks, cstacks then makes a catalog of the short-read sequences from the ustacks output (Catchen et al. 2013).

Two variables are required to run ustacks: M, the maximum distance between stacks, and m, the minimum coverage depth to make a stack. In cstacks, the only required variable is n—the number of mismatches allowed between different sample loci. The values for the M, m, and n were determined by independently running the STACKs pipeline from ustacks to populations. For each run, one of the three variables was increased by 1, while the other variables remained constant at M=2, m=3, and n=0. The variable M ranged from 0 to 8, m ranged from 1 to 6, and n ranged from 0 to 10. For each run, the number of assembled loci, polymorphic loci and SNPs were compared to determine the variables that gave the highest quality results (Paris et al. 2017).

After all comparisons, the values that gave the highest quality results for M, m, and n were M=2, m=2, and n=0. The final step in the STACKs pipeline is populations; this step is used to analyze populations, compute statistics, and create files for downstream analysis. For the current study, files were exported for STRUCTURE v2.3.4, Populations v1-32.2, and R along with the number of private alleles in per population (P), expected heterozygosity (He), observed heterozygosity (Ho), nucleotide diversity (Π), and inbreeding coefficient (FIS) (Catchen et al. 2013).

- Two Different Genetic Structure Protocols

Two different protocols were used to visualize population structure and provide support; a model-based protocol and a distance-based protocol. Population structure analyses organize genetic variation into a visual format to observe evolutionary processes such as genetic drift, recombination, natural selection, and mutation (Freeland et al. 2011). *A. germinans* in the northern GOM is at the leading edge of range expansion and thus expected to experience evolutionary processes that would lead to increased population differentiation and a decrease in genetic diversity (Excoffier et al. 2009).

The model-based protocol utilizes the statistical software package STRUCTURE. STRUCTURE sorts individuals into clusters based on their genotypes at multiple loci using a Bayesian model known as the "admixture model" (Novembre 2016). The admixture model attempts to account for the presence of Hardy-Weinberg equilibrium and linkage disequilibrium by using population structure. STRUCTURE uses a parameter called K, which determines the number of clusters or the number of source populations (Novembre 2016). To obtain the best value of K, the ΔK method of Evanno et al. (2005) was followed. First, 20 runs were performed for each value of K, from one through two above the number of populations included in the study (16 for the current study). These runs plot the mean likelihood for each, referred to as L(K). Next, the average difference between consecutive L(K) values is calculated: L'(K)=L(K)-L(K-1).

Consecutive values of L'(K) are subtracted from each other and finally the absolute value is taken to calculate $\left|L''(K)\right|$, $\left|L''(K)\right| = \left|L'(K+1)-L'(K)\right|$. Finally, to get the true estimate for ΔK, the average of $\left|L''(K)\right|$ over the 20 runs is divided by the standard deviation of L(K), the full formula is $\Delta K = m(\left|L(K+1)-2L(K)+L(K-1)\right|)/s[L(K)]$ (Evanno et al. 2005). The calculation of ΔK was performed using Microsoft Excel 360. After the calculation, ΔK is plotted vs K to determine the value of K that best fits the data, which is the value with the largest peak. Using this method, the best fit value of K was K=3 for the current study (Fig. 1). A final STRUCTURE analysis was run using K=3 (Fig. 2).

The distance-based protocol uses pairwise genetic distance to construct a phylogenetic network. The first step in this method is to calculate pairwise genetic distance using the Nei's D_A method described below. This was performed using the population genetic software Populations v1.2.32. The Nei's D_A method was developed from the Cavalli-Sforza chord distance method. The maximum value for Nei's D_A is one and occurs when two populations have no alleles in common at any locus. The formula for the calculation of Nei's D_A is as follows:

$$D_A = 1 - \sum_{i=1}^{m}\sum_{f=1}^{r}\frac{\sqrt{x_{ij}y_{ij}}}{r}.$$

(Kalinowski 2002)

The Nei's D_A pairwise genetic distance output was then used to create the phylogenetic network using the Neighbor-Net function in the R package phangorn v2.5.3. The Neighbor-Net method is based on the neighbor joining algorithm. According to work by Bryant and Moulton (2004), a phylogenetic network should be used in place of a simple branching phylogenetic tree in studies where the study species does not have an evolutionary history in a tree form or where the species experiences parallel evolution, model heterogeneity, and/or the study contained

sampling error. The study species that do not have a simple branching phylogenetic tree form can occur due to any of the following: recombination, hybridization, gene conversion, and gene transfer (Bryant and Moulton 2004). Mangrove population evolutionary history is not fully understood, but the underlying ecology of mangroves suggests that they meet some of the above criteria. Thus, the Neighbor-Net method was chosen over a traditional branching phylogenetic tree.

- Identity by State (IBS)

To give secondary support to the similarity between populations and individuals observed in the genetic structure analyses, an identity by state (IBS) analysis was performed using the R package SNPRelate (Zheng et al. 2012). IBS is used to create a single variable that measures the similarity between segments of DNA or alleles. Identical DNA segments or alleles are given a value of zero in this analysis and can occur either through close familial relationship or mutation (Stevens et al. 2011). To calculate and create a plot of IBS values, the SNPRelate package tutorial provided by Zheng (2018) was used. This protocol uses the function snpgdsIBS() to calculate IBS as a matrix. Afterwards, the function snpgdsHCluster() runs a hierarchical cluster analysis on the matrix. To create a dendrogram of the results by population the function snpgdsCutTree() was used. The output after snpgdsCutTree() was made into a visual plot using the plot() command in R.

Results

- FastQC and STACKS

FastQC showed poor data quality for the raw Illumina reads. In FastQC every modular analysis is listed with a check mark, exclamation point, or a “x”, which indicates whether the data is in an acceptable range, questionable range, or poor-quality range. All of the files showed some modular analyses in the poor-quality range with mean sequence quality (Phred Score)

ranging from 32-33 and total sequences ranging from 12,102,672- 31,173,431 (Table 2). For process_radtags in STACKS the total number of reads was 532,665,610 with only 40,068,765 reads being retained after cleaning (Table 3). After the removal of problematic reads, 183 individuals were removed leaving 393 individuals in the population map used for downstream processing. This loss in individuals from the removal of reads is in line with other studies using ddRAD-seq by Kobayashi et al. (2018) and Kess et al. (2018). For the final run of the STACKS pipeline, the mean expected heterozygosity and inbreeding coefficient were 0.13 and 0.01, respectively. The standard error for expected heterozygosity and inbreeding coefficient were below 0.05 with the average standard error being 0.003 and 0.028 respectively. All populations exhibited low genetic diversity with the highest expected heterozygosity being observed at GI. The only population with a negative inbreeding coefficient, suggesting that individuals within the population are less related than expected under random mating, was BC. The population with the highest inbreeding coefficient was PF (Table 4).

- Genetic Structure

The STRUCTURE analysis for K=3 revealed that the three clusters appear in similar proportions among individuals and populations (Fig. 2). The only populations to show any discrepancies from this pattern were the populations at EPP and at BC. The results of the Neighbor-Net differed from the STRUCTURE analysis. In both analyses most populations were clustered together; however, unlike STRUCTURE, Neighbor-Net showed that SD, BC, and CS differentiated themselves from the central cluster (Fig. 3). The only population that differentiated itself in both analyses was BC, which is supported by the negative inbreeding coefficient. This suggests that BC could be experiencing a number of various genetic processes that are not experienced in the other populations or are experienced in smaller amounts. These processes could include but are not limited to less genetic exchange with the other northern

GOM populations, a different source population, and is experiencing genetic exchange with different populations outside of the northern GOM.

- IBS

To run an IBS analysis no missing values can exist at any locus; this led to a number of loci being edited out of the data set. After loci with missing data values were removed, only 20 loci were left. For a typical IBS analysis, this number of loci is low, but it provides a preliminary assessment of *A. germinans* for future research on genetic structure. The results of the IBS analysis found that across all study sites, a large proportion of individuals had an IBS of zero with only a select few individuals having IBS above zero (Fig. 4). This means that individuals across all populations show similar or identical DNA segments at the 20 loci, suggesting that all populations in the northern GOM have a close familial relationship from either constant genetic exchange between the populations or that populations may have the same source population.

Discussion

This preliminary study used the new genetic technique of ddRAD-seq to assess the population genetics in the northern GOM. The ddRAD-seq requires higher DNA quality than other RADseq techniques (”Illumina” n.d.). For the current study, achieving high DNA quality presented challenges. The modifications to DNeasy Mini Plant Kit achieved results within a range that was, by field standards, deemed high enough in quality and quantity to give downstream results. The DNA quality, however, led to downstream complications with many samples not meeting standards for library construction. In the end, a number of individuals were removed during the process_radtags step in the Stacks pipeline due to ambiguous barcodes, low quality reads, and/or ambiguous rad-tags. Future studies should explore different DNA extraction techniques to achieve both higher DNA concentrations and quality. Additionally, experimentation

with other sets of enzymes for library construction may help to achieve better genotyping and SNP recovery results.

In this study ddRAD-seq provided a preliminary snapshot of mangrove population history in the GOM. This snapshot suggests that most populations of *A. germinans* in the GOM have similar genetic structure and highly related individuals. Understanding these relationships can help determine mangrove regulations and develop future conservation efforts. In order to better understand these relationships, future studies should seek to improved DNA extraction techniques to provide datasets on which in-depth relatedness analyses can be performed to improve understanding of the genetic structure within and between populations.

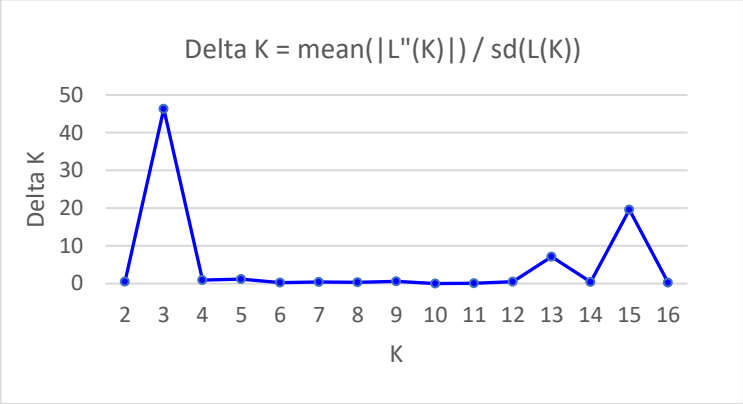

Figure 1. Plot of the delta K analysis to estimate the

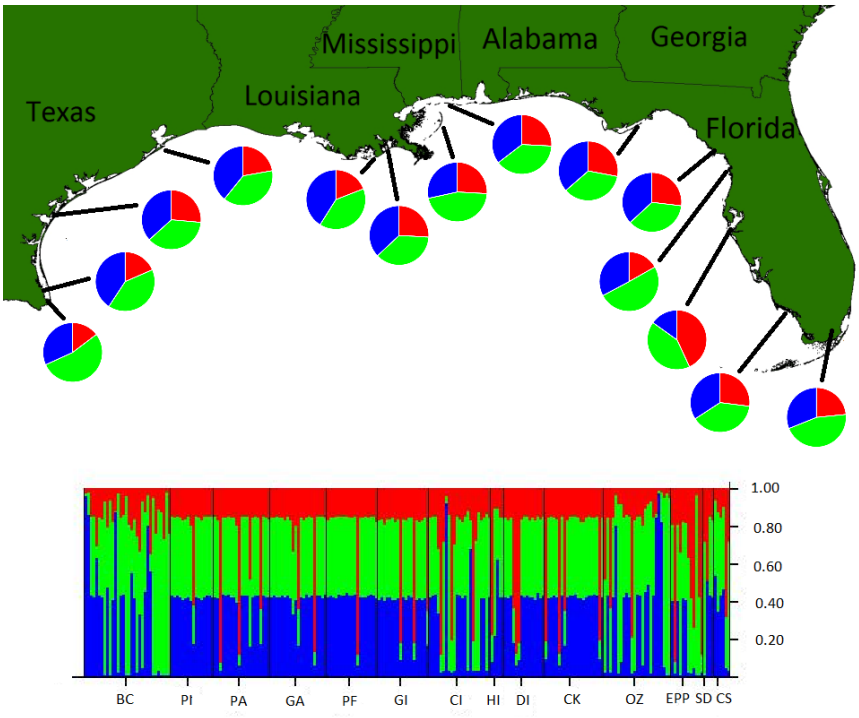

Figure 2. Map of the proportion of each of the 14 *A. geminans* populations assigned to three clusters identified in the program STRUCTURE. The pie charts showing the frequency of each cluster in the population (a). The bar plot showing the frequency of each cluster in the individuals for each population (b).

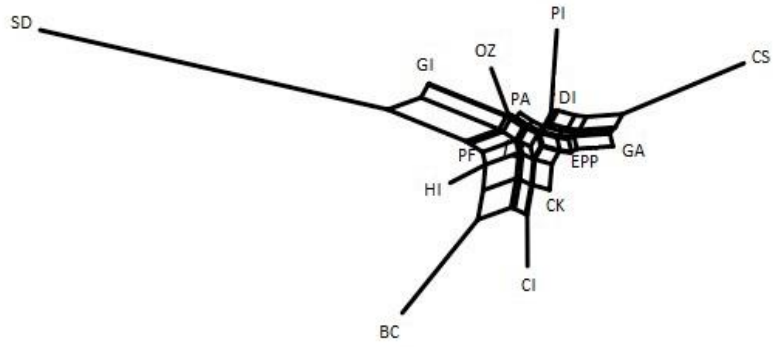

Figure 3. The NeighborNet tree of 14 *A. germinans* populations using

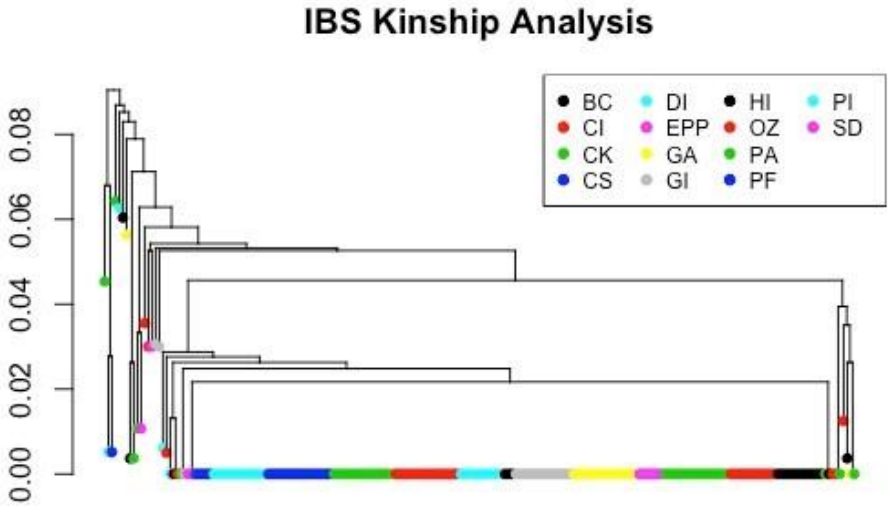

Figure 4. The Identity by state analysis of 14 *A. germinans* populations with a legend identifying the color assignment of each of the 14 populations

Pop ID	Population Name	County/State	Lat/Long
CS	Card Sound Road	Miami-Dade/FL	25º19'33.7"N 80º24'12.4"W
SD	Smallwood Drive	Collier/FL	25º49'4.0"N 81º21'51.2"W
EPP	Emerson Point Preserve	Manatee/FL	27º31'56.8"N 82º37'46.3"W
OZ	Ozello	Citrus/FL	28º52'04.1"N 82º39'55.6"W
CK	Cedar Key	Levy/FL	29º08'09.4"N 83º01'48.0"W
DI	Dog Island	Franklin/FL	29º48'48.1"N 84º35'05.3"W
HI	Horn Island	Jackson/MS	30º14'22.1"N 88º40'17.7"W
CI	Chandeluer Islands	St. Bernard/LA	29º55'08.5"N 88º49'21.0"W
GI	Grand Isle	Lafourche/LA	29º15'51.9"N 89º57'10.6"W
PF	Port Fourchon	Lafourche/LA	29º06'21.3"N 90º11'15.2"W
GA	Galveston	Galveston/TX	29º20'00.1"N 94º44'39.8"W
PA	Port Aransas	Aransas/TX	27º52'04.7"N 97º05'15.8"W
PI	Port Isable	Cameron/TX	26º00'59.8"N 97º16'25.6"W
BC	Boca Chica	Cameron/TX	25º59'53.9"N 97º09'31.8"W

Table 1. Sampling locations, their codes used in figures, and GPS

Plate #	Total Sequences	Sequences flagged as poor quality	Sequence Length	Q	Retained Reads	Low Quality	Ambiguous Barcodes	Ambiguous RAD-Tag	Total
Plate 1	14515960	0	35-76	33	2060467	2077293	20815784	4078376	29031920
Plate 2	16086683	0	35-76	33	2262725	2588906	22466762	4854973	32173366
Plate 3	12102672	0	35-76	32	1757254	1908402	16473108	4066580	24205344
Plate 4	14805827	0	35-76	33	1856926	1980781	22002956	3770991	29611654
Plate 5	17234921	0	35-76	33	3117075	2855321	22748934	5748512	34469842
Plate 6	17780492	0	35-76	33	3141472	2759546	23884806	5775160	35560984
Plate 7	13732915	0	35-76	33	2506072	1820919	18548796	4590043	27465830
Plate 8	15020903	0	35-76	33	2823175	2401395	19407090	5410146	30041806
Plate 9	14561094	0	35-76	33	3424672	2184868	15853506	7659142	29122188
Plate 10	15468991	0	35-76	33	1592156	1690365	24321824	3333637	30937982
Plate 11	14762736	0	35-76	32	1807688	1927203	21597676	4192905	29525472
Plate 12	23577990	0	35-76	33	4389117	3191619	31451584	8123660	47155980
Plate 13	21858307	0	35-76	33	3948364	2924772	30166896	6676582	43716614
Plate 14	31173431	0	35-76	33	5314947	3046581	45270454	8714880	62346862
Undetermined	23649883	0	35-76	32	66655	66567	47006058	160486	47299766

Table 2. Quality information for the raw Illumina Hiseq reads from FastQC and process_radtags.

Total Sequences	532665610
Ambiguous Barcodes	382016234
Low Quality	33424538
Ambiguous RAD-Tag	77156073
Retained Reads	40068765

Table 3. Compiled values from the STACKS process_radtags log.

	Ap	N	H_o	Var	StdErr	HO	Var	StdErr	H_e	Var	StdErr	HE	Var	StdErr	Fis	Var	StdErr
CS	81	1.4288	0.1562	0.1244	0.0077	0.8438	0.1244	0.0077	0.0867	0.0345	0.0041	0.9133	0.0345	0.0041	0.0027	40.199	0.0149
SD	67	1.1733	0.1126	0.0962	0.0066	0.8874	0.0962	0.0066	0.0604	0.0259	0.0034	0.9396	0.0259	0.0034	0.0032	42.958	0.0082
EPP	135	2.3001	0.148	0.1085	0.0057	0.852	0.1085	0.0057	0.0914	0.0332	0.0031	0.9086	0.0332	0.0031	0.0072	38.604	0.0304
OZ	563	2.9353	0.2196	0.141	0.0049	0.7804	0.141	0.0049	0.1371	0.0423	0.0027	0.8629	0.0423	0.0027	0.015	32.721	0.0381
CK	392	2.9283	0.2008	0.1346	0.0052	0.7992	0.1346	0.0052	0.1241	0.0399	0.0029	0.8759	0.0399	0.0029	0.011	34.037	0.0428
DI	639	1.8779	0.2907	0.1829	0.0057	0.7093	0.1829	0.0057	0.1656	0.0503	0.003	0.8344	0.0503	0.003	0.011	31.215	0.0198
HI	74	1.207	0.1375	0.1134	0.0071	0.8625	0.1134	0.0071	0.0746	0.0308	0.0037	0.9254	0.0308	0.0037	0.0042	41.507	0.0089
CI	492	2.3423	0.2304	0.1507	0.0052	0.7696	0.1507	0.0052	0.1358	0.0433	0.0028	0.8642	0.0433	0.0028	0.0057	33.463	0.0297
GI	1001	2.5852	0.3224	0.1887	0.0049	0.6776	0.1887	0.0049	0.1866	0.0514	0.0026	0.8134	0.0514	0.0026	0.015	28.175	0.0306
PF	551	2.4678	0.2107	0.1391	0.0048	0.7893	0.1391	0.0048	0.1358	0.043	0.0026	0.8642	0.043	0.0026	0.027	33.519	0.0294
GA	328	2.5587	0.1889	0.131	0.0053	0.8111	0.131	0.0053	0.1166	0.0394	0.0029	0.8834	0.0394	0.0029	0.012	35.711	0.0368
PA	822	2.6832	0.2564	0.1609	0.0047	0.7436	0.1609	0.0047	0.1568	0.0463	0.0025	0.8432	0.0463	0.0025	0.02	30.865	0.0305
PI	606	2.1695	0.2637	0.17	0.0053	0.7363	0.17	0.0053	0.156	0.0481	0.0028	0.844	0.0481	0.0028	0.017	31.912	0.0233
BC	799	3.4709	0.2764	0.1576	0.0049	0.7236	0.1576	0.0049	0.1632	0.045	0.0026	0.8368	0.045	0.0026	-0.012	28.501	0.0474
MEAN	467.857	2.295	0.215	0.143	0.006	0.785	0.143	0.006	0.128	0.041	0.003	0.872	0.041	0.003	0.010	34.528	0.028

Table 4. The statistical output from STACKS that includes the number of private alleles (Ap), the number of individuals included (N), the observed Heterozygosity (Ho), the observed Homozygosity (HO), the expected Heterozygosity (He), the expected Homozygosity (HE), and the inbreeding coefficient (Fis)

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