Rhizophora mangle (Red Mangrove) Population Genetic Structure in The Bahamas

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Rhizophora mangle (Red Mangrove) Population Genetic Structure in The Bahamas

AGNESSA LAURELLE LUNDY

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Department of Natural Sciences

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I hereby declare that this work has been done by myself and no portion of the work contained in this thesis has been submitted in support of any application for any other degree or qualification in this or any other university or institution of learning. This research received no external funding and I declare no conflict of interest.

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Abstract

Rhizophora mangle, a red mangrove, grows naturally throughout the Bahamian archipelago. Genetic research on the species shows The Bahamas is likely historically linked to both Florida and wider Caribbean populations, however, this conclusion is based on data from a single sample site in The Bahamas. The current study aims to identify the population genetic structure of *R. mangle* throughout the country by sampling multiple islands. A total of 327 trees were sampled across fifteen sites from twelve islands in the archipelago and analyzed using 15 microsatellite markers. At least four genetically distinct clusters of *R. mangle* were identified in The Bahamas. The clusters appeared to align with the geography of the country in two main ways, by the Köppen-Geiger climate classification and by the latitude and longitude. The presence of the shallow sand banks, the Great Bahama Bank and the Little Bahama Bank, also appears to have some influence on the population structure. The most genetically distinct sites were Inagua, the most southern island in the archipelago, and West Grand Bahama, one of the northern islands. These sites were geographically the furthest apart at a minimum distance of 514 miles. This indicates that isolation by distance may play a role in the population structure. Conversely some sample sites that are in close proximity to each other range from moderately to significantly different. For instance, the sample sites on Conception Island and San Salvador island are approximately 42 miles apart but the sites are in different genetic clusters. This indicates that isolation by environment may also be playing a role in the population structure in the country. The findings of this research should be used to inform restoration projects focused on planting red mangroves in The Bahamas to ensure maintenance of the underlying population genetic structure.

Introduction

Mangroves are a halotolerant group of plants belonging to several genera that grow in the tropical belt worldwide (Parida and Jha 2010). They have evolved a phytomorphology resulting in ecological, economic, and social benefits that range from biodiversity preservation, fishery production and erosion control, to functioning as a foundation species. For example, mangrove roots, provide structure for filter feeders, trap sediment that then becomes suitable habitat for crabs and other detritovores, and shelter some species from predation (Hutchison et al., 2014). Mangroves have also been shown to attenuate wave energy, through the structure of their roots and trunks, the density of the forest formed and water depth or slope within the mangrove forest (McIvor et al., 2012). In addition, the mangrove canopy has been shown to reduce wind damage to houses during storms (Das and Crépin, 2013). Finally, mangrove ecosystems are a significant contributor to global carbon sequestration, accounting for 3% of carbon sequestered by the world's tropical forests, and 14% of carbon sequestered in the world's ocean (Alongi, 2012).

The Bahamas, an independent Small Island Developing State (SIDS) in the tropical Atlantic Ocean, relies heavily on the sea for survival. According to a World Bank and the United Nations Department of Economic and Social Affairs (2017) report, the marine resources of SIDS contribute significantly to food security, tourism and other building blocks within their economy. The report also describes SIDS as highly vulnerable to tropical storms. Studies show mangroves support fisheries (Hutchison et al., 2014; Hammerschlag and Layman, 2012) and reduce the impact of storms (McIvor et al., 2012; Das and Crépin, 2013). As a result, mangroves can be expected to help support food security of SIDS, like The Bahamas, and help mitigate the impacts of storms in The Bahamas.

Parida and Jha (2010) define true mangroves as plants that occur only in the mangrove habitat and mangrove associates as plants that are non-exclusive to the mangrove habitat. Three types of true mangroves occur in The Bahamas: white mangrove, *Laguncularia racemose;* black mangrove, *Avicennia germinans*; and red mangrove, *Rhizophora mangle*; along with one mangrove

associate, the buttonwood, *Conocarpus erectus.* Each of these species can be found on all of the larger islands in the country and some of the smaller islands, called Cays. Buttonwood can be found along the landward margin of true mangrove habitat and can tolerate salt spray (Bahamas National Trust, 2008) and is used in landscaping in non-saline soil environments (Gilman, 1993). Red mangrove is found growing along the coast and is the predominant foundation species along Bahamian coastlines. The black and white mangrove grow in the range between the land and red mangroves (Bahamas National Trust, 2008).

Many economically and ecologically important species in The Bahamas rely on mangrove habitat. For example, the Spiny Lobster, (*Panulirus argus*), which contributes $23.5 million per annum to the local economy (Arkema et al., 2017), utilizes mangrove prop roots for shelter (Acosta and Butler, 1997), while the Schoolmaster and Grey Snappers (*Lutjanus spp.*), which are harvested for local consumption and export, both use mangrove lined creeks as a nursery and to feed (Hammerschlag and Layman, 2012). Bonefish (*Albula vulpes*) feed in mangroves at high tide (Hutchison et al., 2014). They are targeted by recreational sport fishers on shallow shoals called flats near coastal mangroves, and flats fishing contributes $141 million per annum to the economy of the country (Fedler, 2019). The land crab, *Cardisoma guanhumi*, feeds on mangrove leaves and another species, *Ucides cordatus*, feeds on red mangrove propagules. Four endemic birds including the white cheeked pintail (*Anas bahamensis*) and the Bahama yellowthroat (*Geothlypsi rostrata*) use mangrove habitat, and the endangered green turtle (*Chelonia mydas)* forages and seeks shelter in mangrove creeks (Gillis, 2018 and Bjorndal et al., 2005). In addition, a 2019 study showed that coastal and nearshore ecosystems, which include mangroves, provide protection from hazards in The Bahamas (Silver et al., 2019). Finally, mangroves contribute to carbon sequestration within tidal creeks (Daneshgar et al., 2016) and marine protected areas (Arkema at al., 2017).

A number of studies have examined population genetic structure and genetic variation of red mangrove populations within the neotropics. These tend to show structure among populations at broad geographic scales, for example

between Brazil and Florida (Ribeiro et. al., 2013) and where geographic impediments to geneflow exist, i.e. the Gulf of California and the Pacific Coast of Baja California (Sandoval-Castro et al., 2012). Low levels of allelic diversity and high levels of inbreeding have been reported at two natural populations in Guayaquil and Esmeraldas in Ecuador (Basyuni et al., 2017), but along the northern Pacific coast of Nicaragua, Bruschi et al. (2013) showed high levels of allelic diversity at four sample sites. Earlier work by Rosero-Galindo (2002) suggested these sites should not be considered a single panmictic group. Very limited work has been undertaken on the population genetic structure and levels of genetic diversity of red mangrove within and among Bahamian islands. One exception is the inclusion of samples from San Salvador in a wider study of red mangrove population genetic structure across the Caribbean and Southern USA (Kennedy et. al. 2016). This study found the trees sampled in The Bahamas to be distinct from those sampled in Belize, Panama, and Florida. Another study tested microsatellite amplification of previously identified *R. mangle* markers and showed microsatellite sequences were conserved among geographically distant populations on San Salvador (Cross, 2011). However, there remains no information about the population genetic structure of this important foundation species among the islands of The Bahamas, which according to Sealey (2006), has a complex network of straits, channels and eddies. This complex network might be influencing the genetic structure.

Multiple studies show that the genetic variation within populations of a host plant, and especially those that can be characterized as foundation species, can influence the diversity and structure of other associated species (Wimp et. al., 2004; Tovar-Sanchez & Oyama, 2006; Wimp et. al., 2007; and Zyntynska et al., 2011). Based on these studies, it may be expected that the genetic variation of Bahamian mangroves, could impact the populations of other species that rely on them, such as the spiny lobster, snappers, bonefish and green turtles.

The primary purpose of this study was to describe the population structure of the foundation species, *Rhizophora mangle,* in The Bahamas using microsatellite markers. The expectation was that there would be genetic

differentiation observed across the archipelago. Population genetic studies have been conducted in Florida and in several Caribbean countries to date, but none have explored the genetic structure within The Bahamas. Describing the population structure of the *R. mangle* may also help inform management of the species that have been shown to rely on *R. mangle,* reveal potential population connectivity within the archipelago, provide baseline data for the country and fill an important gap in knowledge for *R. mangle* in the region.

Materials and Methods

Study Site

The Bahamas is an archipelago in the Northwest Atlantic Ocean formed by sedimentation on a seafloor platform beginning in the Triassic period (Sealey, 2006). While the islands are in proximity to North America, they are not a part of the North American plate. The island chain comprises over 700 islands and cays that extend over 550 miles in a south easterly direction toward the insular Caribbean from South Florida. Their combined landmass makes up 5,382 square miles in total area (Albury, 1975). The most northern island, Walker's Cay, is east of the Florida coastline located at 27°15'58.95" N, 78°24'40.38" W. Great Inagua Island is the most southerly island located at 21°0'51.72"N, 73°16'37.35" W; it is northeast of Cuba and northwest of Hispaniola. The cays of the Cay Sal Bank are the most western islands and are 65 miles south east of the Florida Keys and 65 miles north of Cuba. Mayaguana Island is the most eastern island located 41 miles north west of the Turks and Caicos Islands at 22°21'34.36"N, 72°49'59.13" W.

Submerged carbonate sand banks are a distinctive feature of The Bahamas (Sealey, 2006). The Great Bahama Bank is the largest of the three banks and is wrapped around Andros Island, the largest of the islands at 2,300 square miles. Several other islands in the country (Bimini, New Providence, Eleuthera, Exuma Islands, Long Island and Ragged Islands) sit on the Great Bahama Bank as well. Abaco and Grand Bahama islands sit on the Little Bahama Bank. The Cay Sal Bank is due south of the Florida Cays and due west of the Great Bahama Bank. A cluster of small islands sits on the Cay Say Bank.

On the Köppen-Geiger climate classification system, which classifies the

climate based on precipitation and heat, the predominant climate type in The Bahamas is Equatorial savanna with dry winter (Aw). Beck et al. (2018) refined the Köppen Geiger classification on a 1km scale and incorporated the modelled future conditions of climate change under the Representative Concentration Pathway (RCP) 8.5 global warming scenario. The 8.5 value refers to the predicted concentration of greenhouse gases in the atmosphere in the year 2100 (CoastAdapt, no date). According to Beck et al. (2018), The Bahamas climate classifications are Tropical rainforest (Af), Tropical monsoon (Am), Tropical savannah (Aw) and Arid, steppe hot (BSh). Figure 1 shows three of the four different climate classifications in The Bahamas (where samples were collected from), and the three sand banks within the country.

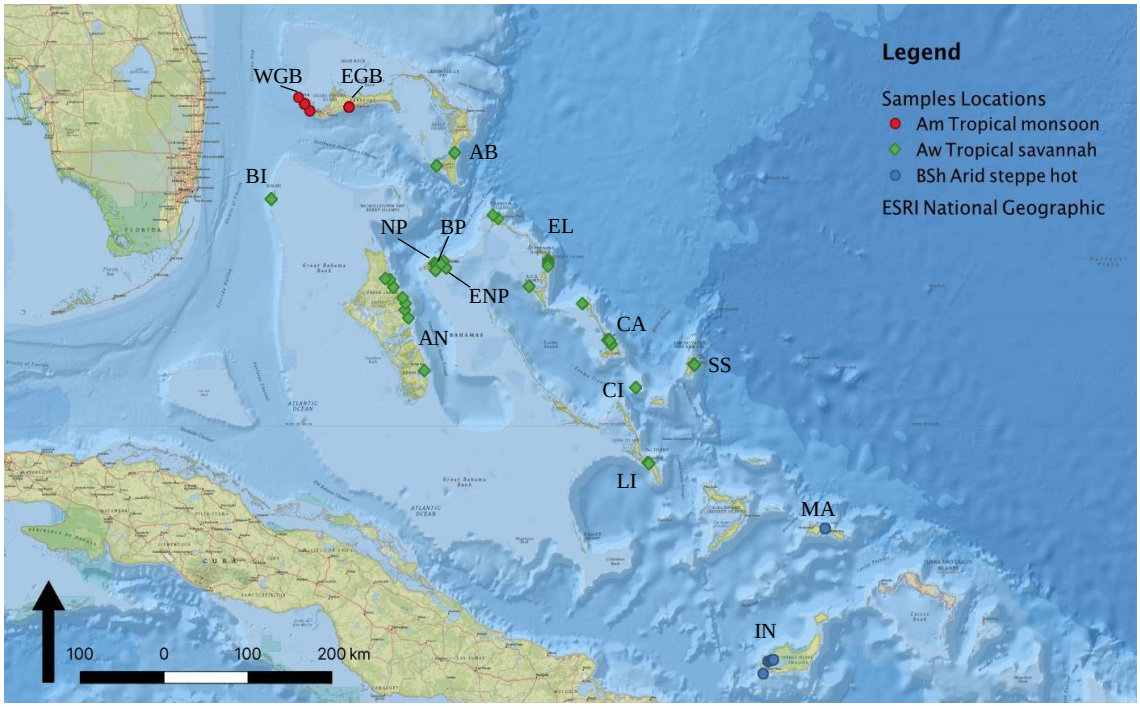

Figure 1. Map of The Bahamas. Map shows proximity to Florida, United States of America and Cuba, with sample sites. Red circles represent samples collected in the Tropical monsoon (Am); Green diamonds represent samples collected in the Tropical savannah (Aw) climate; Blue circles represent samples collected in Arid steppe hot (BSh) climate. The fourth climate type, Af Tropical rainforest is located on North Abaco only and no samples were collected from this area. Island abbreviations follow in Table 1.

Sample Collection

Rhizophora mangle leaves were collected from 327 trees in fifteen (15) locations on twelve (12) islands in The Bahamas from three of the four climate types on the Köppen-Geiger classification scale (Figure 1, Table 1). Two of the

youngest leaves were collected from each tree. These were located at the end of a branch and usually were a pale green color compared to the more mature leaves further down the stem. Every effort was made to select unmarked, healthy leaves. Both leaves from the same tree were then dehydrated and preserved in a storage bag filled with fine grade silica gel, granular size of 0.06 - 0.80 mm (AGM Container Controls Inc., Tuscon, AZ, USA). Leaves were completely covered by the silica to ensure uniform desiccation across the leaf surface. In some cases, where the leaves were too large, they were torn in half, perpendicular to the leaf midrib. The leaves were stored in the silica gel at room temperature before shipping to the Manchester Metropolitan University (MMU) Ecological Genetics Laboratory where they were stored at room temperature until DNA extraction.

DNA Extraction

DNA was extracted by adapting the Quick-Start Protocol from Qiagen DNeasy® Plant Mini Kit. Leaves were removed from the sample bag and cleaned by lightly dusting silica from the surface. 20mg leaf tissue was taken from the apex of the leaf and near the margin of the leaf blade. This sample was then disrupted using a Mixer Mill (MM 400, Retsch, Haan, Germany) at 30 Hz for 2 x 1 minute. The manufacturer's protocol was followed until incubation. Incubation time was then increased from 10 minutes to 90 minutes at 65°C, and incubating samples were mixed at 30 minute intervals and at the end of 90-minute incubation period. Following incubation, the lysate was stored on ice for a minimum of 20 minutes and then the remainder of the protocol was followed. Extracted DNA concentrations were confirmed using a Nanodrop 2000 spectrophotometer (Thermofisher, Walham, MA, USA) and stored at -20°C until amplification.

Multiplexing and Amplification

The Typeit Multiplexing Kit (Qiagen, Germantown, MD USA) was used to create two multiplexes to identify 15 loci as described by Kennedy et al. (2020). Amplification was conducted in 6-µL reaction volumes as follows: 2.5 µL Multiplex PCR Master Mix (Qiagen Type-it® Microsatellite PCR Kit), 0.5 µL multiplex primer mix, 1 µL dH_2O, and 2 µL DNA (~20 ng).

PCR conditions followed the “PCR Method for a Single Set of Cycles” recommended by Culley et al. (2013) using the Prime Thermal Cycler 5PrimeG/02 (Techne, Staffordshire, UK). The PCR conditions were: Initial temperature 95°C for 15 minutes, followed by 35 cycles of: 94°C for 30 seconds; 57°C for 90 seconds; 72°C for 60 seconds; and a final extension 60°C for 30 minutes.

Fragment & Genotype Analysis

Fragment analysis was conducted on an Applied Biosystems 3730 DNA Analyzer at the NERC Biomolecular Analysis Facility, Sheffield. For each sample reaction, 2µL PCR product, 0.15µL GeneScan 600Liz standard, 10µL Hi Di formamide was used.

Statistical Analysis

A series of programming packages run in the R Statistical Platform (R Core Team, 2019) through R Studio Cloud (RStudio Team, 2020) were used to conduct the genotype analyses. Alleles were identified using the Fragman package (Covarrubias-Pazaran, 2015). Linkage Disequilibrium was determined using Genepop On the Web (Raymond and Rousset, 1995; Rousset 2008). Observed and expected heterozygosities were calculated in adegenet (Jombart, 2008; Jombart and Ahmed, 2011). PopGenReport version 3.0.4 (Adamack and Gruber, 2014; Gruber and Adamack, 2015) calculated summary genetic data including allele frequencies, tested for Hardy-Weinberg Equilibrium, F statistics and Nei’s pairwise Fst Genetic distance using the Smouse and Peakall (1999) methodology and the absence of Null Alleles was confirmed using both the Chakraborty et. al. (1992) and the Brookfield (1996) methodologies. Correspondence Analysis (CA) and Discriminant Analysis of Principal Components (DAPC), including cluster assignments in DAPC, were conducted using the Adegenet package (Jombart, 2008; Jombart and Ahmed, 2011). Population assignment clusters were also determined using a structure-like approach within the LEA package (Frichot & Francois 2014) and via Unweighted Pair Group Mean Average (UPGMA) on Nei’s genetic distance in the phangorn package (Schliep 2011).

Results

Descriptive Statistics

All loci were in linkage equilibrium, except for RM50 and RzMg28 and locus RM50 was removed from further analysis as it had fewer alleles than RzMg28. The remaining analysis with 14 loci identified 75 alleles across the 327 samples collected from 15 sites on 12 islands in The Bahamas. Appendix A shows the genetic diversity for each of the loci and the Hardy-Weinberg Equilibrium significance values. RzMg15 was the least informative locus because only one allele was observed at 14 of the 15 sites; the only island with a different allele at this locus was Cat Island. Both RmBra19 and RzMg28 had the highest variation with nine alleles each observed across all sites. Private alleles were observed at seven of the 15 sites, with Cat Island having the most (five) private alleles (Table 1). San Salvador and Long Island each had one private allele. No null alleles were identified. Andros, Cat Island and Abaco each had 46 alleles identified.

Observed heterozygosity (Ho) ranged from 0.11 in Inagua to 0.41 in Bimini (Table 1). Bimini was the only site where Ho was the same as the expected heterozygosity (He). At all other sample sites, Ho was lower than He, indicating lower genetic diversity than expected. West Grand Bahama had the largest difference between Ho and He.

*Table 1. Genetic Diversity. Twenty individuals were collected from each population except for those indicated with *, where 33 individuals were collected. A denotes the number of Alleles, PA denotes Private Alleles, A_R denotes Mean Allelic Richness, Ho denotes mean observed heterozygosity and He mean expected heterozygosity.*

Population	Code	Longitude, Latitude	A [PA]	A_R	Ho	He
Eleuthera	EL	-76.12201, 25.09905	41	2.77	0.34	0.40
East Grand Bahama	EGB	-78.40026, 26.60427	40	2.78	0.34	0.39
San Salvador	SS	-74.45312, 24.05875	31 [1]	2.13	0.17	0.30
Conception Island	CI	-75.12130, 23.82379	33	2.23	0.29	0.33
Abaco	AB	-77.39843, 26.02780	46 [3]	3.05	0.29	0.39
Bimini	BI	-79.2933, 25.6989	40 [2]	2.76	0.41	0.41
Long Island	LI	-74.98183, 23.07022	39 [1]	2.65	0.27	0.35
East New Providence	ENP	-77.29125, 25.01831	38	2.63	0.31	0.34
Mayaguana	MA	-72.95434, 22.40417	29	2.02	0.25	0.28
New Providence	NP	-77.32019, 25.08577	38 [2]	2.60	0.30	0.34
Cat Island	CA	-75.44361, 24.29888	46 [5]	3.09	0.29	0.41
West Grand Bahama	WGB	-78.84928, 26.56681	37	2.52	0.21	0.36
Andros	AN	-77.53923, 23.99368	46 [4]	3.08	0.36	0.46
Bonefish Pond National Park *	BP	-77.41034, 24.99242	40	2.67	0.35	0.40
Inagua *	IN	-78.63090, 26.61027	30	1.95	0.11	0.21

Nei's pairwise F_{ST} values revealed genetic differentiation among sample sites (Table 2). The highest F_{ST} values were between Inagua and Abaco (0.36) and Inagua and West Grand Bahama (0.37), and Inagua was the most distinct site over all, with values greater than 0.25 when compared to nine of the sites (East Grand Bahama, Conception Island, Abaco, Bimini, East New Providence, New Providence, West Grand Bahama, Andros and Bonefish Pond National Park). Most of the sites were moderately genetically distinct from each other with F_{ST} values ranging from 0.05 to 0.14. Nine sites (Abaco, Bimini, Long Island, East New Providence, Mayaguana, New Providence, Cat Island, West Grand Bahama, Andros) had F_{ST} values ranging between 0.15 - 0.25 when compared to Conception Island. The lowest F_{ST} values were between Bonefish Pond National Park and East New Providence (0.027) and Bonefish Pond National Park and Andros (0.028). Overall, Bonefish Pond National Park showed the lowest levels of genetic differentiation from the other sites with values less than 0.05 when compared to Andros, New Providence, East New Providence and Eleuthera.

Correspondence Analysis

The first three axes of the Correspondence Analysis separate the sites according to their geographic location. For example, plotting the first and second axes separates the sites as they are situated from North to South along the archipelago (Figure 2). Plotting the first and third axes separates the sites as they are situated from East to West and most closely aligns with the geography of the archipelago (Figure 3). In all of the Correspondence Analysis plots the New Providence sites (BP, ENP, NP) are close to each other. The Abaco and East Grand Bahama sites are also always near one another. In two of the three comparisons, Conception Island is far removed from the other sites (Figure 2 and Figure 4).

Cluster Analysis

Based on the DAPC, the sample sites were best grouped into either four or six distinct clusters. The DAPC with four clusters showed defined groups with less overlap than the DAPC version with six clusters (Figure 5 and 6). The structure-

like LEA analysis also showed four genetic grouping to describe the data well (Figure 7).

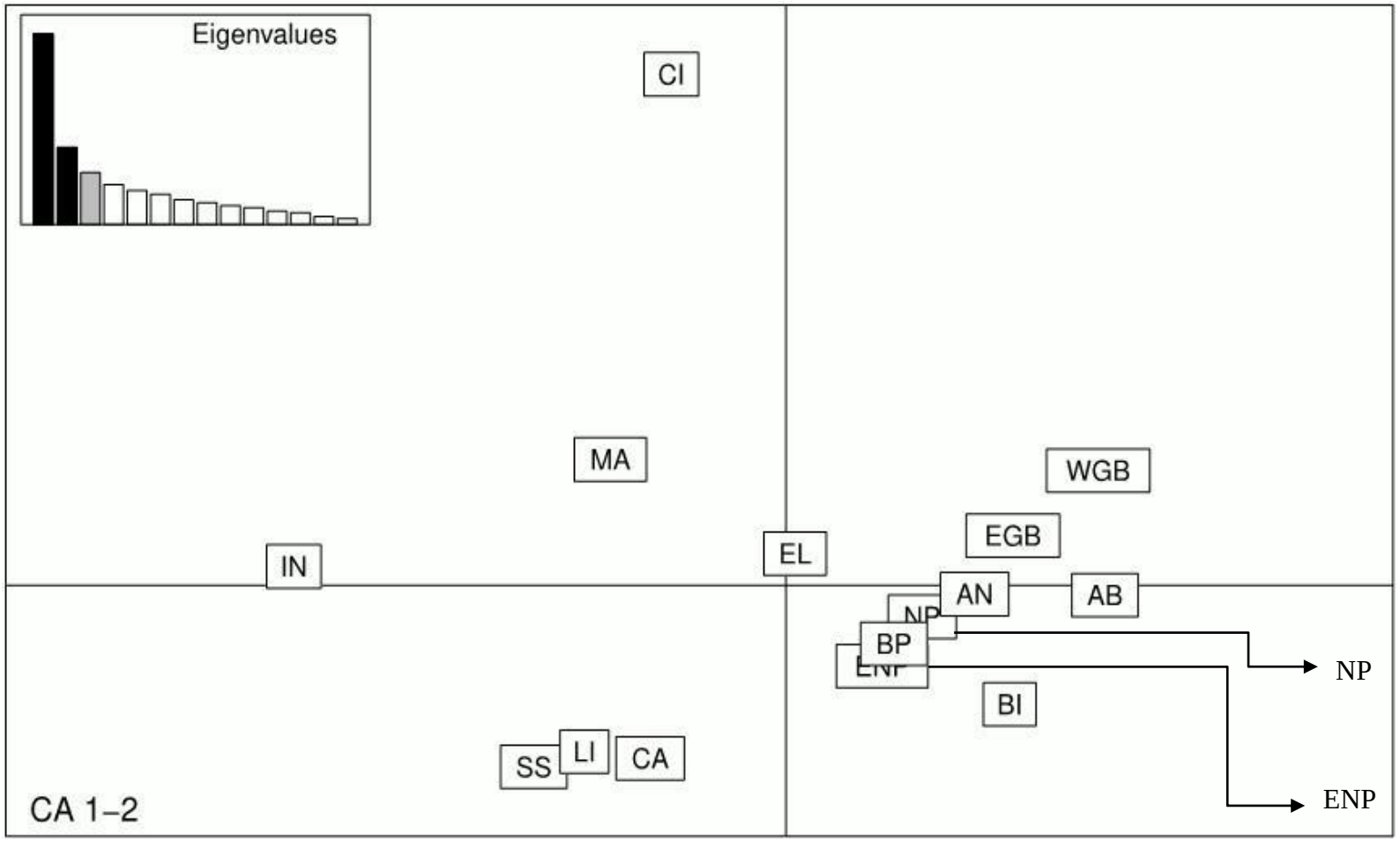

Figure 2. Correspondence Analysis: Axes 1 and 2. Plotting the first and second axes separates the sites as they are situated from North to South along the archipelago of The Bahamas. Sample sites are denoted by abbreviations used in Table 1.

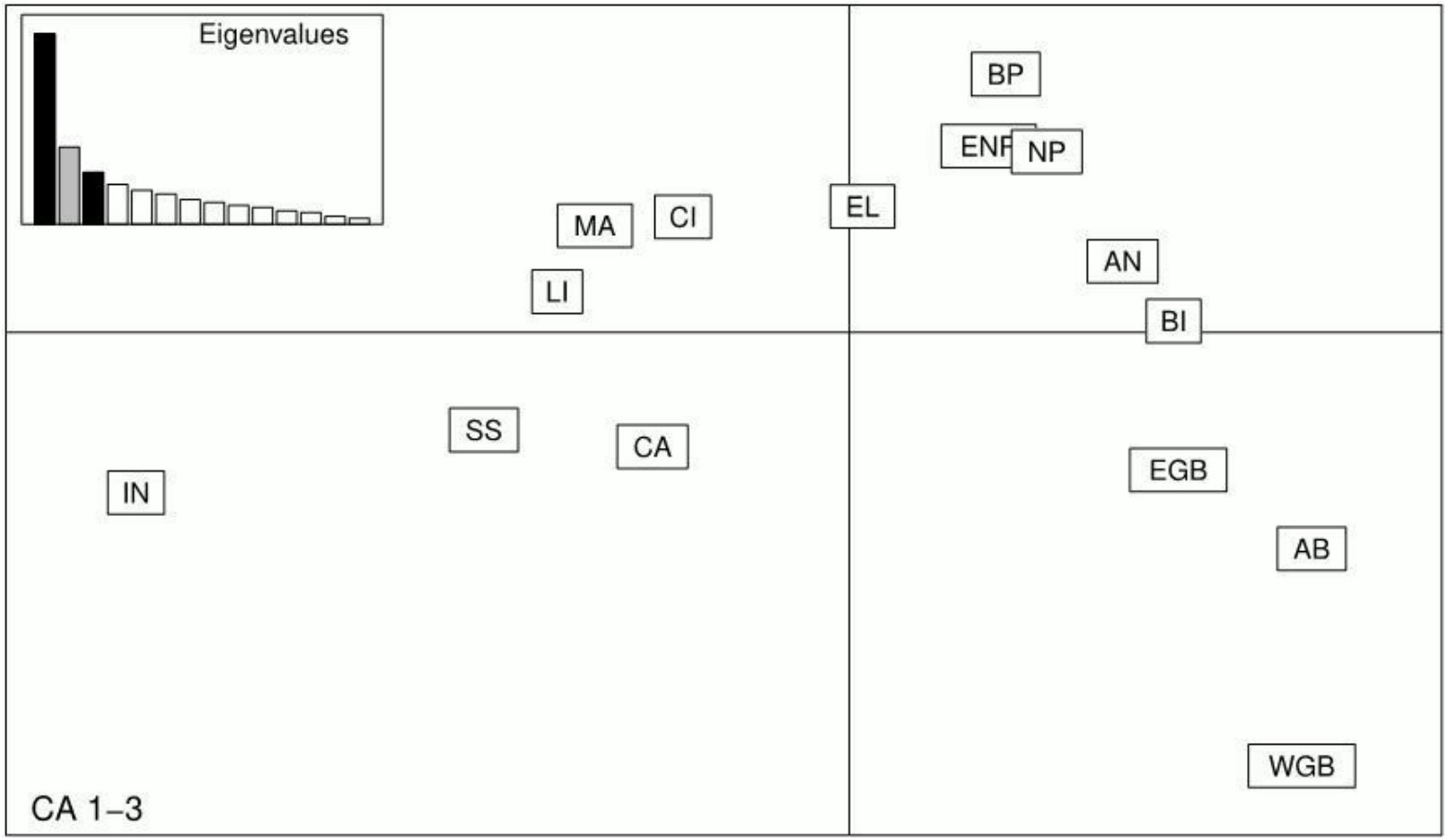

Figure 3. Correspondence Analysis: Axes 1 and 3. Plotting the first and third axes separates the sites as they are situated from East to West and most closely aligns with the geography of the archipelago of The Bahamas. Sample sites are denoted by abbreviations used in Table 1.

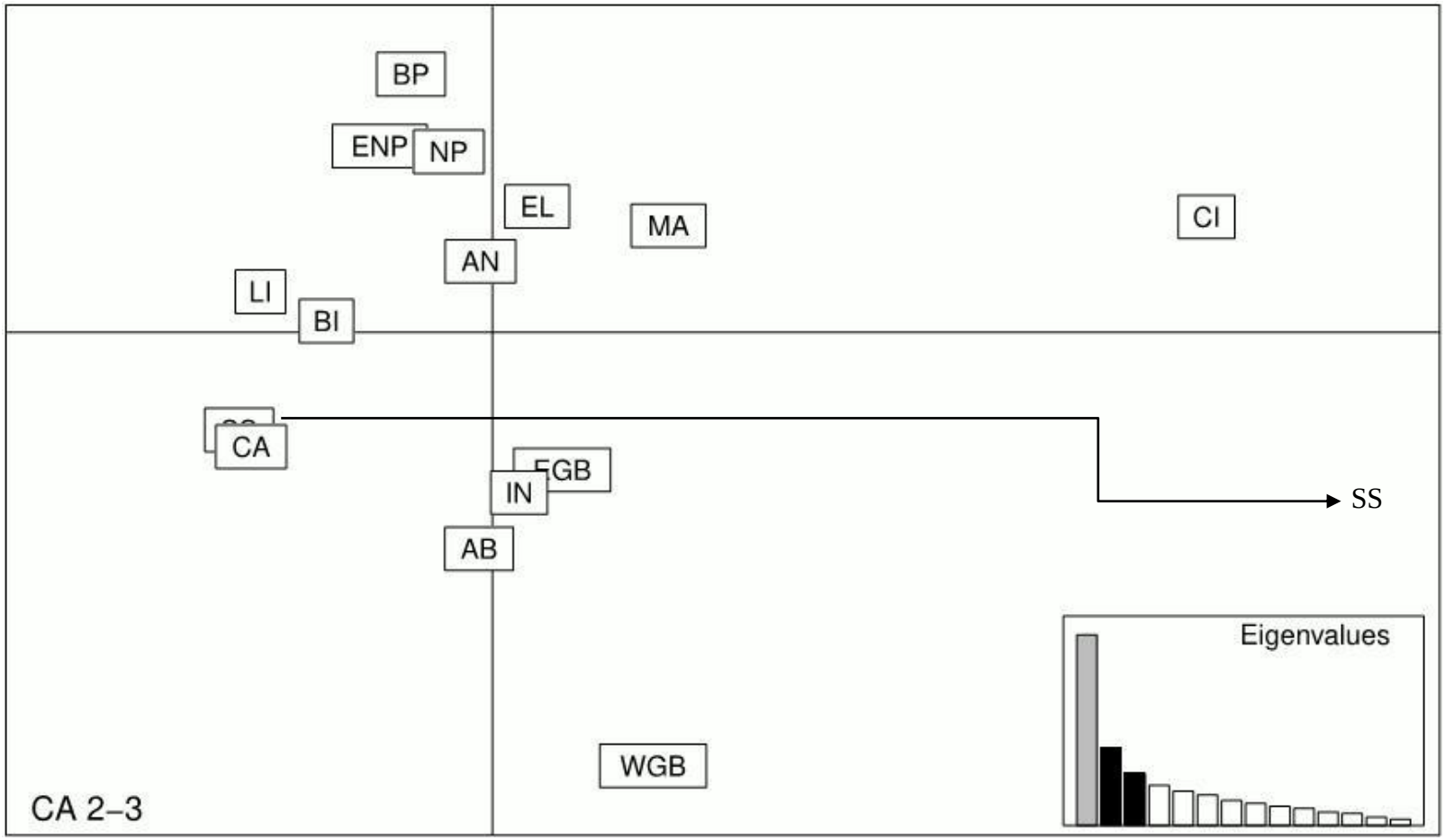

Figure 4. Correspondence Analysis: Axes 2 and 3. Plotting the second and third axes shows Conception Island (CI) is closely related to Mayaguana (MA) and also shows CI is more isolated from the other sites. Sample sites are denoted by abbreviations used in Table 1.

Table 2. Nei's pairwise FST values. Little to no difference (White) < 0.05; Moderate difference (Orange) 0.05-0.14; Great difference (Blue) 0.15-0.25; Significant difference (Green) > 0.25

	EL	EGB	SS	CI	AB	BI	LI	ENP	MA	NP	CA	WGB	AN	BP	IN
EL	0.000														
EGB	0.087	0.000													
SS	0.123	0.156	0.000												
CI	0.135	0.189	0.256	0.000											
AB	0.093	0.058	0.201	0.215	0.000										
BI	0.094	0.055	0.162	0.196	0.060	0.000									
LI	0.092	0.153	0.078	0.219	0.185	0.146	0.000								
ENP	0.087	0.090	0.157	0.204	0.095	0.081	0.109	0.000							
MA	0.108	0.180	0.178	0.165	0.219	0.170	0.081	0.140	0.000						
NP	0.076	0.073	0.147	0.202	0.061	0.082	0.121	0.065	0.168	0.000					
CA	0.068	0.118	0.074	0.188	0.137	0.091	0.030	0.080	0.077	0.108	0.000				
WGB	0.132	0.047	0.243	0.208	0.072	0.099	0.232	0.168	0.241	0.149	0.180	0.000			
AN	0.061	0.041	0.142	0.149	0.047	0.047	0.104	0.047	0.132	0.036	0.080	0.084	0.000		
BP	0.039	0.061	0.120	0.144	0.060	0.048	0.089	0.028	0.114	0.040	0.068	0.122	0.028	0.000	
IN	0.229	0.314	0.178	0.282	0.360	0.321	0.121	0.284	0.189	0.323	0.153	0.366	0.264	0.256	0.000

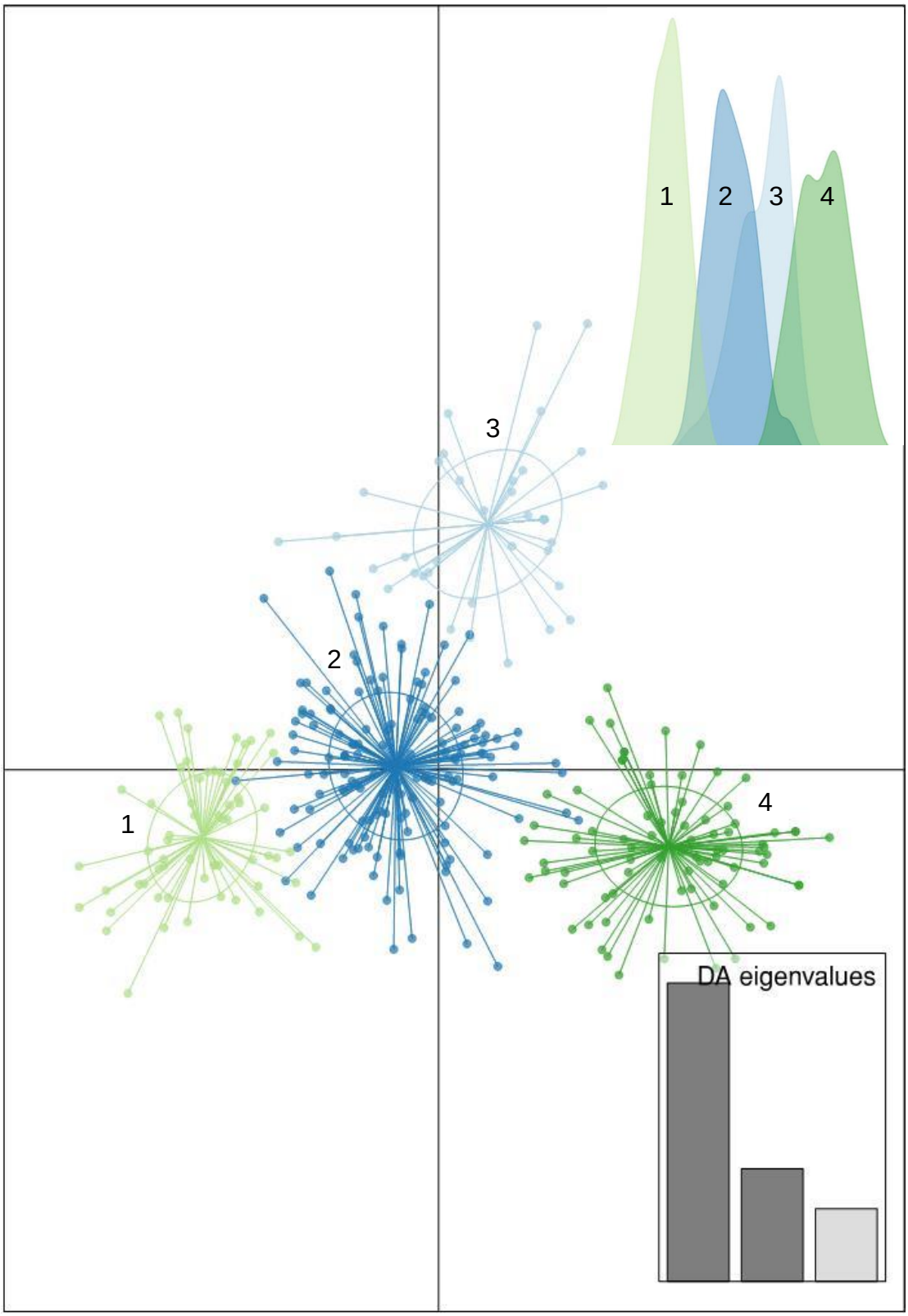

Figure 5. 4 Cluster Discriminate Analysis of Principal Components (DAPC) of Rhizophora mangle *samples in The Bahamas. 327 samples collected from 15 sites on a total of 12 islands in The Bahamas were assigned into the four previously determined clusters in the structure-like LEA analysis. The inset (top right) illustrates the differentiation and overlap between the four groups.*

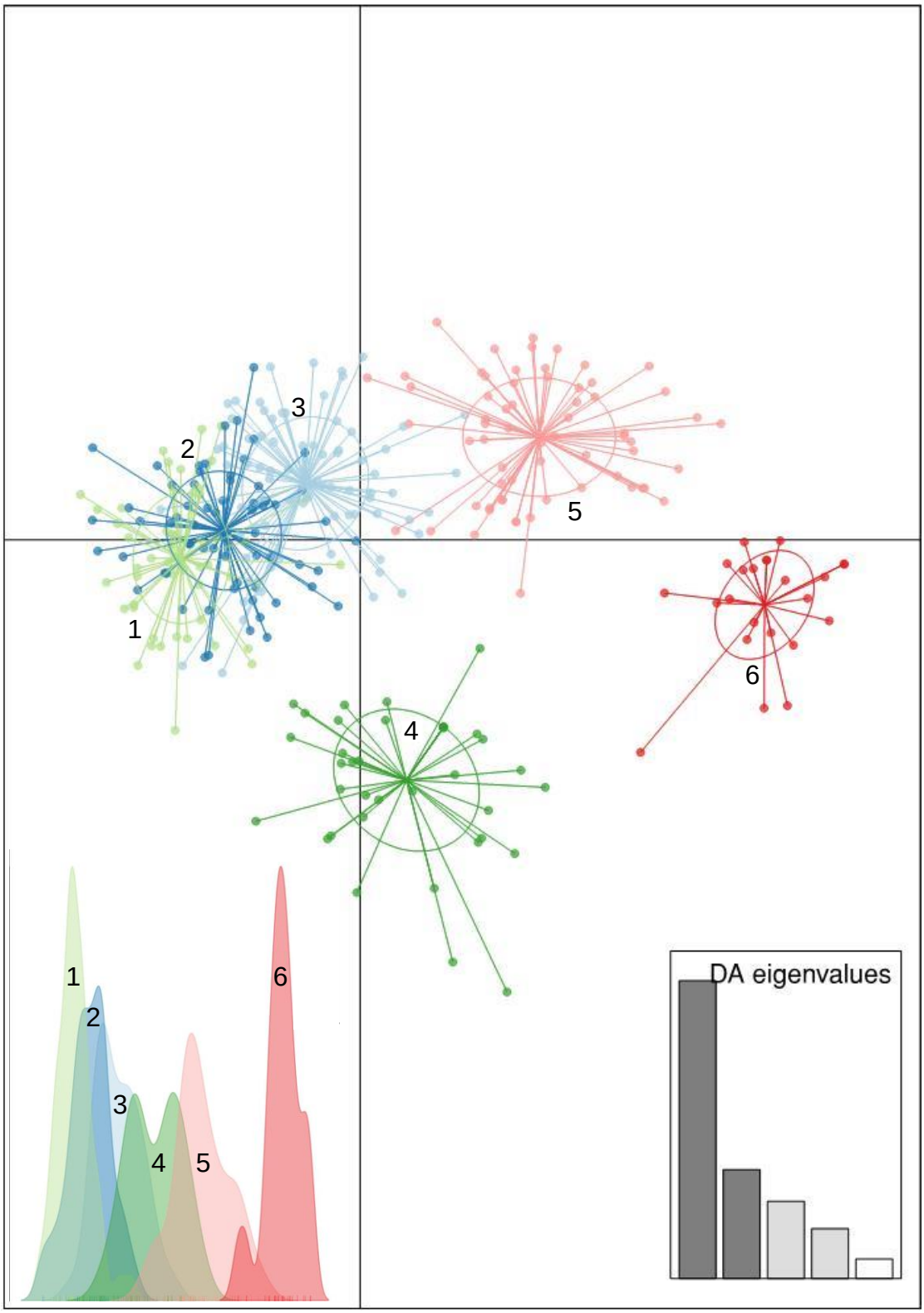

Figure 6. 6 Cluster Discriminate Analysis of Principal Components (DAPC) of Rhizophora mangle *samples in The Bahamas. 327 samples collected from 15 sites on a total of 12 islands in The Bahamas were assigned into the six clusters. The inset (bottom left) illustrates the differentiation and overlap between the four groups.*

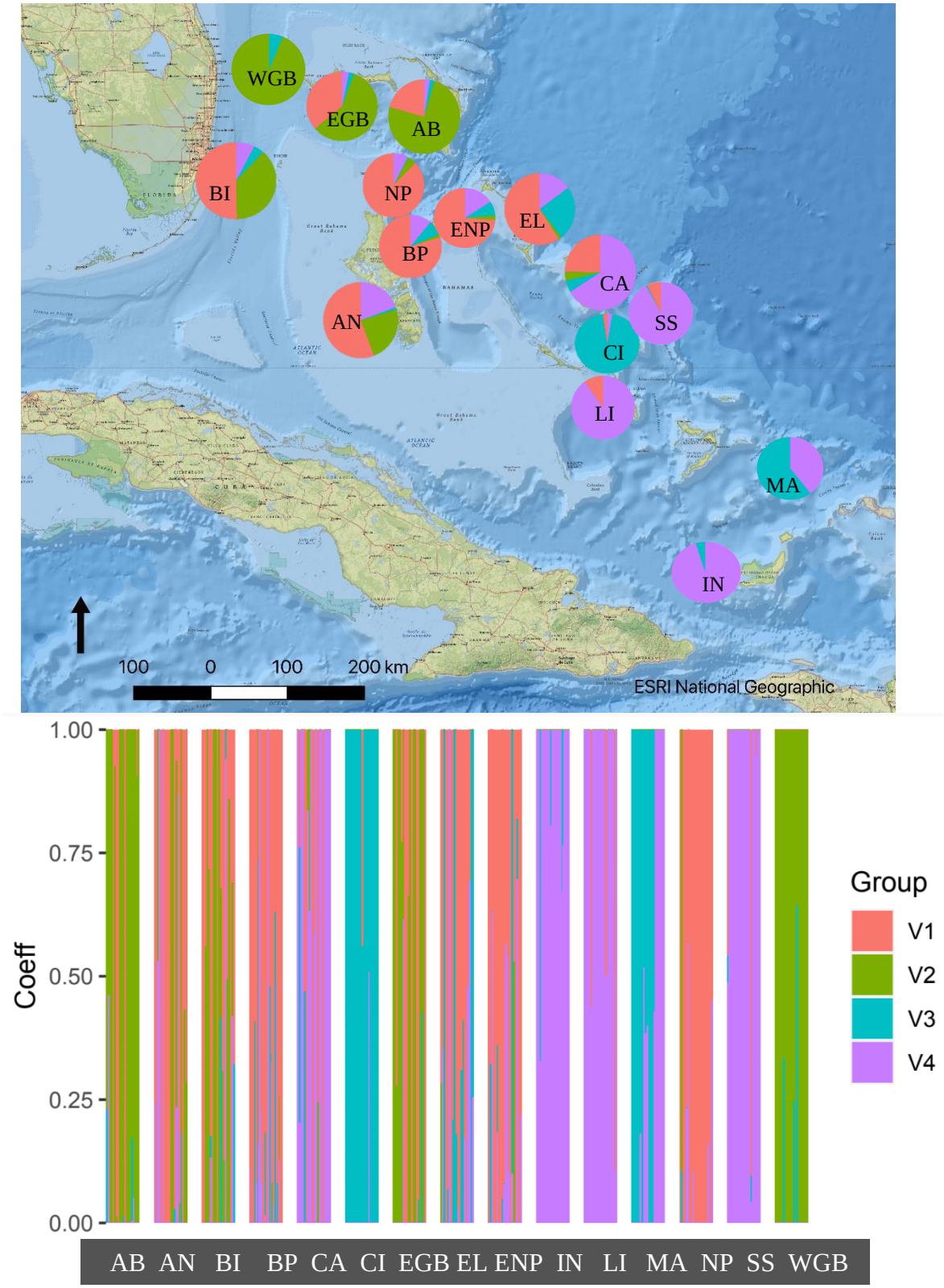

Figure 7. Cluster Assignments. (Top) Map of The Bahamas showing samples sites grouped into four clusters (V1-4) using the structure-like LEA Analysis. (Bottom) Structure-like graph assigning probability of individual samples to different clusters.

Clustering by Unweighted Pair Group Mean Average (UPGMA) showed three main clusters as opposed to four clusters shown in the DAPC and Structure-like analyses (Figure 8). Conception Island (CI) remains separated from the other groups, West and East Grand Bahama while in the same cluster group, are more closely related to each other than to the other groups. Bonefish Pond National Park (BP), New Providence (NP), and East New Providence (ENP) samples sites are located on one island, but the NP site appears to be more closely related to Andros (AN) than BP and ENP.

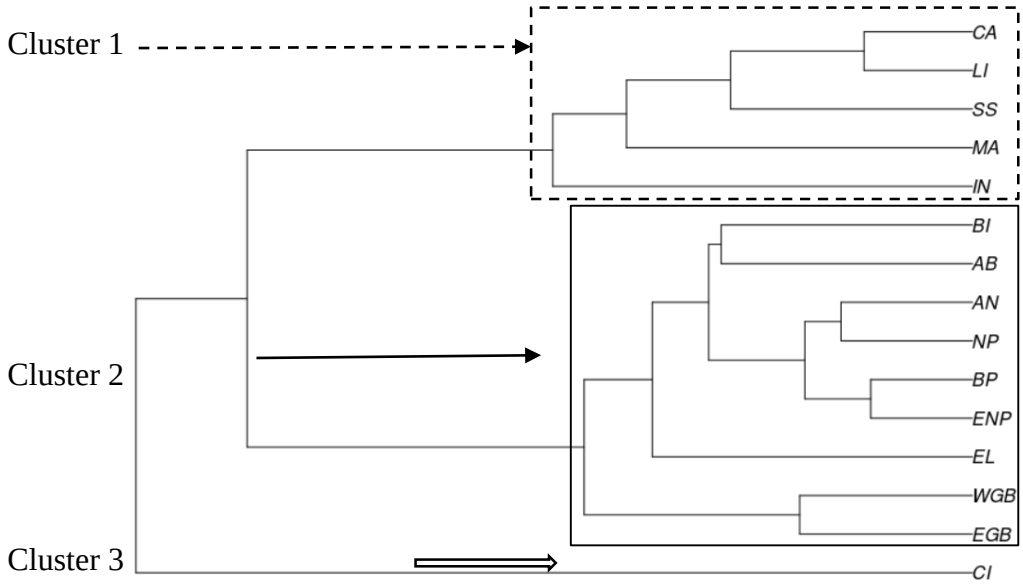

Figure 8. Unweighted Pair Group Mean Average (UPGMA) Cluster Assignment using Nei's genetic distance. Clustering by UPGMA shows three clusters. Cluster 1 – Cat Island (CA), Long Island (LI), San Salvador (SS), Mayaguana (MA), and Inagua (IN); Cluster 2 – Bimini (BI), Abaco (AB), Andros (AN), New Providence (NP), Bonefish Pond National Park (BP), East New Providence (ENP), Eleuthera (EL) West Grand Bahama (WGB), and East Grand Bahama (EGB). Cluster 3 - Conception Island (CI).

Discussion

The purpose of the study was to assess the genetic diversity of natural populations of *Rhizophora mangle* throughout The Bahamas. While different methods were used to assess the genetic diversity in The Bahamas, each method agreed that the *R. mangle* population in The Bahamas is not panmictic. The Correspondence Analyses showed there was some genetic distance among sample sites and that these seemed to correspond with the broad geography of the archipelago. Both the DAPC and Structure-like analysis showed there were at least four distinct genetic clusters, while the UPGMA showed three distinct genetic clusters with The Bahamas.

The results show there are likely at least four distinct genetic groups of *R. mangle* in the country. Of these four groups, the most northern cluster comprises Grand Bahama and Abaco, which are both located on the Little Bahama Bank. The second cluster, slightly further south, groups Bimini, Andros, New Providence, and Eleuthera. This cluster is entirely situated on the Great Bahama Bank. The third cluster comprises Long Island, Cat Island, San Salvador, and Inagua. One island in the third cluster is located on the Great Bahama Bank, Long Island, but the others are not located on a Bank. The fourth cluster only includes two islands, Conception Island and Mayaguana. Conception Island is surrounded by shallow water, but Mayaguana is surrounded by deeper water.

Isolation by distance may be influencing the population structure observed in The Bahamas. Islands closer together were genetically similar and generally classified within the same cluster. For example, the Abaco and Grand Bahama sample sites are about 88 miles apart and form a distinct cluster and Cat Island and San Salvador which are also adjacent and 62 miles apart, are classified within the same cluster. Islands further away from one another were generally genetically distinct. Grand Bahama and Inagua are the two furthest apart at 512 miles and they are represented by two different clusters. Similarly, Bimini and Long Island are also some not in proximity at about 319 miles apart and they are genetically distinct. Isolation by distance was also observed by Kennedy et. al. (2020) in *Rhizophora mangle* populations in Florida and The Bahamas but isolation by distance was not observed by Núñez-Farfán et. al. (2002) in Mexican *Rhizophora mangle* populations. Cisneros de la Cruz et. al. (2018) found that ecological barriers promoted genetic differentiation within sites in the Yucatan Peninsula.

Isolation by environment may also be influencing the population structure observed in The Bahamas in different ways. Firstly, islands geographically isolated on the Little Bahama Bank were genetically similar to each other and most of the sample sites located on the Great Bahama Bank formed a coherent genetic cluster. Water movement over the Banks is limited predominantly to

tidal flow. Paris et. al. (2016) shows the ocean currents move around the perimeter of the Banks, not across the Banks, making it unlikely that propagules could be transported on and off the Banks. Currents resulting from an excursion of the Florida Current has been observed on the Great Bahama Bank in the lee of Andros (Harris et. al., 2014). Secondly, the environment may be influencing the observed genetic structure by climate classification on the Köppen-Geiger system. Mayaguana and Inagua are the two most southern islands in The Bahamas and are both a part of the climate classification BSh (Arid steppe hot). While all samples collected from these islands fall within two clusters, the ratio of samples represented in the different clusters is not the same. This means there is, or has likely been some historical exchange of genetic material. The three sample sites on New Providence, Andros and Eleuthera Islands share the same climate classification Aw. Most of the samples from each of these sites are represented in the same cluster.

Additionally, the ocean currents within and surrounding the archipelago may be facilitating and restricting propagule dispersal, therefore connecting islands which do not appear to be in proximity to each other, and isolating islands that are geographically close. *R. mangle* propagules remain buoyant and viable for several months after separating from the maternal tree enabling them to be transported large distances before settling (Rabinowitz, 1978). There are main ocean currents along the western and eastern boundaries of the islands, but the shallow Banks and presence of coral reefs and small islands lead to the development of smaller current and eddies throughout The Bahamas (Beccario, 2020 and Paris et al., 2016). For example, Long Island, San Salvador and Cat Island appear to be genetically similar and a strong ocean current passes between these islands periodically (Paris et al., 2016). Mayaguana is more genetically similar to Conception Island than it is to Inagua, even though it is geographically closer to Inagua. Beccario (2020) shows ocean currents move between Mayaguana and Conception Island.

The observed genetic differentiation among islands may be influenced by each of the explanations but it is important to note that these factors are not independent of each other, one may be driving the other. For example, the

Climate Classification may be related to the ocean currents and or the islands position on or near to a sand bank.

The genetic diversity (Ho; mean=0.29; range 0.11-0.41) in The Bahamas is similar to the results found in other studies. Low genetic diversity was observed in two *R. mangle* populations in Ecuador by Basyuni et. al. (2017) (mean Ho=0.28; range 0.19-0.41) in the northwestern coast of Mexico by Sandoval-Castro et. al. (2012) (mean Ho=0.17; 0.05-0.27). Núñez-Farfán et. al. (2002) found high inbreeding in fourteen *R. mangle* populations sampled from both the Pacific (mean Ho=0.08; range 0.07-0.10) and Atlantic (mean Ho=0.06; range 0.00-0.11) coast of Mexico. Deviations from Hardy–Weinberg equilibrium were also observed in the Colombian Pacific coast of *R. mangle* (Arbeláez-Cortés et. al., 2007).

Over the last decade, multiple restoration and conservation projects in The Bahamas have focused on *Rhizophora mangle* (Ball, 2020; Sweeting, 2017; Sealey and Bowleg 2015; Lewis, 2020), and as a result, propagules and seedlings have been moved both among and within islands and from Florida in the United States to help restore *R. mangle* habitats. Because the results of this study show there are genetically distinct groups of mangroves in The Bahamas, it is likely that previous projects have introduced mangroves from a different genetic cluster to a restoration site. The benefit of introducing new genotypes to a site is an increased diversity to the restoration site, enhancing the available genotypes available for natural selection and possibly contributing to heterosis (Zavodna et. al., 2015). Zavodna et. al. (2015) also summarized the negative impacts of introducing genetically distant individuals to a restoration site such as introducing maladapted genes and outbreeding depression. The disadvantage of introducing new genotypes is introducing individuals that are not genetically fit for that environment. This can result in diluting the population with individuals not suitable to the site. Furthermore, studies have shown the genetic structure of foundation species to be linked to the wider biodiversity of a site (Wimp et al. 2004; Wimp et al. 2007). This means that any genetic changes to the foundation species can influence populations of other species that rely on it (Angelini et. al., 2011). In the case of *R. mangle*

in The Bahamas, altering the population's natural genetic structure, may impact the species that rely on the habitat, such as the Spiny Lobster (*Panulirus argus*), a variety of snappers *(Lutjanus spp*), Bonefish *(Albula vulpes*), Land crabs *(Cardisoma guanhumi & Ucides cordatus*), Green Turtles *(Chelonia mydas)*, the white cheeked pintail (*Anas bahamensis*) and the Bahama yellowthroat (*Geothlypsi rostrata*) (Arkema et al., 2017; Hammerschlag and Layman, 2012, Hutchison et al., 2014; Fedler, 2019, Gillis, 2018 and Bjorndal et al., 2005).

Marine Protected Areas (MPAs) have been created to help improve nearshore marine habitat representation within the MPA network in The Bahamas (Anderson et. al., 2018). However, the population genetics of the species in the nearshore environment was not considered in these efforts, because the information was not available. Therefore, it is possible the genetic diversity of *R. mangle* was negatively impacted when mangrove habitat was lost. An effective MPA network should be representative of the biodiversity in the country and have built in redundancies to prevent loss of biodiversity in the event that one protected area is compromised. By considering the genetic structure of the *R. mangle* population in the country in the design of the MPA network, the MPA managers can ensure there is representation and redundancy of each of the clusters identified and that the breadth of genetic diversity of the mangroves are protected and maintained.

Information from this research has been shared with the Bahamian government and non-government organizations to help inform future conservation efforts. In light of these new findings, restoration projects focused on replanting *R. mangle* should consider the genetic diversity of the restoration site and introduced plants. Long term monitoring of the site as Zavodna et. al. (2015) conducted could lead to more informed mangrove restoration projects in country. Additionally, Marine Protected Areas (MPAs) that target *R. mangle* could focus on including sites within the MPA network that are representative of the different genetic clusters to help maintain the genetic diversity of the population in The Bahamas.

Recommendations

The information in this study can help guide conservation efforts in the country as environmental managers and community groups are actively harvesting red mangrove propagules from different islands to help enhance restoration efforts. For example, restoration efforts focused on one island could focus on sourcing propagules from an island with a similar genetic cluster. Research that investigates the long term impacts of introducing propagules to a restoration site from similar and distinct genetic clusters is also recommended. To further inform mangrove conservation and restoration efforts in The Bahamas, the study could be expanded to include islands where samples were not collected, in particular samples from the Cay Sal Bank and Exuma. This would help improve the national dataset for genetic information. Genetic diversity of the other species of mangroves in The Bahamas should also be explored to help inform best management practices throughout the mangrove ecosystem.

Finally, an inventory of restoration projects that have moved *Rhizophora mangle* around the country should be conducted and a monitoring programme established for those sites to determine any long term impacts of introducing individuals from a genetically distinct cluster to a different environment.

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Appendices

Appendix A – Tables

TableA 1. Genetic Diversity by Locus A number of Alleles; He Expected Heterozygosity, Ho Observed Heterozygosity, FST fixation index, FIS Inbreeding Coefficient.

Locus	A	H_e	H_o	F_{ST}	F_{IS}
RM19	6	0.4473	0.2791	0.1608	0.2659
RM38	5	0.5727	0.3742	0.2330	0.1629
RmBra18	6	0.3750	0.1754	0.2788	0.3650
RmBra19	9	0.6404	0.3681	0.3091	0.1869
RmBra20	8	0.5735	0.3773	0.1847	0.2046
RmBra50	3	0.3374	0.2209	0.1259	0.2589
RmBra59	7	0.5542	0.3650	0.1784	0.2094
RzMg09	4	0.5046	0.3569	0.1080	0.2143
RzMg15	2	0.0061	0.0000	0.0043	1.0000
RzMg21	5	0.6511	0.3642	0.2610	0.2579
RzMg28	9	0.7551	0.5031	0.2437	0.1352
RzMg32	3	0.0910	0.0706	0.0822	0.1611
RzMg33	4	0.5013	0.2791	0.3112	0.2101
RzMg34	3	0.4608	0.2117	0.3430	0.3184

TableA 2. Significance values for Hardy-Weinberg Equilibrium (HWE) for each loci across the 15 sample sites on 12 islands in The Bahamas. The Bonferroni adjustment was used to reduce Type I error, α = 0.00024. Blue highlighting represents the loci that significantly differed from HWE after adjustment. The site key is shown below the table.

Site	RmBra19	RM19	RmBra18	RmBra59	RzMg15	RzMg28	RmBra20	RmBra50	RM38	RzMg09	RzMg32	RzMg33	RzMg21	RzMg34
EL	0.391	0	1	0.758	1	0.096	0.063	0.22	1	1	1	0.579	0.007	0.118
EGB	0.058	0.89	1	0.05	1	0.436	0.462	0.465	0.875	0.353	1	0.354	0.023	1
SS	1	1	0	0.039	1	0.007	0.059	1	0.114	0.091	1	0.146	0.019	1
CI	1	0.422	0.073	0.483	1	0.093	0.282	1	1	0.364	0.572	1	0.361	1
AB	0.445	0.005	1	0.019	1	0.255	0.013	0.064	0.171	0.002	0.085	0.021	0.032	0.002
BI	1	0.505	0.55	0.365	1	0.052	1	0.129	0.805	0.375	1	0.669	0.815	1
LI	1	1	0.35	0.149	1	0.033	0.083	0.028	0.099	0.064	0.167	1	0.14	0.117
ENP	0.068	1	0.03	0.011	1	0.696	1	1	1	0.331	1	0.132	0.268	1
MA	0.006	1	1	0.185	1	0.015	1	1	0	0.025	1	1	1	0.066
NP	0.006	0	1	1	1	0.03	0.647	0.203	0.691	0.345	1	0.638	0.476	0.014
CA	0.055	0.038	0.004	0.158	0.029	0.099	0.049	0.239	0.069	0.37	1	1	0.012	0.063
WGB	0.009	0.156	1	1	1	0.801	0	0.022	0.003	0.026	1	0.036	0	0.096
AN	0.018	0.015	0.006	0.002	1	0.617	0.157	0.616	0.872	0.165	1	0.088	0.005	1
BP	0.521	1	0.106	0.263	1	0.831	0.291	0.746	0.099	0.377	1	0.007	0.034	0.649
IN	1	0.022	0.009	0	1	0.008	1	0.018	0.017	0.073	1	0.042	0.027	0.024

TableA 3. Site code description

Code	**Island**	**Code**	**Island**
EL	Eleuthera	MA	Mayaguana
EGB	East Grand Bahama	NP	New Providence
SS	San Salvador	CA	Cat Island
CI	Conception Island	WGB	West Grand Bahama
AB	Abaco	AN	Andros
BI	Bimini	BP	Bonefish Pond National Park
LI	Long Island	IN	Inagua
ENP	East New Providence		

Appendix B – Published Articles Contributed to during MRes Studies

My contribution to the following published article includes securing the required research permits from The Bahamas Environment, Science & Technology (BEST) Commission, the Department of Marine Resources (DMR), the Department of Agriculture (DoAg) and the Bahamas National Trust (BNT). Once permits were approved, I collected samples from New Providence, and arranged for the collection of samples from Inagua. I exported the samples to the MMU lab and extracted the DNA. I assisted with the amplification of the samples and testing the microsatellite markers. I reviewed the article and provided some reference material that was incorporated in the article.

The article and a summary document has since been submitted to the Department of Environmental Planning and Protection (DEPP), which was formerly known as the BEST Commission, and the BNT. The information has been used to help inform management of the mangrove resources in country and mangrove restoration in the northern Bahamas.

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METHODS AND RESOURCES ARTICLE

Multiplex microsatellite PCR panels for the neotropical red mangrove, *Rhizophora mangle*: combining efforts towards a cost-effective and modifiable tool to better inform conservation and management

John Paul Kennedy[1] · Hayley Craig[2] · Antonella Jara-Cavieres[1] · Agnessa Lundy[1] · Richard F. Preziosi[1] · Jennifer K. Rowntree[1]

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Abstract

Better-informed mangrove conservation and management practices are needed as the ecosystem services provided by these intertidal forests continue to be threatened by increasing anthropogenic pressures and climate change. Multiple layers of knowledge are required to achieve this goal, including insights into population genetics of mangrove species. Understanding the importance of population-genetic insights to conservation, multiple research groups have developed microsatellite loci for the widespread, neotropical red mangrove, *Rhizophora mangle*. However, although a wealth of genetic markers exist, empirical research is limited in the number of these loci employed. Here, we designed two multiplex PCR panels that combine seven novel loci developed for this work and eight previously-developed loci from three research groups to generate 15-locus genotypes, more than twice the average number of loci used in previous research, in only two PCR. We demonstrated utility in *R. mangle* from four sites across ~2500 km near this species' northern latitudinal limits, and that these multiplex panels were better able to delineate populations than data subsets with numbers of loci comparable to previous research. We focus our discussion on how this tool is a more-informative, efficient (both in terms of time and resources), and easily-modifiable alternative to address many pressing conservation and management issues, such as the generation of baseline genetic data for areas not yet studied, better defining management units, and monitoring genetic effects of restoration projects. We also provide a quick protocol that outlines each step in this procedure to facilitate the use of this tool by others.

Keywords Coastal management · Mangroves · Microsatellites · Multiplex PCR · Population genetics · *Rhizophora*

Introduction

Mangroves provide ecosystem services of both ecological and economic importance to coastal ecosystems worldwide (Lee et al. 2014). However, these intertidal forests are highly susceptible to increasing anthropogenic pressures and climate change (Alongi 2015; Friess et al. 2019). Effective conservation and management of mangrove ecosystems will require multiple layers of knowledge across diverse disciplines, including improved estimates of temporal changes in mangrove cover, standardized protocols to monitor forests, and insights into connectivity across local and regional scales (Canty et al. 2018). Population genetic data can provide insights necessary to understand and continue to monitor species for conservation and management purposes, including estimates of population structure, effective population sizes, and gene flow (Kramer and Havens 2009).

The pantropical genus *Rhizophora* (Rhizophoraceae) consists of nine species and hybrids (Duke et al. 1998) whose large propagules are commonly used in reforestation projects. Three members of this genus exist in the Neotropics, where *Rhizophora mangle* is the most widespread, with a distribution that covers both the Pacific and Atlantic coasts

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✉ John Paul Kennedy
john.p.kennedy@stu.mmu.ac.uk; kennedy3jp@gmail.com

[1] Department of Natural Sciences, Faculty of Science and Engineering, Ecology and Environment Research Centre, Manchester Metropolitan University, Chester Street, Manchester M1 5GD, UK

[2] Department of Earth and Environmental Sciences, Faculty of Science and Engineering, University of Manchester, Manchester, UK

of the Americas and the Atlantic coast of Africa. Understanding the importance of population-genetic insights to mangrove conservation, multiple research groups have developed *R. mangle* microsatellite loci (Rosero-Galindo et al. 2002; Takayama et al. 2008; Ribeiro et al. 2013; Francisco et al. 2018a) and 17 peer-reviewed publications since 2007 have utilized these loci to characterize *R. mangle* population genetics from across this species' distribution (Table 1). However, although we possess a wealth of genetic markers, this field still lacks cohesion in the implementation of these microsatellite loci. Empirical research is limited in the number of these loci employed (6.6 ± 2 loci; mean ± SD), presumably because most studies continue to amplify loci individually (Table 1). Amplification in singleplex can be excessively expensive and time consuming, and limit either the number of markers used or samples genotyped, as expressed in a recent study (Bologna et al. 2019). In addition, many studies limit themselves to loci developed by a single research group and do not use previous research to inform their choice of loci from across all available microsatellites.

Here, we developed a new set of *R. mangle* microsatellite loci and then designed two multiplex PCR panels that combine these novel loci with those of three other research groups. These multiplex panels generate 15-locus genotypes, more than twice the average number of loci used in previous research, in only two PCR. We demonstrate the utility of these multiplex panels in *R. mangle* from four collection sites across ~ 2500 km towards this species' northern latitudinal limits and how this increased number of loci can improve our ability to differentiate among populations of this species. We focus our discussion on how this tool can be an efficient alternative (both in terms of time and resources) to provide necessary baseline genetic data for pressing conservation and management questions, and how these multiplex can be easily modified to incorporate alternative loci from the pool of available microsatellites for this species.

Materials and methods

Novel microsatellites

Rhizophora mangle leaf tissue was collected from a single individual in Fort Pierce, Florida, USA (27.4974, − 80.3057) and immediately dried in silica gel. Genomic DNA from this individual was isolated from 20 mg of dried leaf tissue with the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol, with an extended incubation of 45 min. DNA for sequencing was purified with the High Pure PCR Product Purification Kit (Roche, Penzberg, Germany). We used 2×250-bp paired-end Illumina MiSeq genome sequencing and developed microsatellite markers with the Galaxy-based pipeline outlined by Griffiths et al. (2016), which identified 61,130 sequences with microsatellite motifs and designed primers for 358 loci, of which 42

Table 1 Research articles on *Rhizophora mangle* genetics that employed microsatellite loci

Authors	Year	Region	Loci	Publication
Bologna et al.	2019	St. John, USVI	7	https://doi.org/10.3390/d11040065
Cisneros-de la Cruz et al.	2018	Atlantic Mexico	9	https://doi.org/10.1002/ece3.4575
Francisco et al.	2018a	Brazil	8	https://doi.org/10.1002/ece3.3900
Francisco et al.	2018b	Brazil	4	https://dx.doi.org/10.1590/01047760201824042575
Kennedy et al.	2017	Florida, USA	7[a]	https://doi.org/10.1111/jbi.12813
Hodel et al.	2016	Florida, USA	8	https://doi.org/10.3732/ajb.1500260
Kennedy et al.	2016	Caribbean and Florida, USA	7	https://doi.org/10.3732/ajb.1500183
Cerón-Souza et al.	2015	Across distribution	6	https://doi.org/10.1002/ece3.1569
Cerón-Souza et al.	2014	Pacific Panama	10	https://doi.org/10.1007/s11258-014-0315-1
Sandoval-Castro et al.	2014	Atlantic and Pacific Mexico	6	https://doi.org/10.1371/journal.pone.0093358
Bruschi et al.	2014	Pacific Nicaragua	3	https://doi.org/10.1111/j.1756-1051.2013.00138.x
Takayama et al.	2013	Across distribution	9[a]	https://doi.org/10.3732/ajb.1200567
Cerón-Souza et al.	2012	Atlantic and Pacific Panama	6	https://doi.org/10.1186/1471-2148-12-205
Sandoval-Castro et al.	2012	Pacific Mexico	6	https://doi.org/10.1016/j.aquabot.2012.01.002
Pil et al.	2011	Brazil	8	https://doi.org/10.3732/ajb.1000392
Cerón-Souza et al.	2010	Across distribution	6	https://doi.org/10.3732/ajb.0900172
Arbeláez-Cortes et al	2007	Pacific Colombia	3	https://doi.org/10.1007/s10750-007-0622-9
		Mean	6.6	
		SD	2.0	

[a]Authors utilized multiplex PCR reactions

had assembled read sequences. A subset of 34 of these loci was selected from across all possible perfect repeat motifs (di-, tri-, tetra-, and pentanucleotides) and tested with 16 *R. mangle* individuals from two collection sites in Florida, USA (Avalon: 27.5468, -80.3297; Pine Island: 28.4841, −80.7237; n = 8 per site). We used the DNeasy Plant Mini Kit to isolate genomic DNA from these 16 individuals, as described above.

We aimed to combine multiple loci into a limited number of multiplex reactions, so we performed singleplex testing for all loci with identical PCR conditions. We followed the PCR method for a single set of cycles outlined in Culley et al. (2013): 95 °C for 15 min; 35 cycles at 94 °C for 30 s, 57 °C for 90 s, 72 °C for 60 s; 60 °C for 30 min. We used the Type-it® Microsatellite PCR Kit (Qiagen, Valencia, California, USA) with a total volume of 6 µL per reaction, with 2.5 µL Multiplex PCR Master Mix, 0.5 µL primer mix (0.2 µM of each forward and reverse), 1 µL dH_2O, and 2 µL of genomic DNA (~20 ng). We performed PCR on a Prime thermal cycler (Techne, Straffordshire, UK), and assessed amplification via electrophoresis on 1.5% agarose gels. Of the 34 loci, 18 produced consistent bands, whereas others did not amplify or produced multiple size bands. For these 18 loci, we ordered new forward primers with additional sequences at the 5′ end that correspond to universal primers with fluorescent labels (6-FAM, HEX, or PET), and used a three-primer method to fluorescently label PCR products, as described in Culley et al. (2013). We analysed fragments on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA) and scored alleles in the R-package Fragman (Covarrubias-Pazaran et al. 2016). A total of 15 loci produced easily-identifiable peaks and nine were polymorphic in the 16 Florida samples (Table 2).

Multiplex design

Utilizing the same PCR conditions and three-primer method outlined above, we initiated multiplex testing with 28 loci (15 developed here and 13 published). We selected RM19, RM38, RM41 (Rosero-Galindo et al. 2002), and RM50, RM86 (Takayama et al. 2008) based on our previous experience (Kennedy et al. 2016, 2017), and selected eight loci developed by Ribeiro et al. (2013): RmBra18, RmBra19, RmBra20, RmBra25, RmBra50, RmBra59, RmBra64, RmBra66. RmBra25 was discarded due to inconsistent singleplex amplification. We combined the remaining 27 loci into three initial multiplexes based simply on fragment length differences. Loci were discarded due to inconsistent multiplex amplification (RzMg07, RzMg30, RM86), difficult-to-score peaks (RmBra66, RM41) or monomorphism (RzMg04, RzMg05, RzMg08, RzMg16, RzMg18, RzMg25, RmBra64).

We combined the remaining 15 loci into two multiplex reactions with seven and eight loci each (Table 3). We used the same PCR volumes and conditions described above (see Appendix S1 for a protocol outline).

Multiplex testing

We assessed multiplexes with 103 *R. mangle* individuals from four collection sites: one site in Florida, USA with 31 samples (Jupiter: 26.8179, −80.0480), two sites in The Bahamas, at either end of the archipelago, with 35 samples (New Providence: 24.9920, −77.3868) and 33 samples (Inagua: 21.0954, −73.6300), and one site at this species' northern limit in Texas, USA where only four trees were found (Río Bravo: 25.9526, −97.1513) (Fig. 1). Distances between collection sites range from approximately 335 km (Jupiter–New Providence) to 2500 km (Río Bravo–Inagua). We used the DNeasy Plant Mini Kit to isolate genomic DNA from these individuals, as described above, and voucher material from each collection site was deposited at the Manchester Museum Herbarium (Table 4). We analysed PCR products and scored alleles as described above (see Appendix S2 for genotype data). For each collection site, we determined the number of alleles and private alleles per locus, calculated observed and expected heterozygosity, calculated inter-site genetic differentiation (F_{ST}), and tested for linkage disequilibrium and deviations from Hardy–Weinberg equilibrium after adjusting for multiple comparisons with FSTAT 2.9.3.2 (Goudet 2002). Only allele numbers were determined for the four Texas individuals.

We then evaluated the ability of the 15-locus genotypes generated from these multiplexes to differentiate among the three collection sites from Florida and the Bahamas (inter-site distances ranged from 335–900 km; n = 99 individuals) compared to genotypes with fewer loci, comparable to numbers utilized in previous research (Table 1). To do this, we performed a discriminant analysis of principal components (DAPC) (Jombart et al. 2010) in the R-package adegenet 2.1.1 (Jombart and Ahmed 2011) in R v3.4.2 (R Core Team 2013). DAPC first transforms data with a principal components analysis and then performs a discriminant analysis on the principal components retained (Jombart and Collins 2015). We performed an initial analysis on the complete data set (i.e., individuals with 15-locus genotypes from both multiplexes), and then subsequent analyses on subsets with 7-locus genotypes (i.e., only data from multiplex 1) and with 8-locus genotypes (i.e., only data from multiplex 2). For each analysis, we retained the minimum number of principal components that explained ~90% of the total variance, which corresponded to 15, 9, and 8 principal components, respectively, and then retained both discriminant functions. We extracted each individual's coordinates on the two

Table 2 Characteristics of 15 microsatellite loci developed for *Rhizophora mangle*

Locus	Primer sequences (5′-3′) (*,**,*** indicate additional sequence at 5′ end)		Repeat motif	Approx size range (bp)	Fluorescent label	GenBank accession no
RzMg04	F:	*GGAGAGTTTGCTCCAAAGTCCAAACC	$(ATT)_{27}$	378	6-FAM	MN256326
	R:	GGTGATGGAAATGAAGAGAATAATGGC				
RzMg05	F:	**CTAATGCATCGTCCATCATCGC	$(AAC)_{39}$	272–275	HEX	MN256327
	R:	AGGTCTCTGAGATAGCAAATACATAACG				
RzMg08	F:	***TGGGATTCATTCATTTCTGAGTAGGC	$(ATT)_{24}$	295	PET	MN256328
	R:	GAAAGAAGCTTGCTTCATCTTAGAACC				
RzMg09	F:	*AATTTTGTTTCCACACACGATTCCG	$(ATT)_{39}$	336–340	6-FAM	MN256329
	R:	CAATAAACGAGTCACCATATAGGAACC				
RzMg10	F:	*GTGCTTTAACCGTAATGCATCTATCC	$(AAAT)_{32}$	317–325	6-FAM	MN256330
	R:	ATGTCCCTCAATGTGACTCTTGGC				
RzMg15	F:	**GCAATTAGGTGCAGACCAGGATGG	$(AAAT)_{32}$	343	HEX	MN256331
	R:	TGGCTCTGTTTCGTTTTGATCATGG				
RzMg16	F:	*TGTAATCTCAAATCGTAGCATAGCG	$(ATT)_{33}$	266	6-FAM	MN256332
	R:	GAACTGTCTCAATTGTTTCAAGTCTGC				
RzMg18	F:	***ACTACCACCAGTGGCAAATCACTGC	$(TCC)_{24}$	338	PET	MN256333
	R:	GACAAATGACAACGGGAAAGCAAGC				
RzMg21	F:	*CAAACGTCGCTCCTATTTCCGTACC	$(TTC)_{30}$	427–431	6-FAM	MN256334
	R:	TTTATGACTGGAGGCAGCAAAGTGG				
RzMg25	F:	**AGATCACTAGCCGAGTTGCTTTGGC	$(AAC)_{27}$	337	HEX	MN256335
	R:	TGTCTCTCTCATCTGCTTACGAAGTGC				
RzMg28	F:	*CACGACAAATACGGAAATAGAAGGG	$(ATC)_{30}$	355–378	6-FAM	MN256336
	R:	TCGAACTGCAATGGAAATAAAGTCG				
RzMg30	F:	***AGATTCGCCGTCCCACTAATCTGG	$(CGG)_{27}$	305–314	PET	MN256337
	R:	AAAACTAGAGCCGTACCGTTGTTGC				
RzMg32	F:	***TAGAGCAATGGCTGCCGTGATATGG	$(TC)_{26}$	386–388	PET	MN256338
	R:	AAGATGAAGGGACGGGATTTAAGCG				
RzMg33	F:	**ACTGTCCACTGAAGAATCCAAACGC	$(TC)_{34}$	390–400	HEX	MN256339
	R:	CCACAGTTTAATGCTACTTCAAAAGCC				
RzMg34	F:	***TCTCGATCTCGTCAAGTGTAACATGC	$(TC)_{22}$	436–438	PET	MN256340
	R:	ACCTCTAGCTCCCTGCTCCTTCAGC				

Note Additional sequences at 5′ end and corresponding fluorescent labels outlined in Culley et al. (2013)

*M13(-21) tail: TGTAAAACGACGGCCAGT

**T7term tail: CTAGTTATTGCTCAGCGGT

***M13 modified B tail: CACTGCTTAGAGCGATGC

principal axes of the DAPC (i.e., ind.coord) to then plot in ggplot2 (Wickham 2011).

Results

We found that 14 of the 15 loci in the two multiplexes were polymorphic across these four collection sites (Table 4). We identified 57 total alleles (Table 3), with a range from 44 alleles (Jupiter, Florida) to only 20 alleles (Río Bravo, Texas). Alleles per locus within sites ranged from 1 to 6, with expected heterozygosity from 0.00 to 0.72, and 16 private alleles were identified (Table 4). F_{ST} indicated considerable genetic differences, with a range from 0.22 (Jupiter–New Providence) to 0.52 (Jupiter–Inagua). Expected heterozygosity was generally higher than observed, but few loci deviated from Hardy–Weinberg equilibrium (Table 4). We found no evidence of linkage disequilibrium.

DAPC with the complete dataset of 15-locus genotypes (14 loci were polymorphic), identified clear delineations between the three collection sites in Florida and the Bahamas (Fig. 2A). In contrast, subsets with 7-locus and 8-locus genotypes (6 and 8 loci were polymorphic, respectively) identified a similar pattern, but were unable to clearly differentiate these collection sites (Fig. 2B, C).

Table 3 Multiplex PCR panels for *Rhizophora mangle*. Refer to Appendix S1 for a protocol outline

	Locus	Primer sequence (5′-3′) (*,**,*** indicate additional sequence at 5′ end)		Repeat motif	Approx size range (bp)	A	F Primer (μM)	R Primer (μM)	Tail (μM)	Fluorescent label	References
Rm Multiplex1	RmBra19	F:	*GAGGCAGAGTCAGGTCAGAA	$(CT)_{14}$	122—140	5	0.075	0.250	0.250	6-FAM	Ribeiro et al. (2013)
		R:	CACTGGTCCACTGACAGCAA								
	RM19	F:	*TGCCCTCTACGTTGTGAATG	$(AG)_{26}$	154—170	5	0.050	0.200	0.200	6-FAM	Rosero-Galindo et al. (2002)
		R:	CTGTCTGAGCTTGCATCATTG								
	RmBra18	F:	**TCAAGGACAGGTCAACAG CA	$(TC)_{18}$	181—187	4	0.050	0.200	0.200	HEX	Ribeiro et al. (2013)
		R:	TGAATTAAGACGTCAAAT CATCG								
	RmBra59	F:	*GTGAACGGTCTGGACTGGAG	$(AG)_{24}$	193—211	5	0.075	0.250	0.250	6-FAM	Ribeiro et al. (2013)[a,b]
		R:	TCACCGATCCCCTAGAACTG								
	RM50	F:	***ACACACACACACAGAGAG AGAG	$(AC)_6(AG)_{17}$	223—229	3	0.050	0.200	0.200	PET	Takayama et al. (2008)[a,c]
		R:	ATGGTTTCACACATTCAACAT								
	RzMg15	F:	**GCAATTAGGTGCAGACCA GGATGG	$(AAAT)_{32}$	343	1	0.050	0.200	0.200	HEX	Present study
		R:	TGGCTCTGTTTCGTTTTGATC ATGG								
	RzMg28	F:	*CACGACAAATACGGAAAT AGAAGGG	$(ATC)_{30}$	354—359	8	0.075	0.250	0.250	6-FAM	Present study
		R:	TCGAACTGCAATGGAAATAAA GTCG								
Rm Multiplex2	RmBra20	F:	**TCAGCACAATACATCAGG ACAA	$(AG)_{16}$	164—176	5	0.050	0.200	0.200	HEX	Ribeiro et al. (2013)[a,b]
		R:	GGCGCTATCCATCCTGAGT								
	RmBra50	F:	**ATCGTGGAAGAACGGGGTTT	$(AG)_{18}$	227—231	3	0.050	0.200	0.200	HEX	Ribeiro et al. (2013)[a,b]
		R:	TCAAGAAGTCCAGGGTGCTT								
	RM38	F:	*CTCATGCACACGGATACA GACAC	$(CA)_8$	234—238	3	0.050	0.200	0.200	6-FAM	Rosero-Galindo et al. (2002)
		R:	TGTTCCTATTCCTCAAATGAT GCC								
	RzMg09	F:	*AATTTTGTTTCCACACACGAT TCCG	$(ATT)_{39}$	427—433	3	0.050	0.200	0.200	6-FAM	Present study
		R:	GACAATAAACGAGTCACCATA TAGGAACC								

Table 3 (continued)

Locus	Primer sequence (5′-3′) (*,**,*** indicate additional sequence at 5′ end)		Repeat motif	Approx size range (bp)	A	F Primer (μM)	R Primer (μM)	Tail (μM)	Fluorescent label	References
RzMg32	F:	***TAGAGCAATGGCTGCCGTGATATGG	$(TC)_{26}$	386—388	2	0.050	0.200	0.200	PET	Present study
	R:	AAGATGAAGGGACGGGATTTAAGCG								
RzMg33	F:	**ACTGTCCACTGAAGAATCCAAACGC	$(TC)_{34}$	390—400	4	0.050	0.200	0.200	HEX	Present study
	R:	CCACAGTTTAATGCTACTTCAAAAGCC								
RzMg21	F:	*CAAACGTCGCTCCTATTTCCGTACC	$(TTC)_{30}$	333—342	4	0.050	0.200	0.200	6-FAM	Present study
	R:	TTTATGACTGGAGGCAGCAAAGTGG								
RzMg34	F:	***TCTCGATCTCGTCAAGTGTAACATGC	$(TC)_{22}$	436—438	2	0.050	0.200	0.200	PET	Present study
	R:	ACCTCTAGCTCCCTGCTCCTTCAGC								

Note Additional sequences at 5′ end and corresponding fluorescent labels outlined in Culley et al. (2013)

A number of alleles identified across 103 *R. mangle* individuals

*M13(-21) tail: TGTAAAACGACGGCCAGT

**T7term tail: CTAGTTATTGCTCAGCGGT

***M13 modified B tail: CACTGCTTAGAGCGATGC

[a]Locus also amplified in neotropical *R. racemosa*;

[b]In neotropical *R. harrisonii*;

[c]In multiple Indo-West Pacific *Rhizophora* species in the corresponding reference

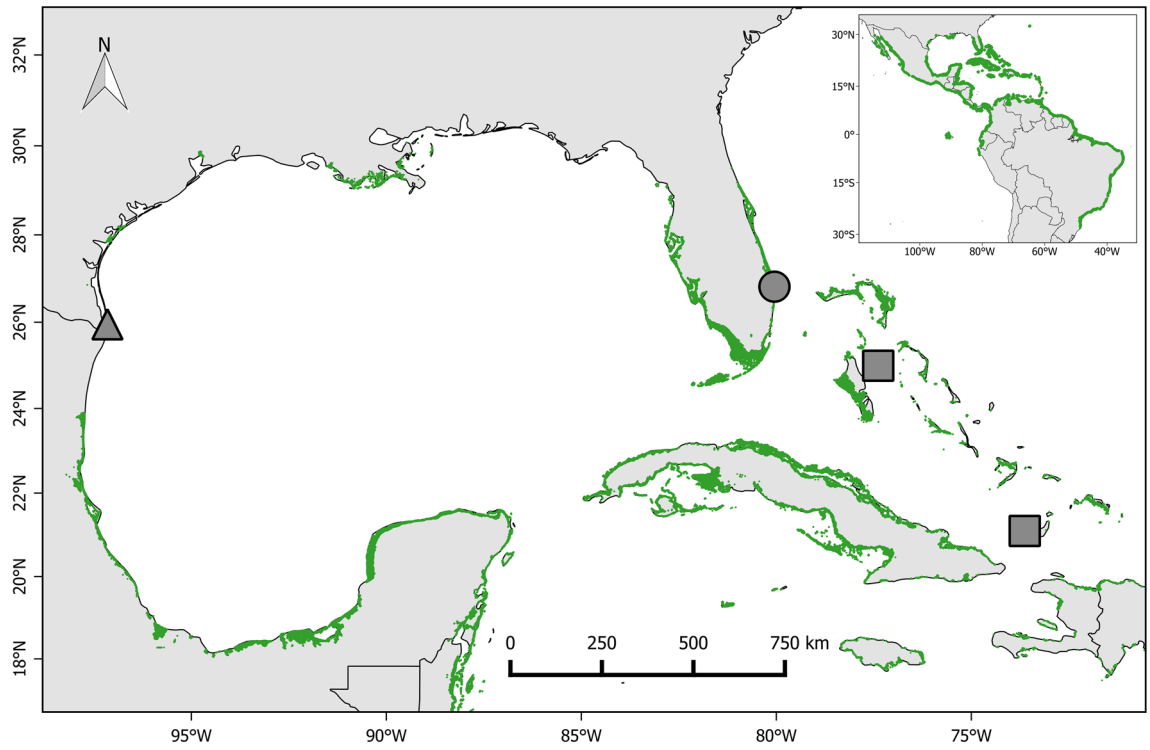

Fig. 1 Four *Rhizophora mangle* collection sites towards this species' northern latitudinal limits. From left to right: Río Bravo, Texas (triangle), Jupiter, Florida (circle), New Providence, The Bahamas and Inagua, The Bahamas (squares). Neotropical mangrove distribution shown in green (Giri et al. 2011)

Discussion

Better-informed mangrove conservation practices are needed as the ecosystem services provided by these intertidal forests continue to be threatened by increasing anthropogenic pressures and climate change (Friess et al. 2019). Population genetic data can provide insights necessary to understand and continue to monitor species for conservation and management purposes (Schwartz et al. 2007; Kramer and Havens 2009). Understanding the importance of population-genetic insights to mangrove conservation, researchers have made a substantial effort to develop genetic markers for the widespread neotropical red mangrove, *Rhizophora mangle*. There are now a total of 57 microsatellite loci available for *R. mangle*, with 42 previously-published loci (Rosero-Galindo et al. 2002; Takayama et al. 2008; Ribeiro et al. 2013; Francisco et al. 2018a) and 15 novel loci from this work. Yet, empirical research, on average, employs less than seven of these available loci, likely because few studies have incorporated multiplex reactions (Table 1). Here, we outlined multiplex PCR panels that combine efforts of four geographically-distant research groups into a tool that should enable us to better outline genetic patterns in this widespread species, and do so with considerable less investment in time and resources. In this discussion, we highlight the continued utility of genetic data in mangrove conservation in the era of next-generation sequencing and urge researchers to use, modify, and improve upon this genetic tool to characterize *R. mangle* population genetics across the Neotropics and answer pressing conservation questions.

Conservation research seems to be in a transition from genetics to genomics as we continue to improve our ability to generate and analyse high-throughput sequence data (Puckett 2017). Genomics will enable researchers to address many new questions and, in certain contexts, provide greater resolution, but the investment in increased data is not always needed (Shafer et al. 2015). To address certain questions, and at certain spatial-scales, genetic data sets may prove sufficient and much more cost effective (Shafer et al. 2015; Puckett 2017), and this certainly seems true in terms of many outstanding questions in mangrove conservation. A reliable panel of microsatellites would be more appropriate for smaller-scale studies with moderate sample sizes, which constitutes most *R. mangle* research to date (Table 1), or when repeated measures are needed, as in the case of monitoring ongoing reforestation projects, as genome sequencing is most cost effective with large numbers of samples (Puckett 2017). Low quantity and quality DNA, as is often the case in mangrove species because leaf tissues are rich in molecular by-products (Huang et al. 2002), can also be

Table 4 Genetic diversity of multiplex PCR panels for *Rhizophora mangle* from four collection sites: Jupiter, Florida (USA); New Providence, The Bahamas; Inagua, The Bahamas; and Río Bravo, Texas (USA)

	Locus	Jupiter (n = 31)				New Providence (n = 35)				Inagua (n = 33)				Río Bravo (n = 4)	
		A	PA	H_O[a]	H_E	A	PA	H_O	H_E	A	PA	H_O[a]	H_E	A	PA
Rm Multiplex1	RmBra19	4	2	0.29	0.40	3		0.49	0.57	1		0.00	0.00	1	
	RM19	3		0.42	0.58	4	1	0.26	0.24	3		0.18	0.29	2	1
	RmBra18	3	1	0.16	0.21	3	1	0.11	0.16	2		0.24	0.47	1	
	RmBra59	3	1	0.32	0.43	4	1	0.40	0.48	3		0.27*	0.58	1	
	RM50	3		0.16	0.31	3		0.69	0.67	3		0.42	0.57	1	
	RzMg15	1		0.00	0.00	1		0.00	0.00	1		0.00	0.00	1	
	RzMg28	6	1	0.32*	0.53	5	1	0.69	0.72	4		0.24*	0.44	1	
Rm Multiplex2	RmBra20	4	1	0.26	0.36	3	1	0.43	0.53	1		0.00	0.00	2	
	RmBra50	2		0.23	0.25	3	1	0.37	0.43	2		0.00	0.06	1	
	RM38	2		0.45	0.49	3		0.63	0.55	2		0.00	0.06	2	
	RzMg09	3	1	0.39	0.50	2		0.34	0.39	2		0.31	0.50	2	
	RzMg32	2		0.19	0.32	1		0.00	0.00	2		0.03	0.03	1	
	RzMg33	3	1	0.52	0.57	3	1	0.40	0.53	2		0.03	0.09	1	
	RzMg21	3		0.48	0.64	3		0.43	0.61	3		0.06	0.12	2	
	RzMg34	2		0.06	0.06	2		0.40	0.44	2		0.15	0.28	1	
	Total	44	8			43	7			33	0			20	1
	Mean	2.93		0.28	0.38	2.87		0.38	0.42	2.20		0.13	0.23	1.33	
	SD	1.16		0.15	0.19	1.06		0.21	0.23	0.86		0.14	0.23	0.49	

Voucher accession numbers: EM650682, EM650683, EM650684, and EM650685, respectively

A number of alleles, *PA* private alleles, H_O observed heterozygosity, H_E expected heterozygosity

[a]Significant deviation from Hardy–Weinberg equilibrium (*P < 0.05)

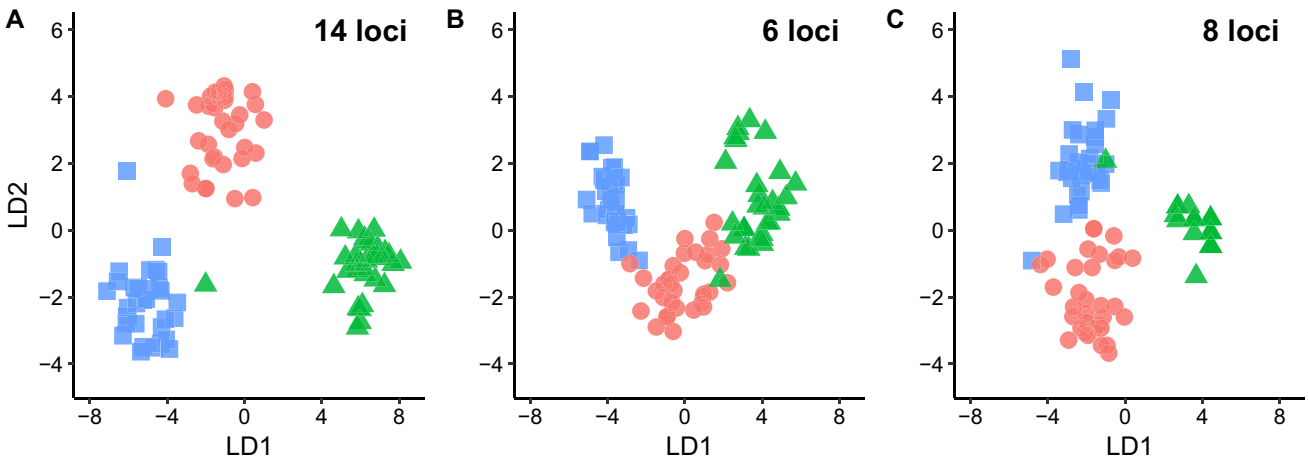

Fig. 2 Multiplex PCR panels performed better than data subsets with numbers of loci comparable to previous research. Scatterplots of discriminant analysis of principal components (DAPC) for **A** the complete data set (both multiplexes with 14 polymorphic loci), **B** only multiplex 1 (6 polymorphic loci), **C** only multiplex 2 (8 polymorphic loci). Individuals from Jupiter, Florida are shown with blue squares, New Providence, The Bahamas are shown with red circles, and Inagua, The Bahamas are shown with green triangles

an impediment to sequence library preparation, but microsatellite amplification often requires limited DNA template. Marker development is the principal investment for microsatellites, but this cost has already been paid by the multiple research groups outlined above, and many of these markers have been tested in multiple published works. The multiplex PCR panels outlined here are the product of these diverse genetic resources and discoveries, and should provide an

easy-to-use and cost-effective (both in terms of time and resources) tool.

Of course, the utility of this tool relies on whether microsatellites provide sufficient polymorphism to answer conservation and management questions across the broad distribution of this species. We demonstrated that the 14 polymorphic loci in these multiplex panels, clearly delineated three populations near the northern limits of this species' distribution and performed better than data subsets with loci numbers comparable to previous research. Although perhaps obvious, greater resolution with these multiplex panels is the result of genotyping twice as many loci as previous studies, congruent with observations based on SNP loci (Hodel et al. 2017), and of utilizing our previous experience to selectively choose loci that have proven informative. However, we have only shown that these multiplex panels are an efficient tool to genotype *R. mangle* from four populations across ~ 2500 km, a fraction of the entire distribution of this species. Although we are confident these multiplex panels will prove informative across a much broader spatial scale, we also envision this tool as a framework that can easily be modified depending on variation in the pool of available microsatellite loci for a particular region. For instance, Kennedy et al. (2016) discarded two loci (RM21, RM46; Rosero-Galindo et al. 2002) due to monomorphism across much of the Caribbean, a pattern also observed in Atlantic Mexico (Cisneros-de la Cruz et al. 2018). These same loci exhibited considerable polymorphism in Pacific *R. mangle* from Panama, Nicaragua, and Mexico (Cerón-Souza et al. 2012; Sandoval-Castro et al. 2012, 2014; Bruschi et al. 2014). Researchers can modify these multiplex primer mixes (see Appendix S1) to include additional informative loci and/or exclude loci that exhibit monomorphism, while maintaining only two PCR per sample. This framework is much more cost effective than protocols used in previous research and should enable the inclusion of more samples and collection sites in future research. These multiplex panels should also facilitate further genetic studies to address multiple pressing conservation questions, such as generating baseline genetic data from areas that have not been studied [i.e., much of Central America, Pacific South America, Caribbean islands, and West Africa (although considerable work has been done in *R. racemosa*; Ngeve et al. 2016)], better defining management units to prioritize conservation measures (Wee et al. 2019), and monitoring genetic effects of restoration projects (Granado et al. 2018).

Conclusions

We developed multiplex panels with novel and published *Rhizophora mangle* microsatellite loci to generate 15-locus genotypes, more than twice the average number of loci used in previous research, in only two PCR (see Appendix S1 for quick protocol). We demonstrated utility across ~ 2500 km of this species' widespread distribution, and that these multiplex panels were better able to delineate three populations near the northern limits of this species' distribution than data subsets with numbers of loci comparable to previous research. This tool improves our ability to characterize *R. mangle* genetic patterns while saving researchers considerable time and resources, enables future research to include more samples and collection sites, can be easily modified to incorporate alternative informative loci, and should facilitate studies to answer multiple pressing conservation and management questions.

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