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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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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:	**TCAAGGACAGGTCAACAGCA	$(TC)_{18}$	181—187	4	0.050	0.200	0.200	HEX	Ribeiro et al. (2013)
		R:	TGAATTAAGACGTCAAATCATCG								
	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:	***ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_{17}$	223—229	3	0.050	0.200	0.200	PET	Takayama et al. (2008)[a,c]
		R:	ATGGTTTCACACATTCAACAT								
	RzMg15	F:	**GCAATTAGGTGCAGACCAGGATGG	$(AAAT)_{32}$	343	1	0.050	0.200	0.200	HEX	Present study
		R:	TGGCTCTGTTTCGTTTTGATCATGG								
	RzMg28	F:	*CACGACAAATACGGAAATAGAAGGG	$(ATC)_{30}$	354—359	8	0.075	0.250	0.250	6-FAM	Present study
		R:	TCGAACTGCAATGGAAATAAAGTCG								
Rm Multiplex2	RmBra20	F:	**TCAGCACAATACATCAGGACAA	$(AG)_{16}$	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:	*CTCATGCACACGGATACAGACAC	$(CA)_8$	234—238	3	0.050	0.200	0.200	6-FAM	Rosero-Galindo et al. (2002)
		R:	TGTTCCTATTCCTCAAATGATGCC								
	RzMg09	F:	*AATTTTGTTTCCACACACGATTCCG	$(ATT)_{39}$	427—433	3	0.050	0.200	0.200	6-FAM	Present study
		R:	GACAATAAACGAGTCACCATATAGGAACC								

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

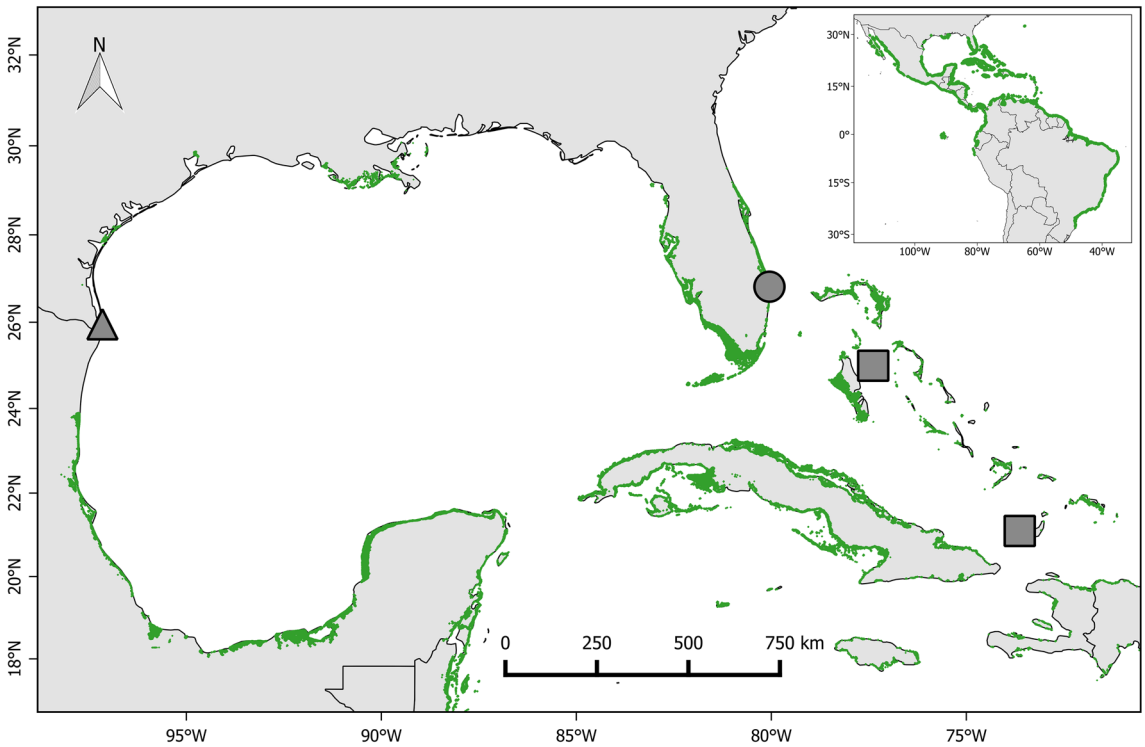

Fig. 1 Four *Rhizophora mangle* collection sites towards this species' northern latitudinal limits. From left to right: Río Bravo, Texas (triangle), Jupiter, Florida (circle), New Providence, The Bahamas and Inagua, The Bahamas (squares). Neotropical mangrove distribution shown in green (Giri et al.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

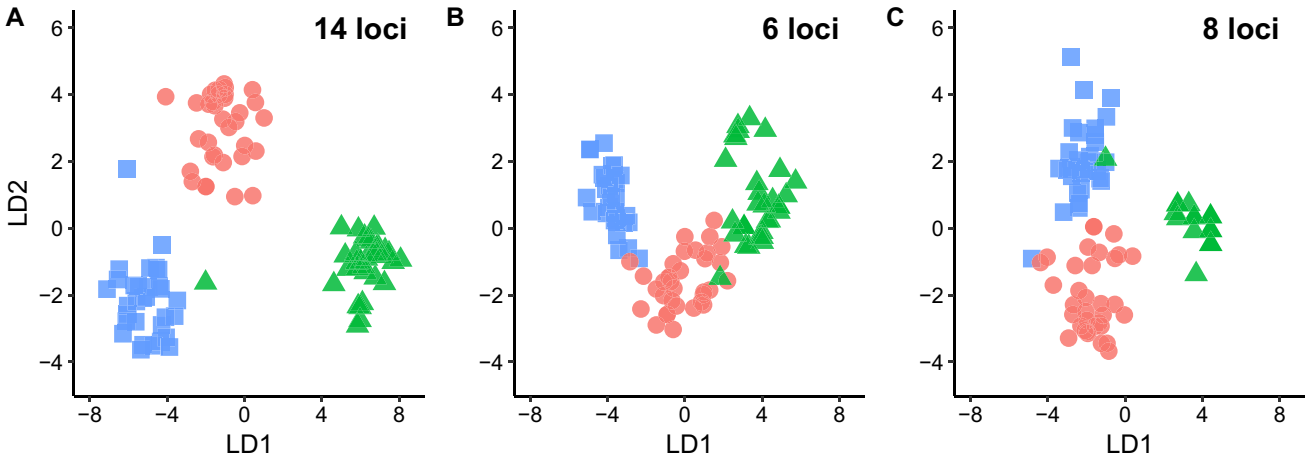

Fig. 2 Multiplex PCR panels performed better than data subsets with numbers of loci comparable to previous research. Scatterplots of discriminant analysis of principal components (DAPC) for **A** the complete data set (both multiplexes with 14 polymorphic loci), **B** only multiplex 1 (6 polymorphic 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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