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GENETIC DIVERSITY AND MATING SYSTEM OF *Rhizophora mangle* L. (RHIZOPHORACEAE) IN NORTHERN BRAZIL REVEALED BY MICROSATELLITE ANALYSIS

FRANCISCO, P. M. TAMBARUSSI, E. V. ALVES, F. M. BAJAY, S. CIAMPI-GUILLARDI, M. SOUZA, A. P. Genetic diversity and mating system of *Rhizophora mangle* L. (Rhizophoraceae) in Northern Brazil revealed by microsatellite analysis. **CERNE**, v. 24, n. 4, p. 295-302, 2018.

HIGHLIGHTS

Rhizophora mangle has low occurrence of self-pollination and low occurrence of mating among relatives.

One to 12 pollen donors fertilized each seed tree. However, Genetic diversity was low.

The number of seed trees required for seed collection ($\hat{m}$) was 62 and ranged from 40 to 107.

This study provides ecological and management implications for *R. mangle*.

Keywords:
Brazilian Atlantic Forest
Conservation genetics
Microsatellite markers
Population genetics
Tropical tree species

ABSTRACT

Rhizophora mangle L. (Rhizophoraceae) grows on aerial roots, which emerge above the water level, giving stands of this tree the characteristic "mangrove" appearance. To produce in situ and ex situ information for genetic conservation programs for this species, we investigated the genetic diversity and mating system of one *R. mangle* population. We sampled 30 adult trees and a total of 349 seeds in Northern Brazil. We genotyped all adult trees and seeds with four microsatellite loci. The average fixation index was -0.222 for adult trees and 0.030 for seeds. The multilocus outcrossing rate (t_m=0.921) was significantly lower than unity (1.0). There was no substantial evidence of null alleles nor genotypic disequilibrium among the loci. The combined power to exclude the first parent probability was 0.921. The average coancestry coefficient ($\overline{\theta}$=0.180) was similar to that expected for half-sib progenies ($\widetilde{\overline{\theta}}$=0.125). Thus, the number of adult trees necessary for seed collection to obtain progeny arrays with an effective size of 150 was estimated to be 62. In conclusion, this study produced important information for the management and conservation of *R. mangle* and will contribute to conservation and management programs for this species.

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INTRODUCTION

The *Rhizophora* genus dominates many tropical mangrove forests worldwide and plays pivotal roles in mangrove ecosystems (Duke & Allen, 2006). Mangroves, which are heterogeneous habitats with an uncommon variety of animals and plants adapted to conditions of high salinity, frequent flooding and muddy anaerobic soil, cover approximately 137,700 km^2 worldwide (Giri et al., 2011).

These mangrove trees have morphological characteristics and adaptations, including aerial roots and a fast-growing canopy, that contribute to efficient nutrient retention mechanisms, water retention and balance sheet carbon estimates (Ezcurra et al., 2016). *Rhizophora* species are mainly pollinated by the wind, as they have considerably more pollen grains than ovaries per flower (Tomlinson, 1986). The viviparous propagules of these species are dispersed by water, and their dispersion occurs under the influence of ocean currents (Francisco et al., 2018).

Rhizophora mangle L. is a key woody plant of the *Rhizophoraceae* mangrove family. Widely distributed throughout the Atlantic East-Pacific biogeographic (AEP) region (Tomlinson, 1986; Takayama et al., 2013), *R. mangle* was present in the Neotropical region 40 million years ago (Graham, 2006). In addition, *R. mangle* is one of the main representative species of this ecosystem in Brazil.

Although important ecosystems, mangroves are being devastated by anthropic actions. In this context, it is urgent to produce data that can help in the conservation of species that are part of mangrove habitats. The development of molecular techniques has generated opportunities to drive research on mangroves in new directions and increase knowledge on the subject of mangrove ecosystems (Triest, 2008). *R. mangle* plants along the eastern coast of South America demonstrate a strong genetic structure due to the influence of oceanic currents, which act both as a barrier to and a facilitator of gene flow depending on the population location (Francisco et al., 2018; Pil et al., 2011; Takayama et al., 2013). The *R. mangle* genetic structure consists of an admixture gradient between two gene pools within the northern coast, whereas populations from the southern coast are homogeneous with a low genetic diversity (Francisco et al., 2018; Pil et al., 2011).

The species is pollinated by anemophily (wind) and by entomophily (insects) (De Menezes et al., 1997). Pollinated *R. mangle* flowers mature in approximately 95 days, producing buoyant hypocotyls also known as propagules (Alongi, 2015). R. mangle is a living species with propagules that germinate and mature on the maternal tree before dropping off. These propagules are large and curved and are dispersed by water fluctuations until they settle, if they find an environment with adequate conditions (McKee & Rooth, 2008). The effective genetic conservation of a species requires knowledge about its mating system and genetic diversity. In this study, we investigated genetic patterns such as the genetic diversity of *Rhizophora mangle* in the equatorial coast of Brazil using microsatellite markers.

MATERIALS AND METHODS

Study Area, Sampling and Genotyping

One population from the northern coast of Brazil in the municipality of Salinópolis, Pará State (47° 22' 25" W, 00° 36' 18" S), was chosen to study the mating system of *R. mangle* (Figure 1).

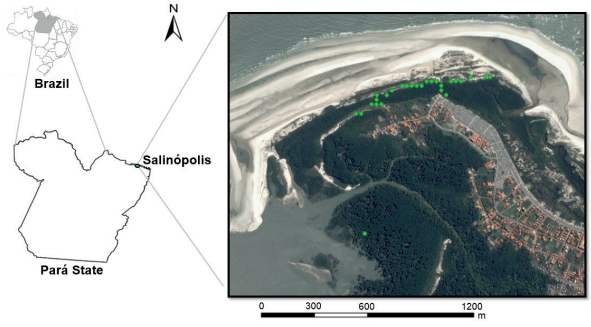

FIGURE 1 Locations of the *Rhizophora mangle* population from the northern coast of Brazil in the municipality of Salinópolis, Pará State. The spatial distribution of the collected genotypes is indicated by green dots.

Leaves were collected from 30 adult trees, and 10 to 12 seeds were collected from each adult tree ($n = 349$), with a minimum distance of 10 m between each adult tree. The geographic coordinates of each adult tree were collected using a GPS receiver (Garmin 76CSx, WGS-84 standard, Garmin International Inc., Olathe, KS, USA). DNA extractions were performed using the DNeasy® Plant Mini Kit (Qiagen, Hilden, DE, Germany). We used four microsatellites loci (Rma3-14, Rma3-23, Rma3-37 and Rma3-38) developed by Francisco et al. (2018), and the procedures for DNA amplification and allele scoring were based on Francisco et al. (2018).

Analysis of Genetic Diversity, Inbreeding, Relatedness and Effective Population Size

To assess the genetic diversity of adults and progenies, genetic diversity was characterized per locus and as an average across all loci using the following

indices: average number of alleles per locus (*k*), observed heterozygosity (H_o), and expected heterozygosity in Hardy-Weinberg equilibrium (H_e). To check whether there was inbreeding in the adults (seed trees) and progeny, we used the within-population fixation index (F_{IS}); 1,000 bootstrap replicates were used to calculate the 95% confidence interval limits for the fixation index. The statistical significance of was tested by permuting alleles among individuals. All analyses were performed using the R (R Development Core Team, 2015) package diveRsity (Keenan et al., 2013), and bootstrapped confidence intervals were also calculated for each F_{IS} value. Linkage was estimated for adult trees using the FSTAT program (Goudet, 1995). We estimated null allele frequencies using FreeNa software (Chapuis & Estoup, 2007). For adult trees, the power to exclude the first parent (when none of the relatives were known) (*P1_parent*) was estimated using Cervus 3.0.3 (Marshall et al., 1998).

The average coancestry coefficient within ($\bar{\theta}_w$) and among ($\bar{\theta}_a$) of all progeny arrays was estimated by the maximum-likelihood estimator, and 1,000 bootstrap replicates were used to calculate the 95% confidence intervals for the coancestry index in Software Coancestry (Wang, 2011). The effective population sizes within and among for the progeny arrays were estimated following Sebbenn (2006).

Mating System Analysis

Mating system analyses were performed with the multilocus analysis program MLTR v.3.4 (Ritland, 2002). This analysis considers the reproduction system based on a mixed model (Ritland & Jain, 1981). The MS (maximization expectation) numerical method was adopted for the families in the analysis with a 95% confidence interval (CI) and 10,000 bootstrap resampling. The multilocus outcrossing rate (t_m), single-locus outcrossing rate (t_s), crossing between related species ($t_m - t_s$), multilocus correlation inbreeding (r_s), and multilocus correlation pollen ($r_{(pm)}$)were estimated for the whole population and each adult tree. Mating system indices were used to estimate other indices as follows. The effective number of pollen donors was calculated by $Nep = 1/rp$. The number of adult trees required for seed collection to yield a progeny array with an effective size of 150 was estimated with the equation $\hat{m} = N_{e(reference)} / \hat{N}_{e(v)}$ (Sebbenn, 2006).

RESULTS

Genetic diversity, inbreeding, relatedness and effective population size

When we evaluated the genetic diversity and inbreeding of the total sample, we found 10 alleles in the adult trees and 12 alleles in the seeds, with two private alleles within the seed arrays (Table 1). The size of the private alleles was 242 (frequency of 0.722) for locus RM7576 and 200 (frequency of 0.003) for locus RM4546.

The observed heterozygosity (H_o) was similar for adult trees and seeds only for locus RM7576; however, the expected heterozygosity (H_e) was similar for all loci, and the average heterozygosity significantly departed from Hardy-Weinberg equilibrium.

Several authors around the world have studied *R. mangle*. Thus, a wide range of research focusing on hybridization and genetic diversity is available in the literature. However, mating system research for this species is still scarce. These authors have used up to 15 markers (ranging from 3 to 15) to obtain similar estimates. For this species, the number of alleles appears to range from 1 to 10. The measures of observed heterozygosity are very similar (ranging from zero to 0.80) among studies published from 2007 until today (Table 2). Therefore, the markers used in this research show robustness in the analyses and can be used for reliable estimates.

TABLE 1 Genetic diversity and fixation index (F) parameters for four microsatellite loci in adults and progeny of the *Rhizophora mangle* population from the northern coast of Brazil in the municipality of Salinópolis, Pará State.

		Locus/Sampled generation		
Adults	*k*	H_o	H_E	*F (95% CI)*
RM7576	2	0.53	0.44*	-0.200* (-0.500 to 0.139)
RM4546	3	0.52	0.49*	-0.052* (-0.401 to 0.301)
RM7374	3	0.67	0.46	-0.465 (-0.668 to -0.306)
RM2728	2	0.10	0.10*	-0.053* (-0.132 to -0.016)
Total	10	-	-	-
Mean	2.5	0.454	0.372*	-0.222 (-0.047 – -0.418)
SD	0.58	0.2133	0.158	-
Seeds		H_o	H_E	*F (95% CI)*
RM7576	3	0.51	0.42	-0.231 (-0.298 to -0.159)
RM4546k	4	0.27	0.50*	0.453* (0.360 to 0.544)
RM7374	3	0.55	0.45	-0.236 (-0.330 to -0.140)
RM2728	2	0.05	0.06	0.261 (-0.026 to 0.511)
Total	12	-	-	-
Mean	3	0.346	0.358	0.030 (-0.026 to 0.084)
SD	0.81	0.201	0.174	-

*Significant departures from Hardy-Weinberg equilibrium at $P < 0.05$; 95% CI indicates the 95% confidence interval.

TABLE 2 Range of observed heterozygosity (Ho) in populations of *Rhizophora mangle*.

Authors	Number of SSRs	Range of number of alleles/locus	Range of observed heterozygosity (Ho)
Rosero-Galindo et al. (2002)	10	2 – 7	0.20 – 0.80
Arbeláez-Cortes et al. (2007)	3	3 – 6	0.34 – 0.60
Pil et al. (2011)*	8	NS	0.01 – 0.26
Ribeiro et al. (2013)	7		
São Paulo population		3 – 10	0.04 – 0.64
Bragança population		3 – 11	0.17 – 0.75
Florida population		1 – 10	0 – 0.52
Kennedy et al. (2016)*	7	NS	0.20 – 0.48
Basyuni et al. (2017)	5	2 – 5	0.189 – 0.405
Francisco et al. (2018)*	15	NS	0 – 0.65
Present study	4	2 – 4	0 – 0.67

*mean for each population, NS: alleles per locus not shown

In terms of the average, the fixation index presented a higher level of heterozygosity for adult trees than for seeds (Table 1; Figure 2). The average fixation index (F_{IS}) had higher values in seeds, with an overall value of 0.03 (ranging from -0.026 to 0.084), than in adult trees, with an overall value of -0.222 (ranging from -0.047 to -0.418) (Figure 2). Thus, these results indicate the occurrence of endogamy in seeds after reproductive event.

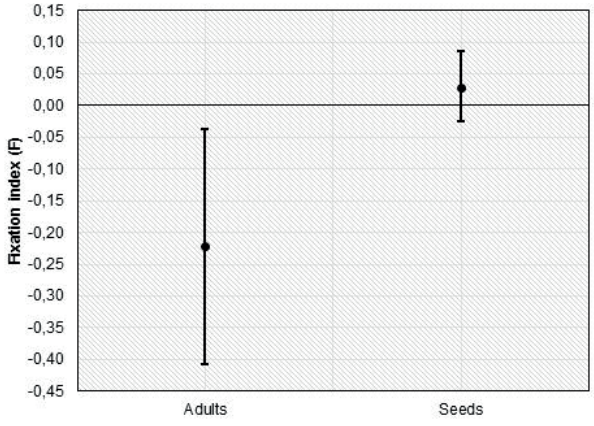

FIGURE 2 Intrapopulation fixation index (FIS) parameters estimated with four microsatellite loci in adult trees and progeny arrays (seeds) of the *Rhizophora mangle* population from the northern coast of Brazil in the municipality of Salinópolis, Pará State.

The FreeNa software analysis revealed null alleles at one locus (RM4546) (Table 3).

No linkage disequilibrium was detected in the studied populations for an adjusted P-value at the 5% nominal level (data not shown). All loci were appropriate for genetic population studies on *R. mangle*. The average power to exclude the first parent (*P1_parent*) was 0.921, and this value indicates that these loci are suitable for future parentage studies.

Mating System

The multilocus outcrossing rate (t_m) was not significantly different from unity (1.0), indicating a low occurrence of self-pollination (Table 3). However, the single-locus outcrossing rate (t_s) was significantly lower than unity (1.0) and significantly lower than the multilocus outcrossing rate, resulting in a nonsignificant difference in $t_m - t_s$. This result suggests the low occurrence of mating among relatives. The multilocus paternity correlation for the population ($r_{p(m)}$) was significantly greater than zero, indicating correlated mating (Table 4). However, the multilocus paternity

TABLE 3 Null allele estimates for four microsatellite loci in progeny arrays (seeds) of the *Rhizophora mangle* population from the northern coast of Brazil in the municipality of Salinópolis, Pará State.

Family	Markers			
	RM7576	RM4546	RM7374	RM2728
F1	0.000	0.000	0.000	0.001
F2	0.001	0.000	0.000	0.001
F3	0.000	0.000	0.000	0.000
F4	0.000	0.000	0.000	0.001
F5	0.000	0.000	0.000	0.001
F6	0.000	0.201	0.000	0.001
F7	0.000	0.167	0.000	0.001
F8	0.000	0.265	0.000	0.001
F9	0.000	0.200	0.000	0.000
F10	0.000	0.001	0.000	0.000
F11	0.000	0.000	0.000	0.000
F12	0.000	0.000	0.000	0.001
F13	0.000	0.000	0.000	0.001
F14	0.000	0.008	0.000	0.001
F15	0.000	0.000	0.000	0.001
F16	0.000	0.000	0.000	0.001
F17	0.000	0.000	0.000	0.001
F18	0.000	0.000	0.000	0.001
F19	0.000	0.001	0.000	0.001
F20	0.000	0.000	0.000	0.001
F21	0.000	0.000	0.000	0.001
F22	0.000	0.001	0.024	0.001
F23	0.000	0.000	0.000	0.024
F24	0.000	0.188	0.000	0.001
F25	0.000	0.001	0.000	0.001
F26	0.000	0.167	0.000	0.001
F27	0.000	0.001	0.000	0.001
F28	0.001	0.000	0.000	0.001
F29	0.000	0.00	0.000	0.001
F30	0.000	0.000	0.000	0.001

Notes: Bold values present evidence of a null allele, assuming a null frequency < 0.20.

correlation was significantly lower among families (Table 5). Consequently, the effective number of pollen donors was significantly lower within progenies, which indicates that between one and thirteen pollen donors fertilized each adult tree. The selfing correlation (r_s) was significantly different from zero, demonstrating variation in the outcrossing rate.

TABLE 4 Mating system parameters for *Rhizophora mangle* populations (95% CI indicates the 95% confidence interval) from the northern coast of Brazil in the municipality of Salinópolis, Pará State.

Parameter	Estimates (95% CI)
Number of adult trees (number of progenies)	30 (349)
Multilocus outcrossing rate:	0.921 (0.911 to 0.930)
Single-locus outcrossing rate:	0.846 (0.805 to 0.886)
Mating among relatives:	0.075 (0.046 to 0.106)
Selfing correlation:	0.166 (0.166 to 0.266)
Multilocus paternity correlation:	0.624 (0.493 to 0.655)
Effective number of pollen donors:	1.6 (1.5 to 2.0)
Number of adult trees: m	62 (40 to 107)

At the progeny level, the multilocus paternity correlation ($r_{p(m)}$) ranged from 0.080 to 0.712, indicating correlated mating (Table 4). Consequently, the effective number of pollen donors presented wide variation among progenies (N_{ep}), which indicated that from one to 12 pollen donors fertilized each adult tree.The average coancestry coefficient among progenies ($\bar{\theta}_a$) was 0.180, and the variance effective size ($N_{e(a)}$) was 2.41. The number of adult trees required for seed collection ($\hat{m}$) was 62 and ranged from 40 to 107. The average coancestry coefficient within progenies ($\bar{\theta}_w$) ranged among adult trees from 0.105 to 0.353, and the variance in effective population size among adult trees ($N_{e(w)}$) ranged from 1.4 to 3.7 (Table 4).

DISCUSSION

Genetic diversity was low for this studied population of *R. mangle*. With the set of *R. mangle* primer pairs that were used in this study, we found a total of 12 alleles. However, no inbreeding was detected using

TABLE 5 Mating system (±SD, standard deviation) indices for open-pollinated progeny arrays from the *Rhizophora mangle* population from the northern coast of Brazil in the municipality of Salinópolis, Pará State.

Progeny	*n*	t_m	t_s	t_m-t_s	r_s	$r_{p(m)}$	N_{ep}	$\bar{\theta}_w$	$N_{e(w)}$
F1	12	0.986±0.011	0.945±0.015	0.041±0.005	0.100±0.005	0.139±0.016	7.2	0.153	2.8
F2	12	0.922±0.020	0.902±0.006	0.020±0.014	0.115±0.008	0.179±0.048	5.6	0.131	3.1
F3	10	0.981±0.014	0.952±0.016	0.029±0.005	0.182±0.032	0.285±0.045	3.5	0.112	3.4
F4	12	0.849±0.031	0.870±0.018	-0.021±0.016	0.105±0.004	0.120±0.017	8.3	0.211	2.1
F5	11	0.928±0.019	0.913±0.009	0.015±0.010	0.108±0.006	0.111±0.016	9.0	0.117	3.4
F6	12	0.982±0.014	0.943±0.016	0.039±0.005	0.106±0.005	0.115±0.015	8.7	0.157	2.7
F7	12	0.981±0.013	0.940±0.014	0.041±0.004	0.111±0.006	0.104±0.016	9.6	0.105	3.7
F8	11	0.934±0.016	0.910±0.007	0.024±0.010	0.106±0.004	0.124±0.015	8.0	0.127	3.1
F9	12	0.964±0.016	0.931±0.012	0.033±0.005	0.120±0.007	0.154±0.015	6.5	0.110	3.6
F10	12	0.975±0.015	0.899±0.010	0.076±0.016	0.160±0.027	0.345±0.084	2.9	0.141	3.0
F11	12	0.970±0.016	0.929±0.012	0.041±0.006	0.109±0.005	0.142±0.026	7.0	0.280	1.7
F12	11	0.942±0.018	0.915±0.009	0.027±0.010	0.125±0.016	0.237±0.085	4.2	0.152	2.8
F13	12	0.924±0.023	0.907±0.009	0.017±0.014	0.127±0.015	0.236±0.084	4.2	0.133	3.0
F14	12	0.906±0.029	0.900±0.007	0.006±0.022	0.128±0.015	0.243±0.082	4.1	0.150	2.8
F15	12	0.967±0.016	0.933±0.013	0.034±0.004	0.104±0.004	0.098±0.011	10.2	0.133	3.1
F16	12	0.662±0.032	0.734±0.074	-0.072±0.046	0.107±0.002	0.101±0.006	9.9	0.168	2.6
F17	12	0.831±0.046	0.890±0.009	-0.059±0.009	0.095±0.003	0.078±0.010	12.8	0.236	2.0
F18	12	0.860±0.029	0.837±0.038	0.023±0.025	0.094±0.004	0.079±0.011	12.6	0.109	3.6
F19	11	0.815±0.069	0.872±0.018	-0.057±0.019	0.137±0.014	0.304±0.019	3.3	0.149	2.8
F20	12	0.930±0.012	0.904±0.007	0.026±0.008	0.121±0.012	0.207±0.067	4.8	0.282	1.6
F21	12	0.916±0.019	0.905±0.006	0.011±0.014	0.114±0.007	0.164±0.040	6.1	0.202	2.2
F22	12	0.991±0.009	0.760±0.048	0.231±0.055	0.481±0.142	0.885±0.102	1.1	0.155	2.8
F23	12	0.488±0.163	0.758±0.069	-0.270±0.003	0.113±0.005	0.120±0.003	8.3	0.132	3.1
F24	12	0.724±0.128	0.870±0.025	-0.146±0.105	0.151±0.028	0.286±0.083	3.5	0.353	1.4
F25	11	0.934±0.016	0.881±0.013	0.053±0.019	0.124±0.012	0.229±0.062	4.4	0.191	2.3
F26	11	0.900±0.017	0.893±0.007	0.004±0.012	0.094±0.003	0.080±0.009	12.5	0.210	2.1
F27	12	0.605±0.130	0.805±0.058	-0.197±0.076	0.130±0.012	0.199±0.034	5.0	0.216	2.1
F28	12	0.669±0.095	0.840±0.035	-0.174±0.062	0.108±0.002	0.116±0.005	8.6	0.274	1.7
F29	11	0.993±0.007	0.950±0.016	0.043±0.010	0.267±0.088	0.712±0.186	1.4	0.218	2.0
F30	10	0.808±0.061	0.885±0.010	-0.077±0.051	0.101±0.001	0.101±0.003	9.9	0.317	1.5

the average fixation index (*F*) estimate at the adult level. Inbreeding was detected in the progeny array (Figure 1). The number of alleles obtained in this study was lower than that obtained by Pil et al. (2011) for the same species from the Brazilian coast, although those authors used a different set of loci and number of individuals across 10 populations. We observed an average heterozygosity of 0.454 and an average expected heterozygosity of 0.372 for adult trees (Table 1), and these results were similar to those of other studies (Forti et al., 2014; Kennedy et al., 2017). Forti. et al. and Kennedy et al. found low outcrossing rates, suggesting that a high rate of selfing decreased the genetic diversity. Evidence of low genetic diversity has been detected by previous studies on mangrove species (Basyuni et al. 2017; Pil et al., 2011).

The effect of the mating system on genetic diversity has been observed in other studies on tropical tree species (Tambarussi et al., 2016). For our results, the mating system analysis presented high outcrossing rates (≥0.900 in 20 families; Table 4) and evidence of mating between relatives only for one progeny (F22) (Table 4). Similar to our study, in Mexico, Sandoval-Castro et al. (2012) found reduced genetic diversity and increased inbreeding in *R. mangle*. Some studies investigating mating systems have found evidence of selfing for *R. mangle* (Lowenfeld & Klekowski, 1992). However, the mating system patterns of a tree species can be controlled by the environment and genetic processes. Thus, some adult trees, populations or even reproductive events may present higher or lower percentages of self-fertilization (Tambarussi et al., 2016; Tambarussi et al., 2017). This result can be observed in the *R. mangle* population of this study, which demonstrated progeny array self-fertilization values ranging from 0.488 (for F16) to 0.993 (F30) (Table 4). Due to the detected selfing, the average coancestry coefficient within progenies ($\overline{\theta}$ =0.180) and effective size variance (N_e=2.41) of this population were similar to those expected for panmictic populations ($\overline{\theta}$ =0.125, $N_e \approx$ 4). Thus, the open-pollinated progenies were composed mainly of half-sibs families. These percentages may have strong implications for the collection of seeds for the conservation, restoration of degraded areas and for the preimprovement of the tree species (Tambarussi et al., 2017).

CONCLUSIONS

This study provides ecological and management implications for *R. mangle*. Despite the small number of microsatellite loci studied in this population, we found an increase in the endogamy of the studied seed generation, even with the observation of high outcrossing rates. This fact can be explained by the process of forest fragmentation. Other studies have shown similar levels of genetic diversity with only five (Basyuni et al., 2017) and seven (Kennedy and Garavelli, 2017) microsatellite loci. The four microsatellite markers in this study presented a high power of parent exclusion, moderate genetic diversity and no linkage disequilibrium. Therefore, all these analyses indicated that this set of microsatellite loci could be used without restriction in studies on the genetic diversity, genetic structure, mating system, and parentage of *R. mangle*.

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