# Isolation of Microsatellite Markers for the Red Mangrove, Rhizophora mangle (Rhizophoraceae)

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PRIMER NOTE

# ISOLATION OF MICROSATELLITE MARKERS FOR THE RED MANGROVE, *RHIZOPHORA MANGLE* (RHIZOPHORACEAE)[1]

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- *Premise of the study:* Three species of the mangrove tree genus *Rhizophora* are found in the New World and along the west coast of Africa. Of these, *R. mangle* is the most abundant and has a complex interbreeding relationship with the sympatric *R. racemosa* and *R. harrisonii*. The development of additional microsatellite markers would permit paternity analyses and investigation of the hybrid origin of these species.
- *Methods and Results:* Using an enriched library method, via hybridization with biotinylated oligonucleotides complementary to repetitive poly AG/TC, primers for 11 microsatellite markers of *R. mangle* were developed and characterized in populations in Pará and São Paulo (Brazil) and Florida (USA). Ten of these markers were transferable to *R. racemosa* and *R. harrisonii*.
- *Conclusions:* The microsatellite markers presented here will be useful in studies of contemporary and historical gene flow between American and West African *Rhizophora* species.

**Key words:** mangrove; microsatellites; *Rhizophora harrisonii*; *Rhizophora racemosa*; Rhizophoraceae.

In the New World and along the west coast of Africa, three species of the mangrove tree genus *Rhizophora* L. (Rhizophoraceae) have been identified. Of these, *R. mangle* L. (red mangrove) is the most widely distributed (Tomlinson, 1986). In terms of interbreeding, *R. mangle* has a complex relationship with the sympatric *R. racemosa* G. Mey. and *R. harrisonii* Leechm. (Céron-Souza et al., 2010), which could potentially be resolved by the use of microsatellite markers. Two studies have previously reported the isolation of microsatellite markers for *R. mangle*. In Rosero-Galindo et al. (2002), 10 polymorphic loci with between two and seven alleles per locus were reported (26 alleles total). In Takayama et al. (2008), 14 simple sequence repeat (SSR) loci were reported and 11 were shown to be polymorphic, with a total of 32 and 27 alleles in populations from the Pacific and Atlantic coasts of Costa Rica, respectively. The allelic diversity reported for previously isolated microsatellite markers for this species is therefore somewhat low, and this is also the case in subsequent studies that made use of these markers. Arbeláez-Cortes et al. (2007) report a total of 17 alleles for three markers in five studied populations, Pil et al. (2011) report 22 alleles for eight markers in 10 populations, and Sandoval-Castro et al. (2012) report 19 alleles for six markers in 10 populations. For this reason, we isolated and characterized new microsatellite markers for *R. mangle*, which were then subsequently tested for transferability to *R. racemosa* and *R. harrisonii*. These markers present higher allelic diversity than has previously been reported. In conjunction with pre-existing markers, these new markers will permit mating systems analysis, paternity tests, and future studies attempting to resolve the potential hybrid origin of *Rhizophora* species.

## METHODS AND RESULTS

Genomic DNA was isolated from leaf tissue of one adult individual of *R. mangle* sampled in the Caeté estuary, Bragança, Pará, northern Brazil (0°52′22″S, 46°39′04″W), and used in the development of the enriched library. Herbarium material was deposited at the herbarium of the Institute for Coastal Research in Bragança (HBRA; accession no. 821). Polymorphism was evaluated using the 24 adult trees of *R. mangle* from Pará, Brazil (0°50′27″S, 46°38′15″W; HBRA 706); São Paulo, Brazil (24°00′21″S, 46°17′59″W); and Florida, USA (25°08′09″N, 80°57′29″W; HBRA 1291). The São Paulo and Florida sites are near the southernmost and northernmost distributional limits of *R. mangle*, respectively. For the transferability test, we genotyped seven individuals of *R. harrisonii* and five individuals of *R. racemosa.*

An enriched library was constructed according to Vinson et al. (2005). Total DNA (50,000 ng) was digested with *Sau*3I and separated on a 2% agarose gel. Fragments of 300–800 bp were recovered with purification of the gel using QIAquick Gel Extraction kit (QIAGEN, São Paulo, Brazil). DNA fragments were ligated to adapters, hybridized to biotinylated $(AG)_{13}$ and $(TC)_{13}$, and the oligofragments separated using streptavidin magnetic beads. Selected fragments were ligated into a pGEM-T Easy Vector (Promega Corporation, Madison, Wisconsin, USA) and transformed in competent *E. coli* XL1-Blue cells that were grown overnight on 1× Luria–Bertani agar plates containing ampicillin, X-gal, and IPTG. Transformants (white colonies) were diluted in water, and the fragment inserted into the plasmid was amplified using the M13 universal primers (M13F[–20] and M13R[–40]) by PCR. PCR products were sequenced on a MegaBACE 1000 instrument (GE Healthcare, Belo Horizonte, Minas Gerais, Brazil) using dye terminator fluorescent chemistry (Sanger method) with the

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Table 1. Characteristics of 11 microsatellite loci developed in *Rhizophora mangle*.

| Loci | Primer sequences (5′–3′) | Repeat motif | Allele size (bp) | $T_a$ (°C) | GenBank accession no. |
|---|---|---|---|---|---|
| RmBra18 | F: TCAAGGACAGGTCAACAGCA | $(TC)_{18}$ | 174 | 54 | HQ850264 |
| | R: TGAATTAAGACGTCAAATCATCG | | | | |
| RmBra19 | F: GAGGCAGAGTCAGGTCAGAA | $(CT)_{14}$ | 162 | 54 | HQ850265 |
| | R: CACTGGTCCACTGACAGCAA | | | | |
| RmBra20 | F: TCAGCACAATACATCAGGACAA | $(AG)_{16}$ | 141–175 | 50 | HQ850255 |
| | R: GGCGCTATCCATCCTGAGT | | | | |
| RmBra25 | F: TCCATTCTGCCAAATTTGATT | $(AG)_{13}$ | 172–226 | 50 | HQ850256 |
| | R: CGAATTCACCGATCACAATG | | | | |
| RmBra27 | F: TCTTTTGTCGAAGCCTCTCC | $(CT)_3(TC)_6(CT)_4(TC)_3$ | 140–184 | 50 | HQ850257 |
| | R: ATCAAGTTGGCGTTGGAAAA | | | | |
| RmBra45 | F: GAAAATGCAAGAGGGCTGAC | $(AG)_{17}(AGG)_3$ | 128–164 | 54 | HQ850258 |
| | R: CATGGGTCTCTCTCCTCTGC | | | | |
| RmBra50 | F: ATCGTGGAAGAACGGGGTTT | $(AG)_{18}$ | 149–215 | 50 | HQ850259 |
| | R: TCAAGAAGTCCAGGGTGCTT | | | | |
| RmBra59 | F: GTGAACGGTCTGGACTGGAG | $(AG)_{24}$ | 145–209 | 54 | HQ850260 |
| | R: TCACCGATCCCCTAGAACTG | | | | |
| RmBra64 | F: CAAGGCTAATCCGAAAATCG | $(AG)_{15}$ | 148 | 54 | HQ850266 |
| | R: ACTCTGTCCCCTGCCTCAC | | | | |
| RmBra65 | F: CCATAGACAATACAGGATACCCAGA | $(AG)_{12}$ | 234 | 52 | HQ850263 |
| | R: CAGCATGAACATCACCTTGG | | | | |
| RmBra66 | F: TCAGCACAATACATCAGGACAA | $(AG)_{19}$ | 138–206 | 54 | HQ850261 |
| | R: GCGCTATCCATCCTGAGTTT | | | | |

*Note*: $T_a$ = annealing temperature.

M13F(−20) primer. The sequences were subsequently edited manually using Sequencher version 4.1.4 software (Gene Codes Corporation, Ann Arbor, Michigan, USA). Primers were designed in the regions flanking AG/CT repeats using Primer3 OUTPUT (Rozen and Skaletsky, 2000) with the following conditions: final amplicon between 100 and 350 bp, average annealing temperature of 55°C, primer length between 18 and 22 bp, and GC content between 40% and 60%. We sequenced 66 clones, and 23 pairs of primers were synthesized. The preliminary polymorphism test was conducted with 12 individuals from the Caeté estuary (Bragança). PCR products were visually compared with the 10-bp ladder in a 10% polyacrylamide gel stained with ethidium bromide.

From the 23 primers, 11 primers produced PCR products, seven loci were polymorphic, and four loci were monomorphic (RmBra18, RmBra19, RmBra64, and RmBra65; Table 1). Forward primers of the seven loci were fluorescently labeled with 6-FAM (RmBra20, RmBra25, and RmBra59; MWG-Biotech, Ebersberg, Germany) or HEX (RmBra27, RmBra45, RmBra50, and RmBra66; MWG-Biotech). Loci were amplified using PCR in a total volume of 13 µL containing: DNA (5 ng), 1× PCR reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), forward primer and reverse primer (0.15 µM), $MgCl_2$ (1.5 mM), bovine serum albumin (BSA; New England Biolabs, Hitchin, Hertfordshire, United Kingdom; 0.75 mg·mL$^{-1}$), dNTP (0.5 mM), and 1.3 U *Taq* polymerase (Invitrogen, Life Technologies, Carlsbad, California, USA). PCR conditions were: denaturation at 96°C for 2 min; 30 cycles of denaturation at 94°C for 15 s, annealing at 50–54°C (Table 1) for 15 s, and extension at 72°C for 15 s; and a final extension at 72°C for 5 min. Transfer of the primers of *R. mangle* to *R. racemosa* and *R. harrisonii* was tested under the same conditions as for *R. mangle* (described above). Fragments were genotyped on a 96-capillary sequencer MegaBACE 1000 (GE Healthcare). Fragments were analyzed using MegaBACE Fragment Profiler version 1.2 (Amersham Bioscience, Belo Horizonte, Minas Gerais, Brazil) using MegaBACE ET 550-R Rox Size Standard (Amersham Bioscience). Number of alleles per locus, observed and expected heterozygosities, coefficient of fixation index, and Hardy–Weinberg and linkage disequilibrium were calculated using FSTAT (Goudet, 2002). Probabilities of paternity exclusion were estimated using CERVUS (Kalinowski et al., 2007). Frequency of null alleles was estimated using MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004).

Results show a total of 90 alleles in the three studied populations (49 in Bragança, 39 in São Paulo, and 42 in Florida), and the number of private alleles varied between one to eight alleles per locus (Table 2). Observed heterozygosity was lower than values of heterozygosity under expected Hardy–Weinberg equilibrium (HWE) for all populations tested, with higher fixation index values for the São Paulo and Florida populations (Table 2). The test of pairwise linkage after the Bonferroni correction showed values higher than the significant value of 0.005, indicating that these loci are not linked and can be used as independent markers. Only one locus, RmBra27, showed significant null alleles using the Oosterhout index (0.26). Fisher's exact test revealed deviations from HWE after Bonferroni correction at two loci for all populations tested, with an excess of homozygotes for the loci RmBra25, RmBra27, and RmBra50 ($P = 0.019$). The combined exclusion power of all seven loci was 0.998, 0.998, and 0.982 for first parent total exclusion probabilities for the Bragança, São Paulo, and Florida populations, respectively. Transfer of the primers was successful, with six polymorphic markers identified for *R. mangle* also polymorphic in *R. racemosa* and *R. harrisonii* (except RmBra25), while the four monomorphic markers identified for *R. mangle* were only tested for amplification.

## CONCLUSIONS

The high number of private alleles in the studied populations indicates that these loci are useful for studies of genetic divergence, genetic structure, and phylogeography. In addition, the parental exclusion probability values are adequate for progeny analysis and paternity tests for further identification of reproduction barriers and identification of hybridization between species of all American and West African *Rhizophora* species.

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TABLE 2. Results of primer screening for seven polymorphic microsatellite loci in three populations of *Rhizophora mangle*.

| | Bragança | | | | | | São Paulo | | | | | | Florida | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Loci | *N* | *A* | $A_p$ | $H_e$ | $H_o$ | $F_{IS}$ | $P_{ex}$ | *N* | *A* | $A_p$ | $H_e$ | $H_o$ | $F_{IS}$ | $P_{ex}$ | *N* | *A* | $A_p$ | $H_e$ | $H_o$ | $F_{IS}$ | $P_{ex}$ |
| RmBra20 | 22 | 10 | 3 | 0.82 | 0.59 | 0.29 | 0.55 | 23 | 6 | 2 | 0.76 | 0.52 | 0.32 | 0.65 | 23 | 10 | 4 | 0.78 | 0.52 | 0.34 | 0.62 |
| RmBra25 | 21 | 6 | 3 | 0.59 | 0.29 | 0.52* | 0.81 | 20 | 7 | 4 | 0.61 | 0. 25 | 0.59* | 0.80 | 15 | 1 | 0 | 0.0 | 0.00 | 0.00 | 1.00 |
| RmBra27 | 20 | 4 | 1 | 0.27 | 0.50 | 0.82* | 0.96 | 24 | 4 | 1 | 0.50 | 0.42 | 0.91* | 0.89 | 15 | 5 | 4 | 0.36 | 0.13 | 0.64* | 0.93 |
| RmBra45 | 22 | 10 | 6 | 0.66 | 0.73 | –0.09 | 0.73 | 24 | 3 | 1 | 0.12 | 0.04 | 0.66* | 0.99 | 19 | 7 | 2 | 0.54 | 0.21 | 0.61* | 0.84 |
| RmBra50 | 24 | 3 | 0 | 0.29 | 0.17 | 0.44* | 0.96 | 22 | 10 | 8 | 0.84 | 0.32 | 0.62* | 0.52 | 17 | 5 | 2 | 0.37 | 0.18 | 0.53* | 0.93 |
| RmBra59 | 20 | 5 | 3 | 0.60 | 0.65 | –0.08 | 0.82 | 22 | 6 | 2 | 0.72 | 0.64 | 0.12 | 0.69 | 21 | 8 | 4 | 0.65 | 0.38 | 0.42 | 0.76 |
| RmBra66 | 24 | 11 | 7 | 0.83 | 0.75 | 0.09 | 0.54 | 23 | 3 | 1 | 0.67 | 0.39 | 0.43 | 0.78 | 20 | 6 | 2 | 0.43 | 0.25 | 0.43 | 0.90 |
| Average | | | 7 | 0.58 | 0.46 | 0.21 | | | 5.6 | | 0.60 | 0.31 | 0.48 | | | 6 | | 0.48 | 0.24 | 0.47 | |

*Note*: *A* = number of alleles; $A_p$ = number of private alleles; $F_{IS}$ = fixation index; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity; *N* = number of samples; $P_{ex}$ = first parental exclusion probability.

* Indicates signification deviations of Hardy–Weinberg equilibrium.

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