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Hybridization and introgression in New World red mangroves, *Rhizophora* (Rhizophoraceae)[1]

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- *Premise of the study:* Hybridization is common in both animals and plants and can lead to a diverse array of outcomes ranging from the generation of new ecotypes or species to the breakdown of morphological differences. Here, we explore the extent of hybridization in the three currently recognized New World *Rhizophora* species—*R. mangle*, *R. racemosa*, and the putative hybrid species *R. harrisonii*.
- *Methods:* We assayed variation across the three recognized *Rhizophora* species using two noncoding chloroplast (cpDNA), two flanking microsatellite regions (FMRs), and six microsatellite loci.
- *Key results:* Gene genealogies of cpDNA and FMRs showed a strong phylogeographic break across the Central American Isthmus, but little relationship to recognized species boundaries. Instead, individuals collected in the same ocean basin and classified as *R. mangle* and *R. racemosa* by morphological characteristics were more closely related to each other than with similar looking individuals collected in the other ocean basin. Nonetheless, there were low, yet significant differences at microsatellite loci among co-occurring populations of *R. mangle* and *R. racemosa* in both ocean basins, suggesting that two taxonomic groups coexist. However, we found no genetic evidence that *R. harrisonii* was a hybrid species. Rather, *R. harrisonii* appears to represent a morphotype produced by ongoing hybridization and backcrossing between *R. mangle* and *R. racemosa*.
- *Conclusions:* Our data support ancient and persistent introgressive hybridization among new world *Rhizophora* and argue for a full revision of the systematic relationships of the group based on much finer morphological, ecological, and genetic analyses.

Key words: Central American Isthmus; evolutionary history; hybridiation; introgression; *Rhizophora harrisonii*; *Rhizophora mangle*; *Rhizophora racemosa*, Rhizophoraceae.

Hybridization is common in both animals and plants, and its role in evolution is becoming more widely appreciated (Mallet, 2005). The evolutionary consequences of these processes are diverse and include increased genetic diversity, the generation of new ecotypes or species, and breakdown or reinforcement of isolation barriers (Rieseberg and Wendel, 1993; Emms and Arnold, 1997, 2004, 2006; Fritz et al., 2006). In addition, the introgression of genes across species provides a pathway for the exchange of potentially adaptive variation (Rieseberg and Soltis, 1991; Baack and Rieseberg, 2007). This possibility promoted our interest in studying the potential role of hybridization in the evolutionary history of the three species of *Rhizophora* in the neotropics.

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Rhizophora L. is an old and widespread diploid genus that has been present in the neotropics since the Early Eocene (50 Ma). Ten million years later, during the Middle Eocene, its distribution expanded around the world (Rico-Gray, 1993; Graham, 1995; Ellison et al., 1999; Duke et al., 2002; Tyagi, 2003). During this period, the neotropics underwent significant geological changes including the independent development of the Greater and Lesser Antilles Islands in the Caribbean (Iturralde-Vinent, 2006), the Pliocene completion of the Central American Isthmus (CAI) (Coates and Obando, 1996), and repeated periods of cooling and sea level change (Graham, 2006).

Today, *Rhizophora* is the most conspicuous genus in tropical, coastal mangrove ecosystems. Currently, three red mangrove species are recognized in the neotropics (i.e., Atlantic-East Pacific biogeographic region—AEP) including *R. mangle* (Linnaeus, 1753), within which Pacific Ocean populations were historically referred to as *R. samoensis* (Hochreutiner, 1925), *R. racemosa* (Meyer, 1818), and a presumed hybrid species *R. harrisonii* (Leechman, 1918). The distribution of these three red mangroves, hereafter referred to as the New World *Rhizophora*, extends across the CAI. *Rhizophora mangle* is the most widespread and the only one of the three species to extend beyond the tropical belt (Afzal-Rafii et al., 1999). *Rhizophora racemosa* and *R. harrisonii* are more restricted to equatorial regions with warmer temperatures and less arid conditions (Duke et al., 1998, 2002; Afzal-Rafii et al., 1999; Dodd and Afzal-Rafii, 2000; Duke and Allen, 2006). The New World *Rhizophora* species overlap in the continental margins of the eastern Atlantic Ocean (i.e., western

Africa and South America, from northern Brazil to Venezuela) and in the east Pacific Ocean (South and Central America from Ecuador to southern Mexico) (Fig. 1) (Salvoza, 1936; Hou, 1960; Tomlinson, 1986; Jiménez, 1987; Duke and Allen, 2006).

The three species of New World *Rhizophora* are primarily distinguished by the number of flowers per inflorescence, although this diagnostic character appears to be extremely plastic and the number of flowers varies within currently recognized species (see Fig. 2) (Hou, 1960). Additionally, where the three species overlap, they appear to be ecologically differentiated and occupy different coastal zones (Leechman, 1918; Savory, 1953; Jonker, 1959; Breteler, 1969; Santos, 1986; Jiménez and Sauter, 1991; Duke et al., 1998; Afzal-Rafii et al., 1999). *Rhizophora mangle* is considered the most salt tolerant of the three species and is found in the lower intertidal zone (Jiménez, 1987; Smith, 1992), while *R. harrisonii* and *R. racemosa* are more typically found at middle intertidal positions with lower salinity (Savory, 1953; Smith, 1992; Afzal-Rafii et al., 1999; Duke et al., 2002; Duke and Allen, 2006). Some authors also suggest that *R. harrisonii* occupies intermediate habitats between *R. mangle* and *R. racemosa* (Leechman, 1918; Savory, 1953; Jonker, 1959). However, the taxonomic position of *R. harrisonii* is more controversial due to conflicting ecological, morphological, and chemical evidence (Afzal-Rafii et al., 1999; Duke and Allen, 2006). This difficulty has prevented botanists from reaching a consensus about how many species are in the genus, the geographical range of these species, and the levels of ecological and reproductive isolation among them (Rico-Gray, 1981; Tomlinson, 1986; Jiménez, 1994; Duke et al., 2002).

In the Indo-West Pacific (IWP) biogeographic region, morphological (Tomlinson and Womersley, 1976; Muniyandi and Natajaran, 1985; Kathiresan, 1995; Duke et al., 1998; Duke, 2006), and genetic evidence (Parani et al., 1997; Tyagi, 2002) indicate significant hybridization among *Rhizophora* species, suggesting that the evolutionary history of the genus in the New World could be similarly complex. Accordingly, we examined population and species boundaries in *R. mangle*, *R. harrisonii*, and *R. racemosa* using a variety of molecular markers with the goal of addressing two important issues. First, we explored the role of changes in geology and climate in the diversification history of new world *Rhizophora*. Second, we tested any possibility and extent of hybridization in the group, and in particular, the hypothesis that *R. harrisonii* is a hybrid species between *R. mangle* and *R. racemosa*.

MATERIALS AND METHODS

Sampling—We focused our collection efforts along the Pacific coast of the CAI, where the three New World *Rhizophora* species co-occur extensively. To create a broader geographic and ecological context, we also sampled sympatric populations of *R. mangle* and *R. racemosa* from one Atlantic locality (San Juan

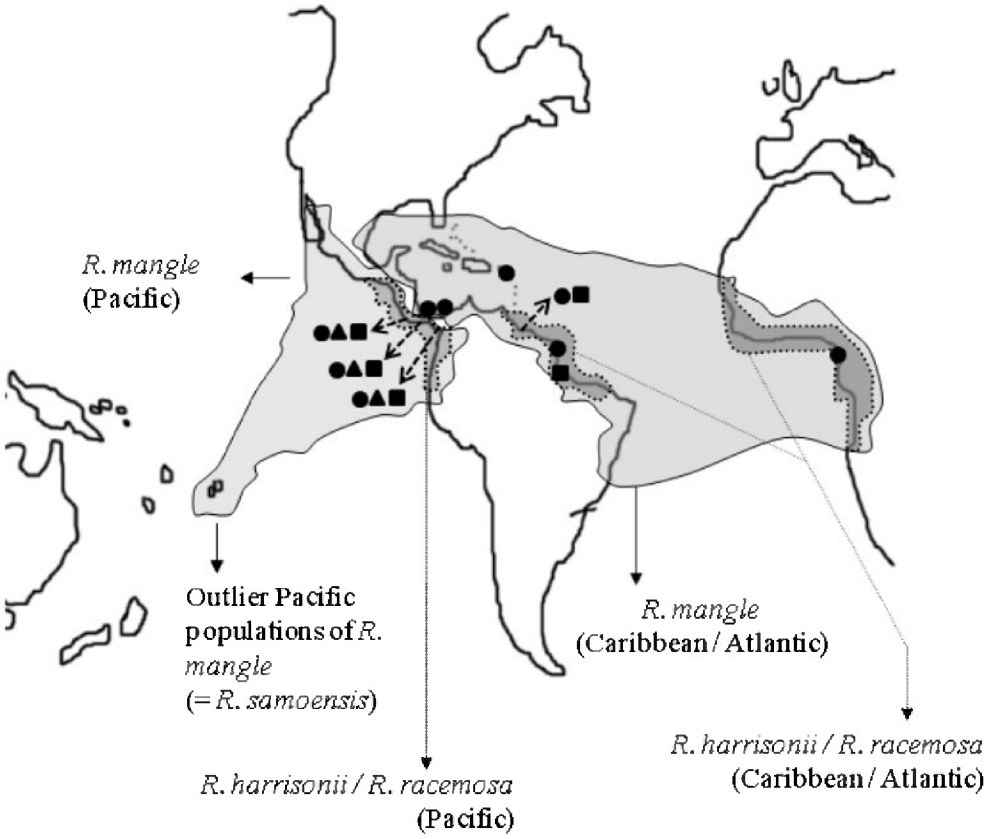


Fig. 1. Geographic distribution of three *Rhizophora* taxa in the neotropics, adapted from Duke and Allen (2006). *Rhizophora mangle* has a wide geographic distribution (light gray) covering Atlantic and Pacific coastal areas on either side of the Central American Isthmus. *Rhizophora racemosa* and *R. harrisonii* have a restricted distribution (dark gray), where they overlap with *R. mangle*. In this study, we analyzed samples from the Atlantic Ocean (i.e., Cameroon, Brazil, French Guiana, Venezuela, Bocas del Toro and Galeta in Panama, and Puerto Rico) and the Pacific Ocean (i.e., Tivives and Damas Island in Costa Rica and San Miguel Gulf in Panama). For each locality, the *Rhizophora* species sampled is indicated (*R. mangle*: circle, *R. harrisonii*: triangle, *R. racemosa*: square. See Appendix 1 for detailed information about sampling collection.

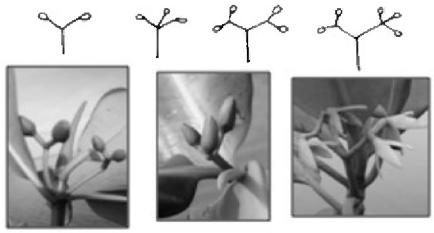
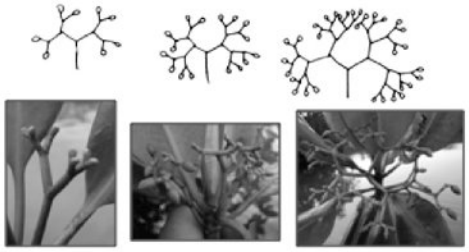
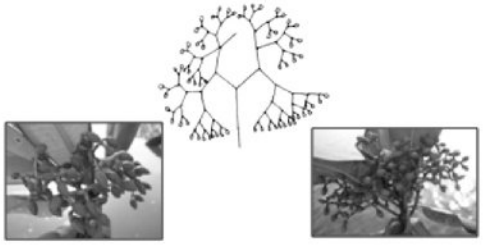
Species name and symbol on Figs. 1 and 3	Number of flowers per inflorescence	Order of bifurcation in the inflorescence	Representation/Photos	Number of individuals analyzed with microsatellites genotyping and sequencing (in parenthesis)	
				Atlantic	Pacific
Rhizophora mangle ○	2-3-4-5	1-2		35 (12)	81 (4)
Rhizophora harrisonii △	8-16-32	3-4-5		-	56* (4)
Rhizophora racemosa □	32-64-128	5-6-7		13 (2)	60 (4)

Fig. 2. Morphological identification of *Rhizophora mangle*, *R. harrisonii*, and *R. racemosa* based on inflorescence type. The number of individuals analyzed with microsatellites genotyping and sequencing for each taxon in Atlantic and Pacific oceans is shown. The number of flowers is directly related to the number of bifurcations in the inflorescence. The suspected hybrid origin of *R. harrisonii* is based on its inflorescence morphology. *Rhizophora harrisonii* has the much-branched, many-flowered inflorescence of *R. racemosa*; but the bud shape, the lax branching, and the longer pedicels of *R. mangle* (Keay, 1953; Hou, 1960; Breteler, 1969; Tomlinson and Womersley, 1976; Breteler, 1977; Tomlinson, 1986). Asterisk (*): One individual collected in the Pacific Ocean (i.e., Damas Island, Costa Rica) had two different inflorescence types and could be classified as *R. harrisonii* or *R. racemosa*.

River, Venezuela) and two monodominant populations of *R. mangle* in the Atlantic (Galeta in Panama and Puerto Rico in the Greater Antilles), where only this morphotype is found. Finally, we obtained several individuals from western Africa (Cameroon), French Guiana, and Brazil (Fig. 1, Appendix 1) Individuals were identified based on inflorescence characteristics following the taxonomic key of Jiménez (1994) (Fig. 2) For each individual, we photographed the inflorescence, recorded its geographic position, and obtained a sample of leaf material, which was placed in Drierite (Xenia, Ohio, USA). Collection location, multilocus genetic information (described later) and inflorescence phenotype are available upon request.

Dried leaves (0.2 g) were ground into a fine powder using a FastPrep FP 120, Bio101 (Qbiogene Carlsbad, California, USA). Genomic DNA from the ground tissue was extracted using a DNeasy 96 plant kit (Qiagen, Valencia, California, USA). The concentration of extracted DNA was estimated on a 1.5% agarose gel by electrophoresis using a Low DNA Mass Ladder (Invitrogen, Madison, Wisconsin, USA). DNA concentrations of samples were consistently low (~0.5 to 10.0 ng/μl), and were used undiluted for template in PCR reactions.

DNA sequencing—We analyzed DNA sequence variation at two chloroplast (cpDNA) noncoding loci, *atpI-atpH* and *psbJ-petA* (Shaw et al., 2007) and at two nuclear flanking microsatellite regions (FMRs), *RM11* and *RM21* (Rosero-Galindo et al., 2002). For detecting heterozygotes within FMRs, PCR products were cloned into TA vectors (Promega, Madison, Wisconsin, USA).

For the two cpDNA regions, primers were redesigned to avoid short-repeat regions and improve sequence quality. Modified primers were F:CGCACCAAAATAAAGAAATGG and R:AAGAAGAGTTCGAATTCAAAGAATG for *atpI-atpH*; and F:ATCCCTGTAGGAATCGGATG, R:GGCGTGATATCGGATTGG for *psbJ-petA*. For both cpDNA regions, the PCR contained ~0.5–10 ng of DNA, 1× PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 μM of each primer, 0.2 mg/mL of BSA and 0.25 U Qiagen *Taq* polymerase in a final volume of 10 μL. Thermocycling conditions were 94°C for 2 min; followed by 35 cycles of 94°C for 30 s, 50°C for 40 s, and 72°C for 40 s; and a final extension step of 72°C for 5 min.

Primers for the amplification of FRMs were also modified from those originally reported (Rosero-Galindo et al., 2002) to increase sequencing length. Primers used were F:TTTCTATTTATGATCCCATCATCTC, R:GCGTTTAACTGCCACAATTC for *RM11*; and F:GCCACACCTGGCAAATCC, R:AGTCAAGATGGTGCGGAAGTC for *RM21*. PCR reactions contained ~0.5–10 ng of DNA, 1× PCR buffer (Qiagen), 0.2 mM of each dNTP, 0.1 μM of each primer, 0.25 U Qiagen *Taq* polymerase, and $MgCl_2$ varied by locus (3.5 mM for *RM11* and 1.5 mM for *RM21*), in a final volume of 10 μL. Amplifications used an initial denaturation cycle of 3 min at 94°C; followed by 35 cycles for 40 s at 94°C, 40 s at 50°C, and 30 s at 72°C; followed by a final extension of 4 min at 72°C.

For both cpDNA and FMRs, we amplified and sequenced two individuals per taxa and per site, covering 10 localities (Appendix 1). Sequences were aligned and edited with the program Sequencher 4.1 (Gene Codes, Ann Arbor, Miami, USA). Nucleotide diversity (π) and the haplotype diversity of cpDNA and FMRs were calculated using the program DnaSP version 4.10.3 (Rozas et al., 2003). Phylogenetic trees for the two combined cpDNA genes and for the two combined FRMs genes were constructed using maximum parsimony (MP) with the program PAUP* version 4.0b10 (Swofford, 2003). For each genome sequence matrix, we calculated the most parsimonious tree using a heuristic search with tree-bisection-reconnection (TBR) branch swapping. The consensus tree was calculated using majority rule, and the confidence of each node was assessed by bootstrapping using 1000 replicates. Bayesian analysis was also applied to estimate the tree that best fit our data using the program MrBayes version 3.04 (Huelsenbeck and Ronquist, 2001). We used an F81 model (Felsenstein, 1981) for the combined cpDNA genes and the HKY+I model for the combined FMR genes as determined by the program MrModelTest (Nylander, 2002) under the Akaike information criterion (Akaike, 1974). We ran 2 000 000 Markov chain Monte Carlo (MCMC) iterations, sampling every 100 iterations with a burn-in of 25% of the first trees.

Multilocus genotyping—Six microsatellite loci, *RM7*, *RM11*, *RM19*, *RM21*, *RM36*, and *RM46* (Rosero-Galindo et al., 2002), were used to genotype 244 *Rhizophora* individuals in areas of sympatry collected across the CAI and two additional monospecific populations of *R. mangle* (i.e., Galeta, Panama, and Puerto Rico) (Appendix 1). All PCR reactions had a final volume of 7 μL and contained ~0.5–10 ng of DNA, 1× Qiagen PCR Buffer, 0.2 mM of each dNTP, 0.25 U Qiagen *Taq* polymerase, and $MgCl_2$ which varied by locus: 2.0 mM for *RM11*, 2.5 mM for *RM7*, 3.0 mM for *RM19* and *RM46* and 3.5 mM for *RM21*. In addition, these reactions contained 0.1 μM fluorescently tagged M13 universal forward primer (Steffens et al., 1993), 0.1 μM forward primer, and 0.03 μM reverse primer, which contained a tail at the 5′ end that was identical to the M13 forward primer sequence. Amplifications used an initial denaturation cycle of 3 min at 94°C; followed by 35 cycles for 40 s at 94°C, 40 s at 50°C (*RM19*, *RM21*, and *RM36*) or 55°C (*RM7*, *RM11*, and *RM46*) and 30 s at 72°C; followed by a final extension of 4 min at 72°C. Products were separated on an ABI 3130xl with the program GeneScan 500LIZ (Life Technologies, Foster City, California, USA) as the size standard. Allele sizes were assigned using the program GeneMapper version 3.7 (Life Technologies).

The number of alleles per locus and the number of unique alleles found in each population of *Rhizophora* taxa were calculated using the program Genetix version 4.03 (Belkhir et al., 2002). Deviation from Hardy–Weinberg equilibrium (HWE) at each locus was tested with the program Genepop version 3.4 (Raymond and Rousset, 1995) using the complete enumeration method (Louis and Dempster, 1987) for loci with less than five alleles and the Markov chain method (Guo and Thompson, 1992) for loci with five or more alleles. Parameters for the Markov chain method were established using 10 000 dememorizations and 400 batches with 5000 iterations per batch. We also examined each locus for the presence of null alleles using the program Microchecker version 2.2.3 (Van Oosterhout et al., 2004). Microchecker also looks for potential scoring problems associated with allelic stuttering and allelic dropout and provided another means of reduce genotyping error across our study. Finally, departure from genotypic linkage equilibrium (LE) between pairs of loci was tested within each population and for all populations using the program FSTAT version 2.9.3.2 (Goudet, 1995, 2001).

We examined the multilocus microsatellite variation across localities on both sides of CAI where *Rhizophora* taxa were in sympatry, using a molecular analysis of variation (AMOVA) (Michalakis and Excoffier, 1996). On the Atlantic side, sampling locations included Venezuela near the mouth of the San Juan River, where both *R. mangle* and *R. racemosa* co-occurred and Panama (Galeta) and Puerto Rico, where only *R. mangle* was found (Fig. 1). Pacific populations included Costa Rica (Tivives and Damas Island) and Panama (San Miguel Gulf), where all three New World *Rhizophora* taxa were found. For the AMOVA, the total variance estimates and pairwise F_{ST} (Weir and Cockerham, 1984) were calculated using the program Arlequin version 3.11 after 10 000 permutations (Excoffier et al., 2005).

In the four localities where two or three *Rhizophora* species were found in sympatry (i.e., Venezuela, two locations in Costa Rica and Panama), we also examined the distribution of microsatellite variation using the program Structure version 2.2 (Pritchard et al., 2000; Falush et al., 2003, 2007). We performed this analysis without information on *Rhizophora* taxonomic classification assuming an admixture model, correlated allele frequencies, and a uniform prior probability of *K*. All runs were performed following 500 000 replicates of MCMC after a burn-in of length 50 000 replicates and the likelihood of *K* was set to vary from $K = 1$ through $K = 10$. We performed 10 runs of the described procedure for each value of *K* and followed the method of Evanno et al. (2005) to generate our estimate of the true number of populations based on the ΔK calculation. Finally, for Pacific localities in Costa Rica (Tivives and Damas Island) and Panama (San Miguel Gulf) where we have geographic coordinates of each *Rhizophora* individual analyzed, we also constructed local maps to determine whether the Bayesian assignment and admixture signal were associated with a particular spatial arrangement of individuals.

RESULTS

For 27 individuals (16 *R. mangle*, four *R. harrisonii*, and seven *R. racemosa*) spanning the geographic range of the three species, we examined sequence variation in two noncoding cpDNA gene regions (164 bp for *atpI-atpH* and 393 bp for *psbJ-petA*). Variation was low, and in the combined data set, we observed three indels of 1 bp, 1 bp, and 11 bp within the *psbJ-petA* region and one indel of 9 bp within the *atpI-atpH* region, plus one transition and four transversions across the two regions that generated four haplotypes. Nucleotide diversity (π) was 0.005 (SD 0.0003), and the haplotype diversity (Hd) was 0.695 (SD 0.050).

Similarly, for 26 individuals (16 *R. mangle*, four *R. harrisonii*, and six *R. racemosa*), we examined sequence variation in two FMRs (570 bp of RM11 and 209 bp of RM21). Variation was greater in this combined data set, where we identified 13 transitions, seven transversions, and no indels, creating 15 FMR haplotypes. Nucleotide diversity was 0.013 (SD 0.0009) and haplotype diversity was 0.867 (SD 0.041). From the 26 samples examined, we detected five heterozygotic individuals including one *R. mangle* and one *R. racemosa* individual from Venezuela (Atlantic) and three *R. harrisonii* individuals from Costa Rica and Panama (Pacific).

Reconstructed genealogies for all loci were strongly concordant. In both the cpDNA and FMRs trees, there were no relationships between branching patterns and currently recognized species boundaries. Instead, genetic variation showed a strong phylogeographic signal unrelated to the morphological differences used to define *Rhizophora* species (Fig. 3). There was a pronounced genetic break between the Atlantic and Pacific populations of both *R. mangle* and *R. racemosa* and, within each ocean basin, the two species shared similar or identical cpDNA or FMR sequences. Similarly, in the Pacific, where we sampled all three recognized species, there were no obvious differences among the species at these loci. However, there appears to be some regional substructure within the Atlantic. Specifically, we observed a Caribbean-West Atlantic lineage and a distinctive trans-Atlantic lineage that included four individuals of both *R. mangle* and *R. racemosa* collected in South America (i.e., Venezuela and French Guiana) and in western Africa (i.e., Cameroon) (Fig. 3).

***Multilocus genotypes in New World* Rhizophora**—We characterized multilocus genotypes at six microsatellite loci among a much larger sample composed of 244 sympatric *R. mangle*, *R. harrisonii*, and *R. racemosa* collected from four populations and individuals of *R. mangle* from two additional populations—Galeta, Panama ($N = 15$), and Puerto Rico ($N = 16$). We observed an average of 13 alleles per locus. However, this is an underestimation of the number of alleles, and there was evidence for extensive null alleles (data not presented) with all but two *Rhizophora* populations showing strong heterozygote deficiencies. Two loci (RM19 and RM46) showed deviations from linkage equilibrium across the wider population sampling.

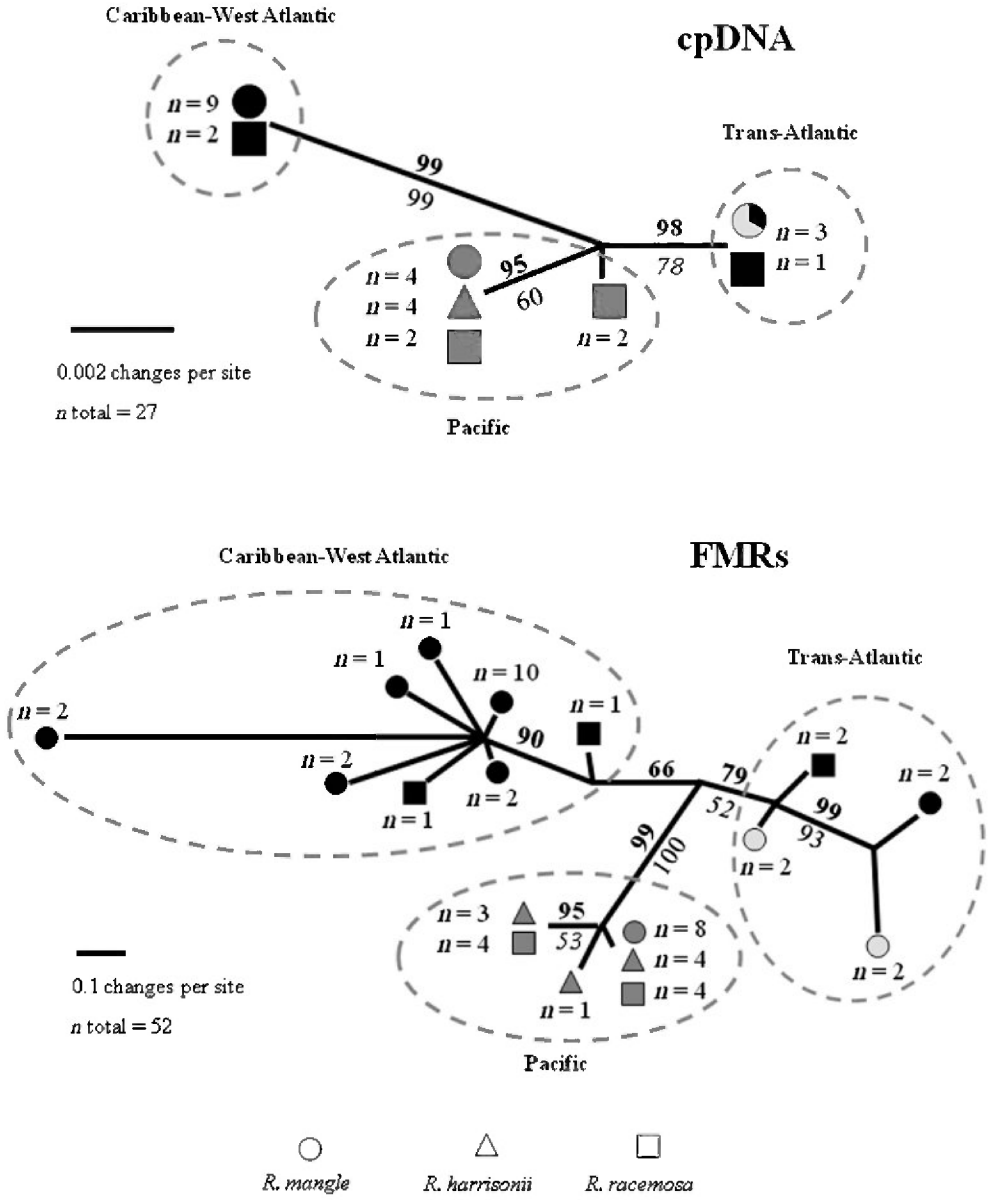


Fig. 3. Unrooted trees for neotropical *Rhizophora* based on (A) cpDNA (*atpI-atpH* and *psbJ-petA*) and (B) nuclear FMR (*RM11* and *RM21*) sequences. All sites containing indels were excluded from analysis. Numbers in boldface above the branches indicate the Bayesian posterior probability as a percentage after 2 000 000 iterations. Numbers below the branches are MP bootstrap support values above 50. Circles represent *R. mangle*, triangles represent *R. harrisonii*, and squares represent *R. racemosa* based on morphology. Colors represent the three main geographic regions collected in this study. Black represents Central and South American samples from the Atlantic basin, light gray represents western African samples, and dark gray represents Central American samples from the Pacific basin. Analysis resulted in three main geographic clades represented by dotted circles: (1) a Caribbean-western Atlantic clade, that includes *R. mangle* and *R. racemosa* samples from Panama (Bocas del Toro and Galeta), Puerto Rico, Venezuela, French Guiana, and Brazil; (2) a trans-Atlantic clade that includes three *R. mangle* samples (two from Cameroon [western Africa] and one from French Guiana) and one *R. racemosa* sample from Venezuela; and (3) a Pacific clade that includes *R. mangle*, *R. harrisonii*, and *R. racemosa* from Costa Rica and Panama (Montijo Gulf and San Miguel Gulf). The number of samples (*n*) for each taxon is indicated for each haplotype.

As seen with the cpDNA and FMR sequences, microsatellite variation was geographically partitioned. There were strong differences among all populations sampled, including a striking genetic discontinuity between Atlantic and Pacific populations of *R. mangle*, *R. harrisonii* and *R. racemosa*. Nearly 73% of the observed alleles were unique to one or the other Ocean (Fig. 4). In general, there were more alleles per locus in the Pacific relative to the Atlantic and these alleles tended to be rare in the population (Fig. 4). These trans-Isthmus differences in allele frequencies generated strong genetic structure between Atlantic and Pacific *Rhizophora* taxa with values ranging from F_{ST} = 0.337 (including both *R. mangle* and *R. racemosa* individuals) and F_{ST} = 0.354 (including only *R. racemosa* individuals) to F_{ST} = 0.479 (including only *R. mangle* individuals).

Within each ocean group, there was also evidence for population differences, albeit less extreme. In Pacific locations, among-population differences accounted for nearly 28% of the observed microsatellite variation, which is approximately twice

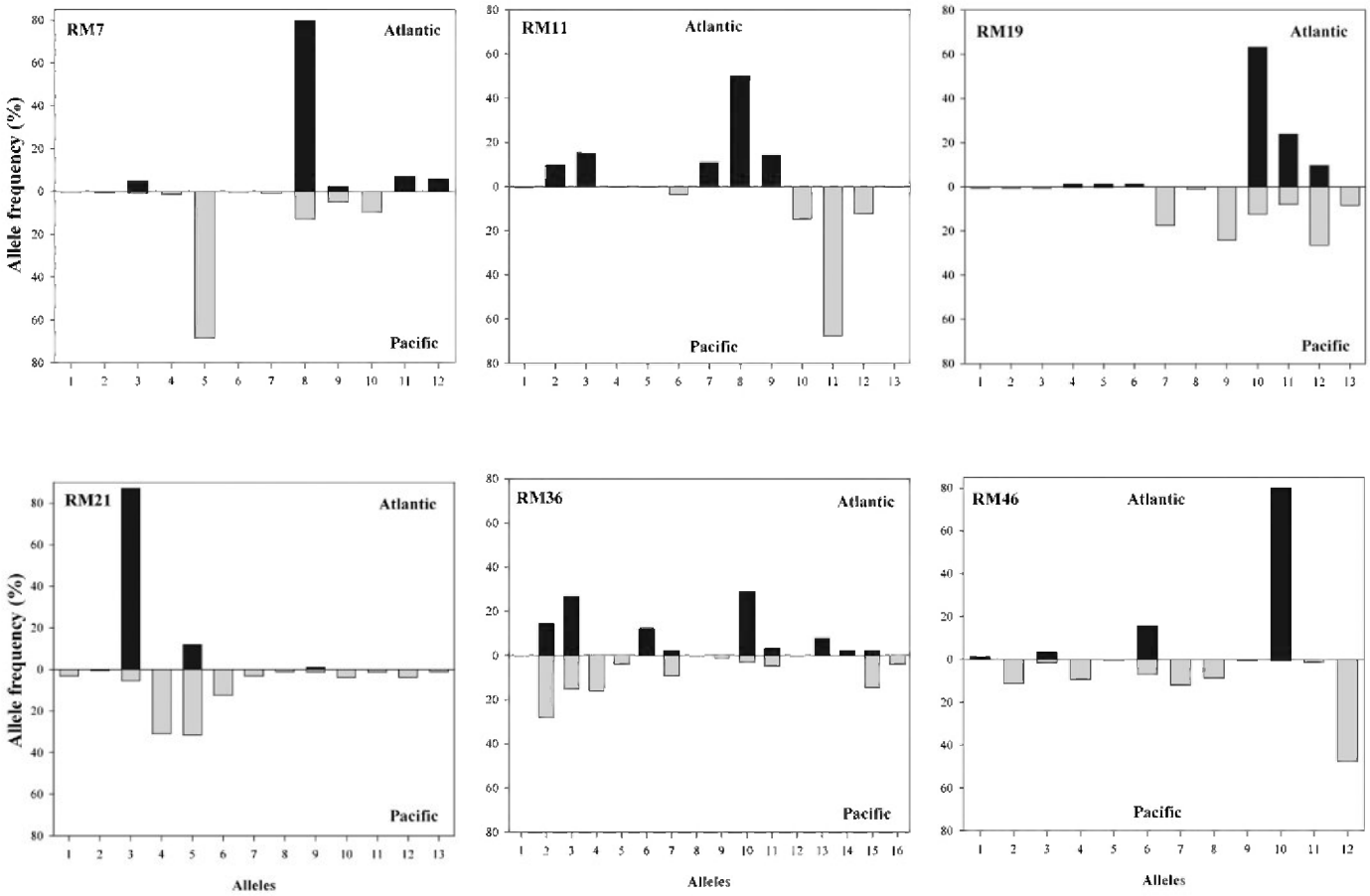


Fig. 4. Allele frequencies of New World *Rhizophora* from the Atlantic (black) compared with those from the Pacific (gray) across the Central American Isthmus based on six microsatellite loci.

the variation explained by taxonomic differences (Appendix 2). For example, all three Pacific *R. mangle* populations differed significantly from each other with F_{ST} values ranging from 0.116 to 0.437. We also observed significant genetic differentiation among populations in our three *R. racemosa* and *R. harrisonii* localities of the Pacific side of CAI (Table 1). A similar pattern was evident in our Atlantic samples of *R. mangle* and *R. racemosa,* where we observed strong genetic structure across Venezuela, Panama, and Puerto Rico (Table 2).

Between-species differences were very similar and, in many cases, lower than among-population differences of the same species (Tables 1, 2). Nonetheless, sympatric populations of *R. mangle* and *R. racemosa* could be distinguished from each other and observed F_{ST} values ranged from 0.251 between *R. mangle* and *R. racemosa* from Panama and 0.485 between *R. mangle* and *R. racemosa* from Costa Rica, Damas Island (Tables 1, 2). The genetic distinction of *R. harrisonii* was less pronounced. *Rhizophora harrisonii* was always distinct from *R. mangle*; however, it differed very little from sympatric individuals of *R. racemosa*. Indeed, in two of the three populations where we sampled all three species, *R. harrisonii* individuals did not differ significantly from *R. racemosa* individuals collected at the same locality.

Individual-based genetic analysis corroborated the population-based analysis of variation. In all cases, Bayesian clustering analysis performed separately in the four sympatric localities demarcated two distinctive groups or clusters ($K = 2$) within each locality (Fig. 5). This value was strongly supported by the evaluation of ΔK, which always had a strong peak of $K = 2$. This analysis was performed independently of inflorescence morphology, but was typically, but not completely, concordant with the taxonomic differences between *R. mangle* and *R. racemosa*. Differences among *R. racemosa* and *R. mangle* were most consistent in the Pacific populations examined. The two genetic clusters were strongly associated with taxonomic designations based on morphology; however, in San Miguel Gulf, Panama, roughly 15% of the *R. mangle* samples possessed a multilocus genotype that clustered with *R. racemosa*. Two clusters largely corresponding to inflorescence morphology were also identified in a larger regional analysis of the three Pacific populations (data not presented). In Venezuela, two distinct clusters were also best supported by the data, but the concordance with taxonomic differences was more blurred. About half of the individuals classed as *R. racemosa* fell within the *R. mangle* cluster with a probability higher than 0.8 (Fig. 5). This pattern contrasted with the two pure *R. mangle* populations, Puerto Rico and Galeta-Panama, where in both cases, a single population cluster was most consistent with the data (data not reported).

In contrast, we found little evidence to support the genetic distinction of *R. harrisonii*. Most *R. harrisonii* had multilocus genotypes that clustered with sympatric *R. racemosa*. The only exceptions were five individuals collected in Tivives (Costa

TABLE 1. Pairwise values of F_{ST} among *Rhizophora* taxa collected in sympatry across three localities in the Pacific Ocean.

		Costa Rica, Tivives			Costa Rica, Damas Island			Panama, San Miguel Gulf		
Location	Taxon	*R. mangle*	*R. harrisonii*	*R. racemosa*	*R. mangle*	*R. harrisonii*	*R. racemosa*	*R. mangle*	*R. harrisonii*	*R. racemosa*
Costa Rica, Tivives	*R. mangle*	—								
	R. harrisonii	0.120*	—							
	R. racemosa	0.345*	0.075*	—						
Costa Rica, Damas Island	*R. mangle*	0.116*	0.286*	0.450*	—					
	R. harrisonii	0.314*	0.093*	0.087*	0.459*	—				
	R. racemosa	0.356*	0.153*	0.102*	0.485*	–0.016	—			
Panama, San Miguel Gulf	*R. mangle*	0.239*	0.307*	0.438*	0.437*	0.420*	0.427*	—		
	R. harrisonii	0.200*	0.055*	0.175*	0.345*	0.209*	0.226*	0.207*	—	
	R. racemosa	0.202*	0.043*	0.171*	0.382*	0.190*	0.201*	0.251*	–0.017	—

Note: * Significantly different from zero ($P < 0.05$) after Bonferroni corrections.

Rica), which showed an admixed pattern and had an equal probability of assignment to either the *R. mangle* or *R. racemosa* cluster (Fig. 5). Differences in the admixing pattern among the four localities were not obviously related to the spatial distribution of individuals. Individuals of apparently mixed ancestry were not found between populations of pure nonadmixed individuals. For example, in our Tivives (Costa Rica) population, admixed *R. harrisonii* individuals were collected next to *R. mangle* individuals and spatially distant (~1000 m) from *R. racemosa* individuals. In addition, within Costa Rica (Damas Island) and Panama (San Miguel Gulf), individuals with different grades of admixture were next to each other without any obvious spatial arrangement.

DISCUSSION

Rhizophora is a pantropical genus and has been a foundation of tropical coastal mangrove ecosystems since the Middle Eocene (Tomlinson, 1986; Kathiresan and Bingham, 2001; Hogarth, 2007). In spite of the ecological importance of the genus, inferring species relationships is complicated due to the extreme phenotypic plasticity of the main diagnostic characters (Jiménez, 1994; Duke et al., 1998, 2002). As a result, questions of synonymy, the exact geographic range of species, and the phylogenetic affinities within the genus have been reconsidered several times since *R. mangle* was described by Linnaeus over 300 years ago (Linnaeus, 1753) and *R. racemosa* by Meyer (Meyer, 1818) roughly 50 years later (Guppy, 1906; Salvoza, 1936; Hou, 1960; Tomlinson, 1986; Duke et al., 2002). In 1960, Hou noted that two distinctive inflorescence types were found concurrently at both sides of the Central American Isthmus (CAI) and in western Africa and recognized two main *Rhizophora* taxa in the New World; the widespread *R. mangle* with single inflorescence and the more restricted *R. racemosa* with complex inflorescence (Figs. 1, 2). The two species were broadly sympatric, and the inflorescence characters used to distinguish them were maintained across their tropical Atlantic and Pacific ranges. Hou's taxonomic view has largely prevailed for the last half century.

Our data contradicts this nomenclature and reopens the debate about the evolutionary history of these ecologically important species. There was no differentiation between *R. mangle* and *R. racemosa* collected within the same ocean basin in phylogenetic trees inferred from the cpDNA and FMR data (Fig. 3). Instead, these data suggest that *R. mangle* and *R. racemosa* individuals from the same ocean basin are more closely related to each other than to conspecifics in the other ocean basin, a conclusion reinforced by our extensive microsatellite genotyping of individuals collected on either side of the CAI. This pattern highlights the major role that the rise of the CAI has had on the evolution of this group and suggests that the uplift severed gene flow between *Rhizophora* populations in the two ocean basins. Whether this separation occurred when the final land bridge formed between 3.1–3.5 Ma (Coates and Obando, 1996), or sometime later, is more difficult to determine with our present data. Both marker types identified three nearly equally divergent lineages—Caribbean-West Atlantic, trans-Atlantic, and Pacific (Fig. 3). The distribution of the two major Atlantic lineages among populations and their relationship with other *Rhizophora* populations in mangrove communities around the world needs much more study.

The conclusion that Pacific and Atlantic *Rhizophora* formed distinctive taxonomic groups and that geography was a better predictor of evolutionary relationships within New World *Rhizophora* than inflorescence morphology was reached by some early mangrove naturalists (Guppy, 1906; Salvoza, 1936; Gregory, 1958). Although these biologists did not recognize a distinction between *R. mangle* and *R. racemosa*, they recognized that the CAI was a significant barrier to interspecies gene flow and made clear taxonomic distinctions between the Atlantic (*R. mangle*) and the Pacific (*R. samoensis*) populations (Guppy, 1906; Salvoza, 1936; Gregory, 1958). An identical

TABLE 2. Pairwise values of F_{ST} among *Rhizophora* taxa across three localities in the Atlantic.

		Panama, Galeta	Venezuela, San Juan River		Puerto Rico
Location	Taxon	*R. mangle*	*R. mangle*	*R. racemosa*	*R. mangle*
Panama, Galeta	*R. mangle*	—			
Venezuela, San Juan River	*R. mangle*	0.293*	—		
	R. racemosa	0.495*	0.262*	—	
Puerto Rico	*R. mangle*	0.400*	0.195*	0.503*	—

Note: * Significantly different from zero ($P < 0.05$) after Bonferroni corrections.

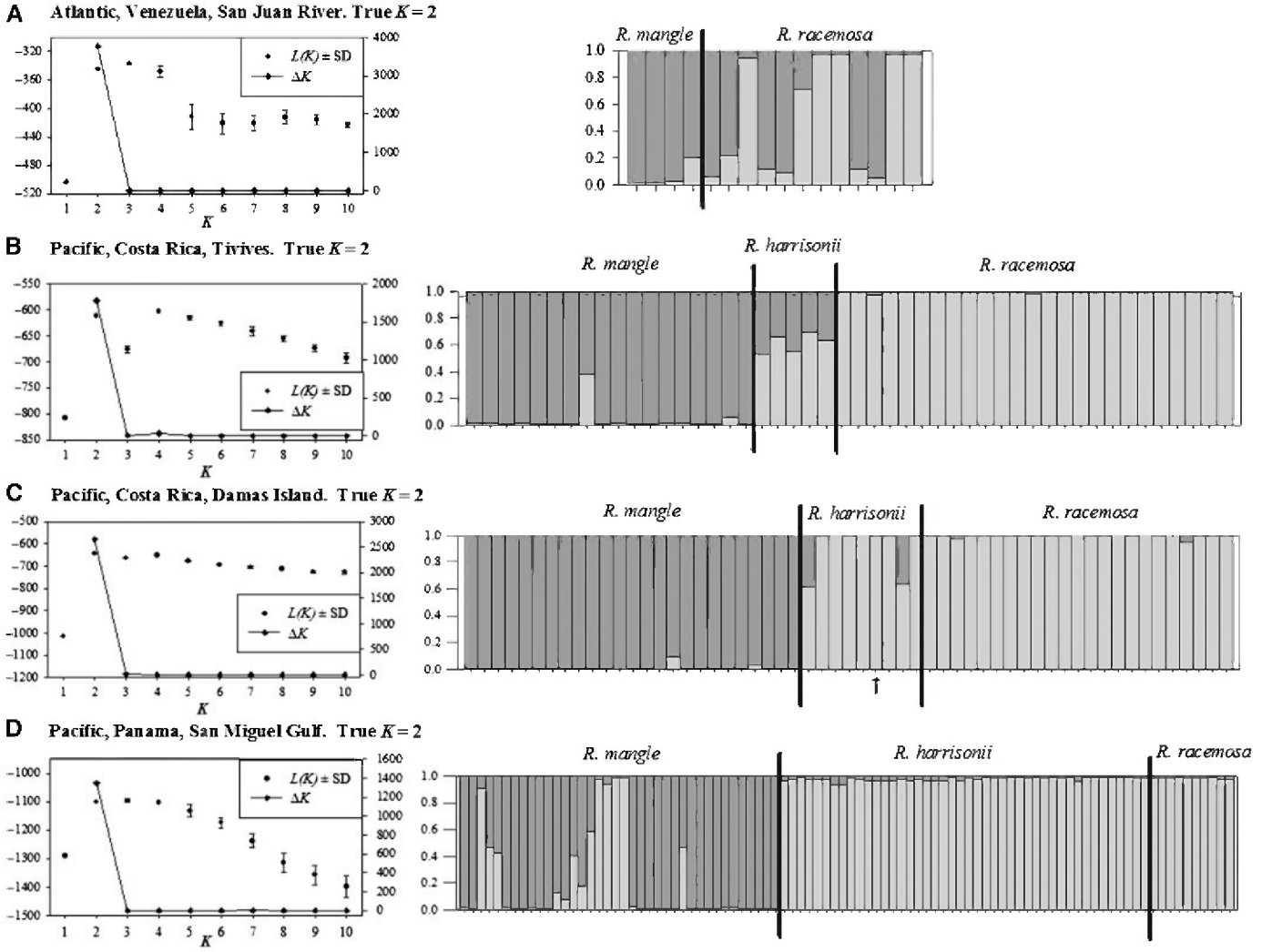


Fig. 5. Bayesian cluster analysis of *Rhizophora* genus using the program Structure 2.2 at four localities where two or three *Rhizophora* taxa were in sympatry. The localities are (A) San Juan River, Venezuela in the Atlantic; (B) Tivives Island, Costa Rica; (C) Damas Island, Costa Rica; and (D) San Miguel Gulf, Panama in the Pacific. The left side shows the maximum likelihood $L(K) \pm$ SD and the ΔK (Evanno et al., 2005) simulating different levels of structure (K), from $K = 1$ to $K = 10$ and over 10 runs of each K. For all localities, the true structure was $K = 2$. The right side shows the assignment of individuals assuming the true $K = 2$ for each locality. Each line represents an individual, and the proportions of color in each line (y-axis) represent the assignment probability for each cluster (i.e., dark gray and light gray). The individual with the arrow had two different inflorescence types and could be classified simultaneously as *R. harrisonii* or *R. racemosa*.

conclusion proceeds logically from the more rapidly evolving microsatellite loci. Each ocean contained a large number of unique alleles (Fig. 4) and estimates of population differentiation were high ($F_{ST} = 0.337$ when pooling all Atlantic and Pacific individuals). Similar results have been observed in two other mangrove or mangrove-associated species, *Avicennia germinans* (black mangrove, Nettel and Dodd, 2007) and *Hibiscus pernambucensis* (Takayama et al., 2006), which also possess sea-drift propagules and have widespread distributions on both sides of the CAI. The trans-Isthmus F_{ST} values observed among different mangrove or mangrove-associated species are more pronounced than the mean differences ($F_{ST} = 0.177$) observed in 42 tropical and terrestrial tree species measured across similar regional geographic distances (Dick et al., 2008), underscoring the importance of the closure of the CAI in the general history of New World mangrove ecosystems.

An impenetrable physical barrier, however, is not required for population differentiation to arise among populations of New World *Rhizophora*. In spite of the potential for long-distance dispersal via floating seed, there was strong differentiation among mangroves at both local and regional scales. Indeed, genetic differentiation among Atlantic *Rhizophora* populations was particularly strong and regional population differences (mean $F_{ST} = 0.377$) were similar to those observed between populations separated by the CAI. Population structure was less extreme among the three Pacific *Rhizophora* populations (mean $F_{ST} = 0.245$), but was still higher than observed in most other tropical tree species across similar geographic ranges (Dick et al., 2008). Nonetheless, long-distance dispersal appears to be important in sporadic colonization events in the recent history of New World *Rhizophora*. In particular, the presence of shared cpDNA haplotypes between western African and South American

populations separated by 7000 km argues for a recent trans-Atlantic migration and underscores the potential of water-borne seeds for long-distance dispersal in *Rhizophora* (Davis, 1940; Rabinowitz, 1978; Ellison, 1996; Steele, 2006). A similar pattern of local population differentiation coupled with evidence for long-distance dispersal was also observed in the black mangrove (*A. germinans*) and the mangrove-associated *H. pernambucensis* (Takayama et al., 2006; Nettel and Dodd, 2007). Both species had a similarly strong regional population genetic structure as well as evidence for recent transatlantic migration. Nettel and Dodd (2007) interpreted this pattern as evidence for recent migration from Africa to the western Atlantic following the predominant equatorial Atlantic current (Renner, 2004). It is interesting that all mangrove species thus far examined show a similar pattern of recent transatlantic migration suggesting that they share a common underlying causal mechanism.

In spite of the poor correspondence between inflorescence morphology and the sequence data presented here, there remains strong genetic evidence for at least two distinctive *Rhizophora* groups within each ocean. This is certainly true in the Pacific where, in all three populations examined, microsatellite loci identified two distinct population clusters. This distinction is preserved along the 700 km of coastline that we surveyed in this study and when all Pacific individuals were analyzed together, two genetic clusters are still identifiable. Our sampling is less intensive in the Atlantic, but we similarly observe two clusters in our Venezuelan collections of *R. racemosa* and *R. mangle*. In most cases, assignment to specific clusters closely followed morphological divisions based on the inflorescence and ecological divisions based on collection location. Thus, at a fine scale, our data support the traditional notion that two distinctive neotropical *Rhizophora* species coexist; however, the relationship between these forms is much more complex than heretofore envisioned. Morphological differences between *R. mangle* and *R. racemosa* appear to be bounded, such that the inflorescence morphology used to define "*mangle*" and "*racemosa*" is found in both the Atlantic and Pacific Oceans. However, these morphological characteristics say very little about the evolutionary history of these individuals, and what is called "*racemosa*" and "*mangle*" in the Pacific has a very distinctive history from what we are calling "*racemosa*" and "*mangle*" in the Atlantic.

In light of the patterns we observed in *R. mangle* and *R. racemosa*, it is interesting to revisit the relationship of *R. harrisonii* within the New World *Rhizophora*. *Rhizophora harrisonii* is found in the Pacific and on both sides of the Atlantic, but only in areas where *R. mangle* and *R. racemosa* currently co-occur (Duke and Allen, 2006). Our data provides little evidence that *R. harrisonii* is a distinct and separately evolving hybrid species. No combination of alleles was unique to *R. harrisonii*, as would be expected if it originated from an old hybridization event and subsequently maintained its genetic and evolutionary distinctiveness (Rieseberg, 1991). Nor did *R. harrisonii* populations consistently possess an admixed genome, as expected if *R. harrisonii* was of recent hybrid origin (Arnold and Bennett, 1993). Rather, in some areas (Tivives, Costa Rica), *R. harrisonii* individuals appear to be recently formed hybrids with admixed genotypes. However, in other areas (e.g., San Miguel Gulf, Panama; Damas Island, Costa Rica), *R. harrisonii* individuals were indistinguishable from *R. racemosa,* a pattern more consistent with a history of repeated hybridization and backcrossing (see below). Given these patterns, we conclude that individuals classified as *R. harrisonii* probably represent extant introgressive hybridization between *R. mangle* and *R. racemosa*. Hybrid ancestry is also evident in individuals that fell into either the "*mangle*" or "*racemosa*" inflorescence groups underscoring the difficulty of relying only on this morphological characteristic to define groups. Indeed, we collected several individuals with branches with that contained both "*racemosa*" and "*harrisonii*" like flowers.

***Hybridization and species boundaries among New World* Rhizophora**—The genetic patterns we observed are most consistent with a history of hybridization within the New World *Rhizophora*. The strong genetic break between Pacific and Atlantic *Rhizophora* populations argues that this history dates back at least as far as the rise of the CAI. Given (1) the long residence time of *Rhizophora* in the New World (Graham, 2006), (2) evidence for widespread distribution of anemophilous pollen fossil of *Rhizophora* in the New World dating back 40 Ma (Muller, 1981; Ellison, 1991; Duke et al., 2002; Graham, 2006), and (3) distinctive *R. mangle* and *R. racemosa* pollen types in the Oligocene–Miocene of Mexico (Muller and Caratini, 1977; Muller, 1981), this history could easily stretch back much further. Rates of hybridization and introgressive gene flow have been extensive enough that morphologically distinct individuals have coupled evolutionary histories on either side of the CAI. Moreover, the repeated occurrence of individuals with a "*harrisonii*" inflorescence in every location where "*racemosa*" and "*mangle*" co-occur indicates that hybridization is ongoing among the species. There are a number of examples where both ancient and ongoing hybridization has been observed in plants; however, in none that we are aware of, has the history of hybridization been so intimate as to generate the coupled evolutionary dynamics we observe in New World *Rhizophora*. In sunflowers, irises, pines, eucalyptus, and oaks, for example, there is likewise evidence for extensive hybridization; however, in none of these taxa has introgressive gene flow led to genome-wide admixture, and parental ecotypes can easily be distinguished at nuclear or at chloroplast markers (see Muller, 1952; Remington, 1968; Potts and Reid, 1990; Wagner et al., 1991; Whittemore and Schaal, 1991; Cruzan and Arnold, 1993; Rieseberg and Wendel, 1993; Rieseberg et al., 1996; Petit et al., 1997; and more recent reviews by Arnold et al., 1999; Arnold, 2004, 2006).

The morphological and genetic patterns we observe raise obvious questions about the maintenance of different ecotypes in the presence of potentially homogenizing levels of gene flow. One explanation is that strong disruptive selection acts directly on the inflorescence morphology. Florescence and flower morphology can evolve rapidly, and there are a number of cases where strong disruptive selection is apparently driving variation in flower morphology (Endress, 1999; Cronk, 2001; Cubas, 2004). However, in all these examples, the plant species are animal pollinated and divergence in flower morphology is hypothesized to be an adaptation to different pollinator guilds (Westerkamp and Classen-Bockhoff, 2007). In contrast, all members of the New World *Rhizophora* complex are wind pollinated (Tomlinson, 1986). Different inflorescence architectures could reflect adaptation to different wind environments, a prediction that needs to be examined further.

Alternatively, strong ecological selection on some other trait may explain the maintenance of morphological distinctions in a scenario of high levels of hybridization. In this case, one must hypothesize a tight linkage between inflorescence morphology and the trait under strong selection. *Rhizophora* with different inflorescence morphologies are not distributed randomly and

independent observations from western Africa to South America have demonstrated consistent differences in the spatial distribution of *R. mangle* and *R. racemosa* (Savory, 1953; Jiménez, 1987; Smith, 1992; Afzal-Rafii et al., 1999; Duke et al., 2002; Duke and Allen, 2006). These differences typically follow salinity gradients, with *R. mangle* consistently found in higher salinity environments. It has been speculated that these distribution patterns reflect underlying adaptive differences in salinity tolerance based on strong differences in cuticular alkaline composition between *R. racemosa* and *R. mangle* (Dodd et al., 1995; Afzal-Rafii et al., 1999), a trait thought to influence salt tolerance. Furthermore, studies have also shown that individuals with a *R. racemosa* inflorescence type are less tolerant to salinity than individuals with the *R. mangle* inflorescence type (Jiménez and Sauter, 1991; Duke et al., 1998; Afzal-Rafii et al., 1999; Gilman et al., 2008). Interestingly, individuals with the intermediate inflorescence type traditionally associated with *R. harrisonii* tend to occupy intermediate salinity environments (Leechman, 1918; Savory, 1953; Jonker, 1959; Breteler, 1969; Santos, 1986). Similarly, inflorescence type may be linked to genes that control flowering time or reproductive success. Phenological differences have been observed between sympatric *Rhizophora* taxa in Costa Rica (Jiménez, 1987, 1988). Moreover, there appears to be differences between pollination efficiency between *R. mangle* and *R. racemosa* and possible reduced fertility of F1 hybrids (Breteler, 1969, 1977; Muller and Caratini, 1977).

Our study documents a history of significant hybridization among New World *Rhizophora* at an unexpected temporal and spatial scale. At the most basic level, these data suggest that the current systematic relationships need to be revisited. Similar to what is being observed in the IWP (Parani et al., 1997; Tyagi, 2002), species boundaries are extremely porous in New World *Rhizophora*, and we found no compelling evidence that evolutionary distinct units reflect current taxonomic boundaries. Nonetheless, genetically distinctive groups coexist in sympatry. These groupings largely follow morphological and ecological boundaries, raising a number of important questions about how differences are maintained in the face of hybridization. In this respect, areas where all three types occur provide a powerful natural laboratory for future focal research into evolutionary and ecological consequences of widespread hybridization in *Rhizophora*.

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APPENDIX 1. Detailed collection and analysis information for the *Rhizophora* species examined in this study. The total number of samples collected per *Rhizophora* species, locations, and the collector are noted. In addition, the total number of samples analyzed for each genetic molecular marker and their respective GenBank accession number are specified. Molecular markers used in this study are six microsatellite loci, two flanking microsatellite regions (FMRs) and two noncoding regions of chloroplast (cpDNA).

Geographic locality	Species	Collector	Total no. of samples per locality	Microsatellites (6 loci)	FMRs			cpDNA		
				N	*N*	*RM11*	*RM21*	*N*	*atpI-atpH*	*PsbJ-PetA*
Atlantic										
Panama, Bocas del Toro	*R. mangle*	I. Cerón-Souza	2	—	2	GU563105, GU563106	GU563134, GU563135	2	GU563051, GU563052	GU563078, GU563079
Panama, Galeta Island	*R. mangle*		15	15	2	GU563107, GU563108, GU563109	GU563136, GU563137	2	GU563053, GU563054	GU563080, GU563081
Puerto Rico	*R. mangle*	E. Rivera-Ocasio	16	16	2	GU563110, GU563111	GU563138, GU563139, GU563140	2	GU563055, GU563056	GU563082, GU563083
Venezuela, San Juan River	*R. mangle*	E. Medina	4	4	2	GU563112, GU563113	GU563141, GU563142	2	GU563057, GU563058	GU563084, GU563085
	R. racemosa		12	12	2	GU563114, GU563115, GU563116	GU563143, GU563144, GU563145	2	GU563059, GU563060	GU563086, GU563087
French Guiana	*R. mangle*	E. Rivera-Ocasio	2	—	2	GU563117, GU563118	GU563146, GU563147	2	GU563061, GU563062	GU563088, GU563089
Brazil	*R. racemosa*	A. Schwarzbach	1	—	—	—	—	1	GU563063	GU563090
Cameroon	*R. mangle*	C. Dick	2	—	2	GU563119, GU563120	GU563148, GU563149	2	GU563064, GU563065	GU563091, GU563092
Pacific										
Costa Rica, Tivives	*R. mangle*	I. Cerón-Souza	18	18	2	GU563121, GU563122	GU563150, GU563151	2	GU563066, GU563067	GU563093, GU563094
	R. harrisonii		5	5	2	GU563123, GU563124	GU563152, GU563153, GU563154, GU563155	2	GU563068, GU563069	GU563095, GU563096
	R. racemosa		25	25	2	GU563125, GU563126	GU563156, GU563157	2	GU563070, GU563071	GU563097, GU563098
Costa Rica, Damas Island	*R. mangle*		25	25	—	—	—	—	—	—
	R. harrisonii		9*	9	—	—	—	—	—	—
	R. racemosa		23	23	—	—	—	—	—	—
Panama, San Miguel Gulf	*R. .mangle*		38	38	2	GU563127, GU563128	GU563158, GU563159	2	GU563072, GU563073	GU563099, GU563100
	R. harrisonii		42	42	2	GU563129, GU563130, GU563131	GU563160, GU563161, GU563162	2	GU563074, GU563075	GU563101, GU563102
	R. racemosa		12	12	2	GU563132, GU563133	GU563163, GU563164	2	GU563076, GU563077	GU563103, GU563104
Total			251	244	26			27		

Note: * One individual had two different inflorescence types and could be classified as *R. harrisonii* or *R. racemosa*.

APPENDIX 2. Analysis of molecular variance (AMOVA) of *Rhizophora* from the Pacific Ocean at two different levels of structure: (1) one group, and (2) three groups—taxa (i.e. *R. mangle* vs. *R. harrisonii* vs. *R. racemosa*).

Level of structure analyzed	Variance	% total	*F* statistics	*P*
One group				
Among populations	0.453	27.67	$F_{ST} = 0.277$	**0.000**
Within populations	1.184	72.33		
Three groups—taxa: *R. mangle* vs. *R. harrisonii* vs. *R. racemosa*				
Among groups	0.243	14.34	$F_{CT} = 0.143$	**0.042**
Within groups	0.266	15.72	$Fsc = 0.184$	**0.000**
Within populations	1.184	69.95	$F_{ST} = 0.301$	**0.000**

Note: Boldface *P* values are significant at 0.05 level.