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Comparative genetic structure of two mangrove species in Caribbean and Pacific estuaries of Panama

Ivania Cerón-Souza^{1,2*}, Eldredge Bermingham¹, William Owen McMillan¹ and Frank Andrew Jones^{1,3}

Abstract

Background: Mangroves are ecologically important and highly threatened forest communities. Observational and genetic evidence has confirmed the long distance dispersal capacity of water-dispersed mangrove seeds, but less is known about the relative importance of pollen vs. seed gene flow in connecting populations. We analyzed 980 *Avicennia germinans* for 11 microsatellite loci and 940 *Rhizophora mangle* for six microsatellite loci and subsampled two non-coding cpDNA regions in order to understand population structure, and gene flow within and among four major estuaries on the Caribbean and Pacific coasts of Panama.

Results: Both species showed similar rates of outcrossing ($t = 0.7$ in *A. germinans* and 0.8 in *R. mangle*) and strong patterns of spatial genetic structure within estuaries, although *A. germinans* had greater genetic structure in nuclear and cpDNA markers (7 demes > 4 demes and $S_p = 0.02 > 0.002$), and much greater cpDNA diversity ($H_d = 0.8 > 0.2$) than *R. mangle*. The Central American Isthmus serves as an exceptionally strong barrier to gene flow, with high levels nuclear ($F_{ST} = 0.3-0.5$) and plastid ($F_{ST} = 0.5-0.8$) genetic differentiation observed within each species between coasts and no shared cpDNA haplotypes between species on each coast. Finally, evidence of low ratios of pollen to seed dispersal ($r = -0.6$ in *A. germinans* and 7.7 in *R. mangle*), coupled with the strong observed structure in nuclear and plastid DNA among most estuaries, suggests low levels of gene flow in these mangrove species.

Conclusions: We conclude that gene dispersal in mangroves is usually limited within estuaries and that coastal geomorphology and rare long distance dispersal events could also influence levels of structure.

Keywords: *Rhizophora mangle*, *Avicennia germinans*, Pollen dispersal, Seed dispersal, Spatial genetic structure

Background

Mangrove communities are composed of phylogenetically unrelated species, each adapted in different ways to the coastal environment where high salinity, diurnal patterns of submergence, wave action, and frequent disturbance create high stress environments [1,2]. Mangrove forests have an extended tropical and subtropical geographic distribution during the last 40 My [3,4]. They are also among the most biologically productive forests and provide key ecosystem services such as breeding grounds for fish, shrimp, and birds and function to protect coasts from tidal surges during hurricanes or

extreme weather [5,6]. However, mangrove forests are being destroyed and fragmented at alarmingly high rates in the tropics due to human development [7-10]. Moreover, mangrove forests may be particularly susceptible to increasing sea levels [11]. The ability of mangroves to adapt and regenerate in the face of disturbance and changing environmental conditions is therefore dependent upon rates of local and long distance dispersal, colonization and genetic diversity found within and among populations.

In most trees, it is generally assumed that rates of pollen gene dispersal are greater, and often much greater, than rates of gene flow via seed. However, several lines of evidence suggest that mangrove species are capable of frequent long distance seed dispersal (LDD) on scales of many km that may exceed rates of gene flow via pollen. For example, exceptionally high levels of gene

* Correspondence: iviceron@gmail.com

¹Smithsonian Tropical Research Institute, Apartado 0843-03092, Panama

²University of Puerto Rico, Rio Piedras Campus, PO BOX 23360, San Juan 00931-3360, Puerto Rico

Full list of author information is available at the end of the article

flow have been reported between West Africa and South America at scales > 6,000 km [12,13]. In addition, mark and recapture experiments in ocean currents in the Caribbean and in the Gulf of Mexico have revealed high dispersal potential for mangrove seeds [14-17]. Direct measurements at very local scales within mature mangrove forests have shown very short seed dispersal distances i.e. less than 10 m in two weeks, [18]. Therefore, the extent of gene flow via seed observed among populations may be due to the frequency at which seeds encounter large open ocean currents when dispersed from more isolated estuaries where water flow is largely due to diurnal tidal action.

A comparison between the extent of pollen vs. seed gene flow in mangroves would improve our understanding of mangrove population structure and reveal the contribution of each to observed spatial patterns of genetic structure [19-21]. In this study, we compare patterns of nuclear and plastid genetic structure in the two dominant mangrove species from the new world, the black mangrove (*Avicennia germinans*, Avicenniaceae) and the red mangrove (*Rhizophora mangle*, Rhizophoraceae), across the four main estuaries of Panama within a scale up to 300 km along same coastline (Figure 1). The focal species currently occur in sympatry on both sides of the Central American Isthmus (CAI) but have different historical biogeographies. *Rhizophora mangle* has been present in the Neotropics for 40 My while *A. germinans* has been present since 16 My [3,4,22]. The two species have contrasting patterns of life-history traits and current demography. *Avicennia germinans* has an entomophilous pollination system with polyphile (i.e. bees, wasps and flies identified as effective pollinators), whereas *R. mangle* is characterized by a simultaneous wind (anemophily) and entomophilous pollination, termed ambophilous pollination [2,23-26]. Although the two mangrove species have perfect flowers, the breeding mechanism seems to be different [2]. The protandry observed in *A. germinans* suggest a mostly outcrossing breeding system, however self-compatibility has not been tested yet in this species [24]. In comparison, *R. mangle* shows a mixed-mating breeding system where self-pollination could be more frequent [23,27]. *Avicennia germinans* has small light ovoid crypto-viviparous propagules with longevity of four months while *R. mangle* seeds are large, elongate and viviparous with longevity of one year [2,28,29]. In addition, while *R. mangle* is the only species from the genus *Rhizophora* to inhabit the Caribbean coast of Panama, on the Pacific side it is in sympatry with *R. racemosa*, generating introgressive hybrid zones where morphological and genetic distinctions between species and hybrids are intricate [13]. Finally, populations of *A. germinans* have a patchy, low-density distribution (e.g. 6 stems/km² in Bocas del Toro, Panama) compared to *R. mangle* populations, which have

a more uniform distribution with extremely high densities (e.g. 1,544 stems/km² in Bocas del Toro, Panama) [30]. Previous analyses of *A. germinans* have reported high levels of genetic diversity of this species on the Pacific side of CAI, including Panama [12,31,32].

Based on these life-history characteristics and previous reports of seed dispersal capacity, we expected that: i) both species would display low genetic structure or little evidence for isolation by distance (IBD) within estuaries and ii) both species would have a higher rate of seed gene flow than pollen gene flow because of their capacity for LDD via sea-drift seeds [12,13]. In addition, at the species level comparison we expected that *Avicennia germinans* would have greater degree of population differentiation than *R. mangle* because a combination of lower seed longevity and viability in ocean water, insect pollen dispersal mechanism, and low-density distribution [19,33].

Results

Genetic differentiation and mating systems

Despite differences in many life history characteristics, the two species did not show significant differences in levels of genetic structure ($F_{ST} = 0.32 \pm 0.04$ SE for *A. germinans* and $F_{ST} = 0.40 \pm 0.05$ SE for *R. mangle*), inbreeding ($F_{IS} = 0.20 \pm 0.03$ SE for *A. germinans* and $F_{IS} = 0.13 \pm 0.13$ SE for *R. mangle*) or outcrossing rates ($t = 0.67 \pm 0.06$ SE for *A. germinans* and $t = 0.77 \pm 0.19$ SE for *R. mangle*) (Kruskal-Wallis ANOVA, $P > 0.05$, $N=4$) (Table 1). Both species showed deviations from HWE, but only *R. mangle* showed evidence of linkage disequilibrium. These deviations could be explained by deme structure within estuaries that we explained below.

General patterns of diversity among four estuaries

Both species showed striking differences in genetic diversity. *Avicennia germinans* showed considerable variation in microsatellite alleles among four estuaries, with the highest H_e observed in the Caribbean Costa Arriba estuary (0.730) and lowest in the Pacific San Miguel Gulf (0.459). In contrast, *R. mangle* showed lower overall gene diversity in Caribbean populations (0.349 and 0.305 for Bocas del Toro and Costa Arriba, respectively) relative to Pacific estuaries (0.654 and 0.610 for Montijo Gulf and San Miguel gulf, respectively), however, no significant differences in outcrossing rates within species were observed among estuaries (Table 1 and Additional file 1). The higher gene diversity and number of alleles in Pacific estuaries of *R. mangle* is complicated by the complex hybridization evident in both Pacific estuaries (Figure 1), which serves as a novel source of genetic diversity in Pacific populations [13].

Chloroplast DNA also revealed striking patterns in diversity and structure in these two species when comparing

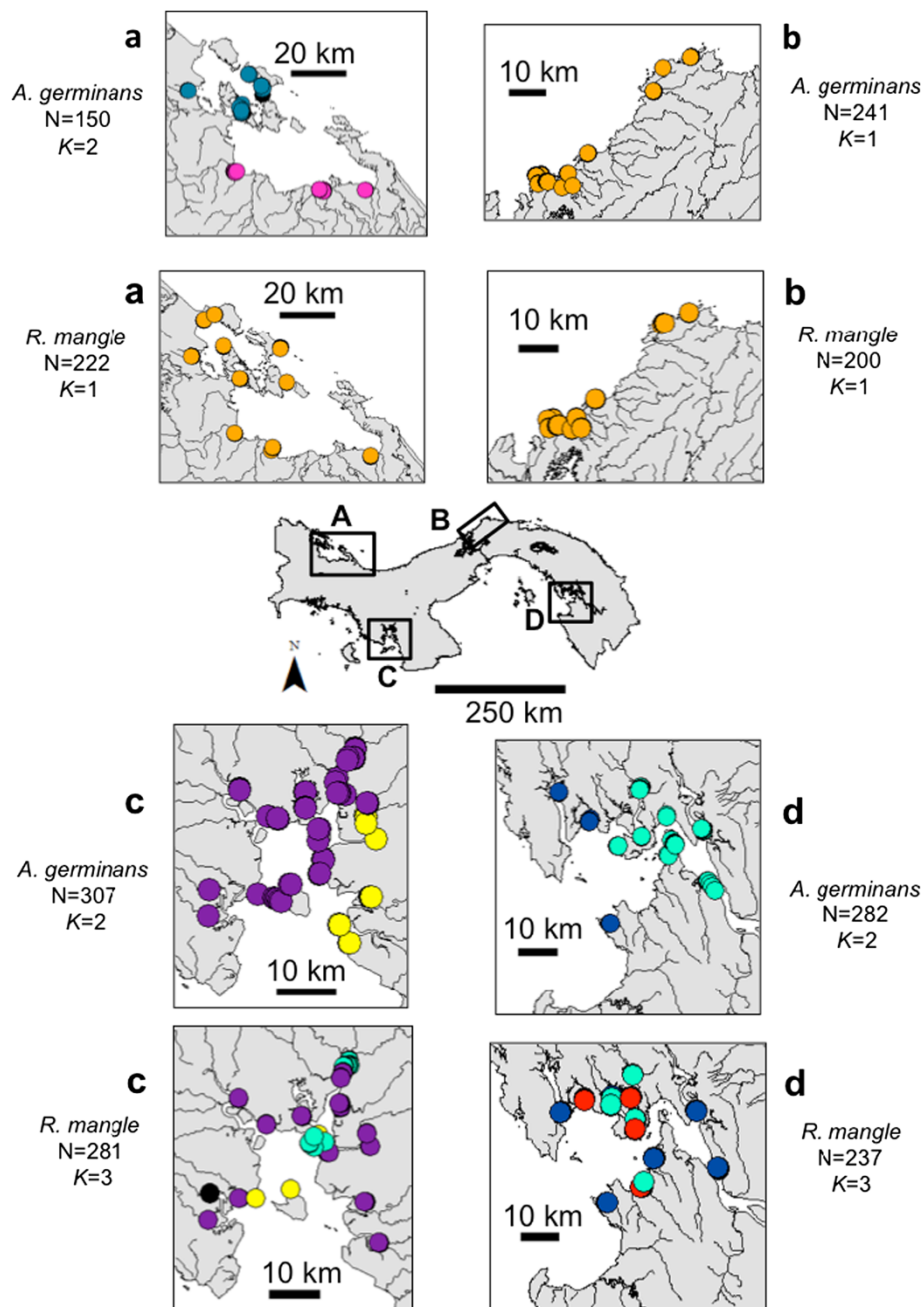


Figure 1 Population structure for two mangrove species within each estuary when analyzed separately using GENELAND 2.0.12. The estuaries under study were Bocas del Toro (a) and Costa Arriba (b) in the Caribbean, and Montijo Gulf (c) and San Miguel Gulf (d) in the Pacific. Points represent individuals and their colors represent the assignment of each one of the inferred clusters (K). Admixed individuals that were simultaneously assigned to two clusters within each estuary with a probability > 0.5 are in black. Due to the scale of estuary maps, all individuals from same transect (i.e. ~30 individuals) are overlapping. However, they were usually assigned to the same cluster (i.e. same color in the map) with very few exceptions.

among estuaries. *Rhizophora mangle* has a single fixed haplotype in the Caribbean coast that include Bocas del Toro and Costa Arriba estuaries ($H_d = 0.00$), and only two haplotypes in the Pacific ($H_d = 0.44$ for Montijo Gulf and $H_d = 0.37$ for San Miguel Gulf) for a total of three

haplotypes. The opposite trend was observed in *A. germinans*, which showed remarkably high genetic diversity of cpDNA haplotypes with 22 haplotypes observed from 58 individuals. For this species, the haplotype diversity was similar among four estuaries ($H_d = 0.84$ for Bocas

Table 1 Nuclear microsatellite F_{ST} and Chloroplast (cpDNA) F_{ST} estimated as G_{ST} in two mangrove species

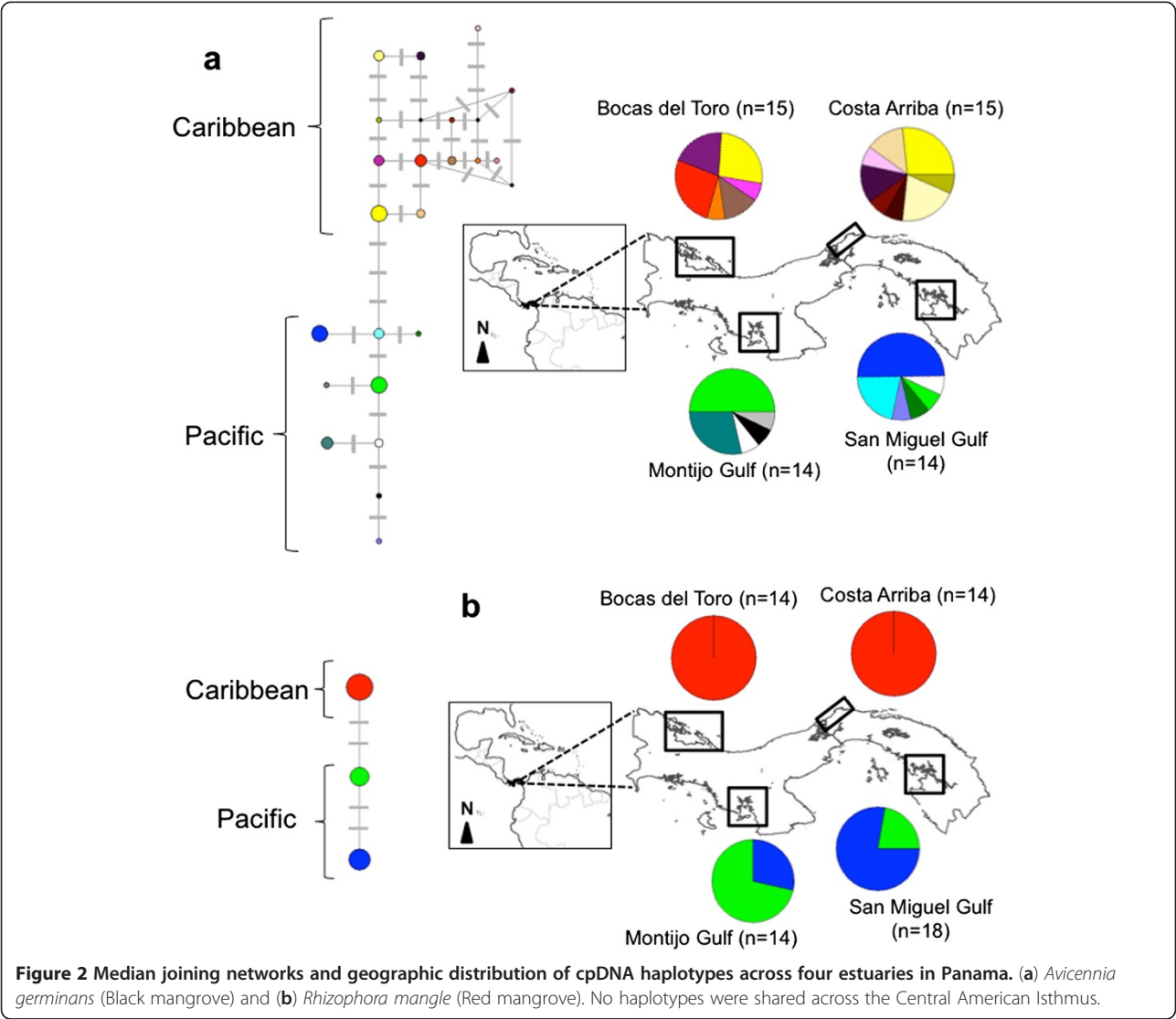
Mangrove species	Microsatellite			CpDNA				Ennos 's method r = Pollen flow/Seed flow
	<i>F_{ST}</i>	<i>F_{IS}</i>	Outcrossing level	Hamilton & Miller's method				
	(SE)	(SE)	(t) (SE)	Expected cpDNA <i>F_{ST}</i>	95% CI	Observed cpDNA <i>G_{ST}</i>	95% CI	
<i>Avicennia germinans</i>	0.3181 (0.0396)	0.1950 (0.0335)	0.6736 (0.0597)	0.5550	0.4758-0.6342	0.3469	0.1407-0.5531	−0.64
<i>Rhizophora mangle</i>	0.4001 (0.0512)	0.1327 (0.1307)	0.7657 (0.1885)	0.6485	0.5461-0.7509	0.8504	0.5888-1.1120	7.65

The estimates of G_{ST} were calculated using the Hamilton and Miller's method [21, eq. 10] and the Ennos's method [43, eq. 5a] in *Avicennia germinans* (black mangrove) and *Rhizophora mangle* (red mangrove) across four Panamanian estuaries. The comparison of F_{ST} , F_{IS} and outcrossing level (t) between *A. germinans* and *R. mangle* did not show significant differences between species (Kruskal-Wallis ANOVA, $P>0.05$, $N=4$).

del Toro, $H_d = 0.89$ for Costa Arriba, 0.70 for Montijo Gulf and 0.73 for San Miguel Gulf). Finally, no cpDNA haplotypes were shared between coasts for either species (Figure 2, Additional file 1, Additional file 2 and Additonal File 3).

Hierarchical structure of genetic variation based on microsatellites

We found evidence of similar levels of genetic structure between species overall ($F_{ST} = 0.32 \pm 0.04$ SE for *A. germinans* $F_{ST} = 0.40 \pm 0.05$ SE for *R. mangle*, Table 1). In addition,



both species showed that majority of microsatellite variation is partitioned within estuaries (70% for *A. germinans* and 64% for *R. mangle*) with differences among estuaries accounting for most of the remaining variation (30% and 36%, Table 2). Thus, pairwise comparison of estuaries separated by the ocean showed exceptionally high levels of differentiation with pairwise F_{ST} ranged between 0.31 to 0.46 in *A. germinans* and between 0.47 to 0.51 in *R. mangle*. Corrected estimates of F_{ST} by null allele presence were highly similar to non-corrected F_{ST} values putatively harboring null alleles (Table 3, Additional file 4).

In spite of these similar strong structures across oceans and estuaries, both species showed differences in how genetic diversity is subdivided between estuaries from the same coast. Pairwise F_{ST} indicates that population differentiation is lower between estuaries in the Caribbean coast than between estuaries in the Pacific coast (i.e. F_{ST} = 0.07 and F_{ST} = 0.008 between two Caribbean estuaries and F_{ST} = 0.134 and F_{ST} = 0.08 between two Pacific estuaries for *A. germinans* and *R. mangle* respectively).

The Bayesian structure analysis supports differences in how structure is organized at two levels (2) Between estuaries from the same coastal line and (3) within estuaries. *Avicennia germinans*, with the exception of the Costa Arriba estuary, shows evidence of two demes within each one of the other three estuaries (Figure 1 and Additional file 5). In comparison, *R. mangle* showed evidence of three demes within each one of the two Pacific estuaries but only one extended population across Caribbean coastline without any differentiation between Bocas del Toro and Costa Arriba estuaries and without any substructure within each one of these two estuaries (Figure 1 and Additional file 6).

At level (3) within estuaries, the two mangrove species showed statistically significant negative slopes (b_{ld}) in the kinship-distance curves (Figure 3 and Table 4). The strength of spatial genetic structure (SGS) measured by S_p statistics for the whole estuary is identical if we compare it with S_p of each deme inferred within the estuary.

The exception was the Montijo Gulf estuary for both *A. germinans* and *R. mangle*. In both cases, S_p calculated for whole estuary was higher than when S_p was calculated for each deme within that estuary. In spite of that, we did not find differences in S_p values across seven *A. germinans*'s demes distributed in the four estuaries (Kruskall-Wallis ANOVA, P = 0.479). However, one of the *R. mangle*'s demes localized in San Miguel Gulf (S_p = 0.04 \pm SE 0.002) showed a stronger SGS compared to only one *R. mangle* deme shared between estuaries from Caribbean coastal line (S_p = 0.01 \pm SE 0.004) (Kruskall-Wallis ANOVA, P = 0.019) (Figure 1, Figure 3 and Table 4).

The extent of SGS of two mangrove species was also different. *Avicennia germinans* showed a range of kinship values almost ten times higher than *R. mangle*. SGS was greater in *A. germinans* (S_p = 0.0186 \pm SE 0.0026, N_{demes} = 7) than for *R. mangle* (S_p = 0.0019 \pm SE 0.0031, N_{demes} = 4) across demes with $N > 50$ on scales up to 10 km (Kruskall-Wallis ANOVA, P = 0.0000) (Figure 3 and Table 4).

Hierarchical structure of genetic variation in cpDNA

Avicennia germinans has lower genetic structure at the cpDNA level (G_{ST} = 0.35) relative to *R. mangle* (G_{ST} = 0.85) (Table 1). However, hierarchical organization of genetic structure of the two species at cpDNA shows more similar patterns than those inferred by nuclear microsatellites. In *A. germinans*, both Caribbean and Pacific coast samples showed strong structure between estuaries, (i.e. F_{ST} = 0.149 between Bocas del Toro and Costa Arriba estuaries in the Caribbean, and F_{ST} = 0.528 between Montijo Gulf and San Miguel Gulf in the Pacific). However, some haplotypes were shared between estuaries along same coastline but separated by ~300 km. In contrast, all *R. mangle* individuals shared a single haplotype on the Caribbean coast, while on the Pacific side although both estuaries shared the same two haplotypes; strong genetic population structure existed between Montijo Gulf and San Miguel Gulf (i.e. F_{ST} = 0.353) (Table 2 and Figure 2). Most of the genetic variation in cpDNA

Table 2 Analysis of molecular variance (AMOVA) for two mangrove species after 10,000 permutations using ARLEQUIN 3.5

Mangrove species	Microsatellite				cpDNA			
	Variance	% total	F_{ST}	P	Variance	% total	F_{ST}	P
<i>Avicennia germinans</i>								
Among estuaries	1.113	30.39	0.304	0.000	2.345	66.80	0.668	0.000
Within Estuaries	2.550	69.61			1.165	33.20		
<i>Rhizophora mangle</i>								
Among estuaries	0.819	35.99	0.399	0.000	2.875	56.23	0.562	0.000
Within Estuaries	1.229	64.01			2.238	43.77		

Analysis of species *Avicennia germinans* (black mangrove) and *Rhizophora mangle* (red mangrove) was performed comparing four estuaries of Panama based on microsatellite and cpDNA polymorphism.

Table 3 Pairwise genetic structure for two mangrove species across four estuaries in Panama using F_{ST}

		Caribbean		Pacific	
		Bocas del Toro	Costa Arriba	Montijo Gulf	San Miguel Gulf
<i>Avicennia germinans</i>	Caribbean				
	Bocas del Toro	-	0.149*	0.817*	0.721*
	Costa Arriba	0.068*	-	0.740*	0.658*
		<u>0.063 (0.035 - 0.099)</u>			
	Pacific				
	Montijo Gulf	0.403*	0.311*	-	0.528*
		<u>0.372 (0.298 - 0.453)</u>	<u>0.287 (0.214 - 0.367)</u>		
	San Miguel Gulf	0.461*	0.369*	0.134*	-
		<u>0.442 (0.340 - 0.549)</u>	<u>0.361 (0.268 - 0.460)</u>	<u>0.140 (0.083 - 0.201)</u>	
<i>Rhizophora mangle</i>					
Caribbean	Bocas del Toro	-	0.000	0.706*	0.504*
	Costa Arriba	-0.008	-	0.706*	0.504*
		<u>0.011 (0.003 - 0.019)</u>			
	Pacific				
	Montijo Gulf	0.471*	0.490*	-	0.353*
		<u>0.453 (0.327 - 0.546)</u>	<u>0.460 (0.334 - 0.553)</u>		
	San Miguel Gulf	0.494*	0.512*	0.078*	-
		<u>0.470 (0.383 - 0.555)</u>	<u>0.479 (0.388 - 0.564)</u>	<u>0.071 (0.046 - 0.104)</u>	

The AMOVA was performed using 10,000 permutations in ARLEQUIN 3.5. It is indicated the F_{ST} values derived from both microsatellite (below the diagonal) and cpDNA data (above the diagonal and in italics) for each pairwise comparison across estuaries. The asterisk indicate $P < 0.05$, after Bonferroni corrections. Underlined values represent the unbiased F_{ST} estimates following the ENA method that correct by the presence of null alleles on F_{ST} estimation (95% CI after 10,000 replicates).

genome is partitioned among ocean basins, with 67% for *A. germinans* and 33% of the variation partitioned within estuaries (Table 2 and Additional file 2). *Rhizophora mangle* shows a similar pattern with 56% of the variation partitioned among oceans and 44% within estuaries (Table 2). Contrary to what was observed across microsatellites, the SGS analysis based on cpDNA did not show significant slopes within each one of the four Panamanian estuaries analyzed, perhaps due to limited sample size (Additional file 3).

Comparison of pollen and seed migration rates

In *A. germinans* the pollen-to-seed gene flow ratio was negative, or basically zero ($r = -0.64$), which indicates one to two times higher gene flow via hydrochorous seed dispersal than entomophilous dispersed pollen gene flow. In comparison, the ratio obtained in *R. mangle* indicates approximately seven times higher ambophilous pollen gene flow than hydrochorous seed gene flow ($r = 7.65$). However, because the confidence limits of each species overlap with the expected value of equal seed and pollen flow, we could not reject the null hypothesis of $m_{pollen} = m_{seed}$ gene flow in either species (Table 1).

Discussion

Mangrove communities are critically important ecosystems that are high in aquatic and terrestrial biological diversity in tropical and subtropical ecosystems worldwide [2,5]. Today they are threatened by high rates of anthropogenic disturbance, including habitat destruction, pollution,

fragmentation, and changes in oceanic and estuarine environments due to climate change [9,10,34]. The goal of this study was to document spatial genetic structure of two dominant Neotropical mangrove species at three spatial levels (1) among four estuaries in Panama (2) Between two estuaries from same coastal line and (3) within each one of the four estuaries. These data provide critical information to understanding how genetic diversity is structured and maintained within mangrove species and communities.

Both mangrove species showed a strong genetic break across the CAI. However, the patterns of diversity observed in this study were the opposite of what we had expected for both species. We found significant differences between estuaries from same coastline and also IBD within estuaries in both mangrove species. In addition, both mangrove species showed comparable outcrossing rates, contrary to other reports on their mating systems. Further, *A. germinans* showed much higher levels of genetic diversity, especially in plastid genomes, than *R. mangle*, in spite of the fact that *R. mangle* populations have a more continuous distribution. Finally, although there is documented evidence for extreme LDD in both mangrove species, our evidence mostly indicates restricted gene dispersal overall and largely equivalent rates of seed and pollen dispersal. Below, we interpret the observed population genetic differences of these species as well as the combined effects of species' life histories, gene dispersal limitation, and biogeographic history.

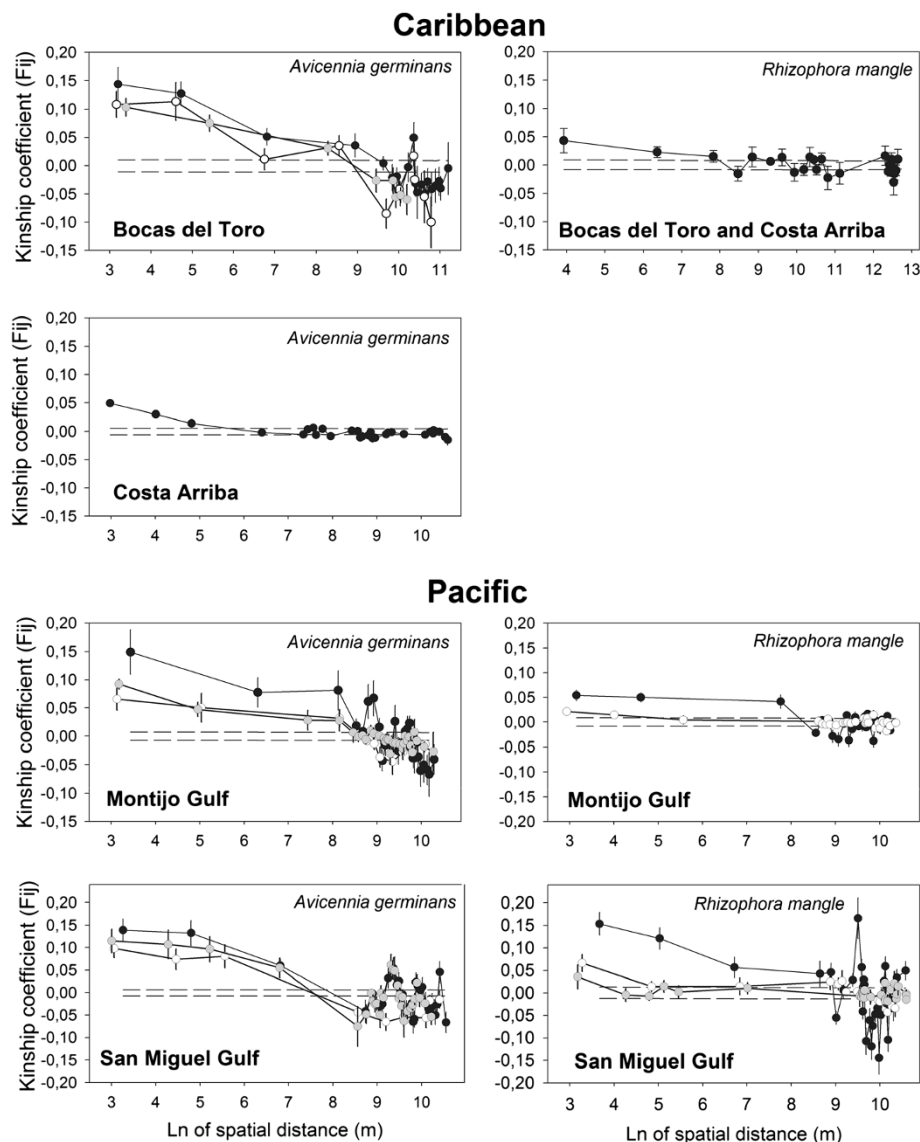


Figure 3 Spatial autocorrelation of average Kinship coefficients (F_{ij}) against the natural logarithm of spatial distance inside four estuaries in Panama. The Kinship-curve for the whole estuary is represented in black. Where GENELAND detected internal substructure, Kinship-curves for each genetic pool were calculated when $N > 50$ (white and grey curves). Dashed lines represent a 95% confidence interval around the hypothesis of no genetic structure for the whole estuary based on 10,000 permutations. We generated uneven lags with constant number of individuals inside distance classes ($N > 100$) with $> 90\%$ of pairwise relationships among nearest neighbors included within the first interval.

The ecological imprint on genetic structure: mating system and Pollen vs. seed movement

The two mangrove species showed strong genetic structure across four estuaries analyzed, including evidence of substructure and IBD within estuaries. Nevertheless, the patterns of genetic structure were very different between species. Microsatellites revealed lower gene diversity and lower genetic structure in *R. mangle* than *A. germinans*. This contradicts the predictions based upon an outcrossing mating system where assumed outcrossing species, such as *A. germinans*, are expected to have lower genetic structure than the mixed-mating *R. mangle* [33,35].

The protandry reported in *A. germinans* is the main evidence that supports an outcrossing mating system in this species because the flower-developing mechanism makes autogamy unlikely (i.e. within-flower pollination) [24]. However, absence of self-pollination (autogamy) is not equivalent to self-incompatibility because pollination could occur between different flowers on the same plant (geitonogamy). Other *Avicennia* species from Indo-West Pacific region including *A. marina* and *A. officinalis* show this pattern [36,37]. There is no direct evidence of self-incompatibility in *A. germinans* that we know of. It is possible that the patchy spatial distribution of *A.*

Table 4 Spatial Genetic Structure (SGS) parameters for two mangrove species across four estuaries in Panama

Mangrove species/ Ocean	Estuary	N	b_{ld} (R^2ld)	F_A (SE)	S_p (SE)
<i>Avicennia germinans</i>					
Caribbean	Bocas del Toro	150	-0.0217*** (0.0639)	0.144*** (0.030)	0.025 ^a (0.007)
	Bocas del Toro (P)	76	-0.0330*** (0.1038)	0.108*** (0.023)	0.037 ^a (0.009)
	Bocas del Toro (MG)	72	-0.0145*** (0.4050)	0.104*** (0.016)	0.016 ^a (0.006)
	Costa Arriba (O)	241	0.0081*** (0.0222)	0.049*** (0.006)	0.008 (0.001)
Pacific	Montijo Gulf	307	-0.0256*** (0.0853)	0.149*** (0.039)	0.030 ^a (0.010)
	Montijo Gulf (Y)	85	-0.0147*** (0.0602)	0.065*** (0.020)	0.016 ^b (0.005)
	Montijo Gulf (P)	222	-0.0150*** (0.0370)	0.092*** (0.009)	0.017 ^{ab} (0.004)
	San Miguel Gulf	282	-0.0308*** (0.1919)	0.139*** (0.025)	0.036 ^a (0.007)
	San Miguel Gulf (LL)	80	-0.0280*** (0.2058)	0.098*** (0.020)	0.031 ^a (0.011)
	San Miguel Gulf (TG)	202	-0.0271*** (0.1454)	0.115*** (0.026)	0.031 ^a (0.006)
<i>Rhizophora mangle</i>					
Caribbean	Bocas del Toro and Costa Arriba (O)	422	-0.0095*** (0.0039)	0.043*** (0.022)	0.010 (0.004)
Pacific	Montijo Gulf	281	-0.0131*** (0.0241)	0.054*** (0.011)	0.014 ^a (0.003)
	Montijo Gulf (TG)	37	-	-	-
	Montijo Gulf (P)	233	-0.0033** (0.0026)	0.021** (0.006)	0.003 ^b (0.001)
	Montijo Gulf (Y)	10	-	-	-
	San Miguel Gulf	237	-0.0276*** (0.0579)	0.154*** (0.025)	0.033 ^a (0.008)
	San Miguel Gulf (R)	41	-	-	-
	San Miguel Gulf (TG)	71	-0.0000108	0.066*** (0.019)	0.004 ^a (0.002)
	San Miguel Gulf (LL)	125	-0.00002175	0.036** (0.027)	0.008 ^a (0.011)

It is indicated the slopes (b_{ld}) of the regression of kinship coefficient values on the natural logarithm of distance (dl_{ij}) for each estuary, the coefficient of determination R^2 , the average and standard error (SE) of the kinship coefficient among individuals separated by less than 100 m (i.e. 0.1 km) (F_A) for each estuary, and the intensity of SGS (S_p) calculated for pairwise distance among individuals up to 10 Km within each estuary is shown. When GENELAND detected spatial genetic discontinuities within estuaries, the SGS parameters were recalculated for each genetic cluster when $N > 50$. Each one of the genetic clusters were named based on color assignment from Figure 1 including pink (P), malachite green (MG) and orange (O) within Caribbean estuaries and yellow (Y), purple (P), tourmaline green (TG), lapis lazuli (LL) and red (R) in Pacific estuaries.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

germinans populations observed in Panama has an effect on the levels of outcrossing and therefore on the genetic structure in this species. In isolated individuals or low density populations geitonogamy could be an advantageous breeding system [36]. Moreover, the $r = -0.64$ estimates of pollen vs. seed flow suggests that although seed dispersal is likely equivalent to pollen dispersal in *A. germinans*, in a patchy matrix, this seed dispersal still is usually very local, leading to greater biparental inbreeding, and increasing population genetic differentiation and spatial genetic structure in nuclear genomes [38,39].

Our data also suggest that historically ambophilous pollen dispersal mechanism of *R. mangle* has been more efficient promoting outcrossing and long-distance gene flow than entomophilous pollination system of *A. germinans*. Based on previous genetic studies, it is predicted to have higher dispersal potential and therefore low genetic structure in species with wind pollination system over species with insect pollinator system [33]. However,

reproductive biology studies in other ambophilous species suggest a completely opposite trend indicating that in self-compatible species as is the case of *R. mangle*, wind is actually the mechanism that promotes selfing and that out-crossing is associated with insect pollen distribution. The reason is that abiotic mechanisms as wind do not target distant receptive flowers as efficiently as insects [40,41].

Although we do not know the rates of wind-to-insect pollen dispersal in *R. mangle*, the ratios of pollen-to-seed dispersal of $r = 7.7$ estimated in this study are lower than those estimated in exclusively wind-dispersed plants ($r = 17$ [42] and $r = 200$ [43]). In addition, based on the Hamilton and Miller's method calculations, pollen vs. seed was not significantly different from each other meaning that ambophilous pollen dispersal is less efficient than exclusively anemophilous pollen dispersal and/or that seed dispersal in this species is comparatively higher than in other exclusively wind pollinated species.

The contrast of seed viability between the two species may also have a strong influence in levels of genetic structure. *Rhizophora mangle* species have the highest longevity seeds of any mangrove genus [15,29]. Direct experiments regarding establishment of seeds after long periods of floating exposure in sea water have showed a 60% success rate after 247 days floating for *R. mangle*, higher than any other Rhizophoracea species [44]. There is no similar quantitative data on establishment success after dispersal for *A. germinans*; however, its seed longevity is shorter than *R. mangle* [29]. The local genetic structure observed in Panama largely corroborates this pattern, especially in the Caribbean, where two estuaries separated by 300 km showed identical chloroplast and nuclear genetic diversity in *R. mangle* but strong structure in *A. germinans*. Although *A. germinans* showed evidence of shared cpDNA haplotypes among estuaries on the same coast, there are also some cpDNA haplotypes and nuclear alleles that are restricted to each estuary, generating strong structure, even within estuaries. Thus, in a variable estuarine environment where seed movement is stochastic [18], our data suggests that higher propagule longevity leads to a greater chance of successful establishing at long distances, increasing gene flow and decreasing population structure [45,46].

The historical imprint on genetic structure

The Isthmus of Panama represents a 20 My to three My old barrier to seed gene flow between the Atlantic and Pacific oceans, and is the narrowest terrestrial area in the New World that separates mangrove populations in each ocean [47,48]. Based on microsatellite and chloroplast genetic diversity observed across the Isthmus, this land mass has created high levels of genetic structure and a strong barrier for contemporary seed gene flow. Moreover, our results indicate that the Isthmus also represents a strong barrier to pollen flow in each species. This is likely due to the absence of a continuous terrestrial population that spans the entire distance between coasts. Thus, the strong isolating effect that the rise of the CAI has had on population differentiation for these two and other species highlights the role of restricted seed dispersal in creating spatial genetic structure in hydrochorious marine species [49-51].

Population differentiation observed between different oceans is exceptionally high compared to other tropical tree species. For example, Dick and Huertz [52] report an average $F_{ST} = 0.14$ for microsatellites variation from the Neotropical tree *Symphonia globulifera*. Within Panama, *S. globulifera* averaged $F_{ST} = 0.11$ for samples taken across the Isthmus of Panama, which is within the range of observations that we see when comparisons are made within oceans for the mangrove species here. However, even trans-Andean F_{ST} between Mesoamerican

populations of bird pollinated, mammal dispersal *S. globulifera* and those in the Amazon separated by > 3,000 km showed maximum $F_{ST} = 0.27$, still lower than lowest pairwise F_{ST} for *A. germinans* made between trans-Isthmian populations of Montijo Gulf and Costa Arriba ($F_{ST} = 0.31$) separated by < 100 km. Furthermore, populations of insect pollinated and wind dispersed mahogany (*Sweitenia macrophylla*) across Mesoamerica also show a lower overall $F_{ST} = 0.10$, with the largest pairwise differences ($F_{ST} = 0.238$) observed between Panamanian and Guatemalan populations at a distance of > 1,600 km [53].

In spite of similar effects of CAI in the genetic structure of these two mangroves, we found unexpectedly high levels of cpDNA diversity and structure in *A. germinans* that suggests a level of diversity that could be more influenced by population history and demography than current gene flow [54]. The cpDNA diversity observed in *A. germinans* is remarkable given the small sample size and short geographic distances separating the populations both within and between coasts. This high level of diversity could be indicative of historical processes determining spatial genetic structure of populations. Although the fossil record of mangroves in Pleistocene is scarce and therefore the reconstruction of current mangrove distribution is very speculative, several lines of evidence suggest that mangrove ecosystem were under episodic crises during the Quaternary, specially associated to sea-level and temperature/humidity fluctuations [55-57]. In particular, it is possible that current populations of *A. germinans* represent remnants of refugial populations created during the Pleistocene [58]. In fact, Panamanian populations are genetically diverse compared to other regions, and it has been suggested that both ancient introgressive hybridization and secondary contact between *A. germinans* and its sister species *A. bicolor* has occurred in Panama (especially on the Pacific side), generating a hotspot of genetic diversity [31].

Our results in *A. germinans* contrast strongly with *R. mangle*, where very little cpDNA diversity was observed. These two species differ in their density and distribution, with *R. mangle* forming extremely dense, continuous forests near to shore, and *A. germinans* forming patchily distributed, lower-density stands at low and middle intertidal zones. One hypothesis that could explain the current distribution of cpDNA diversity is that mangrove populations represent relicts of much larger ancestral populations but that after Pleistocene-Holocene sea-level fluctuations, the mangrove composition shifted to a *R. mangle*-dominated community [55,57,59]. Under this scenario, *R. mangle* could have resulted in a more efficient colonizer than *A. germinans* follow a stepping-stone dispersion pattern. This process combined with self-fertilization observed in *R. mangle* could be the responsible of the current continuous and dense populations and the low

cpDNA and nuclear diversity observed in this species [57]. Alternatively, it is also possible that only certain older lineages of *R. mangle* that are well adapted to current conditions survived in the Pleistocene-Holocene sea-level fluctuations and that only those exclusive lineages recolonized available habitats during the Holocene or that longer time of *R. mangle* presence in neotropics generated more loss of diversity via genetic drift than younger *A. germinans*.

Regardless of the explanation, *A. germinans* joins the ranks of tropical tree species in Panama that show complex biogeographic history with disproportionately high levels of cpDNA diversity relative to other parts of their range [60]. The historical complexity of Panama was also evident when we analyzed the geographical variation in structure with greater population differentiation in Pacific versus Caribbean estuaries for both species in nuclear and plastid genetic markers. In the case of *R. mangle*, introgressive hybridization between *R. mangle* and its sister species, *R. racemosa*, is a novel source of genetic variation exclusive to the Pacific [13]. Thus, the levels of genetic structures and patterns of biodiversity in *R. mangle* are completely different between Caribbean and Pacific due to independent historical processes in the occurrence and sympatry of *R. mangle* and *R. racemosa*.

The influence of coastal morphology

Hydrochory *per se* is assumed to be one of the most efficient mechanisms for LDD in plants. Therefore, it is expected that hydrochorous plant species should have high gene flow among populations and low genetic structure, especially in local geographic areas [61-63]. Our data contradict this hypothesis because the two mangrove species resulted genetically structured among and within estuaries, indicating local restrictions to both pollen and seed dispersal, especially in *A. germinans* species. Currently, one of the major threats in mangroves is fragmentation and sea-level changes associated with climate changes [10,11]. Historically, both mangrove species have experienced sea-level fluctuations at several times and climate changes [3,4,55,56], thus, our results suggest that historically *R. mangle* have maintained more gene dispersal of both pollen and seed dispersal than *A. germinans*. However, our results also suggested that geographic location is important in predicting levels of genetic structure in mangroves. Pacific populations proved to be more structured than Caribbean populations in both mangrove species. One possible explanation is biological. The Pacific coast has been characterized by ancient hybridization between *A. germinans* and *A. bicolor*, but there is also the current scenario of introgressive hybridization between *R. mangle* and *R. racemosa*. Both hybridization processes are apparently complicating current levels of structure compared with Caribbean populations [13,31]. The other possible explanation is abiotic, including

basin geomorphology and connectivity due to ocean currents. Our results suggest that current or historical landscape characteristics of Pacific estuaries are in some way enhancing pollen and seed dispersal limitations, generating more structure compared to Caribbean estuaries. Similarly, the density of *A. germinans* is very variable and in some places, for example in French Guiana *A. germinans* could be more dense and extended than *R. mangle* [64]. In consequence, patterns of genetic structure among and within estuaries in that region could be completely different to observed in Panama. Thus, although life-history traits are important to predict expected genetic structure, landscape settings are generating a variety of situations on local scales that complicate any prediction in terms of expected levels of genetic structure [20]. Therefore, although long distance gene flow between South America and West Africa has been observed in both *A. germinans* and *R. mangle* [12,13], estuarine geomorphology and ocean currents in Panama, especially on the Pacific side, seem to be more complex, preventing pollen and seed dispersal.

Methods

Study sites and sampling strategy

We collected leaf samples from *A. germinans* and *R. mangle* trees within the four largest estuaries in Panama: Bocas del Toro and Costa Arriba in the Caribbean and Montijo Gulf and San Miguel Gulf in the Pacific (Figure 1). Within each estuary, we selected ten equidistant sites across the geographic contour for sampling. The sampling sites included rivers, streams, channels and shoreline areas, each with riverine or fringe mangrove forests. Within each sampling site we established transects parallel to the water's edge, and randomly selected a maximum of 30 adult trees for each mangrove species with a dbh ≥ 10 cm and spaced with a minimum distance of 5 m between sampled trees. All selected trees were geo-referenced using a GPS or a compass and measuring tape.

Microsatellite analysis

We used 11 and 6 microsatellite loci for the analysis of 980 *A. germinans* individuals and 940 *R. mangle* individuals respectively, following established protocols [13,65-67] but with modifications [68] (See Additional file 4 for details). We calculated the average number of alleles per locus, observed heterozygosity (H_o), expected heterozygosity (H_e), and the fixation index (F_{IS}) for each species across the four estuaries using GENALEX 6.0 [69]. In addition, we calculated the outcrossing rate by hand as $1 = F_{IS}/1 + F_{IS}$ [70]. For each locus, we tested deviations from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium (LE) with GENEPOP 3.4 [71]. Also, we tested for the presence of null alleles and scoring problems associated with allelic stuttering or allelic dropout using MICROCHECKER 2.2.3 [72].

This analysis found no stutter bands or genotyping errors across microsatellites. However, some loci showed a positive presence of null alleles, ranged from -0.04 to 0.26 in *A. germinans* and from -0.18 to 0.29 in *R. mangle* (Additional File 4).

We compared the hierarchical genetic structure of *A. germinans* and *R. mangle* at three geographic levels: (1) Among four estuaries, (2) Between two estuaries along same coastline and (3) Within estuaries. For (1) among estuaries, we used a F_{ST} based AMOVA [73] in ARLEQUIN 3.5 [74] after 10,000 permutations. We compare this result with the *ENA* method implemented in *FREENA* [75] to correct the bias induced by the presence of null alleles on the F_{ST} estimation after 10,000 replicates (Additional File 4). For levels (2) Between two estuaries along same coastline and (3) Within estuaries we used STRUCTURE 2.2 [76-78], GENELAND 3.1.4 [79,80] and, for mixed-mated *R. mangle*, INSTRUCT [81].

In STRUCTURE 2.2 we assumed an admixed model and a uniform prior probability of the number of populations, K . All the runs were performed with 500,000 MCMC replicates after a burn-in of 50,000 replicates. We used a model of correlated allele frequencies varying the level of structure from $K = 1$ to 6 populations. Ten independent runs were done for each value of K to generate our estimate of the true number of demes [82]. Previous empirical and simulation analysis using STRUCTURE showed that null allele presence have a low effect in the accuracy of assignment tests [83,84]. This effect was moderate even in populations with a frequency of null alleles > 0.917 for a single locus [83]. Therefore we performed the STRUCTURE 2.2. analysis with no correction to the raw data to account for null alleles. Nevertheless, the GENELAND 3.1.4. analysis was performed using a explicit null alleles presence model as we explain below.

The GENELAND 3.1.4 model assumes that population membership is structured across space. Thus, if this assumption is correct, the power of inferring clusters based upon the combination of genetic and geographical information increases compared with using STRUCTURE alone [85,86]. However, in the case of weak spatial organization, the inferred structure of GENELAND is expected to be similar to STRUCTURE inference [80]. For each run in GENELAND, we used the spatial D-model to calculate allele frequencies and set the maximum rate of the Poisson process and the maximum number of nuclei (i.e. three times the total number of individuals) according to the total number of individuals collected within each coast. In addition, we set an uncertainty attached to spatial coordinates fixed to 50 m. We performed ten independent runs of GENELAND allowing K to vary from 1–10 populations using

the simultaneously uncorrelated allele frequency model, the spatial model, and the null allele model due to the presence of null alleles detected in both mangrove species (Additional File 4). We completed 100,000 iterations for each independent run, saving every 10th iteration using a burn-in of 5,000 iterations. Finally, as mating systems may be different between the two species under study and selfing rates could influence deme structure in mixed-mating species, we used INSTRUCT to infer simultaneously the selfing rates of *R. mangle* and its genetic structure at two levels: (2) Between two estuaries along same coastline and (3) Within estuaries. In INSTRUCT, HWE is not assumed as it is in STRUCTURE; rather the expected genotype frequencies are calculated based on selfing rates. We ran five independent chains, each chain having 500,000 iterations steps, 250,000 burn-in iterations, a thinning interval of 10 and assuming a Dirichlet process of mixture.

Spatial genetic structure within estuaries

We investigated spatial genetic structure (SGS) at level (3) within estuaries using a spatial autocorrelation analysis [87]. For this analysis, we calculated the pairwise kinship coefficient between all individuals (F_{ij}) separated at different distance classes following [88] up to 10 km. Kinship coefficients (F_{ij}) were regressed using the logarithm of the spatial distance between individuals (ld_{ij}) within each one of the estuaries analyzed. Standard errors were assessed by jackknifing data over each locus. We generated uneven lags with constant number of individuals inside distance classes ($N > 100$) with $> 90\%$ of pairwise relationships among nearest neighbors included within the first interval. Using SPAGeDi 1.3 [89], we calculated the regressions of kinship coefficients (F_{ij}) vs. natural logarithm of distance classes (ld_{ij}) to provide the regression slope (b_{ld}). We tested the significance of SGS and the IBD in two dimensions using the observed slope (b_{ld}) of the linear regression of the kinship coefficient on the logarithm of the distance class against the null hypothesis $H_0: b_{ld} = 0$ (i.e. the overall absence of SGS) by comparing the observed values with those obtained after 10,000 permutations of individuals between locations. Where GENELAND detected a spatial deme within an estuary, this procedure was applied to each inferred deme that was represented by at least 50 individuals, excluding admixed individuals.

To compare the extent of SGS between the two mangrove species over the same geographic scales and across different estuaries, we calculated the S_p statistics on spatial scales of up to 10 km. The S_p statistics were calculated from the slope of the regression (b_{ld}) of the Kinship coefficient [F_{ij} of 88] against the logarithm of the distance, $S_p = -b_{10km}/(1 - F_A)$ where the regression slope, b_{10km} is less than 10 km and F_A is the average

kinship coefficient between individuals belonging to the first distance class (< 100 m in all cases). This first distance class included all pairs of neighbors [90]. In the cases where we found demes within estuaries we repeated this procedure for each one of the subpopulations if $N > 50$.

Chloroplast analysis

A subsample of 58 individuals of *A. germinans* and 60 individuals of *R. mangle* and distributed across the four estuaries (i.e. 14–18 individuals per estuary) was sequenced using two chloroplast (cpDNA) non-coding regions *atpI-atpH* and *psbJ-petA* [91]. We redesigned primers to avoid short-repeat regions and improve sequence quality in all regions except for the *atpI-atpH* region in *A. germinans* [91]. Modified primers for the *psbJ-petA* region in *A. germinans* were F: AGATTGATC GATATCGGGTTC and R: GGAAAACCGAAACCCA GAC. Modified primers for *R. mangle* analysis, PCR and sequencing specifications for both species were described previously [13]. For each mangrove species we calculated the haplotype diversity using DNASP 4.5 [92]. In addition we constructed a median joining network [93] for the combined cpDNA regions using NETWORK 4.5.1.0 (fluxus-engineering.com). We calculated population structure using ARLEQUIN 3.5 [74] at level (1) among four estuaries and (2) between two estuaries from same coastal line. Finally, we used SPAGED1 1.3 [89] to investigate SGS at level (3) within estuaries, using a spatial autocorrelation analysis of cpDNA variation. For this analysis, we calculated the pairwise kinship analogue coefficient between all individuals based on the genetic distances between haplotypes (N_{ij}) separated at different distance classes vs. natural logarithm of distance classes (ld_{ij}) to provide the regression slope (b_{ld}) on spatial scales up to 10 km in a procedure similar to that used for microsatellites [89].

Comparison of pollen and seed migration rates

In order to compare pollen and seeds migration rates across four estuaries we estimated F_{ST} and F_{IS} from microsatellite data and G_{ST} from cpDNA data using SPAGED1 1.3 [89]. These F -estimates were used to calculate the ratio (r) of pollen migration (m_p) to seed migration (m_s) (i.e. m_p/m_s) for each mangrove species following the equation $r = [(1/F_{ST}^{bipar} - 1)(1 + F_{IS})] - 2(1/G_{ST}^{mat} - 1)/(1/G_{ST}^{mat} - 1)$ [Eq. 5a, 43]. In addition, the same F -statistics based on microsatellite and cpDNA variations were used to test the null hypothesis $m_{seed} = m_{pollen}$ comparing the expected maternal F_{ST} predicted from microsatellite variation (i.e. biparental F_{ST}) vs. the observed maternal F_{ST} estimated as G_{ST} from actual cpDNA variation [21]. The expected maternal F_{ST} was calculated following $F_{ST}^{mat} = (a_{bipar}F_{ST}^{bipar})/[a_{mat} + (a_{bipar} - a_{mat})F_{ST}^{bipar}]$ where $a_{mat} = 2.0$ and

a_{bipar} change depending of outcrossing rate (t) calculated for each species from microsatellite data [Eq. 10, 21]. Under this procedure, the null hypothesis is rejected if confidence intervals ($\pm 2SE$) of observed and expected values fail to overlap [21].

Conclusions

Although there is documented direct and genetic evidence for extreme LDD in mangrove species *A. germinans* and *R. mangle*, our data across estuaries in Panama showed restricted gene dispersal overall and equivalent rates of seed and pollen dispersal in both mangrove species. *Rhizophora mangle* showed lower gene diversity and lower genetic structure than *A. germinans*. This suggest that an amphophilous pollen syndrome combined with a higher propagule longevity leads to a greater chance of successful establishing at long distances, increasing gene flow and decreasing gene diversity and population structure. However, species density, coastal geomorphology as well as ocean currents could vary across ocean basins and estuaries, generating a variety of situations on local scales that complicate any prediction in terms of expected levels of genetic structure.

Additional files

Additional file 1: Genetic diversity of nuclear (i.e. microsatellites) and chloroplast genomes for *Avicennia germinans* (black mangrove) and *Rhizophora mangle* (red mangrove) in Panama. The number of individuals analyzed per estuary, the number of alleles, observed heterocigosity (H_o), and the expected heterocigosity (H_e) of microsatellites calculated with GENALEX 6.0 is shown. In addition, the number of individuals analyzed per estuary, the number of haplotypes and the haplotype diversity found in chloroplast using DNASP 4.5 and NETWORK 4.5.1.0 (fluxus-engineering.com), and the GenBank accession number of each individual analyzed is indicated.

Additional file 2: Median joining network indicating and geographic distribution of cpDNA haplotypes found in *Avicennia germinans* (Black mangrove). Within the network, the haplotype name and the number of individuals per each haplotype is indicated. In addition, for each estuary, the geographic distribution of haplotypes and their frequency (i.e. pie) is indicated.

Additional file 3: Median joining network indicating and geographic distribution of cpDNA haplotypes found in *Rhizophora mangle* (Red mangrove). Within the network the haplotype name and the number of individuals per each haplotype is indicated. In addition, for each estuary the geographic distribution of haplotypes and their frequency (i.e. pie) is indicated.

Additional file 4: PCR conditions for 11 microsatellite loci for *A. germinans* (black mangrove) [65,66] and six microsatellite loci for *R. mangle* (red mangrove) [67] following established protocols [13,65,66] with modifications including three primers in the PCR thus: a dye tagged M13 universal forward primer (5'-CACGACGTTGTAACGAC-3') [68], primer Forward (F) and primer Reverse (R) where either F or R primer (indicated with asterisk *) had a tail at the 5' end that was identical to the M13 universal forward primer sequence. Amplified fragments from both mangrove species were electrophoretically separated on ABI 3130xl and analyzed using ABI PRISM® GeneMapper™ software version 3.7. For each estuary is indicated the frequency of null alleles calculated by the Brookfield method 1 in MICROCHECKER 2.2.3 [72].

Additional file 5: Bayesian genetic assignment of *Avicennia germinans* (black mangrove) from two Caribbean (Bocas del Toro and Costa Arriba) and two Pacific (Montijo Gulf and San Miguel Gulf) estuaries in Panama based on STRUCTURE ver. 2.2 and GENELAND ver. 2.0.12. The true K for each procedure after simulations is indicated.

Additional file 6: Bayesian genetic assignment of *Rhizophora mangle* (Red mangrove) from two Caribbean (Bocas del Toro and Costa Arriba) and two Pacific (Montijo Gulf and San Miguel Gulf) estuaries in Panama based on STRUCTURE ver. 2.2, INSTRUCT and GENELAND. 2.0.12. The true K for each procedure after simulations is indicated. In addition, INSTRUCT was used to help to simultaneously infer the selfing rates of this mixed mating species and the demic structure on both sides of the Isthmus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ICS, EB and WOM defined the research topic and the experimental design. ICS carried out the laboratory experiments and generate the data. ICS and AJ analyze data and wrote the manuscript. All authors have contributed to, seen and approved the manuscript.

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Author details

¹Smithsonian Tropical Research Institute, Apartado 0843-03092, Panama.
²University of Puerto Rico, Rio Piedras Campus, PO BOX 23360, San Juan 00931-3360, Puerto Rico. ³Department of Botany and Plant Pathology, Oregon State University, 2070 Cordley Hall, Corvallis, OR 97331, USA.

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