Plant Ecol (2014) 215:481–493
DOI 10.1007/s11258-014-0315-1

Reproductive phenology and physiological traits in the red mangrove hybrid complex (*Rhizophora mangle* and *R. racemosa*) across a natural gradient of nutrients and salinity

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Received: 31 July 2013 / Accepted: 27 January 2014 / Published online: 5 March 2014

Abstract Factors modulating introgressive hybridization between the red mangrove species *Rhizophora mangle* and *R. racemosa* in spatially defined sites are poorly understood. To investigate this, we evaluated the reproductive phenology and the nutrient and physiological traits in those two species and their F_1 hybrids genotyped with microsatellite data across a natural hybrid zone from the Pacific coast of Panama. We found no evidence that reproductive phenology represents a barrier to gene flow, because *R. mangle* and the F_1 hybrids produced flowers and propagules throughout the annual cycle, while *R. racemosa* flowered only in the dry season. Soil nutrient concentrations decreased landward, while soil salinity varied only slightly. Foliar nutrients and $\delta^{15}N$ signatures varied according to the soil nutrient gradient, but only foliar phosphorus and carbon varied among species. In contrast, two structural variables (height and trunk diameter) and leaf variables related to salinity tolerance (Na, Cl:Na, K:Na, cation:anion) and water-use efficiency (i.e., $\delta^{13}C$) differed among species, suggesting higher salinity tolerance for *R. mangle* and F_1 hybrids compared with *R. racemosa*. We conclude that parental species and F_1 hybrids differ in salinity tolerance and water-use efficiency, which could be associated with adaptive evolution of the red mangrove hybrid complex.

Keywords Introgressive hybridization · Salinity tolerance · Nutrient limitation · Flowering time · Microsatellites · Water-use efficiency

Communicated by Siegy Krauss.

Electronic supplementary material The online version of this article (doi:10.1007/s11258-014-0315-1) contains supplementary material, which is available to authorized users.

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Introduction

Hybridization is a common process in animals and plants. Defined as the crossbreeding between individuals of different species, or in more general terms of populations that differ in heritable characters, it is estimated that 25 % of plant species hybridize with

other taxa (Mallet 2005). This process has a strong influence on the evolution of plants, because it can reinforce the mechanism of reproductive isolation between species, or lead to hybrid speciation or exchange of potentially adaptive variation between species when introgression occurs (Rieseberg and Wendel 1993; Baack and Rieseberg 2007; Rieseberg and Soltis 1991; Arnold 2006; Arnold et al. 2012).

Red mangrove (*Rhizophora* spp.) is one of the most conspicuous mangrove genera worldwide due to its broad distribution in the intertidal zone (Duke et al. 2002). The genus has two species in the neotropics: *R. mangle* is widely distributed in tropical and subtropical coastlines, while *R. racemosa* is restricted to specific regions in the tropical belt in both the Atlantic and Pacific oceans, including West Africa, Central, and South America (Duke and Allen 2006). These two species experienced ancient hybridization and introgression, which continues today when they occur in sympatry (Cerón-Souza et al. 2010). The natural hybrids are known as *Rhizophora* × *harrisonii* and correspond to what has been considered erroneously as a third species in the red mangrove complex in the neotropics (Cornejo 2013; Cerón-Souza et al. 2010).

Despite the introgressive hybridization, the distinctive inflorescence types of *R. mangle* and *R. racemosa* are genetically identifiable and coexist in sympatry, usually following a clear spatial zonation apparently associated with variation in salinity across the intertidal zone. Where zonation occurs, *R. mangle* usually grows in areas with high salinity, suggesting that it tolerates salinity to a greater extent than *R. racemosa*, which usually occurs in areas with significant input of fresh water. Further, hybrids have been observed to occupy intermediate positions between *R. mangle* and *R. racemosa*, suggesting intermediate salinity tolerance compared with the parent species (Afzal-Rafii et al. 1999; Savory 1953; Jiménez 1987; Smith 1992).

Field studies indicate that *R. mangle* is extremely plastic in its nutrient demand and salinity tolerance (Fry 2000; McKee et al. 2002). The $\delta^{13}C$ signature of dwarf and tall *R. mangle* in Florida demonstrated that isotopic differences between height-morphotypes lack any genotypic imprint. The dwarf condition was reversed by nutrient addition, thus supporting phenotypic plasticity associated with nutrient supply and high soil salinity (Fry 2000; Lin and Sternberg 1992a, b). Although *R. racemosa* seems to be also plastic in its nutrient requirements (Medina et al. 2008), the restriction of this species to the tropical belt and its consistent association with fresh water input in riverine mangrove systems suggest that it might require greater nutrient supply than *R. mangle* (see Fig. 1 in Medina and Francisco 1997).

The spatial pattern in *Rhizophora* species raises a number of questions concerning the physiological and environmental factors that contribute to the maintenance of the genetic identity and spatial genetic structure of *Rhizophora* in hybrid zones (Cerón-Souza et al. 2010), as well as the importance of introgressive hybridization in the adaptive evolution of this group to tolerate salinity and nutrient limitation (Arnold et al. 2012). In this study, we examined reproductive phenology, soil chemistry, and leaf physiological traits, including stable isotopic ratios ($\delta^{13}C$, $\delta^{15}N$) in a hybrid *Rhizophora* zone on the Pacific coast of Panama. We asked the following questions: (1) Are there differences in the timing of flowering and the production of propagules among *R. mangle*, *R. racemosa* and hybrids? (2) Are parental *Rhizophora* species and hybrids distributed along gradients of nutrients and salinity in the intertidal zone? If so, (3) do parental *Rhizophora* species and hybrids show a physiological response associated with the taxonomic group?

Materials and methods

Study plot and forest characterization

We established a study site at the mouth of Guabitas River, on the Pacific coast of Panama (08°18.151′ N, 80°16.443′ W), along a hybrid zone between *R. mangle* and *R. racemosa*. The area is characterized by flooding twice per day. We established three replicate transects 150–200 m long and 5–100 m apart perpendicular to the shoreline and traversing three distinct zones: a shoreline zone dominated by *R. mangle*, a middle zone where most potential hybrids occur, and an inner zone dominated by *R. racemosa*. Three plots (50 × 50 m) were established at 5–20 m intervals along each transect for a total of nine plots in the study area (Fig. 1a). Within each of the nine plots, we randomly selected ten adult *Rhizophora* individuals with diameter at breast height (DBH) ≥ 0.1 m. These 90 individuals were mapped, tagged, measured (height and DBH), and identified as *R. mangle*, *R. racemosa*,

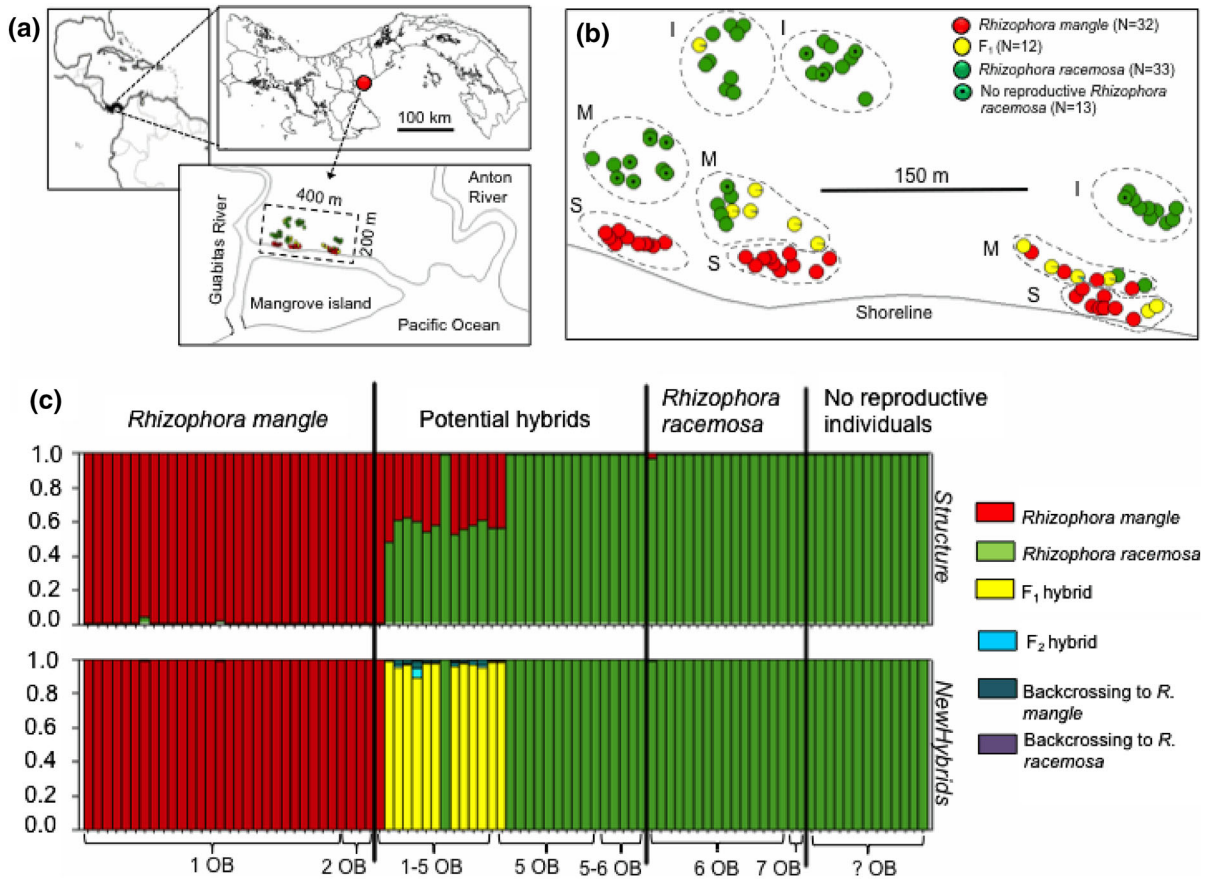

Fig. 1 Study plot and the genetic structure and Bayesian assignment analysis of 90 *Rhizophora* individuals using 10 microsatellite molecular markers. **a** The study plot is localized in the mouth of Guabitas River, in the Pacific coast in Panama. **b** The study area was subdivided in three replicate plots per each intertidal position: shoreline (S), middle (M), and inner (I). Within each one of the plots, 10 individuals were marked and identified taxonomically (i.e., using the order of Bifurcation in the inflorescence—OB) and genotyped using 10 microsatellite molecular markers. The genetic analysis identified 32 *R. mangle* individuals, 12 F_1 hybrids, and 46 *R. racemosa* individuals (13 of them without inflorescence during 19 months of study). **c** Detailed results of the Bayesian assignment analysis of the study plot using *Structure* and *NewHybrids*. Each vertical line represents one of the 90 individuals. The individuals were separated in three taxonomic groups following the inflorescence type (OB): *R. mangle*, potential hybrids and *R. racemosa*. The colours indicate the genetic group to which each individual is assigned. Using *Structure*, the best genetic partition consisted in two clusters ($K = 2$) (see Online Source 1) that matched with *R. mangle* and *R. racemosa* inflorescence type. Using *NewHybrids*, the individuals were assigned to six genetic groups, namely pure *R. mangle*, pure *R. racemosa*, F_1 hybrids, F_2 hybrids, backcrossing to *R. mangle*, and backcrossing to *R. racemosa*

or potential hybrid based on inflorescence characteristics (Cerón-Souza et al. 2010). Vouchers of selected individuals were deposited at the University of Panama herbarium except for 13 individuals that showed no inflorescence or propagules during the 19 months of study.

Genetic classification of individuals

The 90 *Rhizophora* individuals were genotyped using 10 microsatellite loci. Six loci (i.e., RM07, RM11, RM19, RM21, RM36 and RM46) followed the same PCR methodology used previously (Cerón-Souza et al. 2010). The other four loci (i.e., RM05, RM50, RM59, RS67) followed the procedure described by Takayama et al. (2008), but included a fluorescently tagged M13 universal forward primer in the PCR reaction (Steffens et al. 1993) following Cerón-Souza et al. (2010). We determined the genetic composition of individuals and, in the case of hybrids, the hybrid class using two Bayesian statistic analyses for inference of population structure and assignment of

individuals, *Structure* 2.3.3 (Pritchard et al. 2000; Falush et al. 2003, 2007) and *NewHybrids* 1.1 beta (Anderson and Thompson 2002).

We performed the *Structure* analysis without considering information on *Rhizophora* taxonomic identification based on inflorescence-types, 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 made ten runs of the above procedure for each value of K and followed the criteria of Evanno et al. (2005) to select the best estimate of the number of genetic groups based on ΔK calculation.

The *NewHybrids* analysis was performed five times, starting each time with a different random number of seeds, using 500,000 iterations and 50,000 burn-in steps of the MCMC chain. In the analysis, 90 individuals were assigned to one of six genotype classes: pure *R. mangle* parent, pure *R. racemosa* parent, F_1 hybrid (50 % of the genome originated from *R. mangle* and 50 % from *R. racemosa*), F_2 hybrid (50 % originated from F_1 hybrid and 25 % from each of the parents *R. mangle* and *R. racemosa*), back-crosses with *R. mangle* (50 % originated from F_1 hybrid and 50 % from parent *R. mangle*), and back-crosses with *R. racemosa* (50 % originated from F_1 hybrid and 50 % from parent *R. racemosa*).

Reproductive phenology

From May 2010 to February 2012, we recorded presence/absence of inflorescence and propagules (i.e., germinated seeds in the mother plant through viviparism) for each of the 90 identified individuals. This was done once per month, except for three months from November 2010 to January 2011 when extreme rainfall prevented access to the study area. We calculated the monthly frequency of inflorescences and propagules, grouping individuals by genetic categories inferred by Bayesian analysis of microsatellite polymorphism and compared this data with meteorological information for the Pacific coast of Panama recorded monthly for the last 20 years (data per year are provided by the *Autoridad de Recursos Acuáticos de Panama* upon request). This information included mean precipitation (mm), temperature (°C), wind speed (m s^{-1}), and insolation (kWh m^{-2} day^{-1}).

Soil and leaf sampling

Across the nine plots classified in the intertidal zone as shoreline ($n = 3$), middle ($n = 3$), or inner ($n = 3$), we measured 13 edaphic variables at two depths (0–10 and 30–40 cm) (Fig. 1b), covering the rainy season (in October 2010) and the dry season (in February 2011). Samples were always taken during low tide. Each sample was a composite of five cores distributed equidistantly within the plot.

Three mature and healthy leaves from the canopy of each of the 90 selected individuals were collected in the rainy season (in October 2010). We determined the leaf area for each of the fresh leaves without the petiole. Leaves where dried in a ventilated oven at 60 °C for 48 h and weighed after cooling to room temperature. The three leaves per individual tree were pooled and ground.

Soil and plant analysis

Measurements of redox potential, pH, and temperature where made in situ using the Oyster™ Portable pH/Conductivity/TDS/ORP Salinity kit. Pore water salinity was measured with a refractometer (VEE GEE Scientific, Inc). Conductivity was measured in a dilution of 5 g of soil (previously dried at 30 °C) in 15 ml of deionized water using the Oyster™ Portable kit after 14 h. Extractable ammonium and nitrate concentrations were determined using extraction in 2 M KCl on the day of collection, with detection by automated colorimetry using a Lachat Quikchem 8500 flow injection analyzer (Hach Co., Loveland, CO, USA). Ammonium was measured at 660 nm following reaction with phenolate, while nitrate was measured at 520 nm following cadmium-catalyzed reduction to nitrite and reaction with sulfanilamide at pH 8.5. Resin-extractable phosphate (Resin P) was determined using anion-exchange membranes with detection by means of automated molybdate colorimetry (Turner and Romero 2009).

Total carbon (C), total nitrogen (N), and stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) of soils and leaves were determined simultaneously using stable isotope ratio

mass spectrometry using a Flash HT Elemental Analyzer coupled through a Conflo III interface to a Delta V Advantage continuous flow isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). The base cations calcium (Ca), magnesium (Mg), potassium (K), and sodium (Na) in leaves were determined by digestion under pressure in concentrated HNO_3 with detection using inductively-coupled plasma optical-emission spectrometry (Optima 7300 DV, Perkin Elmer Inc., Shelton, CT, USA). Foliar chloride (Cl) was determined by means of titration using a Salt Analyzer (SAT-500; DKK-TOA Corporation, Japan).

Statistical analysis

To test seasonality of flowering and fruiting for each genetic group, we used a Chi square test under the null hypothesis of same frequency of inflorescence and propagules across 19 months of study. In addition, to test differences in the physicochemical conditions and macronutrient concentrations across the intertidal soils where *Rhizophora* taxa show spatial zonation, we used an Analysis of covariance (ANCOVA) for one covariate (i.e., the distance from shoreline) and two factor variables, season (dry and rainy), and depth (0–10 and 30–40 cm). Similarly, to test the physiological differences across *Rhizophora* individuals, we performed an ANCOVA for one covariate (i.e., the distance from shoreline) and one factor variable, the genetic groups, 32 *R. mangle*, 12 F_1 hybrids, and 46 *R. racemosa*. For each one of the soil and foliar variables, the initial models included interaction among all factors. Models were simplified using a stepwise backward selection and the Akaike's Information criterion model (Crawley 2007). In the cases where analysis did not meet the assumption of homogeneity of slopes, we divided the distance from shoreline into three intervals: shoreline, middle, and inner, and we focused on the effect of the categorical variables (Logan 2010). Post-hoc multiple pairwise comparisons were made according to the Tukey method under the best AIC model. Results were considered significant at $p < 0.05$. Relationships within and between foliar and edaphic variables were examined using linear regression analysis. All statistical analyses were performed in R 3.0.0 (R Core Team 2013). Variables that were not normally distributed were transformed using either Log_{10}, square root, or the Johnson transformation under the *Johnson* package in R (Santos-Fernandez 2011). Untransformed means ± standard errors are presented for all variables.

Results

Spatial and genetic structure of mangrove forest

In total, we identified 31 *R. mangle* individuals, 16 *R. racemosa* individuals, and 30 potential hybrids based on the order of bifurcation (OB) in the inflorescence. In addition, 13 individuals remained unidentified due to the absence of reproductive organs (i.e., inflorescences or propagules) during the study. The *Structure* analysis identified two clusters ($K = 2$) as the best genetic partition of the data (Online Resource 1), which largely supported morphological classification. In both Bayesian analyses (*Structure* and *NewHybrids*), individuals showing "mangle" inflorescence-type (i.e., OB between 1 and 2) were assigned to the pure parental *R. mangle* genetic group and individuals with "racemosa" inflorescence-type (i.e., 6–7 OB) were assigned to the pure parental *R. racemosa* group. Likewise, all 13 individuals without inflorescence were genetically assigned to the pure *R. racemosa* group. In comparison, potential hybrids based on inflorescence-type were split into different categories after Bayesian analysis. Twelve individuals were assigned to the F_1 progeny group (50 % *R. mangle* and 50 % *R. racemosa*), one individual was assigned to the pure *R. mangle* group and 17 individuals were assigned to the pure *R. racemosa* group. Those 18 individuals with intermediate inflorescence that were identified as pure paternal species suggest more than two advanced generations of backcrossing that could not be accurately detected in the *NewHybrids* analysis or, alternatively, greater ranges of variation in parental species, which would imply less diagnostic value for morphological characters (Fig. 1b, c).

Rhizophora individuals followed a spatial zonation at the intertidal zone: pure *R. mangle* individuals were located exclusively in the shoreline and middle plots, while pure *R. racemosa* individuals were located exclusively in the middle and inner plots. In comparison, the 12 F_1 hybrids were spread across the three intertidal zones, two in the shoreline, nine in the middle, and one in the inner zone. Thus, the middle plots were transition zones where parental species and F_1 hybrids coexist (Fig. 1b, c).

Phenological patterns

The comparison of presence (%) of inflorescences and propagules in pure *R. mangle* ($N = 32$), F_1 hybrids ($N = 12$) and pure *R. racemosa* individuals (i.e., 33 reproductive and 13 non-reproductive) indicated strong differences in reproductive phenology. For the pure *R. mangle* individuals, >84 % maintained inflorescences and >70 % maintained propagules throughout the study ($X^2 = 2.2$, df = 18, $p = 1$ and $X^2 = 5.0$, df = 18, $p = 0.99$, respectively). In comparison, inflorescence and propagules for the 33 reproductive *R. racemosa* individuals showed strong seasonal peaks in the dry and rainy seasons, respectively ($X^2 = 111.5$, df = 18, $p < 0.001$, and $X^2 = 131.1$, df = 18, $p < 0.001$, respectively). More than 80 % of individuals flowered during the dry season (January–April), when insolation and wind speed were greatest. In comparison, the presence of inflorescences decreased dramatically at the onset of the rainy season between June and September. Accordingly, >60 % of *R. racemosa* individuals produced propagules during wetter months (June–July). All 12 F_1 hybrids were fertile and, similar to *R. mangle* phenology, inflorescences and propagules were present throughout the year with no sign of seasonality ($X^2 = 6.8$, df = 18, $p = 0.99$ and $X^2 = 15.8$, df = 18, $p = 0.61$) (Fig. 2).

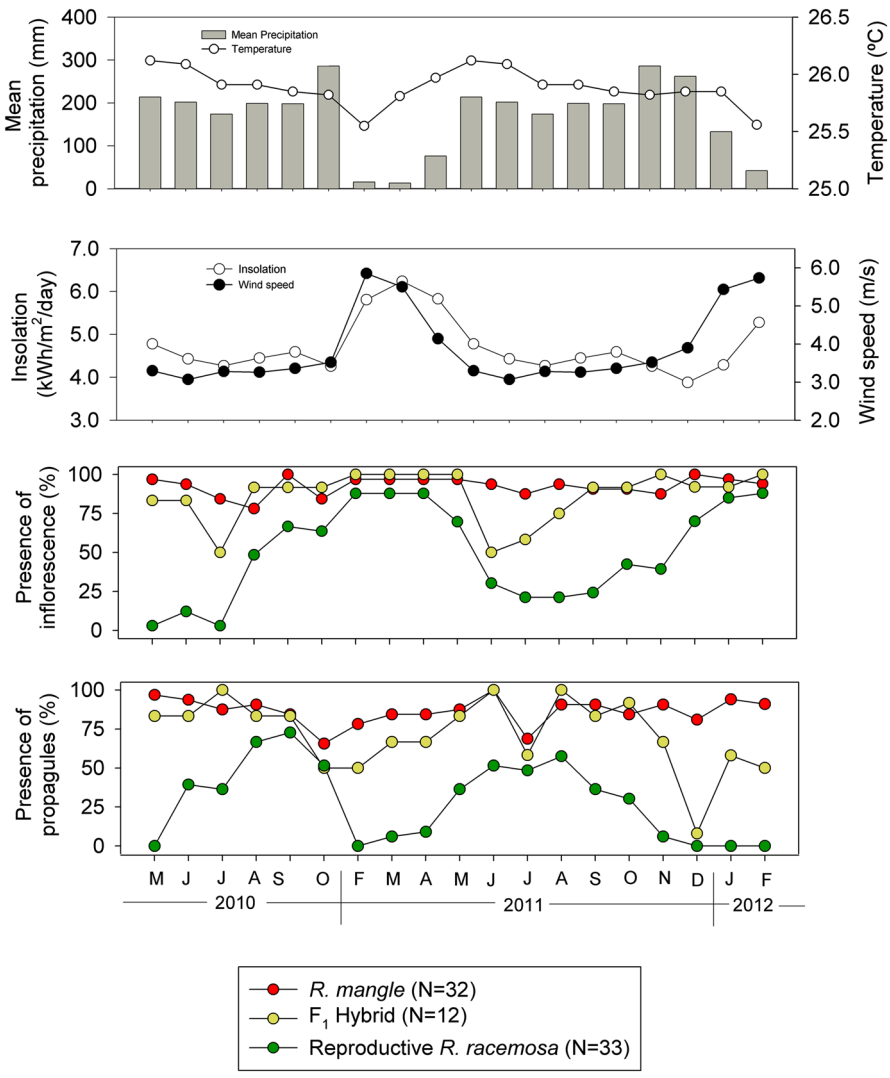

Fig. 2 Flowering and fruiting phenology (percentage of presence of inflorescence and presence of propagules) recorded in *R. mangle*, *R. racemosa*, and F_1 hybrids during 19 months of study, from May 2010 to January 2012. The upper two graphics show the means of four meteorological variables in the Pacific coast of Panama during the last 20 years (i.e., precipitation, temperature, insolation, and wind speed). The lower two graphics show the proportion of inflorescence and propagules in 77 red mangrove individuals (32 *R. mangle*, 33 reproductive *R. racemosa* and 12 F_1 hybrids). All monitored individuals were adults with a DBH ≥0.1 m

Physicochemical conditions and soil fertility

The physicochemical conditions in the soil (i.e., redox potential, conductivity, pH and pore water salinity) fluctuated in response to the interaction between intertidal position and season. The exception was temperature, which varied only according to season (Online Resource 2). These measurements suggested that the shoreline zone where *R. mangle* was dominant was flooded for longer than the inner zone where *R. racemosa* was dominant. Consequently, the redox potential (mV), a measure of the concentration of oxidants in the soil, tended to increase landward, and the conductivity and the pH tended to decrease landward. Pore water salinity varied along the flooding gradient, but the pattern varied seasonally. During the wet season salinity was greater in the shoreline zone. However, during the dry season salinity concentrations were greater than in the wet season, but the gradient was reversed, and the salinity was lower in the shoreline compared with inner areas (Fig. 3; Online Resource 2 and 3).

The concentration of soil nutrients across the intertidal zone was explained by either the distance from shoreline or the season, with no interaction between the two factors (Online Resource 2). All nutrient measurements suggest that the shoreline zone where *R. mangle* was dominant contained greater nutrient concentrations than the inner zone where *R. racemosa* was dominant. Thus, total C, total N, C:N, and nitrate in the soils decreased landward. Total P, soil $\delta^{13}C$, and $\delta^{15}N$ also decreased landward, and varied according to either soil depth (i.e., higher concentration of total P at 0–10 cm than at 30–40 cm) or season (i.e., higher values of both $\delta^{13}C$ and $\delta^{15}N$ in the dry season comparing with the rainy season) (Fig. 3; Online Resource 2 and 3). Moreover, $\delta^{15}N$ was correlated positively with total C, total N, and total P, suggesting that $\delta^{15}N$ reflects nutrient supply in the intertidal zones (Online Resource 6). Ammonium and resin P varied only seasonally, with higher concentrations during rainy season than during dry season (Online Resource 2 and 3).

Foliar nitrogen and $\delta^{15}N$

Foliar N, $\delta^{15}N$, and the C:N ratios varied with distance to the shoreline, independently of genetic groups (Fig. 4; Online Resource 4 and 5). Except for N, these

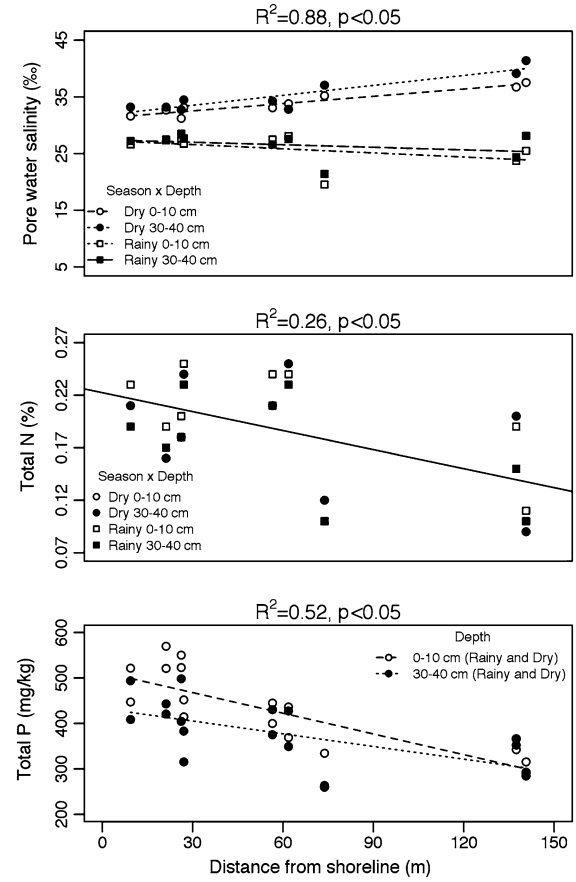

Fig. 3 ANCOVA analysis of pore water salinity, total N, and total P in the soils. For each variable, the R^2 value and the *p* value of the best model is indicated

foliar variables decreased in a landward direction, corresponding to the soil nutrient gradient, although correlations between soils nutrients and foliar nutrients were not significant (Online Resource 6).

Foliar nutrients, foliar $\delta^{13}C$, and tree size

Foliar Cl, Mg, K, and the K:Mg ratio varied across the intertidal zone independently of genetic group. Moreover, foliar calcium and the Ca:Mg ratio showed a significant interaction between distance from shoreline and genetic group suggesting variation across the intertidal zone within and among the genetic groups (Fig. 5; Online Resource 4 and 5).

Despite this general pattern, the variation in foliar Na, the cation:anion ratio, Cl:Na, K:Na, P, C, and $\delta^{13}C$ (which is an indirect measurement of water-use efficiency) were associated only with genetic groups

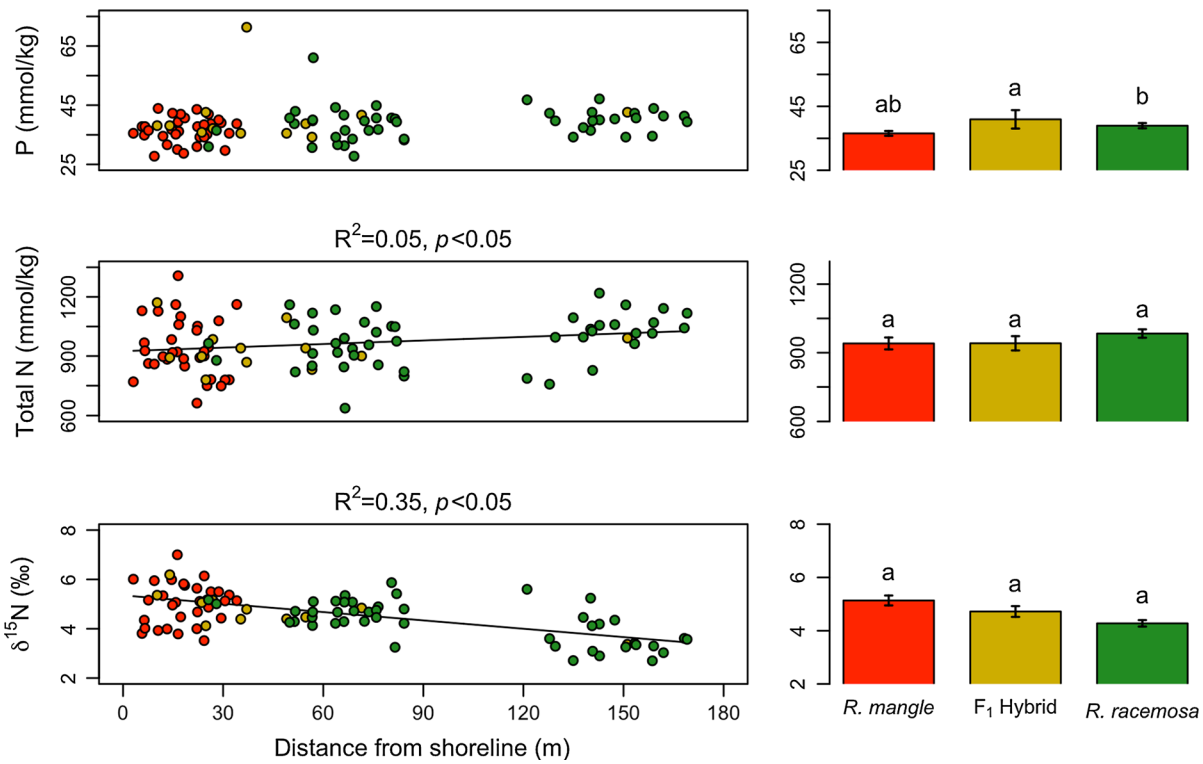

Fig. 4 ANCOVA analysis of three foliar variables measured in 90 *Rhizophora* individuals associated with soil nutrient gradients: Phosphorus (P), total nitrogen (N), and δ^{15}N. Except for P, the final model showed a significant covariance with the distance from shoreline without differences associated with the taxonomy. Each bar represents the mean (±SE) values by taxonomic/genetic groups such as *R. mangle* (red), F_1 hybrids (yellow), *R.* racemosa (green). The *letters* indicate significant differences at $p < 0.05$ after post-hoc Tukey analysis

(Figs. 4, 5). The cation:anion ratio in leaves was higher in *R. racemosa* and F_1 hybrids compared with *R. mangle* (2.7 ± 0.1 and $2.2 \pm 0.2 > 1.8 \pm 0.1$). Foliar Na was greater in *R. racemosa* compared with both *R. mangle* and F_1 hybrids ($975.4 \pm 13.7 > 748.5 \pm 30.2$ and 797.5 ± 67.7 mmol kg^{-1}, respectively). The Cl:Na ratio was lower in *R. racemosa* compared with *R. mangle* and F_1 hybrids ($0.7 \pm 0.1 < 1.1 \pm 0.1$ and 0.9 ± 0.1, respectively); and the K:Na ratio was lower in *R. racemosa* than *R. mangle* and F_1 hybrids ($0.19 \pm 0.0 < 0.22 \pm 0.01$ and 0.21 ± 0.01). Moreover, F_1 hybrids showed greater foliar P, total C, and δ^{13}C than *R. racemosa*, but neither of these two genetic groups differed significantly from *R. mangle* (Figs. 4, 5; Online Resource 5). We found positive correlations between the foliar δ^{13}C, foliar Na, and the Cl:Na ratio, and also between foliar P and N (Online Resource 6).

The height and DBH of the trees varied only depending on genetic group. Assuming that our selection of trees is representative of the wider adult community at the site, this data show that *R. racemosa* and F_1 hybrid trees were taller than *R. mangle* trees (19.4 ± 1.2 and $17.8 \pm 0.6 > 14.0 \pm 0.6$ m, respectively). Moreover, the DBH of F_1 hybrid trees were greater than the parental species *R. mangle* and *R. racemosa* ($0.22 \pm 0.03 > 0.15 \pm 0.01$ and 0.18 ± 0.01 m, respectively) (Fig. 6; Online Resource 5).

Discussion

Hybrid zone structure and reproductive phenology

Genetic analysis based on 10 microsatellite loci confirms a zonation of *Rhizophora mangle, R. racemosa*, and their hybrids along the intertidal zone in our study site. Such zonation based on genetic data is consistent with observations from morphological characters of the inflorescence that are used to separate

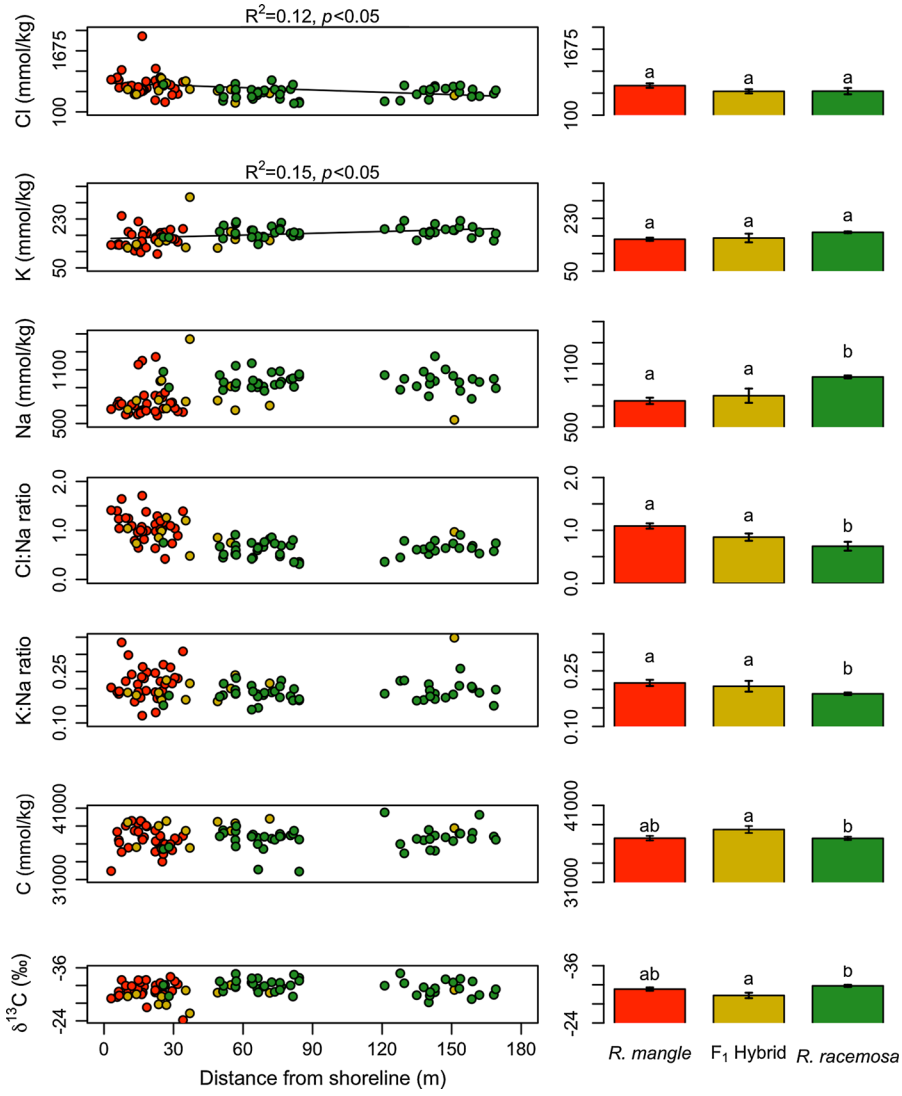

Fig. 5 ANCOVA analysis of seven variables measured in 90 *Rhizophora* individuals associated with salinity tolerance and water-use efficiency. Except for Chloride (Cl) and Potassium (K), all the variables were explained by differences in the taxonomic/genetic group without effect of the distance from shoreline. Each bar represents the mean (±SE) values by means of taxonomic/genetic groups such as *R. mangle* (red) F_1 hybrids (yellow), *R. racemosa* (green). The *letters* indicate significant differences at $p < 0.05$ after post-hoc Tukey analysis

the two hybridizing species. Finding the causes for such zonation would help to understand the structure of red mangroves and could provide clues for the differentiation of two sister species (*R. mangle*, and *R. racemosa*) in the presence of gene flow.

Flowering time is one important pre-zygotic barrier in plants (Lowry et al. 2008), but our data reveal that this barrier is permeable for the mixed-mated *Rhizophora* characterized by a simultaneous anemophily and entomophily (ambophilous) pollination syndrome (de Menezes et al. 1997; Sánchez-Núñez and Mancera-Pineda 2012). The flowering patterns of 32 *R. mangle* trees in the Pacific coast of Panama indicate that this species flowers throughout the annual cycle (Gill and Tomlinson 1971). This is consistent with observations of *R. mangle* individuals in the Caribbean and in the Atlantic Ocean basin, although some fluctuations may exist mainly associated with levels of insolation, water balance, and salinity (Gill and Tomlinson 1971; Mehlig 2006; Fernandes 1999; Agraz-Hernández et al. 2011; Sánchez-Núñez and Mancera-Pineda 2011). In contrast, reproductive *R. racemosa* individuals showed a conspicuous peak of inflorescence and propagule production in the dry and in the rainy seasons, respectively (Fig. 2). This pattern is identical to that observed in the Pacific coast of Costa

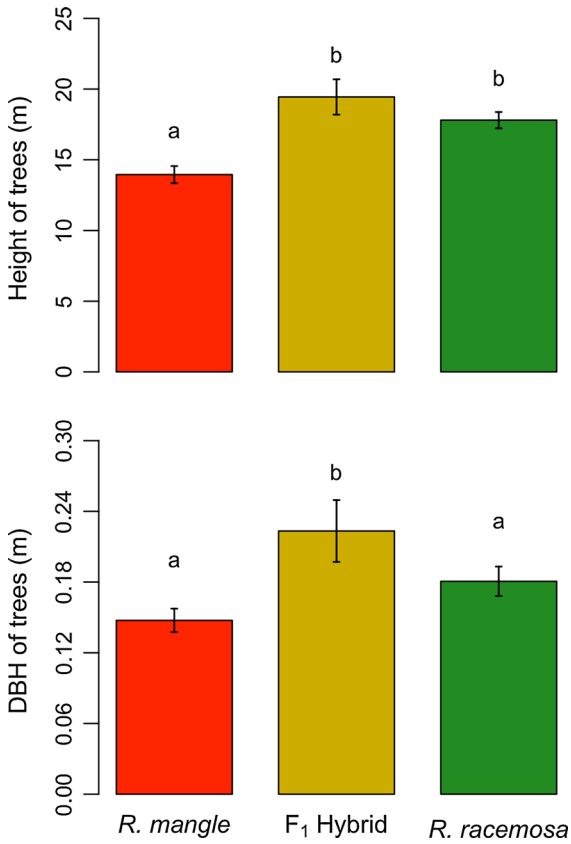

Fig. 6 ANCOVA analysis of two structural variables, Height and DBH, measured in 90 *Rhizophora* individuals. Both variables were explained by differences in the taxonomic genetic/group without effect of the distance from shoreline. Each bar represents the mean (±SE) values by means of taxonomic/genetic groups such as *R. mangle* (red) F_1 hybrids (yellow), *R. racemosa* (green). The *letters* indicate significant differences at $p < 0.05$ after post-hoc Tukey analysis

Rica (Jiménez 1988), suggesting that the reproductive phenology of *R. racemosa* throughout the East Pacific coast is more sensitive to environmental fluctuations than the reproductive phenology of *R. mangle*.

It has been speculated that the flowering-time of *R. racemosa* during the dry season is associated with windier conditions that improve pollen dispersal and therefore enhance out-crossing (Jiménez 1988), a hypothesis that fits our data since wind speed in the Pacific of Panama is high during the dry season. The permeability of the phenological barrier during the dry season is consistent with the formation of F_1 hybrids and backcrosses at the study site, and also with previous results in other geographic regions where hybrid progeny and different levels of backcrossing have been observed (Cerón-Souza et al. 2010).

It has been hypothesized that a high proportion of malformed pollen could prevent fertility of F_1 hybrids (Breteler 1969, 1977; Muller and Caratini 1977). In our study, all 12 trees identified as F_1 hybrids through Bayesian analyses were reproductively active, producing both flowers and propagules. This provides direct evidence that reduced F_1 pollen viability is not an effective post-zygotic barrier. Even if a high proportion of F_1 hybrid pollen is malformed, F_2 progeny and backcrossing occur, as has been inferred using molecular markers (Cerón-Souza et al. 2010). In sum, independently of the factors that may influence the reproductive phenology of the two species, flowering time does not represent an effective pre-zygotic barrier. However, it is possible that a consistent annual cycle of environmentally framed interspecific gene flow influences the formation of hybrids and introgressants, a topic that deserves further research.

Responses to hydro-edaphic and nutritional soil conditions

We found a clear gradient of flooding and nutrients in the intertidal zone of our study plot. More commonly flooded areas (i.e., shoreline and middle zones) contained greater nutrient concentrations than less flooded areas (i.e., inner zones). Within this hydro-edaphic and nutritional gradient, *R. mangle* individuals and F_1 hybrids occurred in zones with higher nutrient input than *R. racemosa*, yet foliar nutrients showed no response associated with taxonomy. On the contrary, distance from the shoreline was the only factor that explained variation in foliar nutrient concentrations. It seems that foliar N and $\delta^{15}N$ reflect nutrient availability at the site that is independent of genetic group (Medina et al. 2001; Fry 2000; McKee et al. 2002; Troxler 2007; Ellison et al. 1996). We conclude that foliar nutrients are not directly involved in the maintenance of *Rhizophora* hybrid zones or in the spatial zonation along which the two species often occur.

Salinity tolerance of *R. mangle* and *R. racemosa* in the hybrid zone

Mangroves grow over a broad range of salinity levels under natural conditions. Field and greenhouse experiments support the hypothesis that interspecific differences in salt tolerance might contribute to the

maintenance of an interspecific spatial zonation across the salinity gradients (Ball 1988, 1996). During water uptake through the roots, *Rhizophora* species take up Na and Cl (Medina et al. 1990). Here, foliar Na was greater in *R. racemosa* compared with *R. mangle* and F_1 hybrids. This pattern is opposite to the soil salinity gradient observed during rainy season, which decreased landward (Fig. 3). Thus, as we measured foliar variables in the rainy season, our data suggest that *R. mangle* has a greater capacity to restrict Na uptake and its subsequent accumulation in leaves compared with *R. racemosa*, which also generates a difference in the Cl:Na ratio (Li et al. 2008). Moreover, our data suggest that the greater ability of *R. mangle* to restrict Na uptake is inherited by the F_1 hybrids.

It has been reported that halophyte species accumulate less Cl than Na and K, so it is assumed that chloride has greater toxicity than organic anions for protein synthesis (Flowers and Colmer 2008). This tendency was corroborated in *R. stylosa* from Australia (Clough 1984), but not in the red mangrove hybrid complex from this study, where Cl:Na ratios were >1. Our data suggest a broad range in the ability of *Rhizophora* species to regulate the uptake of Na and Cl.

Potassium is important for photosynthesis and other metabolic processes in plants (Huber 1985; Ball 1988). Thus, the Na:K ratio is an index of the capacity of the plant to take up K for metabolic requirements in the presence of high concentrations of Na (Medina & Francisco 1997). Our data suggest that *R. mangle* has greater selectivity for K compared with *R. racemosa,* and that this quality is also inherited by F_1 hybrids.

Finally, foliar C, $\delta^{13}C$, and P differed among the genetic groups. Previous analyses of $\delta^{13}C$ among species, and comparing different environments within species (i.e., riverine vs. fringe mangrove, or dwarf vs. tall trees), have demonstrated that $\delta^{13}C$ reflects water-use efficiency associated to salinity stress through an effect on stomatal conductance and intracellular CO_2 concentration during photosynthesis (Ball and Farquhar 1984; Lin and Sternberg 1992a, b). Therefore, less negative $\delta^{13}C$ values indicate a greater water-use efficiency (Medina and Francisco 1997; Medina et al. 2001; Kao and Chang 1998; Cernusak et al. 2013).

Our results suggest that F_1 hybrids occur in more salt-stressed areas than at least one of the parental species, *R. racemosa*. If this is so, it indicates that (1) introgression might be a rapid alternative avenue of adaptive evolution in red mangroves to enlarge the range of tolerance to salinities in *R. racemosa* (Whitney et al. 2006, 2010; Martin et al. 2005; Martin et al. 2006; Arnold et al. 2012), and (2) the patterns of hybridization and introgression between *R. mangle* and *R. racemosa* could be asymmetrical toward *R. mangle* (Scascitelli et al. 2010). Future work should experimentally assess the physiological responses of parental species and advanced hybrids across controlled salinity conditions, as well as the possible interactive effect of salinity with other environmental factors including light and nutrients that could promote *R. mangle* and *R. racemosa* coexistence (Lopez-Hoffman et al. 2006; Lovelock and Feller 2003).

Acknowledgments This study was supported by grants from the BBVA Foundation in Spain, Secretaria Nacional de Ciencia, Tecnología e Innovación—SENACYT (FID-0927) and Smithsonian Tropical Research Institute—STRI in Panama. The authors thank to Autoridad Nacional del Ambiente—ANAM in Panama for study permits and logistic support in the province of Anton-Cocle; J. Chavarría, G. Jaramillo, G.J. Jaramillo, L. Jaramillo, S. Martínez and L. Rodríguez for field assistance; D. Agudo for laboratory support; J. Touchon and S. Dennis for their suggestions in the statistical analysis and L. Santiago for his comments to improve the manuscript.

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