Do inbreeding depression and relative male fitness explain the maintenance of androdioecy in white mangrove, *Laguncularia racemosa* (Combretaceae)?

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Summary

• Mathematical models predict that to maintain androdioecious populations, males must have at least twice the fitness of male function in hermaphrodites. To understand how androdioecy is maintained in *Laguncularia racemosa* (white mangrove), outcrossing, inbreeding depression, and relative male fitness were estimated in two androdioecious populations and one hermaphroditic population.

• Outcrossing was estimated based on length of pollinator foraging bout and pollen carryover assumptions. Inbreeding depression was measured at three life stages: fruit set, seedling emergence, and seedling survivorship. The relative fitnesses of males and the male component of hermaphrodites were compared at these three stages and at the pollen production stage. Male frequency predictions generated by Lloyd's model were compared with observed frequencies in two androdioecious subpopulations.

• Outcrossing estimates were moderate for all populations (0.29–0.66). Inbreeding depression varied among populations (−0.03–0.86), but the strength of inbreeding depression did not increase with male frequency. Males produced significantly more flowers/inflorescence than hermaphrodites, but pollen production/flower did not differ. Male and hermaphroditic progeny did not differ significantly at other life stages.

• Populations of white mangrove with male plants were functionally androdioecious. Lloyd's model accurately predicted male frequency in one androdioecious subpopulation, but underestimated male frequency in the second subpopulation.

Introduction

Biologists have been intrigued by the amazing diversity of plant breeding systems since Darwin (1888) wrote *Different Forms of Flowers on Plants of the Same Species*. Much research has been done describing breeding systems and trying to understand the evolutionary processes responsible for the origin and maintenance of different breeding systems (Richards, 1997), particularly those potentially important in the evolution of

dioecy. Androdioecy is an extremely rare breeding system with two breeding types, males and hermaphrodites (Darwin, 1888). In androdioecious systems, hermaphroditism can be expressed in individual plants in one of two ways: either through the production of hermaphroditic (perfect) flowers, or through the production of both male and female unisexual flowers (i.e. monoecious individuals).

Given its rarity, androdioecy has received much attention by theorists (Lloyd, 1975; Ross & Weir, 1976; Charlesworth

& Charlesworth, 1978; Ross, 1982; Charlesworth, 1984; Pannell, 1997a; Vassiliadis *et al.*, 2000; Pannell, 2002; Wolf & Takebayashi, 2004) who address one fundamental question: How can males persist in populations with hermaphrodites that have both male and female function? Theoretical models of androdioecy based on Evolutionarily Stable Strategy (ESS) assumptions require that male plants have at least twice the fitness incurred by hermaphrodites through male function. The large fitness requirement necessary for male persistence may explain why androdioecy is such a rare breeding system. Until recently, the few descriptions of androdioecy in plants were based on flower morphology, not on sexual function (Charlesworth, 1984). However, form does not always indicate function. In a critical re-examination of androdioecious species, Charlesworth (1984) argued that most, if not all, androdioecious species are cryptically dioecious. In most cases, either the anthers are not dehiscent or the pollen is not viable in the hermaphroditic plants, so morphological hermaphrodites function only as females. Therefore, to establish whether a plant population is functionally androdioecious, pollination tests are necessary. Such tests have demonstrated that only a few plant species are functionally androdioecious (for a review see Pannell, 2002).

Models predict that males with the required twofold fitness advantage can be maintained when hermaphrodites are self-incompatible or when outcrossing is high (Lloyd, 1975; Charlesworth, 1984). However, in species that employ mixed mating strategies, inbreeding depression can cause a reduction in fitness of self-compatible hermaphrodites relative to males, thereby allowing males to be maintained despite moderate or low levels of outcrossing (Rieseberg *et al.*, 1993). High levels of inbreeding depression could also allow males to be maintained at higher frequencies than outcrossing levels would otherwise predict. Further, when outcrossing is held constant at low to moderate levels, male frequency should increase as inbreeding depression increases because: (1) the fitness of self-compatible hermaphrodites decreases as inbreeding depression increases; (2) the relative fitness of males increases as hermaphrodite fitness decreases; and (3) male frequency increases as male fitness increases. The twofold fitness advantage required of male plants is a minimum requirement that allows the persistence of male plants at low frequencies; the fitness advantage required of males increases to infinity as male frequency increases to 50%, so androdioecious populations are expected to have low male frequencies (Vassiliadis *et al.*, 2000).

Previous research indicated that white mangrove (*Laguncularia racemosa*) is androdioecious on San Salvador, Bahamas (Rathcke *et al.*, 1996, 2001b). Here we examine populations of white mangrove in Florida with a range of male frequencies (0–42%) to test whether or not the populations with male plants are functionally androdioecious, to examine factors that might allow the maintenance of males in these populations, and to compare observed male frequencies with those generated by Lloyd's model (Lloyd, 1975).

To determine whether morphologically hermaphroditic plants have both male and female function, pollen viability, fruit set, seedling emergence, and survival of seedlings with hermaphroditic pollen parents were examined. To determine whether males incur a fitness advantage relative to male function in hermaphrodites, males and hermaphrodites were compared at four different stages of the life cycle: pollen production, fruit set, seedling emergence and seedling survivorship. Males could incur the twofold fitness advantage predicted by theoretical models (Lloyd, 1975; Charlesworth, 1984) at any of these stages, or smaller fitness advantages incurred at each stage could, in combination, result in the minimum fitness required of males.

Theoretically, males must incur the fitness advantage at some stage, so we predicted that male plants would produce more pollen than hermaphrodites, and that crosses made with the pollen of male plants would result in greater fruit set than crosses made with hermaphroditic pollen. Further, we predicted that a larger proportion of seedlings would emerge from the fruits produced through crosses made with male pollen relative to hermaphroditic pollen, and that those seedlings would be more likely to survive. Inbreeding depression was also measured at three stages: fruit set, seedling emergence and seedling survivorship. Inbreeding depression has a stronger effect on self-compatible hermaphrodites than on male plants that are incapable of selfing, so males could incur a fitness advantage through a reduction in hermaphrodite fitness caused by low to moderate outcrossing levels coupled with inbreeding depression. We also predicted that male frequency would be positively correlated with the strength of inbreeding depression if outcrossing levels were similar among androdioecious populations.

Parameter values for Lloyd's (1975) model were estimated from field studies and male frequency predictions generated by the model were compared with observed frequencies in two subpopulations of one androdioecious population in South Florida. Lloyd's model calculates male frequency (q) using the following formula:

$$q = (t - 2lv(t + I - ti))/(2iv(1 - t)(1 - l) + t(1 + v - 2lv)),$$

(v is the relative viability of zygotes to reproductive maturity (hermaphrodite : male); l is the relative intrinsic pollen fecundity (number of pollen grains produced per inflorescence, hermaphrodite : male); i is the relative fitness of seeds due to inbreeding depression (selfed : outcrossed); and t is the proportion of seeds resulting from cross-fertilization (outcrossing index)).

A range of male frequency predictions was generated by the model using a series of outcrossing indices (for explanation see the 'Materials and Methods' section, Outcrossing estimates). The predictions were qualitatively compared to observed male frequencies in the two subpopulations.

Materials and Methods

Study species

Laguncularia racemosa (L.) Gaertn. f is a shrub or tree found in mangrove forests throughout the Neotropics and the coast of western Africa (Correll & Correll, 1982; Tomlinson, 1994). In Florida, most plants flower in June and July, although flowering has been reported May–December in Florida (Tomlinson, 2001) and year-round in the Bahamas (Correll & Correll, 1982; L. B. Kass, pers. comm.). The small, white flowers are displayed on branching inflorescences. Both hermaphroditic and male flowers have one style, one stigma, and 10 stamens arranged in two whorls of five, but male flowers do not have ovaries (Tomlinson, 2001). The urn-shaped hermaphroditic flowers are sessile on the rachis, while the smaller, cup-shaped male flowers are very weakly held on the rachis by a short pedicel. Hermaphroditic flowers are open for 2 d; the anthers dehisce on the first day and the stigma is fully receptive on the second day. Male flowers are open for only 1 d. Both flower types produce nectar and are visited by many insect species, including bees, wasps, flies, and butterflies (Rathcke *et al.*, 2001a; Landry, 2005; Landry *et al.*, 2005). Each flower has two ovules, only one of which produces a seed. Plants are semiviviparous, with seeds germinating inside the fruit but not emerging from the fruit until after dispersal. The spongy fruits are water-dispersed (Tomlinson, 2001; Kass, 2005; C. L. Landry, pers. obs.).

White mangrove was first described as morphologically androdioecious on San Salvador Island in the Bahamas (Rathcke *et al.*, 1996); subsequent preliminary pollination tests indicated that it was also functionally androdioecious, and that plants could self-pollinate and self-fertilize (Rathcke *et al.*, 2001b). Previously, Tomlinson (1980, 2001) had described white mangrove in Florida as functionally dioecious because some plants never produce fruit (i.e. are female sterile). However, he further described white mangrove flowers as 'either male or perfect (sometimes female)', and he noted, 'the stamens of the perfect flowers apparently contain functional pollen' (Tomlinson, 2001).

Study sites

The breeding system of white mangrove was examined in three populations on the east coast of Florida, USA: two populations in south Florida, at Coral Gables (CG) just south of Miami and at Hollywood (HOL) approx. 10 miles south of Fort Lauderdale; and one population in central Florida, at Wabasso Island (WAB) approx. 50 miles south of Cape Canaveral. Male frequency varied among these populations. At Coral Gables, two local subpopulations, less than 3.22 km apart, were studied: a South Coral Gables (CGS, 18% male) subpopulation in 1998 and 1999; and a North Coral Gables (CGN, 23% male) subpopulation in 2000. Both CGS and CGN are parts of a large, somewhat fragmented population that stretches along the coast in south Miami-Dade County, and will be referred to as 'subpopulations' throughout this paper. The Wabasso Island population (0% male) was studied in 1999 and 2000, and the Hollywood population (42% male) was studied in 2000.

Plants were also grown in the greenhouse at the Matthaei Botanical Gardens at the University of Michigan, Ann Arbor MI, USA. Most plants flowered after 1–3 yr but 40% of the plants did not flower even after 5 yr. Nearly all plants survived for the 5-yr duration of the study.

Pollination experiments: hermaphrodite vs male pollen donors

To determine whether populations with males were functionally androdioecious or cryptically dioecious, we tested whether pollen from hermaphrodites would produce viable seed. Because each fruit contains a single seed (Tomlinson, 2001), fruit set can be used to estimate seed set.

Pollination experiments were done in the field to compare the average relative fitness of pollen from males vs hermaphrodites. Budded inflorescences on hermaphroditic plants were enclosed in fine mesh pollination bags to exclude floral visitors. One inflorescence on each plant was assigned to each of the following two treatments: hand-pollination with pollen from other hermaphroditic plants (H × H); or hand-pollination with pollen from male plants (H × M). In 1999 and 2000, the anthers were removed from hermaphroditic flowers (ovule donors) on the first day and pollen was transferred to the stigmas on the second day, when the stigmas were receptive. At CGS (1998), anthers were not removed from ovule donors, but pollen was still applied to the stigma on the second day. In all years, male and hermaphroditic inflorescences used as pollen donors were bagged to exclude floral visitors. Immediately before the pollinations were performed, pollen was collected and then applied to the stigmas using cotton swabs (1998) or glass microtubes (1999 and 2000). In all cases, each ovule-donating flower received pollen from a single pollen donor. Different plants were used between years at WAB and CGS.

Following pollinations, the mesh bags were replaced and fruits were allowed to mature in the bags on the trees until September, when the bagged inflorescences were cut from the plants and the fruits within the bags were counted. Fruit set was calculated as

$$\text{Fruit Set} = ((\text{total number of fruits produced})/(\text{total number of flowers pollinated})).$$

Seedling emergence and survivorship

We estimated the relative viability parameter (v) in Lloyd's model as the product of seedling emergence and seedling

survivorship to 1 yr. Fruits collected from the 1998 H-self, H × H and H × M treatments at CGS were used in seedling emergence and seedling survivorship experiments performed in Michigan. Although the H-self treatment was not hand-pollinated at CGS in 1998, the method of self-pollination is not relevant to seedling emergence and survivorship, so the H-self treatment from 1998 could be compared to the outcross treatments (H × H and H × M) at these life-history stages. Fruits from known crosses (i.e. both ovule and pollen donors were known) were pressed into trays of commercial potting soil, watered daily, and exposed to sodium vapor lights 12 h d^{-1}. The temperature at the soil surface was kept near 26.7°C. Seedling emergence from the fruit was checked daily for 10 weeks and is reported as the number of emerged seedlings/number of fruits planted. Seedling survival was recorded for 1 yr and is reported as the number of yearlings/number of emerged seedlings.

Pollen and flower production: hermaphrodite vs male

The relative pollen fecundity parameter (l) in Lloyd's model was estimated as the product of pollen production and flower production. To estimate pollen production, one flower per plant was randomly selected from plants grown in the greenhouse and two anthers were removed, one from each of the two whorls of stamens. Anthers were crushed and stained with lactophenol aniline blue, which stains callose in the intine and thus indicates pollen viability (Kearns & Inouye, 1993); pollen grains were counted at ×100 using a compound microscope. To determine whether flower size was related to pollen production, a flower size index for each flower was also calculated as the product of the corolla length and width. While conducting pollen counts, occasional small hermaphroditic flowers were found clustered on the distal ends of the inflorescences with fewer pollen grains than the larger hermaphroditic flowers, so four flower categories were created for the statistical analysis: small hermaphrodite (herm-sml); large hermaphrodite (herm-lrg); all hermaphrodite (herm-all); and male. To estimate flower production, young inflorescences were bagged in the field and all flowers and fruits were counted at the end of the season.

Inbreeding depression

To estimate inbreeding depression, average fruit set from an additional pollination treatment, hermaphroditic flowers hand-pollinated with geitonogamous pollen (H-self) and the two outcrossed treatments (H × H and H × M) were compared for each population. The H-self treatment was not performed at CGS in 1998; instead, unopened flowers were bagged and left unmanipulated to test for autogamous self-pollination. Inflorescences used for the H-self treatment in all other populations and years were on the same trees as the other pollination treatments and treated as already described

(see earlier 'Pollination experiments: hermaphrodite vs male pollen donors'). The inbreeding index (i) in Lloyd's model was estimated as the ratio of the proportion fruit set from the H-self treatment and the proportion fruit set from the outcross treatments for paired treatments using the same maternal plant. In addition, the inbreeding coefficient was also calculated for paired treatments using the same maternal plant (inbreeding coefficient = 1 − (proportion fruit set from H-self treatment/proportion fruit set from outcross treatment), where outcross treatment is either H × H or H × M). Because the H-self treatment was not hand-pollinated at CGS in 1998, reduced fruit set in this treatment could reflect poor pollination success of the autopollination mechanism as well as inbreeding depression; therefore, these results are excluded from the fruit set analysis.

Outcrossing estimates

We estimated the outcrossing index (t) in Lloyd's model based on the length of pollinator foraging bouts within plants and assumptions concerning pollen carryover. The foraging bout length, defined as the number of flowers visited by each insect before that insect left the plant, was recorded for approx. 220 insects in each population. Insects were observed on trees that were not used in pollination experiments in case insects behaved differently on trees with bagged inflorescences.

Pollen carryover is the amount of pollen on the pollinator from outcross sources that is available for pollination. Other studies have shown that pollen carryover drops exponentially as the pollinator moves between flowers within a plant and that, depending on the species involved, the probability of outcrossing decreases to zero somewhere between the third and seventh flowers visited (Harder & Barrett, 1996; Snow *et al.*, 1996). Based on these studies, low estimates of pollen carryover (i.e. the first two or three flowers are outcrossed) are considered more biologically realistic.

A series of outcrossing indices were calculated for each subpopulation based on pollen carryover assumptions that allowed the first two to six flowers on a plant to be outcrossed before the fraction of outcross pollen in the pollinator's load was exhausted. After the specified numbers of flowers were outcrossed, all other flowers visited during the foraging bout in that tree were assumed to be selfed. The summation of flowers selfed vs the summation of flowers outcrossed for each pollen carryover assumption was used to estimate the proportion of flowers outcrossed. This method does not account for competition among pollen types; if outcross pollen outcompetes self pollen, then the method will underestimate outcrossing.

Male frequency predictions generated by Lloyd's model

All parameters for Lloyd's model for androdioecy were estimated for the two Coral Gables subpopulations (CGS and CGN).
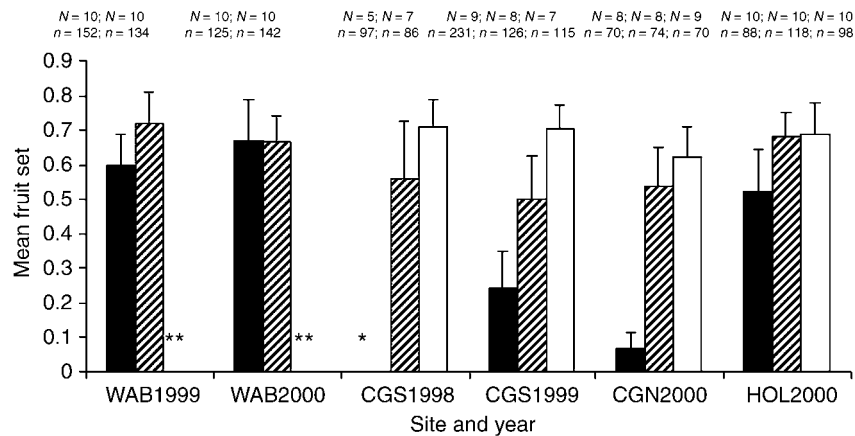
Fig. 1 Average (+ SE) fruit set for all hand-pollination treatments using pollen either from hermaphrodites (H-Self, closed bars; and H × H, hatched bars) or from males (H × M, open bars) of *Laguncularia racemosa* (white mangrove). Significantly fewer fruits developed from forced self treatments in the CGN population relative to the WAB and HOL populations ($P < 0.01$ and $P < 0.05$, respectively), and relative to H × H and H × M treatments at CGN ($P < 0.05$ and $P < 0.01$, respectively). Fruit set from the CGS forced self treatment (H-self) was significantly lower relative to WAB ($P < 0.05$) and relative to the H × M treatment at CGS ($P < 0.01$). N, number of inflorescences; n, number of flowers; *, missing data; **, cross not possible. Population codes: CGN, Coral Gables North, FL, USA; CGS, Coral Gables South, FL, USA; HOL, Hollywood, FL, USA; WAB, Wabasso, FL, USA. Treatment codes: H-Self, crosses made using a single hermaphroditic plant acting as both ovule-donor and pollen-donor (geitonogamous pollen); H × H, crosses made with two hermaphroditic plants, one acting as ovule-donor, the other as pollen-donor; H × M, crosses made with a hermaphroditic plant acting as ovule-donor and a male plant acting as pollen-donor.

The model was used to generate a range of male frequency predictions using a series of outcrossing indices (for a description of the model, see the 'Introduction' section; for methods used to estimate the parameters, see the preceding 'Materials and Methods' sections). The predictions were qualitatively compared with observed male frequencies in these two subpopulations. The model could not be used to generate male frequency predictions for the HOL population because there was no relative pollen fecundity or relative viability data for this population. Further, the model is inappropriate for the WAB population because it does not include male plants.

Statistical analyses

For the statistical analyses, proportional data were arcsine square root transformed to normalize their distributions. Significant differences were tested with ANOVAs using SYSTAT 10 (Systat Software Inc. San Jose, CA, USA). Initially, a two-way ANOVA was done on the combined fruit set data for all sites to determine if significant interactions existed between pollination treatments and site. Two-way ANOVAs were also performed independently on the WAB and CGS fruit set data to determine if significant interactions existed between pollination treatments and years. Data from each site were analysed separately using one-way ANOVAs (followed by Tukey HSD *post hoc* tests) to test for significant differences among all pollination treatments at three life-history stages: fruit set, seedling emergence and seedling survivorship. A two-way ANOVA was used to determine if there were significant differences in inflorescence size between males and hermaphrodites at all

sites. One-way ANOVAs (followed by Tukey HSD *post hoc* tests) were also performed to determine if male flowers produced significantly more pollen than hermaphroditic flowers, if flower production by males and hermaphrodites was consistent among populations and to test for significant differences in inbreeding coefficients among populations.

Results

Pollination experiments: hermaphrodite vs male pollen donors

Fruit set varied significantly among populations (two-way ANOVA, *F*-ratio = 3.380, df = 10, $P = 0.001$; test of hypothesis, $F = 3.097$, df = 2, $P = 0.049$) and among pollination treatments across populations (test of hypothesis, $F = 4.150$, df = 2, $P = 0.018$) (Fig. 1), but there was no significant interaction between population and treatment (test of hypothesis, $F = 1.612$, df = 6, $P = 0.149$). Fruit set did not vary significantly between years at Wabasso (two-way ANOVA, test of hypothesis, $F = 0.004$, df = 1, $P = 0.950$) or Coral Gables South (two-way ANOVA, test of hypothesis, $F = 0.190$, df = 1, $P = 0.666$) (Fig. 1). In addition, no significant interactions between treatment and year were found in either population (Wabasso two-way ANOVA, test of hypothesis, $F = 1.331$, df = 1, $P = 0.256$; Coral Gables South two-way ANOVA, test of hypothesis, $F = 0.018$, df = 1, $P = 0.895$), so data from both years were pooled for these populations for further analysis. Fruit set differed significantly among pollination treatments in the Coral Gables North and Coral
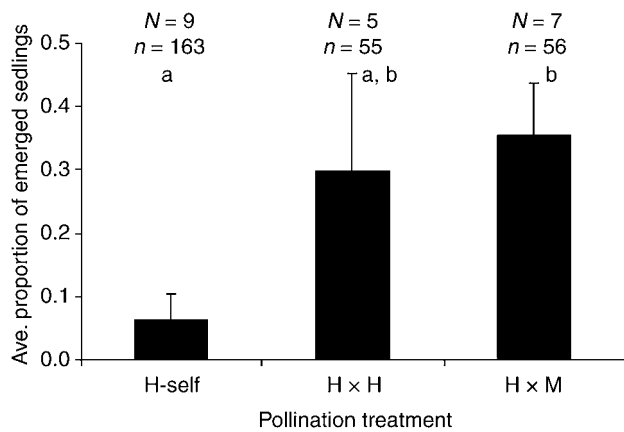
Fig. 2 Average (+ SE) proportion of *Laguncularia racemosa* (white mangrove) seedlings that emerged from fruits produced from pollination treatments. Seedling emergence in the H × M treatment was significantly greater than seedling emergence in the H-self treatment. Seeds were produced through controlled crosses performed at CGS (Coral Gables South, FL, USA) in 1998. Treatments with different letters differ significantly (*P* < 0.05). N, number of inflorescences; n, number of fruits. Treatment codes: H-Self, crosses made using a single hermaphroditic plant acting as both ovule-donor and pollen-donor (geitonogamous pollen); H × H, crosses made with two hermaphroditic plants, one acting as ovule-donor, the other as pollen-donor; H × M, crosses made with a hermaphroditic plant acting as ovule-donor and a male plant acting as pollen-donor.
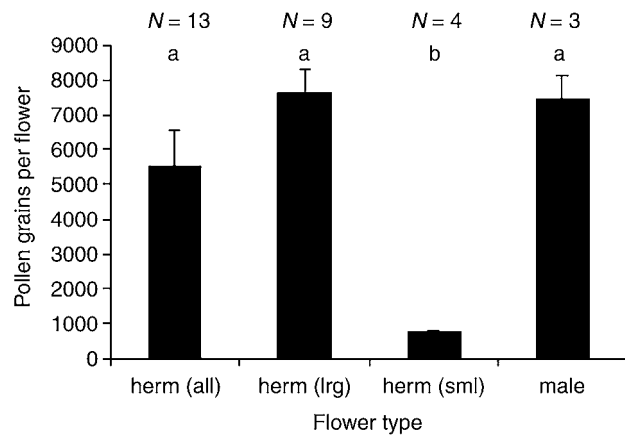

Fig. 3 Average (+SE) number of pollen grains for all hermaphroditic flowers (all), for large hermaphroditic flowers only (lrg), for small hermaphroditic flowers only (sml), and for male flowers of *Laguncularia racemosa* (white mangrove). Hermaphroditic flowers with indices < 11.00 mm² (herm-sml) have significantly fewer pollen grains per flower relative to hermaphroditic flowers with indices ≥ 11.00 mm² (herm-lrg) or male flowers. N, number of plants sampled. Flower types with different letter designations differ significantly (*P* < 0.001).

Gables South subpopulations (one-way ANOVAs: CGN *F*-ratio = 7.059, df = 2, *P* = 0.004; CGS *F*-ratio = 6.283, df = 2, *P* = 0.005), but not in the Hollywood and Wabasso populations (one-way ANOVAs: HOL *F*-ratio = 0.451, df = 2, *P* = 0.642; WAB *F*-ratio = 0.047, df = 1, *P* = 0.830).

Pollination experiments demonstrated that the two south Florida populations (CG and HOL) are functionally androdioecious; average fruit set was > 50% for crosses made with hermaphroditic pollen. By contrast, the central Florida population (WAB) appears to be strictly hermaphroditic as no male plants were ever observed and all flowering plants produced fruit. Fruit set did not differ significantly among populations for the H × H treatments (one-way ANOVA: *F*-ratio = 1.086, *P* = 0.364) or the H × M treatments (one-way ANOVA: *F*-ratio = 0.137, df = 2, *P* = 0.873) (Fig. 1). In the androdioecious populations, fruit set from crosses made with male pollen and hermaphroditic pollen did not differ significantly (*P* > 0.20) (Fig. 1).

Seedling emergence and survivorship

In the androdioecious CGS population, seedling emergence differed significantly among all pollination treatments (one-way ANOVA: *F*-ratio = 3.924, df = 2, *P* = 0.039), but the difference was between self and outcross treatments (see later, 'Inbreeding depression'), not between H × H and H × M treatments (Tukey HSD *post hoc* test, *P* = 0.897) (Fig. 2). In

addition, there was no difference in survivorship of the progeny of H × H and H × M crosses (one-way ANOVA: *F*-ratio = 0.450, df = 2, *P* = 0.651; Tukey HSD *post hoc* test, *P* = 0.727). Nearly all seedlings survived the first year of the study (N_{Total} = 60, N_{H-self} = 13, $N_{H×H}$ = 27, $N_{H×M}$ = 20); only one seedling died over the winter (progeny of H × M cross). Subsequent mortality was low and appeared to be related to glasshouse temperatures, which are affected by extremely low outdoor temperature conditions common in Michigan. Of the progeny that flowered during the course of the study (*N* = 32), progeny from crosses with hermaphroditic pollen parents were all hermaphroditic (*N* = 18), while crosses with male pollen parents resulted in both male and hermaphroditic progeny (*N* = 5 and *N* = 9, respectively).

Pollen and flower production: hermaphrodite vs male

Pollen production per flower differed significantly among the four flower types (Fig. 3) (one-way ANOVA: *F*-ratio = 5.913, df = 3, *P* = 0.003). Small hermaphroditic flowers produced significantly less pollen than either male (Tukey HSD *post hoc* test, *P* = 0.023) or large hermaphroditic flowers (Tukey HSD *post hoc* test, *P* = 0.002), but when all hermaphroditic flowers were pooled, male and hermaphroditic flowers produced approximately the same number of pollen grains per flower (Tukey HSD *post hoc* test, *P* = 0.724). Pollen was equally viable in hermaphrodites and males; nearly all pollen grains (> 95%) from hermaphrodites and males stained dark blue with lactophenol aniline blue.

When plants from all populations were pooled, male plants produced significantly more flowers per inflorescence than
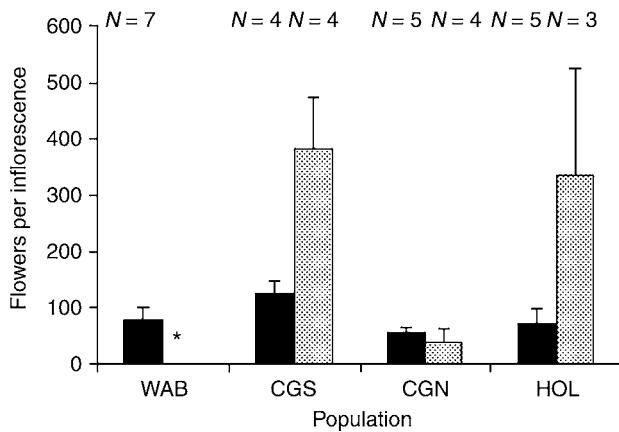
Fig. 4 Average (+ SE) flower production per inflorescence for hermaphroditic (black bars) and male (grey bars) *Laguncularia racemosa* (white mangrove) plants. Males produce significantly more flowers per inflorescence than hermaphrodites (*P* = 0.006); flower production in males and hermaphrodites does not differ among populations (*P* > 0.05). N, number of inflorescences; *, number of male plants (hermaphroditic population). Population codes: CGN, Coral Gables North, FL, USA; CGS, Coral Gables South, FL, USA; HOL, Hollywood, FL, USA; WAB, Wabasso, FL, USA.
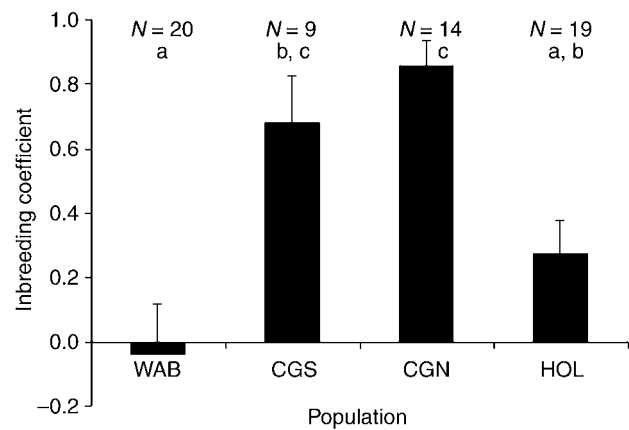

Fig. 5 Average (+ SE) inbreeding depression coefficients for all *Laguncularia racemosa* (white mangrove) populations. Inbreeding depression was stronger in the androdioecious populations (CGS, CGN, HOL) than in the hermaphroditic population (WAB). N, number of forced self: outcross ratios, paired by maternal plant. Populations with different letters differ significantly (*P* < 0.05). Population codes: CGN, Coral Gables North, FL, USA; CGS, Coral Gables South, FL, USA; HOL, Hollywood, FL, USA; WAB, Wabasso, FL, USA.

hermaphroditic plants (two-way ANOVA: *F*-ratio = 5.470, df = 6, *P* = 0.001; test of hypothesis: *F* = 9.189, df = 1, *P* = 0.006) (Fig. 4). A significant interaction between population and gender was due to very low male flower production at CGN (test of hypothesis: *F* = 3.774, df = 2, *P* = 0.037), which was mainly caused by herbivory and external damage to inflorescences. There was no significant difference in flower production by males among populations (one-way ANOVA: *F*-ratio = 3.361, df = 2, *P* = 0.087) or by hermaphrodites among populations (one-way ANOVA: *F*-ratio = 1.658, df = 3, *P* = 0.214).

Inbreeding depression

Inbreeding depression varied significantly among populations (one-way ANOVA: *F*-ratio = 10.358, df = 3, *P* < 0.001) (Fig. 5). Inbreeding coefficients were significantly greater for the two androdioecious Coral Gables subpopulations than for the hermaphroditic Wabasso population (Tukey HSD *post hoc* tests: CGS, *P* = 0.016; CGN, *P* < 0.001), but the coefficient for the androdioecious Hollywood population did not differ from the Wabasso coefficient (Tukey HSD *post hoc* test: *P* = 0.919). There was no difference between the inbreeding coefficients for CGS and CGN (Tukey HSD *post hoc* test: *P* = 0.693). The coefficient for CGN was significantly greater than the Hollywood coefficient (Tukey HSD *post hoc* test: CGN, *P* < 0.001); the coefficient for CGS was also greater but not significantly different than the Hollywood coefficient (Tukey HSD *post hoc* test: CGS, *P* = 0.061).

Because fruit set in the two outcrossed treatments (H × H, H × M) did not vary among populations, differences in the strength of inbreeding depression were caused by differences in fruit set in the H-self treatments (one-way ANOVA: *F*-ratio = 6.369, df = 3, *P* = 0.001). Fruit set in the self-pollination treatment was significantly reduced in the CGN subpopulation compared with the HOL and WAB populations, and in the CGS subpopulation compared with the WAB population (*P* < 0.05) (Fig. 1). Fruit set did not differ significantly in any other pairwise comparison.

Inbreeding depression in the CGS subpopulation was also evident at the seedling stage (Fig. 2). Significantly fewer seedlings emerged from fruits produced through the self-pollination treatment (H-self) than emerged from the H × M treatment (Tukey HSD *post hoc* test: df = 18, *P* = 0.042). There was also a nonsignificant reduction in seedling emergence in the H-self treatment relative to the H × H treatment (Tukey HSD *post hoc* test: df = 18, *P* = 0.164).

Outcrossing estimates

Outcrossing estimates are intermediate in all populations for all pollen carryover assumptions (0.29–0.66, Table 1). There is little difference among populations in outcrossing estimates calculated using the same pollen carryover value (Table 1), despite differences in the frequency of short pollinator foraging bouts between the hermaphroditic population (WAB) and the two androdioecious populations (HOL and CG). More insects engaged in short foraging bouts (insects visited one to two flowers during the foraging bout) at HOL and CG than at WAB (Fig. 6).

Table 1 Parameter values for populations used to generate male frequency predictions (*q-calc*) for Lloyd's (1975) model of androdioecy[a]

Population	*t*	*i*	*l*	*v*	*q-calc*	*q-obs*
CGS	0.33	0.3195	0.3277	0.8924	0.02	0.18
	0.44				0.10	
	0.53				0.15	
	0.60				0.19	
	0.66				0.21	
CGN	0.33	0.1438	0.4154	0.8924	0.03	0.23
	0.44				0.09	
	0.53				0.13	
	0.60				0.15	
	0.66				0.17	
HOL	0.32	0.7262	No data	No data	–	0.42
	0.42					
	0.50					
	0.57					
	0.63					
WAB	0.29	1.0312	No data	No data	–	0.00
	0.40					
	0.49					
	0.57					
	0.65					

[a]Population codes: CGN, Coral Gables North, FL, USA; CGS, Coral Gables South, FL, USA; HOL, Hollywood, FL, USA; WAB, Wabasso, FL, USA. *t*, proportion of seeds resulting from cross-fertilization (outcrossing index); *i*, relative fitness of seeds due to inbreeding depression (selfed : outcrossed); *l*, relative intrinsic pollen fecundity (number of pollen grains produced per inflorescence, hermaphrodite : male); *v*, relative viability of zygotes to reproductive maturity (hermaphrodite : male); q_{obs}, observed male frequency. For each subpopulation, a series of outcrossing values (*t*) were estimated using a range of plausible pollen carryover values. Male frequency predictions are compared with observed male frequencies (q_{obs}) for the CGS and CGN subpopulations. WAB is a hermaphroditic population.

Fig. 6 Trend lines show the frequency of insect visitors that made foraging bouts of different lengths; foraging bout length ranged from one flower visited to > 35 flowers visited before the insect left the plant. More insects made short foraging bouts of one to two flowers in the two androdioecious populations (CG, HOL) than in the hermaphroditic population (WAB) of *Laguncularia racemosa* (white mangrove). Population codes: CG, Coral Gables, FL, USA; HOL, Hollywood, FL, USA; WAB, Wabasso, FL, USA.

Male frequency predictions generated by Lloyd's model

For CGS, the male frequency prediction (q_{calc}) generated using a moderately high outcrossing estimate (0.60) approximated the observed male frequency (q_{obs}) (Table 1: predicted, 0.19; observed, 0.18). For CGN, all male frequency predictions generated by the model were less than the observed frequency (Table 1: maximum predicted, 0.17; observed, 0.23).

Discussion

Our study confirms that white mangrove is functionally androdioecious in the Coral Gables and Hollywood populations in south Florida, but hermaphroditic in the Wabasso population in central Florida. The variability in male frequency among these populations allows us to address the following questions about androdioecy: Do males persist in androdioecious populations because they hav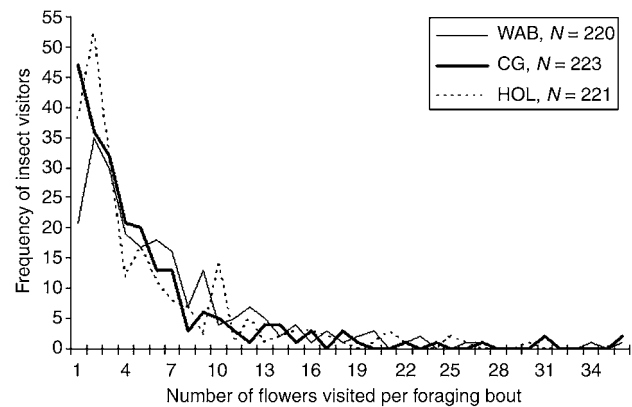e at least twice the fitness contributed by the male component of hermaphrodites, as predicted by theoretical models (Lloyd, 1975; Charlesworth, 1984), and if so, which characteristics impart the fitness advantage?

Males produced nearly three times more flowers per inflorescence than hermaphrodites (Fig. 4), but did not produce more pollen per flower (Fig. 3). Male plants of other insect-pollinated androdioecious species produce more flowers per inflorescence than hermaphrodites (Appanah, 1982; Ishida & Hiura, 1998; Muenchow, 1998; Akimoto *et al.*, 1999), and in at least one case, pollen production per flower did not differ between male and hermaphroditic flowers (Ishida & Hiura, 1998). In insect-pollinated plants, greater pollen production per flower does not necessarily increase pollination success, whereas increased flower production can result in increased attractiveness to pollinators and lead to increased pollination success (Willson, 1983; Wyatt, 1983; Harder & Barrett, 1996). In contrast, the male plants in wind-pollinated androdioecious species can maximize their fitness gain strictly through an increase in pollen number per flower, reducing the investment in floral structures (Willson, 1979). This strategy has been reported in male plants in wind-pollinated *Datisca glomerata* (Philbrick & Rieseberg, 1994; Spencer & Rieseberg, 1995), but not in *Mecurialis annua* (Pannell, 1997b) or *Ulnus major* (Lopez-Almansa *et al.*, 2003), which have greater pollen production per plant because of increased flower production, not increased pollen production per flower. The last two species appear to have adopted alternative strategies involving vegetative and secondary sexual characteristics to maximize fitness. In *M. annua*, male plants are taller than hermaphroditic plants, the architecture of male inflorescences increases the likelihood of pollen dispersal, and the hermaphroditic

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plants are strongly protogynous (Pannell, 1997b). *Ulnus minor* appears to reproduce primarily through vegetative propagation, and male plants may gain an advantage by producing more ramets than hermaphrodites, which bear heavier costs of fruit production resulting from sexual reproduction (Lopez-Almansa *et al.*, 2003).

The fertility success of male pollen in the androdioecious white mangrove populations was not significantly different from that of hermaphroditic pollen; fruit set was slightly but not significantly higher (1.0–1.4 times higher) in H × M vs H × H crosses (Fig. 1). By contrast, other studies of androdioecious species have shown that males have higher pollen fertility than hermaphrodites. Pollen tube growth in *D. glomerata* may be faster in males relative to hermaphrodites (Philbrick & Rieseberg, 1994). In *Phillyrea angustifolia*, pollen tube growth was nearly 1.5 times more likely in male pollen vs hermaphroditic pollen (Vassiliadis *et al.*, 2000), and in several populations, fruit set from crosses made with male pollen was significantly higher (1.9–3.9 times higher) relative to fruit set from crosses made with hermaphroditic pollen (Lepart & Dommee, 1992; Traveset, 1994; Vassiliadis *et al.*, 2000). Male pollination success was also significantly higher than hermaphroditic success in *Fraxinus ornus* (Dommee *et al.*, 1999), leading Verdu *et al.* (2006) to suggest that *F. ornus* is functionally cryptically dioecious. In *Fraxinus lanuginosa*, males had a significantly higher pollen germination rate (2.8–3.4 times higher) and siring success (2.7 times higher) relative to hermaphrodites (Ishida & Hiura, 1998). For these species, higher fertility success of males increases male fitness more than twofold, satisfying theoretical requirements for the maintenance of androdioecy.

Male advantage was also not evident in white mangrove at seedling emergence (Fig. 2) or seedling survival stages. Seedlings derived from H × M crosses emerged with slightly greater frequency (1.19 times greater), but survived with slightly less frequency (1.06 times lower), than seedlings derived from H × H crosses, and the differences were not significant. In *D. glomerata*, seeds with male and hermaphroditic pollen parents germinated at the same frequency, but the seedlings with male pollen parents were significantly larger than seedlings with hermaphroditic pollen parents (Rieseberg *et al.*, 1993). While we did not measure white mangrove seedlings at the time of emergence, there were no obvious differences in the size of the seedlings regardless of treatment.

White mangrove hermaphrodites are self-compatible (Figs 1 and 2) and can self-pollinate (Rathcke *et al.*, 2001b; Landry, 2005). Selfing should make it more difficult to maintain males in hermaphroditic populations because males will have fewer mating opportunities (Lloyd, 1975; Charlesworth, 1984). Self-compatibility has been found in several androdioecious species: *D. glomerata* (Liston *et al.*, 1990), *M. annua* (Pannell, 1997b), *Schizopepon bryoniaefolius* (Akimoto *et al.*, 1999), and *F. ornus* (Dommee *et al.*, 1999). White mangrove fruits are water dispersed, and the ability of white mangrove

to self-pollinate and self-fertilize may be advantageous for colonization of open habitats (Baker, 1955; Charlesworth, 2006).

Males can overcome the disadvantage imposed by selfing hermaphrodites if inbreeding depression is strong because this will reduce the reproductive success of selfing hermaphrodites. As predicted, inbreeding depression was evident in the androdioecious populations, which would be favorable to the maintenance of male plants, but not in the hermaphroditic Wabasso population. The Wabasso population did not exhibit inbreeding depression (inbreeding coefficient = −0.03), which may indicate that deleterious alleles have been purged from this population due to high levels of selfing.

The strength of inbreeding depression did not increase with increasing male frequency as we had predicted (Fig. 5). Inbreeding depression was strongest in the two Coral Gables subpopulations (inbreeding coefficients: CGS, 0.68; CGN, 0.86), which have male frequencies less than 0.25, whereas the Hollywood population, with a male frequency of 0.42, exhibited much weaker inbreeding depression (inbreeding coefficient = 0.27). The correlation between inbreeding depression and male frequency is not expected if outcrossing levels differ between the two populations. To calculate outcrossing, we had to assume an average pollen carryover value for all pollinators; however, pollen carryover can vary between pollinator species. Differences in the species composition or abundance of the pollinator community found in the two white mangrove populations could result in different outcrossing rates due to differences in pollen carryover. The pollen carryover values used in this study represent the range of pollen carryover values reported for other plants and their pollinators (Harder & Barrett, 1996). The differences in outcrossing estimates calculated for each population using low vs. high pollen carryover estimates demonstrate that pollen carryover can have a strong influence on outcrossing levels (Table 1). Further, outcrossing levels could differ between the two populations if outcross pollen has a greater competitive advantage over self pollen in one population than in the other.

The parameters of Lloyd's (1975) model were estimated from field data collected in the Coral Gables subpopulations and male frequency predictions were generated for the range of outcrossing values calculated for each subpopulation (Table 1). Lloyd's model predicted male frequency to be 0.19 at the CGS subpopulation when outcrossing was moderately high (0.60), which closely matched the observed male frequency of 0.18. However, at CGN, Lloyd's model underestimated male frequency for all outcrossing values (Table 1: predicted, 0.03–0.17; observed, 0.23), perhaps because the effects of pollen competition were not considered in the outcrossing estimates.

Other factors could also increase outcrossing opportunities for males relative to hermaphrodites, providing males with a fitness advantage. For example, males tend to flower before hermaphrodites (C. L. Landry, unpublished), making male pollen relatively more available early in the season, which

increases the probability of male pollen success. Earlier male flowering has also been reported in *D. glomerata* (Spencer & Rieseberg, 1995) and in *Xerospermum intermedium* (Appanah, 1982). In addition, male white mangrove flowers are loosely held on the inflorescence and insect visitors often fall from the plant with the flower they visit, and then subsequently move to another inflorescence, often on nearby trees. Further, we have evidence that pollinator visitation rate is higher to male plants than hermaphroditic plants, which would increase mating opportunities to males (Landry *et al.*, 2005; Landry, 2005). The combination of these traits in white mangrove and the interaction of these traits with the foraging behaviors of pollinating insects could increase male pollen success.

The sex determination system in white mangrove is unknown; however, our preliminary results suggest that maleness is dominant. Of the progeny that flowered during the course of the survivorship study (*N* = 32), progeny from crosses with hermaphroditic pollen parents were all hermaphroditic (*N* = 18), while crosses with male pollen parents resulted in both male and hermaphroditic progeny (*N* = 5 and *N* = 9, respectively). Limited evidence also suggests that androdioecy may have evolved from hermaphroditism in this species, in contrast to other species where androdioecy appears to have evolved from dioecy (*D. glomerata*, Rieseberg *et al.*, 1992; *M. annua*, Pannell, 1997b,c). We have occasionally found fruits developing at the basal end of inflorescences on male plants both in Florida and the Bahamas, and we have never found flowers on hermaphroditic plants that lack pollen, although we have occasionally found small hermaphroditic flowers with reduced pollen production at the distal end of inflorescences (Fig. 3). Unfortunately, the molecular systematics of the Combretaceae has not been studied (for the last systematic study of this group see Exell, 1931), so the ancestral breeding system and the evolution of androdioecy of *Laguncularia*, a monotypic genus, cannot yet be determined.

Acknowledgements

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