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Cover image - Patch reef near the wall off Grotto Beach (photo by Lee Florea).

Do reproductive barriers exist between two closely-related species of *Croton* that occur in sympatry?

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1. Abstract

Croton linearis and *Croton discolor* are common plant species in coastal communities in The Bahamas. They are distinguished primarily by leaf morphology, not floral characteristics; however, we found individuals in the community that were intermediate with respect to leaf morphology. To determine whether these morphological intermediates could be hybrids produced from crosses between the two species, we investigated potential reproductive barriers that could separate the species. We observed floral visitors to determine if the two species shared pollinators or if they were reproductively isolated due to a lack of shared pollinators. Pollen on insects that visited the plants was removed and identified, and following insect visitation, the stigmas of virgin female flowers were collected, fixed, and screened for *Croton* pollen. To determine whether the species were cross-compatible, the female flowers of each species were hand-pollinated in March and June 2014; flowers received either conspecific pollen or pollen from the other species. We compared average fruit and seed set, and seed germination rates for the four treatments.

During 26 hours of observation, a total of 11 dipteran and hymenopteran species were observed on flowers of the two species or their intermediates. Five insect species were responsible for most visits and did not discriminate between plant species, but of these, only four dipteran species visited flowers on both male and female plants: *Chrysanthrax maculipennis*, *Geron salmonus*, *Ligyra cereberus*, and *Villa lateralis* complex. *Croton* pollen was also found on specimens of these insects, and one *L. cereberus* individual

observed visiting a virgin flower deposited at least one pollen grain on that flower's stigma. There were no significant differences among treatments in average fruit set or in the average number of seeds produced per fruit, and seeds from interspecific crosses germinated. The lack of significant differences among pollination treatments and evidence that hybrid seeds are viable suggest that the two species are cross-compatible. Further, the two species may be hybridizing because the primary floral visitors did not differentiate between the two species or their morphological intermediates.

2. Introduction

Studying the dynamics of pollination within communities is necessary for successful conservation (Kearns et al. 1998). *Croton linearis* Jacq. and *Croton discolor* Willd. (Euphorbiaceae) are common, closely-related, insect-pollinated plants native to The Bahamas (Landry et al. 2014a). They are distinguished primarily by differences in leaf morphology; Correll and Correll (1982) describe *C. discolor* as “a venose, broad-leaved form of *C. linearis*”. On San Salvador, the distribution of *C. discolor* appears to be more restricted than *C. linearis*, but they do co-occur at several locations on the island. In at least one location where their distributions overlap, morphologically intermediate individuals were previously observed (Landry, pers. obs.). We investigated potential reproductive barriers between *C. linearis* and *C. discolor* to determine whether the morphological intermediates could be hybrid individuals.

In order to prevent hybridization between species found in the same community, at least one reproductive barrier must exist

(Hopkins 2013). In this study, we investigated mechanical, gametic, and post-zygotic barriers. Mechanical barriers physically prevent reproduction between two species; animal-pollinated plant species that do not share pollinators are isolated from one another via mechanical barriers. If gametic or post-zygotic barriers exist between the *Croton* species, they would prevent successful fertilization or the development of viable seeds, respectively (Lepais et al. 2013). If strong gametic or post-zygotic reproductive barriers exist, we do not expect interspecific crosses to set fruit or seed. If these barriers exist but are weak, we expect lower fruit and seed set, and lower frequency of seed germination in interspecific crosses versus crosses made with conspecific pollen. Finally, if gametic or post-zygotic barriers do not exist, we expect similar fruit set, seed set, and germination rates in all treatments.

3. Methods

We distinguished the two species and their intermediates by leaf shape and an estimate of the leaf width-to-length ($W : L$) ratio of each individual. Plants were categorized as *C. linearis* if the leaves were linear and the $W : L$ ratio was < 0.25 , and as *C. discolor* if the leaves were oblong to elliptic and the $W : L$ ratio was > 0.33 ; intermediates were “narrowly oblong” or “widely linear” and had intermediate $W : L$ ratios.

We made pollinator observations on all plants in flower that were growing along a 1 km transect. For each plant observed, we recorded the plant category (as defined above), plant gender, and all floral visitors. Insects collected while visiting the plants were sacrificed and their pollen loads were removed and transported to the lab at OSU-Mansfield. Pollen was clarified via acetolysis and bleached with KOH before mounting in basic fuschin jelly (Kearns and Inouye 1993). Pollen was also removed from insect specimens that Landry and Elliott had previously collected on the two *Croton* species. Pollen species were identified using

reference slides prepared from known samples by Landry; the two species have identical pollen morphologies, so pollen identification was restricted to genus.

To determine whether pollinators were able to deposit pollen on the stigma, we observed visitors to virgin flowers. Female flower buds were covered with mesh bags until the flowers opened. Bags were removed during watches, and any virgin flowers visited by insects were tagged, re-bagged, and collected at the end of the experiment; the insect visitors were also collected when possible. Flowers were carried back to the lab at OSU-Mansfield; the stigmas were dissected from the flowers, mounted on slides in decolorized aniline blue, and pollen tubes were visualized using fluorescent microscopy (Kearns and Inouye 1993).

To test for gametic and post-zygotic barriers, we hand-pollinated the female flowers of each species with either conspecific pollen or with pollen from the other species. There were four treatments: $D \times D$, $D \times L$, $L \times D$, and $L \times L$ (female \times male crosses; $D = discolor$, $L = linearis$). All open flowers in the same inflorescence were treated with pollen from the same source. Following pollination, the flowers were covered with mesh bags to exclude insect visitors and to protect the fruits and seeds until maturation. Mature fruits and seeds from flowers pollinated in March were collected in June, and those from flowers pollinated in June were collected in December. One-way ANOVA was used to test for differences in fruit and seed set among treatments. The seeds were carried back to OSU-Mansfield, where they were planted in cactus blend potting mix and allowed to germinate in a growth chamber.

4. Results

During March and June, 68 insects representing 11 species visited *Croton* flowers during approximately 26 hours of observation time (Table 1). Five insect species were responsible for most visits and did not discriminate among plant categories, but of these, only four dipterans

Table 1. Insect visitors to male and female *C. linearis*, *C. discolor*, and their intermediates, and presence or absence of *Croton* pollen on insect specimens. * = pollen loads not investigated; ** = pollen reported in nest cells (Landry et al. 2014b).

Insect species	Pollen present (# specimens)	# Visitors to male and female plants (M/ F)		
		<i>linearis</i>	intermediate	<i>discolor</i>
Diptera				
Bombyliidae				
<i>Chrysanthrax maculipennis</i> Scarborough & Davidson	Yes (4)	1 / 2	0 / 1	1 / 0
<i>Geron salmonus</i> Scarborough & Davidson	Yes (3)	3 / 1	1 / 1	2 / 0
<i>Ligyra cereberus</i> (F.)	Yes (4)	4 / 1	5 / 2	10 / 4
<i>Villa lateralis complex</i> (Say)	Yes (2)	0 / 0	1 / 0	0 / 1
Syrphidae				
<i>Ocyrtamus cylindricus</i> (F.)	No (1)	—	—	—
<i>Palpada albifrons</i> (Wied.)	Yes (4)	0 / 0	0 / 0	1 / 0
Hymenoptera				
Apidae				
<i>Xylocopa cubaecola</i> Lucas	*	0 / 0	0 / 0	1 / 0
Crabronidae				
<i>Microbembex</i> sp.	Yes (1)	—	—	—
Eumenidae				
<i>Pachodynerus scrupeus</i> Zavattari	*	0 / 0	0 / 1	3 / 1
Megachilidae				
<i>Megachile alleni</i> Mitchell	Yes**	2 / 0	3 / 0	10 / 0
Scoliidae				
<i>Campsomeris t. trifasciata</i> Fabr.	*	0 / 0	0 / 0	1 / 0
Sphecidae				
<i>Stictia signata</i> (L.)	No (1)	1 / 0	0 / 0	1 / 0
Vespidae				
<i>Polistes bahamensis</i> Beq. & Salt	*	0 / 0	1 / 0	0 / 1

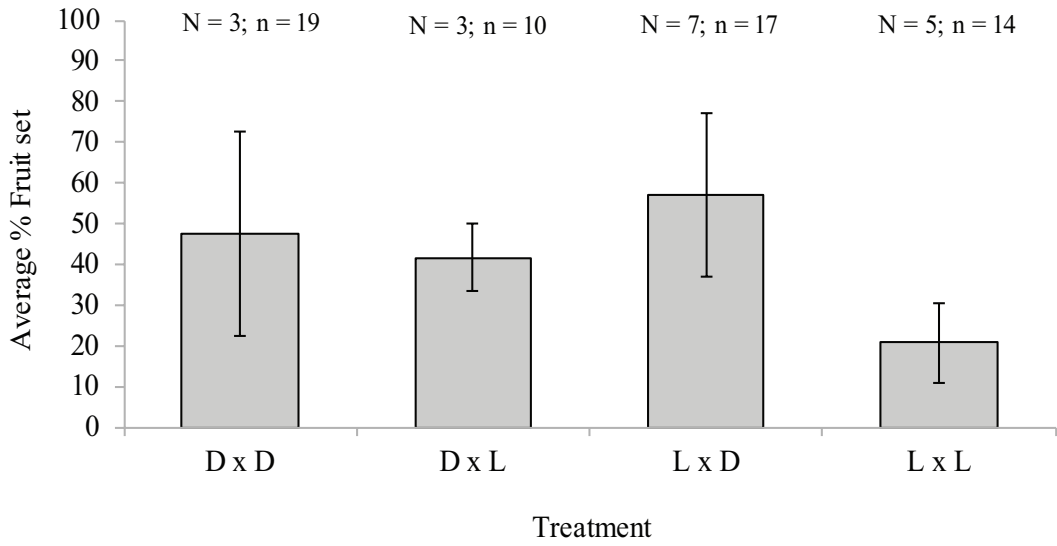


Figure 1. Average percent fruit set for hand-pollinated crosses between *C. discolor* individuals (D x D), *C. linearis* individuals (L x L), and reciprocal crosses between *C. discolor* and *C. linearis* (D x L and L x D), with SE bars. N = # mating pairs; n = # flowers.

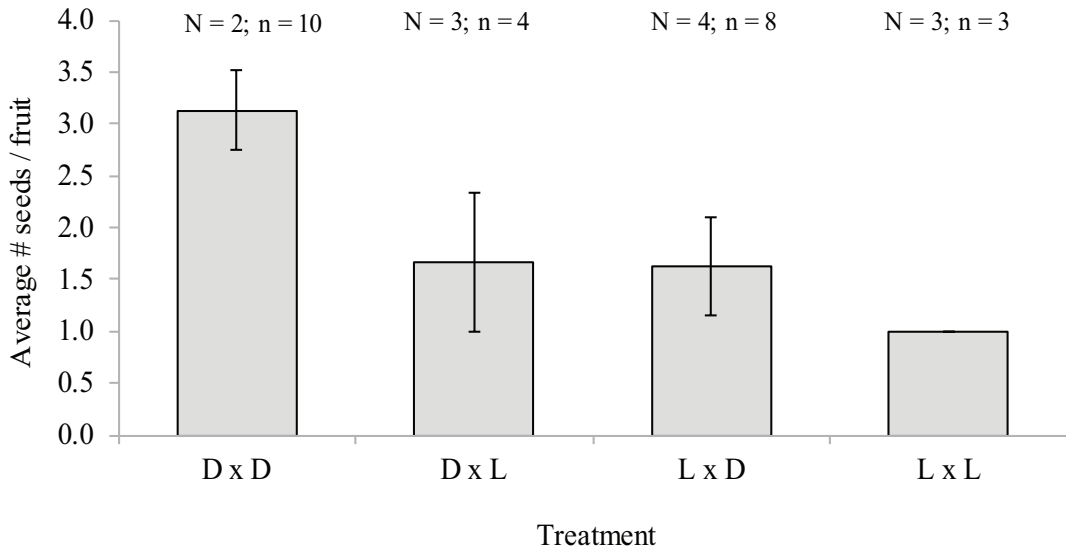


Figure 2. Average number of seeds per fruit for hand-pollinated crosses between *C. discolor* individuals (D x D), *C. linearis* individuals (L x L), and reciprocal crosses between *C. discolor* and *C. linearis* (D x L and L x D), with SE bars. N = # of mating pairs; n = # of fruits.

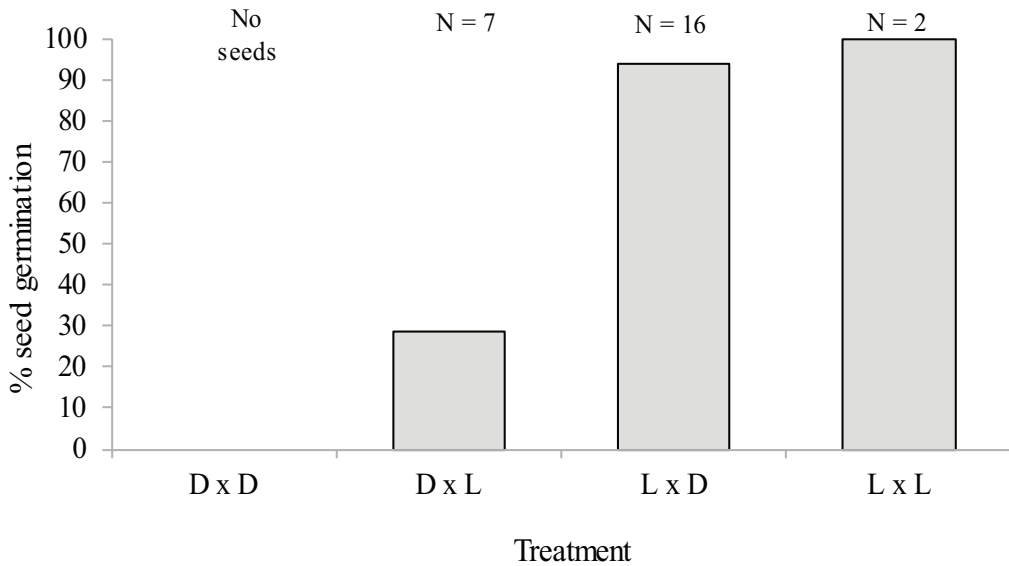


Figure 3. Percent germination for seeds resulting from crosses between *C. linearis* individuals (L x L), and reciprocal crosses between *C. discolor* and *C. linearis* (D x L and L x D). N = # seeds.

(*Chrysanthrax maculipennis*, *Geron salmonus*, *Ligyra cereberus*, and *Villa lateralis* complex) visited flowers on both male and female plants. Some insects were observed moving between male and female plants (data not shown). Six of the seven insect species screened were carrying *Croton* pollen as well as pollen from other plant species. Further, one pollen grain was found on the stigma of a virgin flower that was visited by *Ligyra cereberus*; this insect was collected as it flew away from the plant, and additional *Croton* pollen was found on its body.

Flower buds that were assigned to the D x D treatment in March failed to open, so the only D x D crosses made were performed in June. Fruit and seed set for the other three treatments were consistent for crosses made in March and June, so these data were joined for the analysis. All four types of crosses produced fruit; average fruit set ranged from 21% – 57% (Figure 1), but there were no statistically significant differences (ANOVA, F-ratio = 0.508, df = 3, p = 0.683). Average seed set ranged from 1.00 – 3.13 seeds/fruit (Figure 2), but these differences were not statistically significant (ANOVA, F-ratio = 2.625, df = 3, p = 0.122). The only

seeds that germinated were from crosses made in March, so there were no seeds from the D x D treatment in the germination experiment. Germination rates ranged from 29% – 100% for the three remaining treatments (Figure 3).

5. Discussion

There were no differences in fruit or seed set among the four treatments, and the germination experiment demonstrates that seeds from interspecific crosses are viable, which together suggests that there are not strong gametic or post zygotic barriers between *C. linearis* and *C. discolor*. Further, the fly species that visited both male and female plants did not distinguish between the two species or their intermediates, so mechanical barriers do not appear to exist. Bombyliid flies, in particular *Ligyra cereberus*, were common visitors to both male and female plants and carried pollen from *Croton*, therefore they are probably the principle pollinators of these *Croton* species.

This is an ecologically important study because *Croton* flowers are visited by many insects, providing them with both pollen

and nectar throughout the year (Landry et al. 2014a; Landry and Elliott, unpubl. data). Animal visitor species that have been observed on *Croton* flowers also visit other species, so *Croton* may facilitate successful pollination of other plant species by supporting pollinator populations in coastal communities.

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