

# NATURAL SELECTION REINFORCES SPECIATION IN A RADIATION OF NEOTROPICAL RAINFOREST PLANTS

Kathleen M. Kay<sup>1,2,3</sup> and Douglas W. Schemske<sup>1,4</sup>

<sup>1</sup>Department of Plant Biology, 166 Plant Biology Building, Michigan State University, East Lansing, Michigan 48824

<sup>2</sup>E-mail: kay@biology.ucsc.edu

<sup>4</sup>W. K. Kellogg Biological Station, 3700 E. Gull Lake Dr., Hickory Corners, Michigan 49060

Received March 26, 2008

Accepted June 23, 2008

The importance of reinforcement, that is, natural selection that strengthens reproductive isolation between incipient species, remains controversial. We used two approaches to test for reinforcement in a species radiation of Neotropical gingers in the genus *Costus*. First, we conducted an intensive study of *Costus pulverulentus* and *Costus scaber*, two recently diverged species that co-occur and share hummingbird pollinators. The hummingbird pollinators transfer pollen between these *Costus* species, but hybrids are rarely found in nature. By performing pollinations between populations of *C. pulverulentus* and *C. scaber* from three sites across the species' geographic ranges, we find that pollen–pistil incompatibilities acting prior to fertilization have evolved only between locally sympatric populations, whereas geographically distant populations within the region of sympatry and allopatric populations remain fully interfertile. Second, we conducted a comparative study of isolating mechanisms across the genus. We find lower seed set due to pollen–pistil incompatibility between species pairs that co-occur and experience pollen transfer in nature compared to species pairs that are otherwise isolated, regardless of genetic distance. Taken together, these studies indicate that crossing barriers prevent potentially maladaptive hybridization and effectively reinforce the speciation process. Our results add to mounting evidence for reinforcement from animal studies and show that plant speciation may also involve complex mate recognition systems. Reinforcement may be particularly important in rapidly diverging lineages where ecological factors play a primary role in reproductive isolation, as may often be the case in tropical communities.

**KEY WORDS:** *Costus*, crossing barriers, differential fusion, pollen–pistil incompatibility, pollination, reinforcement, reproductive character displacement, tropical diversity.

The process of reinforcement operates when the production of less fit hybrid offspring directly causes selection for stronger prezygotic reproductive isolation (reviewed in Dobzhansky 1940). Whereas there is broad support for reproductive barriers evolving as an indirect consequence of divergent adaptation during geographic isolation (Coyne and Orr 2004), the concept of reinforcement has been highly controversial. It was once proposed

as a common final stage in speciation when previously isolated lineages experience secondary geographic contact (Dobzhansky 1940; Lewontin 1974), but it has been criticized on theoretical grounds, and clear empirical support is limited (reviewed in Butlin 1987; Noor 1999; Servedio and Noor 2003). Recent studies have provided strong empirical evidence for reinforcement of mating discrimination behavior in at least a few cases of animal speciation (Noor 1995; Coyne and Orr 1997; Saetre et al. 1997; Rundle and Schluter 1998; Higgie et al. 2000), and theoretical studies have identified a broader range of parameters under which

<sup>3</sup>Current address: Department of Ecology and Evolutionary Biology, EMS A308, University of California, Santa Cruz, CA 95064

reinforcement is plausible (Liou and Price 1994; Kelly and Noor 1996; Cain et al. 1999; Kirkpatrick and Servedio 1999; Servedio 2000; Kirkpatrick 2001). Now debate focuses on how to reliably distinguish reinforcement from alternative explanations, the relative importance of reinforcement across taxa, the conditions under which it is likely to occur, and its underlying genetic basis (Coyne and Orr 2004; Servedio 2004).

Establishing an empirical case for reinforcement is difficult, and there is no example that fulfils all possible criteria. In addition to a pattern of stronger prezygotic isolation in sympatry, it would be desirable to (1) establish that there is some gene flow between species, (2) quantify hybrid disadvantage in nature, (3) estimate the contribution of the putatively reinforced barrier to the total reproductive isolation between the species in nature, and (4) rule out alternative explanations that could result in a similar pattern of sympatric isolation. The latter criterion has proved problematic, especially the challenge of distinguishing reinforcement from the more general case of reproductive character displacement (RCD) and from differential fusion, both of which can result in patterns of stronger isolation in sympatry. RCD, in which reproductive traits evolve larger differences in sympatry, may result from selection against competition, mating interference, and/or gamete wastage. Importantly, it can occur between even distantly related species. Reinforcement results in the same pattern of sympatric character displacement, but only occurs between closely related lineages and involves some gene flow. This is a critical distinction because reinforcement functions as a late stage in the speciation process whereas RCD occurs between taxa that are already reproductively isolated (Butlin 1987). Moreover, much of the theoretical debate about reinforcement centers on the difficulty of reconciling trait divergence with ongoing gene flow and recombination, which are not factors in RCD. For closely related species with low levels of gene flow, however, RCD and reinforcement may be difficult to distinguish empirically. RCD has been reported for a wide variety of organisms (Coyne and Orr 2004), but we do not understand how often this pattern may be caused by reinforcement.

In contrast, the differential fusion hypothesis posits that stronger isolation does not evolve in sympatry, but rather that weakly isolated taxa will either fuse or go extinct upon sympatric contact, leaving a pattern of greater isolation between sympatric species than allopatric species (Templeton 1981). If differential fusion is operating, as opposed to reinforcement, one would expect both pre- and postzygotic isolation to be stronger in sympatry, because either should prevent fusion/extinction (Coyne and Orr 1989). Moreover, the prezygotic barriers found between at least a subset of allopatric species should be comparable in strength to those found between sympatric species, because differential fusion posits that sympatric species were once strongly isolated allopatric species (Coyne and Orr 1989). Both of these distinc-

tions between differential fusion and reinforcement require comparative studies of reproductive isolation across many sympatric and allopatric species pairs and so have rarely been evaluated (but see Coyne and Orr 1997; Moyle et al. 2004; van der Niet et al. 2006). However, differential fusion is difficult to invoke for within-species geographic variation in the strength of isolation unless intraspecific gene flow is so low that populations fuse or go extinct independently (Noor 1999).

The best-supported cases for reinforcement to date come from animal studies; the importance of reinforcement in plant speciation is relatively unknown. Many of the early studies cited as support for plant reinforcement are more appropriately considered examples of RCD. Grant (1965, 1966) claimed that strong post-pollination incompatibilities between sympatric leafy-stemmed *Gilia* species were the product of reinforcement. He showed that allopatric species were more likely to produce F1 seeds in hand pollinations than sympatric species, but the phylogenetic relationships among these species were unknown and all the F1s were either inviable or sterile with poor chromosome pairing, precluding any gene flow. Other potential examples in plants came from studies of flower color in pairs of partially sympatric *Phlox* species. It was shown that a difference in flower color reduces the movement of pollinators between species and therefore reduces both pollen transfer and the frequency of hybridization (Levin and Kerster 1967; Levin 1978). However, hybrids between the *Phlox* species in these studies are completely infertile and all appear to be F1s, so there is no evidence of gene flow. Another often-cited example is the series of artificial selection experiments conducted by Pater-niani (1969) on two varieties of maize. After five generations of planting mixed fields and selecting for individuals with the lowest proportion of hybrid seeds, a marked increase in prezygotic isolation occurred through both a shift in flowering time and an increase in pollen–pistil incompatibility. During this experiment, all hybrid seeds were discarded, and no gene flow was allowed. Thus the *Gilia*, *Phlox*, and maize examples are more consistent with RCD caused by selection to avoid wasting gametes and less with reinforcement per se, although for *Gilia* and *Phlox* low levels of gene flow may be difficult to detect. An additional example is found in the recently evolved metal tolerance of plants growing on mine tailings. Tolerant populations of several species have evolved traits that reduce maladaptive hybridization with adjacent nontolerant populations, such as increased self-pollination and shifts in flowering time (Antonovics 1968; McNeilly and Antonovics 1968). These cases clearly involve gene flow, but are perhaps best explained as evidence of microspatial adaptive differentiation within species rather than reinforcement.

More recently, two comparative studies have shown patterns consistent with reinforcement. A study of 41 sister species pairs across three plant families in the Cape flora of South Africa shows that shifts in pollination system that confer reproductive isolation

are significantly associated with sympatric species pairs that also differ in edaphic habitat affinity—presumably to avoid the production of hybrids that may be less fit due to physiological trade-offs involved in edaphic adaptation (van der Niet et al. 2006). Differential fusion is unlikely in this system, because pollination shifts are not associated with sympatric pairs sharing edaphic conditions. Reinforcement also appears to be a better explanation than RCD, because the study involves sister species, but detailed mechanistic studies of putatively reinforced pairs are lacking. A study of reproductive isolation across many species pairs in the diverse Mediterranean deceptive orchids found that strong postpollination prezygotic crossing barriers have evolved between sympatric species that share guilds of pollinators, presumably as a mechanism to avoid maladaptive hybridization in the face of pollen transfer, but not between sympatric species that differ in highly specialized pollinators (Scopece et al. 2007). These results are intriguing but do not rule out alternative explanations because they do not examine pollinator sharing at the level of individual pollinators, as opposed to pollinator guilds, or present data on interspecific pollen transfer for the putatively reinforced sympatric species, nor do they evaluate allopatric species pairs for comparison. Moreover, molecular evidence suggests that there is no gene flow between sympatric pairs (Cuzzolino and Widmer 2005; Moccia et al. 2007). Taken together, all the putative cases of plant reinforcement show that key pieces of the reinforcement process are possible, and perhaps common, but our understanding is incomplete.

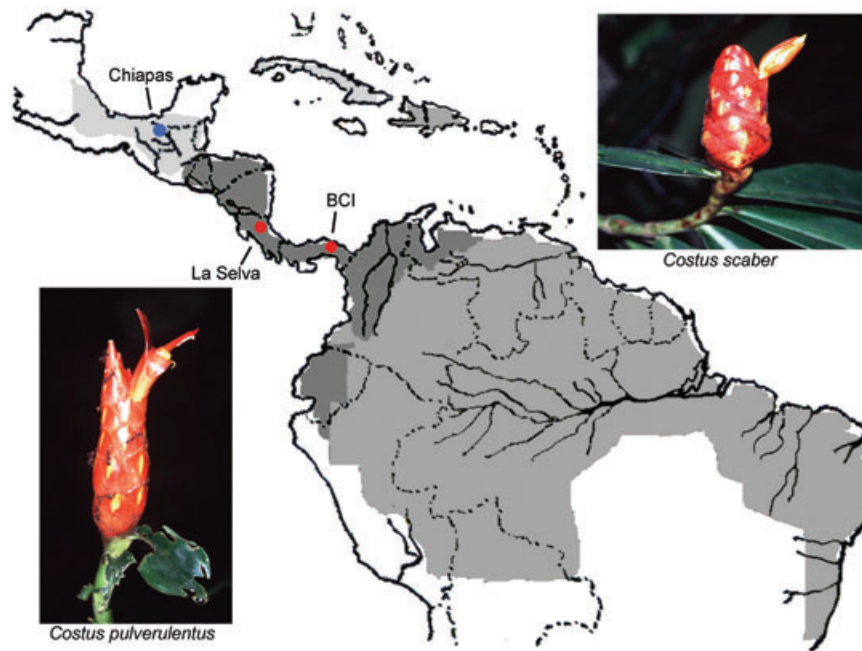
Here we examine the hypothesis of reinforcement in a diverse and recent radiation of understory Neotropical rainforest herbs in the genus *Costus* (Costaceae)—a genus that has been our focus for previous studies of speciation patterns and mechanisms (Schemske 1981; Kay and Schemske 2003; Kay et al. 2005; Kay 2006). We compare the strength of reproductive isolation between sympatric and allopatric populations of a partially sympatric species pair and place our results in the context of isolating mechanisms across a broader sample of species in the genus.

We focus in particular on postpollination isolation, because a previous study of two closely related and partially sympatric species, *Costus pulverulentus* and *Costus scaber*, identified strong pollen–pistil incompatibility as an important mechanism of reproductive isolation where the species co-occur (Kay 2006). This incompatibility functions prezygotically to prevent hybridization that might otherwise occur because of heterospecific pollen transfer from *C. pulverulentus* to *C. scaber* by their shared hummingbird pollinators. This incompatibility was striking in light of their close phylogenetic relationship and thus presented a putative case of reinforcement. We test the hypothesis of reinforcement by first estimating the strength of the incompatibility between populations of *C. pulverulentus* and *C. scaber* with varying geographic over-

lap and then by conducting an extensive study of other pairings of *Costus* species across a broad range of genetic distances to determine the distribution of pollen–pistil incompatibility throughout the genus. Reinforcement predicts that postpollination isolation should be strongest between populations or species that experience heterospecific pollen transfer. We consider two different null hypotheses: (1) that some postpollination isolation occurs between species pairs in the genus but is independent of whether the species experience heterospecific pollen transfer in sympatry, and (2) that postpollination isolation accumulates as a function of genetic distance.

If the incompatibility between *C. pulverulentus* and *C. scaber* has evolved in response to selection against hybridization, it should be stronger between locally sympatric populations of the two species than between geographically distant or allopatric populations. Conversely, if it is simply a byproduct of evolutionary divergence between species, the strength of the incompatibility should either be consistent across all interspecific pairings or any variation should be independent of the geographic origin of the populations. To test this, we performed pollinations on *C. scaber* using *C. pulverulentus* pollen from plants collected at the same locality, from plants collected at a geographically distant site within the region of sympatry, and from plants collected outside the region of sympatry. These pollination treatments were replicated on *C. scaber* plants collected from two different geographic regions (Fig. 1). *Costus scaber* was used as the seed parent in these crosses because field experiments reveal that interspecific pollen transfer by hummingbirds is exclusively from *C. pulverulentus* to *C. scaber* (Kay 2006).

The reinforcement hypothesis predicts that the strong pollen–pistil incompatibility found between *C. pulverulentus* and *C. scaber* is unusual for *Costus* species, and is a result of selection to reduce gene flow rather than a byproduct of divergence. To test this, we compared the crossing results for *C. pulverulentus* and *C. scaber* to controlled crosses for 15 additional pairings of species of Neotropical *Costus*. Like *C. pulverulentus* and *C. scaber*, one of these pairings (*C. allenii* and *C. laevis*) also shares pollinators in sympatry and experiences extensive interspecific pollen transfer in the field (Schemske 1981). Other pairings either differ in pollination syndrome, thereby precluding pollen transfer (Kay and Schemske 2003), are broadly sympatric but inhabit different habitats, and are unlikely to come into direct contact, or are allopatric. We asked whether species pairs known to experience pollen transfer in nature have lower crossing success, consistent with reinforcement, whether crossing relationships are congruent with genetic distance, or whether crossing relationships are independent of both genetic distance and heterospecific pollen transfer. We also include differences in style length between the male and female parent as a covariate, as flower length differences commonly contribute to asymmetrical results from



**Figure 1.** Geographic ranges of *C. pulverulentus* and *C. scaber*. The species both attract the same hummingbird pollinators, and differences in flower shape and size prevent interspecific pollen movement from *C. scaber* to *C. pulverulentus*, but not in the reverse direction. The two species are sympatric in Central America (dark gray), but are allopatric elsewhere (*C. pulverulentus*, light gray; *C. scaber*, medium gray). Source populations for crossing studies are indicated with red- and blue-filled circles, for sympatric and allopatric sites, respectively.

reciprocal crosses in plants because of constraints on pollen tube growth (Tiffin et al. 2001).

## Materials and Methods

### THE STUDY SYSTEM

The genus *Costus* comprises approximately 51 species in the Neotropics and has undergone a rapid radiation since dispersing from Africa roughly 1.5–7.1 Ma (Kay et al. 2005). Its primary center of diversity is in Costa Rica, Panama, western Colombia, and Ecuador, although it ranges from Mexico to Brazil. There are a few widespread species, but many are locally restricted, with several known only from their type locality. All species are herbaceous, diploid, and self-compatible. They occur in a variety of habitats, including streamsides, treefall gaps, and limestone outcrops, typically growing at low density in primary forest. Species are found from low to mid elevation, but are most common in wet, low-elevation sites in which it is typical to find several species growing in sympatry.

The Neotropical *Costus* species are remarkable for their floral biology. The characteristic spiralling stems support terminal inflorescences that generally produce a single large showy flower each day of an extended flowering season. Each species is specialized for pollination by either hummingbirds or orchid bees, and differences in pollination syndrome often function as effective premating isolating mechanisms for sympatric species (Kay and

Schemske 2003). Hummingbird pollination has evolved repeatedly from bee-pollinated ancestors in the Neotropics (Kay et al. 2005).

*Costus pulverulentus* and *C. scaber* are very closely related (Kay et al. 2005) and sympatric throughout much of Central and northwestern South America (Fig. 1). Both species have showy red flowers (Fig. 1) pollinated almost exclusively by the Long-billed Hermit hummingbird (*Phaethornis longirostris*, formerly *Phaethornis superciliosus*) at sympatric sites in Costa Rica and Panama. The Long-billed Hermit comprises 100% of the flower visitation to *C. pulverulentus* and 74% of the visitation to *C. scaber* across multiple years and locations (Kay and Schemske 2003). Birds move between flowers of the two species on their foraging routes, but differences in floral morphology affect pollen placement on the bird and effectively eliminate pollen transfer from *C. scaber* to *C. pulverulentus*. Pollen does move in the other direction, from *C. pulverulentus* to *C. scaber*. In experimental arrays of plants using dye to track pollen, 28% of the dye deposited on *C. scaber* stigmas was from *C. pulverulentus* anthers (Kay 2006). Nevertheless, hybrids are rarely found in nature. Experimental transfer of pollen from *C. pulverulentus* to *C. scaber* during controlled hand pollinations in the greenhouse results in very low seed set because pollen grains generally fail to adhere to the stigma or germinate (Kay 2006). This pollen–pistil incompatibility is sufficient to explain the rarity of hybrids in the face of ongoing

pollen transfer yet is surprising in light of their close phylogenetic relationship.

#### POPULATION-LEVEL CROSSES BETWEEN *C. PULVERULENTUS* AND *C. SCABER*

##### Plant collections

Plants of both species were collected as seeds or rhizomes from La Selva Biological Station (La Selva) in Heredia Province, Costa Rica (10°25'N, 84°00'W) and from Barro Colorado Island Nature Monument (BCI) in the Canal Zone, Panama (9°09'N, 79°51'W). These sites represent the region of sympatry and are approximately 470 km apart. Allopatric plants of *C. pulverulentus* were also collected from the Lacandon region of Chiapas, Mexico (16°43'N, 91°08'W). Sample sizes were as follows for *C. pulverulentus*: 15 plants from La Selva, 15 from BCI, and 11 from Chiapas, and for *C. scaber*: nine plants from La Selva and nine from BCI. Representative vouchers were made from each population and are deposited in the Michigan State University (MSC) herbarium.

##### Greenhouse crosses

We grew the plants to flowering in the greenhouses of University of Washington (Seattle) and Michigan State University (East Lansing) and crossed them according to previously published methods (Kay 2006). Each plant typically produces a single one-day flower per day when flowering, and plants flowered sporadically, preventing a balanced crossing design. Nevertheless, over the course of four years, all combinations of dams and sires were attempted to estimate seed set. A total of 868 flowers were crossed, and sample sizes for each cross type are reported in

Table 1. All statistical tests were performed using JMP version 6.0 (SAS Institute, Inc., Cary, NC).

We quantified seed set in crosses between two populations of *C. scaber* (La Selva and BCI) and three populations of *C. pulverulentus* (La Selva, BCI, and Chiapas) with *C. scaber* as the maternal parent, thus mimicking the observed direction of pollen dispersal between species. For each maternal population of *C. scaber*, we compared the number of seeds per fruit among paternal *C. pulverulentus* source populations with a nonparametric Kruskal–Wallis test, and we made posthoc comparisons of means with Tukey HSD.

To test whether the local interspecific incompatibility between species is associated with any decrease in intraspecific crossing success between La Selva and BCI, we performed intraspecific pollinations for each species among plants from the same site and between plants from different sites. Seed set per pollination was examined separately for each species with a mixed-model analysis of variance (ANOVA) including the following main effects: maternal population, maternal plant within maternal population (random), paternal population, paternal plant within paternal population (random), and the maternal population  $\times$  paternal population interaction. This model tests for population level incompatibility while accounting for general differences among populations in female fertility (maternal population) or siring ability (paternal population). Model fitting was done with restricted maximum likelihood.

Crossing relationships were also examined between the allopatric population of *C. pulverulentus* from Chiapas and one of the sympatric populations (from La Selva) of *C. pulverulentus* to ensure that the geographically distant Chiapas population

**Table 1.** Summary of crossing results for *C. pulverulentus* and *C. scaber*. Numbers are relative seed set in bold, calculated as the mean seed set per pollination in that cross type divided by mean seed set in outcrossed, intraspecific crosses within that maternal population. The absolute mean seed set per fruit  $\pm$  1SE and the sample size of pollinations is given in parentheses.

Maternal popn.	Paternal population				
	<i>C. scaber</i>		<i>C. pulverulentus</i>		
	La Selva	BCI	La Selva	BCI	Chiapas
<i>C. scaber</i>					
La Selva	<b>1.0</b> (6.1 $\pm$ 0.9, 53)	<b>1.1</b> (6.7 $\pm$ 1.4, 21)	<b>0.1</b> (0.8 $\pm$ 0.3, 46)	<b>1.1</b> (6.5 $\pm$ 0.7, 60)	<b>0.9</b> (5.2 $\pm$ 1.1, 23)
BCI	<b>1.1</b> (9.1 $\pm$ 3.6, 7)	<b>1.0</b> (8.0 $\pm$ 1.1, 45)	<b>0.8</b> (6.0 $\pm$ 0.7, 61)	<b>0.3</b> (2.1 $\pm$ 0.6, 39)	<b>0.7</b> (5.4 $\pm$ 1.0, 23)
<i>C. pulverulentus</i>					
La Selva	<b>0</b> (0 $\pm$ 0, 59)	<b>0</b> (0 $\pm$ 0, 11)	<b>1.0</b> (23.6 $\pm$ 2.2, 71)	<b>0.9</b> (20.9 $\pm$ 2.6, 48)	<b>0.9</b> (20.3 $\pm$ 1.8, 73)
BCI	<b>0.1</b> (1.0 $\pm$ 0.9, 20)	<b>0.05</b> (0.9 $\pm$ 0.3, 52)	<b>0.7</b> (12.8 $\pm$ 3.0, 44)	<b>1.0</b> (19.2 $\pm$ 3.2, 41)	–
Chiapas	<b>0</b> (0 $\pm$ 0, 12)	–	<b>0.8</b> (13.0 $\pm$ 2.2, 23)	–	<b>1.0</b> (16.4 $\pm$ 1.7, 36)



represented the same biological species. Seed set was analyzed with the same ANOVA model as above.

### Pollen germination and tube growth

Postpollination events were examined using epifluorescent microscopy of La Selva *C. scaber* according to previously published methods (Kay 2006). Reduced seed set in interspecific crosses has been shown to be the result of differences in pollen adhesion and percent germination that together result in fewer germinated pollen grains (Kay 2006). For *C. scaber* from La Selva, interspecific pollinations were compared for these measures using *C. pulverulentus* from both BCI and La Selva as sires ( $N = 11$  and 22 pollinations, respectively) with nonparametric Wilcoxon rank-sum tests.

*Costus* have wet stigmas, and we hypothesized that interspecific incompatibility might result from pollen–pistil interactions mediated by biochemical factors secreted by the stigma. Thus, we attempted to overcome the pollen–pistil incompatibility by performing a series of pollinations in which we transferred the stigmatic exudates from La Selva *C. pulverulentus* to La Selva *C. scaber* stigmas before pollinating them with La Selva *C. pulverulentus* pollen. These pollinations were paired with crosses in which the stigmatic exudates were not manipulated within inflorescences on representatives from four maternal families. We compared seed set with a paired *t*-test.

### GENUS-WIDE CROSSING STUDY

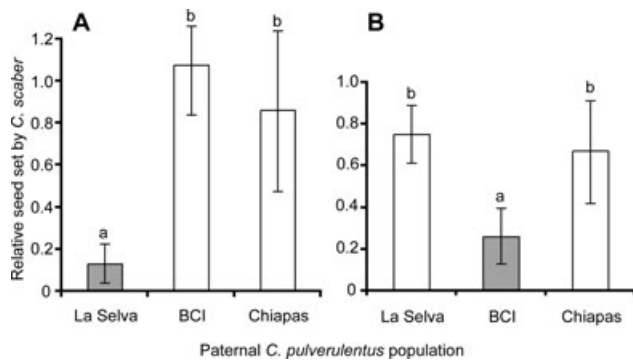
For the larger crossing study, crosses were either performed in the field or plants were collected and grown to flowering in the greenhouses at the University of Washington, Seattle, and/or Michigan State University, East Lansing. Fifteen pairings of species were included in this study. These involved a total of 10 species of *Costus* from various sites in Costa Rica and Panama, with reciprocal crosses performed for nine species pairs and unidirectional crosses performed for six species pairs, totalling 24 cross types. A total of 915 flowers were crossed, not including *C. pulverulentus* and *C. scaber*. Results for six of the cross types performed in the field were taken from a previous study (Schemske 1981). A summary of the crosses is presented in Appendix 1. Source locations and representative voucher specimens are described in Kay et al. (2005). Mean seed set in interspecific crosses was divided by mean seed set in concurrently performed intraspecific crosses to calculate measures of relative seed set that are comparable across pairings. Relative seed set values were truncated at 1.0 and arcsine square root transformed before statistical tests. Pairings were categorized by whether they experience pollen transfer in nature based on geographic distribution, pollination system, gross habitat differences, and field data on pollen transfer (Maas 1972; Schemske 1981; Kay and Schemske 2003). We included results from *C. pulverulentus* and *C. scaber* in the analyses by taking the

mean values of relative seed set and genetic distance for La Selva and BCI.

In addition to reinforcement caused by pollen transfer, we hypothesized that relative seed set may be affected by genetic distance and/or differences in style length between the male and female parent, and we first explored the effects of these factors separately. We compared relative seed set between species pairs experiencing pollen transfer in nature to those without pollen transfer with a *t*-test. We performed linear regression between relative seed set and genetic distance, which was estimated as branch lengths from a Bayesian phylogenetic analysis of rDNA internal and external transcribed spacers (Kay et al. 2005). We also calculated the Pearson product-moment correlation between relative seed set for reciprocal crosses, which by definition are separated by the same genetic distance. Because the relative seed set from reciprocal crosses is uncorrelated ( $P > 0.10$ ) and our work on *C. pulverulentus* and *C. scaber* has shown that reciprocal crossing barriers can have independent causes, we used each direction of cross as a separate datapoint in our analyses. The effect of differing style lengths on relative seed set between male and female parents of a reciprocal cross was compared with a two-tailed sign test for the nine species pairs with significantly different style lengths. Relative seed set then was analyzed in a combined analysis of covariance (ANCOVA) model, incorporating a categorical variable indicating pollen transfer and continuous variables for genetic distance and the difference in style length between parent species. Pollen adhesion and germination was examined for *C. allenii* and *C. laevis*, the only other sympatric pair of species known to experience pollen transfer in nature, with the same methods detailed above for *C. pulverulentus* and *C. scaber*, and results were compared between intra- and interspecific crosses with nonparametric Wilcoxon rank-sum tests.

## Results

For the crosses between *C. scaber* (seed parent) and *C. pulverulentus* (pollen parent), we find that seed set per pollination is only reduced in crosses between plants from the same geographic site (Fig. 2; Table 1). For *C. scaber* from La Selva, pollinations by *C. pulverulentus* from either BCI or Chiapas result in more than fivefold higher seed set than pollinations by *C. pulverulentus* from La Selva. Similarly, for *C. scaber* from BCI, pollinations by *C. pulverulentus* from either La Selva or Chiapas result in more than twofold higher seed set than pollinations by *C. pulverulentus* from BCI. Examination of pollen adhesion and germination on stigmas of *C. scaber* from La Selva shows that the pollen–pistil incompatibility is significantly weaker when *C. scaber* is pollinated by the geographically distant *C. pulverulentus* from BCI. Although pollen adhesion is not significantly higher for *C. pulverulentus* pollen from BCI compared to *C. pulverulentus* from



**Figure 2.** Mean relative seed set ( $\pm 2$  SE) for *C. scaber* from (A) La Selva, Costa Rica and (B) BCI, Panama following interspecific pollination by *C. pulverulentus* from La Selva, BCI, and Chiapas, Mexico. Relative seed set is the absolute seed set per fruit divided by the mean seed set obtained from outcrossed, intraspecific pollinations within that maternal population and is comparable among all cross types. Shaded bars indicate locally sympatric pairings. For each maternal *C. scaber* population, seed set differed significantly by the site of origin of *C. pulverulentus* (La Selva:  $\chi^2 = 34.7$ ,  $df = 2$ ,  $P < 0.0001$ ; BCI:  $\chi^2 = 16.0$ ,  $df = 2$ ,  $P = .0003$ ). Within each graph, lowercase letters represent significant Tukey HSD differences in means.

La Selva (62.0 vs. 31.9 pollen grains,  $t(33) = -1.747$ ,  $P = 0.09$ ), a higher percentage of the adhered pollen grains from BCI germinate (75.2 vs. 28.8 percent germination,  $t(33) = -4.25$ ,  $P = 0.0002$ ), leading to a higher number of overall germinated pollen grains (47.1 vs. 11.4,  $t(33) = -2.749$ ,  $P = .01$ ). We also find a marked increase in hybrid seed set when *C. scaber* stigmas from La Selva are first coated with the stigmatic exudate of *C. pulverulentus* from La Selva (mean seeds per pollination  $\pm 2$  SE:  $3.95 \pm 1.38$  with exudate added vs.  $0.35 \pm 0.36$  without exudates;  $N = 20$  paired pollinations; paired  $t$ -test,  $P < 0.001$ ). Taken together, these results show that there are very strong prezygotic incompatibility barriers between the stigma of *C. scaber* and the pollen of *C. pulverulentus* and that these barriers only operate between locally sympatric populations, providing strong support for the reinforcement hypothesis.

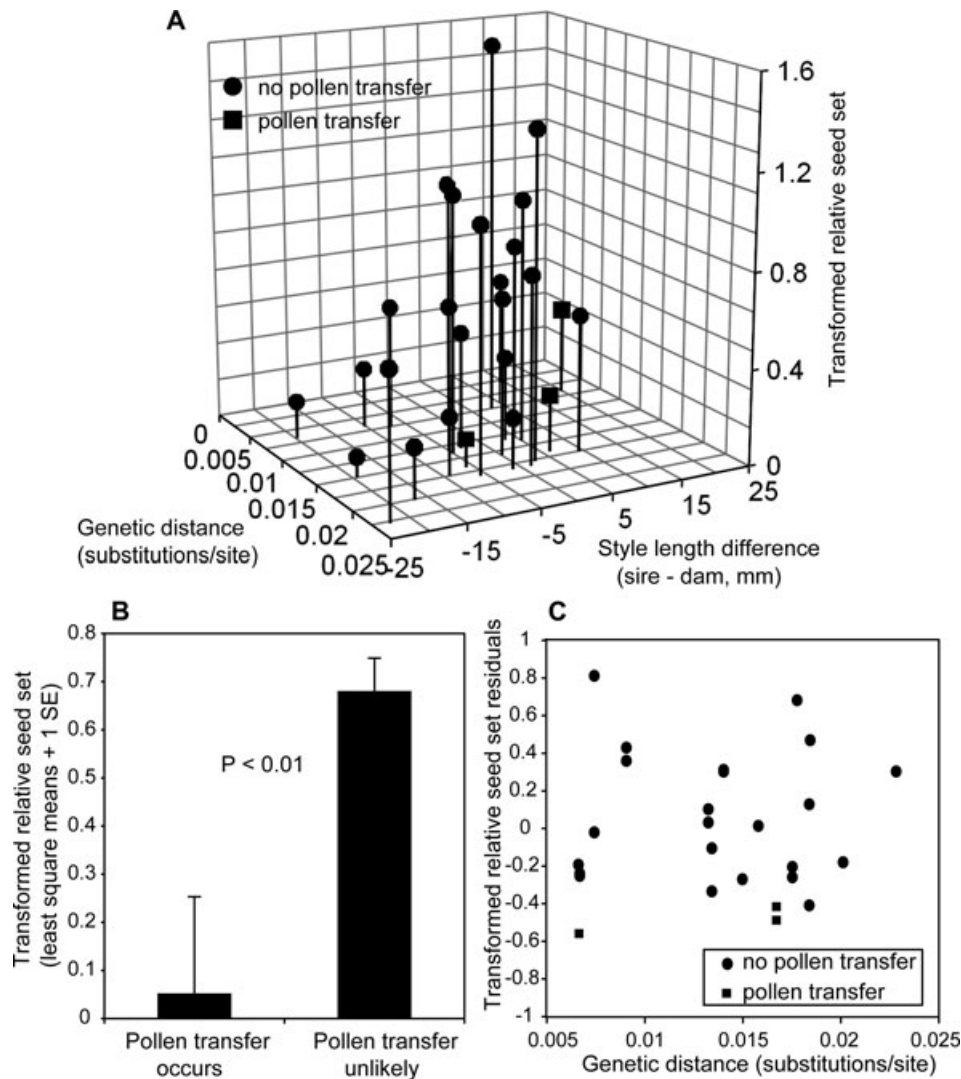
There is also a strong crossing barrier when *C. pulverulentus* is pollinated by *C. scaber*. The style of *C. pulverulentus* is approximately 2 cm longer, and the pollen tubes of *C. scaber* stop short of the ovary (Kay 2006), a typical result when crossing between flowers differing in length (Tiffin et al. 2001). Because there is no evidence for natural pollen transfer from *C. scaber* to *C. pulverulentus*, and thus no opportunity for reinforcement, we did not predict that seed set would differ according to the geographic source of the *C. scaber* pollen donors. Accordingly, interspecific pollinations of the various populations of *C. pulverulentus* by *C. scaber* from both La Selva and BCI result in effectively no hybrid seed set, similar to locally sympatric interspecific pollinations (Table 1).

Remarkably, the local crossing barriers that have evolved between *C. pulverulentus* and *C. scaber* have not been accompanied by any significant decrease in crossing success between populations within a species. Reciprocal greenhouse crosses between conspecific plants from La Selva and BCI show no incompatibility (Table 1; seed set per pollination; *C. pulverulentus* maternal population  $\times$  paternal population interaction:  $F_{1,143} = 2.58$ ,  $P = 0.11$ ; *C. scaber* maternal population  $\times$  paternal population interaction:  $F_{1,87} = 0.54$ ,  $P = 0.46$ ). Seed set per pollination in reciprocal crosses between *C. pulverulentus* from La Selva and Chiapas also does not show any incompatibility (Table 1; maternal population  $\times$  paternal population interaction:  $F_{1,161} = 2.25$ ,  $P = 0.14$ ). Thus there is no evidence of postpollination reproductive isolation among geographically disparate populations within either species.

### GENUS-WIDE CROSSING STUDY

We find that many Neotropical *Costus* cross easily, but that crosses between species pairs that experience pollen transfer in nature have significantly lower relative seed set compared to those without pollen transfer ( $t$ -test assuming unequal variance,  $df = 9.1$ ,  $t = -3.7$ ,  $P = 0.005$ , Fig. 3A, 3B; Appendix 1). This is consistent with the reinforcement hypothesis. Furthermore, crossing success, measured as relative seed set, is not explained by genetic distance (linear regression,  $N = 26$ ,  $R^2 = 0.01$ ,  $P = 0.65$ ; Fig. 3C), and there is no significant correlation between the relative seed set of reciprocal pairs that share a genetic distance (Pearson product-moment correlation,  $N = 10$ ,  $P > 0.10$ ). For the nine reciprocal crosses for which style length is significantly different, relative seed set is lower when the female parent has a longer style in eight cases (one-tailed sign test,  $P = 0.020$ ). A combined model including the difference in style length between the female and male parent, genetic distance, and a categorical variable indicating whether the pair experiences pollen transfer in nature explains a significant portion of the variance in relative seed set across the genus, with pollen transfer and style length differences showing significant effects (Table 2; Fig 3A).

To ensure that our results are not sensitive to our decision to use each direction of cross as a separate datapoint, we performed two parallel analyses on the subset of species pairs that we had crossed reciprocally, using the transformed mean of the relative seed set. Although *C. scaber* and *C. pulverulentus* only experience pollen transfer in one direction, we included the pair in the group with pollen transfer. The two species pairs that experience pollen transfer in nature have significantly lower mean relative seed set compared to those without pollen transfer ( $t$ -test assuming unequal variance,  $df = 8$ ,  $t = 4.4$ ,  $P = 0.002$ ). The relationship between genetic distance and relative seed set was insignificant (linear regression,  $N = 10$ ,  $R^2 = 0.05$ ,  $P = 0.54$ ).



**Figure 3.** Genus-wide crossing study. (A) Relative seed set between Neotropical *Costus* species plotted against genetic distance and the difference in style length between the male and female parent. The three cross types known to experience pollen transfer in nature are indicated with squares, and include *C. scaber*  $\times$  *C. pulverulentus*, *C. allenii*  $\times$  *C. laevis*, and *C. laevis*  $\times$  *C. allenii*. Both pollen transfer and style length differences significantly affect relative seed set. (B) Least square mean relative seed set ( $\pm 1$  SE) between species pairs with pollen transfer in nature compared to between species pairs without pollen transfer in nature. This graph shows the estimated effect of pollen transfer while controlling for style length difference and genetic distance. (C) Transformed relative seed set residuals from a regression analysis with style length differences plotted against genetic distance. This graph shows the lack of relationship between genetic distance and crossing success, while controlling for style length differences.

*Costus allenii* and *C. laevis* are the only other pair of *Costus* species we have studied that share pollinators and experience pollen transfer in sympatry (Schemske 1981). We find that the strong crossing barrier between these species is caused by prezygotic pollen–pistil incompatibility, consistent with the reinforcement hypothesis. For *C. allenii* as the maternal parent, we compare intra- to interspecific crosses and find significant differences in the number of pollen grains adhering to the stigma (129 vs. 50,  $Z = 2.09$ ,  $P = 0.037$ ) and percent pollen germination (95% vs. 49%,  $Z = 2.09$ ,  $P = 0.037$ ). For *C. laevis* as the maternal parent, we again compare intra- to interspecific crosses and find signifi-

cant differences in the mean number of pollen grains adhering to the stigma (190 vs. 79,  $Z = 3.28$ ,  $P = 0.001$ ) and percent pollen germination (99% vs. 63%,  $Z = 3.37$ ,  $P = 0.001$ ).

## Discussion

### TESTING THE REINFORCEMENT HYPOTHESIS

During a prior study quantifying different components of reproductive isolation between *C. pulverulentus* and *C. scaber*, Kay (2006) found that premating isolation in sympatry was incomplete, but that strong postpollination barriers prevented most



**Table 2.** ANCOVA for genus-wide crosses.

Source	df	SS	MS	F	P
Model	3	1.49	0.50	4.48	0.013
Error	22	2.43	0.11		
Effects					
Genetic distance	1	0.01		0.11	0.739
Style length difference	1	1.00		9.08	0.006
Pollen transfer experienced in nature	1	0.93		8.42	0.008

hybridization. The species are both pollinated largely by hummingbirds and individual birds move between both plant species. Because of differences in flower shape and size that affect pollen placement on the birds, pollen is not transferred from *C. scaber* to *C. pulverulentus* but is transferred from *C. pulverulentus* to *C. scaber*. Nevertheless, strong incompatibility between the pistil of *C. scaber* and the pollen of *C. pulverulentus* prevents this pollen transfer from resulting in substantial hybridization. We found this prezygotic incompatibility striking, given that the two species are very closely related (Kay et al. 2005), and hypothesized that it evolved by reinforcement in the face of sympatric pollen transfer. We also considered two alternative hypotheses, 1) that pollen–pistil incompatibility may evolve sporadically between species irrespective of pollen transfer in sympatry and 2) that pollen–pistil incompatibility may accumulate with genetic divergence.

The hypotheses for the origin of pollen–pistil incompatibility make different predictions about the strength of the barrier both at the population and species level. Under reinforcement, the incompatibility should be strongest between populations that have most recently experienced pollen transfer and weaker between populations that are geographically distant. For the population-level crosses, this means that *C. scaber* pistils should show stronger incompatibility with *C. pulverulentus* pollen from the same geographic location than from either a geographically distant site within the region of sympatry or from an allopatric site. For the species-level crosses, sympatric species that experience pollen transfer should have lower crossing success than species pairs that are allopatric or otherwise isolated. The second hypothesis predicts that at both the population and species level some incompatibility may occur, but the strength of incompatibility should not vary with respect to whether pollen transfer occurs. The third hypothesis not only predicts that more distantly related species pairs in the genus will show stronger incompatibility, but also that *C. pulverulentus* and *C. scaber* are consistently incompatible, regardless of the geographic origin of the populations tested.

To distinguish among these hypotheses, we conducted a series of controlled crossing experiments between several populations of our two focal species from a wide geographic range and between many species of *Costus* representing the range of

genetic distances in the genus. We tested each of two populations of *C. scaber* from the region of sympatry with pollinations from locally sympatric, regionally sympatric, and allopatric *C. pulverulentus*. We found that for both populations of *C. scaber*, pollinations by the locally sympatric *C. pulverulentus* had strikingly low seed set, whereas the pollinations by other *C. pulverulentus* were highly successful and similar to intraspecific crosses in seed set. An additional 24 sets of controlled crosses were made between *Costus* species to examine the distribution of postpollination isolation across the genus. Most of these species pairs either were allopatric or were sympatric but isolated by differences in pollinators or gross habitat affinities, but the reciprocal crosses between *C. allenii* and *C. laevis* represented an additional pairing of sympatric species that experience pollen transfer by shared pollinators (Schemske 1981). For these species-level crosses, we found lower seed set between pairs experiencing pollen transfer than between pairs otherwise isolated. We also established that the incompatibility between *C. allenii* and *C. laevis* acts prior to fertilization, as also observed in crosses between *C. scaber* and *C. pulverulentus*. *Costus allenii* and *C. laevis* are bee-pollinated and are phylogenetically distinct from the hummingbird-pollinated *C. pulverulentus* and *C. scaber*, thus they represent a putative second case of reinforcement that involves convergent evolution of pollen–pistil incompatibility. Although crossing success showed no relationship to genetic distance, we did find that style length difference contributed significantly to the success of interspecific crosses, with pollen from short-styled species less successful at fertilizing long-styled species.

Taken together, these results strongly support the reinforcement hypothesis and reject the hypothesis that postpollination incompatibility accumulates with genetic distance in this clade. They also provide some support for our alternative hypothesis that other factors may affect postpollination incompatibility in *Costus*. Differences in style length certainly confer postpollination isolation in one direction of many reciprocal crosses, most likely due to intrinsic aspects of pollen tube growth rather than a more active form of incompatibility. Even considering style length differences, however, we find that our combined model explains only 38% of the variation in crossing success across the 26 cross types. Thus there are clearly other prezygotic or early-acting postzygotic factors that affect postpollination compatibility in *Costus*. To better understand these factors, it will be necessary to examine pollen germination and tube growth for the partially compatible species pairs and to add more species pairs to the crossing study.

Alternative explanations for the strong incompatibility between populations experiencing pollen transfer are implausible. It is easy to imagine that sympatric differences in other isolating traits, such as flowering phenology or floral morphology, can be an indirect result of differential evolutionary responses of the

species to an array of ecological variables, making reinforcement difficult to interpret. Yet an ecological variable that would cause such a locally specific postpollination incompatibility without affecting other crossing relationships is highly improbable. We can also exclude the more general case of RCD, which is often difficult to distinguish from reinforcement *per se*. In *Costus*, the barrier operates after pollen deposition, fertile hybrids can be made at a low frequency, and putative hybrids have been observed in nature, albeit rarely (Kay et al. 2005; Kay 2006). Finally, the pattern of stronger prezygotic isolation between sympatric species could result from the differential fusion or extinction of less isolated lineages in sympatry, so that only lineages that happen to be strongly isolated may coexist (Templeton 1981). Although this is difficult to rule out, differential fusion was originally proposed to explain an among-species pattern of increased isolation and is an unlikely explanation of a within-species pattern because of the extremely low levels of intraspecific gene flow necessary for it to operate (Noor 1999; Coyne and Orr 2004). This explanation also predicts that strong pollen–pistil incompatibility is likely to be found between a subset of species pairs throughout the genus. Although there are other pairings that show extremely low relative seed set, all but one of these exhibit a large difference in style length and do not require the more active incompatibility that we found for the putatively reinforced pairs. The only exception is the cross between the similarly sized flowers of *C. laevis* (seed parent) by *C. guanaiensis* (pollen parent). Although we do not think these species experience pollen transfer on the level of *C. pulverulentus*/*C. scaber* or *C. allenii*/*C. laevis* because of striking habitat differences, they are broadly sympatric and share a bee pollination syndrome, so we cannot rule out pollen transfer. We currently have no examples of other species pairs without pollen transfer that show the same type of pollen–pistil incompatibility observed for *C. pulverulentus*/*C. scaber* or *C. allenii*/*C. laevis*.

It also is possible that phylogenetic constraints on crossing relationships affect our results for the broader crossing study. Because of the logistical difficulties in using live plants, we did not set up our crossing study to use only phylogenetically independent contrasts and often used the same species in multiple pairings. However, our results suggest that crossing relationships are highly labile. *Costus pulverulentus* and *C. scaber* only show pollen–pistil incompatibility at the scale of locally sympatric populations, and phylogenetic evidence indicates that the incompatible populations are no more distantly related than other population pairs (Kay et al. 2005). Our broader crossing study also shows that all species involved in putative cases of reinforcement are able to cross with other species that either are allopatric or differ in pollination syndrome or habitat (Appendix 1). For example, *C. scaber* shows incompatibility when pollinated by sympatric *C. pulverulentus* but has full seed set when pollinated by two more distantly related allopatric species, *C. lasius* and *C. woodsonii*,

and *C. pulverulentus* is able to successfully pollinate three more distantly related bee-pollinated species. *Costus allenii* and *C. laevis* are both successful as seed and pollen parents in other pairings not involving natural pollen transfer (Appendix 1). Thus there is no evidence that the putatively reinforced species are generally less crossable.

## REINFORCEMENT IN THE CONTEXT OF OTHER ISOLATING MECHANISMS

Reinforcement requires that postzygotic reproductive isolation causes selection for stronger prezygotic isolation. Compared to nonhybrid controls, greenhouse-created F1 hybrid seeds between *C. scaber* and *C. pulverulentus* from La Selva have significantly lower rates of germination, and F1 hybrids between plants from BCI have significantly lower pollen fertility. Moreover, La Selva hybrid backcrosses to either parental species have substantially lower pollen fertility compared to nonhybrid controls or F1s (Kay 2006). Nonetheless, these differences are relatively small, with many hybrids growing vigorously and producing at least partially fertile flowers. Because the species show habitat segregation, with *C. pulverulentus* found in drier soil under a more open canopy (K. M. Kay, unpubl. data), postzygotic isolation may be determined more by the reduced ecological performance of hybrids than by strong intrinsic genetic incompatibility, a phenomenon that has been empirically demonstrated in other systems (Hatfield and Schluter 1999) and that can theoretically contribute to reinforcement (Kirkpatrick 2001). We have found five putative hybrids in the field that morphologically matched F1 hybrids created in the greenhouse and were growing in areas in which *C. pulverulentus* and *C. scaber* were the only hummingbird-pollinated *Costus* species present. Of these hybrids, all were found in areas of recent deforestation, in which ecological factors contributing to isolation may have broken down. Because the species differ in their sites of pollen placement on the shared hummingbird pollinator, hybrids also may suffer from reduced mating success because of their intermediate floral morphology. Field trials of F1 and advanced generation hybrids will be necessary to determine how ecological factors contribute to postzygotic isolation.

Much of the debate over reinforcement centers on the conditions under which it is likely to occur. Dobzhansky (1940) envisioned reinforcement as a process that brings speciation closer to completion after substantial reproductive isolation has evolved in geographic isolation, and that appears to be the case for *C. pulverulentus* and *C. scaber*. Total prezygotic reproductive isolation has been measured between these species by summing the multiplicative effects of barriers that act sequentially to limit potential gene flow. These include broad-scale geographic isolation, microhabitat differences, flowering phenology, pollinator use, floral mechanical isolation and pollen–pistil interactions (Kay 2006). Reproductive isolation is 99.0% complete at the prezygotic stage

for *C. scaber* as the maternal parent. Without the contribution of pollen–pistil incompatibility, however, prezygotic isolation is estimated to be only 95.0% complete, which would allow a significant amount of hybridization. The initial amount of reproductive isolation and the extent of interspecific gene flow are parameters central to the theory of reinforcement (Liou and Price 1994; Servedio and Kirkpatrick 1997; Kirkpatrick 2001), and this study is unique in providing estimates of reproductive isolation in nature both with and without the putatively reinforced mechanism.

#### POLLEN–PISTIL INCOMPATIBILITY AND SPECIATION

Pollen–pistil interactions have long been recognized as a potential source of reproductive isolation in plants (Stebbins 1950), but have received relatively little attention in this context compared to floral differences involved in pollinator attraction (but see Howard 1999). They are often considered analogous to animal interactions between sperm and the female reproductive tract and/or eggs (Coyne and Orr 2004), and some of the species-specific proteins involved in those interactions are known to undergo extremely rapid evolution, indicating a likely role in speciation (Metz and Palumbi 1996; Hellberg and Vacquier 1999; Clark et al. 2006). Although the pollen–pistil incompatibility observed in *Costus* involves population-level differences in both the pollen and pistil, we can consider it as primarily a mate choice phenomenon that operates through female function, because selection on mate choice through male function should act prior to pollen deposition. Thus our results conform to one of the predictions Coyne and Orr (2004) recently put forth to distinguish reinforcement from alternative hypotheses, that reinforcement should cause greater changes in female traits in sympatry because females pay a larger cost for disadvantageous matings.

Nevertheless, there are aspects of the *C. scaber*/*C. pulverulentus* incompatibility mechanism that are poorly understood. We only tested *C. scaber* with pure loads of *C. pulverulentus* pollen, whereas in nature mixed pollen loads may be more likely. If there is conspecific pollen precedence (Howard 1999), mixed pollen loads may amplify the effects of postpollination isolation. Conversely, conspecific pollen could facilitate fertilization by *C. pulverulentus* pollen if it reduces the active incompatibility response in the *C. scaber* stigma, akin to the mentor effect that has been shown to override the incompatibility response in self-incompatible plants (Richards 1986). In addition, the physiological mechanism of interspecific incompatibility is entirely unknown in *Costus*. Most knowledge of pollen–pistil interactions at the physiological or molecular level involves species that are self-incompatible and/or have dry stigmas (Nasrallah 2002; Clark et al. 2006), neither of which applies to *Costus*. *Costus* would be an excellent system for further studies of the molecular basis of pollen–pistil interactions.

#### SPECIATION IN *COSTUS* AND THE NEOTROPICAL FLORA

Reinforcement between *C. pulverulentus* and *C. scaber*, and possibly between *C. allenii* and *C. laevis*, is perhaps not surprising in light of the emerging pattern of speciation in Neotropical *Costus*. Phylogenetic evidence suggests a recent and rapid radiation for this clade (Kay et al. 2005). There has been marked divergence in habitat use (Maas 1977), and the biogeographic distribution of species suggests extensive range shifts (Kay et al. 2005). Strong intrinsic postzygotic isolation between species, however, has yet to evolve. We have attempted to grow F1 progeny from 13 different crossing combinations, and all are viable and most show little reduction in pollen fertility (Schemske and Kay, unpublished data). Approximately one-fifth of the speciation events in the Neotropical *Costus* have involved a shift in specialized pollination from orchid bees to hummingbirds (Kay et al. 2005), which effectively prevents any potential pollen flow between species that are otherwise interfertile (Kay and Schemske 2003, Appendix 1). However, the majority of speciation events do not involve a change in pollination syndrome, and this sets up a scenario in which direct natural selection could act to reinforce postpollination isolation in cases when incipient species come into secondary contact. We hope to add more pairs of *Costus* species to our crossing study to better understand the importance of reinforcement to this species radiation.

We do not know how typical our results are for the Neotropical flora. The causes of tropical diversity are complex (Mittelbach et al. 2007), but some patterns are emerging that motivate this type of investigation into the evolution of isolating mechanisms in tropical lineages. Broad phylogenetic studies have shown higher diversification rates at lower latitudes for both flowering plants (Davies et al. 2004) and birds (Cardillo et al. 2005), and paleontological studies conducted across disparate lineages of marine animals further show higher origination rates in the tropics (reviewed in Mittelbach et al. 2007). The Neotropics are home to more plant species than tropical Asia and Africa combined (Raven and Axelrod 1974), and many species-rich Neotropical plant lineages, in addition to *Costus*, have undergone bursts of extremely rapid and recent diversification (Richardson et al. 2001; Bell and Donoghue 2005; Kay et al. 2005; Hughes and Eastwood 2006; Erkens et al. 2007; Sarkinen et al. 2007). The speed of these radiations and the common sympatric occurrence of several related species in Neotropical forests lead to the hypothesis that speciation may arise less by the gradual accumulation of genetic incompatibilities over time but rather as a consequence of rapid ecological differentiation during short periods of isolation. We suggest that if reinforcement is an important factor in plant speciation, its signature is likely to be evident in floral biodiversity hot spots, including the Neotropical forests where ecological factors may play a primary role in reproductive isolation.

# "REINFORCEMENT OF REPRODUCTIVE ISOLATION: PLANTS VERSUS ANIMALS" REVISITED

Given the paucity of cases of plant reinforcement in the literature, Levin (1970) posed the question, "Has natural selection played a more immediate role in the evolution of reproductive isolating mechanisms in animals than in plants?" Almost four decades later, we still do not know the answer. Although Moyle et al. (2004) found no evidence for reinforcement in a survey of postpollination reproductive isolation in *Glycine* and *Silene*, the lack of information on premating barriers and natural pollen transfer rendered their results concerning reinforcement inconclusive. More recent comparative studies have suggested an important role for reinforcement in plants (van der Niet et al. 2006; Scopece et al. 2007) but have not clearly ruled out alternative explanations. Here we present the first study in plants that can convincingly differentiate reinforcement from RCD. We show how the reinforced postpollination barrier contributes substantially to the suite of isolating mechanisms between the species in nature, and we place the results for our focal species in the context of a study of post-pollination isolation across the genus. We find that reinforcement can contribute to speciation in plants, that it may evolve locally when incipient species experience geographic contact, and that, as in animal systems, it may involve complex mate recognition systems.

## ACKNOWLEDGMENTS

We thank K. Ward, M. Cooper, and G. Chen for field and greenhouse assistance, D. Ewing and M. Hammond for greenhouse support, and H. D. Bradshaw, J. Conner, P. Fine, R. Lenski, D. Plachetzki, A. Prather, J. Schuetz, C. Voelckel, J. Whittall, and P. Wilson for comments on early versions of the manuscript. This work was supported by a fellowship and dissertation improvement grant from the National Science Foundation to KMK, a grant from the Andrew W. Mellon Foundation to DWS, and fellowships from the Garden Club of America, the Organization for Tropical Studies, and the Environmental Science and Policy Program at MSU to KMK.

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Associate Editor: J. Kohn

**APPENDIX.** Summary of crossing results for a broader sample of *Costus* species.

Seed parent	Pollen parent	Location	N—Intrasp. (crosses, plants)	Intrasp. seeds/fruit ( $\pm 2$ SE)	N—Intersp. (crosses, plants) <sup>1</sup>	Intersp. seeds/fruit ( $\pm 2$ SE)	Relative seed set	Category	Sire — dam style length difference (mm)	Genetic distance
<i>C. allenii</i> , BCI	<i>C. guanaensis</i> , BCI	Field*	39, 4	50.4 $\pm$ 5.4	38, 7	26.60 $\pm$ 4.7	0.53	sympatric, different habitat	4.7	0.01324
<i>C. allenii</i> , BCI	<i>C. laevis</i> , BCI	Field*	39, 4	50.4 $\pm$ 5.4	40, 6	2.90 $\pm$ 1.9	0.06	sympatric, pollen transfer occurs	6.0	0.01668
<i>C. allenii</i> , BCI	<i>C. pulverulentus</i> , BCI	Field	10, 4	38.7 $\pm$ 10.4	9, 3	14.40 $\pm$ 9.9	0.37	sympatric, different pollinators	0.1	0.01579
<i>C. allenii</i> , BCI	<i>C. scaber</i> , BCI	Field	10, 4	38.7 $\pm$ 10.4	8, 2	1.75 $\pm$ 3.5	0.05	sympatric, different pollinators	—16.8	0.02011
<i>C. allenii</i> , BCI	<i>C. villosissimus</i> , BCI	Field	36, 9	29.6 $\pm$ 6.3	38, 9	22.7 $\pm$ 4.8	0.77	sympatric, different habitat	—0.3	0.00906
<i>C. bracteatus</i> , La Selva	<i>C. lasius</i> , El Valle	Greenhouse	11, 2	22.7 $\pm$ 5.3	22, 2	1.41 $\pm$ 1.0	0.06	allopatric	—10.2	0.00668
<i>C. guanaensis</i> , BCI	<i>C. allenii</i> , BCI	Field*	29, 7	109.8 $\pm$ 12.4	33, 4	35.3 $\pm$ 11.3	0.32	sympatric, different habitat	—4.7	0.01324
<i>C. guanaensis</i> , BCI	<i>C. laevis</i> , BCI	Field*	29, 7	109.8 $\pm$ 12.4	31, 6	55.3 $\pm$ 10.2	0.50	sympatric, different habitat	1.3	0.01839
<i>C. guanaensis</i> , BCI	<i>C. villosissimus</i> , BCI	Field	5, 2	73.2 $\pm$ 3.3	7, 2	43.4 $\pm$ 22.7	0.59	sympatric, different habitat	—5.0	0.01402
<i>C. laevis</i> , BCI	<i>C. allenii</i> , BCI	Field*	48, 6	53.4 $\pm$ 4.4	53, 4	0.8 $\pm$ 0.6	0.02	sympatric, pollen transfer occurs	—6.0	0.01668
<i>C. laevis</i> , BCI	<i>C. guanaensis</i> , BCI	Field*	48, 6	53.4 $\pm$ 4.4	29, 7	2.4 $\pm$ 1.9	0.04	sympatric, different habitat	—1.3	0.01839
<i>C. laevis</i> , BCI	<i>C. pulverulentus</i> , BCI	Field	6, 3	6.7 $\pm$ 11.4	7, 3	4.9 $\pm$ 6.4	0.73	sympatric, different pollinators	—6.0	0.01844
<i>C. laevis</i> , BCI	<i>C. scaber</i> , BCI	Field	6, 3	6.7 $\pm$ 11.4	6, 2	2.17 $\pm$ 4.3	0.33	sympatric, different pollinators	—22.9	0.02282

Continued.

## APPENDIX. Continued.

Seed parent	Pollen parent	Location	N—Intrasp. (crosses, plants) <sup>1</sup>	Intrasp. seeds/fruit ( $\pm 2$ SE)	N—Intersp. (crosses, plants) <sup>1</sup>	Intersp. seeds/fruit ( $\pm 2$ SE)	Relative seed set	Category	Sire—dam style length difference (mm)	Genetic distance
<i>C. laevis</i> , La Selva	<i>C. malortieanus</i> , La Selva	Field	5, 2	80.2 $\pm$ 28.7	6, 6	4.7 $\pm$ 8.6	0.06	sympatric, different habitat	-9.3	0.01752
<i>C. lasius</i> , El Valle	<i>C. bracteatus</i> , La Selva	Greenhouse	21, 3	8.2 $\pm$ 3.0	23, 2	2.2 $\pm$ 3.9	0.26	allopatric	10.2	0.00668
<i>C. malortieanus</i> , La Selva	<i>C. laevis</i> , La Selva	Field	17, 6	32.3 $\pm$ 6.0	8, 2	9.5 $\pm$ 9.8	0.29	sympatric, different habitat	9.3	0.01752
<i>C. malortieanus</i> , La Selva	<i>C. pulverulentus</i> , La Selva	Field	17, 6	32.3 $\pm$ 6.0	8, 2	3.9 $\pm$ 3.4	0.12	sympatric, different pollinators	3.2	0.01342
<i>C. malortieanus</i> , La Selva	<i>C. scaber</i> , La Selva	Field	17, 6	32.3 $\pm$ 6.0	13, 2	0.2 $\pm$ 0.3	0.01	sympatric, different pollinators	-19.5	0.01498
<i>C. pulverulentus</i> , BCI & La Selva	<i>C. scaber</i> , BCI & La Selva	Greenhouse	115, 30	19.8 (averaged)	110, 18	0.47 (averaged)	0.02	sympatric, pollen transfer occurs	-19.8	0.00661
<i>C. pulverulentus</i> , La Selva	<i>C. malortieanus</i> , La Selva	Field	5, 2	27.2 $\pm$ 23.3	5, 3	6.0 $\pm$ 7.8	0.22	sympatric, different pollinators	-3.2	0.01342
<i>C. scaber</i> , BCI	<i>C. lasius</i> , El Valle	Greenhouse	45, 6	12.8 $\pm$ 8.2	47, 3	12.2 $\pm$ 2.2	0.96	allopatric	2.6	0.01777
<i>C. scaber</i> , BCI & La Selva	<i>C. pulverulentus</i> , BCI & La Selva	Greenhouse	99, 18	7.0 (averaged)	77, 30	1.5 (averaged)	0.13	sympatric, pollen transfer occurs	19.8	0.00661
<i>C. scaber</i> , La Selva	<i>C. woodsonii</i> , Bocas & Tortuguero	Greenhouse	53, 9	6.1 $\pm$ 1.8	9, 3	6.5 $\pm$ 3.1	1.08	allopatric	8.0	0.00742
<i>C. villosissimus</i> , BCI	<i>C. allenii</i> , BCI	Field	28, 8	30.6 $\pm$ 11.8	36, 11	21.8 $\pm$ 8.1	0.71	sympatric, different habitat	0.3	0.00906
<i>C. villosissimus</i> , BCI	<i>C. guanaiensis</i> , BCI	Field	5, 3	57.2 $\pm$ 7.0	8, 2	41.4 $\pm$ 14.0	0.72	sympatric, different habitat	5.0	0.01402
<i>C. woodsonii</i> , Bocas	<i>C. scaber</i> , La Selva	Greenhouse	5, 3	8.8 $\pm$ 7.9	7, 2	2.1 $\pm$ 2.8	0.24	allopatric	-7.1	0.00742

Field locations are as follows: BCI, Barro Colorado Island, Panama; La Selva, La Selva Biological Station, Costa Rica; El Valle, El Valle de Anton, Panama; Bocas, Bocas del Toro, Panama; Tortuguero, Tortuguero National Park, Costa Rica. A voucher specimen from each population has been deposited in the herbarium at Michigan State University (MSC). Asterisks indicate crosses performed in the field and reported in Schemske (1981). Results for *C. pulverulentus* and *C. scaber* were averaged between La Selva and BCI; see Table 1 for details. Relative seed set values presented here were transformed prior to analysis.

<sup>1</sup>The number of plants in the sample size column for interspecific crosses represents the number of pollen parents; the number of seed parents is the same as for the intraspecific crosses.