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REPRODUCTIVE ISOLATION BETWEEN TWO CLOSELY RELATED HUMMINGBIRD-POLLINATED NEOTROPICAL GINGERS

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Abstract.—Empirical estimates of the relative importance of different barriers to gene flow between recently diverged species are important for understanding processes of speciation. I investigated the factors contributing to reproductive isolation between *Costus pulverulentus* and *C. scaber* (Costaceae), two closely related hummingbird-pollinated understory Neotropical herbs. I studied broad-scale geographic isolation, microhabitat isolation, flowering phenology, overlap in pollinator assemblages, floral constancy by pollinators, mechanical floral isolation, pollen-pistil interactions, seed set in interspecific crosses, and postzygotic isolation (hybrid seed germination, greenhouse survival to flowering, and pollen fertility). Aside from substantial geographic isolation, I found evidence for several factors contributing to reproductive isolation in the sympatric portion of their geographic ranges, but the identity and relative strength of these factors varied depending on the direction of potential gene flow. For *C. pulverulentus* as the maternal parent, mechanical floral isolation was the most important factor, acting as a complete block to interspecific pollen deposition. For *C. scaber* as the maternal parent, microhabitat isolation, pollinator assemblage, mechanical floral isolation, and postpollination pollen-pistil incompatibility were important. Overall, prezygotic barriers were found to be strong, resulting in 100% reproductive isolation for *C. pulverulentus* as the maternal parent and 99.0% reproductive isolation for *C. scaber* as the maternal parent. Some postzygotic isolation also was identified in the F_1 generation, increasing total isolation for *C. scaber* to 99.4%. The results suggest that ecological factors, including habitat use and plant-pollinator interactions, contributed to speciation in this system and evolved before extensive intrinsic postzygotic isolation.

Key words.—Floral isolation, hummingbird pollination, plant speciation, pollen-pistil interactions, tropical biology.

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Biological diversity is a direct consequence of speciation, and the evolution of reproductive isolation is central to the speciation process. Understanding the types of barriers that contribute to reproductive isolation will help to elucidate the conditions under which speciation is likely to occur and the role of natural selection in speciation, and it can motivate studies of the genetic basis of speciation. Barriers can be classified as operating either before or after fertilization. Prezygotic barriers include ecological differences, mating discrimination, and postmating interactions between the male and female reproductive tracts (Mayr 1963; Grant 1981), whereas postzygotic barriers can be intrinsic, including low hybrid viability and fertility (Dobzhansky 1937; Muller 1942), or extrinsic, including hybrid ecological inferiority and low mating success (Rundle et al. 2000; Schluter 2000). Although reproductive isolation is generally thought to evolve as an incidental consequence of phenotypic and genotypic divergence in allopatry (Dobzhansky 1937; Mayr 1959), much of our knowledge of isolating mechanisms necessarily comes from cases in which the taxa currently are found in at least partial sympatry. It is in these cases that we are able to examine the barriers sufficient for the effective cessation of gene flow between related taxa.

For a good understanding of speciation processes, it is important to study reproductive isolation across the full range of isolating mechanisms and across a range of divergence, from differentiated populations to distinct species. Not all divergence among populations will lead to speciation, however, and deeply diverged species will have continued to accumulate ecological and morphological differences, potentially obscuring the relative contributions of various isolating mechanisms during species formation. Studying incipient or very closely related species can minimize these problems and may provide the most relevant data on the conditions necessary for speciation. Furthermore, while the evolutionary literature is replete with studies of isolating mechanisms, few have systematically explored the contribution of most or all potential mechanisms to total isolation for any pair of taxa (but see Chari and Wilson 2001; Husband and Sabara 2003; Ramsey et al. 2003). In nature, isolating mechanisms act sequentially, so that a given barrier can reduce only the potential gene flow not precluded by earlier acting barriers. Thus, studying a limited number of barriers may skew our view of the relative importance of various stages. Reproductive isolation may also evolve asymmetrically between taxa, with the identities and relative strengths of mechanisms differing between the directions of potential gene flow (Levin 1978; Arnold et al. 1996; Coyne and Orr 1998; Tiffin et al. 2001). To understand the traits responsible for reproductive isolation, it is therefore necessary to evaluate each direction separately, with each species acting as either the female or male parent in a potential hybridization event.

While thorough empirical studies of reproductive isolation are difficult, plants provide an excellent opportunity because they can be relatively easy to study in nature and to manipulate in a laboratory or greenhouse setting. Local adaptation and ecotypic differentiation are well known in plants (Clausen et al. 1940; Stebbins 1950), suggesting an important role for ecological isolation, both pre- and postzygotic. Intrinsic postzygotic isolation, in the form of hybrid inviability and sterility, has also been found in various plant groups (Clausen et al. 1945; Grant 1981). Plant-pollinator interactions, however, arguably have received the most attention as mecha-

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nisms of prezygotic reproductive isolation (Grant and Grant 1965; Hiesey et al. 1971; Stebbins 1974; Levin 1978; Grant 1981; Schemske and Bradshaw 1999; Kay and Schemske 2003). Still, their importance to speciation is controversial because specialization in plant-pollinator relations has been questioned (Ollerton 1996; Waser et al. 1996; Waser 1998). Different pollination syndromes have been shown to confer reproductive isolation among closely related sympatric species in several cases (Grant 1994b; Fulton and Hodges 1999; Schemske and Bradshaw 1999), but many plant speciation events do not involve a shift in pollination syndrome. The role of plant-pollinator interactions in reproductive isolation for species sharing pollinators is less understood (but see Grant 1994a,b; Husband and Sabara 2003).

There also may be a geographic bias in our understanding of plant speciation. Most evolutionary studies of plants to date have been conducted in the temperate zone, yet the tropics harbor most of the world's plant diversity. This bias could give a misleading picture of the existence or importance of various mechanisms of reproductive isolation. Range sizes, population sizes, habitat patchiness, the strength and variability of selective pressures, and dispersal and mating systems are all important for determining evolutionary trajectories. Despite a scarcity of empirical evidence, all of these parameters have been proposed to differ between tropical and temperate zones (Dobzhansky 1950; Fedorov 1966; Ashton 1969; Rapoport 1982; Stevens 1989; Givnish 1999; Schemske 2002). Furthermore, Neotropical forests specifically have been suggested to be a hotspot of plant speciation, yet very little is known about the nature of reproductive isolation for the many species radiations that have been identified (but see Stiles 1975; Kress 1983; McDade 1984).

Here I examine mechanisms of reproductive isolation for a pair of closely related pollinator-sharing Neotropical rainforest herbs. I quantify the contributions of numerous potential isolating mechanisms, including broad-scale geography, microhabitat differences, flowering phenology, overlap in pollinator assemblage, floral constancy, mechanical floral isolation, pollen-pistil interactions, interspecific seed set, and the seed germination, greenhouse survival, and pollen fertility of F_1 hybrids. Following Coyne and Orr (1989, 1997) and Ramsey et al. (2003), I combine estimates from each stage to calculate total isolation and the relative contribution of each component.

Materials and Methods

Costus pulverulentus and *C. scaber* (Costaceae) are large understory monocot herbs that grow in the rainforests of Central and South America. They are typically found along streams and in small forest gaps. Like many tropical plants, they grow as isolated individuals within a highly diverse matrix of other plant species, often with large distances (>50 m) between conspecific individuals. Phylogenetic evidence from the rDNA ITS and ETS regions suggests that they are putative sister species, very closely related, and part of a larger Neotropical species radiation of *Costus* subgenus *Costus* characterized by rapid and recent diversification (Kay 2004; Kay et al. 2005). They are sympatric throughout much of Central and northwestern South America, but the range of *C. pulverulentus* extends further north into Mexico and Cuba and the range of *C. scaber* extends further south and east into Amazonian Brazil and Bolivia and coastal Brazil.

The species are similar in vegetative appearance but can be distinguished in the field by floral characters. Both have unbranched spiraling stems that grow to a height of approximately 1–2 m with long elliptical leaves arranged in a spiral around the stem. They are capable of clonal growth either by underground rhizomes or by the rooting of fallen stems. Both species have bright red floral bracts and tubular red flowers, although the flowers of *C. pulverulentus* are longer (~5 cm vs. 3 cm), more open, and less sharply decurved than the flowers of *C. scaber* (Maas 1972). The flowers of *C. pulverulentus* also have exserted anthers and stigma, whereas those of *C. scaber* are inserted. *Costus pulverulentus* and *C. scaber* both exhibit traits typical of a hummingbird pollination syndrome, and this is reflected in their floral visitors. At sites in Costa Rica and Panama, both are primarily visited by the long-tailed hermit hummingbird, *Phaethornis superciliosus*. In an allopatric part of its geographic range, outside the range of *P. superciliosus*, *C. scaber* is visited by other hummingbirds in the genus *Phaethornis* (Kay and Schemske 2003).

All components of reproductive isolation except geographic isolation were studied within the region of sympatry, at one or more of the following lowland sites: La Selva and Sirena Biological stations in Costa Rica and Barro Colorado Island in Panama. La Selva (10°25′N, 84°00′W) is a 1536-ha reserve in the Atlantic lowlands of Heredia Province, Costa Rica, that shares a boundary with the extensive Braulio Carillo National Park. Sirena (8°29′N, 83°35′W) is located along the Pacific Coast of Costa Rica in the expansive Area Conservación de Osa. Both La Selva and Sirena consist of mature, tropical wet forest and regenerating agricultural lands (McDade and Hartshorn 1994). Barro Colorado Island (BCI; 9°09′N, 79°51′W) is a 16-km^2 island located in Gatun Lake in the Panama Canal that consists of mature, tropical moist forest (Croat 1978).

For each stage-specific mechanism that I examined, I first determined whether there was a statistically significant effect on potential interspecific gene flow. If so, I then quantified the effect of that mechanism, using stage-specific indices of reproductive isolation (*RI*, with a subscript indicating the stage; Table 1). These indices are constructed generally to vary from zero to one, with zero representing no barrier to interspecific gene flow and one representing a complete barrier. Negative values could occur for stages having a positive impact on interspecific (relative to intraspecific) gene flow, such as higher hybrid fitness. *RI* indices at each stage were estimated separately for both directions of potential gene flow. For stages that I was able to quantify for plants from more that one geographic location, I averaged any significant *RI* among sites, always noting first any differences.

Geographic Isolation

Following Ramsey et al. (2003), I examined both elevational overlap and two-dimensional geographic coexistence. I gathered data on *C. pulverulentus* and *C. scaber* from herbarium specimens in the Missouri Botanical Garden's online database

TABLE 1. Equations used to quantify components of reproductive isolation. Details of how the variables were constructed are given in the text.

Barrier	Equation for calculating *RI*
Prezygotic	
Geographic	1 − [no. heterospecific quadrats/(no. heterospecific quadrats + no. conspecific quadrats)]
Microhabitat	1 − [no. heterospecific quadrats/(no. heterospecific quadrats + no. conspecific quadrats)]
Pollinator species assemblage	1 − proportion of the visitation rate composed of shared pollinator species
Floral mechanical	1 − (mean index of interspecific dye deposition/mean index of intraspecific dye deposition)
Postpollination	1 − (no. seeds per interspecific pollination/no. seeds per intraspecific pollination)
Postzygotic	
Viability	1 − (viability of F_1 hybrids/viability of parentals)
Fertility	1 − (fertility of F_1 hybrids/fertility of parentals)

(http://mobot.mobot.org/W3T/Search/vast.html) and at Chicago's Field Museum. My confidence in the species determinations for these specimens was high, since most were made by P. J. M. Maas, the taxonomic expert for the group. I also visually inspected the Field Museum specimens. Only one specimen from a particular site was analyzed. Elevation was recorded from 363 specimens of *C. pulverulentus* and 360 specimens of *C. scaber*. Latitude and longitude were recorded from 362 specimens of *C. pulverulentus* and 324 specimens of *C. scaber* and transformed into *xy* coordinates. Differences in elevation were tested with a nonparametric Mann-Whitney *U*-test. Broad-scale spatial isolation was determined by randomly placing a series of virtual quadrats across the combined geographic ranges of the species and examining the co-occurrence of the species within the quadrats. Because the plants generally grow at very low density and little is known about their dispersal, the amount of geographic isolation, or allopatry, is difficult to quantify. This method of randomly placing quadrats allowed me to sample from a distribution of possible dispersal areas. I explored a variety of quadrat sizes, from 10 × 10 km to 100 × 100 km, placing 100,000 quadrats per size category. For each species, I compared the number of quadrats in which the two species co-occurred (heterospecific quadrats) to the number of quadrats in which there were at least two collection sites of that particular species but none of the other species (conspecific quadrats). This analysis is sensitive to the size of the quadrat, that is, with a small enough quadrat, each individual plant will be completely isolated from all others, while with a large enough quadrat, the species will always co-occur. Therefore, I used the distances between specimens in my dataset to judge the appropriate quadrat size for the calculation of geographic isolation. Specifically, I chose the minimum size for which quadrats containing singletons were less common than quadrats containing two or more specimens. The index of geographic isolation ($RI_{geographic}$; Table 1) varies from zero for complete sympatry to one for complete allopatry and, in contrast to most of the other indices, can never be negative, since greater than complete sympatry is illogical. I used a delete-*d* jackknife resampling method to construct 95% confidence intervals for a given quadrat size, with 1000 replicate analyses and *d* set at one-fifth. This simultaneously resamples the specimen locations and the location of quadrats and, in contrast to bootstrapping, does not introduce repeated datapoints that would affect counts of co-occurrence. (The C++ source code for these analyses is available from the author upon request.)

Microhabitat Isolation in Sympatry

I examined the fine-scale spatial isolation in sympatry that may be caused by microhabitat differences by mapping the distribution of each species at La Selva. During the flowering seasons of 1999–2001, I hiked all the trails and most of the streams in the older part of the reserve with more mature forest and noted all individuals visible with binoculars (approximately 25 m on either side of my path). I recorded the precise location of all individuals using the permanent gridposts, and mapped them using ArcInfo (ESRI, Redlands, CA) on the station's geographical information system. Only flowering or fruiting individuals were mapped, as vegetative individuals cannot be assigned unambiguously to species. Stems occurring within 5 m of each other were considered part of the same individual, since *Costus* is able to grow clonally from rhizomes or fallen stems. Overall, I found and mapped 44 individuals of *C. pulverulentus* and 90 of *C. scaber*. For the contribution of spatial distribution to reproductive isolation, the parameter of interest is the opportunity for interspecific mating relative to intraspecific mating. Randomly placed virtual quadrats were used to determine the extent of small-scale spatial isolation similarly to ecogeographic isolation, except in this case the datapoints were individuals and not herbarium specimen collection sites. To accommodate the large interplant distances, I used 100 m on a side as the minimum quadrat size and increased it by intervals of 100 m up to 500 m on a side, and I randomly placed 10,000 quadrats each per size category.

Phenological Isolation

When in flower, individuals of *C. pulverulentus* and *C. scaber* typically produce a single inflorescence at a time, each opening approximately one flower per day over an extended period. Each flower opens at dawn and drops off or wilts by midafternoon. These species are known to flower in the wet season at La Selva, with peak flowering occurring May through August (Stiles 1978b). To quantify the overlap in flowering phenology, I censused plants at La Selva and BCI during the wet season of 1999 and at La Selva in 2001. Because of the highly dispersed distribution of plants and the long flowering season, it was impractical to census the num-

ber of flowering individuals throughout the reserves on a regular basis. Instead, I estimated the flowering span of individual inflorescences using the sequential order of flowering that occurs along the inflorescence. Early in the wet season, I located as many developing or mature inflorescences as possible, and for those in flower, I marked the bract subtending the current day's flower. Marked plants were revisited approximately two weeks later, and the bracts between the current day's flower and the marked bract were counted to estimate a plant specific rate of flower production. The total number of bracts on the inflorescence below and above the mark was used to estimate the start and end dates of flowering for that plant. Later in the wet season, I used the same technique on plants not flowering during the first census. Inflorescences damaged by falling branches and debris were dropped from the study. For each species-site-year combination, the proportion of individuals flowering was plotted across time. To estimate the consistency of flowering time between species for a given site and year, the Julian dates representing the midpoints of each individual's flowering duration were tested with Wilcoxon rank sum tests.

Floral Isolation

Premating floral isolation

Pollinator assemblages.—To quantify isolation due to differences in pollinator species, I calculated the proportion of the total visitation rate (visits per flower per hour) to each plant species contributed by shared pollinator species. Kay and Schemske (2003) found that *C. pulverulentus* and *C. scaber* share their primary pollinator, the long-tailed hermit hummingbird (*Phaethornis superciliosus*), at La Selva, Sirena, and BCI. For that study, observations were made in 1998–2000 at La Selva, 1998–1999 at BCI, and 2002 at Sirena, for a total of 511 h for *C. pulverulentus* and 519 h for *C. scaber*. Across all site-year combinations of observations reported in Kay and Schemske (2003), 48 individuals of *C. pulverulentus* and 44 individuals of *C. scaber* were observed, and visitation rates were generally low (less than one visit per flower per hour). Plants without any observed legitimate pollinator visits ($N = 10$) were excluded from the calculations. *Costus pulverulentus* was exclusively pollinated by *P. superciliosus*, while *C. scaber* also was visited by the hummingbirds *Amazilia tzacatl* and *Thalurania columbica* and rarely by orchid bees (*Euglossa* sp.). Most of the variation in visiting species was among individual plants and not among sites or years. Therefore, I quantified isolation due to differences in pollinator species using individual plant as the unit of replication. This index varies from zero for complete overlap in pollinator assemblage to one for no overlap ($RI_{pollinator}$; Table 1). The confidence interval was constructed by bootstrapping the mean 1000 times.

Floral constancy.—For shared pollinator species, I attempted to determine whether floral constant behavior by individual birds reduces the opportunity for pollen flow. I observed the behavior of *P. superciliosus* at natural mixed patches and followed marked individuals at isolated plants to ascertain whether individual pollinators preferentially visited one species over the other. I found three natural sites at La Selva and one at Sirena in which individuals of both species were simultaneously visible with binoculars. These natural sites contained limited numbers of flowers, often spaced more than 10 m apart; thus, they do not represent typical choice tests but are examples of natural foraging routes. To determine whether birds travel between isolated individuals of the two plant species, *P. superciliosus* at La Selva were captured using mistnets in 2000 and given individually recognizable colored paint markings according to the protocol of Stiles and Wolf (1973). Over three weeks of netting, 42 individuals were marked and released, and video cameras set up at plants were used to observe flower visitation.

Mechanical isolation.—Reductions in pollen flow due to differences in flower shape and size were estimated by allowing pollinators to visit experimental arrays of *C. pulverulentus* and *C. scaber* and then following pollen movement. At La Selva in 2000 and 2001, I grew plants of each species in the shadehouse and placed them in mixed arrays in the primary or mature secondary forest. This allowed me to better control the relative numbers and the spatial arrangement of flowers than would have been possible using the natural distribution of plants. Arrays typically consisted of four to eight plants, split evenly between the species and assigned at random to positions within the array. I rotated the arrays through a total of seven different sites to expose the plants to a diversity of hummingbird individuals. Pollen is not unambiguously distinguishable between *Costus* species, so I coated the dehiscent anthers with colored powder to track pollen movement. Before I used any dye on the anthers, I left the plants out for two to three days at a site to allow the hummingbirds to discover and become accustomed to them. During this time, arrays were videotaped to ensure that hummingbirds were present and that they were not showing any obvious constancy or preference for one species. Flowers at each array were marked with unique randomly assigned colors at dawn, and the stigmas were examined in the mid to late afternoon. If there was no evidence that any of the flowers in an array had been visited (i.e., no pollen deposition or removal), that site/date combination was dropped from all further analysis. For arrays that had been visited, I constructed an index of dye deposition, calculated as $(P \times C)/N$, where P is the proportion of the stigma covered in either intra- or interspecific dye, C is the concentration of that dye on a qualitative scale from 1 to 3, and N is the number of marked flowers in the array that could have contributed that dye. This allowed me to examine for each pollen donor the amount of intraspecific (both outcrossed and self) and interspecific dye that a particular stigma received. Intraspecific and interspecific dye deposition indices per stigma were compared with a Wilcoxon paired-sample test. In the absence of floral constancy, the relative value of these deposition indices were used to quantify mechanical floral isolation ($RI_{floralmech}$; Table 1). The confidence interval for the measure of $RI_{floralmech}$ was constructed by bootstrapping the mean, calculated per stigma, 1000 times.

I also attempted to track pollen flow among the naturally occurring plants at Sirena in the wet season of 2002. Over the course of four days, I marked the anthers of as many flowers as I could find of both species in the morning, using one color per species, and I checked the stigmas of these

same plants in the afternoon. Because of the large distances between plants, simultaneous marking was impossible and the number of possible dye donors varied throughout the day. Therefore, I did not use the above index of dye deposition, but simply scored each stigma for presence or absence of each color. In total, I marked 38 flowers of *C. pulverulentus* and 35 of *C. scaber*.

Postpollination isolation

Plants of both species were collected as seeds or cuttings from La Selva and BCI in 1997 and 2000 and brought back to the greenhouse, where they were grown to flowering for crossing studies. Sample sizes were as follows: 15 individuals from across six different maternal families for La Selva *C. pulverulentus*, nine individuals from six families for La Selva *C. scaber*, 15 individuals from four families for BCI *C. pulverulentus*, and nine individuals from seven families for BCI *C. scaber*. For the plants from each site, I compared the success of interspecific crosses to intraspecific crosses. The plants flowered sporadically, so it was not possible to follow an established crossing design. However, from 1999 to 2003 all possible interspecific and intraspecific combinations of families were attempted multiple times for the plants from each site. To control for any problems with plant health, I conducted intraspecific crosses on all inflorescences used for interspecific crosses. If the intraspecific crosses failed to set seed, data from that inflorescence were dropped from the study.

I determined postpollination isolation by quantifying seed set per pollination; to determine whether any differences in seed set were pre- or postzygotic, I further examined pollen germination and pollen tube growth with epifluorescent microscopy for the La Selva populations. Flowers were pollinated and either left to set seed or harvested after 2 h (for pollen adhesion and germination) or after 9 h (for pollen tube growth). In the field, flowers typically open just before dawn and drop off in the mid to late afternoon, so 9 h represents the maximum time for pollen tubes to grow to the base of the style. All crosses were done in the morning, from 0600 to 1000 h, to mimic the peak time of natural pollinator visitation. Crosses harvested for pollen germination and tube growth were not used to assess seed set. The number of pollen grains applied was standardized for each maternal species by completely saturating the stigmas with pollen far in excess of the number of ovules. I estimate that a typical pollination involved a minimum of several hundred pollen grains. Pollen was removed and applied with flat wooden toothpicks and carried between plants in microcentrifuge tubes. To control for any unintended pollen deposition, null pollinations were performed on several flowers per population with a clean toothpick. *Costus pulverulentus* from BCI are known to set some seed through autogamy; therefore, when these plants were pollen recipients, they were emasculated prior to anthesis. Harvested pistils were fixed in a solution of 3 parts 95% ethanol to 1 part glacial acetic acid for 24 h, gently rinsed in distilled water, softened and cleared in 4 M NaOH for 24 h, gently rinsed in distilled water, and stained in decolorized aniline blue (0.01% in 0.02 M K_3PO_4) for 24 h, following a modified procedure of Martin (1959) and Goodwillie (1997). Pistils were mounted in a drop of stain, gently squashed with a cover slip, and viewed with an epifluorescent microscope using UV transmission filters for the illuminator and UV absorption filters in the ocular tubes. Fluorescence of the pollen grains and tubes was clearly distinguishable from the stigma and stylar tissue, and *Costus* pollen grains are large enough (approximately 100 μm in diameter) to count individually. For pollen adhesion, I used flowers harvested after 2 h and counted as adhered any pollen grains remaining on the stigma after the multiple rinsings and squashing during the microscopy preparations. For pollen germination, I counted the numbers of germinated and ungerminated pollen grains on the stigma after 2 h. For pollen tube growth, I measured the length of the longest pollen tube and the number of pollen tubes reaching the ovary after 9 h.

Seed set for reciprocal crosses was examined separately for La Selva and BCI using two-way ANOVA with restricted maximum-likelihood model fitting. The effects were as follows: maternal species, maternal plant as a random factor nested within maternal species, paternal species, paternal plant as a random factor nested within paternal species, and the maternal species × paternal species interaction. The interaction term indicates incompatibility between the species (Husband et al. 2002). Pollen germination and tube growth measures were compared between intra- and interspecific crosses with Mann-Whitney *U*-tests for each maternal species for the La Selva populations.

For the calculation of reproductive isolation due to postpollination crossing barriers ($RI_{postpollination}$; Table 1) results were averaged between the La Selva and BCI plants. Confidence intervals were constructed by first bootstrapping mean intra- and interspecific seed set for La Selva and BCI separately 1000 times each, then randomly drawing a bootstrap mean from each site and cross-type category, calculating $RI_{postpollination}$ for each site and then averaging between sites. This resampling procedure was replicated 1000 times, and 95% of the range was taken as the confidence interval.

Postzygotic Isolation

Hybrid viability: seed germination and survival to flowering

I attempted to germinate all the hybrid seeds and a portion of the intraspecific seeds from the above crosses as they ripened in the greenhouse, to quantify relative fitness of hybrids. For this experiment, additional hybrid seeds were created by transferring stigmatic exudates from *C. pulverulentus* to *C. scaber* stigmas and then pollinating with *C. pulverulentus* pollen, a method that increases interspecific seed set more than fourfold (Kay 2004). For La Selva, only hybrids with *C. scaber* as a maternal parent could be produced, while for BCI, I sampled hybrids made in both directions. F_1 hybrids with *C. pulverulentus* and *C. scaber* as maternal parent are denoted hereafter H(P) and H(S), respectively. Intraspecific fruits were chosen for germination so that each maternal family contributed several fruits, except that none of the intraspecific fruits from *C. scaber* for BCI were germinated. Seeds were sown into soil in bottom-watered plug trays in an incubator set on a 12-h light-dark cycle, with the temperature kept between 24°C and 27°C. All the seeds from a particular fruit were sown at the same time; therefore, I cal-

culated a germination rate per fruit and compared germination rates among cross types for La Selva and BCI separately with Kruskal-Wallis tests. Once germinated, seeds were transferred to pots in the greenhouse, and their survival to flowering was monitored.

For the calculation of postzygotic isolation caused by differences in seed germination and survival to flowering ($RI_{viability}$; Table 1), results were averaged between data from the La Selva and BCI plants, and confidence intervals were constructed in the same way as they were for $RI_{postpollination}$.

Hybrid male fertility

Percent pollen stainability, a common measure of pollen viability, was used as a proxy for male fertility, according to the methods of Kearns and Inouye (1993). Pollen samples were taken in the greenhouse from F_1 hybrids and outcrossed lines of both *C. pulverulentus* and *C. scaber* from both the La Selva and BCI populations. Also, for BCI, no outcrossed lines of *C. scaber* were made, so pollen stainability was measured on the wild-collected plants that were grown to flowering in the greenhouse. Two to four flowers were sampled per plant from 10–20 individuals per cross type. I sampled fresh pollen from flowers in the morning and immediately placed it in a microcentrifuge tube with several drops of 2% lactophenol aniline blue. The tubes were mixed thoroughly and allowed to sit for at least 3 h, after which I placed approximately 50 μl of the solution on a microscope slide with a cover slip on top and sealed the edges of the coverslip with a heated mixture of paraffin and petroleum jelly. Slides were then laid flat for an additional 2–3 h to enhance staining. Slides were placed on top of graph paper on a dissecting microscope, and pollen grains counted grid by grid in a predetermined pattern for a minimum of 200 grains. The frequency of dark, fully-stained grains was estimated and compared among cross types for La Selva with a Kruskal-Wallis test. Since I had both types of hybrids from BCI, I compared H(P) with *C. pulverulentus* and H(S) with *C. scaber* separately with Mann-Whitney *U*-tests. For the calculation of postzygotic isolation due to male fertility ($RI_{fertility}$; Table 1), results were averaged between data from the La Selva and BCI plants, and confidence intervals were constructed in the same way as they were for $RI_{postpollination}$.

Total Reproductive Isolation

I estimate total reproductive isolation (*T*) between *C. pulverulentus* and *C. scaber* following the methods of Coyne and Orr (1989, 1997) and Ramsey et al. (2003), as the product of individual isolating mechanisms that act sequentially to prevent gene flow. The strength of reproductive isolation for each mechanism is estimated independently (*RI*), and the absolute contribution of that mechanism (*AC*) is the proportional reduction in gene flow that has not been eliminated by previous stages of reproductive isolation. To make comparisons across isolating mechanisms, the relative contribution (*RC*) of each component is further estimated as the *AC* of that component divided by *T*. Confidence intervals were constructed by resampling the distributions of means for each stage of *RI*. A mean was drawn at random for each stage and used in a calculation of total isolation and the relative contributions of each stage. This resampling was performed 1000 times to generate a distribution of total isolation and relative contributions for each stage.

RESULTS

Geographic Isolation

Both species are common in lowland forest and do not differ significantly in elevation (*C. pulverulentus*: mean = 435 m, range = 0–2860 m; *C. scaber*: mean = 385 m, range = 0–1500 m; Mann-Whitney *U*-test, $P = 0.31$). Although their elevational ranges do not completely overlap, only six of the 363 specimens of *C. pulverulentus* were found outside the elevational range of *C. scaber*. Therefore, elevational differences are not considered a component of reproductive isolation.

The computer simulations of virtual quadrats using the herbarium specimen collection localities showed that the relative frequency of heterospecific to conspecific quadrats increased with quadrat size, as expected. Across the five quadrat sizes examined (10, 20, 60, 80, and 100 km on a side), the mean $RI_{geography}$ varied from 0.686 to 0.298 for *C. pulverulentus* and from 0.560 to 0.468 for *C. scaber*. For the overall calculation of reproductive isolation, I used the results from 80 × 80 km quadrats, because that is the smallest size for which quadrats containing two or more collection sites outnumbered singletons. For this quadrat size, mean $RI_{geography}$ was 0.348 (95% CI: 0.322–0.417) for *C. pulverulentus* and 0.478 (95% CI: 0.437–0.526) for *C. scaber*.

Microhabitat Isolation

My search for individuals of both species was not systematic in sampling the entire forest at La Selva, but it suggests that *C. scaber* is more abundant than *C. pulverulentus*, although both species exhibit a very dispersed distribution. For individuals of *C. pulverulentus*, the mean distance to the nearest conspecific neighbor was 70 m (median = 42 m), and for *C. scaber* the mean was 54 m (median = 36 m). The relative frequency of heterospecific quadrats increases with the size of the quadrat, as expected, and for both species, mean $RI_{habitat}$ decreased as the quadrat size increased, for *C. pulverulentus* ranging from 0.446 to 0.129 (mean = 0.300) and for *C. scaber* ranging from 0.720 to 0.438 (mean = 0.597). Regardless of quadrat size, $RI_{habitat}$ was always higher for *C. scaber* compared to *C. pulverulentus*, a result consistent with *C. scaber*'s higher abundance and shorter interplant distance. The appropriate quadrat size for the overall calculation of reproductive isolation depends on the foraging patterns of shared pollinators and the amount of pollen carryover between flower visits. I chose 500 × 500 m to use in the overall calculation, because individuals of *P. superciliosus* are known to travel long distances on their foraging flights and in their intensive *P. superciliosus* marking studies at La Selva, Stiles and Wolf (1979) found that more than half of the birds marked at a particular site were observed more than 500 m away. Therefore, $RI_{habitat}$ was set at 0.129 (95% CI: 0.101–0.180) for *C. pulverulentus* and 0.438 (95% CI: 0.396–0.538) for *C. scaber*.

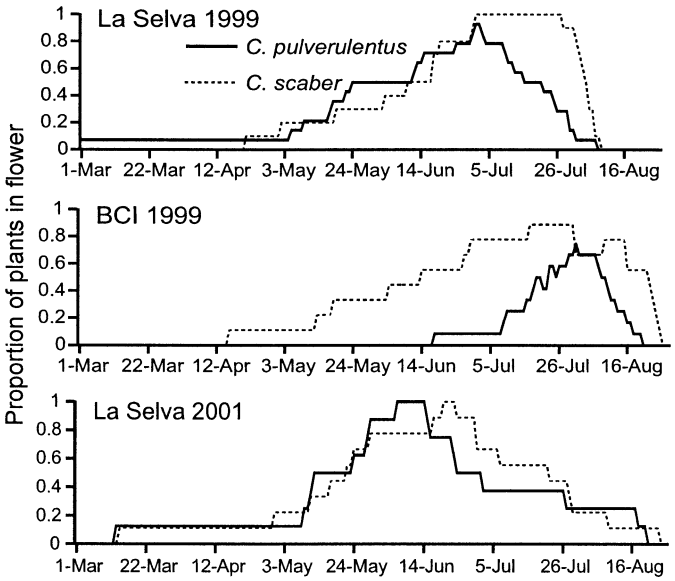

FIG. 1. The proportion of plants of *Costus* each species in flower plotted across time, for 1999 and 2001 at La Selva and for 1999 at BCI.

Phenological Isolation

The flowering phenology of the two species was highly overlapping and in all cases peaked between May and August (Fig. 1). At La Selva in 1999, *C. pulverulentus* (N = 14 individuals) had a mean start date of May 30 and a mean end date of July 18, while *C. scaber* (N = 10) had a mean start date of June 5 and a mean end date of August 3. The midpoints of individual flowering times for each species were not significantly different (Wilcoxon rank sum: $Z = 1.41$, $P = 0.16$). At BCI in 1999, flowering peaked slightly later. *Costus pulverulentus* (N = 12) had mean start and end dates of July 18 and August 6, while *C. scaber* (N = 9) had a longer season with mean start and end dates of June 12 and August 16, and the midpoints of individual flowering times did not differ (Wilcoxon rank sum: $Z = -1.64$, $P = 0.10$). In 2001, flowering at La Selva was highly consistent between species and peaked slightly earlier than in 1999. Mean start dates were May 13 and May 17 and end dates were July 10 and July 25 for *C. pulverulentus* (N = 8) and *C. scaber* (N = 9), respectively, and the midpoints of individual flowering times did not differ (Wilcoxon rank sum: $Z = -0.82$, $P = 0.41$). Because of the high overlap in flowering phenology across sites and years, this factor is unlikely to contribute to reproductive isolation and was not used in the overall calculations of *RI*.

Floral Isolation

Premating isolation

Pollinator assemblages and floral constancy.—For *C. pulverulentus*, *P. superciliosus* was the only pollinator observed, while for *C. scaber*, *P. superciliosus* comprised an average of 0.743 of the total visitation rate across all individual plants observed at La Selva, BCI, and Sirena. For *C. pulverulentus*, mean $RI_{pollinator}$ was zero, while for *C. scaber* it was 0.257 (95% CI: 0.146–0.379).

No floral constancy by *P. superciliosus* was found at the natural mixed patches. At two of the La Selva mixed patches, there were no floral visitors in 4.0 and 5.5 h of observation, respectively. At the remaining patch, with one to six flowers per species, there were nine *P. superciliosus* foraging bouts in 11 h of observation spread over four days. During each bout, the bird visited each of the flowers exactly once, except for one bout where the only two *C. pulverulentus* flowers open that day were unvisited. At Sirena in 2002, there was a large patch with 11 *C. scaber* and seven *C. pulverulentus* flowers visible. In 4 h of observation, one *P. superciliosus* visited six *C. scaber* and five *C. pulverulentus* flowers during a single foraging bout. Sample sizes were too low within bouts to test whether observed visitation frequency was different than expected based on relative abundance, but over 10 total bouts, nine involved visits to both species. The order of flower visitation for these foraging bouts was not analyzed because it could reflect the nonrandom spatial distribution of plants instead of floral preference. Of the color-marked *P. superciliosus* at La Selva, eight were distinguishable on videotape visiting *Costus* flowers for a total of 40 separate marked visits. All eight birds were seen at *C. scaber* (N = 35 visits, 360 h of observation, 21 individual plants), while three of these were also seen at *C. pulverulentus* (N = 5 visits, 260 h of observation, 19 individual plants). From all of these sources, I conclude that there is no evidence of pollinator constancy.

Mechanical isolation.—At the mixed species arrays at La Selva in 2000 and 2001, no transfer of dye occurred from the anthers of *C. scaber* to *C. pulverulentus*, while there was substantial transfer from *C. pulverulentus* to *C. scaber* (Fig. 2). At two array sites, there was no evidence for any pollinator visitation. At the other five, there were 21 array-date combinations (array-days) with pollinator visitation in 2000 and 19 in 2001. Across these 40 array-days, I examined a total of 52 *C. pulverulentus* and 44 *C. scaber* stigmas in 2000 and 42 *C. pulverulentus* and 40 *C. scaber* stigmas in 2001. Although there was interspecific dye transfer from *C. pulverulentus* to *C. scaber*, when paired by stigma it was significantly less than intraspecific dye transfer, regardless of whether self pollen was included (Wilcoxon paired sample tests: $P < 0.01$). Self dye deposition by the hummingbirds was substantial and the species are self-compatible, but the contribution of selfed progeny to fitness may be limited by considerable inbreeding depression, assuming *C. pulverulentus* and *C. scaber* are similar to other Neotropical *Costus* species (Schemske 1983). Furthermore, self-pollination similarly increases both intraspecific and interspecific isolation and should therefore not effect net reproductive isolation (Coyne and Orr 2004, p. 212). For these reasons and to make a conservative estimate of the contribution of mechanical isolation to total *RI*, self dye transfer was excluded from analysis of intraspecific dye transfer.

At Sirena in 2002, results from the flower marking of naturally distributed plants were qualitatively similar to results from La Selva. Of the 38 marked flowers of *C. pulverulentus*, 19 had no dye deposited on the stigma and 19 had intraspecific dye. Of the 35 marked *C. scaber* flowers, 13 had no dye

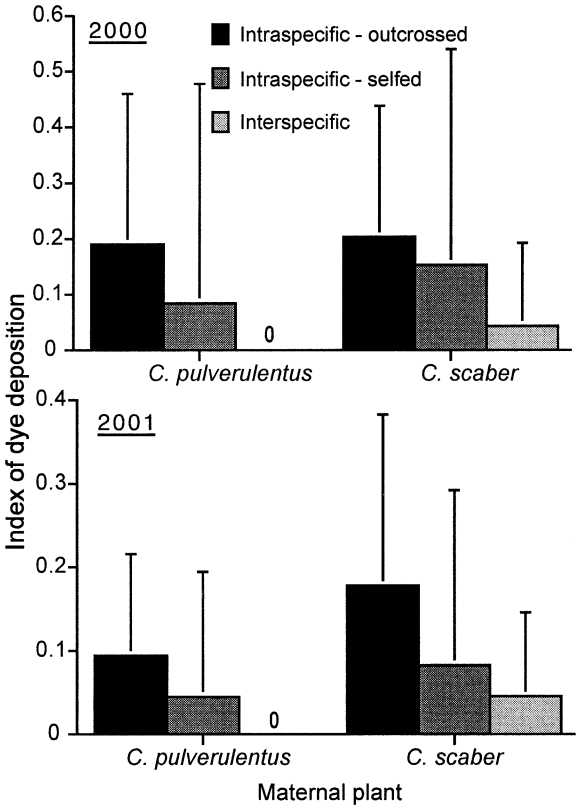

FIG. 2. Mean intra- and interspecific dye deposition on stigmas of *Costus pulverulentus* and *C. scaber* in experimental arrays at La Selva in 2000 and 2001. The index of dye deposition was calculated as $(P \times C)/N$, where P is the proportion of the stigma covered in intra- or interspecific dye, C is the concentration of that dye on a qualitative scale from 1 to 3, and N is the number of marked flowers in the array that could have contributed that dye. Error bars represent 2 standard errors.

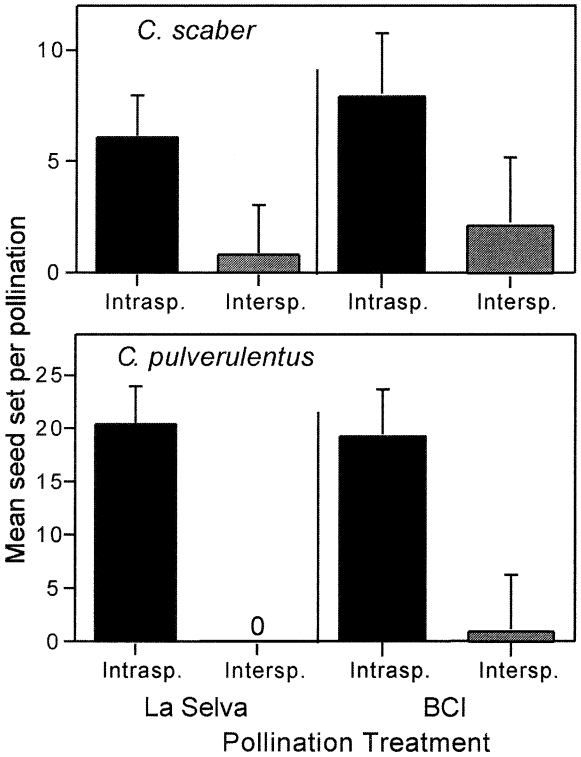

FIG. 3. Mean seed set per pollination for intra- and interspecific pollination treatments on plants of each species from La Selva and BCI. Error bars represent 2 standard errors. ANOVA results are summarized in Table 2.

deposited, 19 had intraspecific dye only, and three had interspecific dye only. Thus, similar to the experiments at La Selva, there was evidence of interspecific pollen transfer from *C. pulverulentus* to *C. scaber* but not in the other direction. However, because the results from naturally occurring plants at Sirena confound the effects of spatial distribution and mechanical floral isolation, only the results from the experimental arrays at La Selva were used in the quantitative calculation of reproductive isolation caused by mechanical floral isolation. Because of the consistency of results between years at La Selva, I combined the data from 2000 and 2001. For *C. pulverulentus*, with no interspecific pollen deposition, $RI_{floralmech}$ was calculated as a complete barrier of 1.00. For *C. scaber*, the mean intraspecific dye deposition index was 0.118 and the mean interspecific index was 0.045, resulting in a value for $RI_{floralmech}$ of 0.769 (95% CI: 0.708–0.884).

Postpollination Isolation

Seed set

For both species from both La Selva and BCI, seed set per pollination was lower in interspecific crosses compared to intraspecific crosses (0.79 vs. 6.076 seeds in *C. scaber* from La Selva; 2.13 vs. 7.96 seeds in *C. scaber* from BCI; 0 vs. 20.4 seeds in *C. pulverulentus* from La Selva; 0.94 vs. 19.20 seeds in *C. pulverulentus* from BCI; Fig. 3). ANOVA results are summarized in Table 2. For plants from both sites, there

TABLE 2. Summary of ANOVA results for seed set in reciprocal crosses, reported separately for La Selva and BCI populations. The significance of each random effect (denoted [R]) was judged by the 95% confidence interval of the variance component. All nested random effects had variance ratios of less than one, and therefore the MSE was used as the denominator in F-tests for the fixed effects.

	La Selva			BCI		
Source of variation in seed number	df	df_D	F	df	df_D	F
Maternal sp.	1	9	15.1**	1	6	4.5
Maternal plant (maternal sp.) [R]	21	206	*	6	161	ns
Paternal sp.	1	10	12.1**	1	9	6.1*
Paternal plant (paternal sp.) [R]	22	206	*	9	161	*
Maternal sp. × paternal sp.	1	206	56.4***	1	161	33.7***

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

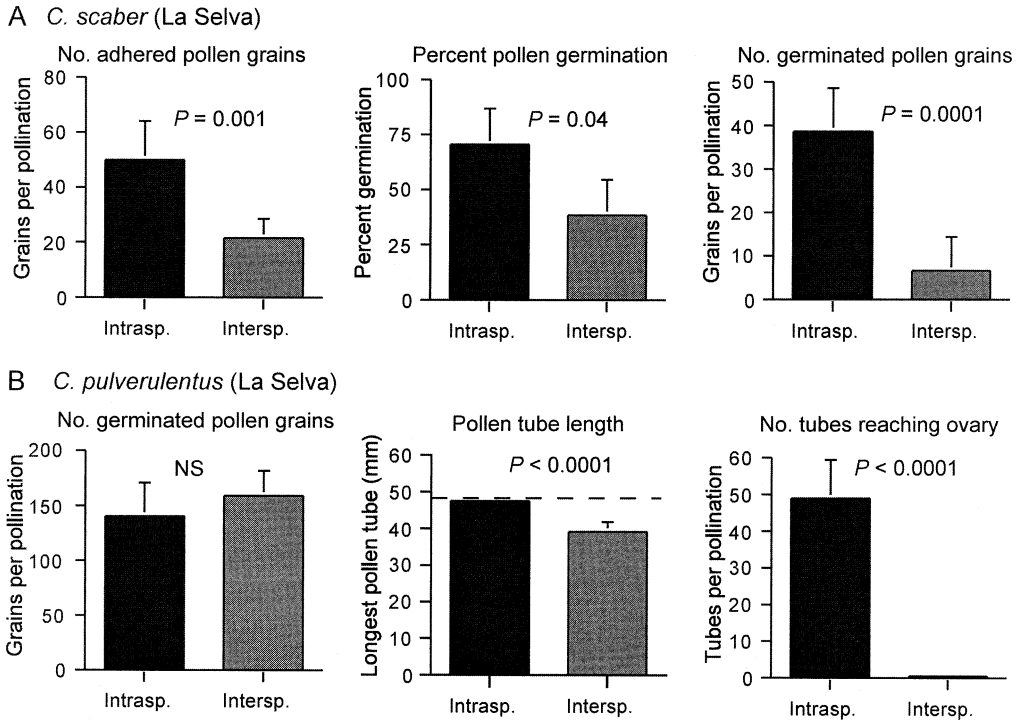

FIG. 4. Measures of postpollination prezygotic isolation for *Costus scaber* (A) and *C. pulverulentus* (B) from La Selva as pollen recipients. All columns represent means, and error bars are +2 standard errors. For *C. scaber*, differences in pollen adhesion and percent germination combine to give an overall difference in the number of germinated pollen grains per pollination. For *C. pulverulentus*, differences in the final length of the pollen tubes contribute to a large difference in the number of pollen tubes reaching the ovary. In the graph showing the length of the longest pollen tube for *C. pulverulentus* as pollen recipient, the dashed line represents the average style length of *C. pulverulentus*.

was a significant maternal species × paternal species interaction term, indicating reciprocal incompatibility in seed set. None of the null pollinations set any seed, indicating that unintended selfing or pollen transfer in the greenhouse did not occur.

Pollen germination and tube growth

Examination of pollen germination and tube growth for the La Selva populations showed that lower interspecific seed set is the result of prezygotic isolation. For pollinations on *C. scaber*, the number of germinated pollen grains after 2 h was significantly lower in interspecific pollinations compared to intraspecific pollinations (6.6 vs. 38.6 mean pollen grains, $N = 23$ inter- and 14 intraspecific pollinations, Mann-Whitney U-test: $P = 0.0001$; Fig. 4A). This was a product of significant differences in both the number of pollen grains that adhered to the stigma (21.5 vs. 49.9 mean pollen grains, Mann-Whitney U-test: $P = 0.001$) and the percentage of those grains that germinated (38.3 vs. 70.5 mean percentage germination, Mann-Whitney U-test: $P = 0.04$). The decrease from 38.6 to 6.6 mean germinated pollen grains per pollination was sufficient to explain the decrease in interspecific compared to intraspecific seed set. For pollinations on *C. pulverulentus*, there was no difference in pollen germination between intra- and interspecific crosses (140.5 vs. 156.9 grains, $N = 15$ intra- and 40 interspecific pollinations, Mann-Whitney U-test: $P = 0.28$; Fig. 4B). However, there was a significant difference in pollen tube growth after 9 h, with the length of the longest pollen tube shorter in interspecific pollinations (39.2 mm vs. 47.5 mm, $N = 22$ inter- and 16 intraspecific pollinations, Mann-Whitney U-test: $P < 0.0001$) and fewer pollen tubes reaching the ovary in these pollinations (0.5 vs. 48.9 pollen tubes, Mann-Whitney U-test: $P < 0.0001$). The differences in pollen tube growth were sufficient to explain the decrease in interspecific compared to intraspecific seed set.

For *C. pulverulentus*, I estimated $RI_{postpollination}$ as 1.0 for La Selva and 0.951 for BCI. For *C. scaber*, I estimated $RI_{postpollination}$ as 0.870 for La Selva and 0.732 for BCI. I averaged the estimates for each species between sites for the overall calculation of postpollination isolation. Therefore, my estimate was 0.976 (95% CI: 0.939–0.994) for *C. pulverulentus* and 0.801 (95% CI: 0.558–0.940) for *C. scaber*.

Postzygotic Isolation

Seed germination and survival

For La Selva, the rate of seed germination was significantly different among cross types (Kruskal-Wallis test, $N = 45$ fruits, $P = 0.02$; Table 3), with a mean germination rate of 0.06 for H(S) hybrids, compared to 0.34 for *C. pulverulentus* and 0.32 for *C. scaber*. At BCI, however, there was no difference in germination among the two hybrid cross types and the *C. pulverulentus* intraspecific fruits (Kruskal-Wallis test, $N = 26$ fruits, $P = 0.27$; Table 3). The mean germination rate was 0.33 for *C. pulverulentus*, 0.39 for H(S), and 0.48

TABLE 3. Relative performance of *Costus pulverulentus*, *C. scaber*, and F_1 hybrids produced with *C. pulverulentus* (H[P]) or *C. scaber* (H[S]) as the maternal parent. Asterisks denote cases in which the hybrids performed significantly worse than the parentals with the same maternal parent.

	C. pulverulentus	H(P) F_1	*C. scaber*	H(S) F_1
Germination rate				
La Selva	0.34	n/a	0.32	0.06*
BCI	0.33	0.48	n/a	0.39
Proportion viable pollen				
La Selva	0.93	n/a	0.94	0.93
BCI	0.95	0.92**	0.92	0.96

* $P < 0.05$, ** $P < 0.01$.

for H(P). Some seeds died shortly after germination or being transplanted to the greenhouse. Once established in the greenhouse, however, there was essentially no natural mortality, although plants were culled or severely trimmed several times to conserve greenhouse space. All plants not culled eventually flowered during the next four years, and hybrids were generally observed to grow vigorously and produce abundant flowers. Therefore, $RI_{viability}$ was calculated solely from the results for seed germination.

For *C. pulverulentus*, $RI_{viability}$, which depends on the relative germination rates of *C. pulverulentus* parentals versus H(P) hybrids, was taken as zero, since H(P) hybrids could not be made for the La Selva plants and there were no significant differences for the BCI plants. For *C. scaber*, I estimated $RI_{viability}$ as 0.82 for La Selva and zero for BCI. I averaged the estimates between sites for the overall calculation of postzygotic viability isolation. Therefore, my estimate was 0.41 (95% CI: 0.01–0.50) for *C. scaber*.

Hybrid fertility

The proportion of fully stained pollen grains did not differ for the three cross types from La Selva ($N = 78$, $H = 0.53$, $P = 0.769$; Table 3). For BCI, the pollen stainability of hybrids with *C. pulverulentus* as a maternal parent was lower than outcrossed *C. pulverulentus* (0.92 vs. 0.95 proportion stained, $N = 80$, $Z = -3.092$, $P = 0.002$; Table 3), while the pollen fertility of hybrids with *C. scaber* as a maternal parent did not differ from that of wild-collected *C. scaber* ($N = 33$, $Z = -1.70$, $P = 0.09$; Table 3). Therefore, $RI_{fertility}$ was taken as zero for *C. scaber* from both sites and for *C. pulverulentus* from La Selva and as 0.035 for *C. pulverulentus* from BCI. Averaging between sites gave a value of 0.017 (95% CI: 0.001–0.034) for $RI_{fertility}$ for *C. pulverulentus*.

Total Reproductive Isolation

I summarized components of reproductive isolation separately for each species in Figure 5. With total reproductive isolation calculated as a multiplicative function of sequential isolating mechanisms, reproductive isolation is estimated to be nearly complete at the prezygotic stage, with values of 1.0 for *C. pulverulentus* as the maternal parent and 0.990 (95% CI: 0.982–0.998) for *C. scaber* as the maternal parent. Including the effects of intrinsic postzygotic isolation increased total isolation for *C. scaber* as the maternal parent to 0.994 (95% CI: 0.984–0.999). I calculated the absolute and relative contributions to total reproductive isolation both including the effects of large-scale geographic isolation and only using measures from sympatry. Excluding geographic isolation resulted in a reduction of total reproductive isolation for *C. scaber* to 0.989 (95% CI: 0.978–0.999).

DISCUSSION

Spatial Isolation

The importance of geography in speciation has long been recognized (Mayr 1959), and limited range overlap between closely related species may indicate that geographic isolation was important in initiating speciation (Barraclough and Vogler 2000). Nevertheless, many studies of reproductive isolation focus only on regions of sympatry, disregarding geography as part of total reproductive isolation. Current geographic isolation can be a holdover from a historical allopatric distribution with limited dispersal and range expansion or can indicate broad-scale ecological differences (Mayr 1947; Ramsey et al. 2003). In the latter case, geographic isolation indicates an important ecological contribution to speciation and should be considered. For *C. pulverulentus* and *C. scaber*, it is unclear what limits geographic range overlap. The species show no altitudinal segregation and are found in similar enough habitats that are likely to co-occur throughout Central and South America. Still, I found evidence of substantial geographic isolation. Limits to dispersal are a likely cause of this isolation, since the allopatric regions of both species occur beyond major topographic features. *Costus scaber* is found by itself to the south and east of the northern Andes, and the allopatric region of *C. pulverulentus* occurs in Cuba and to the north of the Mayan and Lacandon mountains in Belize and Mexico. Because of the probability of purely historical causes, I hesitate to classify broad-scale geographic isolation as ecogeographic isolation (sensu Ramsey et al. 2003), and I calculate total reproductive isolation both with and without the effects of geography. Transplant experiments, in which the fitness of each species is quantified in the allopatric region of the other species, would be required to definitively address this issue.

At a local scale at La Selva, *C. pulverulentus* and *C. scaber* still exhibit significant spatial isolation, and this is more likely the result of adaptation to different habitats and the higher abundance of individuals of *C. scaber*. Although I did not plot the spatial distribution of plants at either BCI or Sirena, the differences in their distributions are qualitatively similar at these sites (pers. obs.). At all sites, *C. pulverulentus* is found at small isolated treefall gaps, while *C. scaber* is found in wetter areas, often near swamps and streams (K. M. Kay, unpubl. data). It is clear that *P. superciliosus* fly between *C. pulverulentus* and *C. scaber* habitat, but the habitat differentiation likely results in less pollinator movement between species than would be expected otherwise. This is underscored by the occurrence of occasional hybrids in areas of recent deforestation, in which microhabitat isolation may have broken down. No hybrids have been reported from undisturbed mature forest, but over the five years of this study I have seen five putative F_1 hybrids in highly disturbed, open areas with an unusually high density of *Costus* plants. In

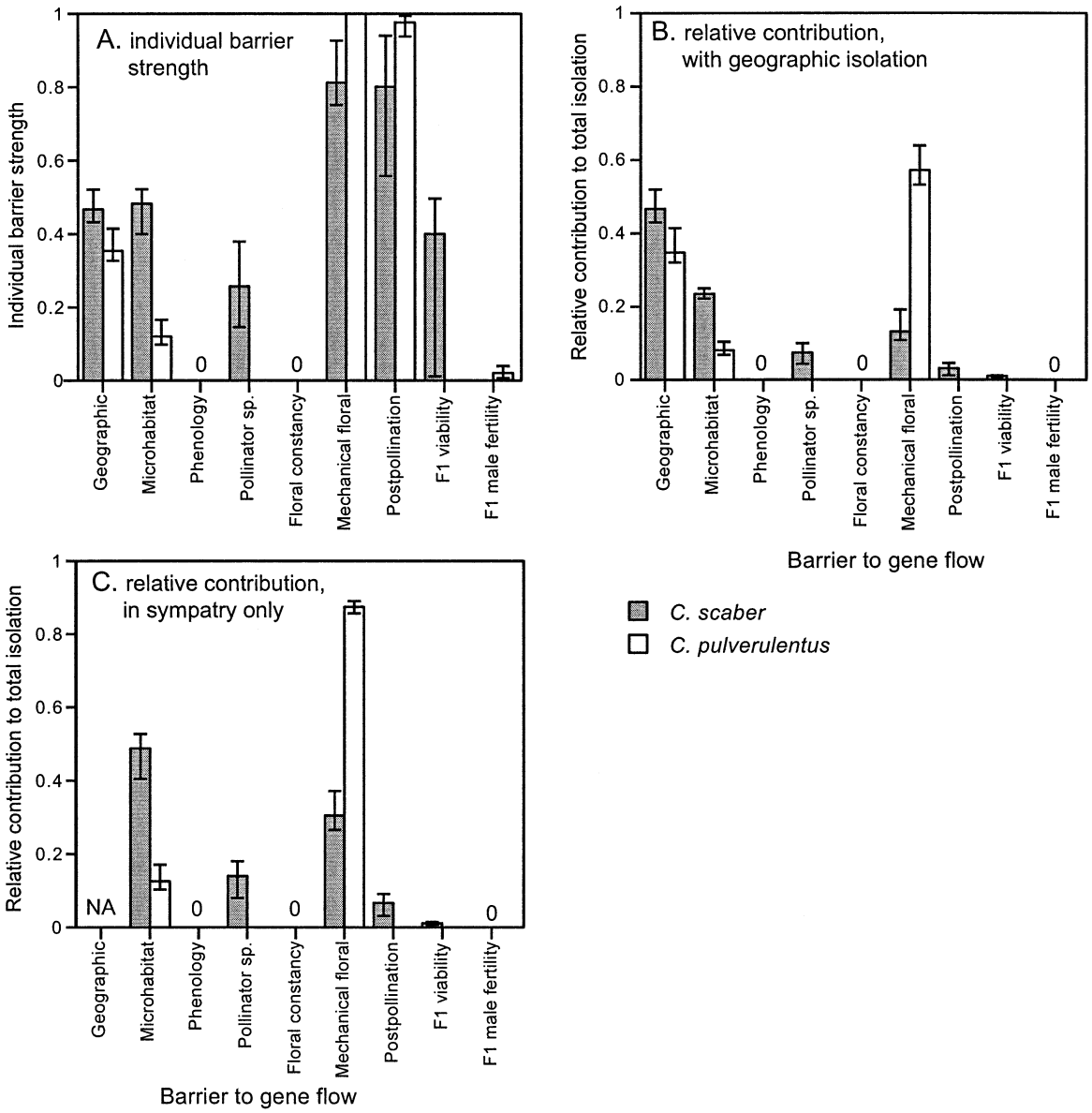

FIG. 5. All components of reproductive isolation calculated separately for each species. (A) The strengths of individual isolating mechanisms calculated separately (RI_n). (B) The relative contribution of each mechanism to total reproductive isolation (AC_n/T), including geographic isolation. (C) The relative contribution of each mechanism in sympatry, without geographic isolation. All error bars represent 95% confidence intervals of the means.

addition to limiting the opportunity for interspecific mating, this differential habitat affinity may also contribute to extrinsic postzygotic isolation by limiting the successful establishment of hybrids. If my calculations are correct, there should be a low rate of hybrid seed production with *C. scaber* as the maternal parent, yet these hybrids apparently do not survive to flowering in primary forest.

Floral Isolation

One of the striking features of this system is the importance of floral isolation, despite the fact that both species are specialized on the same hummingbird pollinator. Floral isolation caused by shifts between entirely different pollination syndromes has been shown to be important in this genus (Kay and Schemske 2003) and in other plant genera (Fulton and Hodges 1999; Chari and Wilson 2001; Ramsey et al. 2003), but these results also indicate the importance of floral isolation for speciation events that involve only subtle changes in floral characters. A similar result was found for diploid and tetraploid fireweeds (Husband and Sabara 2003), with bumblebees showing fidelity to one cytotype over another. Together, these results suggest that floral isolation should not be discounted even when floral traits have not diverged substantially.

Overall, most of the floral isolation in this system is mechanical. *Costus scaber* has a much shorter, more closed flower, with the stigma and anthers inserted just inside the opening of the tubular corolla (Maas 1972). It is apparent in video

recordings (Kay and Schemske 2003) that when *P. superciliosus* inserts its long decurved bill into the flower, pollen is deposited on the upper portion of the distal half of the bill (pers. obs., 182 flower visits). In contrast, *C. pulverulentus* has a longer (by ~2 cm), more open flower, with reflexed petals and exserted stigma and anthers (Maas 1972). When *P. superciliosus* is observed visiting, it inserts its bill without touching it to the stigma or anthers. As the bird pushes into the flower to reach the nectar, its forehead contacts the stigma and anthers, sometimes knocking clumps of pollen down the corolla tube, so that pollen may also be deposited on the distal portion of the bill (pers. obs., 73 flower visits). In this way, pollen is occasionally transferred from *C. pulverulentus* to *C. scaber*, but apparently not in the reverse direction.

Mechanical floral isolation has long been considered a mechanism of reproductive isolation in plants (reviewed in Grant 1994b), but its relative importance compared to other isolating mechanisms is not well understood. Here I find that in sympatry, mechanical floral isolation makes the largest contribution to total isolation for *C. pulverulentus* and the second largest for *C. scaber* (after microhabitat isolation). Furthermore, it appears to be a complete barrier to potential gene flow for *C. pulverulentus* as a maternal parent. Studies of Central American *Heliconia*, another genus of large understory monocots, many of which are also specialized on hermit hummingbirds like *P. superciliosus*, have found significant but incomplete mechanical isolation caused by differing sites of pollen placement on the birds (Stiles 1975, 1979; Kress 1983). It may be that growing at low density, with a strategy of producing few nectar-rich flowers, requires pollination by nonterritorial long-distance foragers like hermit hummingbirds (Stiles 1978a). Since there are relatively few species of hermits in the Neotropical forests compared to other hummingbirds, the numerous plant species relying on the hermits may be under strong selection to partition the sites of pollen placement.

I also found strong postpollination isolation in both directions of the crosses, although the mechanism appears to differ. With *C. pulverulentus* as the maternal parent, *C. scaber* pollen adheres and germinates but the pollen tubes fail to reach the ovary. This may be the result of an intrinsic inability of *C. scaber* pollen tubes to grow long enough, since there is a difference of approximately 2 cm in style length. Reduced pollination success of long flowers by pollen from short flowers is well-documented across many plant taxa (e.g., Emms et al. 1996; Howard 1999; Tiffin et al. 2001). In the other direction of cross, the barrier appears to act earlier and involves pollen adhesion and germination. In both directions, the strength of the barrier is close to unity, but considering that only *C. scaber* appears to receive interspecific pollen in nature, its relative contribution to total isolation differs. I also note that the contribution of postpollination isolation to total isolation for *C. scaber* could be affected by pollen competition. When *C. scaber* receives pollen from *C. pulverulentus* in nature, it may be in a mixture with conspecific pollen. If there is conspecific pollen precedence (Howard 1999) it may amplify the effects of postpollination isolation. Conversely, the conspecific pollen could facilitate fertilization by *C. pulverulentus* pollen if it reduces an 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). These potential factors were not quantified in this study and their effects are unknown.

While some mechanical isolation was anticipated in this system, the postpollination isolation was surprising. Within this recent and rapid species radiation, widespread crossability in artificial crosses has been found, and both *C. pulverulentus* and *C. scaber* can be crossed with more distantly related species (D. W. Schemske and K. M. Kay, unpubl. data). The strong postpollination barrier in *C. scaber* may have evolved by reinforcement in the face of interspecific pollen deposition. Reinforcement, in which direct natural selection strengthens prezygotic isolation to avoid hybridization, is predicted to occur upon secondary geographic contact between incipient species that have acquired substantial, but incomplete, reproductive isolation in geographic isolation (Dobzhansky 1940). *Costus pulverulentus* and *C. scaber* may fit this model. Indeed, without taking into account the effects of pollen-pistil incompatibility, total isolation for *C. scaber* as a maternal parent is calculated as only 97.0% (95% CI: 94.3–98.7%) complete, which would allow a limited but significant amount of hybridization. With reinforcement, postpollination barriers should be strongest between sympatric populations of *C. scaber* and *C. pulverulentus*, a prediction that should be straightforward to test. The selective origin of the pollen-pistil incompatibility is further suggested by the results of greenhouse crossing experiments among various species in the genus. Of nine other interspecific pairings, eight are easily crossable, and these are all pairs that are either allopatric in distribution or use different pollinators (D. W. Schemske and K. M. Kay, unpubl. data). The only other incompatible pairing is between *C. allenii* and *C. laevis*, species that are sympatric in Panama, attract the same species of bee pollinators and experience substantial interspecific pollen movement (Schemske 1981). From these patterns in crossing relationships, I further predict that postpollination barriers are likely to be found between other sympatric species pairs that have incomplete premating isolation but not between allopatric species or between sympatric species using different pollinators.

Postzygotic Isolation

Although postzygotic isolation was only roughly estimated in the greenhouse, it is clear that it is possible to make viable and fertile F_1 hybrids between *C. pulverulentus* and *C. scaber*. My measures of postzygotic isolation are admittedly the weakest part of this study, in part because of the difficulty of producing large numbers of hybrids in the face of strong prezygotic crossing barriers, and the lack of statistical power may explain the different results for plants from La Selva and BCI. My results for hybrid seed germination were compromised by the low and sporadic rate of fruit production over the years and the lack of seed dormancy that would allow me to start a large cohort of seeds at the same time under identical conditions. Once past the germination stage, hybrids grew vigorously and had high pollen fertility. However, even if F_1 hybrids are viable and fertile in the greenhouse environment, they may be poorly adapted to the avail-

able habitats in the natural environment (Hatfield and Schluter 1999), suffer from reduced mating ability because of pollinator attraction or pollen placement, or experience hybrid breakdown in later generations (reviewed in Coyne and Orr 1998, 2004). In fact, preliminary measures of pollen viability for a first generation backcross population constructed for genetic mapping showed a decrease in pollen viability compared to the high viability of parentals or the F_1 generation. Backcrosses to *C. scaber* average 76.6% pollen stainability (1 SE = 2.1% , N = 25 plants, 61 flowers), while backcrosses to *C. pulverulentus* average 68% (1 SE = 8.2%, N = 3 plants, 9 flowers). It may be simplistic, however, to quantify postzygotic isolation, especially beyond the F_1 generation, as the average relative fitness of hybrids compared to parentals. Hybrids may vary widely in phenotype and fitness (reviewed in Rieseberg et al. 1999; Burke and Arnold 2001), and their effects on genetic introgression may be complex (Gavrilets and Cruzan 1998; Barton 2001). Much more work will be required to fully understand the contribution of postzygotic factors to reproductive isolation in this system.

Relative Importance of Isolating Mechanisms

I can draw some general conclusions about the relative importance of different isolating mechanisms from this study. Prezygotic isolation, including habitat differences and floral isolation, in this case appears to be far more important in the early stages of species formation than intrinsic postzygotic isolation. Although I measured a small decrease in hybrid fitness in the greenhouse, F_1 hybrids generally appear to be vigorous and fertile. Furthermore, it is unlikely that strong intrinsic postzygotic isolation was important at the early stages of speciation but has been lost as species diverged post speciation, because it appears that an increase in hybrid incompatibility with time since divergence is the dominant pattern across a wide variety of organisms (reviewed in Bolnick and Near 2005). This result is consistent with an emerging pattern of stronger prezygotic, compared to intrinsic postzygotic, isolating mechanisms between closely related species. Nosil et al. (2005) surveyed systems for which multiple isolating mechanisms had been quantified. Across 20 study systems, including both plants and animals, they found a striking pattern of generally strong prezygotic isolation combined with weak postzygotic isolation.

One of the major gaps in this study concerns extrinsic postzygotic isolation, and thus I cannot directly compare pre- and postzygotic isolation. The divergence in habitat affinity suggest that extrinsic postzygotic isolation may be substantial, with hybrids potentially not adapted to any available habitat. If this is the case, however, it may be more relevant to compare ecological isolation to isolation without an apparent underlying ecological cause. *Costus* shows strong ecological isolation, including microhabitat and floral mechanical isolation. The major exception is the postpollination isolation, which I suggest may be a physiological limitation or a product of reinforcement, depending on the direction of the cross. The importance of ecological speciation has received much attention (e.g., Schluter 2000), and my results are consistent with the pattern of a large role for ecology, and ultimately natural selection, in the speciation process.

Systematic investigation into the nature of reproductive isolation clearly is necessary to understand the process of speciation. While my study is nearly comprehensive in evaluating different isolating mechanisms, it represents one case study in which speciation is already effectively complete, and therefore some of the differences identified may have been acquired postspeciation. To better estimate the relative importance of various isolating mechanisms during species formation, it would be ideal to investigate reproductive isolation across a range of evolutionary divergence in a phylogenetic context. This has been attempted for a variety of organisms (reviewed in Coyne and Orr 2004), but typically only for measures of postzygotic isolation. When prezygotic isolation is included, it is represented by the one or two stages that are easy to measure in a controlled environment, such as laboratory mating trials or greenhouse crosses, thus excluding many potentially important stages of reproductive isolation (Coyne and Orr 1997; Mendelson 2003; Moyle et al. 2004). Plants, which are particularly amenable to field studies of habitat differentiation and floral isolation and greenhouse crossing studies, would provide an excellent opportunity to study the accumulation of isolating mechanisms during speciation.

Evolution in the Tropics

Dobzhansky (1950) suggested that differences in patterns and mechanisms of speciation may be responsible for the great differences in species diversity between temperate and tropical regions and that biotic interactions may play an especially important role in tropical diversification. Today, we still have relatively few data to address these ideas, partly as a result of the added difficulties associated with studying tropical organisms. This is especially true for those organisms that exist at low density, that are poorly collected, and whose taxonomy, phylogeny, and natural history are not well known. Unfortunately, these features are common in the tropics. Here I present the most comprehensive study to date of reproductive isolation in tropical plants. My findings are consistent with the prediction of strong biotic interactions, in this case plant-pollinator interactions, promoting evolutionary divergence in the tropics (Dobzhansky 1950; Corner 1954; Ashton 1969; Gentry 1982; Schemske 2002). The lack of strong intrinsic postzygotic isolation also suggests that divergence occurred relatively recently, consistent with the idea of the Neotropical forests as a site of ongoing speciation as opposed to a collection of relictual species (Fischer 1960; Gentry 1989; Schemske 2002). This work also helps to show that many of the challenges of studying evolution in the tropics can be overcome and that a reasonably complete study of isolating mechanisms is feasible.

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