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THE EVOLUTION OF FLORAL CHARACTERS AND REPRODUCTIVE ISOLATION IN NEOTROPICAL *COSTUS* (COSTACEAE)

By

Kathleen Marie Kay

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ABSTRACT

THE EVOLUTION OF FLORAL CHARACTERS AND REPRODUCTIVE ISOLATION IN NEOTROPICAL *COSTUS* (COSTACEAE)

By

Kathleen Marie Kay

Speciation, defined as the process by which populations acquire reproductive isolation, is the foundation of biological diversity. Nevertheless, the mechanisms contributing to speciation for most groups of organisms are poorly understood. I investigate patterns and processes of speciation in a group of Neotropical understory herbs, *Costus* subgenus *Costus*, specifically focusing on pollination biology and mechanisms of prezygotic reproductive isolation. Through pollinator observations of 11 species throughout the Neotropics, I show that each species is specialized on either orchid bees or hummingbirds for pollination, and that pollinator visitation is accurately predicted by the species' pollination syndrome. Pollinator discrimination is sufficient to prevent most potential pollen flow between sympatric species that differ in floral syndrome. In a phylogenetic study using evidence from the internal and external transcribed spacers of nuclear ribosomal DNA, I find that the Neotropical species form a natural group within the subgenus. I estimate that shifts between the bee and hummingbird pollination syndromes have occurred a minimum of eight times in the Neotropics. A molecular clock approach suggests that diversification among the Neotropical clade has been recent and rapid following long distance dispersal from Africa, and has coincided with dramatic climatic and geologic changes in Pliocene and Pleistocene epochs.

To better understand the traits contributing to speciation, I detail components of reproductive isolation between two recently diverged and partially sympatric species, *C. pulverulentus* and *C. scaber*. I find that reproductive isolation is nearly complete at the prezygotic stage. For *C. pulverulentus* as the maternal parent, floral mechanical isolation, in which differences in floral morphology prevent heterospecific pollen deposition by their shared hummingbird pollinator, is the most important barrier. For *C. scaber* as the maternal parent, I find that microhabitat isolation and floral mechanical isolation prevent most heterospecific pollen deposition, but that postpollination pollen-pistil interactions further prevent hybrid seed set. The pollen-pistil incompatibility is consistent with reinforcement, in which prezygotic isolation is strengthened in sympatry to prevent maladaptive hybridization. In experimental crosses, the crossing barrier only functions to prevent hybrid seed set by the sympatric population of *C. pulverulentus*, and not by geographically distant or allopatric populations of *C. pulverulentus*.

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TABLE OF CONTENTS

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Kay, K. M., and D. W. Schemske. 2003. Pollinator assemblages and visitation rates for 11 species of Neotropical *Costus* (Costaceae). Biotropica 35: 198-207.

Images in this dissertation are presented in color.

Pollinator Assemblages and Visitation Rates for 11 Species of Neotropical *Costus* (Costaceae)[1]

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ABSTRACT

Most plant species in tropical forests are pollinated by animals, and yet the diversity and specificity of pollinator assemblages are poorly documented. Here, we investigated pollinator relationships for 11 species of understory herbs in the genus *Costus*, with the goal of documenting visitation rates and pollinator assemblages among a variety of habitats. For a subset of species, we documented pollinator visitation for multiple years and/or multiple sites to examine temporal and spatial variation in pollinator relationships. Furthermore, we examined the extent to which specialization in pollination systems can contribute to reproductive isolation for sympatric species. Each species was primarily pollinated by either euglossine bees or hummingbirds. Total visitation rates were generally low, averaging 3.2 visits per flower per hour for bee-pollinated species and 0.5 visits per flower per hour for hummingbird-pollinated species. All of the higher elevation species studied were hummingbird-pollinated, while low elevation species were pollinated either by euglossine bees or hummingbirds. Spatial and temporal variation in visitation rates and pollinator identities was minimal. Pollinator specificity was found to contribute strongly to reproductive isolation for the 11 pairwise combinations of sympatric species differing in pollination syndrome, in some cases functioning as a complete barrier to potential pollen flow.

RESUMEN

La mayoría de las especies vegetales de los bosques tropicales son polinizadas por animales, pero la diversidad y las especies de los gremios de polinizadores son poco conocidas. En este trabajo investigamos las interacciones de los polinizadores de 11 especies de hierbas del sotobosque pertenecientes al género *Costus*, con el objetivo de documentar la frecuencia de visitas y los gremios de polinizadores en distintos hábitats. Para un subconjunto de las especies, documentamos las visitas de los polinizadores en varios años y/o sitios para examinar la variación espacial y temporal de las interacciones de los polinizadores. Además, examinamos como la especialización de los sistemas de polinización puede contribuir al aislamiento reproductivo de especies simpátricas. Cada especie fue polinizada principalmente ya sea por abejas euglosinas o colibríes. Las frecuencia de visitas fue baja en general, con un promedio de 3.2 visitas/flor/hora en las especies polinizadas por abejas y 0.5 visitas/flor/hora en las especies polinizadas por colibríes. Las especies localizadas a mayor altitud fueron polinizadas por colibríes, mientras que las de bajas elevaciones fueron polinizadas por ambos. La diferencia espacial y temporal en frecuencia de visitas e identidad de polinizadores fue mínima. Se encontró que la especificidad de los polinizadores contribuye al aislamiento reproductivo, en las 11 combinaciones reciprocas de especies simpátricas se observaron síndromes de polinización diferentes, que en algunos caso sirvieron como una barrera efectiva al flujo potencial de polen.

Key words: Bolivia; Costa Rica; Costus; *euglossine bees; hummingbirds; Panama; pollination biology; pollination syndromes; reproductive isolation.*

Relationships between plants and their pollinators have been the subject of much interest because of their implications for the evolution of floral characters, patterns of gene flow, and the number, strength, and variance of species interactions in communities. Tropical regions harbor a spectacular diversity of plant species and floral phenotypes, and tropical pollination systems are often assumed to be more highly specialized than temperate systems (Feinsinger 1983, Johnson & Steiner 2000), although recent reviews of specialization across latitudinal gradients have reached conflicting conclusions (Olesen & Jordano 2002, Ollerton & Cranmer 2002). Pollinator specificity can also contribute to reproductive isolation between closely related plant species (Stebbins 1970, 1974; Grant 1981; Fulton & Hodges 1999; Schemske & Bradshaw 1999), and so specialization in pollination systems could promote speciation. Nevertheless, detailed information on pollinator relationships is lacking for most tropical plant taxa. Furthermore, the po-

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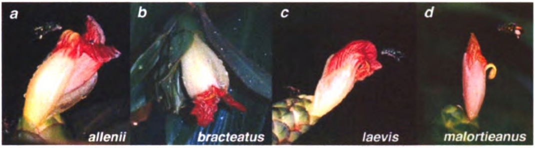

FIGURE 1. *Costus* species included in the study that were classified by Maas (1977) as bee-pollinated.

tential role of pollinator specificity in reproductive isolation has not been directly quantified for any tropical plant group.

The deficiency of data on pollination in the tropics is understandable in light of the special challenges presented by tropical forests. While plant diversity is high, the density of individual plant species is often extremely low (Janzen 1970), making large sample sizes difficult to obtain. Trees and epiphytes in the canopy and subcanopy often possess abundant displays of flowers, but are logistically difficult to observe, while understory herbs often produce only one or a few flowers at a time. Interestingly, for plant species growing at low density, specialization may be favored to ensure pollen transfer between highly dispersed conspecifics while minimizing deposition of heterospecific pollen (Feinsinger 1978). To better understand tropical pollination systems, information is needed on pollinator identities, visitation rates, and relative efficiencies of pollen transfer, preferably measured throughout a species' range and across time. Unfortunately for most plant taxa, not even the identity of pollinators is known. Often plants are quickly categorized based on sparse observations and suites of floral characters that correspond to recognized pollination syndromes, but it is unclear how well these syndromes predict actual pollinators (Waser *et al.* 1996, Ollerton 1998, Ollerton & Watts 2000).

Maas (1977) used floral characters and scant data on pollinator visitation to classify Neotropical members of the genus *Costus* into pollination syndromes. In the Neotropics, the genus comprises *ca* 60 species of understory terrestrial herbs (Maas 1972). Although a few pollinator observations have been published (Maas 1977, Stiles 1978, Schemske 1981, Grove 1985, Sytsma 1985), there has been no systematic investigation into pollinator relationships in the genus. Thus, this group provided an opportunity to identify the pollinator assemblages for a diverse set of tropical species and test the pollination syndromes proposed by Maas (1977).

In undisturbed forest, *Costus* generally grow at extremely low density, sometimes with hundreds of meters between flowering individuals. Most have spiral stems with terminal inflorescences that produce only one flower per day (rarely two) over an extended flowering period. Maas (1977) proposed that Neotropical *Costus* species are pollinated by either euglossine bees or hummingbirds. Flowers of the putatively bee-pollinated *Costus* have a short, broad labellar tube with a distinct white or yellow limb; the lateral lobes of the limb are often striped with red or purple; entry to the flower is blocked by the stamen; and the floral bracts are green (Fig. 1). The putatively hummingbird-pollinated species have flowers with a narrow, tubular labellum without an attached limb, and the labellum and floral bracts are yellow, orange, or red (Fig. 2). Flowers in both pollination categories are odorless and diurnal, and they produce relatively large quantities of nectar.

Evolutionary specialization by plants on their pollinators has been considered important to the diversification of many angiosperm lineages because it may drive adaptive divergence in floral morphology and can contribute to the origin and maintenance of reproductive isolation among close relatives (Stebbins 1970, 1974; Grant 1981). Much attention has been paid to evolutionary shifts in pollination syndromes as isolating mechanisms between sympatric congeners (Grant 1994, Fulton & Hodges 1999, Schemske & Bradshaw 1999). This attention is justified, however, only if shifts in syndrome accurately indicate specialization on different pollinators and other strong isolating mechanisms are absent. Other potentially important prezygotic mechanisms of reproductive isolation in plants include habitat segregation, differences in

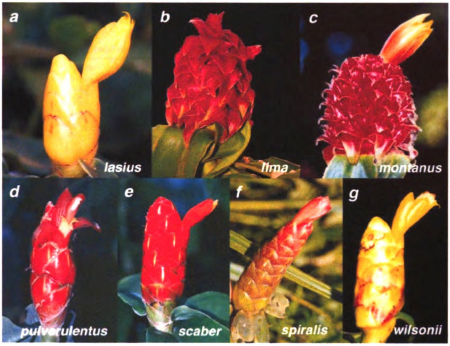

FIGURE 2. *Costus* species included in the study that were classified by Maas (1977) as **hummingbird-pollinated.**

flowering phenology, floral constancy of shared pollinator species, mechanical inefficiency of pollen transfer, and incompatible pollen–pistil interactions, while post-zygotic mechanisms may include hybrid inviability, infertility, or reduced fitness. *Costus* presented an excellent system to investigate the role of plant–pollinator interactions in reproductive isolation because it has undergone a species radiation in the Neotropics involving multiple shifts in pollination syndrome (Specht *et al.* 2001). Furthermore, it is common to find multiple *Costus* species, representing both bee and hummingbird syndromes, in sympatry at any given Neotropical lowland rain forest site.

We documented pollinator assemblages and visitation rates for 11 species of *Costus*. For a subset of these species, observations were made for multiple years and/or at multiple sites, and intensive observations were made of sympatric species at three sites. We explored the following questions: (1) How well do the suites of floral characters proposed by Maas (1977) for bee- and hummingbird-pollinated *Costus* predict their actual pollinators?; (2) How much variation in pollinator visitation is there across time and space?; and (3) Does pollinator specificity contribute to the reproductive isolation of sympatric *Costus*?

MATERIALS AND METHODS

Study species and sites.—Observations were made at five lowland sites and three mid-elevation sites. Lowland sites included Barro Colorado Island Nature Monument (BCI) in Panama, La Selva Biological Station (La Selva) and Sirena Biological Station (Sirena) in Costa Rica, and along the Rio Moile (Moile) and near the town of Asunción de Guarayos (Guarayos) in Bolivia. BCI (9°09′N, 79°51′W) is a 16 km² island located in Gatun Lake in the Panama Canal that consists of mature, tropical moist forest (Croat 1978). La Selva (10°25′N, 84°00′W) is a 1536 ha reserve in the Atlantic lowlands of Costa Rica that shares a boundary with the extensive Braulio Carillo National Park. It consists of mature tropical wet forest as well as regenerating agricultural lands (McDade & Hartshorn 1994). Sirena (8°29′, 83°35′W) is located along the Pacific Coast of Costa Rica in the expansive Area Conservación de Osa, and the surroundings consist of mature, tropical wet forest and regenerating agricultural lands. The Moile site (17°01′S, 64°02′W) was located on the border of the 430,000 ha Parque Nacional y Área de Uso Múltiple Amboró in Bolivia, in fragments of seasonal moist forest. The Guarayos site (15°23′S,

TABLE 1. *Pollination syndromes and observation periods of* Costus *species.*

Species	Syndrome[a]	Site[b]	Peak flowering[c]	Year observed	Month(s) observed	No. plants	No. hours
C. allenii	bee	BCI	June–Aug.	1998	July	2	24
C. bracteatus	bee	LS	Apr.–June	2000	May–Aug.	4	113
C. laevis	bee	BCI	May–Aug.	1998	July	2	26
		Sirena	May–Aug.	2002	July	6	41
C. lima	hummingbird	Sirena	May–July	2002	July	10	69
C. malortieanus	bee	LS	Aug.–Dec.	1997	Aug.	3	20
				1999	Feb.	2	12
				2000	July–Aug.	4	50
				2002	Oct.	6	47
C. lasius	hummingbird	EV	NA	1999	July	3	13
C. montanus	hummingbird	MV	May–July	1999	July	3	11
C. pulverulentus	hummingbird	BCI	June–Aug.	1998	July	4	65
				1999	July–Aug.	7	36
		LS	May–Aug.	1998	July	3	43
				1999	July	5	24
				2000	May–Aug.	19	282
		Sirena	May–July	2002	July	10	61
C. scaber	hummingbird	BCI	May–Aug.	1999	July–Aug.	4	28
		LS	May–Aug.	1999	July	5	62
				2000	May–Aug.	21	351
		Moile	NA	2001	Mar.	8	31
		Sirena	May–July	2002	July	14	78
C. spiralis	hummingbird	Guarayos	NA	2001	Apr.	8	8
C. wilsonii	hummingbird	LA	NA	2000	Sept.	3	9

[a] Based on Maas (1977).

[b] Sites: BCI: Barro Colorado Island, Panama; EV: El Valle de Antón, Panama; Guarayos: Asunción de Guarayos, Bolivia; LA: Las Alturas, Costa Rica; LS: La Selva Biological Station, Costa Rica; Moile: Rio Moile, Bolivia; MV: Monteverde, Costa Rica; Sirena; Sirena Biological Station, Costa Rica.

[c] Flowering phenology of all species is based on unpublished data and personal observations, except for *Costus malortieanus* and *C. pulverulentus* at La Selva (Stiles 1978), and *C. allenii* and *C. laevis* at BCI (Schemske 1981). NA: information not available.

63°00′W) was outside of the town of Asunción de Guarayos in forest patches and along small creeks, in a patchwork matrix of semievergreen moist forest and cleared rangeland. Our mid-elevation sites included (1) El Valle, Cocle Province, Panama, (800–1000 m; 8°38′N, 80°07′W), (2) Monteverde, Puntarenas Province, Costa Rica (1200–1400 m; 10°17′N, 84°47′W), and (3) Parque Nacional La Amistad near the Las Alturas Field Station, Puntarenas Province, Costa Rica (1500–1600 m; 8°57′N, 82°50′W). These sites represent lower montane wet forest, premontane wet forest, and montane wet forest, respectively.

Our study included four species classified by Maas (1977) as bee-pollinated (Fig. 1a–d) and seven classified as hummingbird-pollinated (Fig. 2a–g). At La Selva, we studied *C. bracteatus* (Fig. 1b), *C. malortieanus* (Fig. 1d), *C. pulverulentus* (Fig. 2d), and *C. scaber* (Fig. 2e), and at BCI, we studied *C. allenii* (Fig. 1a), *C. laevis* (Fig. 1c), *C. pulverulentus*, and *C. scaber*. At Sirena, we studied all species, including *C. laevis*, *C. lima* (Fig. 2b), *C. pulverulentus*, and *C. scaber*. *Costus scaber* was studied at Moile, and *C. spiralis* (Fig. 2f) at Guarayos. All species studied at mid-elevation sites exhibited hummingbird pollination syndromes and included *C. lasius* (Fig. 2a) at El Valle, *C. montanus* (Fig. 2c) at Monteverde, and *C. wilsonii* (Fig. 2g) at Las Alturas.

FLOWER VISITATION.—We made 1504 total hours of observations from 1997 to 2002, primarily during May through August when *Costus* was at its peak flowering in the Central American rainy season. Dates and sites of observations and sample sizes are summarized for each species in Table 1. Peak months of flowering, when known, are also noted for each species, although it is typically possible to find a few individuals flowering throughout the year. Because of low plant density and sporadic flower production, we were unable to sample plants for a standardized amount of time. The number of individuals observed typically represents what we could find during the time spent at each site, and

TABLE 2. *Pollinator visitation rates*[a] *for bee-pollinated* Costus.

			Pollinator species[b]					
Plant species	Site	Year	*Euglossa*	*Eulaema*	*Exaerete*	*Trigona*	Other bees	*P. s.*
C. allenii	BCI	1998	**9.66** (216)	—	—	—	—	—
C. bracteatus	LS	2000	**0.18** (42)	—	—	—	—	—
C. laevis	BCI	1998	**7.61** (176)	—	—	—	—	—
	Sirena	2002	**0.55** (25)	—	—	**0.06** (2)	**0.02** (1)	**0.08** (2)
C. malortieanus	LS	1997	**0.51** (52)	—	**0.01** (1)	—	—	—
		1999	**0.55** (9)	**0.19** (3)	—	—	—	—
		2000	**0.56** (20)	**0.10** (6)	**0.03** (2)	—	—	—
		2002	**0.27** (13)	**0.06** (3)	—	**0.04** (2)	—	**0.33** (26)

[a] Rates presented in bold are population means per flower per hour. Parentheses under each rate represent total number of legitimate visits.

[b] Pollinators: *P. s.*: *Phaethornis superciliosus.*

every effort was made to spread the hours of observation evenly among them. Because *Costus* has the ability to spread clonally, plants within 5 m of each other possibly represent the same genet; therefore, only one was chosen for observation. Distances between conspecific individuals observed at a site typically ranged from tens of meters to a few kilometers. The majority of our observations was made by video, using cameras set on tripods and covered with camouflage plastic covers and/or vegetation. Cameras ran for four hours between tape changes, which allowed us to observe multiple isolated plants for a major portion of the day. Direct observations were made with binoculars from a distance sufficient to avoid disturbing pollinators. The flowers of each *Costus* species open at dawn and last for only a single day, and observations were made from dawn until mid-afternoon, at which point the flowers either fell off or wilted and were no longer visited. Visitors were considered legitimate pollinators if they appeared to contact the anthers and stigma. We did not quantify the pollination success of the legitimate visitors. Hummingbirds were identified to species and bees were identified to genus. Identification of bees to species would have required collecting, which was impractical for video observations.

Data analysis.—Visitation rates are first presented separately by plant species, site, and year. For each type of visitor, a population mean visitation rate (per flower per hour) was calculated from the individual rates of each observed plant. To estimate the potential contribution of pollinator specificity to reproductive isolation, we determined the proportional similarity (PS) in pollinator assembly for pairs of sympatric congeners, calculated as $PS = 1 - 1/2 \sum_{i=1}^{n} |P_{ai} - P_{bi}|$, where P_{ai} and P_{bi} are the proportion of the total visitation rate made up by taxon i for plant species a and b, respectively (modified from Schemske & Brokaw 1981). This index, which ranges from 0 to 1, takes into account both the identity of pollinators and their relative visitation rates. Data were combined across years for populations sampled more than once. Lower values indicate less overlap in pollinator use, and therefore, a larger potential contribution of pollination system to reproductive isolation. Although the *Costus* species studied appeared to have subtly distinct habitat preferences, all species growing at a site were considered sympatric, as distances between conspecifics were similar to distances between heterospecifics.

RESULTS

Bees (Apoidea; Table 2) and hummingbirds (Trochilidae; Tables 3 and 4) were the sole pollinators of the *Costus* in our study, and each *Costus* species was specialized on one of these two groups of pollinators. The pollination syndromes proposed by

TABLE 3. *Pollinator visitation rates*[a] *for low elevation, hummingbird-pollinated* Costus.

Plant species	Site	Year	Hermits[b]				Non-hermits[b]					Bees[b]
			G. h.	*P. p.*	*P. r.*	*P. sub.*	*P. sup.*	*A. t.*	*A. n.*	*T. c.*	*T. f.*	*Eug.*
C. lima	Sirena	2002	—	—	—	—	**0.29** (19)	—	—	—	—	—
C. pulverulentus	BCI	1998	—	—	—	—	**0.13** (8)	—	—	—	—	—
		1999	—	—	—	—	**0.09** (4)	—	—	—	—	—
	LS	1998	—	—	—	—	**0.16** (11)	—	—	—	—	—
		1999	—	—	—	—	**0.23** (5)	—	—	—	—	—
		2000	—	—	—	—	**0.16** (30)	—	—	—	—	—
	Sirena	2002	—	—	—	—	**0.25** (15)	—	—	—	—	—
C. scaber	BCI	1999	—	—	—	—	**1.13** (51)	—	—	**0.08** (6)	—	**0.06** (2)
	LS	1999	—	—	—	—	**0.06** (6)	**0.03** (11)	—	**0.22** (81)	—	—
		2000	—	—	—	—	**0.25** (111)	—	—	**0.05** (23)	—	—
	Moile	2001	—	**0.03** (1)	**0.75** (32)	**0.21** (9)	—	—	—	—	**0.01** (1)	—
	Sirena	2002	—	—	—	—	**0.22** (14)	—	—	**0.10** (10)	—	—
C. spiralis	Guarayos	2001	**1.34** (18)	—	—	—	—	—	**0.25** (15)	—	—	—

[a] Rates presented in bold are population means per flower per hour. Parentheses under each rate represent total number of visits.

[b] Pollinators: *G. h.*: *Glaucis hirsuta*; *P. p.*: *Phaethornis pretrei*; *P. r.*: *Phaethornis ruber*; *P. sub.*: *Phaethornis subochraceous*; *P. sup.*: *Phaethornis superciliosus*; *A. t.*: *Amazilia tzacatl*; *A. n.*: *Anthracothorax nigricollis*; *T. c.*: *Thalurania columbica*; *T. f.*: *Thalurania furcata*; *Eug.*: *Euglossa*.

TABLE 4. *Pollinator visitation rates*[a] *for high elevation, hummingbird-pollinated* Costus.

			Pollinator species[b]				
Plant species	Site	Year	*C. h.*	*E. e.*	*E. c.*	*P. l.*	*P. g.*
C. lasius	EV	1999	—	—	**0.21** (5)	**0.14** (4)	—
C. montanus	MV	1999	**0.40** (4)	**0.08** (34)	—	—	—
C. wilsonii	LA	2000	—	—	—	—	**0.16** (2)

[a] Rates presented in bold are population means per flower per hour. Parentheses under each rate represent total number of legitimate visits.

[b] Pollinators: *C. h.*: *Campylopterus hemileucurus*; *E. e.*: *Eupherusa eximia*; *E. c.*: *Elvira chionura*; *P. l.*: *Phaethornis longuemareus*; *P. g.*: *Phaethornis guy*.

Maas (1977) were effective at predicting the pollinators for all 11 species. Of the 601 legitimate visits to the 4 species predicted to be bee-pollinated, 573 were by bees and 568 were specifically by euglossine bees (Euglossini). Of 532 legitimate visits to the 7 species predicted to be hummingbird-pollinated, 530 were by hummingbirds. Of the 12 site/species combinations for the 7 putatively hummingbird-pollinated species, the mean percentage of visits by hummingbirds was 99.6 (range = 96.6–100). Of the 5 site/species combinations representing the 4 putatively bee-pollinated species, the mean percentage of visits by bees was 94.9 (range = 81.0–100). Only *C. malortieanus* received a substantial number of visits by both bees and hummingbirds, and this was only observed during the fall of 2002. In all other years, bees were the only legitimate visitors to *C. malortieanus*. Illegitimate visits, primarily by shorter-billed hummingbirds that pierced the base of the corolla, were common for both bee- and hummingbird-pollinated species (KMK, pers. obs.).

Within the hummingbirds, there was a variety of pollinating species, including both Phaethornine (hermit) and Trochiline (non-hermit) hummingbirds. Hermits included *Glaucis hirsuta*, *Phaethornis guy*, *P. longuemareus*, *P. pretrei*, *P. ruber*, *P. subochraceous*, and *P. superciliosus*. Non-hermits included *Amazilia tzacatl*, *Anthracothorax nigricollis*, *Campylopterus hemileucurus*, *Elvira chionura*, *Eupherusa eximia*, *Thalurania columbica*, and *T. furcata*. Most bees belonged to three genera in the tribe Euglossini (Apidae), *Euglossa*, *Eulaema*, and *Exaerete*, with *Euglossa* by far the most common. Overall, there was a mean of 2.5, a median of 2, and a mode of 1 visiting taxa per plant species, and for every species/site combination sampled, the most frequent visitor made up at least 60 percent of the total visitation rate. The visitation rates to the bee-pollinated species at BCI were considerably higher than those at La Selva or Sirena. In general, the hummingbird-pollinated species, especially those with only one visiting taxon, had extremely low visitation rates. Across plant species, visitation rates were generally slightly higher at mid-morning and declined after noon, but no patterns in the identity of visitors across the day were detected (KMK, pers. obs.).

There were only minor fluctuations in total visitation rate among years and sites, except in the case of *C. scaber*, for which BCI and Moile had much higher visitation rates than either Costa Rican site, and *C. laevis*, for which the bee visitation rate at BCI was 12 times higher than at Sirena. At the level of visiting taxa, there was little temporal and spatial variation in the composition of the pollinator assemblage and the relative visitation rates for most *Costus* species. One notable exception was the difference between Central and South American populations of *C. scaber*. The observed hummingbird species do not occur both in Central America and at Moile in Bolivia; however, while the species of hummingbird visitors at Moile were different than in Central America, the genera were the same. Also, the pollination system of *C. malortieanus* exhibited some temporal variation at La Selva, with *P. superciliosus* only observed visiting during the October 2002 sampling.

Proportional similarity in pollinator assemblage was consistently low ($\bar{x} = 0.08$) for sympatric congeners differing in pollination syndrome, while it was consistently high ($\bar{x} = 0.81$) for pairs sharing a syndrome (Table 5). These values indicate that there was little overlap in pollinator use for species living at the same site but exhibiting different pollination syndromes. In contrast, specialization by

TABLE 5. *Mean proportional similarity*[a] *± 1SD in pollinator assemblage for sympatric* Costus *species.*

Site	Same syndrome	Different syndrome
BCI	**0.95** ± 0.078 (2)	**0.03** ± 0.029 (4)
LS	**0.70** ± 0.071 (2)	**0.10** ± 0.114 (4)
Sirena	**0.79** ± 0.180 (3)	**0.11** ± 0 (3)
All sites	**0.81** ± 0.152	**0.08** ± 0.076

[a] Proportional similarity (PS) in pollinator assemblage is calculated as $1 - ½ \sum_{i=1}^{n} |Pai - Pbi|$, where P_{ai} and P_{bi} are the proportion of the total visitation rate made up by taxon *i* for species *a* and *b*, respectively. PS was calculated separately for each pairwise combination of species at a site using composite visitation rates calculated across all individual plants regardless of sampling year, and then averaged across all pairs in each category. Sample sizes of pairwise combinations in each category follow PS values in parentheses. Some pairwise combinations of species occurred at more than one site.

Costus species within functional groups of pollinators did not greatly reduce overlap in pollinator use.

DISCUSSION

SPECIALIZATION AND POLLINATION SYNDROMES IN *COSTUS*.—Of the scores of potential pollinators in the tropical forest, each *Costus* species is pollinated by a limited number of taxa, indicating a high level of specialization by these plants to their pollinators. Although sample sizes for some *Costus* species were low, we probably have not missed many important pollinators for a given species at the site and time period sampled. Species accumulation curves constructed for well-sampled site/year combinations saturate quickly. Curves were computed using EstimateS (Colwell 1997) to randomize across sample order, and they give Michaelis–Menten estimates of the asymptote equal to the total observed number of pollinating taxa with just one to three individual plants observed. For most species, we found that sampling at multiple sites or across multiple years uncovered no or only very rare new visitors. For *C. scaber*, however, adding a geographically distant site in South America doubled the number of hummingbird species but did not increase the number of genera. Further, we found that sampling *C. malortieanus* at the end of the wet season at La Selva uncovered previously unobserved hummingbird visits. In general, we do not know the relative success of each pollinator at effecting seed set, which may or may not correspond to visitation rates (Schemske & Horvitz 1984); thus, it is possible that we are underestimating specialization by including ineffective visitors. Finally, specialization in these systems was greater for plants than for pollinators because most of the visiting taxa are known to visit other plant species (Dressler 1968, Snow & Snow 1972, Feinsinger 1976, Stiles and Wolf 1979).

Our results support the idea that suites of characters associated with bee and hummingbird pollination syndromes indicate specialization by plants to these pollinators. Although the *Costus* species exhibit traits generally associated with bee and hummingbird pollination syndromes, more detailed knowledge of their natural history and floral morphology may have predicted that many would be further specialized on euglossine bees or hermit hummingbirds. Plants growing at low density and displaying only one or a few flowers at a time are unlikely to be pollinated by animals defending foraging territories. Both euglossine bees and hermit hummingbirds have been noted for their long foraging routes among isolated flowers in tropical forests (Skutch 1964, Janzen 1971, Stiles & Wolf 1979). *Costus* species growing in denser patches, such as *C. montanus*, *C. spiralis*, and some individuals of *C. scaber*, were visited by more territorial non-hermit hummingbirds. The long, curved corolla tubes of the hummingbird-pollinated species also suggests some specialization on hermits, which generally have long, decurved bills, and the large size of the bee-pollinated flowers fits with the large body sizes and long tongues of euglossine bees. Nevertheless, pollination syndromes did not predict all visits for all species. For example, the putatively bee-pollinated *C. malortieanus* at La Selva displayed substantial temporal variation in pollinator assemblage, with bees composing 100 percent of the visitation rates from 1997 to 2000 when it was observed during the dry season or at the peak of the wet season, but only 53 percent of the visitation rate in the late wet season of 2002 when the hummingbird *P. superciliosus* was a common visitor. *Phaethornis superciliosus* had previously been observed visiting *C. malortieanus* at that time of year (Stiles 1978), and this has been interpreted as a response to a seasonal nectar shortage in the forest (Grove 1985). The uncommon visits of *P. superciliosus* to bee-pollinated species (Table 2) and *Euglossa* sp. to a hummingbird-pollinated species (Table 3) show that specialization is not absolute and suggest potential mechanisms by which evolutionary shifts in pollination syndrome could occur. Phylogenetic evidence suggests that there have been multiple pollinator shifts in the genus (Specht *et al.* 2001).

Plant–pollinator interactions and reproductive isolation.—Our studies of sympatric *Costus* differing in pollination syndrome allowed us to explicitly quantify the potential contribution of pollination syndromes to reproductive isolation. The sympatric pairs of species differing in syndrome (Table 5) included in this analysis all showed very little or no overlap in pollinator use. Comparatively, sympatric pairs sharing a syndrome all had high overlap in pollinator use. Since there was extensive overlap in flowering phenology and spatial distribution for sympatric pairs (Table 1; KMK & DWS, pers. obs.), specialization in pollination syndrome is an important mechanism for preventing pollen flow between sympatric congeners. Furthermore, we have conducted artificial interspecific crosses in the greenhouse for 5 of the 11 sympatric pairs differing in syndrome and found that all 5 readily set seed and that hybrids show high fertility (KMK & DWS, pers. obs.). Therefore, specialization in pollination system is an important mechanism for preventing actual gene flow between these sympatric *Costus*. Although it is unknown whether there were other important isolating mechanisms in place at an earlier stage of speciation, our studies show that specialization in pollination system is currently a primary mechanism of reproductive isolation for these species pairs.

In our study, five sympatric species pairs shared the same pollination syndrome and major pollinators, and therefore provided an excellent opportunity to investigate reproductive isolation not caused by specialization in pollination systems. What other mechanisms might contribute to preventing gene flow between these species pairs? For *C. bracteatus* and *C. malortieanus* at La Selva, flowering phenology may be important, since typically most *C. bracteatus* finish flowering before *C. malortieanus* starts. For *C. pulverulentus* and *C. scaber*, differences in floral morphology greatly reduce pollen transfer by their shared pollinator, *P. superciliosus* (KMK, pers. obs.). At BCI, *C. allenii* and *C. laevis* flower concurrently, share pollinators, and experience substantial interspecific pollen flow but do not successfully hybridize (Schemske 1981) because of post-pollination interactions between the pollen and pistil (KMK, pers. obs.). All of these mechanisms are currently under investigation to better understand speciation processes throughout the genus.

Our results suggest a limited set of strong and consistent ecological connections between *Costus* species and their pollinators. Further, divergence in floral phenotypes associated with syndromes can contribute to reproductive isolation among close relatives living in sympatry. Although it is not clear how generally our results apply to other groups of plants, geographic regions, or syndromes, they are consistent with emerging evidence for specialized plant–pollinator relationships in other systems (McDade 1984, Armbruster 1993, Hodges & Arnold 1994, Johnson *et al.* 1998, Schemske & Bradshaw 1999), and they greatly contribute to the empirical data available for tropical pollination systems.

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CHAPTER 2

Rapid speciation and the evolution of hummingbird pollination in Neotropical *Costus* subgenus *Costus* (Costaceae): Evidence from nrDNA ITS and ETS sequences

Abstract—We estimate phylogenetic relationships and the biogeographic and pollination history of *Costus* subgenus *Costus* (Costaceae) using sequence data from the internal and external transcribed spacer (ITS and ETS) regions of 18S-26S nuclear ribosomal DNA. The Neotropical species radiation is supported as monophyletic and nested within the basal African members of the subgenus. The Neotropical species exhibit large, showy flowers visited by either euglossine bees or hummingbirds. The hummingbird pollination syndrome is supported as a derived character state from the bee pollination syndrome, and we estimate that it has evolved independently a minimum of eight times in the Neotropics. A molecular clock approach suggests that diversification among the Neotropical clade has been recent and rapid, and that it coincides with dramatic climatic and geologic changes, Andean orogeny, and the closing of the Panama isthmus that occurred in the Pliocene and Pleistocene epochs. We propose a scenario for the diversification of *Costus*, in which rapid floral adaptation in geographic isolation and range shifts in response to environmental changes contribute to reproductive isolation among close relatives. We suggest that these processes may be common to other recently-diversified plant lineages centered in Central America or the Northern Andean phytogeographic region.

Key words: *Costus*; ETS; euglossine bees; hummingbirds; ITS; plant-animal interactions; pollination syndromes; speciation; tropical diversification.

Neotropical forests harbor the highest plant diversity of any tropical region (Gentry 1982; Raven 1976), yet the evolutionary history of diversification for most resident plant lineages is poorly understood. Many ideas have been put forth addressing the history of this spectacular flora. Dynamic climatic and geological changes, especially in recent epochs, have been proposed as a causative agent of allopatric speciation (Bush 1994; Graham 1997; Haffer 1969; Haffer and Prance 2001). Some authors also have suggested that speciation generally may occur at a faster rate in the tropics (Fischer 1960; Gentry 1989; Schemske 2002) perhaps due to a dominant evolutionary influence of strong biotic interactions (Ashton 1969; Corner 1954; Dobzhansky 1950; Schemske 2002). Plant-pollinator interactions specifically have been considered important to the diversification of many angiosperm lineages because they may drive adaptive divergence in floral morphology and can contribute to reproductive isolation among close relatives (Grant 1981; Stebbins 1970; Stebbins 1974). These interactions have been suggested to be more specialized in tropical regions (Johnson and Steiner 2000). In contrast, others have stressed the relative antiquity of Neotropical plant lineages (Dick et al. 2003) and the long term stability of high Neotropical plant diversity (Colinvaux and De Oliveira 2001; Wilf et al. 2003), and have downplayed the importance of Pleistocene refugia in Neotropical diversification (Colinvaux et al. 2001; Haberle and Maslin 1999). These ideas are not mutually exclusive, of course, and the evolutionary history of the Neotropical flora is likely to be complex (Bush 1994; Morley 2000). Overall, however, there is little empirical evidence regarding the biogeographic pattern or timing of plant diversification, or the mechanisms and relative importance of recent or rapid speciation.

The debate over the diversification of the Neotropical flora may be partly resolved by recognizing that different lineages are likely to have different histories and different responses to environmental changes. The flora primarily consists of lineages of Gondwanan origin that dominate the vast lowland forests, along with some of Laurasian origin that are relegated mainly to high montane temperate-like habitats (Raven and Axelrod 1974). Within the Gondwanan groups, Gentry (1982) recognized two main categories by their phytogeographic region of highest diversity and endemism: woody canopy trees and lianas with a center of diversity in the Amazon and epiphytes, understory shrubs, and understory monocots with a center of diversity at the base and along the lower slopes of the Northern Andes or in Southern Central America. The plants in this second category constitute over half of the Neotropical species diversity, and include orchids, bromeliads, gingers, gesneriads, *Ingas*, and *Psychotrias*, among others. Gentry suggests that contrary to a gradual diversification of Amazon-centered trees, these lineages have undergone "explosive speciation and adaptive radiation" in response to dramatic changes in geology and climate. The entire Neotropics experienced climatic oscillations during the Pleistocene epoch, but volcanism in Southern Central America, the closing of the isthmus of Panama approximately 3.5 mya, and the uplift of the Northern Andes from about 5 mya (Gentry 1982; Graham 1997) would have especially affected these lineages. Gentry also notes that specialized pollination, particularly by hummingbirds, is a prominent feature of these groups and that shifts in specific pollinators appear to be a common mode of speciation. The center of hummingbird diversity is in the Northern Andes (Bleiweiss 1998), and this wide array of potential pollinators may have further promoted speciation in plant lineages already experiencing dramatic environmental heterogeneity.

These ideas for the diversification of Northern Andean-centered herb, shrub, small tree, and epiphyte lineages require evidence from phylogenetic studies. Support for rapid and recent speciation has been found for the Andean-centered small tree genus *Inga* (Richardson et al. 2001a), and several genera have been examined for specialized pollination systems (Bruneau 1997; Kay and Schemske 2003; McDade 1992; Stiles 1975). Studies are needed, however, that combine phylogenetic analysis of the biogeographic history and timing of diversification along with an examination of the evolution of ecological features, such as pollination systems, that may contribute mechanistically to speciation. Understanding the diversification of these lineages will contribute fundamentally to resolving the quandary posed by the extraordinary species richness of the Neotropical flora.

Costus subgenus *Costus* provides an excellent opportunity to examine the diversification of species and pollination systems of a Northern Andes/Central America-centered group in a phylogenetic context. The family Costaceae, commonly known as the spiral gingers, has its origin in Africa (Specht et al. 2001) and contains four genera, of which *Costus* is by far the most species rich. *Costus* subgenus *Costus*, with *c.* 51 species in the Neotropics and several species in Africa, comprises most of the diversity in the genus (Garcia-Mendoza 1991; Maas 1972; Maas 1977; Maas and Maas 1990; Maas and Maas-van de Kamer 1997). Its primary center of diversity is in Costa Rica, Panama, western Colombia, and Ecuador, although it ranges from Mexico to Brazil. While there are a few widespread species, many are locally restricted, with several known only from their type locality. The species are all herbaceous and occur in a variety of habitats, including streamsides, treefall gaps, and limestone outcrops. The subgenus is found from low to mid elevation, but is most common in wet, low elevation sites.

The Neotropical *Costus* species are remarkable for their floral biology. The characteristic spiraling stems support terminal inflorescences that generally produce a single large showy flower each day of an extended flowering season. Each species can be classified as having either a bee or hummingbird pollination syndrome based on distinct suites of floral characters. In his monograph for Flora Neotropica, Maas (1977) divided the subgenus into sections, *Costus* and *Ornithophilus*, according to these pollination syndromes. Flowers of the bee-pollinated *Costus* have a short broad labellar tube with a distinct white or yellow limb, the lateral lobes of which are often striped with red or purple, and the floral bracts are green. The hummingbird-pollinated species have flowers with a narrow tubular labellum without an attached limb, and the labellum and floral bracts are yellow, orange, or red. Flowers in both pollination categories are odorless and diurnal, and they produce relatively large quantities of nectar. In a study of 11 species, Kay and Schemske (2003) found that these syndromes accurately predicted pollinators, with species in sections *Costus* and *Ornithophilus* visited almost exclusively by orchid bees (Euglossini: Apidae) and hummingbirds, respectively.

Here we use rDNA ITS and ETS sequence data to examine the hypothesis of explosive recent speciation and pollination specialization within *Costus* subgenus *Costus* using a phylogenetic approach. Although the ITS locus has come under criticism recently because of some features of its evolution (Alvarez and Wendel 2003), it is still the best available tool for species-level phylogenetic inference in many plant groups because of its multicopy nature, the availability of universal primers, and its rapid substitution rate. The ETS locus provides additional characters and typically has a higher substitution rate, making it useful in cases of possible rapid or recent diversification (Baldwin and Markos 1998). In this study, we estimate species-level relationships and reconstruct ancestral states for pollination syndromes and biogeographic distributions using maximum parsimony. Because there are no fossils available for the Costaceae, estimates of divergence times and diversification rates are made with a molecular clock approach from published rates of ITS evolution. We attempt to determine the biogeographic origin of subgenus *Costus*, and whether it has undergone rapid and recent diversification in the Neotropics. We also use the estimated phylogeny to determine whether the diversification is associated with repeated evolutionary shifts in the pollination system.

MATERIALS AND METHODS

Taxon sampling—We sampled leaf tissue from 41 species of *Costus*, 38 of which are classified in subgenus *Costus*. When possible, we attempted to include individuals from different subspecies, varieties, or geographically dispersed populations within each species, and overall we sampled 63 accessions. Leaf tissue was collected on silica gel in the field or fresh from the collection in the Michigan State University greenhouse. Additional silica-dried samples were contributed by botanical gardens and an extensive private collection. Voucher and collection information, geographic origin, and GENBANK accession numbers are summarized in Appendix A. *Costus laterifolius*, *C. letestui*, and *C. talbotii* were selected as outgroups based on a previous phylogenetic analysis of the Costaceae (Specht et al. 2001).

We sampled the three most geographically widespread and collected African species that likely belong to subgenus *Costus* sensu Specht et al. (2001), but the taxonomy of the Paleotropical *Costus* is poorly understood. Schumann (1904) originally circumscribed the subgenera of *Costus*, and he placed 35 African, Asian and Australian species in subgenus *Eucostus* (= *Costus*), but most of these clearly do not belong to subgenus *Costus* sensu Specht et al. (2001). Ten of these 35 species have been put into synonymy (Hutchinson and Dalziel 1968; Koechlin 1964; Lock 1985; Maas 1979). Maas (1979) identified nine of the remaining as belonging to the Asian *C. globosus* complex, which was placed in a distant part of the Costaceae by Specht et al. (2001), as was the Asian *C. lacerus*. Maas (1979) further suggests that the widespread *C. speciosus*, which is native to Asia but cultivated in the Neotropics, is closely related to *C. lacerus*. Two African species, *C. fissiligulatus* and *C. littoralis*, clearly exhibit the "Cadalvena-type" floral morphology that was excluded from subgenus *Costus* by Specht et al. (2001). Six additional African species are very poorly known, especially regarding floral traits that may indicate their taxonomic placement, and specimens were not found for this study. The other six African taxa include *C. maculatus* (which is sometimes considered a variety of *C. afer*), *C. sarmentosus*, *C. subbiflorus*, and the three species we sampled. These species have been noted for their similarity, apparent in floral phenotype and habitat use (Lock 1985), and therefore the three widespread species we sampled likely represent well the Paleotropical members of the subgenus.

Molecular methods—Total genomic DNA was extracted from fresh or silica-dried tissue using the modified CTAB method (Doyle and Doyle 1987) or the FastDNA Kit (Bio 101, Carlsbad, CA). The ITS region (ITS1, 5.8S, and ITS2) was amplified using the ITS4 and ITS5 primers (Baldwin 1992). To design a *Costus*-specific primer for ETS, the entire ETS region was amplified from a subset of taxa by long polymerase chain reaction (PCR) using the primers 18S-IGS and 26S-IGS (Baldwin and Markos 1998). The 3' region of the ETS was sequenced and a 5' primer (5'-CTTTGTTGTGCTCGGCGGAGTTC -3') was designed. The 3' end of the ETS was amplified from all taxa using this primer and 18S-IGS. Long PCR conditions were as follows: 94°C for 75 sec, 30 cycles of 94°C for 15 sec and 68°C for 8 min, with a final extension of 72°C for 10 min. All other PCR reactions consisted of 2.5 μL Promega (Madison, WI) 10x reaction buffer, 2.5 μL 25mM MgCl, 1.25 μL 40mM dNTPs, 0.25 μL of each primer (25mM), 1.25 μL DMSO, 0.25 μL Promega *Taq* polymerase, approximately 50 ng DNA, and water to 25 μL. PCR was performed in a PT-100 thermocycler (MJ Research, Waltham, MA) as follows: 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 45 sec (adding 3 sec per cycle), followed by a final extension of 72°C for 7 min. Most PCR was preceded by a manual hot start of 94°C for 5 min before the *Taq* polymerase was added to the reaction. The PCR products were purified using Qiagen Qiaquick spin-columns (Qiagen, Valencia, California, USA).

Both strands were sequenced using ABI automatic sequencers (Applied Biosystems, Inc., Foster City, California). The ITS was sequenced using primers ITS4 and ITS5, while the ETS was sequenced using the 18S-E primer of Baldwin and Markos (1998), which is slightly internal to 18S-IGS, and the *Costus*-specific 5' ETS primer. Sequencher version 3.0 (Gene Codes Corporation, Ann Arbor, Michigan, USA) was used to edit the sequences. *Costus dubius*, *C. malortieanus*, and *C. pulverulentus* from Barro Colorado Island in Panama appeared to have multiple alleles of ITS, while *C.*

pulverulentus from the Osa Peninsula in Costa Rica and *C. pictus* showed multiple alleles for both ITS and ETS. These PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Six to ten positive clones were selected from each PCR product for sequencing.

Analyses—ITS and ETS sequences were aligned manually using the program Se-Al version 1.0a1 (A. Rambaut, University of Oxford, Oxford, United Kingdom). Unambiguous insertions and deletions (indels) were scored as missing data and then recoded as unordered multistate characters in a separate matrix following the procedure of Simmons and Ochoterena (2000).

A preliminary parsimony analysis including all sequenced clones and PCR products was conducted using PAUP* 4.0b10 (Swofford 1998) separately for ITS and ETS. A heuristic search strategy with 100 random sequence addition replicates and 10 best trees held per iteration, followed by tree bisection-reconnection (TBR) branch swapping on these trees with MAXTREES set to 10,000 was used to construct a strict consensus tree. If all the clones from one individual were supported as monophyletic or as components of the same polytomy, a representative sequence showing the fewest autapomorphies was chosen for all further analyses. For two cases, the ITS of African *C. dubius* and the ETS of *C. pulverulentus* from the Osa Peninsula of Costa Rica, the sequenced clones composed two distinct clades, and a representative clone with the fewest autapomorphies was chosen from each. To construct a combined ITS/ETS data set, each of the two divergent clones was paired with the same sequence from the other locus, so that those accessions were represented twice in the data set and resulting trees. Congruence of phylogenetic signal among the ITS and ETS data sets was assessed using the partition homogeneity test (Farris et al. 1994) as implemented in PAUP*, with 100 replicate data partitions and using heuristic searches with 10 random stepwise addition sequences each.

Parsimony analysis was conducted for the combined data set with 1000 random sequence addition replicates and 20 best trees held per iteration. The best trees found during the preliminary search were subjected to a second round of tree bisection-reconnection branch swapping with MULTREES = on and MAXTREES = 40,000. A consensus tree was constructed from the resulting most parsimonious trees. Clade support was estimated using bootstrap values (Felsenstein 1985), which were calculated using 1000 heuristic search replicates (10 random additions each, MAXTREES = 20). Maximum likelihood estimates of phylogenetic relationships were also made using a TVM+G model of sequence evolution with four rate categories and a shape parameter for the gamma distribution identified by hierarchical likelihood ratio tests implemented in MODELTEST version 3.06 (Posada and Crandall 1998). Maximum likelihood analysis was implemented in PAUP* for the combined data set (without indels) using a heuristic search with 10 random sequence addition replicates.

To explore the evolutionary history of pollination syndromes, ancestral states were reconstructed using parsimony in MacClade version 4.06 (Maddison and Maddison 2001). We attempted to determine both the location of shifts in pollination syndrome on the tree as well as estimate the total number of shifts that have occurred in the Neotropics. In order to avoid overestimating the number of shifts in pollination syndrome, trees were first pruned so that each species or variety was represented only once. Although different samples of *C. wilsonii* and *C. varzearum* were found in different parts of the tree, only one accession of each was used in the pollination analysis. In both cases, the choice was arbitrary and did not affect the analyses. Each species was scored as either bee or hummingbird pollinated based on pollinator observations or pollination syndrome, which has been shown to be a good predictor of actual pollinators in subgenus *Costus* (Kay and Schemske 2003). Ancestral character states were reconstructed on the consensus tree using parsimony. Unresolved nodes were assumed to be soft polytomies so that MacClade's resolution of them in the way most parsimonious for the pollination character would yield a conservative estimate of the number of shifts.

In our estimates of how many shifts in pollination have occurred, there are two major sources of error: uncertainty in tree topology for the taxa sampled and incomplete sampling (35 out of the *c.* 51 Neotropical species of subgenus *Costus*). To account for uncertainty in tree topology, the number of steps in the pollination character was calculated for each of the trees produced by bootstrapping the combined data set in PAUP 400 times, with 10 random addition sequences and 1000 best trees saved per replicate. These values were then used to calculate a mean number of shifts and a 95% confidence interval. Bootstrap trees were used instead of most parsimonious trees from the original data set in order to consider a wider range of possible tree topologies. To explore the effects of incomplete species sampling, we randomly removed species from the parsimony consensus tree before calculating the number of shifts in pollination. Species were removed in multiples of five, with 100 replicates at each level of species removal. The replicates were used to calculate means and 95% confidence intervals at each level of species sampling. These analyses were conducted in Mesquite version 1.0 (Maddison and Maddison 2003) using the TreeFarm module. We then regressed the number of estimated shifts on the number of taxa sampled, and used this relationship to roughly predict how many shifts in syndrome might be reconstructed with full sampling of all Neotropical species.

To reconstruct the biogeographic history of subgenus *Costus*, trees were pruned so that each species or variety was represented only once. Species were coded according to the following phytogeographic regions: Africa, Central America, the Northern Andes, the Southern Andes, the greater Amazon basin, and the West Indies. Ancestral states for phytogeographic region were reconstructed in MacClade. We were particularly interested in determining the continent of origin of the entire subgenus and the specific biogeographic origin of the Neotropical radiation. Therefore, we examined the sensitivity of the reconstruction of these nodes to both uncertainty in tree topology and the addition of unsampled species that clearly belong in subgenus *Costus*. The inferred character states of the basal nodes of the subgenus and of the Neotropical radiation were examined across all the trees from the bootstrap analysis of pollination syndrome. We also coded 16 unsampled Neotropical species for phytogeographic region and attached them at random to Neotropical branches of the strict consensus tree using the TreeFarm module of Mesquite. With 1000 replications of this tree augmentation, we were able to examine possible phylogenetic scenarios that would change our inference for the biogeographic origin of the Neotropical species.

A test for evolutionary rate constancy across subgenus *Costus* for ITS1 and ITS2 was conducted by using a tree-wide likelihood ratio test for differences between clock-constrained and clock-unconstrained trees (Felsenstein 1988). Likelihood scores were obtained using a HKY+G model of sequence evolution (as chosen by MODELTEST for

ITS 1 and ITS 2) for the strict consensus tree identified through parsimony analysis of the combined data set. The likelihood ratio test statistic (twice the difference in likelihood scores between the clock-constrained and unconstrained trees) was compared to a Chi-square distribution with $s - 2$ degrees of freedom, where s equals the number of sequences in the analysis (Huelsenbeck and Crandall 1997). Clock-like evolution of the ITS allows the possibilities of roughly dating nodes in the phylogenetic tree and calculating a diversification rate based on published rates of ITS substitution. Independently calibrated and published ITS substitution rates from across angiosperms range from $0.38 \text{ X}10^{-9}$ to $8.34 \text{ X } 10^{-9}$ subs/site/year, with a mean of $2.86 \text{ X } 10^{-9}$ subs/site/yr (95% CI: $2.10 \text{ X } 10^{-9}$—$3.57 \text{ X } 10^{-9}$ subs/site/yr; Appendix B). Rates from herbaceous lineages, which are significantly faster than those from long-lived woody plants, range from $1.72 \text{ X}10^{-9}$ to $8.34 \text{ X } 10^{-9}$ subs/site/year, with a mean of $4.13 \text{ X } 10^{-9}$ subs/site/yr (95% CI: $2.35 \text{ X } 10^{-9}$—$6.21 \text{ X } 10^{-9}$ subs/site/yr; Appendix B). We used both the overall mean and the herbaceous mean, with their associated 95% confidence intervals, to roughly date nodes of interest in *Costus* subgenus *Costus* on the clock-constrained tree and to calculate a diversification rate for Neotropical species. A per-lineage rate of diversification was calculated as $[\ln N - \ln N_0]/T$, where N is the current species diversity, N_0 is the starting diversity, and T is the inferred age of the clade (Baldwin and Sanderson 1998). In order to conservatively estimate maximum ages and minimum diversification rates, all nodes dated in our analysis were stem group nodes that gave rise to the most recent common ancestor of the clade of interest, as opposed to crown groups.

RESULTS

The total aligned length of the ITS region was 715 bp, including 290 bp for ITS1, 164 for 5.8S and 261 bp for ITS2, and the aligned ETS region was 580 bp. Forty-seven indels were scored, 24 from ITS and 23 from ETS. The overall dataset included 1342 characters, 230 of which were informative for parsimony. ITS and ETS substitutions yielded 72 and 132 informative substitutions, respectively, while 26 of the indels were informative. Excluding the outgroups reduced the number of informative characters within subgenus *Costus* to 135.

The partition homogeneity test showed that the ITS versus ETS partitions of the data were not significantly different from random pairwise partitions ($p = 0.71$). Strict consensus trees based on separate parsimony analysis of ITS and ETS were examined for hard topological differences, and none were found. All further analyses were conducted on the combined total dataset. Preliminary analyses of the ITS and ETS datasets also included all sequenced clones for polymorphic taxa. All clones from a given individual were resolved as monophyletic or as part of the same polytomy except for the ITS of *C. dubius* and the ETS of *C. pulverulentus* from the Osa Peninsula of Costa Rica. Clones were picked for further analyses according to the guidelines stated in the methods.

Parsimony analysis failed to identify a single most parsimonious tree, and the search was limited to saving 40,000 most parsimonious trees which all had a length of 605, consistency indices of 0.826, retention indices of 0.881, and rescaled consistency indices of 0.728. The strict consensus tree with bootstrap support is presented in Figure 3. Many nodes were resolved with high support. The Neotropical species within subgenus

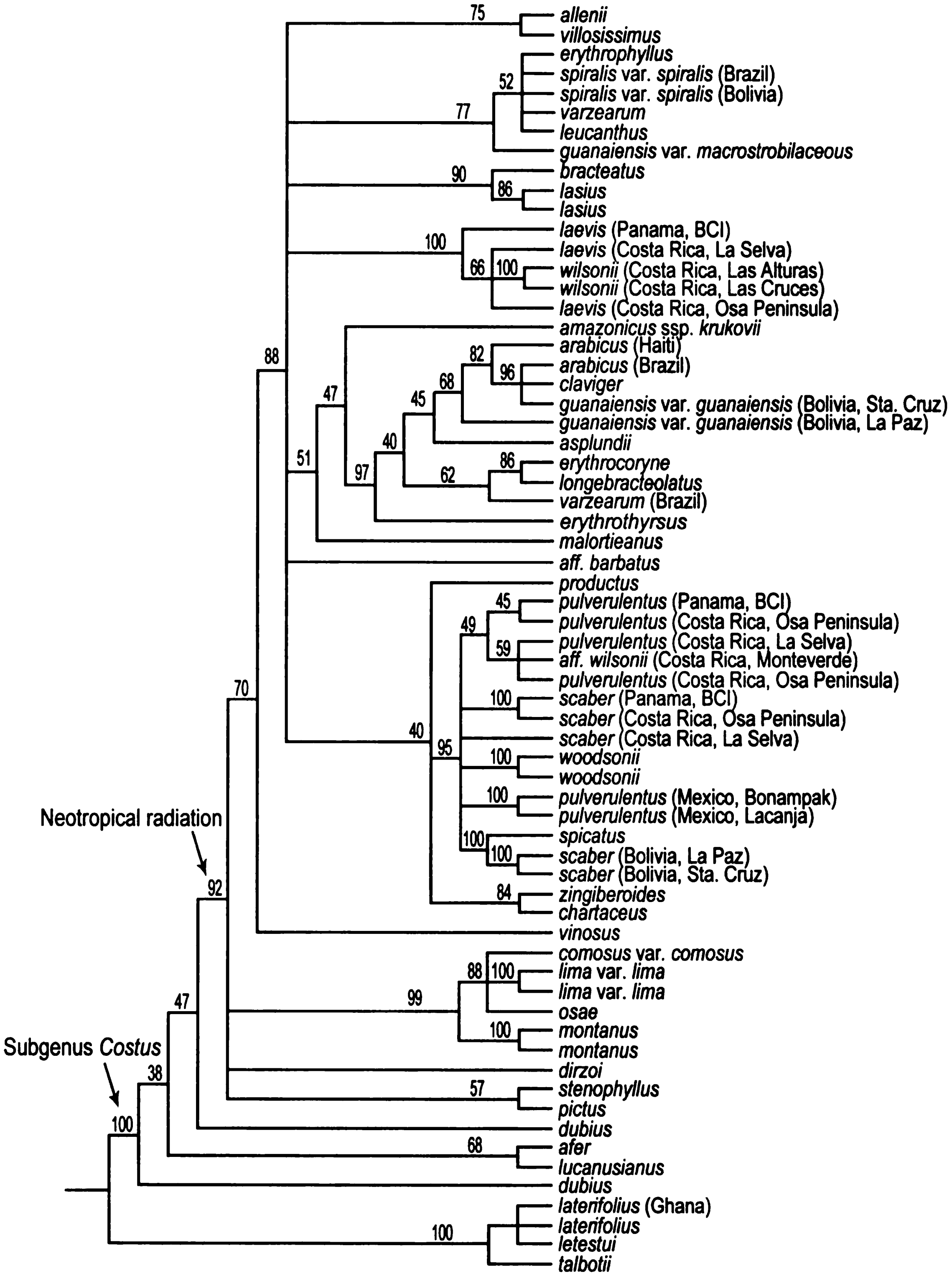

Figure 3. Strict consensus of all most parsimonious trees from the combined analysis of *Costus* ITS and ETS nrDNA sequences. Numbers above the branches indicate support based on 1000 bootstrap replicates.

Costus were supported as monophyletic and nested within the African taxa. Multiple populations sampled for a given species were often not monophyletic, and were typically part of a multi-species polytomy. Maximum likelihood analysis of the full dataset produced two best trees that were topologically equivalent to the consensus tree from the parsimony analysis and are not presented.

Hummingbird pollination has evolved multiple times since subgenus *Costus* colonized the Neotropics. Figure 4 shows the parsimony reconstruction of pollination syndrome on the strict consensus tree. Species for which there are documented pollinator observations are denoted with asterisks. Across all the most parsimonious trees from the bootstrapped combined dataset, either eight or nine shifts are reconstructed (mean = 8.52, median = 9; Figure 5). Because polytomies are considered soft, they are always resolved to require the fewest changes in pollination. For example, *C. aff. barbatus* is resolved to share hummingbird pollination with the clade including *C. pulverulentus* by descent; however, there are no molecular synapomorphies to support this resolution. Subsampling the taxa for removal from the tree suggests that incomplete taxon sampling has a large effect on the estimated number of shifts in pollination syndrome, and that the actual number of shifts occurring could be substantially higher than eight or nine (Figure 5). For the subsampled data sets, we found the number of taxa sampled to be a strong predictor of the estimated number of shifts in pollination syndrome. The linear regression predicts an additional estimated shift in pollination for approximately every five species sampled (number of shifts = 0.199 * number of sampled taxa + 0.57; Figure 5). The hummingbird pollination syndrome is supported as a derived character state from bee pollination, and there is no strong evidence for any reversals, although on some bootstrap trees the

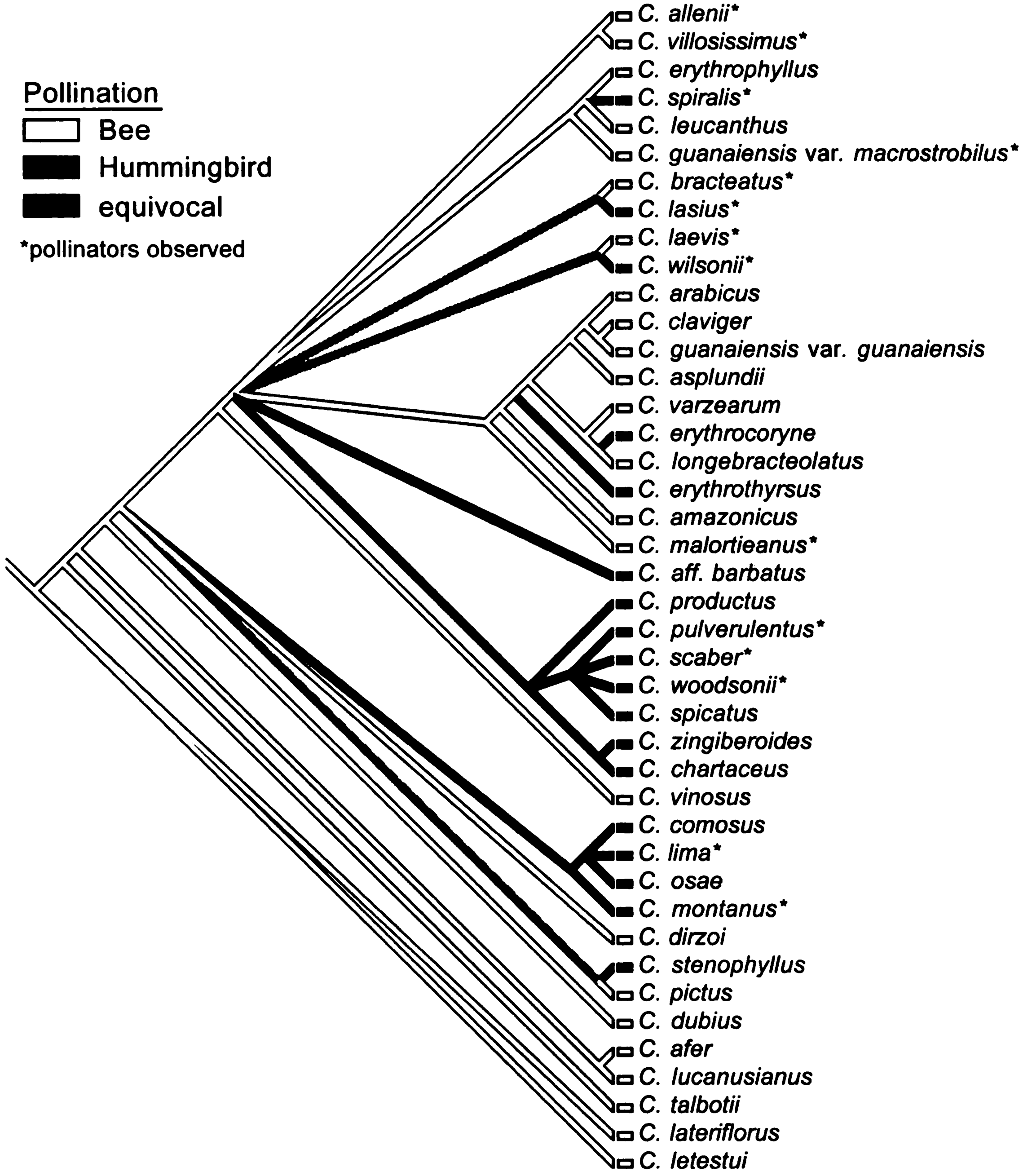

Figure 4. Parsimony reconstruction of ancestral pollination syndromes on the strict consensus tree. Asterisks denote species for which pollinators have been observed.

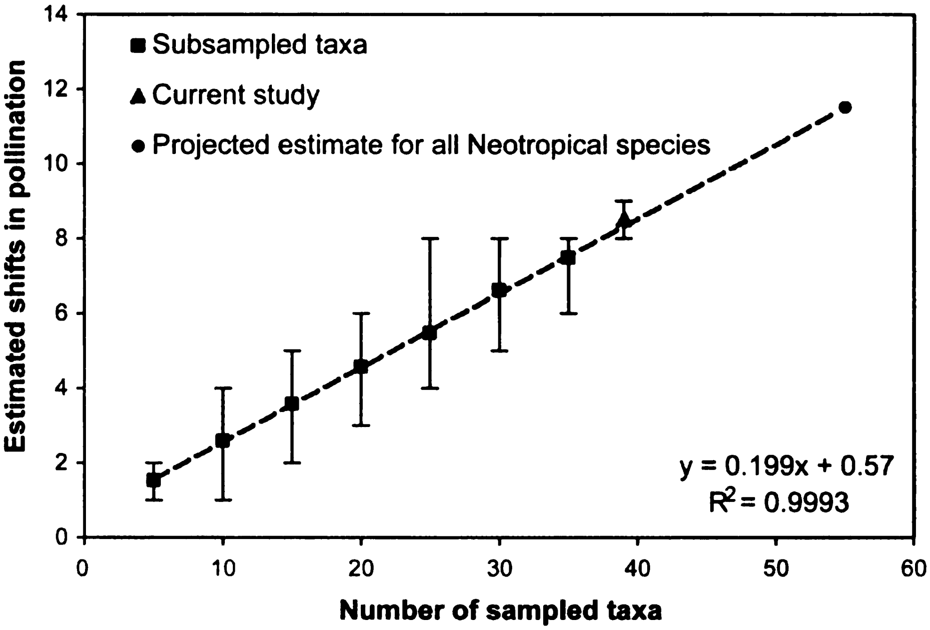

Figure 5. Estimates of the mean number of shifts in pollination syndrome within subgenus *Costus* for various levels of species sampling. For the 38 species included in the current study, the estimate was made across all most parsimonious trees constructed from the bootstrap data sets used in the analysis for Figure 3. Error bars represent the 95% CI of the bootstrap means. For 35 taxa and less, estimates were made by randomly dropping species from the consensus tree. Error bars represent the 95% CI across 100 replicates at each sampling level. For the subsampled taxa, the relationship between the number of taxa sampled and the number of pollination shifts was estimated by linear regression and used to predict the number of shifts for all 54 Neotropical taxa, including 16 unsampled species.

branches leading to differing terminal taxa are reconstructed as equivocal for the pollination character state.

Reconstructing the biogeographic history of the subgenus suggests that Central America was colonized from Africa, with subsequent dispersal to the Andes, the Amazon and the Caribbean (Figure 6). Across all trees from the bootstrapped datasets, the African taxa are resolved as basal to the Neotropical clade, and the base of the Neotropical clade is resolved as Central America. This second result is robust to most topological

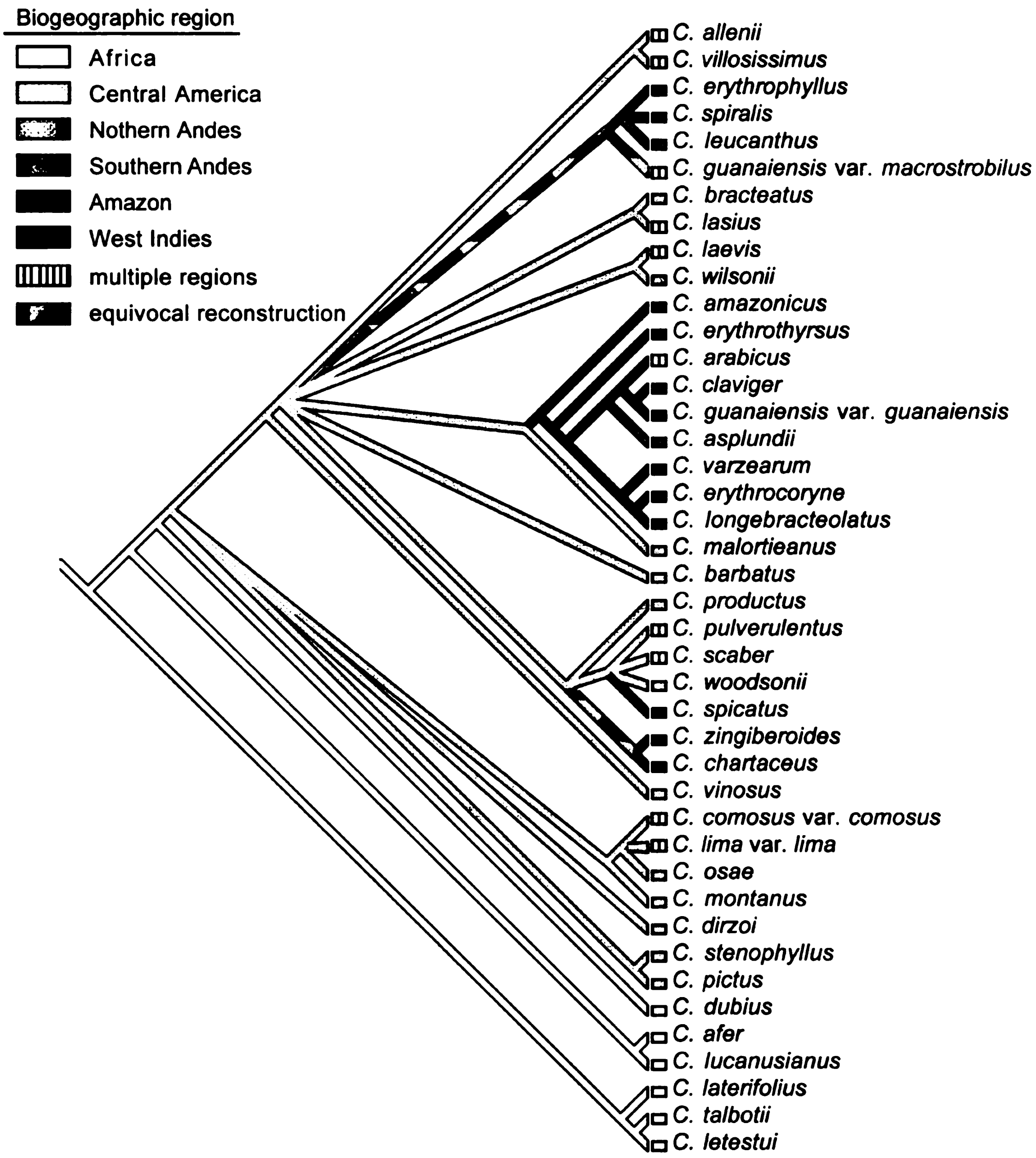

Figure 6. Parsimony reconstruction of biogeographic distribution on the strict consensus tree.

arrangements of unsampled species on the strict consensus tree. The unsampled species included only three from the Amazonian phytogeographic region; the other 13 were either Central American or Andean. Out of 1000 tree augmentation replicates, the origin was resolved unequivocally as Central American 835 times, as Northern Andean 9 times and as Amazonian 2 times. In 154 replicates, the origin was equivocal, but always included Central American as one of the possible states.

Rates of evolution of ITS 1 and 2 within *Costus* were found to be consistent with a molecular clock ($\chi^2 = 51.393$, df = 63, p = 0.85). Maximum likelihood branch lengths show 0.0115 substitutions per site in ITS since the basal divergence between Africa and the Neotropics. This suggests an approximate date for that node of 4.0 mya (95% CI: 3.2—5.5 mya) using the mean of published ITS substitution rates, or 2.8 mya (95% CI: 1.9—4.9 mya) using the mean of ITS rates from herbaceous lineages. Assuming that all *c.* 51 Neotropical species of subgenus *Costus* are monophyletic, including the 16 known but unsampled species, a diversification rate of 0.97 species per million years (95% CI: 0.72—1.22) is estimated from the date for the basal split from African subgenus *Costus* using the overall mean of ITS rates, or 1.41 species per million years (95% CI: 0.80—2.12) using the mean of herbaceous rates. The earliest dispersal to South America is similarly estimated at 3.1 mya (95% CI: 2.5—4.2 mya) using the mean of all ITS rates or 2.2 mya (95% CI: 1.4—3.8) using the mean of herbaceous rates.

DISCUSSION

Phylogenetic analysis and taxonomic considerations—ITS and ETS rDNA sequences proved to be a useful tool for estimating the phylogenetic relationships within *Costus* subgenus *Costus*. Our results are entirely consistent with the broad phylogenetic analysis of the Costaceae (Specht et al. 2001), but provide much more detail for the Neotropical species radiation. As expected with a rapid diversification, not all nodes were resolved even with these relatively fast evolving loci. The New World radiation is supported as monophyletic and nested within the basal African taxa. The result was not surprising, given that there are no species that naturally occur on both continents and that the frequency of intercontinental dispersal is likely to be low. Our study does not clarify the sister species to the Neotropical radiation, because the ITS alleles from the accession of *C. dubius* are polyphyletic, and because of the incomplete sampling of African species. Additional genetic loci and more thorough taxon sampling will be necessary to address that question.

For the species in the study, we often found divergent sequences when more than one accession was sampled. In some cases, accessions from distinct geographic areas were distantly related and we suggest that they may represent independent evolutionary lineages that have converged on a similar phenotype. The varieties of *C. guanaiensis*, for example, deserve further evaluation for elevation to species status, as does the *C. aff. wilsonii* from the Monteverde region. For both of these cases, multiple individuals were sequenced to exclude the possibility of laboratory error, and sequences were found to be identical within a given site (results not presented). For *C. varzearum*, the geographic origin of one of the divergent accessions was unknown, so the results should be interpreted with caution. In other cases, we found that different populations of a species yielded divergent sequences that were not monophyletic but that stemmed from the same polytomous node. For example, our extensive sampling of the geographically widespread *C. pulverulentus* and *C. scaber* resulted in a large polytomy, within which were nested *C. spicatus*, *C. woodsonii*, and the Monteverde accession of *C. aff. wilsonii.* A cautious interpretation is that these five species are very recently diverged, and for such closely related species, multiple unlinked loci and more thorough population sampling would be required to better estimate the evolutionary history of diversification. Another wrinkle we uncovered was the existence of divergent ITS or ETS alleles within a single individual. It is unlikely that these were pseudogenes, since they did not have mutations in the conserved 5.8S region, and DMSO was added to all PCR to increase primer specificity. Other explanations include incomplete concerted evolution, minor replication errors perpetuated during PCR, or gene flow between divergent populations.

Pollination systems—Our reconstruction of ancestral pollination states suggests that changes in pollination biology have been a prominent feature in the Neotropical radiation of *Costus* subgenus *Costus*. Although there is almost no information on the pollination systems of African subgenus *Costus*, they exhibit floral traits characteristic of bee pollination, all possessing white tubular flowers with pink and/or yellow nectar guides and green floral bracts. In the Neotropical clade, the majority of the species exhibit a hummingbird pollination syndrome, and the bee-pollinated species for which there are pollinator observations have further specialized on orchid bees (Kay and Schemske 2003). Neither hummingbirds nor orchid bees occur in Africa, so all Neotropical species likely exhibit derived pollination systems, although the characters involved in hummingbird pollination are especially distinct, including bright reds and yellows, tubular curved corollas, and a reduction in the labellum that bee pollinators use as a landing platform. Our parsimony reconstructions suggest that there have been at least eight shifts in pollination syndrome in the Neotropics and that hummingbird pollination is a multiply derived character state. Although some nodes are reconstructed as equivocal, there is no strong support for any reversals to bee pollination. This direction of change in pollination is consistent with the wide floral phenotypic diversity among the hummingbird pollinated species, but relative consistency in floral traits among the bee pollinated species (c.f. Kay and Schemske 2003). All but three of the unsampled Neotropical species exhibit a hummingbird pollination syndrome, and our exploration of the effects of incomplete taxon sampling suggest that there may be as many as 11 shifts out of 51 species likely belonging to the Neotropical clade. It is possible to calculate a per speciation "hummingbird pollination shift" rate as the number of independent evolutions of hummingbird pollination divided by the number of speciation events. Ignoring extinction and assuming that all polytomies in the phylogeny are soft, the number of speciation events is the current number of species in the clade minus one. For *Costus* subgenus *Costus*, with at least eight independent evolutions across 35 sampled species, the rate is 0.24. In other words, approximately a quarter of the speciation events have involved a shift to hummingbird pollination. This is a high rate compared to other plant lineages that have been studied. For comparison, we calculated hummingbird pollination evolution rates in some other groups that have multiple shifts reported. We found a similarly high rate of 0.33 for *Mimulus* sect. *Erythranthe* (Beardsley et al. 2003), but only 0.11 for *Erythrina* (Bruneau 1997) and 0.03 for Sinningieae (Perret et al. 2003).

In *Costus*, these shifts in pollination syndrome are concentrated in the species of Central American or Andean distribution, suggesting that proximity to montane environments is associated with the evolution of hummingbird pollination. Hummingbird diversity is centered in the Northern Andes, and comprises hundreds of species. The major hummingbird lineages diversified in the Miocene and now comprise over 300 species (Bleiweiss 1998). Thus, there were many potential hummingbird pollinators available when subgenus *Costus* arrived in the Neotropics.

Biogeography and diversification rates— Our dating based on a molecular clock suggests that the rapid speciation in *Costus* subgenus *Costus* coincided with important climatic and geological events in Central America and Northern South America, notably the uplift of the Northern Andes from about 5 mya, the closing of the Panama isthmus at about 3.5 mya, and the dramatic climatic oscillations from warmer and wetter to cooler and drier that were frequent during the Pleistocene epoch. Although fossil evidence is lacking for Costaceae, we use a molecular clock derived from published ITS substitution rates to indirectly estimate the arrival date of *Costus* in the Neotropics at 1.9 to 4.9 mya (using the mean of the herbaceous rates). Even the lower limit of the 95%CI for all ITS rates dates the basal divergence between Africa and the Neotropics at 5.5 mya, near the beginning of the Pliocene, and suggests long distance intercontinental dispersal instead of ancient vicariance (>95 mya) or even Miocene dispersal across tropical Laurasian habitat (Davis et al. 2002). The reconstruction of ancestral biogeography strongly supports Central America as the original site of the Neotropical diversification. The earliest estimated date of spread to South America was 1.4 to 3.8 mya (using herbaceous rates) or 2.5 to 4.2 mya (using all rates), both or which are in line with the closing of the Panama isthmus at approximately 3.5 mya. Although all these dates should be considered rough estimates because of the lack of an independent fossil calibration within *Costus*, rates of ITS evolution in *Costus* would have to be as slow as the slowest known rates (from *Hamamelis* and Winteraceae) to alter our conclusion that Neotropical *Costus* subgenus *Costus* is a recent radiation. Recency of divergence is further supported by the ease with which fertile hybrids can be made between many of the Neotropical species in greenhouse crosses (DWS and KMK, unpubl. data). Our diversification rates of 0.72 –

1.22 species per lineage per million years estimated from the mean of published ITS rates suggest that *Costus* subgenus *Costus* has diversified at a rate comparable to the fastest known plant radiations. For example, the Hawaiian silversword alliance has been estimated at 0.56 ± 0.17 species per lineage per million years (Baldwin and Sanderson 1998), the paleotropical shrub genus *Gaertnera* (Rubiaceae) at 0.717 - 0.832 (Malcomber 2002), and the South African ice plants (Aizoaceae) at 0.77 – 1.75 (Klak et al. 2004).

Diversification in the Neotropics— Our results suggest a recent and rapid radiation in the Neotropics that has gone hand in hand with changes in specialized pollination systems, a key part of Gentry's hypothesis for the diversification of the Central America and Northern Andes-centered Neotropical flora. Questions remain about how these striking shifts in floral traits have occurred, and how they may have contributed mechanistically to speciation. We propose the following scenario for the diversification of *Costus*. African species in subgenus *Costus* have large flowers with a bee pollination syndrome, and the ancestor of the Neotropical radiation quickly adopted orchid bees for pollination. Orchid bees are prominent and species rich in lowland Neotropical rainforest, relatively large in body size, and are known to travel between isolated nectar-rich plants on their long distance foraging routes (Cameron 2004). Climatic fluctuations and geological uplift caused range shifts and occasional isolation of *Costus* populations along the slopes and valleys of the Central American cordillera and Northern Andes. In the tropics, even relatively minor topological features can be effective dispersal barriers (Janzen 1967). Isolated populations at higher elevations adapted to the local hummingbird fauna for pollination, since bees are poor fliers in the cool wet weather common at higher elevations in the tropics, while hummingbirds remain highly active (Cruden 1972). Intermediate floral forms are missing in *Costus*, suggesting that the shifts to hummingbird pollination occur relatively quickly or involve a limited number of genetic changes. In times of climatic cooling, these newly hummingbird-adapted lineages could have migrated to lower and warmer areas, but would have been substantially reproductively isolated from their bee-pollinated relatives. Shifts in specialized pollination systems are well known to contribute to reproductive isolation (Fulton and Hodges 1999; Grant 1994b; Schemske and Bradshaw 1999), and in *Costus*, pollinator specialization is sufficient to prevent most potential pollen flow between sympatric species that differ in syndrome (Kay and Schemske 2003). Climatic oscillations occurred repeatedly as the Andes continued their spectacular rise, creating many opportunities for allopatric speciation and secondary range expansions (Graham 1997). Of course, not all speciation events in *Costus* were accompanied by a dramatic shift in pollination syndrome, but with such a young radiation, it is likely that strong prezygotic isolation maintains the distinctiveness of sympatric species, whether by floral specialization within a pollination syndrome or other ecological differences acquired in such an environmentally heterogeneous landscape.

Putative sister species differing in pollination syndrome suggest that this process may be continually occurring. For example, the low elevation *C. bracteatus* is supported as sister to the cloud forest *C. lasius* (Figure 3), and pollinator observations show that the former is visited exclusively by euglossine bees while a mix of hummingbirds visits the latter. Similarly, *C. wilsonii* is a rare species, restricted to a single region of cloud forest in the Central Cordillera of Southern Costa Rica, and we find its nrDNA alleles nested within the alleles of geographically widespread lowland *C. laevis* (Figure 3). *Costus* *laevis* is primarily pollinated by euglossine bees, with occasional hummingbird visits, while only hummingbirds have been seen at *C. wilsonii* (Kay and Schemske 2003).

Although this scenario for the Neotropical *Costus* radiation is speculative, it integrates much of what is known about pollination biology and the climatic and geologic history of the region with the results from our phylogenetic analysis. Furthermore, it provides a general hypothesis for the diversification of many herbaceous, epiphytic, or shrubby lineages in the region – lineages that altogether make up a substantial portion of Neotropical floristic diversity. Perhaps many of these lineages of relatively short-lived plants have been able to rapidly diverge ecologically, especially in response to the local biotic assemblage, when isolated by rapidly changing topological and climatic barriers, while many longer-lived trees and lianas diversify gradually across the vast and comparatively uniform Amazon basin. Many more time-referenced species-level phylogenies are needed from a diversity of taxa, but this possibility suggests that the tropics may be both a museum of plant diversity, and a cradle for recent and "explosive" speciation involving diversification in plant-pollinator relationships.

CHAPTER 3

Strong and asymmetrical prezygotic reproductive isolation between the hummingbird-pollinated Neotropical spiral gingers *Costus pulverulentus* and *C. scaber* (Costaceae)

Abstract—The process of speciation involves the evolution of barriers to gene flow, and empirical estimates of the relative importance of different isolating mechanisms between recently diverged species are important to the understanding of speciation. I investigated the factors contributing to reproductive isolation between *Costus pulverulentus* and *C. scaber* (Costaceae), two closely related understory Neotropical herbs. I studied broad-scale geographic isolation, microhabitat isolation, 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). 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 postmating pollen-pistil incompatibility were important. Overall, prezygotic barriers were found to be strong, resulting in 100% reproductive isolation for *C. pulverulentus* and 99.2% reproductive isolation for *C. scaber*. No significant postzygotic isolation was identified. These results suggest that ecological factors, including habitat use and plant-pollinator interactions, contributed to speciation in this system, and have evolved before any strong postzygotic isolation.

Keywords: plant speciation, hummingbird pollination, tropical biology, pollen-pistil interactions, floral isolation

Speciation is fundamental to biological diversification, and the evolution of traits that confer reproductive isolation is central to the speciation process. Understanding the types of barriers that contribute to reproductive isolation will better elucidate the conditions under which speciation is likely to occur and the role of natural selection in speciation, and 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 and mating discrimination (Grant 1981; Mayr 1963), while postzygotic barriers include hybrid inviability, infertility (Dobzhansky 1937; Muller 1942) or ecological inferiority (Rundle et al. 2000; Schluter 2000). Reproductive isolation is generally thought to evolve as an incidental consequence of phenotypic and genotypic divergence in allopatry, yet much of our knowledge of isolating mechanisms come 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 cessation of gene flow between related taxa.

A major challenge for the study of speciation is that once speciation has occurred, ecological and morphological differences between the species continue to accumulate and it becomes difficult to identify which differences were important during species formation. However, while barriers continue to accumulate, it is less likely that a mechanism important during the initial stages will be reversed. Thus a thorough survey of mechanisms maintaining reproductive isolation between close relatives will likely identify those involved in the initial stages of speciation. Although the relative contributions of various mechanisms to reproductive isolation could have changed since speciation, by studying incipient or closely related species this problem can be eliminated or greatly reduced.

The evolutionary literature is filled with studies of isolating mechanisms, yet few have systematically explored the contribution of possible mechanisms to total isolation for any pair of taxa, potentially skewing our view of the relative importance of various stages (Chari and Wilson 2001; Ramsey et al. 2003). In nature, isolating mechanisms act sequentially, so that a given barrier can only reduce potential gene flow not precluded by earlier acting barriers. Isolating mechanisms may also operate asymmetrically, preventing gene flow in only one direction between taxa (Arnold et al. 1996; Coyne and Orr 1998; Levin 1978; Tiffin et al. 2001), and therefore it is important to evaluate each direction separately.

Plants provide an excellent study system for the evolution of reproductive isolation because they can be relatively easy to study in nature and to manipulate in a laboratory or greenhouse setting. Furthermore, plant-pollinator interactions have received considerable attention as mechanisms of prezygotic reproductive isolation in plants (Grant 1981; Grant and Grant 1965; Hiesey et al. 1971; Kay and Schemske 2003; Levin 1978; Schemske and Bradshaw 1999; Stebbins 1974). Still, their importance to speciation is controversial because specialization in plant-pollinator relations has been questioned (Ollerton 1996;

Waser 1998; Waser et al. 1996). Different pollination syndromes have been shown to confer reproductive isolation among closely related sympatric species in several cases (Fulton and Hodges 1999; Grant 1994b; 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; Grant 1994b).

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 and densities, habitat patchiness, the strength and variability of selective pressures, 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 (Ashton 1969; Dobzhansky 1950; Fedorov 1966; Givnish 1999; Rapoport 1982; Schemske 2002). Furthermore, the Neotropical forests specifically have been suggested to be a hotbed of speciation, yet very little is known about the nature of reproductive isolation for the many species radiations that have been identified (but see Kress 1983; McDade 1984; Stiles 1975).

Here I examine mechanisms of reproductive isolation for a pair of closely related pollinator-sharing Neotropical rainforest herbs. I quantify sequential stages of isolation, including broad-scale geography, microhabitat differences, flowering phenology, overlap in pollinator species, floral constancy, mechanical floral isolation, pollen-pistil interactions, interspecific seed set, and the seed germination, greenhouse survival, and pollen fertility of F1 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* 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 very closely related, and part of a larger Neotropical species radiation of *Costus* subgenus *Costus* characterized by rapid and recent diversification. 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 (approx. 5 cm vs. 3 cm), more open, and less sharply decurved than the flowers of *C. scaber*. The flowers of *C. pulverulentus* also have exserted anthers and stigma, while those of *C. scaber* are inserted. *C. pulverulentus* and *C. scaber* both exhibit traits typical of a hummingbird pollination syndrome, and this is reflected in their floral visitors (Kay and Schemske 2003). 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*.

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 (http://mobot.mobot.org/W3T/Search/vast.html) and at the Chicago Field Museum. Only one specimen from a particular site was analyzed. Elevation was recorded from 224 specimens of *C. pulverulentus* and 214 specimens of *C. scaber*. Latitude and longitude were recorded from 233 specimens of *C. pulverulentus* and 185 specimens of *C. scaber*. Differences in elevation were tested with a nonparametric Mann-Whitney U test. Broad-scale differences in spatial distribution were examined following the methods of Ramsey et al. (2003), in which virtual quadrats were used to examine co-occurrence of the species. The number of quadrats with incidents of co-occurrence was compared between the natural distribution and artificial distributions in which geographic coordinates were randomly assigned to species, maintaining the same sample sizes as the original data set. Simulations were run with a variety of quadrat sizes, 50 randomizations per quadrat size, and 100,000 virtual quadrats per randomization. I compared co-occurrence in the natural and artificial simulations with a Mann-Whitney U test.

All other components of reproductive 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).

Microhabitat Isolation in Sympatry

I examined 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, California) on the station's Geographical Information System (GIS). Only flowering or fruiting individuals were mapped, as vegetative individuals can not 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*. Computer simulations of virtual quadrats were used to determine the extent of spatial isolation with the natural distribution of plants. Differences in abundance and clumping between the species can lead to asymmetries in the effects of spatial isolation on potential pollen flow; therefore spatial isolation was calculated separately for each species. I explored a variety of quadrat sizes, and simulated 100 sampling bouts of 10,000 quadrats each per size category. For each species within a sampling bout, 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 individuals of that particular species but none of the other species ("conspecific quadrats"). Differences were tested with a Wilcoxon paired sample test.

Phenological isolation

When in flower, individuals of *C. pulverulentus* and *C. scaber* typically produce a single inflorescence at a time, producing approximately one flower per day over an extended period. Each flower opens at dawn and drops off or wilts by mid-afternoon. These species are known to flower in the wet season at La Selva, with peak flowering occurring May through August (Stiles 1978). 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 number of flowering individuals throughout the reserve on a regular basis. Instead, I estimated the flowering span of individual inflorescences using the sequential order of flowering that occurs in a spiral up the inflorescence. Early in the wet season, I located as many developing or mature inflorescences as possible, and for those in flower, marked the bract subtending the current day's flower. Marked plants were revisited in approximately two weeks, 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—C. pulverulentus and *C. scaber* share their primary pollinator, the Long-tailed Hermit hummingbird (*Phaethornis superciliosus*), at La Selva, Sirena, and BCI (Kay and Schemske 2003). Observations were made in 1998-2000 at La Selva, 1998-1999 at BCI, and 2002 at Sirena, for a total of 511 hours for *C. pulverulentus* and 519 hours for *C. scaber*. *Costus pulverulentus* is exclusively pollinated by this species, while *C. scaber* also is visited by the hummingbirds *Amazilia tzacatl* and *Thalurania columbica* and rarely by orchid bees (*Euglossa* sp.). Most of the variation in the identity of floral visitors to *C. scaber* was among individual plants, and not among sites or years. Therefore, to quantify overall isolation due to differences in pollinator species for each plant, I calculated the proportion of the total visitation rate (visits per flower per hour) contributed by the shared pollinator species, *P. superciliosus*. Across all site/year combinations of observations reported in Kay and Schemske 2003, 44 individuals of *C. scaber* were observed. Visitation rates were generally low (<< one visit per flower per hour), and plants without any legitimate pollinator visits (N = 10) were excluded from the calculations.

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, and so 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. Video cameras set up at isolated plants were used to track their individual foraging routes.

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 with colored dyes. At La Selva in 2000 and 2001, I grew plants of each species in the shadehouse and put out mixed arrays of potted plants. This allowed me to better control the relative numbers and that spatial arrangement of flowers than would have been possible using the natural distribution of plants. Over the course of two flowering seasons, I rotated the experimental arrays through seven different sites in the primary or mature secondary forest, in order 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 2-3 days at a site to allow the hummingbirds to discover and become accustomed to them. Flowers at each array were marked with unique randomly assigned colors at dawn, and the stigmas examined for colored powder 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 a dye flow index that took into account both the percent stigma coverage and relative concentration of dye, standardized by the number of open flowers of a particular type on that day. 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. The index of dye deposition was calculated as $(P*C)/N$, 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. Intraspecific and interspecific dye flow indices per stigma were compared with a Wilcoxon paired-sample test.

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 for intra- and interspecific dye deposition 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 of dye. In total, I marked 38 flowers of *C. pulverulentus* and 35 of *C. scaber*.

Postmating 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 raised to flowering for crossing studies. Sample sizes were as follows: 15 individuals from across 6 different maternal families for La Selva *C. pulverulentus*, 9 individuals from 6 families for La Selva *C. scaber*, 15 individuals from 4 families for BCI *C. pulverulentus*, and 9 individuals from 7 families for BCI *C. scaber*. For the plants from each site, I sought to compare 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 was dropped from the study.

I determined postmating isolation by quantifying seed set per pollination, and to determine whether any differences in seed set were pre- or postzygotic, I further examined pollen germination and pollen tube growth with epifluorescent microscopy. Flowers were pollinated, and either left to set seed, or harvested after 2 hours (for pollen germination) or after 9 hours (for pollen tube growth). In the field, flowers typically open just before dawn and drop off in the mid- to late afternoon, so 9 hours represents the maximum time for pollen tubes to grow to the base of the style. All crosses were done in the morning, from 6 to 10 am, 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. Pollen was removed and applied with flat wooden toothpicks, and carried between plants in 1.7 mL microcentrifuge tubes. To control for any unintended pollen deposition, null pollinations were performed on several flowers per population with a clean toothpick. Harvested pistils were fixed in a solution of 3 parts 95% ethanol: 1 part glacial acetic acid for 24 hours, gently rinsed in distilled water, softened and cleared in 4 *M* NaOH for 24 hours, gently rinsed in distilled water, and stained in decolorized aniline blue (0.01% in .02 *M* K_3PO_4) for 24 hours, 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. For pollen germination, I counted the numbers of germinated and ungerminated pollen grains on the stigma after 2 hours. For pollen tube growth, I measured the length of the longest pollen tube, and the number of pollen tubes reaching the ovary.

Seed set for reciprocal crosses was examined separately for La Selva and BCI using two-way ANOVA. The main 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 by paternal species interaction. The nested maternal and paternal plant effects were used as sources of error for the maternal and paternal species effects, respectively, while 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 and for *C. pulverulentus* as maternal species for the BCI populations. Pollen germination and tube growth analyses were less straightforward for *C. scaber* from BCI as maternal parent. Unlike the other crosses that were all completed at University of Washington, over the time period that the BCI pollinations of *C. scaber* were examined, the plants were grown in three different greenhouses (University of Washington, Kellogg Biological Station, and Michigan State University) with unavoidable differences in water, fertilizer, temperature and humidity regimes. Furthermore, pollinations were examined with three different fluorescent microscopes with slightly different filter sets and lenses. To account for any effect of location, intra- and interspecific pollinations on each species were compared with ANOVA with main effects of paternal species, greenhouse/lab location, and a paternal species x location interaction. However, sample sizes were very small for any particular site.

Postzygotic Isolation

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. 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 hour light-dark cycle, with the temperature kept between 24° and 27° C. All the seeds from a particular fruit were sown at the same time; therefore, I calculated 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 was monitored.

Hybrid fertility

Percent pollen stainability was used as a proxy for hybrid male fertility, according to the methods of Kearns and Inouye (1993). Pollen samples were taken in the greenhouse from F1 hybrids and outcrossed lines of both *C. pulverulentus* and *C. scaber* from both the La Selva and BCI populations. For La Selva, only hybrids with *C. scaber* as a maternal parent were available, while for BCI, I sampled hybrids made in both directions. F1 hybrids with *C. pulverulentus* and *C. scaber* as maternal parent are denoted hereafter H(P) and H(S), respectively. Also, for BCI, no outcrossed lines of *C. scaber* were made, so pollen stainability was measured on the wild-collected plants. 2-4 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 hours, 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 hours 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.

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. In order 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.

RESULTS

Geographic Isolation

Both species are common in lowland forest and do not differ significantly in elevation (*C. pulverulentus*: mean = 464 m, range = 0 – 2860 m; *C. scaber*: mean = 404 m, range = 0 – 1500 m; Mann-Whitney U: $p = 0.75$). Although their elevational ranges do not completely overlap, only three of the 224 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 natural and randomized distributions showed that, regardless of quadrat size, *C. pulverulentus* and *C. scaber* co-occur less often than expected by chance. I varied quadrat size by steps of 10 km^2 from 10 km^2 to 150 km^2, and at each level differences were significant (Mann-Whitney U; $p < .001$). Following Ramsey (2003), I calculated the contribution of geography to reproductive isolation as:

$$RI_{geog} = 1 - \frac{\text{no. co-occurrences (natural distr. sim.)}}{\text{no. co-occurrences (random distr. sim.)}}$$

This index varies from zero for complete sympatry to one for complete allopatry. Across the 15 quadrat sizes (from 10 x 10 km to 150 x 150 km), the mean RI_{geog} varied only from 0.374 to 0.462. Therefore, for the overall calculation of reproductive isolation, I used the mean of 0.421 (95% CI: 0.335-0.466). To calculate the confidence interval around this mean, one of the numbers of natural co-occurrences and one of the numbers of random co-occurrences were drawn at random with replacement from within each quadrat size, and the RI_{geog} from each quadrat size used to compute an overall mean. This resampling procedure was repeated 1000 times, and the central 95% of the distribution of means taken as the 95% CI.

Microhabitat isolation

Although my search for individuals of both species was not systematic in sampling the entire forest, it suggests that *C. scaber* is more abundant than *C. pulverulentus*, but that 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 computer simulations of virtual quadrats show that the frequency of mixed species quadrats relative to single species quadrats increases with the size of the quadrat, as expected. To accommodate the large interplant distances, I used 100 m^2 as the minimum quadrat size and increased it by intervals of 100 m^2 up to 500 m^2. For the contribution of spatial distribution to reproductive isolation, the parameter of interest is the opportunity for interspecific mating relative to intraspecific mating. Within a sampling bout of 10,000 virtual quadrats, I first discarded all quadrats in which only one individual was found. Then I compared the number of heterospecific quadrats to the number of conspecific quadrats separately for each species. Across all five levels of quadrat size, conspecific quadrats for *C. scaber* were significantly more numerous than heterospecific quadrats (Wilcoxon paired sample: $p < 0.001$), indicating that *C. scaber* has a clumped distribution. For *C. pulverulentus*, the opposite was true. Regardless of quadrat size, heterospecific quadrats were significantly more numerous than conspecific quadrats for *C. pulverulentus* (Wilcoxon paired sample: $p < 0.001$), indicating that *C. pulverulentus* grows as isolated individuals within a matrix of the more abundant *C. scaber*. For each species, the contribution of spatial distribution to reproductive isolation was calculated as:

$$RI_{habitat} = 1 - \frac{\text{no. heterospecific quadrats}}{(\text{no. heterospecific quadrats}) + (\text{no. conspecific quadrats})}$$

This index ranges from zero if all quadrats are heterospecific to one if all quadrats are conspecific only. For both species, mean $RI_{habitat}$ decreased as the quadrat size increased, for *C. pulverulentus* ranging from 0.475 to 0.108 (mean = 0.317) and for *C. scaber* ranging from 0.762 to 0.507 (mean = 0.628). 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 m^2 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 0.108 (95% CI: 0.098-0.117) for *C. pulverulentus* and 0.507 (95% CI: 0.491-0.522) for *C. scaber*. Confidence intervals were calculated as 95% of the range of $RI_{habitat}$ results for 100 sampling bouts of 10,000 virtual quadrats each.

Phenological isolation

The flowering phenology of the two species was highly overlapping and in all cases peaked between May and August (Figure 7). 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. Mean start dates were May 13 and 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, it is unlikely to contribute to reproductive isolation.

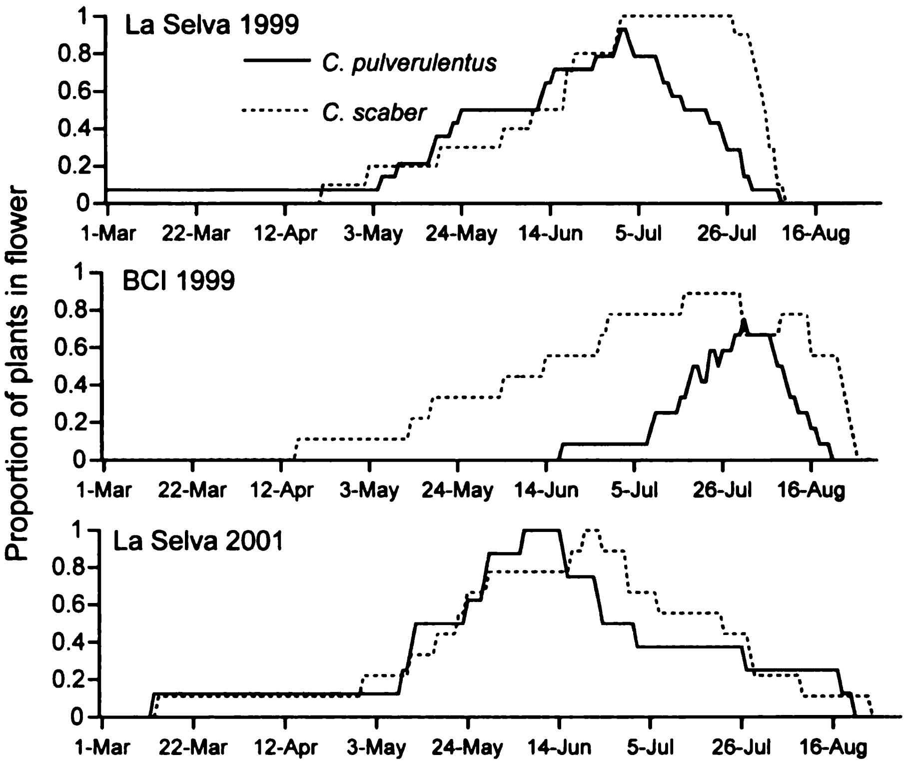

Figure 7. The proportion of plants of each species in flower plotted across time, for 1999 and 2001 at La Selva and for 1999 at BCI.

Floral isolation

Premating isolation

Pollinator assemblages and floral constancy—For *C. pulverulentus*, *Phaethornis superciliosus* is the only pollinator observed, while for *C. scaber*, *P. superciliosus* comprises an average of 0.743 of the total visitation rate across all individual plants observed at La Selva, BCI, and Sirena. The contribution of pollinator species to reproductive isolation was calculated as:

$$RI_{pollsp} = 1 - \left(\text{Prop. of visitation rate composed of shared pollinator species}\right)$$

For *C. pulverulentus*, mean RI_{pollsp} is zero while for *C. scaber* it is 0.257 (95% CI: 0.146-0.379). The confidence interval was constructed by bootstrapping the mean with 1000 replicates.

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 and 5.5 hours of observation, respectively. At the other, with 1-6 flowers per species, there were nine *P. superciliosus* foraging bouts in 11 hours of observation spread over four days. During each bout, the bird visited each of the flowers exactly once, except for one bout where the two *C. pulverulentus* flowers were unvisited. At Sirena in 2002, there was a large patch with 11 *C. scaber* and 7 *C. pulverulentus* flowers visible. In 4 hours 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 ten bouts, nine involved visits to both species. The order of flower visitation for these foraging bouts was not analyzed because it could reflect the non-random 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 hours of observation, 21 individual plants), while three of these were also seen at *C. pulverulentus* (N = 5 visits, 260 hours of observation, 19 individual plants). From all of the evidence of interspecific pollinator movement, isolation due to pollinator constancy is concluded to be negligible.

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* (Figure 8). 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: $p < 0.01$). Although self dye deposition by the hummingbirds was substantial and the species are self-compatible, 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). Because of this, and to make a conservative estimate of the contribution of mechanical isolation to total RI, self dye transfer was excluded from intraspecific dye transfer.

At Sirena in 2002, results from the flower marking of naturally-distributed plants were qualitatively similar to results from La Selva. Out of the 38 marked flowers of *C. pulverulentus*, 19 had no dye deposited on the stigma and 19 had intraspecific dye. Out of the 35 marked *C. scaber* flowers, 13 had no dye deposited, 19 had intraspecific dye only, and 3 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

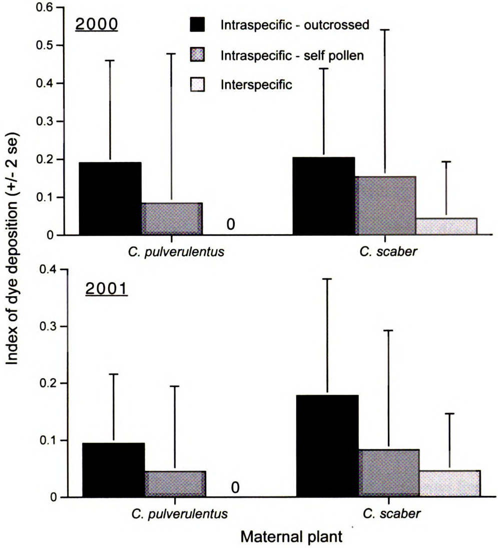

Figure 8. Mean intra- and interspecific dye deposition on stigmas of *C. pulverulentus* and *C. scaber* in experimental arrays at La Selva in 2000 and 2001. The index of dye deposition was calculated as (P*C)/N, where P = the proportion of the stigma covered in intra- or interspecific dye, C = the concentration of that dye on a qualitative scale from 1 to 3, and N = the number of marked flowers in the array that could have contributed that dye. Error bars represent 2 SE.

of reproductive isolation caused by mechanical floral isolation. For each species, this was calculated as:

$$RI_{floralmech} = 1 - \frac{\text{mean index of interspecific dye deposition}}{\text{total of intersp. and intrasp. mean dye deposition indices}}$$

This index ranges from zero, if all dye is interspecific, to one, if all dye is intraspecific. 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, $RI_{floralmech}$ is calculated as a complete barrier of 1.00. For *C. scaber*, the mean intraspecific dye deposition index was 0.193 and the mean interspecific index was 0.045, resulting in a value for $RI_{floralmech}$ of 0.812 (95% CI: 0.751-0.927). The confidence interval was constructed by bootstrapping the mean of measures of $RI_{floralmech}$, calculated per stigma, 1000 times.

Postmating 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; Figure 9). ANOVA results are summarized in Table 6. For plants from both sites, there was a significant maternal species x 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 shows that lower interspecific seed set is the result of prezygotic isolation for the La Selva populations. For pollinations on *C. scaber*, the number of germinated pollen grains after 2 hours was significantly

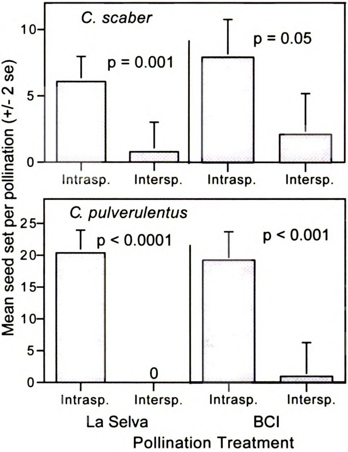

Figure 9. Mean seed set per pollination for intra- and interspecific pollination treatments on plants of each species from La Selva and BCI. Error bars represent 2 SE. ANOVA results are summarized in Table 6.

Table 6. Summary of ANOVA results for seed set in reciprocal crosses, reported separately for La Selva and BCI populations. Maternal plant (maternal species) and paternal plant (paternal species) were designated as random effects, while all other effects were fixed.

	La Selva			BCI		
source of variation in seed number	d.f.	MS	F	d.f.	MS	F
maternal sp.	1	561.9	4.16*	1	144.0	1.26
maternal plant (maternal sp.)	21	232.5	2.27**	19	251.0	2.41**
paternal sp.	1	2255.4	25.72***	1	273.0	2.56
paternal plant (paternal sp.)	22	71.9	0.70	15	108.9	1.04
maternal sp. X paternal sp.	1	1362.6	13.32***	1	504.3	4.84*

*p < 0.05, **p < 0.01, ***p < 0.001

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: $p = 0.0001$; Figure 10a). 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: $p = 0.001$) and the percentage of those grains that germinated (38.3 vs. 70.5 mean percentage germination, Mann-Whitney U: $p = .04$). 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: $p = 0.28$; Figure 10b). However, there was a significant difference in pollen tube growth after nine hours, 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: $p < 0.0001$) and fewer pollen tubes reaching the ovary in these pollinations (0.5 vs. 48.9 pollen tubes, Mann-Whitney U: $p < 0.0001$). The differences in pollen tube growth were sufficient to explain the decrease in interspecific compared to intraspecific seed set.

For the BCI populations, results were less straightforward, but they also indicate that low interspecific seed set is the result of prezygotic mechanisms. For *C. scaber* as the maternal parent, the number of germinated pollen grains per pollination showed a significant effect of greenhouse location (MS = 17,058.4, $F = 5.56$, $p = 0.03$) but not paternal species (MS = 8448.2, $F = 2.75$, $p = 0.12$), although within each location there was a trend of higher germination in intraspecific crosses. For the grains that did

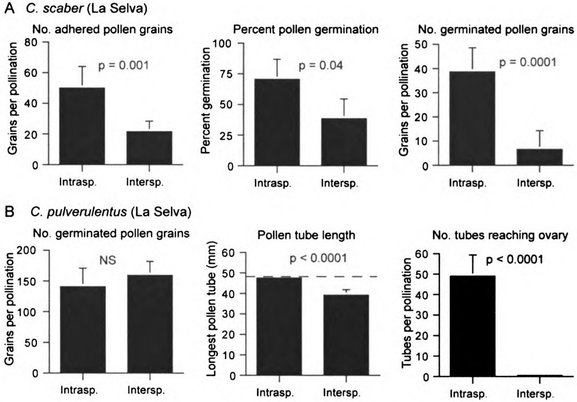

Figure 10. Measures of postmating prezygotic isolation for *C. scaber* (A) and *C. pulverulentus* (B) from La Selva as pollen recipients. All columns represent means, and error bars are +2SE. NS = not significantly different. For *C. scaber*, differences in pollen adhesion and percent germination combine to give an overall difference in the number of germinated pollen grains per pollination. 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*.

germinate, there were also differences in the length of the longest pollen tube after 2 hours, with intraspecific pollen tubes growing farther (MS = 90.5, F = 6.62, p = 0.02). This model showed a significant greenhouse location effect as well (MS = 104.9, F = 7.67, p = 0.01). For the pollinations on *C. pulverulentus* from BCI, which were all completed at the University of Washington, results were similar to those of *C. pulverulentus* from La Selva. There was no difference in the number of germinated pollen grains per pollination in intra- vs. interspecific crosses (81.7 vs. 85.0 grains, N = 16 intraand 24 interspecific pollinations, Mann-Whitney U: $p = 0.98$). However, after nine hours, *C. scaber* pollen tubes generally failed to reach the ovary. The mean length of the longest pollen tube was 48.3 mm (N = 22 pollinations, se = 0.56), while the average style length was 50.8 mm (N = 31 styles, se = 0.48), and in these pollinations an average of only 1.1 pollen tubes reached the ovary (se = 0.53). No intraspecific pollinations at 9 hours were examined for comparison. However, the extremely low numbers of pollen tubes reaching the ovary was sufficient to explain the low seed set in interspecific crosses.

Reproductive isolation due to postmating, but prezygotic, crossing barriers was calculated for each species as:

$$RI_{postmating} = 1 - \frac{\text{No. seeds per intersp. pollination}}{\text{No. seeds per intrasp. pollination}}$$

For *C. pulverulentus*, I estimated $RI_{postmating}$ as 1.0 for La Selva and 0.951 for BCI. For *C. scaber*, I estimated $RI_{postmating}$ as 0.870 for La Selva and 0.732 for BCI. Because of the qualitatively similar patterns in seed set between La Selva and BCI, I averaged the estimates for each species for the overall calculation of postmating isolation. Therefore, my estimate is 0.976 (95% CI: 0.939-0.994) for *C. pulverulentus*, and 0.801 (95% CI: 0.558-0.940) for *C. scaber*. 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/crosstype category, calculating $RI_{postmating}$ for each site and then averaging between sites. This resampling procedure was replicated 1000 times, and 95% of the range taken as the confidence interval.

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), with a mean germination rate of 0.06 for H(S) hybrids, compared to 0.34 for *C. pulverulentus* and 0.31 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). The mean germination rate was 0.33 for *C. pulverulentus*, 0.39 for H(S), and 0.48 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 bench space. All plants not culled eventually flowered during the next four years. Although neither plant size nor flower or inflorescence number was quantified, hybrids were generally observed to grow vigorously and produce abundant flowers.

Because of the conflicting results between the La Selva and BCI plants, the low sample sizes of fruits planted, the generally low rates of germination even for non-hybrid seeds, and the non-random placement of seeds in the incubator, it remains unclear whether the stages of seed germination and early seedling survival in the greenhouse contribute to reproductive isolation. Later stages of survival and flowering in the greenhouse do not contribute to reproductive isolation. For the purposes of the current study, isolation due to all of these factors (seed germination, survival, and reproduction) is considered negligible.

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; Figure 11). For BCI, the pollen fertility 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 = .002; Figure 11), 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 = .09; Figure 11). Because there was no difference for La Selva and only a slight difference for one of the hybrid types for BCI, differences in pollen stainability are considered negligible.

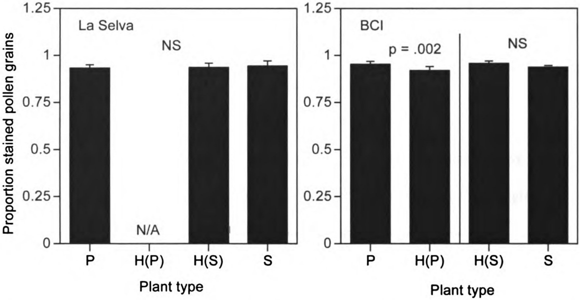

Figure 11. Mean proportion of stained pollen grains for the different cross types for both La Selva and BCI source populations. Error bars represent 2SE. No H(P) hybrids were made, so none were measured. NS = not significantly different.

Total Reproductive Isolation

I summarized components of reproductive isolation separately for each species in Table 7 and Figure 12. 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* and 0.992 (95% CI: 0.983-0.998) for *C. scaber*. 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 results in a reduction of total reproductive isolation for *C. scaber* to 0.986 (95% CI: 0.969-0.997). 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.

DISCUSSION

Geographic 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 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.

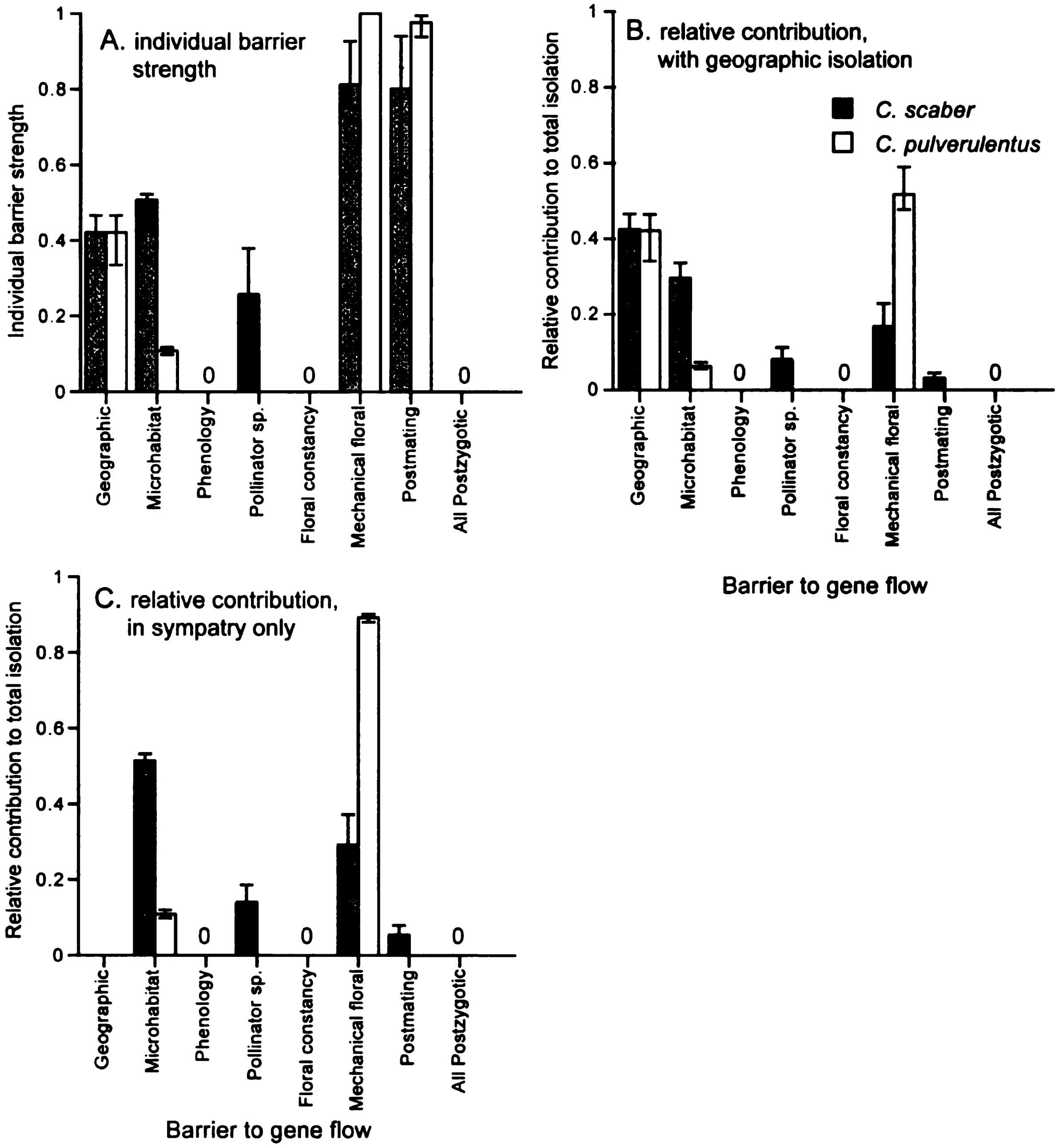

Figure 12. All components of reproductive isolation calculated separately for each species. A) The strengths of individual isolating mechanisms calculated separately (RI_n). B) The relative contribution of each mechanism to total reproductive isolation (AC_n/T), including geographic isolation. C) The relative contribution of each mechanism in sympatry, without geographic isolation. All error bars represent 95% confidence intervals of the means.

Table 7. Summary of all studied mechanisms of reproductive isolation, their individual strengths, and their absolute contributions to total reproductive isolation. Stages are shown separately for each species, and absolute contributions are calculated with and without the effects of geographic isolation.

	Individual barrier strength (RI)		Absolute contribution to total isolation (AC)			
			With geographic isolation		In sympatry	
Stage of Isolation	*C. pulverulentus*	*C. scaber*	*C. pulverulentus*	*C. scaber*	*C. pulverulentus*	*C. scaber*
Prezygotic						
Geographic isolation	0.421	0.421	0.421	0.421		
Microhabitat	0.108	0.507	0.063	0.294	0.108	0.507
Phenology	0	0	0	0	0	0
Pollinator species identity	0	0.257	0	0.073	0	0.127
Pollinator constancy	0	0	0	0	0	0
Mechanical floral isolation	1	0.812	0.516	0.172	0.892	0.297
Postmating	0.976	0.801	0	0.032	0	0.055
Postzygotic						
Seed germination	0	0	0	0	0	0
Hybrid survival	0	0	0	0	0	0
Hybrid reproduction	0	0	0	0	0	0
Hybrid pollen fertility	0	0	0	0	0	0
Total isolation			**1**	**0.992**	**1**	**0.986**

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 that in a range of virtual quadrat sizes from 10 km^2 to 150 km^2, species always co-occurred less often than in the random simulations, with an average value of geographic isolation of 0.421. 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.

Microhabitat Isolation

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*. For both species, virtual quadrats of 500 m^2 at La Selva are often strictly conspecific, although the effect was much stronger for *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.), with *C. scaber* more frequently encountered than *C. pulverulentus*. At all sites, *C.*

pulverulentus grows at extremely low density in undisturbed forest, and is found at small isolated treefall gaps, while *C. scaber* is more abundant and found in wetter areas, often near swamps and streams (pers. obs.). These differences in habitat and abundance translate into important spatial isolation, even at a relatively large scale comparable to the long distance foraging flights of their shared pollinators. Although I assumed that reproductive isolation is linearly related to spatial isolation, the actual effects of this isolation are not straightforward and depend on pollinator foraging routes and the dynamics of pollen carryover from one flower to the next. Although *P. superciliosus* regularly fly between *C. pulverulentus* and *C. scaber* habitat, subtle patterns in their foraging routes are difficult to track and may either increase or decrease the effects of spatial isolation. We also have no information about patterns of pollen carryover.

Floral Isolation

Plant-pollinator interactions have been the focus of many studies of plant speciation, and this study confirms their importance even for species sharing the same pollination syndrome and the same primary pollinator. Occasional visits by other hummingbirds besides *P. superciliosus* to *C. scaber* make a small contribution to reproductive isolation, but most of the floral isolation 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. When *P. superciliosus* inserts its long decurved bill into the flower, pollen deposited on the upper portion of the distal half of the bill. In contrast, *C. pulverulentus* has a longer (by approximately 2 cm), more open flower, with reflexed petals and exserted stigma and anthers. When *P. superciliosus* visits, it inserts its bill without touching it to the stigma or anthers. As it 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. 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. In this study, 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*, although not for *C. scaber*. Interestingly, 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 (Kress 1983; Stiles 1975; Stiles 1979).

Postmating Isolation

I found strong postmating isolation in both directions of the reciprocal crosses for La Selva and BCI, 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, or inhibition of heterospecific pollen tubes by the pistil. Reduced pollination success of long flowers by pollen from short flowers is well-documented across many plant taxa (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. For *C. scaber* it may effectively prevent hybridization from the limited amount of heterospecific pollen deposition.

The contribution of postmating isolation to total isolation for *C. scaber* may also be affected by pollen competition, which I did not quantify in this study. When *C. scaber* receives pollen from *C. pulverulentus* in nature, it is likely to be in a mixture with conspecific pollen. If there is conspecific pollen precedence (Howard 1999) it may amplify the effects of the reductions in heterospecific pollen adhesion and germination, leading to stronger effective postmating 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" which has been shown to override the incompatibility response in self-incompatible plants (Richards 1986).

Postzygotic Isolation

Although stages of postzygotic isolation were only roughly estimated in the artificial environment of the greenhouse, it is clear that there are no strong intrinsic viability or fertility barriers in the first generation of hybridization. My results for hybrid seed germination were compromised by the low and sporadic rate of fruit production over the years, the lack of seed dormancy that would allow me to start a large cohort of seeds at the same time under the same conditions, and my general lack of knowledge of the conditions necessary for good *Costus* seed germination. The result was extremely high fruit to fruit variability in germination rate and overall low germination, even for non-hybrid seeds. Once past the germination stage, hybrids grew vigorously and had high pollen fertility. However, the fitness of hybrids in nature may be quite different than in the greenhouse, and at this point is unknown. Even if F1 hybrids are viable and fertile in a laboratory or greenhouse environment, they may be poorly adapted to the available habitats in the natural environment (Hatfield and Schluter 1999), suffer from reduced mating ability because of pollinator attraction or pollen placement, or may experience hybrid breakdown upon backcrossing (Coyne and Orr 1998).

Because of strong prezygotic isolation, any difference in hybrid fitness would contribute very little to total reproductive isolation. Furthermore, because intrinsic genetic incompatibility once acquired is unlikely to be reversed, it is unlikely that intrinsic postzygotic isolation was important at the early stages of speciation. This result is similar to findings of strong prezygotic barriers combined with weak postzygotic barriers in several other plant speciation studies (reviewed in Ramsey et al. 2003).

Total Isolation

I found total reproductive isolation to be nearly complete at the prezygotic stage. Including the effects of geographic isolation, I measured 100% total isolation for *C. pulverulentus* as the maternal parent and 99.2% isolation for *C. scaber* as the maternal parent. Considering reproductive isolation in sympatry reduces total isolation to 98.6% for *C. scaber*. Although this study did not account for the effects of pollen carryover or pollen competition in mixed pollinations, and hybrid fitness was only crudely estimated in the greenhouse, these results are consistent with the very rare occurrence of hybrids in nature. No hybrids have been reported from undisturbed mature forest, but over the five years of this study I have seen five probably F1 hybrids in areas of recent deforestation. These hybrids may be the result of a breakdown of microhabitat isolation in deforested areas, and they underscore my finding that microhabitat isolation has a high relative importance to total isolation. My results also generally support the idea that *C. pulverulentus* and *C. scaber* are good biological species, although the utility of the Biological Species Concept for plants has been debated (Mayr 1992; Mishler and Donoghue 1982).

One of the striking features of this system is the floral isolation, despite the fact that both species are specialized on the same hummingbird pollinator. For *C. pulverulentus*, mechanical floral isolation appears to be a complete barrier to potential gene flow, while for *C. scaber*, floral isolation comprises the sequential barriers of incomplete overlap in pollinator assemblage, floral mechanical isolation, and pollen-pistil incompatibility. Floral isolation caused by shifts between entirely different pollination syndromes (orchid bee and hummingbird) has been shown to be important in this genus (Kay and Schemske 2003), but these results also indicate the importance of floral isolation for speciation events that involve only subtle changes in floral characters.

While some effects of floral shape on mechanical isolation were anticipated, the postpollination isolation found in this system 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 KMK, 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 96.0% (95% CI: 94.3-98.5%) complete, which would allow a limited but significant amount of hybridization. The selective origin of the pollen-pistil incompatibility is further supported by the results of greenhouse crossing experiments among various species in the genus. Out of 9 other interspecific pairings (besides *C. pulverulentus* and *C. scaber*), 8 are easily crossable, and these are all pairs that are either allopatric in distribution or use different pollinators. The only 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 1981b). From these patterns in crossing relationships, I can speculate 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.

General Conclusions

Systematic investigation into the nature of reproductive isolation is necessary to understand the process of speciation. The results presented here represent one case study in which speciation is already effectively complete, and therefore some of the differences identified may have been acquired post-speciation. To better estimate the relative importance of various isolating mechanisms at the time of 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 the fruit fly genus *Drosophila*, in a study that found prezygotic isolation evolved at a faster rate in sympatric taxa (Coyne and Orr 1989; Coyne and Orr 1997), but has not been attempted for any plant groups.

Nevertheless, 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 postzygotic isolation. Although postzygotic isolation is well-known in plants (Stebbins 1950), there are many studies of closely related plants that exhibit strong prezygotic isolation but weak postzygotic isolation (reviewed in Grant 1981; Ramsey et al. 2003). My results are also consistent with the prediction of strong biotic interactions, in this case plant-pollinator interactions, promoting evolutionary divergence in the tropics (Dobzhansky 1950; Schemske 2002). Finally, my findings could motivate studies of the genetics of speciation. To date, the field has been dominated by studies of the genetic basis of hybrid inviability and infertility (Coyne and Orr 1998). While understanding the evolution of these phenomena is important, in many cases we also need to focus on the evolutionary genetics of ecological divergence to better understand the process of speciation.

CHAPTER 4

Reinforcement of postmating isolation contributes to speciation in two Neotropical rainforest herbs

Abstract—The role of natural selection in speciation has been debated since the time of Darwin. Reinforcement is the process whereby natural selection strengthens reproductive isolation between incipient species to reduce maladaptive hybridization. Although once thought to be a common final stage in speciation (Dobzhansky 1940; Lewontin 1974), reinforcement has been controversial and clear empirical support has been limited (Butlin 1987; Hostert 1997; Noor 1999; Spencer et al. 1986). Recent studies in animals have found reinforcement of mating discrimination behavior (Coyne and Orr 1997; Higgie et al. 2000; Noor 1995; Rundle and Schluter 1998; Saetre et al. 1997); however, its existence and importance in plant speciation is unknown. Here I present evidence for reinforcement of postmating isolation between two recently diverged tropical rainforest herbs that share the same hummingbird pollinator. In field studies I demonstrate that there is substantial interspecific pollen transfer by their shared hummingbird pollinator, and in greenhouse studies I show that pre-fertilization incompatibility between pollen and pistil reduces hybrid seed production from this pollen transfer. I further show that the pollen-pistil incompatibility is unique to locally sympatric populations, compared to interspecific crosses between geographically distant populations. This study suggests that reinforcement can contribute to speciation in plants, that it may evolve locally when incipient species experience sympatric contact, and that, as in animal studies, it may involve complex mate recognition systems.

RESULTS AND DISCUSSION

Costus pulverulentus and *C. scaber* (Costaceae) are closely related understory rainforest herbs sympatric throughout much of Central and Northwestern South America. They are part of a recent and rapid species radiation of the Neotropical spiral gingers (*Costus* subgenus *Costus*) that has been marked by the evolution of specialized hummingbird and orchid bee pollination. Both have bright red flowers (Figure 2d and 2e) pollinated almost exclusively by the Long-tailed Hermit hummingbird (*Phaethornis superciliosus*) at sympatric sites in Costa Rica and Panama (Kay and Schemske 2003), and the birds move between flowers of the two species on their traplining foraging routes (Chapter 3). Differences in floral morphology that affect pollen placement on the bird effectively eliminate pollen transfer from *C. scaber* to *C. pulverulentus*, but not from *C. pulverulentus* to *C. scaber* (Figure 13). Nevertheless, hybrids are rarely found in nature. In controlled greenhouse crosses, pollination of *C. scaber* by *C. pulverulentus* results in very low seed set because of reduced pollen adhesion and percent pollen germination compared to intraspecific crosses.

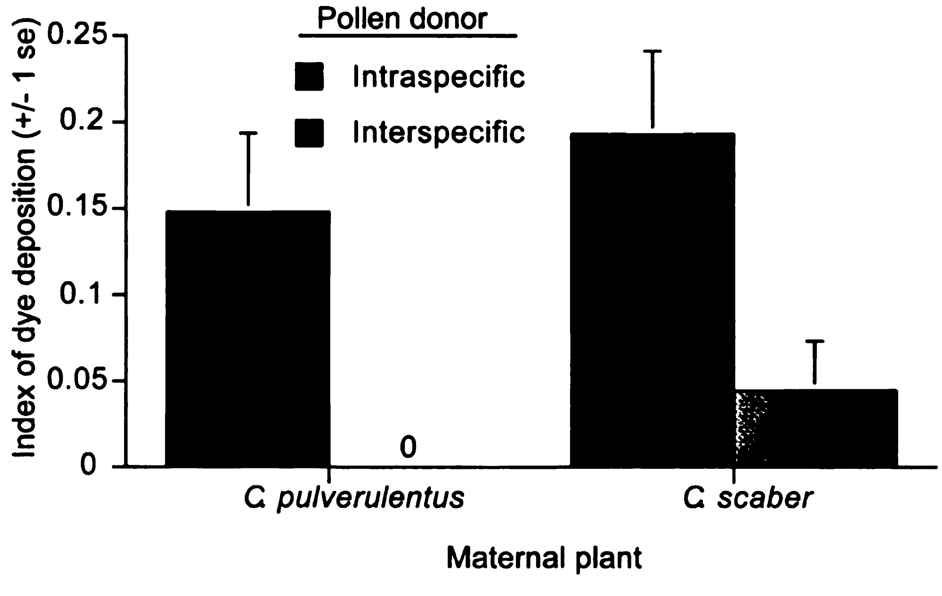

Figure 13. Estimates of intra- and interspecific dye deposition for each species.

If the pollen-pistil incompatibility has evolved under a model of reinforcement (i.e., to limit hybridization in the face of sympatric pollen flow) incompatibility should be stronger between local populations than between geographically distant populations. To test this hypothesis, I performed pollinations on *C. scaber* using *C. pulverulentus* pollen from plants collected at the same locality, from plants collected at a geographically disparate site within the region of sympatry, and from plants collected outside the region of sympatry in Chiapas, Mexico. These pollination treatments were replicated on *C. scaber* plants collected from two different sympatric sites approximately 470 km apart, La Selva Biological Station in Costa Rica, and Barro Colorado Island (BCI) in Panama, so that *C. scaber* from each of the sites was pollinated by *C. pulverulentus* from La Selva, BCI, and Chiapas.

I found that seed set per pollination depends on the specific combination of maternal *C. scaber* source population and paternal *C. pulverulentus* source population, and is only reduced in interspecific crosses between plants from the same geographic site (Figure 14). For *C. scaber* from La Selva, pollinations by *C. pulverulentus* from either BCI or Chiapas result in more than five-fold higher seed set than pollinations by *C. pulverulentus* from La Selva. Conversely, for *C. scaber* from BCI, pollinations by *C. pulverulentus* from either La Selva or Chiapas result in more than two-fold higher seed set than pollinations by *C. pulverulentus* from BCI. There were no significant overall effects of maternal or paternal population on seed set that would indicate general differences among populations in ovule fertility or siring ability. Comparison of pollen adhesion and germination on stigmas of *C. scaber* from La Selva shows that the number of pollen

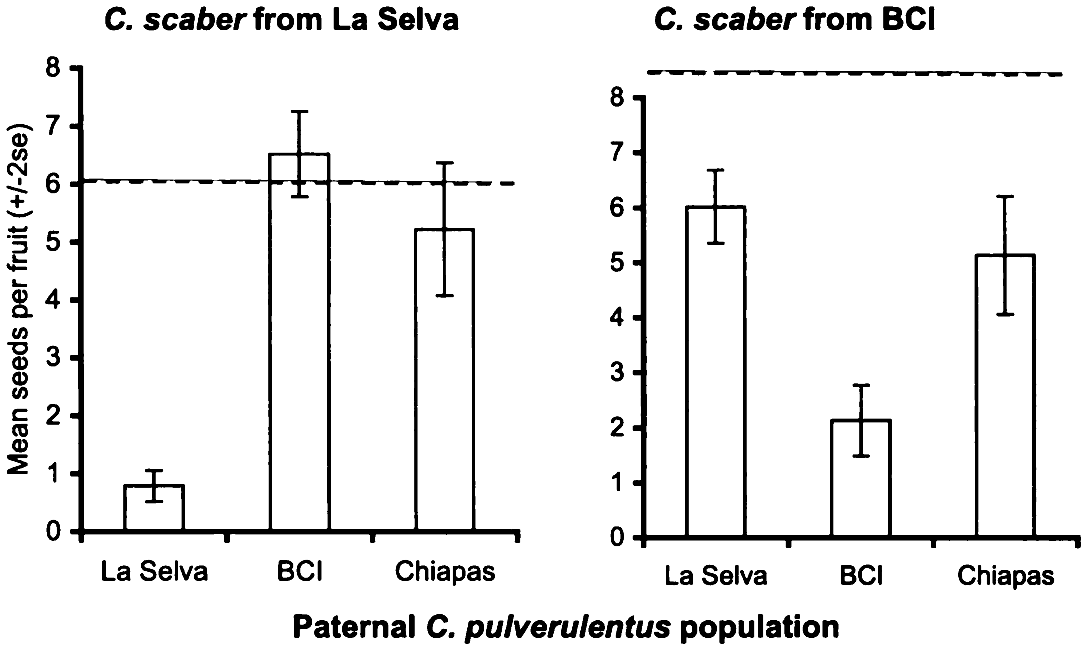

Figure 14. Mean seed set per fruit (+/- 2 SE) in interspecific pollinations on plants of *C. scaber* from La Selva and BCI. The dashed line represents mean seed set in intraspecific crosses.

grains adhering and the number of germinated pollen grains per pollination is higher for *C. pulverulentus* pollen from BCI than from La Selva.

There is also a strong crossing barrier in the other direction, in which *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, a typical result when crossing between flowers differing in length (Emms et al. 1996; Tiffin et al. 2001). Because there is no evidence for natural pollen movement in that direction, it was not predicted that seed set would differ according to the geographic source of the *C. scaber* pollen donors. Interspecific between-site pollinations of *C. pulverulentus* were attempted with *C. scaber* pollen from La Selva and BCI, and resulted in effectively no hybrid seed set, similarly to within-site interspecific pollinations (Table 8).

Table 8. Summary of crossing results for C. *pulverulentus* and C. *scaber*. Numbers are relative seed set, 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, followed by the absolute mean seed set and the sample size of pollinations in parentheses.

	Paternal population				
	C. scaber		*C. pulverulentus*		
Maternal population	La Selva	BCI	La Selva	BCI	Chiapas
C. scaber					
La Selva	1.0 (6.1, 53)	1.1 (6.7, 21)	0.1 (0.8, 46)	1.1 (6.5, 60)	0.9 (5.4, 23)
BCI	1.3 (10.7, 6)	1.0 (8.0, 44)	0.8 (6.0, 61)	0.3 (2.1, 39)	0.6 (5.1, 23)
C. pulverulentus					
La Selva	0 (0, 59)	0 (0, 11)	1.0 (20.4, 71)	1.0 (20.9)	0.9 (19.2, 77)
BCI	0.1 (1.0, 20)	0.05 (0.9, 51)	0.7 (12.8, 44)	1.0 (19.2, 41)	--
Chiapas	0 (0, 12)	--	0.8 (13.0, 23)	--	1.0 (16.4, 36)

These results suggest that incompatibility has evolved locally to prevent seed set from interspecific pollen flow and involves population-level differences in both the pollen and pistil. Surprisingly, this trait divergence has not been accompanied by any significant decrease in crossing success among populations within a species (Table 8). Pollen-pistil interactions in plants are known to be complex, both in cases of self-recognition (Nasrallah 2002) and interspecific interactions (Howard 1999). The mechanism of incompatibility is unknown in the case of *Costus*, but likely acts at the stigma-pollen interface. This idea is further supported by 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 ± 1SE: 3.7 ± 0.72 with exudate added vs. 0.8 ± 0.13 without exudates; N = 16 and 46 pollinations, respectively; Mann-Whitney U, $p < 0.01$).

Reinforcement posits that postzygotic reproductive isolation causes selection for stronger prezygotic isolation. F1 hybrids are viable and fertile in the greenhouse. The species show habitat segregation, so hybrid fitness may be determined by their ecological performance instead of any intrinsic genetic incompatibility (Hatfield and Schluter 1999; Kirkpatrick 2001).

Alternate explanations for the crossing results are unlikely. Sympatric divergence to avoid mating interference not involving hybridization (the idea of reproductive character displacement, which can occur between even unrelated species; Noor 1999), is improbable in this case since the barrier operates after pollen deposition, fertile hybrids can be made, and hybrids have been observed in nature, albeit rarely. Phenotypic differences contributing to reproductive isolation in sympatry, such as flowering phenology or floral morphology, also can be an indirect result of differential evolutionary responses to an array of ecological variables. Yet it is difficult to imagine an ecological variable that would cause such a locally specific postmating incompatibility without affecting other crossing relationships. Finally, there phylogenetic evidence indicates that populations exhibiting crossing barriers are no more distantly related than other population pairs.

Much of the debate over the role of 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 reproductive isolation has been measured between these species, using the methodology of Coyne & Orr (1997) and Ramsey et al.(2003), and found to be 99.2 percent complete (95% CI: 98.2-99.6) at the prezygotic stage for *C. scaber* as the maternal parent (Chapter 3). Without the contribution of postmating pollen-pistil incompatibility, however, I estimate it to be only 96.0% (95% CI: 94.3-98.5%) complete. The initial amount of reproductive isolation and the extent of interspecific gene flow are parameters central to the theory of reinforcement (Kirkpatrick 2001; Liou and Price 1994; Servedio and Kirkpatrick 1997), and this study is unique in providing estimates of reproductive isolation both with and without the putatively reinforced mechanism.

Evidence of reinforcement between these species is not surprising in light of the emerging pattern of speciation in this clade. Phylogenetic evidence suggests a recent and rapid radiation in species (Chapter 2). There also has been marked divergence in habitat use (Maas 1977), and the biogeographic distribution of species suggests extensive range shifts (Chapter 2). Many speciation events in the clade have involved a shift in specialized pollination between orchid bee and hummingbird, which effectively prevents any potential pollen flow between species which are otherwise easily crossable (Kay and Schemske 2003). However, the majority of speciation events do not involve a complete change in pollination syndrome, and this sets up a scenario in which direct natural selection could act to reinforce speciation in cases when incipient species come into secondary contact. In fact, the only other known case of a crossing barrier in the subgenus is between a pair of orchid bee-pollinated species, *C. allenii* and *C. laevis*, which are sympatric, share pollinators, and experience extensive pollen flow (Schemske 1981b).

This study presents the first compelling evidence of reinforcement in plants. Interestingly, the first suggestion of reinforcement in plant speciation, in the genus *Gilia* (Polemoniaceae), also proposed postpollination crossing barriers as the target of selection (Grant 1966), although in that case there were complete sterility barriers and unknown phylogenetic relationships among the species. Here I show both a pattern of crossing relationships consistent with reinforcement, and that the prezygotic crossing barriers counteract the effects of interspecific pollen movement in nature that would otherwise result in some hybridization.

MATERIALS AND METHODS

Pollen transfer

I estimated intra- and interspecific pollen transfer in mixed-species arrays of potted plants at five different sites in the forest at La Selva Biological Station in Heredia Province, Costa Rica (10°25'N, 84°00'W). Because pollen is not unambiguously distinguishable, I coated the anthers with colored dye to track pollen movement, according the methods detailed in ref. 12. I constructed an index of dye deposition, calculated as (P*C)/N, where P equals the proportion of the stigma covered in either intra- or interspecific dye, C equals the concentration of that dye on a qualitative scale from 1 to 3, and N equals the number of marked flowers in the array that could have contributed that dye. From 2000 to 2001, there were 40 site/date combinations with pollinator visitation, and I examined a total of 94 *C. pulverulentus* and 84 *C. scaber* stigmas for dye deposition.

Plant collections

Plants of both species were collected as seeds or rhizomes from La Selva and Barro Colorado Island Nature Monument (BCI) in the Canal Zone, Panama (9°09'N, 79°51'W) to represent the region of sympatry. 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 LS, 15 from BCI, and 11 from Chiapas, and for *C. scaber*: 9 plants from LS and 9 from BCI. Representative vouchers were made from each population and are deposited in the Michigan State University herbarium (MSC).

Seed set

Plants were grown to flowering in the greenhouses of University of Washington, Seattle, and Michigan State University, East Lansing, and were crossed according to the methods detailed in Chapter 3. Each plant typically produces a single one-day flower per day when flowering, and plants flowered sporadically, preventing a balanced crossing design with even sample sizes. Nevertheless, over the course of four years, all combinations of dams and sires were attempted to estimate seed set. For pollen-pistil interactions, only a subset of combinations was examined.

Seed set was quantified in pollinations on the two populations of *C. scaber* by *C. pulverulentus* from La Selva, BCI, and Chiapas. The number of seeds per fruit was analyzed with a mixed-model ANOVA including the following main effects: maternal population, maternal plant (maternal population) (random), paternal population, paternal plant (paternal population) (random), and the maternal population x paternal population interaction. This model tests for population level incompatibility that could be the result of sympatric reinforcement (maternal population x paternal population interaction), while accounting for general differences among populations in female fertility (maternal population) or siring ability (paternal population). The plant effects are nested and used as sources of error for the population effects. Model fitting was done with restricted maximum likelihood. The only significant effect in the model was the interaction term (d.f. = 2, MS = 96.4, F = 5.38, p = 0.005).

To test whether the local interspecific incompatibility between species is associated with any decrease in intraspecific crossing success between La Selva and BCI, I 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 the same ANOVA model as for interspecific crosses. I found no significant effects for either species (whole model, *C. pulverulentus*: MS = 211.7, $F_{52,143} = 0.640$, p = 0.967; *C. scaber*: MS = 20.0, $F_{39,87} = 0.43$, p = 0.998).

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 represented the same biological species. Using the same ANOVA model as above, I found no significant effects on seed set per pollination (whole model, MS = 200.3, $F_{42,161} = 0.93$, p = 0.60).

Pollen germination and tube growth

Postpollination events were examined using epifluorescent microscopy for the La Selva *C. scaber* according to the methods detailed in Chapter 3. Reduced seed set in interspecific has been shown to be the result of differences in pollen adhesion and percent germination that together result in fewer germinated pollen grains. 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. Pollen load was not significantly higher for BCI compared to La Selva (30.6 vs. 22.2 pollen grains, Z = 1.43, p = 0.15), but a higher percentage of the adhered pollen grains germinated (75.3 vs. 39.3 percent germination, Z = 2.27, p = 0.02), leading to a higher number of overall germinated pollen grains (24.3 vs 6.7, Z = 3.30, p = 0.001).

APPENDICES

APPENDIX A

Taxon sampling for phylogenetic analysis

Table A1. Taxon sampled, voucher/source information, and geographic origin.

Taxon	Voucher / Source[a]	Geographic origin
C. afer Ker-Gawl	Utrecht 68GR00199 ESW, 10A.100 HHK46G	Ivory Coast
C. allenii Maas	Kay 0314 (MSC) / field collection	Barro Colorado Island, Panama
C. amazonicus (Loes.) Macbride ssp. *krukovii* Maas	Utrecht 72GR00353 E S P 12826 16B.210 HHK45F	Arce Rio Moa, Brazil
C. arabicus Linn.	Kay 037 (MSC), UW #1924	Haiti
C. arabicus Linn.	Utrecht 95GRO1263EMAAS, 16B.220HHK04	Amazonus, Brazil
C. asplundii Maas	Schemske 031 (MSC) / T. Wood living collection	Ecuador
C. aff. barbatus Suess	Kay 0311 (MSC) / UW #1696	Costa Rica
C. bracteatus Rowlee	Kay 0316 (MSC) / field collection	La Selva Biological Station, Costa Rica
C. chartaceus Maas	T. Wood living collection, received from NMNH GH 90-016	Colombia
C. claviger Benoist	Utrecht 91GR01861 ESMAAS, 16A.100 HHK04	Guyana
C. comosus Rosc. var. *comosus*	Schemske 032 (MSC) / T. Wood living collection	unknown
C. curcumoides Maas	Jardin Botanique National de Belgique 95-0116-29	French Guiana
C. dirzoi García-Mendoza & G.Ibarra-Manríquez	Utrecht 80GR00128 ESV.RD., 14A.530 HHK45F	Los Tuxtlas Biological Station, Mexico

C. dubius (Afzel.) K.Schum.	Utrecht 68GR00199 ESW, 10A.100 HHK46G	Africa
C. *erythrocoryne* K.Schum	Utrecht 94GR02117 EIMAAS, 17A.500 HHK04	Alpahuayo, Peru
C. erythrophyllus Loes.	Kay 038 (MSC) / T. Wood living collection	South America
C. erythrothyrsus Loes.	Kay 0339 (MSC) / T. Wood living collection	South America
C. glaucus Maas	Utrecht 74GR00415 ESMAAS, 14B.600 HHK45F	San Vito, Costa Rica
C. guanaiensis Rusby var. *guanaiensis*	Kay 0318 (MSC) / field collection	Depto. Santa Cruz, Bolivia
C. guanaiensis Rusby var. *guanaiensis*	Kay 0317 (MSC) / field collection	Depto. La Paz, Bolivia
C. guanaiensis Rusby var. *macrostrobilus* (K.Schum.) Maas	Kay 0319 (MSC) / field collection	Barro Colorado Island, Panama
C. laevis Ruiz & Pav.	Kay 0320 (MSC) / field collection	La Selva Biological Station, Costa Rica
C. laevis Ruiz & Pav.	Kay 0310 (MSC) / field collection	Barro Colorado Island, Panama
C. laevis Ruiz & Pav.	Kay 024 (MSC) / field collection	Osa Peninsula, Costa Rica
C. lasius Loes.	UW 1926 94-3738	unknown
C. lasius Loes.	Kay 0321 (MSC) / field collection	El Valle de Anton, Panama
C. laterifolius Baker	Utrecht 89GR0087 ES, 10A.120 HHK04	Ghana
C. laterifolius Baker	Schemske 035 (MSC) / T. Wood living collection	Africa
C. letestui Pellegr.	Schemske 036 (MSC) / T. Wood living collection	Gabon
C. leucanthus Maas	Utrecht 86GR00130 ESMAAS, 17A.200 HHK04	Colombia
C. lima K.Schum var. *lima*	Jardin Botanique National de Belgique 75-0400	Colombia

C. lima K.Schum var. *lima*	Kay 023 (MSC) / field collection	Osa Peninsula, Costa Rica
C. longebracteolatus Maas	T. Wood living collection	Costa Rica
C. lucanusianus J.Braun et K.Schum.	Utrecht 68GR00220 NS 10B.030 HHK46G	Cameroon
C. malortieanus H.Wendl.	Kay 0322 (MSC) / field collection	La Selva Biological Station, Costa Rica
C. malortieanus H.Wendl.	MSU living collection / field collection	La Selva Biological Station, Costa Rica
C. montanus Maas	Jardin Botanique National de Belgique 72-6433	Costa Rica
C. montanus Maas	Kay 0323 (MSC) / field collection	Monteverde, Costa Rica
C. osae Maas & H.Maas	Kay 0324 (MSC) / Marie Selby Botanical Gardens #1997-0373, T. Wood living collection	Osa Peninsula, Costa Rica
C. pictus D.Don ex Lindl.	Jardin Botanique National de Belgique 00-5272	Central America
C. productus Gleason ex Maas	Kay 039 (MSC) / T. Wood living collection (labeled *C. curvibracteatus*)	unknown
C. pulverulentus Presl	Kay 0328 (MSC) / field collection	Barro Colorado Island, Panama
C. pulverulentus Presl	Kay 0326 (MSC) / field collection	La Selva Biological Station, Costa Rica
C. pulverulentus Presl	Kay 022 (MSC) / field collection	Osa Peninsula, Costa Rica
C. pulverulentus Presl	Kay 031 (MSC) / field collection	Bonampak, Chiapas, Mexico
C. pulverulentus Presl	Kay 0327 (MSC) / field collection	Lacanja, Chiapas, Mexico
C. pulverulentus Presl	MSU living collection / field collection	Barro Colorado Island, Panama

C. scaber Ruiz & Pav.	Kay 0329 (MSC) / field collection	Depto. La Paz, Bolivia
C. scaber Ruiz & Pav.	Kay 0325 (MSC) / field collection	Barro Colorado Island, Panama
C. scaber Ruiz & Pav.	Kay 0330 (MSC) / field collection	La Selva Biological Station, Costa Rica
C. scaber Ruiz & Pav.	Kay 021 (MSC) / field collection	Osa Peninsula, Costa Rica
C. scaber Ruiz & Pav.	Kay 032 (MSC) / field collection	Depto. Santa Cruz, Bolivia
C. spicatus Sesse & Moc.	Fairchild #81-424B, in cult. MSU	unknown
C. spiralis Rosc. var. *spiralis*	T. Wood living collection, received from Marie Selby Botanical Gardens 1996-0700	Edo Rio de Janeiro, Brazil
C. spiralis Rosc. var. *spiralis*	Kay 033 (MSC) / field collection	Depto. Santa Cruz, Bolivia
C. stenophyllus Standl. & L.O.Williams	Kay 0331 (MSC) / Hort.	Osa Peninsula, Costa Rica
C. talbotii Ridley	Schemske 038 (MSC) / T. Wood living collection	Africa
C. varzearum Maas	Utrecht 71GR00153 ESP, 16B.000 HHK04	Acre, Brazil
C. varzearum Maas	Kay 0333 (MSC) / T. Wood living collection	unknown
C. villosissimus Jacq.	Kay 0313 (MSC) / field collection	Barro Colorado Island, Panama
C. vinosus Maas	T. Wood living collection	Panama
C. aff. wilsonii Maas	Kay 0336 (MSC) / field collection	Monteverde, Costa Rica
C. wilsonii Maas	Kay 0335 (MSC) / field collection	Las Alturas Biological Station, Costa Rica
C. wilsonii Maas	Kay 0334 (MSC) / field collection	Las Cruces Biological Station, Costa Rica

C. *woodsonii* Maas	Fairchild Tropical Garden 82-252A	Tortuguero, Costa Rica
C. *woodsonii* Maas	Kay 034 (MSC) / field collection	Tortuguero, Costa Rica
C. *zingiberoides* Macbr.	Jardin Botanique National de Belgique 86-0010	Peru

[a] Sources for plant material are as follows: University Botanic Gardens, P.O. Box 80.162, NL-3508 TD Utrecht, The Netherlands (Utrecht); Jardin Botanique National de Belgique, Nationale Plantetuin van Belgie, Domein van Bouchout, B-1860 Meise, Belgium; Tom Wood living collection, Archer, Florida; and the University of Washington Botany Greenhouse living collection, Department of Biology, Box 351800, Seattle, Washington 98195-1800 (UW). Field collections were made by KMK or DWS.

APPENDIX B

Published rates of nrITS substitution

Literature Survey

We surveyed *Systematic Botany*, *Molecular Phylogenetics and Evolution*, *The American Journal of Botany*, *Evolution* and any references therein, from January 1995 through December 2003. We recorded rates for any study that independently calibrated and reported a rate for ITS1 and ITS2. If multiple rates were reported, we followed the author's recommendation on which was likely to be most accurate, unless their reasoning was based on the rate's similarity to other published rates. In such cases, we took the average of the reported rates. When separate rates were reported for ITS1 and ITS2, a weighted average based on the average number of base pairs in each nrITS region was calculated. For each rate, we also recorded the predominant growth form/life history (Hickman 1993; Mabberley 1997), rate constancy test results, calibration type, and calibration age.

We identified 29 independent nrITS substitution rates ranging from 0.38 x 10^{-9} subs/site/yr in *Hamamelis* to 19 x 10^{-9} subs/site/yr in *Gentiana* Sect. *Ciminalis* (Table B1). The latter rate was excluded as an outlier from all of our analyses since the calibration was based on only a single base-pair substitution and the rate was greater than twice the second highest rate of 8.34 x 10^{-9} subs/site/year in *Soldanella*. The mean of the 28 remaining rates was 2.86 x 10^{-9} subs/site/yr (95% bootstrap CI: 2.10 x 10^{-9}—3.57 x

10^{-9} subs/site/yr). Because of non-normality we obtained CI of means by bootstrapping 1000 replicates. Of those studies reporting results of rate constancy tests, approximately two thirds passed or partially passed (Table B1).

Phylogenetic Signal

Twenty-one different angiosperm families are represented among the 29 rates. Frequently, authors that utilize published rates in their own studies base their choices on phylogenetic relatedness. In order to determine whether such choices are warranted, we tested for any phylogenetic signal in the rates. First we constructed a phylogeny of the taxa in Table B1 using the web-based program Phylomatic (Webb and Donoghue 2003) and then manually edited the tree to reflect recent and more detailed phylogenetic hypotheses for some families (APG II 2003; Bayer and Starr 1998; Doyle et al. 1997) (tree available upon request). The Test for Serial Independence was implemented in the program Phylogenetic Independence v. 2.0 (Abouheif 1999; Reeve and Abouheif 2003) with 1000 randomizations. This test compares the autocorrelation of adjacent branches of the original tree to that of a series of randomized trees. No significant phylogenetic signal was detected ($p = 0.393$), suggesting that phylogenetic relatedness is not an appropriate justification when choosing rates from the literature. Indeed, rates from the family Asteraceae alone span almost the entire range found in Table B1, with 2.5 x 10^{-9} subs/site/year in *Eupatorium* and 7.83 x 10^{-9} subs/site/year in *Robinsonia.*

Life History Effects

Generation time has been suggested to affect rates of molecular evolution (Gu and Li 1992; Ohta 1993), and thus we classified each rate according to the predominant life history of the clade, either annual/herbaceous perennial or woody perennial, as a proxy for generation time. The herbaceous category (N = 10) consists of relatively short-lived plants that reach sexual maturity within one to a few years, while the woody perennial life history category (N = 18) consists of long-lived lineages. The average nrITS substitution rates for these two categories are significantly different ($p = 0.0013$, Wilcoxan Rank Sum Test;). The herbaceous annual/perennial mean is 4.13×10^{-9} subs/site/yr (95% bootstrap CI: 2.35×10^{-9} — 6.21×10^{-9} subs/site/yr), and the woody perennial mean is 2.15×10^{-9} subs/site/yr (95% bootstrap CI: 1.26×10^{-9} — 3.34×10^{-9} subs/site/yr).

Table B1. Independently calibrated nrITS substitution rates sorted by magnitude of rate.

Target Taxon	**Family**	**Life History**	**Clock Test**	**Calibration Type**	**Calibration Date (mya)**	**Rate (subs/site/year x 10^{-9})**	**Reference**
Hamamelis	Hamamelidaceae	Woody	NA	Geographic vicariance	8.5	0.38	Wen and Shi 1999
Winteraceae	Winteraceae	Woody	NA	Geographic vicariance	65[a]	0.45[b]	Suh et al. 1993
Nothofagus	Nothofagaceae	Woody	NA	Fossil	83	0.50	Manos 1997
Salicaceae	Salicaceae	Woody	NA	Fossil	50[a]	0.60	Leskinen and Alstrom-Rapaport 1999
Aralia sect. *Dimorphanthus*	Araliaceae	Woody	NA	Geographic vicariance	12	1.07	Wen 2000
Echium	Boraginaceae	Woody	Passed	Geographic vicariance	20	1.10	Boehle et al. 1996
Alnus	Betulaceae	Woody	Rejected	Fossil	70	1.10	Savard et al. 1993
Empetraceae	Empetraceae	Woody	Passed	Fossil	37	1.44	Li et al. 2002
Saxifraga	Saxifragaceae	Herbaceous	NA	NA	5.5[a]	1.72	Vargas et al. 1999
Aesculus	Hippocastanaceae	Woody	Passed	Fossil	65	1.72	Xiang et al. 1998

Gaertnera	Rubiaceae	Woody	Passed	Fossil	54	1.99	Malcomber 2002
Ormocarpum	Fabaceae	Woody	Rejected	Geographic vicariance	35	2.00	Thulin and Lavin 2001
Inga	Fabaceae	Woody	Rejected	Geographic vicariance	3.5	2.34	Richardson et al. 2001a
Phylica	Rhamnaceae	Woody	Rejected	Geographic vicariance	2	2.44	Richardson et al. 2001b
Adansonia	Bombacaceae	Woody	Passed	Fossil	47^{a}	2.48	Baum et al. 1998
Eupatorium	Asteraceae	Herbaceous	Ingroup passed	*ndh*F dated node	14.8	2.51	Schmidt and Schilling 2000
Tarweeds/ Hawaiian silverswords	Asteraceae	Woody	Rejected	Climatic	15	3.00	Baldwin and Sanderson 1998
Robinioid legumes	Fabaceae	Woody	Rejected	Fossil	39.4^{a}	3.30	Lavin et al. 2003
Lupinus	Fabaceae	Herbaceous	NA	Fossil	60	3.46^{b}	Kass and Wink 1997
Astragalus	Fabaceae	Herbaceous	NA	Fossil	35	3.50	Wojciechowski et al. 1999
Cucurbitoideae	Cucurbitaceae	Herbaceous	NA	Fossil	40	3.62	Jobst et al. 1998
Ehrharta	Poaceae	Herbaceous	Rejected	*rbc*L dated node & Fossil	41	3.81	Verboom et al. 2003

Plantago	Plantaginaceae	Herbaceous	Rejected	Geographic vicariance	0.6	4.27	Rahn 1996
Gentianella	Gentianaceae	Herbaceous	Ingroup passed	Geological evidence	3	4.52	von Hagen and Kadereit 2001
Dendroseris	Asteraceae	Woody	Passed	Geographic vicariance & cpDNA dated node	3.3[a]	5.00	Sang et al. 1994
Gossypium	Malvaceae	Herbaceous	NA	Fossil & cpDNA dated node	8.5[a]	5.50[b]	Wendel et al. 1995
Robinsonia	Asteraceae	Woody	Passed	Geographic vicariance	4	7.83	Sang et al. 1995
Soldanella	Primulaceae	Herbaceous	Passed	Fossil	23.3[a]	8.34	Zhang et al. 2001
Gentiana sect. *Ciminalis*	Gentianaceae	Herbaceous	Ingroup passed	Geographic vicariance	0.1	19.00	Hungerer and Kadereit 1998

[a] Calibration age and associated substitution rate are averages of two reported ages.

[b] Substitution rate is a weighted average of ITS1 and ITS2 rates reported separately.

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