EXPERIMENTAL STUDIES OF ADAPTATION AND SPECIATION IN TWO NEOTROPICAL *COSTUS* SPECIES

By

Grace Fu-chun Chen

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ABSTRACT

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To understand the extraordinary diversity and complexity of organisms requires comprehensive studies of the mechanisms by which populations diverge to become different species. The importance of ecology in speciation has long been recognized; however, there is little direct evidence that ecological factors are the principal isolating barriers between closely related species. To examine how adaptation to ecological factors may contribute to reproductive isolation and lead to speciation, I studied the isolating mechanisms between two closely related Neotropical herbs, *Costus allenii* and *C. villosissimus*. These perennial species occur in the same geographic region, but occupy distinct habitats. *Costus allenii* is located along shady ravines in mature forests, while *C. villosissimus* is found in drier, open sites along forest edges. Both species are pollinated by euglossine bees and can be crossed to produce fully fertile hybrids. I conducted field studies in central Panama, where the two species co-occur, to quantify the strength of multiple isolating barriers and total reproductive isolation. I also conducted reciprocal transplant experiments in the field that were coupled with greenhouse studies to determine whether the two species are locally adapted to ecological factors in their respective habitats, and to identify putative adaptive traits that may contribute to local adaptation and speciation.

There are four chapters in my dissertation. Chapter 1 discusses the mechanisms contributing to sexual isolation in these species. I found that pollinator-mediated barriers and gametic isolation restrict heterospecific gene flow asymmetrically between *C. allenii* and *C. villosissimus*. Chapter 2 describes the parapatric distribution of the two species along a water

availability gradient. The results of reciprocal transplant experiments at two life stages, seeds and juveniles, suggest that local adaptation contributes to strong microhabitat isolation and asymmetrical extrinsic postzygotic isolation between *C. allenii* and *C. villosissimus*. As habitat isolation has been found to be strong in this system, the environmental factors contributing to this form of isolation are of great interest. Chapter 3 summarizes comparisons of the two parental habitats and shows differences in putative adaptations to these habitats among *C. allenii*, *C. villosissimus*, and their F1 hybrids in the greenhouse. Higher leaf mass per area was found in *C. allenii*, which occupies habitats with lower light availability, while higher drought tolerance was found in *C. villosissimus*, which occupies habitats with lower soil moisture. The F1 hybrids had leaf mass per area similar to that of *C. villosissimus*, although hybrid fitness was not reduced in *C. allenii* habitats compared to pure *C. allenii* transplants. The F1 hybrids had intermediate drought tolerance, which is consistent with their lower seedling survival in *C. villosissimus* habitats. Chapter 4 presents an examination of multiple isolating barriers and comparisons of their relative contribution to speciation in *C. allenii* and *C. villosissimus*. Total reproductive isolation was found to be high between the two species, and the major isolating barriers are ecogeographic and microhabitat isolation. Because ecogeographic isolation represents spatial isolation based on genetic differences between species, presumably due to local adaptation, and microhabitat isolation is found to be a consequence of local adaptation (Chapter 2), I conclude that local adaptation is the primary mechanism of speciation in *C. allenii* and *C. villosissimus*.

In this research, I used a comprehensive approach to the study of speciation by linking adaptation to the origin of reproductive isolation. My dissertation provides empirical evidence for how local adaptation to different environmental conditions contributes to reproductive isolation and lead to speciation.

DEDICATION

This dissertation is dedicated to my parents, who have always been supportive of me.

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ABSTRACT

Examining different reproductive isolating barriers and comparing their importance in gene flow reduction are essential for understanding processes of speciation. Sexual isolation has been found to contribute to speciation in many sympatric taxa, while its role in parapatric taxa where interspecific gene flow is plausible is poorly understood. I investigated sexual isolation in *Costus allenii* and *C. villosissimus*, two closely related Neotropical understory species which occupy different and often adjacent habitats, yet are within cruising range of their shared insect pollinators. Individual barriers investigated include pollinator isolation, floral mechanical isolation, and gametic isolation. A pollination array was used to test whether individual pollinators travel between species, to estimate the proportion of hetero- and conspecific pollen deposited on the stigmas, and to examine the proportion of hybrid progeny. In comparison to *C. villosissimus*, *C. allenii* produces flowers with smaller labella, shorter stamen-labellum distances, greater stigma-to-corolla-aperture ratios, and shorter styles. Pollinators preferred *C. villosissimus*, but visited both species, and displayed low constancy. In *C. allenii,* heterospecific pollinator transitions were more common than conspecific transitions, but floral mechanical isolation greatly reduced the likelihood of heterospecific gene flow. *Costus villosissimus*, however, was isolated from *C. allenii* largely because it is preferred by pollinators. The contribution of gametic isolation was weak in *C. allenii* and moderate in *C. villosissimus*. Combining estimates for pollinator isolation, floral mechanical isolation, and gametic isolation, I conclude that sexual isolation is weak in *C. allenii*, restricting heterospecific gene flow by 12%, but moderate in *C. villosissimus*, where gene flow from *C. allenii* is reduced by 58%. To understand the relative contribution of sexual isolation to total isolation in this parapatric species pair requires

estimating the importance of other prezygotic isolating barriers such as ecogeographic, phenological and habitat isolation, and both extrinsic and intrinsic postzygotic barriers.

INTRODUCTION

In accordance with the biological species concept (Mayr, 1942), species are formed as a consequence of accumulated reproductive isolation limiting gene flow between populations. A number of barriers have been identified as components of reproductive isolation (Dobzhansky, 1937; Mayr, 1947, 1963; Schluter, 2000; Coyne and Orr, 2004; Nosil et al., 2005) and their strength has been estimated in a variety of organisms (e.g., Palumbi and Metz, 1991; Hatfield and Schulter, 1999; Hurt et al., 2005; Whiteman and Semlitsch, 2005; Mallet, 2006; Nosil, 2007; see review in Lowry et al., 2008 for examples in flowering plants). To assess the overall strength of isolation and to estimate the relative importance of different barriers, the strength of multiple isolating barriers is estimated sequentially, and summed over the life history of the organism (Ramsey et al., 2003; Coyne and Orr, 2004; Husband and Sabara, 2004; Kay, 2006; Matsubayashi and Katakura, 2009; Dopman et al., 2010; Schemske, 2010; Sobel et al., 2010).

The relative importance of pre- vs. postzygotic isolating barriers has been extensively debated. Because the strength of intrinsic postzygotic isolation increases with time since divergence (Coyne and Orr, 1989, 1997; Presgraves, 2002; Price and Bouvier, 2002; Bolnick and Near, 2005), it is unclear whether the barriers which eliminate interspecific gene flow today are the same as those which were important during speciation. Schemske (2010) has suggested that prezygotic barriers such as ecogeographic, habitat and sexual isolation may often play the major role in speciation, while intrinsic postzygotic isolation typically evolves after speciation is complete.

Sexual isolation has received considerable attention due to its direct relationship to reproduction. Sexual isolation (i.e., nonecological isolation in Coyne and Orr, 2004; chapter 6) involves prezygotic isolating barriers such as mating preference, mechanical isolation, and

gametic isolation. Because reproduction between specific individuals can be better experimentally controlled when the subjects are plants, this study focuses on studying the barriers in plants rather than in animals. Mating preference contributes to reproductive isolation when conspecifics mate more often than is expected by chance. Assortative mating has been observed in systems including sticklebacks (e.g., Nagel and Schulter, 1998; McKinnon et al., 2004), fruit flies (e.g., Coyne and Orr, 1989, 1997; Rundle et al., 2005), African cichlids (e.g., Seehausen et al., 1999; Couldridge and Alexander, 2002), birds (Slabbekoorn and Smith, 2002), and plants (Grant, 1994; Ramsey et al., 2003). Although plants do not directly choose their mates, the process of specialized pollination is similar to mating preference in animal systems. When flowering synchronously, plant species with distinct flower color, shape, odor, and/or nectar production may utilize different pollinators, thereby reducing heterospecific gene flow (e.g., Ramsey et al., 2003; Campbell, 2008; Peakall et al., 2010; but see Wesselingh and Arnold, 2000). Even if different plant species are visited by the same pollinator assemblage, fidelity of individual pollinators to a single plant species will increase the degree of reproductive isolation between taxa (Fulton and Hodges, 1999; Husband and Sabara, 2003; Yang et al., 2007; Smith et al., 2008).

When two individuals from different species attempt to mate with each other, mechanical isolation may hinder heterospecific gene flow if their reproductive structures are mismatched (e.g., Niklas and Buchmann, 1987; Sota and Kubota, 1998). In plants, individual pollinators traveling interspecifically may not efficiently transfer pollen from one to the other. This may be due to differences among plant species in the locations of stigmas and anthers, and as a consequence, differences among pollinators in the sites of pollen deposition. Such mechanical

isolation has been shown to prevent hybridization in a number of taxa (Grant, 1994; Fulton and Hodges, 1999; Kay, 2006; Yang et al., 2007).

Gametic isolation occurs when heterospecific gametes have a reduced fertilization rate (e.g., Palumbi, 1998; Price et al., 2000, 2001). There are relatively few studies of gametic isolation, perhaps because this barrier is difficult to study, especially in internally fertilizing animals and plants, in which the gametic interactions are cryptic. In plant systems, the differences between con- and heterospecific pollen-stigma (Heslop-Harrison, 1982; Pellegrino et al., 2010), pollen tube-style (Levin, 1978), and/or pollen tube-ovule interactions (Escobar-Restrepo et al., 2007) may cause gametic isolation and prevent heterospecific zygote formation.

The relative importance of pollinator isolation, mechanical isolation, and gametic isolation varies across study systems. Ramsey et al. (2003) found that *Mimulus cardinalis* and *M. lewisii* were almost completely isolated by their distinct pollinators, hummingbirds and bees, respectively. Floral mechanical isolation was not estimated in this system, but gametic isolation was strong. Fulton and Hodges (1999) studied reproductive isolation in *Aquilegia formosa* and *A. pubescens*, and found evidence of strong floral isolation. Although both plant species were visited by multiple pollinator guilds, the fidelity of individual pollinators paired with their differential pollination effectiveness caused substantial sexual isolation (Fulton and Hodges, 1999). Kay (2006) found that the Neotropical herb species *Costus scaber* and *C. pulverulentus* shared the same pollinator, the hummingbird *Phaethornis superciliosus*, and that individual pollinators showed no constancy. Nevertheless, floral mechanical isolation was almost complete: pollinators carried pollen of *C. scaber* on the distal half of their bills and that of *C. pulverulentus* on their forehead. Gametic isolation was also strong between these two *Costus* species (Kay,

2006). Pellegrino et al. (2010) found weak pollinator and mechanical isolation between the orchids *Orchis italica* and *O. papilionacea*, but gametic isolation was complete.

All of these studies were conducted with species pairs which are currently sympatric--the importance of sexual isolation between parapatric species located within the cruising range of their pollinators is largely unknown. In general, the distribution of a species refers to its cruising range where the organisms can reproduce. Thus, only individuals of sympatric species have the opportunity of encountering heterospecific mates. Unsuitable habitats beyond the cruising range of pollinators inhibit dispersal and cause geographic isolation between allopatric species (Mayr, 1947). Sexual isolation is irrelevant to contemporary gene flow between allopatric species that are geographically isolated at a large spatial scale. However, many species exhibit parapatric distributions, and although largely isolated by ecogeographic barriers, such taxa may still have an opportunity for heterospecific gene flow. Furthermore, although different plant species may occupy distinct, nonoverlapping habitats, the cruising range of their pollinators may be greater than that of the plants. If two plant species share the same assemblage of pollinators and the pollinators are able to carry pollen beyond the geographic boundaries of the parents, ecogeographic barriers may not be sufficient to eliminate all heterospecific gene flow. In such parapatric species, studying sexual isolation becomes important for understanding whether sexual isolation may further reduce heterospecific gene flow. This is especially of interest when hybrids are found in nature, as this proves that heterospecific gene flow is possible.

Here I investigated the strength of sexual isolation in *Costus allenii* and C. *villosissimus*, a pair of closely related, parapatric species that in Central Panama use the same pollinators and grow within the pollinators' cruising range. Flowering individuals of these two species are in close contact (within 1 km), but are not sympatric, and there is no hybrid zone (see Ch. 2).

Nevertheless, hybrids are observed within the parental habitats on rare occasions (see Ch. 2), suggesting that a small amount of gene flow may permeate the strong isolating barrier of habitat differentiation. The pollinators of both *C. allenii and C. villosissimus*, Euglossine bees, can fly long distances (Janzen, 1971; Dressler, 1982; Wikelski et al., 2010), and may be capable of carrying pollen beyond the distribution of the parental species. Studying sexual isolation in such a system becomes a critical step towards understanding the pattern of reproductive isolation in parapatry. The objectives of this study are 1) to measure the strength of sexual isolation, and 2) to determine the relative contribution of pollinator isolation, floral mechanical isolation, and gametic isolation to sexual isolation. Sexual isolation has been shown to play an important role in many sympatric systems. Here I show that sexual isolation can also contribute to reproductive isolation in parapatric species.

MATERIALS AND METHODS

Study System

The pantropical family Costaceae, also known as the spiral gingers, comprises four genera and about 100 species. *Costus* is the largest genus (~60 species), with its greatest diversity centered in the Neotropics (Maas, 1972; Specht et al. 2001; Kay et al., 2005). Recent and rapid speciation in the genus coincided with climatic fluctuations and geographic uplift in central and northern South America (Kay et al., 2005). In general, *Costus* are perennial herbs which are found in the understory of primary forests. Their large, entire leaves are spirally arranged along the deciduous stems. Most species are clonal, with several stems sprouting from an underground rhizome. A terminal inflorescence produces morphologically complex flowers, and species are pollinated either by euglossine bees or hummingbirds.

A molecular phylogeny of Neotropical *Costus* (Kay et al., 2005) shows that the two study species are very closely related, and are probably sister taxa. They occur in the same geographic region (Maas, 1972) but occupy distinct habitats. *Costus allenii* is located in shady habitats along ravines in old forests, while *C. villosissimus* inhabits drier, open sites along forest edges. These habitat differences result in a parapatric distribution. Both species flower approximately from May to August, which is the early wet season in Central America, and the fruits mature in late September through early October. Individuals of both species typically produce one flower per day and may flower for several months. Although their flowers are morphologically distinct, both species are pollinated by female euglossine bees. *C. allenii* produces cream-colored flowers with a small, red-striped labellum (Fig. 1.1A) and *C. villosissimus* has larger, yellow flowers with a broad labellum (Fig. 1.1B). Flowering individuals of the two species have not been observed in sympatry, but individuals on the edges of their respective distributions are sometimes within pollinator flight distances (Chen, pers. observ.). The two species can be easily crossed to produce fully fertile F1 and F2 hybrids, suggesting weak intrinsic postzygotic isolation. Natural hybrids between the two species are rare in the study region, representing 2% of the natural populations of the two species (see Ch. 2).

Study Sites

The study was conducted in central Panama, the center of distribution of Neotropical *Costus* (Maas, 1972; Kay et al., 2005). In this region there is a sharp rainfall gradient across the Isthmus of Panama, from the wet Atlantic side to the north to the much drier Pacific side to the south. According to the Panama Canal Authority Meteorological and Hydrological Service weather station network, the average annual precipitation is 3234 mm at Cristobal, which is close to the Caribbean Sea, and 1798 mm at Balboa Heights, which is close to the Pacific Ocean. The

species composition of the plant community differs substantially across this gradient (Engelbrecht et al., 2007), as does the distribution of *C. allenii* and *C. villosissimus*. *Costus allenii* occupies wetter, northern regions, while *C. villosissimus* is found in southern, drier regions (Ch. 2). The two species exhibit a parapatric distribution in the region.

The study was conducted largely along Pipeline Road (PLR), which runs south to north through primary forest in Soberania National Park (Fig. 1.2). The southern end of PLR is wide and the canopy is open. As the road progresses northward, the canopy closes gradually and the road narrows until becoming nearly inaccessible. Natural populations of both species are found in the vicinity of the road. Most plants included in the study were located within approximately 10 meters of the road, and the sampled individuals occupied microhabitats which are typical of the overall populations of each species.

Most (> 95%) of the flowering *C. villosissimus* included in the study were found in the southern stretch of PLR, from the entrance near Gamboa to approximately 5.8 km northwards. In contrast, most flowering *C. allenii* were found in the northern stretch beyond the 7.8 km point of PLR. The closest flowering heterospecific individuals were approximately 75 m apart, while more than 80% of the flowering plants of the two species were at least 500 m apart.

A pollination array was established on PLR in a region closest to the edges of the natural distributions between the two species (approximately 6.9 km north of the road entrance near Gamboa). No flowering individuals of the two species were found in the immediate vicinity, and the experimental plants were transplanted from the natural populations in the vicinity of PLR. The location of the pollination array thus represents the geographic scale at which gene flow by pollinators might be expected if flowering individuals of the two species did coexist in the area.

Species Differences in Floral Traits

To explore the potential mechanisms of pollinator isolation, floral mechanical isolation, and gametic isolation, four floral traits were compared between natural populations of the two species (Fig. 1.1C and 1.1D). Labellum width (LW) was measured as an indicator of flower size, as it may be relevant to pollinator attraction. The ratio of the width of the stigma to the width of the corolla aperture (SA) was calculated to represent the likelihood of a pollinator making contact with the stigma while entering the flower. The shortest distance between the petaloid stamen and the limb of the labellum (PL) was measured to determine the likelihood of anther-pollinator contact as a pollinator enters the corolla tube. Style length (SL) was measured to estimate the distance that pollen tubes must grow in order to reach the ovule. LW, SA and SL were measured, with a few exceptions, in 16 *C. allenii* and 18 *C. villosissimus* flowers in 2007. PL was measured in another 34 *C. allenii* and 65 *C. villosissimus* flowers in 2008. All four traits were compared between species using two-tailed t tests.

In addition to these morphological traits, nectar production may also contribute to sexual isolation if pollinators prefer flowers containing more nectar. Because nectar production can have a large environmental component (Zimmerman and Pyke, 1988; Boose, 1997), and is inducible upon removal (Pyke, 1991), an indirect but noninvasive measurement was taken on the flowers used in the pollination and floral mechanical isolation experiment described in later sections. Handling time of a pollinator has been shown to be positively correlated with nectar production in nectar-rich flowers (Harder, 1982, 1983) and was used here to estimate the standing crop of nectar available to pollinators. The duration of a bee's visit to a flower, from the time the bee entered the corolla tube until it left, was recorded for the first visit to each flower on each observation day. Because the flowers were bagged before the observation period on each

day, nectar accumulated in the flower until it was removed by the first visitor. Therefore, pollinator handling time for the first visit to each flower on each observation day was measured as an indirect assessment of nectar production. For each species, the mean pollinator handling time was calculated on each day (mean of two flowers/day), and a paired t-test was used to compare the difference in handling time between species.

To determine if pollinators visiting the two species differed in the mechanics of pollen transfer or deposition, video observations were made on modified flowers in the pollinator array. An elliptical window, approximately 3 cm long and 1 cm wide, was cut from one side of the corolla tube. The lobe of the labellum was left intact so that the image of the flower as viewed by an approaching pollinator did not differ between manipulated and un-manipulated flowers. The corolla tube was then wrapped with transparent plastic to prevent pollinators from exiting through the hole in the corolla and to provide an unobstructed view for video observation. Video recordings were made only on days when no other experiments were conducted in the arrays.

Pollinator Isolation

The pollination array consisted of 5 *C. allenii* and 9 *C. villosissimus* in 2007, and 4 of each species in 2008. These plants were originally collected from natural populations along PLR and they were chosen mainly due to their accessibility, i.e., to allow transportation without damaging the flowering stems. The densities of both species are very low in natural populations--rarely are there more than a few individuals in flower in a local area. To mimic natural densities, I used two flowers per species per day in the pollination array to estimate pollinator isolation. This number of flowers was small enough to be monitored in an area where pollinator activities could be closely observed but not disturbed. Since plants often do not flower every day, multiple plants of each species were required to provide enough flowers for daily observation. Flowers

which were not included in the pollinator isolation experiment were removed before the daily observation or were bagged to prevent natural pollination. The focal inflorescences were approximately two meters apart, and the locations of the inflorescences were randomly reassigned daily by moving the pots to prevent pollinators from memorizing the locations. The inflorescences were bagged before the daily observations began to ensure that all pollination events were documented.

The two *Costus* species have very similar pollinator assemblages and thus sexual isolation caused by differential pollinator specialization is negligible in this system. Orchid bees in the tribe Euglossini are the primary pollinators. Video observation in the natural populations of the two species on Barro Colorado Island (BCI) (n = 6 flowers for each species) and in the pollination array on PLR (n = 3 *C. allenii* flowers and n = 7 *C. villosissimus* flowers) revealed that 97% and 89% of the pollinator visits on *C. allenii* and *C. villosissimus*, respectively, were bees in the genus *Euglossa*. Most of these visits were by *Euglossa imperialis*. The remaining 3% of visits to *C. allenii* were made by bees in the genus *Eulaema*, while 3% of the visits on *C. villosissimus* were by *Eulaema*, 7% by *Exaerete*, and 1% by the hummingbird, *Phaethornis superciliosus* (Chen unpublished data). Schemske (1981) also found that *Euglossa imperialis* was the only visitor to *C. allenii* flowers on BCI.

Pollinator observations were conducted in June and July in 2008 and 2009, from 7 A.M. to 11 A.M., the period with the highest pollinator visitation. Each pollination bout consisted of a pollinator visiting one or more flowers. The pollinator was recorded and followed from its first visit until it left the array. For each bout, I recorded the plant species visited flowers in sequence to determine whether individual pollinators visit randomly or exhibit constancy to one or the other species (Waser, 1986; Jones, 1997; Smith et al., 2008). Pollinator preference was

determined by comparing the number of bouts in which the pollinators visited *C. allenii* first to those in which they visited *C. villosissimus* first using a G-test of goodness of fit.

To estimate flower constancy, i.e., the tendency of a pollinator to move sequentially between different flowers of the same species, only bouts with more than two visits and only the first two visits of those bouts were analyzed. These criteria were implemented because with just two flowers of each species in the array, any pollinator visiting the two flowers of a single species in sequence during a bout must switch to a different species for a third or fourth visit. In this case, analyzing pollinator transitions from the second to the third visits and the subsequent transitions would unrealistically reduce the estimated flower constancy. This would not be an issue if there were many flowers of each species presented in the pollination array, but as discussed above, flower density was kept low to mimic the natural conditions. Thus, only the transition from the first to the second visited flower of each bout was included in the analysis of flower constancy. G-tests of independence were performed to determine whether the plant species of the succeeding visit was independent of the species of the preceding visit (Flanagan et al., 2009).

A constancy index (*CI*) was calculated for each species as suggested by Gegear and Laverty (2005) to represent the extent to which pollinators traveled between conspecific flowers. The values of *CI* range from -1 (complete inconstancy) to 0 (random transition) to 1 (complete constancy). Because the second-visited flowers served as the maternal parent of the potential hybridization, a *CI* was calculated with pollinator transitions of which the second-visited flowers belong to a given species. This approach allows pollinator isolation to be analyzed in the direction of gene flow from male to female, as in the analyses of floral mechanical and gametic isolation. In the calculation of *CI*, the observed numbers of transitions were compared to the

expected values, which were calculated by multiplying the total number of transitions by the pollinator preference calculated above. Such comparisons eliminate the effects of pollinator preference on constancy. For example, if pollinators prefer species A to species B with a ratio of 80:20, then before a second visit to species A, the expected probability of the first visit to A is 0.8. By chance, 80% of the pollen received by species A would be from a conspecific flower. Flower constancy represents the case where the probability of the first flower visited being species A is significantly greater than 0.8 when species A serves as the maternal parent of the potential hybridization.

Floral Mechanical Isolation

Following Kay's (2006) protocol, I marked newly dehiscent anthers on flowers in the pollination array using radiant color dye (Magruder Color Co., NJ) as a pollen analog and observed dye deposition on the stigmas to estimate floral mechanical isolation; N = 18 flowers per species in 2007, and N = 44 flowers per species in 2008. This experiment was conducted on the same flowers used for pollinator observations and on flowers produced when pollinator observations were not conducted. On each day, one flower per species was marked as the pollen donor and the other flower of the same species was marked as the pollen recipient. The donor flowers of each species were randomly assigned the color pink or blue daily; the stigmas of the recipient flowers of both species were examined in the afternoon when the flowers were about to wither. For each species, the proportion of the stigma covered by con- and heterospecific dye was measured visually in a three-step process: first, the stigmatic surfaces were divided into four areas; second, each section was assigned either 0 (no dye deposition), 1 (faint coloration), or 2 (strong coloration) for each color; and finally, the numbers assigned to each area for each color were summed separately, yielding scores ranging from 0 (no dye deposition) to 8 (entire

stigmatic surface covered by dye). The amount of con- and heterospecific dye deposition on stigmas was compared using paired t-tests for each species.

Gametic Isolation

Gametic isolation was measured in 2008 using fruits from hand-pollinated flowers produced by plants in the pollination array. Only flowers not involved in other experiments were used for this purpose. On each day of hand pollination, a 50:50 pollen mixture was made in early morning with pollen collected from one flower of each species. Plants used as pollen donors were from the natural *C. allenii* and *C. villosissimus* populations on PLR, and they were chosen for their accessibility and flower production on the day when hand pollination was carried out. All pollen donors and recipients were bagged to prevent natural pollination. A total of 14 *C. allenii* and 14 *C. villosissimus* flowers were hand pollinated. Fruits were collected in late September and seeds were germinated shortly thereafter (October - November) in the greenhouse at Michigan State University. The environmental conditions of the greenhouse were set to be near a maximum of 26°C during the day and a minimum of 15°C during the night, with the actual temperature being subject to change depending on the conditions outside the greenhouse. Supplemental light was used from 6 A.M. to 6 P.M to mimic the natural photoperiod in Panama. Seeds from each fruit were sowed in potting soil (High Porosity Professional Mix, Baccto) in a 4-L pot. Once a seed germinated and the first true leaf was fully expanded, the seedling was transplanted to a 5 cm x 5 cm pot. All the plants were fully hydrated daily with fertilized water (18-9-18 pH Reducer Fertilizer, 100 ppm N, PLANTEX®).

F1 hybrids of these two species cannot be distinguished from the parental species by their morphology in seed or seedling stages, and it would require too much time and space to grow plants until flowering. Therefore, I assessed the frequency of F1 hybrids by genotyping up to ten

seedlings per fruit, using amplified fragment length polymorphisms (AFLP). Fruits that produced less than three seeds or seedlings were excluded from the analyses. For the remaining fruits, seedlings were chosen randomly with respect to germination times. In total, 90 seedlings from 12 *C. allenii* fruits and 96 seedlings from 11 *C. villosissimus* fruits were genotyped.

Apical leaf tissue was collected into microcentrifuge tubes (FastDNA® kits, MP Biomedicals) and DNA was extracted following the standard FastPrep® procedures. An initial marker screen was conducted with 6 EcoR1 and 3 Mse1 primers resulting in 18 primer pairs. Six plants per species and two F1s from each direction of reciprocal crosses were screened. The three primer pairs which generated the most species-specific markers were used to distinguish hybrids from the parental species: AGG + CCG, ACG + CGG, and ACT + CCG. From these three primer pairs, a total of 12 polymorphic markers, 6 diagnostic for *C. allenii* and 6 for *C. villosissimus*, were used to distinguish hybrids from the parental species. Seedlings with 6 species-specific markers of a given parental species were identified as pure species and those with all 12 markers of both species were identified as hybrids. Following digestion, ligation, pre-selective amplification, and final amplification were performed by G. Chen, and Genescan for AFLPs was performed by the Genomics Technology Support Facility at Michigan State University.

The relative proportions of hybrids and pure species were compared within each fruit using repeated G tests of goodness of fit (Husband and Schemske 2000). The expected ratios were set to be 0.50 for both hybrids and pure species as the pollen mixtures were 50:50 (heterospecific:conspecific). Three *G* values were reported for each repeated G test: $G_{Heterogeneity}$, G_{Pool}, and G_{Total}. $G_{Heterogeneity}$ gives the test of heterogeneity among replicates. G_{Pool} represents the significance of the overall differences between con- and heterospecific gene

flow as compared to the expected proportions. G_{Total} is the sum of $G_{Heterogeneity}$ and G_{Pool}, and indicates whether the data set conforms to the null expectation as a whole (Sokal and Rohlf, 1981).

Gametic isolation was estimated from the frequencies of F1 hybrids produced in the hand-pollinated fruits. If there was no gametic isolation, the frequencies of F1 hybrids in the seedlings should be equal to the frequencies of heterospecific pollen deposited on the stigmas. The frequency of hybrid formation observed could include the effects of both gametic isolation, a prezygotic barrier, and early-acting, intrinsic postzygotic barriers such as seed abortion (see review by Johnson, 2010) and differences between hybrids and parents in seed germination rate, and/or seedling mortality (Martin and Willis, 2007). However, there is no evidence of F1 genetic incompatibilities in crosses between these species--fruits which were hand pollinated with heterospecific or conspecific pollen produce similar seed set, and there are no noticeable differences in germination and mortality rates between the hybrids and the parental species (Chen, unpublished data). Therefore, the estimates of gametic isolation in this study are due mainly to prezygotic mechanisms.

Strength of Individual Barriers

The procedures of Sobel and Chen (in prep) were used to estimate the strengths of reproductive isolating barriers. In brief, their approach generates isolation indices which have a biologically and mathematically meaningful range of -1 (complete heterospecific mating or heterosis) to 0 (no isolation) to 1 (complete isolation). Isolation indices calculated by this approach are comparable to each other. These indices indicate the relative under-representation of heterospecific reproduction or the relative over-representation of conspecific reproduction relative to expectations under random mating. To consider the possibility of asymmetric isolation

as suggested by Kay (2006) and Martin and Willis (2007), I calculated the isolation indices for *C. allenii* and *C. villosissimus* separately.

Pollinator isolation ($RI_{pollinator}$) of each species was calculated using the first pollinator transitions of all the bouts in which at least two flowers were visited (see above), as

$$RI = 1 - 2 * \left(\frac{H}{C + H} \right) \quad (1)$$

(Sobel and Chen in prep.). The probability of conspecific gene flow (C) was estimated as the proportion of pollinator transitions between flowers of the same species, and the probability of heterospecific gene flow (H) was estimated as the proportion of pollinator transitions between flowers of different species. By calculating $RI_{pollinator}$ in this way, both pollinator preference and constancy were taken into account simultaneously. If only a small proportion of pollinators travel between species, isolation would occur whether it is caused by preference or by constancy. The significance levels of the $RI_{pollinator}$ values were determined with G-tests of goodness of fit on the number of con- and heterospecific pollinator transitions. The expected values used in the G-tests were calculated by multiplying the total number of transitions by 0.5, assuming pollinators visited all flowers randomly.

The strength of floral mechanical isolation ($RI_{flormech}$) was estimated using the proportion of con- and heterospecific dye deposition. Because the dye was carried by natural pollinators, the deposition ratios were dependent on both pollinator preference and constancy. To eliminate the effects of pollinator isolation on floral mechanical isolation, the isolation index of each flower was calculated as

$$RI_{flormech} = 1 - \frac{2(\text{observed } H / \text{expected } H)}{(\text{observed } C / \text{expected } C) + (\text{observed } H / \text{expected } H)} \quad (2)$$

(Sobel and Chen, in prep.). Observed *C* and *H* were estimated from dye coverage on the stigmas (see above). Expected *C* and *H* were calculated by multiplying the proportion of con- and heterospecific pollinator transition (previous barrier) and the total amount of dye observed on each stigma. The products give the expected amount of the respective dye which would be deposited on the stigma by the pollinators if there was no floral mechanical isolation. $RI_{flormech}$ of individual flowers was then averaged within each species. The significance levels of $RI_{flormech}$ values were determined by repeated G tests of goodness of fit conducted on the amounts of con- and heterospecific dye deposition on stigmas of the species with the expected ratios calculated from pollinator isolation, treating each stigma as an independent replicate.

For each species, gametic isolation ($RI_{gametic}$) was estimated using the proportion of parental and hybrid seedlings in the hand-pollinated fruits. Because equal proportions of con- and heterospecific pollen were deposited on the stigmas, the measurement of gametic isolation was not affected by floral mechanical isolation. Therefore, I computed $RI_{gametic}$ with equation (1) as suggested by Sobel and Chen (in prep.). The proportion of conspecific seedlings in each individual fruit was used to represent the probability of conspecific gene flow (*C*), while the probability of heterospecific gene flow (*H*) was the proportion of heterospecific seedlings in the same fruit. Estimates of $RI_{gametic}$ from individual fruits were then averaged and the mean $RI_{gametic}$ was calculated for each species.

Sexual Isolation

The three reproductive isolating barriers measured in this study act sequentially -- pollinator isolation is followed by floral mechanical isolation, and then by gametic isolation. Cumulative sexual isolation (*SI*) between *C. allenii* and *C. villosissimus* was computed as a multiplicative function of the three individual barriers as suggested by Sobel and Chen (in prep):

$$SI = 1 - \frac{2 \times H_{pollinator} \times H_{flormech} \times H_{gametic}}{C_{pollinator} \times C_{flormech} \times C_{gametic} + H_{pollinator} \times H_{flormech} \times H_{gametic}} \quad (3).$$

This approach assumes independence of the proportion of heterospecific gene flow reduced by each barrier.

To verify the accuracy of the estimates of cumulative sexual isolation, the overall strength of sexual isolation was measured in the field in 2008 as the proportion of hybrid seed production in fruits from naturally-pollinated flowers. Fruits from the flowers observed in the floral mechanical isolation experiment (n = 22 for each species) were used to estimate the overall strength of sexual isolation. These flowers were naturally pollinated in the pollination array, and the reduction in the proportion of hybrid seed production in these fruits thus represents the overall strength of pollinator, mechanical, and gametic isolation. These fruits were collected and their seeds were germinated and grown in the greenhouse at Michigan State University under the same conditions as described above (see gametic isolation). Eighty-one seedlings from 15 *C. allenii* fruits and 131 seedlings from 19 *C. villosissimus* fruits were genotyped following the protocol described above to determine the proportion of hybrid seedlings in each fruit. Equation (1) was used to calculate the strength of sexual isolation (RI_{sexual}) in which the proportion of hetero- and conspecific gene flow were represented by the proportion of hybrid and parental species seedling formation in these naturally-pollinated fruits (Sobel and Chen, in prep.).

Therefore, sexual isolation represents the summation of barriers that occur from the point of heterospecific pollinator transition to the production of hybrid seedlings. To assess the significance level of the cumulative isolation indices, repeated G tests of goodness of fit were employed to compare the differences between the observed and expected (in this case, 0.5 for all directions of gene flow) proportion of hybrids in the naturally pollinated fruits.

To dissect the individual contribution of each isolating barrier to RI_{sexual}, the absolute contribution (AC) of each barrier was calculated as

$$AC_i = \frac{2 \times \prod H_{i-1}}{\prod C_{i-1} + \prod H_{i-1}} - \frac{2 \times \prod H_i}{\prod C_i + \prod H_i} \quad (4)$$

where the subscripted i denotes the order of an individual barrier: 1 for pollinator isolation, 2 for floral mechanical isolation, and 3 for gametic isolation (Sobel and Chen, in prep.). Because pollinator isolation was the first-acting barrier measured in this study, $AC_{pollinator}$ was set to be the same as $RI_{pollinator}$ as suggested by Ramsey et al. (2003). To calculate $AC_{flormech}$ and $AC_{gametic}$, the same data set used for the calculation of $RI_{flormech}$ was analyzed again except that this time the expected proportion of gene flow was set at 0.5. Thus the comparisons of the observed proportion of dye deposition represent the degree of pollinator-mediated isolation ($RI_{pollmed}$) caused by the combined effects of pollinator and floral mechanical isolation. To obtain an overall view of the strength of pollinator-mediated barriers, data for the two years were pooled. Equation (1) was used to calculate $RI_{pollmed}$, in which C and H were, respectively, the amount of con- and heterospecific dye coverage on the stigmas of each species. $AC_{flormech}$ was

calculated as the difference between $RI_{pollmed}$ and $RI_{pollinator}$, and $AC_{gametic}$ was calculated as the difference between RI_{sexual} and $RI_{pollmed}$.

RESULTS

Species Differences in Floral Traits

The flowers of *C. allenii* and *C. villosissimus* are morphologically different (Table 1.1). The labellum (LW) is significantly wider in *C. villosissimus* flowers than in *C. allenii*. The ratio of the width of the stigma to the width of the corolla aperture (SA) is smaller in *C. villosissimus* than in *C. allenii*. The petaloid stamen-labellum distance (PL) is significantly shorter in *C. allenii* flowers than in *C. villosissimus*. In fact, in half of the *C. allenii* flowers, the petaloid stamens contacted the labellum (distance = 0). The style (SL) is significantly shorter in *C. allenii* than in *C. villosissimus*. No significant difference was found between species in pollinator handling time (40.77 ± 10.76 sec in *C. allenii*, 33.00 ± 11.38 in *C. villosissimus*, $t = 1.15$, $p = 0.26$), suggesting that the two species produce similar levels of nectar.

Video recordings of pollinator visitation of both species showed that, after a bee landed on the limb of the labellum, it walked through the space between the petaloid stamen and the labellum to enter the corolla tube to collect the nectar. The stigma lobes were pushed open as the bee crawled down the corolla tube and the bee's dorsal thorax touched the receptive side of the stigma and the anther. During this process, the bee would deposit the pollen it carried from previous visits on the stigma and then the pollen from this flower would be deposited on its back. In flowers of *C. allenii*, which have a shorter stamen-labellum distance than that of *C. villosissimus*, a bee would be forced to lift the tips of the petaloid stamens, and to squeeze through the tight space, causing its dorsal thorax to brush against the stigma and then the anther. In flowers of *C. villosissimus*, the bee also contacts the anther and stigma with its dorsal thorax,

but for only a brief time. In both species, after the bee has finished feeding on the nectar, it exits by crawling backward, at which time more pollen may be deposited on its back. When the bee exits the flower, the receptive side of the stigma remained closed and self-fertilization would be avoided. Examples of the pollinator videos on *C. allenii* and *C. villosissimus* are available at http://dl.dropbox.com/u/12153878/20080714allenii.avi and http://dl.dropbox.com/u/12153878/20080620villosissimus.avi, respectively.

Pollinator Isolation

Pollinators were observed at the arrays for 18 hours over 9 days in 2007 and for 37 hours over 17 days in 2008. In 2007, a total of 240 bouts consisting of 425 visits were recorded. Analysis of the first visit by pollinators in each bout indicates a nearly-significant preference for *C. villosissimus* (55% of visits) ($G = 2.82$, df = 1, $p = 0.09$). When the second flower visited was *C. allenii* (n = 48), 40% of the transitions between flowers were conspecific and 60% were heterospecific (Fig. 1.3A). The low proportion of conspecific transitions resulted in a *CI* of -0.10 after the insignificant pollinator preference to *C. villosissimus* was taken into consideration. For bees that visited *C. villosissimus* as their second flowers (n = 78), the probabilities of their previous visits to *C. allenii* or to *C. villosissimus* were 36% and 64%, respectively (Fig. 1.3A). A positive *CI* of 0.18 suggests a low degree of constancy in bees visiting *C. villosissimus*. The G test of independence of whether the species of the second visited flowers depend on the species of the first visits suggests no significant flower constancy ($G = 0.17$, df = 1, $p = 0.68$). $RI_{pollinator}$ for *C. allenii* in 2007 was calculated as -0.208 ($G = 2.10$, df = 1, $p = 0.15$), while that of *C. villosissimus* was 0.282 ($G = 6.29$, df = 1, $p = 0.01$). The negative value of $RI_{pollinator}$ for *C. allenii* confirmed the field observations that pollinators traveled more frequently from *C. villosissimus* to *C. allenii* than from *C. allenii* to *C. allenii*; i.e., heterospecific transitions were

more prevalent than conspecific transitions. In contrast, significant isolation was observed in *C. villosissimus*, suggesting more heterospecific pollinator transitions to this species in the pollination array in 2007.

In 2008, 478 bouts consisting of 803 visits were observed. A significant preference for *C. villosissimus* was observed, with 69% of the first flower visits to *C. villosissimus* and 31% to *C. allenii* ($G = 67.91$, df = 1, $p < 0.001$). Taking pollinator preference for *C. villosissimus* into consideration, the proportion of heterospecific pollinator transitions was higher than that of conspecific transitions in *C. allenii* (28% conspecific and 72% heterospecific, $CI = -0.09$, Fig. 1.3B) but lower in *C. villosissimus* (77% conspecific and 23% heterospecific, $CI = 0.20$, Fig. 1.3B). The G test of independence indicates no significant flower constancy ($G = 0.58$, df = 1, $p = 0.45$). In 2008, $RI_{pollinator}$ for *C. allenii* was calculated as -0.449 ($G = 19.45$, df = 1, $p < 0.001$), while that of *C. villosissimus* was 0.532 ($G = 43.92$, df = 1, $p < 0.001$). The negative value of $RI_{pollinator}$ for *C. allenii* was consistent with the observation in 2007, and again confirmed the field observations of frequent heterospecific pollinator transitions from *C. villosissimus* to *C. allenii*. The significant level of pollinator isolation in *C. villosissimus* in 2008 was also consistent with observations in 2007 and suggests a high frequency of conspecific transitions in the species.

Combining the two years (Fig. 1.3C), $RI_{pollinator}$ for *C. allenii* was -0.366 ($G = 19.49$, df = 1, $p < 0.001$), while that for *C. villosissimus* was 0.446 ($G = 46.26$, df = 1, $p < 0.001$). In brief, pollinators preferred *C. villosissimus* but displayed low constancy, and as a result, pollinator isolation reduced heterospecific gene flow from *C. allenii* to *C. villosissimus*, but not in the reverse direction.

Floral Mechanical Isolation

Floral mechanical isolation was measured for 9 days in 2007 and 22 days in 2008. In 2007, there was no significant difference between species in dye deposition on *C. allenii* stigmas ($t = 0.90$, $df = 8$, $p = 0.40$; mean ± 95% CI deposition index of *C. allenii* dye on *C. allenii* stigmas = 3.00 ± 1.39, *C. villosissimus* dye on *C. allenii* = 1.89 ± 1.86; Fig. 1.4A). A similar pattern was observed on *C. villosissimus* stigmas; the mean ± 95% CI deposition index of *C. villosissimus* dye was 3.44 ± 1.28 and that of *C. allenii* was 2.22 ± 1.37 (Fig. 1.4A), with no significant difference between species ($t = 1.69$, $df = 8$, $p = 0.13$). Using the proportion of con- and heterospecific pollinator transitions from the pollinator isolation experiment in 2007 (*C. allenii*: conspecific = 0.40, heterospecific = 0.60; *C. villosissimus*: conspecific = 0.64, heterospecific = 0.36) to calculate the expected values of deposition indices, average $RI_{flormech}$ of *C. allenii* was 0.443, and that of *C. villosissimus* was 0.079 in 2007 (Table 1.2; see individual flower data in Appendix). Although no significant difference was detected when the amounts of con- and heterospecific dye deposited on stigmas of *C. allenii* were compared directly with a paired t-test, a significant $RI_{flormech}$ was found in this species. This is because the paired t-test only compared the observed amount of dye deposition while $RI_{flormech}$ and the corresponding repeated G test of goodness of fit also considered the expected dye deposition due to the different frequencies of pollinator transitions. Despite significant pollinator infidelity in *C. allenii*, floral structures favored conspecific pollen deposition on stigmas of *C. allenii* in 2007.

In 2008, the mean ± 95% CI deposition index of *C. allenii* dye on *C. allenii* stigmas was 2.27 ± 0.70, and that of *C. villosissimus* dye on *C. allenii* stigmas was 2.73 ± 0.57 (Fig. 1.4B). There was no significant difference between the amounts of dye from the two species deposited

on *C. allenii* stigmas ($t = 1.23$, $p = 0.23$). A significant difference was observed on *C. villosissimus* stigmas; more conspecific dye (2.91 ± 0.78) was deposited than heterospecific dye (1.32 ± 0.62) (Fig. 1.4B; $t = 4.23$, $p < 0.001$). Using the proportions of pollinator transitions from the pollinator isolation experiment in 2008 (*C. allenii*: conspecific = 0.28, heterospecific = 0.72; *C. villosissimus*: conspecific = 0.77, heterospecific = 0.23) to calculate the expected values of deposition indices, $RI_{flormech}$ of *C. allenii* was 0.164, and that of *C. villosissimus* was 0.081 (Table 1.2; see data for individual flower in Appendix). Although a slightly higher proportion of heterospecific dye was found on the stigmas of *C. allenii*, floral structural differences between the two species may have increased the proportion of conspecific dye deposition compared with the proportion of conspecific pollinator transitions. Despite higher conspecific dye deposition, reproductive isolation due to floral mechanical isolation per se was not significant for *C. villosissimus*. This suggests that the species difference in dye deposition on stigmas of *C. villosissimus* was because *C. villosissimus* flowers were more frequently visited by pollinators carrying conspecific pollen rather than conspecific pollen being transported more efficiently.

When data for the two years are combined (Fig. 1.4C) and compared with the combined proportion of con- and heterospecific pollinator transitions in the pollinator isolation experiment (*C. allenii*: conspecific = 0.32, heterospecific = 0.68; *C. villosissimus*: conspecific = 0.72, heterospecific = 0.28), significant floral mechanical isolation was found for *C. allenii* ($RI_{flormech} = 0.220$) but not for *C. villosissimus* ($RI_{flormech} = 0.095$) (Table 1.2; see data for individual flowers in Appendix).

Gametic Isolation

$RI_{gametic}$ was estimated from hand-pollinated fruits which received a 50:50 pollen mixture. The average proportion (± 95% CI) of hybrids per fruit was 0.13 ± 0.17 (n = 12 fruits) in *C. allenii* and 0.32 ± 0.18 (n = 11 fruits) in *C. villosissimus*. The overall frequency of hybrids was significantly lower than the expected 50% in both species (Table 1.2). In addition, there was also significant heterogeneity in hybrid frequency among fruits in *C. villosissimus* but not in *C. allenii* (Table 1.2; see individual fruit data in Appendix). $RI_{gametic}$ was measured as 0.892 in *C. allenii* and 0.310 in *C. villosissimus*.

Sexual Isolation

The cumulative sexual isolation (*SI*) estimated from the three individual barriers was 0.858 for *C. allenii* and 0.720 for *C. villosissimus* (Table 1.3). In contrast, the estimate of sexual isolation obtained simply from the frequency of hybrid formation in naturally-pollinated flowers was substantially lower than *SI* for *C. allenii* and moderately lower for *C. villosissimus*. The average proportion (± 95% CI) of hybrid formation following natural pollination in the array was 0.44 ± 0.22 for *C. allenii* (n = 15 fruits) and 0.21 ± 0.14 for *C. villosissimus* (n = 19 fruits). Using these proportions of hybrid formation as the frequencies of heterospecific gene flow, RI_{sexual} was 0.121 for *C. allenii* and 0.581 for *C. villosissimus* (Table 1.2; see individual fruit data in Appendix). For *C. allenii*, the proportion of hybrid seedling formation was not significantly lower than 0.5; i.e., sexual isolating barriers did not reduce heterospecific gene flow from *C. villosissimus* to *C. allenii*. For *C. villosissimus*, a significant reduction in the proportion of hybrid seedling formation was observed, indicating that sexual isolation significantly reduced heterospecific gene flow from *C. allenii* to *C. villosissimus*. The values of RI_{sexual} represent

more direct and conservative estimates for the cumulative strength of the three barriers than the values of *SI*, which were computed from the estimates of indices of individual barriers. Therefore, values of RI_{sexual}, which indicated that sexual isolation is significant in *C. villosissimus* but not in *C. allenii*, were used to calculate the contribution of each barrier in each species.

The contribution of each barrier to total sexual isolation (*AC*) is reported in Table 1.3. In *C. allenii*, negative pollinator isolation ($AC_{pollinator}$ = -0.366) and positive floral mechanical isolation ($AC_{pollinator}$ = 0.337) essentially canceled each other out to give a weak $RI_{pollmed}$ of -0.029. $AC_{gametic}$ of *C. allenii* was 0.150, calculated as the difference between RI_{sexual} and $RI_{pollmed}$. In *C. villosissimus*, both pollinator and gametic isolation contribute to cumulative sexual isolation ($AC_{pollinator}$ = 0.446, $AC_{gametic}$ = 0.207), although floral mechanical isolation did not ($AC_{flormech}$ = -0.072). $RI_{pollmed}$ was 0.374 in *C. villosissimus*, with pollinator isolation contributing the most.

DISCUSSION

Based on the proportion of hybrid seedling observed in naturally-pollinated fruits, sexual isolation was stronger in *C. villosissimus* (RI_{sexual} = 0.581) than in *C. allenii* (RI_{sexual} = 0.121), suggesting asymmetrical isolation between this parapatric species pair. Pollinators preferred *C. villosissimus*, but visited both species and exhibited low flower constancy. The strong preference for *C. villosissimus* coupled with low constancy means that pollinators regularly moved between species, but pollinator transitions from *C. villosissimus* to *C. allenii* were more common than those from *C. allenii* to *C. villosissimus*. Floral mechanical and gametic barriers significantly reduced the probability of heterospecific gene flow in *C. allenii*, but these barriers barely

counteracted the effects of frequent heterospecific pollinator transitions (Table 1.3). In *C. villosissimus*, pollinator isolation and gametic isolation restricted heterospecific gene flow, while floral mechanical isolation was not significant. These three barriers together resulted in significant sexual isolation in *C. villosissimus* (Table 1.3).

Mechanisms of Sexual Isolation

Pollinator Isolation. The strong preference of pollinators for *C. villosissimus* reduces gene flow from *C. allenii* to *C. villosissimus*, but not in the reciprocal direction. This is primarily due to strong pollinator preference for *C. villosissimus*. It is unclear why *C. villosissimus* was favored in the common garden, but here I propose two alternative explanations. First, C. *villosissimus* may be truly preferred over *C. allenii* across their geographic range. Pollinator preference is usually determined by floral display size and/or reward size (Makino and Sakai, 2007). Pollinator handling time was not different between species, which implies that the reward size is unlikely to be the key trait causing pollinator preference. *Costus villosissimus* produces larger flowers which may be more visible to pollinators. In addition to flower size, the bright, yellow color of *C. villosissimus* flowers (Fig. 1.1B) and the red-striped, cream-colored flowers of *C. allenii* (Fig. 1.1A) may also display different attractiveness to pollinators. In other systems, it has been suggested that bumble bees prefer yellow to red (Schemske and Bradshaw, 1999) and solitary *Hylaeus* bees prefer white to yellow (Campbell et al., 2010). How flower color may affect preference of euglossine bees is unclear. *Costus allenii* and *C. villosissimus* do not produce an obvious floral scent (Chen, personal observation), thus differences in scent probably do not contribute to pollinator preference. Although male Euglossine bees display strong preference for different floral scents (Zimmermann et al., 2006), all pollinators observed on the two *Costus* species in this study were females.

A second explanation for the pollinator preference for *C. villosissimus* is a result of the difference in flowering phenology of the two species. Euglossine bees are known to have good memory and display "traplining" foraging behavior (Dressler, 1982). *Costus villosissimus* flowers slightly earlier than *C. allenii* (see Ch. 4), thus bees may be more familiar with *C. villosissimus*. Once a bee finds a *C. villosissimus* inflorescence, it might continue to visit this species repeatedly. However, the finding that bees exhibit low flower constancy to *C. villosissimus* suggests that minor differences in flowering phenology contribute little to pollinator preference. During the experiment, the inflorescences were randomly relocated on each observation day, which should have further reduced the opportunity for learning. Nevertheless, pollinators preferred *C. villosissimus* but showed low constancy, resulting in substantial heterospecific gene flow.

Floral Mechanical Isolation. The degree of floral mechanical isolation was also asymmetrical between *C. allenii* and *C. villosissimus*, but in a different direction from that of pollinator isolation. Significantly less *C. villosissimus* dye was deposited on *C. allenii* stigmas than expected, but this was not the case for *C. allenii* dye deposited on *C. villosissimus* stigmas. Pollinators of *C. allenii* are more likely to enter the corolla tube under the petaloid stamen because flowers of *C. allenii* have a smaller stigma/aperture ratio (SA). In addition, the shorter stamen-labellum distance (PL) in *C. allenii* flowers further facilitates contact between the plants' sexual structures and the pollinators when the bees lift the stamen on their way to the nectaries (see supplemental video). Both floral traits can affect the efficiency and accuracy of pollen transport to the stigma.

When a pollinator enters a *C. villosissimus* flower, it may touch the stigma and anther briefly without transferring a substantial amount of pollen. If the same bee then visits a *C. allenii*

flower, it would deposit less pollen on the stigma than if it has previously visited *C. allenii*. Thus, pollen transport in pollinator transitions from *C. allenii* to *C. allenii* may be more efficient than those from *C. villosissimus* to *C. allenii*. When a bee moves from *C. allenii* to *C. villosissimus*, it may carry more pollen from *C. allenii* due to close contact between the bee's dorsal thorax and the anther. In comparison with a flower of *C. allenii*, there is more space between the labellum and the petaloid stamen in a flower of *C. villosissimus*, and therefore less contact between the bee's dorsal thorax and the stigma and anther. The bee would only deposit a small proportion of *C. allenii* pollen on the stigma of *C. villosissimus*, presumably a similar amount as if it carries pollen from a flower of *C. villosissimus*. The differences in floral structure may explain why floral mechanical isolation is significant in *C. allenii* but not in *C. villosissimus*.

Gametic Isolation. Both species displayed strong gametic isolation, as estimated by the relatively low frequency of hybrids in experimental pollinations conducted with a 50:50 ratio of pollen from the two parental species, ($RI_{gametic}$ = 0.892 for *C. allenii* and 0.310 for *C. villosissimus*). The styles of *C. allenii* were 2.3 mm shorter than those of *C. villosissimus*. Gametic isolation caused by differences in pistil length between species has been found in many systems (Williams and Rouse, 1990; see review in Yost and Kay, 2009). However, whether the gametic isolation observed between *C. allenii* and *C. villosissimus* is because *C. allenii* have shorter styles, and presumably shorter pollen tubes, is unknown. To dissect the mechanisms of gametic isolation between the two species will require *in vitro* investigations of pollen tube competition.

One important caveat in the interpretation of the strength of gametic isolation regards the methods used to measure this barrier. Gametic isolation is defined as the reduction in hybrid formation that is caused by mechanisms operating after mating but before fertilization (Coyne and Orr, 2004). However, it is often difficult to distinguish gametic isolation from early-acting,

intrinsic postzygotic isolation. Specifically, if hybrid zygotes have reduced survival, it is technically difficult to determine if the cause of reduced hybrid formation is due to prezygotic or postzygotic barriers (Klips, 1999; Chari and Wilson, 2001). As gametic isolation in this study was measured by comparing the expected and observed hybrid production from heterospecific crosses, there is the possibility that some of the isolation observed at this stage is due to intrinsic postzygotic barriers. However, the seed set of pure interspecific crosses is not significantly less than that of intraspecific crosses between *C. allenii* and *C. villosissimus* (see Ch. 4), suggesting that gametic isolation is the major post-pollination mechanism of isolation between these species. Reduced hybrid formation due to gametic isolation, but not intrinsic postzygotic isolation, has also been shown in *Helianthus* (Rieseberg et al., 1995), *Hibiscus* (Klips, 1999), and *Silene* (Rahme et al., 2009).

*Temporal Variation in Sexual Isolation***.** Substantial differences were observed between years in the magnitude of some isolating barriers. In *C. villosissimus,* pollinator isolation was stronger in 2008 than in 2007 ($RI_{pollinator}$ = 0.534 vs. 0.282), and in *C. allenii*, floral mechanical isolation was weaker in 2008 than in 2007 ($RI_{flormech}$ =0.164 vs. 0.443). These differences may be caused by temporal variation in any number of environmental factors which influence floral display and thus pollinator visitation (Mothershead and Marquis, 2000; Carroll et al., 2001; Halpern et al., 2010). Since the pollinators visiting the pollination array were not naïve, differences between years in the availability of other floral resources may also affect pollinator preferences. It is also worth noting that the sample size in 2008 was approximately two-fold larger than that in 2007 (478: 240 bouts in the pollinator isolation observation and 22: 9 days in the floral mechanical isolation experiment). Thus, data from 2008 may be more indicative of the preference, constancy, and efficiency of the pollinator assemblage.

Contribution of Individual Barriers to Sexual Isolation

While pollinator isolation has been shown to be an important barrier in some other systems (Ramsey et al., 2003; Grant, 1994), the data presented here suggest that pollinator-mediated isolation between plant species specializing on the same pollinators may also restrict heterospecific gene flow. Similar results have been found between diploid and tetraploid fireweeds (Husband and Sabara, 2003), bee-pollinated *Pedicularis* species (Yang et al., 2007), and hummingbird-pollinated *Costus* species (Kay, 2006). In these three studies, subtle differences in floral traits caused differences in pollinator preference, constancy, and efficiency, respectively, and contributed to strong sexual isolation.

For *C. villosissimus* as the maternal parent of the potential hybridization, the cumulative strength of sexual isolation computed from pollinator, floral mechanical, and gametic barriers (SI = 0.720) was slightly greater than that measured from naturally pollinated fruits in the pollination array (RI_{sexual} = 0.581). The value of SI (SI = 0.858) is much higher than the value of RI_{sexual} (RI_{sexual} = 0.121) for *C. allenii* as the maternal parent of the potential hybridization. The difference between the two estimates of sexual isolation, SI and RI_{sexual}, mainly results from the disproportional contribution of gametic isolation to RI_{sexual}.

Although strong gametic isolation was observed in *C. allenii* ($RI_{gametic}$ = 0.892) when the barrier was measured with fruits produced by hand pollination with a 50:50 pollen mixture, its contribution to sexual isolation ($AC_{gametic}$ = 0.150) was much smaller than one would expect from the high $RI_{gametic}$ value. The strength of gametic isolation may depend on the degree of competition for ovules, and this is likely to vary with the amount of pollen deposited on the

stigmas. In this study, flowers were hand pollinated with far more pollen grains than there are ovules. Thus, if the pollen of one species is competitively superior to the other, it could fertilize all the ovules, and the gametic isolation measured would be essentially complete. However, as the pollen load decreases, the opportunity for competitive exclusion is reduced. For example, if a flower contains 30 ovules and is pollinated with 15 pollen grains from each of two species, the fruit may produce 15 hybrid and 15 pure species seeds, and no gametic isolation would be detected. If the same flower is pollinated with 100 pollen grains from each species, all the ovules could be fertilized by conspecifics. This phenomenon has also been observed in *Mimulus* in which stronger gametic isolation was detected when more pollen was deposited on the stigma (Sobel 2010).

Because the degree of gametic isolation estimated in experimental crosses may vary with the size of the pollen load, some studies have precisely controlled the amount of pollen applied (Rieseberg et al., 1995; Ramsey et al., 2003; Rahme et al., 2009). Nevertheless, these studies were all conducted under greenhouse conditions and did not mimic the composition or amount of pollen applied. This approach is much more difficult in field experiments, and field studies of gametic isolation have often used excess pollen (Klips, 1999; Kay, 2006), as was the case here. The low density of flowers of both *Costus* species in the present study made it impossible to test different proportions and densities of pollen.

Sexual Isolation between Parapatric Species

Mechanisms for the evolution of reproductive isolation have been discussed by Albert and Schluter (2004). Drawing largely on examples and theory based upon animal systems, they proposed three mechanisms: reproductive isolation can evolve by 1) direct selection on mating preference, 2) as a byproduct of adaptation to different niches, and 3) through reinforcement.

Their findings support the mechanism of direct selection on mating preference in the evolution of reproductive isolation between sympatric stickleback populations. The proposed mechanisms can also be applied to plant systems analogously. First, speciation could be driven by sexual isolation when direct selection acts upon mating preference. Harder and Johnson (2009) proposed three modes of pollination-mediated diversification, including 1) pairwise coevolution of specific floral-pollinator interaction, 2) divergent use of the same pollen vector, and 3) pollinator shifts. In each case, floral traits are the direct targets of selection exerted by pollinators, and adaptation to pollinators is the major isolating mechanism which contributes to the initial divergence between populations (Harder and Johnson, 2009; Peakall et al., 2010).

A second mechanism is that sexual isolation is not the direct target of natural selection, but an incidental consequence of the adaptive evolution of other traits, such as habitat preference. This mechanism may be more prevalent in allopatric species in which adaptation to different environmental factors could have pleiotropic effects on sexual isolation. For example, Searcy and MacNair (1990) suggested that edaphic adaptation to different copper concentrations caused pollen-pistil incompatibility between copper-sensitive and copper-tolerant populations of *Mimulus guttatus*.

In contrast, reinforcement might be responsible for the evolution of increased sexual isolation between taxa that evolved in allopatry but have come back together in a zone of secondary contact. In this case, sexual isolation might show geographic variation, with sympatric populations evolving stronger sexual isolation than allopatric populations (Kay and Schemske, 2008; Yost and Kay, 2009).

For *C. allenii* and *C. villosissimus*, sexual isolation was weak in *C. allenii* (RI_{sexual} = 0.121) and moderate in *C. villosissimus* (RI_{sexual} = 0.581), thus this barrier alone is not sufficient

to prevent heterospecific gene flow. The two species are pollinated by the same pollinators, and there is no clear evidence of divergent use of the same pollinators or pairwise coevolution between the plants and their pollinators. Therefore, the direct-selection mechanism is unlikely to be the case here. The currently parapatric distribution of these species probably reduces selection for reinforcement, suggesting that the traits involved in sexual isolation between *C. allenii* and *C. villosissimus* are more likely to be a byproduct of local adaptation to their distinct habitats.

Pollinator shifts from bees to hummingbirds have evolved independently multiple times in Neotropical *Costus* (Kay et al., 2005). However, pollinator isolation alone does not explain diversification in the genus because many of the speciation events have occurred within the clades in which species share the same pollinators (Kay et al., 2005). In some systems, e.g., *C. bracteatus* and *C. lasius*, a pollinator shift has occurred between sister species occupying different habitats (Kay et al., 2005). In other systems, e.g., the broadly sympatric *C. pulverulentus* and *C. scaber*, species share the same pollinators, yet sexual isolation contributes significantly to speciation (Kay, 2006).

The results presented here for *C. allenii* and *C. villosissimus* are very different from those observed in *C. pulverulentus* and *C. scaber*. In contrast to the parapatric distribution of *C. allenii* and *C. villosissimus*, *C. pulverulentus* and *C. scaber* are regularly sympatric, but like *C. allenii* and *C. villosissimus*, they flower at the same time and share the same pollinator. As a result, heterospecific pollen flow between *C. pulverulentus* and *C. scaber* is common, yet hybrids are rare, and this is due principally to strong sexual isolation that includes both gametic and mechanical isolation (Kay, 2006). Kay and Schemske (2008) suggested that pollen-pistil incompatibilities have evolved between these species through reinforcement. However, in parapatric *C. allenii* and *C. villosissimus*, there is little pollinator isolation, and gametic and floral

mechanical isolation are not sufficient to isolate the species. Sexual isolation has evolved as a byproduct of local adaption, and local adaptation to spatially distinct habitats is a key element of speciation in *C. allenii* and *C. villosissimus*. That these two congeneric species pairs display such striking differences in their isolating barriers suggests that speciation can be achieved through a diversity of mechanisms.

For allopatric and parapatric speciation, sexual isolation may not be a crucial barrier if ecogeographic and habitat isolation have eliminated most, if not all, heterospecific gene flow. If two species are completely isolated by their distinct geographic ranges as a consequence of genetically-determined adaptive traits, there would be no direct selection for sexual isolation. If two species are partially sympatric, the contribution of sexual isolation to the total isolation may still be greatly reduced by ecogeographic isolation. For example, *M. cardinalis* and *M. lewisii* are almost completely isolated by their distinct pollinators, but they are also strongly isolated by heritable differences in their ecogeographic ranges (Ramsey et al., 2003; Schemske, 2010). A similar pattern was also observed in phytophagous ladybird beetles (*Henosepilachna vigintioctomaculata* and *H. pustulosa*), where females of both species exhibit high degrees of assortative mating indicating strong sexual isolation, yet habitat isolation was also substantial (Matsubayashi and Katakura, 2009). These examples suggest that the relative contribution of sexual isolation to the total isolation between taxa depends on the degree of ecogeographic isolation. This dependency is due to the fact that the isolating barriers act in a specific sequence, and only organisms living in the same geographic range have the opportunity to exchange genes (Ramsey et al., 2003; Coyne and Orr, 2004).

Different habitats may support different pollinator assemblages (Herrera, 1988), and different pollinator assemblages may further result in pollinator isolation (e.g., Ramsey et al.,

2003). In the present study of *C. allenii* and *C. villosissimus*, this is unlikely because most plants were pollinated by a single species, *Euglossa imperialis*. The observation that some hybrids are produced in nature indicates that flowering individuals of the two species may sometimes occur within the traveling range of individual pollinators. In these rare circumstances, sexual isolation is an important, though incomplete, barrier to heterospecific gene flow.

On PLR, flowering individuals of the two species are rarely located within 1 km of each other (see Ch. 2). During the floral mechanical experiments, the stigmas of flowers on nearby *Costus* of both species were also inspected. The closest natural plants were approximately 1 km away from the array, and no dye was ever observed on the flowers of these plants. These preliminary observations suggest that the habitats of *C. allenii* and *C. villosissimus* may be sufficiently displaced to virtually eliminate pollinators traveling between species. In the relatively rare cases when flowering individuals of the two species are located within the traveling range of a single pollinator, sexual isolation may substantially reduce heterospecific gene flow.

Foraging bees typically visit neighboring plants (see review in Mitchell et al., 2009) and may groom pollen from each previous visit, thus reducing the likelihood of long distance gene flow by pollen (Thomson, 1986). Orchid bees also groom pollen from their bodies and move pollen away from their dorsal thorax (Kimsey, 1984), where pollen can be deposited on a heterospecific stigma. Thus pollinators may not transfer pollen as far as their travel distance, and plant species such as *C. allenii* and *C. villosissimus* that are adapted to different habits may be functionally isolated by virtue of their largely non-overlapping spatial distributions.

Few other studies have investigated sexual isolation between parapatric species. Svensson et al. (2006) studied population divergence and premating isolation in parapatric

damselfly (*Calopteryx splendens*) populations. Courtship success in crosses of males and females from the same populations was higher than that of males and females from different populations, indicating sexual isolation between populations. Takami et al. (2007) studied reproductive isolation between the parapatric ground beetles *Carabus yamato* and *C. albrechti*. Reproductive isolation is strong but incomplete and asymmetric in this system. For *C. yamato* as the maternal parent, genital mismatching, a type of mechanical isolation, contributes most to the total reproductive isolation. For *C. albrechti* as the maternal parent, male mate choice was the major barrier. The results from these animal systems are similar to those obtained for *C. allenii* and *C. villosissimus*, suggesting that sexual isolation reduces heterospecific gene flow and contributes to total reproductive isolation between parapatric species.

Conclusion

In summary, the parapatric Neotropical herb species *C. allenii* and *C. villosissimus* are partially and asymmetrically isolated by sexual isolating barriers. Sexual isolation is probably a byproduct of local adaptation to distinct habitats, as opposed to the direct result of selection against hybrid formation, i.e., reinforcement. Further research on the cruising ranges of both plants and their pollinators, on the correlation between floral traits and pollinator behaviors, and on the mechanisms of pollen-pistil interactions would shed light on the biology of sexual isolation in this system. Given the fact that *C. allenii* and *C. villosissimus* are unambiguously distinct species in nature, the modest level of sexual isolation observed suggests that other barriers must contribute to reproductive isolation. To better understand the relative contribution of each barrier to speciation requires measurements and comparisons of the strength of all potential isolating barriers between *C. allenii* and *C. villosissimus*. These include isolation that results from differing ecogeographic ranges and local habitat preferences, sexual isolation, and

extrinsic and intrinsic hybrid unfitness. Such a comprehensive study of perennial plants requires long term experiments involving a variety of approaches. In addition to the results of sexual isolation presented here, the degree and the mechanisms of habitat isolation and the comparisons among all isolating barriers will be further investigated to provide a more comprehensive understanding of speciation in *C. allenii* and *C. villosissimus*.

Table 1.1. Comparisons of floral traits between *C. allenii* and *C. villosissimus*.

Trait	mean ± 95% CI (n)		t-value	P
	C. allenii	*C. villosissimus*		
Labellum width (mm)	55.61 ± 2.84 (18)	34.55 ± 2.27 (16)	12.26	< 0.001
Stigma-aperture ratio	0.22 ± 0.01 (15)	0.19 ± 0.01 (15)	3.24	0.003
Petaloid stamen-labellum distance (mm)	1.14 ± 0.48 (34)	5.39 ± 0.68 (65)	10.27	< 0.001
Style length (mm)	55.88 ± 1.28 (16)	58.14 ± 1.60 (14)	2.36	0.025
Pollinator handling time (sec)	40.77 ± 10.76 (24)	33.00 ± 11.38 (24)	1.15	0.260

Table 1.2. Reproductive isolation index values and the corresponding results of repeated G tests of goodness of fit on floral mechanical isolation, gametic isolation, and the cumulative estimate of sexual isolation. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Isolating Barrier	Year	Species	*RI*	$G_{Heterogeneity}$	G_{Pool}	G_{Total}
Floral mechanical isolation	2007	*C. allenii*	0.443	27.87 (8) ***	8.47 (1) **	36.35 (9) ***
		C. villosissimus	0.079	11.36 (8)	0.24 (1)	11.60 (9)
	2008	*C. allenii*	0.164	18.64 (21)	15.79 (1) ***	34.43 (22) *
		C. villosissimus	0.081	23.71 (21)	3.03 (1)	26.74 (22)
	Comb.	*C. allenii*	0.220	49.71 (30) *	22.17 (1) ***	71.89 (31) ***
		C. villosissimus	0.095	36.01 (30)	2.78 (1)	38.79 (31)
Gametic isolation	2008	*C. allenii*	0.892	11.74 (11)	80.68 (1) ***	92.42 (12) ***
		C. villosissimus	0.310	38.27 (10) ***	9.53 (1) **	47.80 (11) ***
Sexual isolation	2008	*C. allenii*	0.121	66.42 (14) ***	0.01 (1)	66.43 (15) ***
		C. villosissimus	0.581	60.38 (18) ***	53.90 (1) ***	114.27 (19) ***

Table 1.3. Components of reproductive isolation and absolute contributions to sexual isolation for the reproductive barriers studied. Isolating components range from -1 (complete disassortative mating) to 0 (random mating) to 1 (complete assortative mating). For the barriers of pollinator isolation and floral mechanical isolation, data from 2007 and 2008 were combined to calculate the isolation indices for each species. *SI* represents the cumulative sexual isolation index computed from the individual components of RI, and RI_{sexual} represents the sexual isolation index measured from the proportion of hybrid seedling formation in the naturally-pollinated fruits from the pollination array. G-tests of goodness of fit were used to assess the significance. Repeated G-tests of goodness of fit were used to assess the significance of mechanical and gametic isolation and RI_{sexual}. The numbers in bold indicate a p value of less than 0.05 in these statistical analyses.

Isolating barriers	Components of *RI*		*AC* to sexual isolation	
	C. allenii	*C. villosissimus*	*C. allenii*	*C. villosissimus*
Pollinator isolation	**–0.366**	**0.446**	–0.366	0.446
Floral mechanical isolation	**0.220**	0.095	0.337	–0.072
Gametic isolation	**0.892**	**0.310**	0.150	0.207
	Magnitude of *SI*		Magnitude of RI_{sexual}	
Sexual isolation	0.858	0.720	0.121	**0.581**

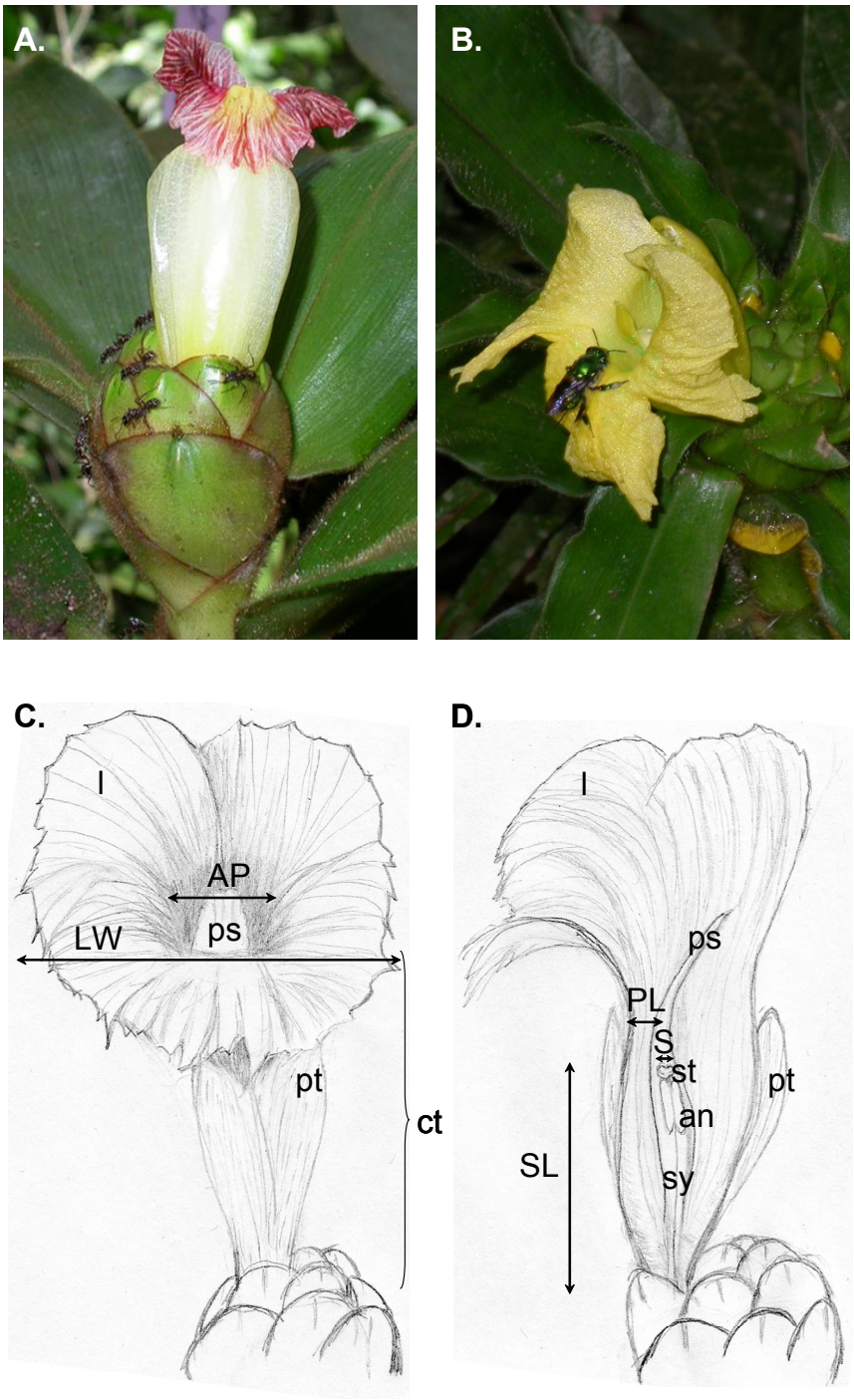

Figure 1.1. Flowers of (A) *Costus allenii* and (B) *C. villosissimus* with the pollinator *Euglossa imperialis* and graphic illustrations of floral structures and measurements of the morphological traits in a front view (C) and a longitudinal section view (D). Trait measurements: LW: labellum width; SA = S (stigma width) / A (corolla aperture width); PL: petaloid stamen-labellum distance; SL: style length. Floral structures: an: anther; ct: corolla tube; l: labellum; p: petal; ps: petaloid stamen; st: stigma; sy: style. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

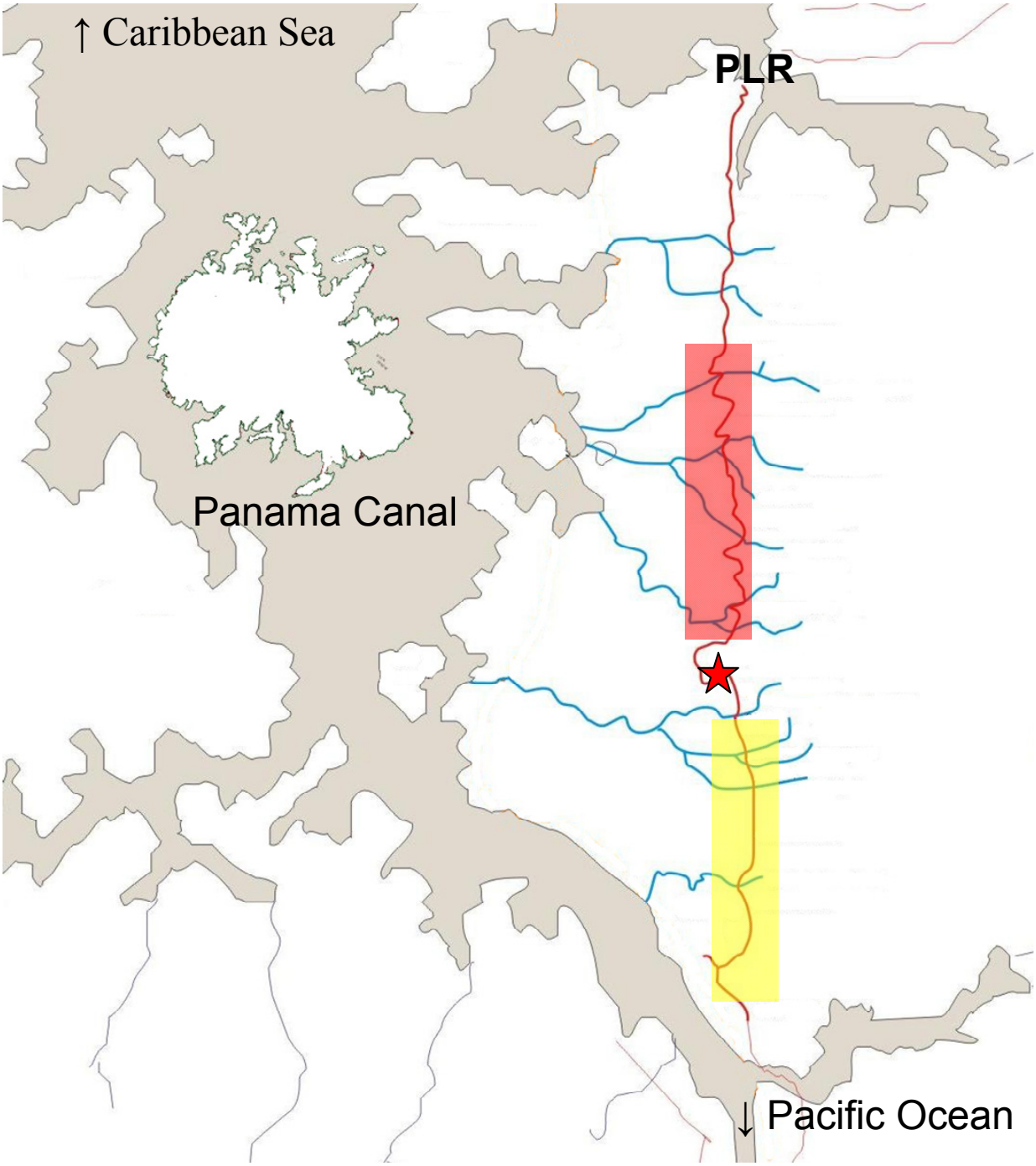

Figure 1.2. Map of the study site in Central Panama (modified from http://www.stri.si.edu./images/Mapas/Pipeline_BarroColorado.jpg). Pipeline Road (PLR) is marked by the red solid line on the right side of the map. The red asterisk indicates the location of the common garden. The red and yellow shaded areas are the locations of the natural populations of *C. allenii* and *C. villosissimus*, respectively.

A.

Species of the 2nd visited flower	Species of the 1st visited flower: *C. allenii*	Species of the 1st visited flower: *C .villosissimus*
C. allenii	19	29
C. villosissimus	28	50

B.

Species of the 2nd visited flower	Species of the 1st visited flower: *C. allenii*	Species of the 1st visited flower: *C .villosissimus*
C. allenii	26	68
C. villosissimus	34	112

C.

Species of the 2nd visited flower	Species of the 1st visited flower: *C. allenii*	Species of the 1st visited flower: *C .villosissimus*
C. allenii	45	62
C. villosissimus	97	162

Figure 1.3. Pollinator transitions between *C. allenii* and *C. villosissimus* in the pollination array in 2007 (A), 2008 (B), and for the two years combined (C).

A.

Species of the pollen recipient	Species of the pollen donor	
	C. allenii	*C .villosissimus*
C. allenii	3.00	1.88
C. villosissimus	2.22	3.44

B.

Species of the pollen recipient	Species of the pollen donor	
	C. allenii	*C .villosissimus*
C. allenii	2.27	2.72
C. villosissimus	1.32	2.91

C.

Species of the pollen recipient	Species of the pollen donor	
	C. allenii	*C .villosissimus*
C. allenii	2.48	2.48
C. villosissimus	1.58	3.06

Figure 1.4. Dye deposition scores measured on stigmas of *C. allenii* and *C. villosissimus* in the pollination array in 2007 (A), 2008 (B), and for the two years combined (C).

APPENDIX

Table A.1. Results of repeated G tests of goodness of fit on floral mechanical isolation, gametic isolation, and the overall strength of sexual isolation. When an observed value equals zero, *ln*(0) was replaces by *ln*(1e-10) in the calculation of G.

Floral mechanical isolation – estimated in 2007

C. allenii flowers	Observed conspecific dye index	Observed heterospecific dye index	Expected conspecific dye ratio	Expected heterospecific dye ratio	G
1	0	6	0.3958	0.6042	6.0469
2	3	2	0.3958	0.6042	0.8461
3	3	6	0.3958	0.6042	0.1502
4	2	0	0.3958	0.6042	3.7070
5	4	1	0.3958	0.6042	3.4179
6	2	1	0.3958	0.6042	0.8958
7	2	1	0.3958	0.6042	0.8958
8	6	0	0.3958	0.6042	11.121
9	5	0	0.3958	0.6042	9.2676
	$G_{heterogeneity}$ = 27.8747 df = 8, p = 0.0004		G_{pool} = 8.4737 df = 1, p = 0.0036		G_{total} = 36.3484 df = 9, p < 0.0001

C. villosissimus flowers	Observed conspecific dye index	Observed heterospecific dye index	Expected conspecific dye ratio	Expected heterospecific dye ratio	G
1	0	0	0.6410	0.3590	0.0000
2	3	4	0.6410	0.3590	1.3034
3	4	4	0.6410	0.3590	0.6632
4	6	3	0.6410	0.3590	0.0260
5	4	2	0.6410	0.3590	0.0173
6	4	3	0.6410	0.3590	0.1438
7	4	0	0.6410	0.3590	3.5575
8	2	4	0.6410	0.3590	2.3366
9	4	0	0.6410	0.3590	3.5575
	$G_{heterogeneity}$ = 11.3643 df = 8, p = 0.1819		G_{pool} = 0.2410 df = 1, p = 0.6242		G_{total} = 11.6053 df = 9, p = 0.2364

Table A.1. (cont'd)

Floral mechanical isolation – estimated in 2008

C. allenii flowers	Observed conspecific dye index	Observed heterospecific dye index	Expected conspecific dye ratio	Expected heterospecific dye ratio	G
1	0	4	0.2766	0.7234	2.5903
2	2	1	0.2766	0.7234	1.9693
3	2	3	0.2766	0.7234	0.3534
4	3	3	0.2766	0.7234	1.3361
5	6	4	0.2766	0.7234	4.5524
6	3	2	0.2766	0.7234	2.2762
7	2	1	0.2766	0.7234	1.9693
8	4	4	0.2766	0.7234	1.7815
9	0	4	0.2766	0.7234	2.5903
10	1	2	0.2766	0.7234	0.0465
11	4	3	0.2766	0.7234	2.6636
12	4	4	0.2766	0.7234	1.7815
13	2	2	0.2766	0.7234	0.8908
14	3	3	0.2766	0.7234	1.3361
15	4	2	0.2766	0.7234	3.9386
16	3	2	0.2766	0.7234	2.2762
17	0	1	0.2766	0.7234	0.6476
18	2	6	0.2766	0.7234	0.0289
19	3	3	0.2766	0.7234	0.3534
20	2	3	0.2766	0.7234	0.3534
21	0	1	0.2766	0.7234	0.6476
22	1	2	0.2766	0.7234	0.0465
	$G_{heterogeneity} = 18.6372$ $df = 21, p = 0.6084$		$G_{pool} = 15.7922$ $df = 1, p < 0.0001$		$G_{total} = 34.4295$ $df = 22, p = 0.0443$

Table A.1. (cont'd)

Floral mechanical isolation – estimated in 2008

C. villosissimus flowers	Observed conspecific dye index	Observed heterospecific dye index	Expected conspecific dye ratio	Expected heterospecific dye ratio	G
1	0	0	0.7671	0.2329	0.0000
2	4	4	0.7671	0.2329	2.6885
3	4	2	0.7671	0.2329	0.3117
4	2	2	0.7671	0.2329	1.3442
5	6	5	0.7671	0.2329	2.5956
6	5	2	0.7671	0.2329	0.1043
7	3	1	0.7671	0.2329	0.0065
8	4	3	0.7671	0.2329	1.3036
9	2	0	0.7671	0.2329	1.0604
10	6	1	0.7671	0.2329	0.3542
11	2	1	0.7671	0.2329	0.1558
12	6	1	0.7671	0.2329	0.3542
13	2	0	0.7671	0.2329	1.0604
14	3	1	0.7671	0.2329	0.0065
15	1	1	0.7671	0.2329	0.6721
16	3	0	0.7671	0.2329	1.5906
17	3	0	0.7671	0.2329	1.5906
18	0	3	0.7671	0.2329	8.7435
19	2	0	0.7671	0.2329	1.0604
20	3	1	0.7671	0.2329	0.0065
21	1	1	0.7671	0.2329	0.6721
22	2	0	0.7671	0.2329	1.0604
	$G_{heterogeneity}$ = 23.7114 df = 21, p = 0.3072		G_{pool} = 3.0308 df = 1, p = 0.0817		G_{total} = 26.7421 df = 22, p = 0.2212

Table A.1. (cont'd)

Floral mechanical isolation – for the two years combined

C. allenii flowers	Observed conspecific dye index	Observed heterospecific dye index	Expected conspecific dye ratio	Expected heterospecific dye ratio	G
1	0	6	0.3169	0.6831	4.5734
2	3	2	0.3169	0.6831	1.6893
3	3	6	0.3169	0.6831	0.0111
4	2	0	0.3169	0.6831	4.5967
5	4	1	0.3169	0.6831	4.9515
6	2	1	0.3169	0.6831	1.5398
7	2	1	0.3169	0.6831	1.5398
8	6	0	0.3169	0.6831	13.790
9	5	0	0.3169	0.6831	11.492
10	0	4	0.3169	0.6831	3.0489
11	2	1	0.3169	0.6831	1.5398
12	2	3	0.3169	0.6831	0.1532
13	3	3	0.3169	0.6831	0.8639
14	6	4	0.3169	0.6831	3.3788
15	3	2	0.3169	0.6831	1.6893
16	2	1	0.3169	0.6831	1.5398
17	4	4	0.3169	0.6831	1.1519
18	0	4	0.3169	0.6831	3.0489
19	1	2	0.3169	0.6831	0.0037
20	4	3	0.3169	0.6831	1.9193
21	4	4	0.3169	0.6831	1.1519
22	2	2	0.3169	0.6831	0.5759
23	3	3	0.3169	0.6831	0.8639
24	4	2	0.3169	0.6831	3.0796
25	3	2	0.3169	0.6831	1.6893
26	0	1	0.3169	0.6831	0.7622
27	2	6	0.3169	0.6831	0.1727
28	3	3	0.3169	0.6831	0.1532
29	2	3	0.3169	0.6831	0.1532
30	0	1	0.3169	0.6831	0.7622
31	1	2	0.3169	0.6831	0.0037
	$G_{heterogeneity} = 49.71$ df = 30, p = 0.0132		$G_{pool} = 22.1739$ df = 1, p < 0.0001		$G_{total} = 71.8888$ df = 31, p < 0.0001

Table A.1. (cont'd)

Floral mechanical isolation – for the two years combined

C. villosissimus flowers	Observed conspecific dye index	Observed heterospecific dye index	Expected conspecific dye ratio	Expected heterospecific dye ratio	G
1	0	0	0.7232	0.2768	0.0000
2	3	4	0.7232	0.2768	2.6597
3	4	4	0.7232	0.2768	1.7781
4	6	3	0.7232	0.2768	0.1384
5	4	2	0.7232	0.2768	0.0923
6	4	3	0.7232	0.2768	0.7388
7	4	0	0.7232	0.2768	2.5924
8	2	4	0.7232	0.2768	3.9341
9	4	0	0.7232	0.2768	2.5924
10	0	0	0.7232	0.2768	0.0000
11	4	4	0.7232	0.2768	1.7781
12	4	2	0.7232	0.2768	0.0923
13	2	2	0.7232	0.2768	0.8891
14	6	5	0.7232	0.2768	1.5755
15	5	2	0.7232	0.2768	0.0028
16	3	1	0.7232	0.2768	0.0146
17	4	3	0.7232	0.2768	0.7388
18	2	0	0.7232	0.2768	1.2962
19	6	1	0.7232	0.2768	0.7160
20	2	1	0.7232	0.2768	0.0461
21	6	1	0.7232	0.2768	0.0716
22	2	0	0.7232	0.2768	1.2962
23	3	1	0.7232	0.2768	0.0146
24	1	1	0.7232	0.2768	0.4445
25	3	0	0.7232	0.2768	1.9443
26	3	0	0.7232	0.2768	1.9443
27	0	3	0.7232	0.2768	7.7071
28	2	0	0.7232	0.2768	1.2962
29	3	1	0.7232	0.2768	0.0146
30	1	1	0.7232	0.2768	0.4445
31	2	0	0.7232	0.2768	1.2962
	$G_{heterogeneity}$ = 36.0139 df = 30, p = 0.2076		G_{pool} = 2.7804 df = 1, p = 0.0954		G_{total} = 38.7943 df = 31, p = 0.1585

Table A.1. (cont'd)

Gametic isolation – hand-pollinated *C. allenii* fruits

Fruit No.	Observed conspecific progeny	Observed heterospecific progeny	Expected conspecific progeny ratio	Expected heterospecific progeny ratio	G
1	3	0	0.5	0.5	4.1589*
2	4	0	0.5	0.5	5.5452*
3	5	0	0.5	0.5	6.9315*
4	5	1	0.5	0.5	2.9110
5	6	0	0.5	0.5	8.3178*
6	7	0	0.5	0.5	9.7041*
7	8	0	0.5	0.5	11.090*
8	8	2	0.5	0.5	3.8549*
9	9	1	0.5	0.5	7.3613*
10	9	2	0.5	0.5	4.8182*
11	10	0	0.5	0.5	13.863*
12	10	0	0.5	0.5	13.863*
	$G_{heterogeneity}$ = 11.7399 df = 11, p = 0.3835		G_{pool} = 80.6791 df = 1, p < 0.0001		G_{total} = 92.4190 df = 12, p < 0.0001

Gametic isolation – hand-pollinated *C. villosissimus* fruits

Fruit No.	Observed conspecific progeny	Observed heterospecific progeny	Expected conspecific progeny ratio	Expected heterospecific progeny ratio	G
1	2	1	0.5	0.5	0.3398
2	2	7	0.5	0.5	2.9419
3	2	8	0.5	0.5	3.8549
4	4	4	0.5	0.5	0.0000
5	5	5	0.5	0.5	0.0000
6	6	2	0.5	0.5	2.0930
7	6	3	0.5	0.5	1.0194
8	8	2	0.5	0.5	3.8549
9	9	0	0.5	0.5	12.477
10	9	1	0.5	0.5	7.3613
11	10	0	0.5	0.5	13.863
12	2	1	0.5	0.5	0.3398
	$G_{heterogeneity}$ = 38.2709 df = 10, p < 0.0001		G_{pool} = 9.5339 df = 1, p = 0.0020		G_{total} = 47.8048 df = 11, p < 0.0001

Table A.1. (cont'd)

Sexual isolation – naturally-pollinated *C. allenii* fruits

Fruit No.	Observed conspecific progeny	Observed heterospecific progeny	Expected conspecific progeny ratio	Expected heterospecific progeny ratio	G
1	0	3	0.5	0.5	4.1589
2	0	5	0.5	0.5	6.9315
3	0	10	0.5	0.5	13.863
4	1	2	0.5	0.5	0.3398
5	1	9	0.5	0.5	7.3613
6	2	1	0.5	0.5	0.3398
7	3	0	0.5	0.5	4.1589
8	3	1	0.5	0.5	1.0465
9	3	1	0.5	0.5	1.0465
10	3	2	0.5	0.5	0.2014
11	3	5	0.5	0.5	0.5053
12	5	0	0.5	0.5	6.9315
13	5	1	0.5	0.5	2.9110
14	6	0	0.5	0.5	8.3178
15	6	0	0.5	0.5	8.3178
	$G_{heterogeneity}$ = 66.4184 df = 14, p < 0.0001		G_{pool} = 0.0123 df = 1, p = 0.9116		G_{total} = 66.4308 df = 15, p < 0.0001

Table A.1. (cont'd)

Sexual isolation – naturally-pollinated *C. villosissimus* fruits

Fruit No.	Observed conspecific progeny	Observed heterospecific progeny	Expected conspecific progeny ratio	Expected heterospecific progeny ratio	G
1	1	3	0.5	0.5	1.0465
2	1	5	0.5	0.5	2.9110
3	2	4	0.5	0.5	0.6796
4	3	0	0.5	0.5	4.1589
5	3	0	0.5	0.5	4.1589
6	3	0	0.5	0.5	4.1589
7	3	1	0.5	0.5	1.0465
8	4	1	0.5	0.5	1.9274
9	4	4	0.5	0.5	0.0000
10	6	3	0.5	0.5	1.0194
11	6	3	0.5	0.5	1.0194
12	7	0	0.5	0.5	9.7041
13	8	0	0.5	0.5	11.090
14	8	0	0.5	0.5	11.090
15	8	1	0.5	0.5	6.1977
16	9	0	0.5	0.5	12.477
17	10	0	0.5	0.5	13.863
18	10	0	0.5	0.5	13.863
19	10	0	0.5	0.5	13.863
	$G_{heterogeneity}$ = 60.3787 df = 18, p < 0.0001		G_{pool} = 53.8957 df = 1, p < 0.0001		G_{total} = 114.2744 df = 19, p < 0.0001

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Chapter 2

Local Adaptation to Different Microhabitats in Neotropical *Costus*

ABSTRACT

Local adaptation to different environmental conditions may drive speciation. When the spatial distributions of species do not overlap, the probability of hybridization is reduced. If local adaptation is a primary cause of spatial isolation, such that species are unable to colonize "foreign" habitats, adaptation can be considered a prezygotic isolating barrier. Furthermore, if hybrids cannot perform as well as the parental species in either of the parental habitats, local adaptation may also contribute to extrinsic postzygotic isolation. To understand the relationship between local adaptation and speciation, I studied the distribution of two recently diverged Neotropical plant species, *Costus allenii* and *C. villosissimus*, in central Panama. Here the two species display a parapatric distribution that reflects local environmental differences, particularly in relation to gradients in precipitation across the Isthmus of Panama. I summarized precipitation data from local weather stations and measured soil moisture in populations of both species. The locations of individuals of the two species were mapped to determine their spatial distributions, and these data were used to estimate the strength of habitat isolation. Reciprocal transplant experiments carried out with seeds and cuttings of mature plants of the two species and their hybrids were conducted to determine whether the two species are locally adapted to their home habitats and whether there is evidence of extrinsic postzygotic isolation.

I found that the distinct spatial distributions of these species are strongly associated with local soil moisture in the dry season--*Costus allenii* is found in habitats with higher water availability. Both species performed better in their home habitats than in foreign habitats, indicating that their parapatric distribution is due to local adaptation. There was evidence of extrinsic postzygotic isolation only at the seed stage-- F1 hybrid seeds had reduced fitness when transplanted into *C. villosissimus* habitats. Therefore, local adaptation contributes strongly to

habitat isolation and weakly to extrinsic postzygotic isolation between these two recently diverged species. Because habitat isolation is the major reproductive barrier in this system, I conclude that local adaptation is the primary mechanism of speciation between *C. allenii* and *C. villosissimus*.

INTRODUCTION

Ecology has been recognized as a major factor in speciation (Darwin, 1859; Dobzhansky, 1937; Mayr, 1947, 1963; Schluter, 2001; Coyne and Orr, 2004; Sobel et al., 2010). Local adaptation caused by divergent selection in different environments may lead to reproductive isolation (Schluter, 2001; Rundle and Nosil, 2005; Nosil et al., 2009; Sobel et al., 2010). When locally adapted species occupying different habitats are unable to colonize foreign habitats, the probability of hybridization is reduced (Mayr, 1963; Coyne and Orr, 2004; Nosil et al., 2005). Local adaptation thus reduces heterospecific gene flow and leads to ecogeographic and/or microhabitat isolation (reviewed in Schluter, 2001; Coyne and Orr, 2004; Rundle and Nosil, 2005; Hendry et al., 2007; Schemske, 2010; Sobel et al., 2010). If hybrids are formed, divergent selection against the parental species in the foreign habitats may also reduce survival, growth, and reproduction of hybrids in either of the parental habitats (reviewed in Coyne and Orr, 2004; Rundle and Nosil, 2005). The recombination of divergently adaptive traits may result in unfavorable phenotypes and reduced fitness when hybrids are grown in the parental habitats (e.g., Hatfield and Schluter, 1999; Campbell and Waser, 2007; McBride and Singer, 2011). When hybrids experience reduced fitness in the parental habitats, local adaptation may lead to extrinsic postzygotic isolation and further reduce heterospecific gene flow (reviewed in Coyne and Orr, 2004; Rundle and Nosil, 2005).

Divergent selection in different habitats and subsequent local adaptation in closely related species are often reflected in parapatric or allopatric geographic distribution (Kawecki and Ebert 2004; Fine et al., 2005). When two closely related species display nonoverlapping distributions congruent with environmental differences, it is reasonable to suspect that local adaptation contributes to spatial isolation. However, differences in species' geographic ranges may also be

influenced by historical factors (Endler, 1982; Coyne and Orr, 2004; Thorpe et al., 2008; Sobel et al., 2010). To determine the cause of the nonoverlapping geographic distribution, reciprocal transplant experiments can be conducted to separate ecological from historical factors (Sobel et al., 2010). Local adaptation to home habitats and maladaptation in foreign habitats can be demonstrated by artificially moving organisms to environments where they naturally do not occur (reviewed in Kawecki and Ebert, 2004; Leimu and Fischer, 2008). Organisms usually have low fitness and fail to establish a viable population when they are transplanted beyond their current distribution range (e.g., Angert and Schemske 2005). When reduced fitness is observed in foreign habitats compared to that in home habitats, we conclude that the current distribution is mainly due to biological constraints, not to history (Schemske, 2000; Sobel et al., 2010). However, reciprocal transplant experiments are more difficult to conduct with animals than with plants because animals have greater mobility. Conducting reciprocal transplant experiments with plants have been commonly used to test adaptation to different environments between closely related species (e.g., Clausen et al., 1940; Campbell and Waser, 2001; Angert and Schemske, 2005). To examine whether current species distribution is due to local adaptation, I am more interested in conducting reciprocal transplant experiments with plants.

Just as a reciprocal transplant experiment between closely related species estimates the degree of prezygotic isolation due to habitat, such an experiment conducted with hybrids between the parental species can be a measurement of postzygotic isolation. When hybrids have reduced fitness independent of their environment, intrinsic postzygotic isolation occurs as a consequence of genetic incompatibilities between the parental genomes. In contrast, when hybrids have lower fitness than parental species in either of the parental habitats, heterospecific gene flow is reduced due to this genotype-by-environment interaction, i.e., extrinsic postzygotic

isolation (Rundle and Whitlock, 2001; Coyne and Orr, 2004; Kimball et al., 2008). However, hybrids may also have superior fitness than parental species in the corresponding parental habitats, especially in early generations (Burke and Arnold, 2001). In this case, postzygotic isolation does not exist and heterospecific gene flow is not reduced by natural selection against hybrids. Despite the large number of reciprocal transplant experiments conducted in plant systems, most have not incorporated hybrids, so estimates of extrinsic postzygotic isolation are rare (Widmer et al., 2009).

In their review of plant speciation studies, Lowry et al. (2008a) found that most studies provided evidence of genetically-based habitat isolation, but only a few examined extrinsic postzygotic isolation. Schemske (2010) reviewed speciation studies of both plant and animal systems and concluded that habitat isolation makes a significant contribution to speciation while few studies measured the relative contribution of extrinsic postzygotic isolation. For example, studies of the narrowly sympatric, closely related species *Mimulus cardinalis* and *M. lewisii* show that plants are adapted to different elevation (Angert and Schemske, 2005; Angert et al., 2008), and that habitat isolation is the major isolating mechanism between the two species (Ramsey et al., 2003). However, because of the low likelihood of hybrid formation, hybrid fitness in parental habitats has not been measured and extrinsic postzygotic isolation has not been examined in this system. In another study of the role of local adaptation in speciation, Lowry et al. (2008b) compared inland and coast races of *M. guttatus* by reciprocal transplanting seedlings of parental races and F1 hybrids. They demonstrated local adaptation causing strong habitat isolation but not extrinsic postzygotic isolation between races (Lowry et al., 2008b).

To understand the role of local adaptation in speciation requires studies involving reciprocal transplant experiments of both parental species and hybrids in parental habitats.

Although emerging evidence demonstrates local adaptation in many systems, fitness of parental species and hybrids in parental habitats was rarely compared through life history. Such comparisons in recently diverged species are more likely to reflect the ecological and evolutionary conditions at the time of speciation, before additional adaptive differences accumulate after two species have been completely speciated (Schemske, 2010).

To understand whether local adaption contributes to microhabitat isolation and extrinsic postzygotic isolation, I studied two recently diverged species, *Costus allenii* and *C. villosissimus*. Both *C. allenii* and *C. villosissimus* are found in Central America and northern South America but they occupy different habitats (Maas, 1972; Ch.4). The typical habitats of *C. allenii* are moist forest understory along ravines, while those of *C. villosissimus* are more open sites along forest edges or large gaps (Ch. 3). In this chapter, I examined two environmental factors, precipitation and soil moisture, in central Panama, where the two species co-occur, and determined the spatial distribution of the two species in this region. Habitat differences between the two species may lead to isolation on a large spatial scale, i.e., ecogeographic isolation, as well as on a small spatial scale, i.e., microhabitat isolation. In a geographic region where both species occur within a short distance, the low resolution of ecogeographic distribution limits our ability to properly assess spatial isolation. In such a region, measuring microhabitat isolation is more appropriate to determine the reduction of gene flow due to habitat differences. To this end, the distribution data in central Panama were used to calculate the strength of microhabitat isolation.

I also conducted reciprocal transplant experiments to determine whether the current distribution is a consequence of local adaptation. Parental species and hybrids were reciprocally transplanted to determine whether there is evidence of extrinsic postzygotic isolation. Because *Costus* are perennials and it may take decades to assess the lifetime fecundity of a plant, the

reciprocal transplant experiments were conducted with plants of two life stages: seeds and cuttings of mature plants. This experimental design allows us to compare fitness through life history of these perennials in a reasonable time frame. Given the observed habitat differences between the two species, I hypothesized that 1) precipitation is lower in *C. villosissimus* habitats, 2) the distribution of the two species correlates with soil moisture, 3) the two species are locally adapted to their home habitats, and 4) the hybrids have reduced fitness in parental habitats.

MATERIALS AND METHODS

Study System

The genus *Costus* is a clade of perennial, tropical herbs characterized by their spirally arranged leaves. *Costus* occupy diverse habitats including tree-fall gaps, forest edges, understory streamsides, a range of edaphic conditions such as red clay, white sand, and limestone, and are found from low to mid elevation (Maas, 1972; Schemske, 1983; Kay et al., 2005). Individual plants usually produce one to several upright stems from their rhizome and terminal inflorescences, which produce morphologically complex, bee- or hummingbird-pollinated flowers. The subgenus *Costus*, which comprises the majority of species in the genus *Costus*, has diversified rapidly in the Neotropics during the last three to five million years (Kay et al., 2005).

Costus allenii and *C. villosissimus* are sister taxa found in Central America and northern South America (Kay et al., 2005). *Costus allenii* is found mostly in Panama, Colombia, and Venezuela, while *C. villosissimus* has a wider distribution, including Mexico to the northwest, Ecuador to the south, and Guyana to the east (Maas, 1972; Ch. 4). Although the two species are found in the same geographic region, they occupy different habitats: *C. allenii* is located along ravines in rainforest understory, while *C. villosissimus* inhabits forest edges. Both species flower in the early wet season (late May to August; Ch. 4) and are pollinated by euglossine bees (Ch. 1).

Fully fertile F1 and F2 hybrids of the two species can be easily made by hand-pollination in the field or greenhouse, yet hybrids are rarely found in nature.

Study Sites

This study was conducted at a number of sites in central Panama, where natural populations of both *C. allenii* and *C. villosissimus* are found (Maas, 1972; Ch. 4). Water and light availability differ significantly between microhabitats of the two species (Ch. 3). Across the Isthmus of Panama, a distance of 80 km, more rain falls on the Atlantic side (mean annual precipitation = 3234 mm at Cristobal) than on the Pacific side (mean annual precipitation = 1798 mm at Balboa Heights) (Panama Canal Authority Meteorological and Hydrological Service weather station network). Soberania National Park (SNP), located along the Panama Canal approximately 25 km north of Panama City, served as the primary study area for the research described here. SNP encompasses 22,104 hectares, and includes populations of both of the study species. The spatial distribution of the two species was determined in the vicinity of Pipeline Road (PLR; Fig. 2.1), which runs through SNP from south to north and parallels the Panama Canal. Along the southern end of PLR, the road is wide and the canopy is open. As the road progresses northward, the canopy closes gradually and the road narrows until becoming completely inaccessible. The gradient of water availability and light availability along PLR spans the typical habitats of *C. allenii* and *C. villosissimus*. I conducted studies of spatial distribution along 17 km of PLR. Both species were found along this section, with *C. allenii* predominating in the northern stretch and *C. villosissimus* to the south.

For the reciprocal transplant experiments, the "home" site for *C. villosissimus* (VP) was located between 1 km and 2 km from the entrance of PLR and the "home" site for *C. allenii* (AP) was located between 10 km and 11 km from the entrance (Fig. 2.1). Two additional locations

were used in the reciprocal transplant experiments, 1) Gigante Peninsula (AG) in Barro Colorado Nature Monument was used as representative of a *C. allenii* habitat, and 2) a site near the Chagres River in Gamboa (VG), which is representative of the habitat occupied by *C. villosissimus* (Fig. 2.1). In summary, two sites per species' habitat type were included in the reciprocal transplant experiment: *C. allenii* sites on PLR (AP) and on Gigante Peninsula (AG), and *C. villosissimus* sites on PLR (VP) and in Gamboa (VG) (Fig. 2.1).

Spatial and Seasonal Variation in Water Availability

To compare the differences in precipitation between habitats of *C. allenii* and *C. villosissimus*, I used rainfall data collected by the Panama Canal Authority Meteorological and Hydrological Service weather station network (http://striweb.si.edu/esp/physical_monitoring/data/tesp/acp_rain_mon.zip). Monthly precipitation records from weather stations at Frijolito (9°13'N, 79°43W) and Gamboa (9°06N, 79°42W) were used to represent *C. allenii* and *C. villosissimus* habitats, respectively (Fig. 2.1). From May 1998 to December 2007, the period when precipitation data were available from both weather stations, monthly precipitation was compared between Frijolito and Gamboa using a paired t-test. In central Panama, the wet season usually begins in May and ends in December, while the dry season is from January to April. To examine seasonal differences, I used paired t-tests to compare differences in monthly precipitation between the two locations for months in the wet and dry seasons, respectively. All statistical analyses in this study were done in R, version 2.12.2 (R Development Core Team, 2011).

To examine soil moisture along the distribution of *C. allenii* and *C. villosissimus*, I collected soil samples along PLR and measured gravimetric water content. Two soil cores were extracted from the top 15 cm of soil from each kilometer point along the road, from the south

entrance of PLR (0 km) to the 11 km point, in June 2007 and in March 2008. The samples were collected at these two time points to represent the wet and dry seasons, respectively. The soil samples were sealed in a plastic bag and weighed with a digital balance within 12 hours. Samples were then weighed after being oven dried at 60ºC for 7 days. The gravimetric water content in the soil sample was calculated as: (wet weight – dry weight) / wet weight. Soil moisture for each kilometer section was calculated by averaging gravimetric water content of samples collected at two adjacent kilometer points. The association between gravimetric water content and the location of the corresponding kilometer section was analyzed using Pearson correlation for each season to determine the change in soil moisture along PLR, where natural populations of both species are found.

Spatial Distribution

The spatial distributions of both species were determined by censusing individual plants in the vicinity of PLR from March 2006 to July 2009. Because only flowering plants can contribute to gene flow by pollen, I recorded the reproductive status of each plant as "flowering" or "non-flowering". Plants that had produced at least one inflorescence during the census period were classified as "flowering", and plants without inflorescences were classified as "non-flowering". According to the species descriptions listed in Maas (1972) and from greenhouse observation, plants with broad leaves, brownish trichomes, large extrafloral nectaries, rounded bracts, and small, cream-colored flowers with red stripes were identified as *C. allenii*, while plants with narrow leaves, white trichomes, small extrafloral nectaries, leafy bracts, and large, yellow flowers were identified as *C. villosissimus*. Flowering hybrids were easily distinguished from the parental species by their large yellow flowers with red stripes, and non-flowering plants with narrow leaves but brownish hair or vice versa were identified as putative non-flowering

hybrids. The shortest distance between heterospecific flowering individuals was measured to investigate the maximum likelihood of hybridization. Kolmogorov-Smirnov tests were used to determine whether the spatial distribution of the two parental species (flowering and non-flowering) were significantly different. To conduct Kolmogorov-Smirnov tests, I calculated the proportion of plants of each species and stage located within each kilometer section.

Within each kilometer section, I also determined species composition. Species composition was measured with the proportion of flowering and non-flowering plants which are identified as *C. allenii*, representing the abundance of *C. allenii* in relative to that of *C. villosissimus*. To examine the relationship between water availability and the distribution of *C. allenii* and *C. villosissimus*, the correlation between gravimetric water content in each season and the relative abundance of flowering and non-flowering *C. allenii* was analyzed. Because gravimetric water content was only assessed from samples collected between 0 km and 11 km, only plants found within this section of PLR were included in the analysis.

Reciprocal Transplant Experiments

To compare fitness between the two *Costus* species and their hybrids in the two types of parental habitats, reciprocal transplant experiments were conducted with plants of two life stages: seeds and cuttings of mature plants. The experiment with seeds assesses the differences in seed germination and early seedling survival after one year of transplanting, while the experiment with cuttings examines the differences in plant survival, growth, and reproduction after establishment. This approach was used to increase the likelihood that sample sizes would be sufficient for statistical analysis across the entire life history. Because the study species are perennials with a relatively long pre-reproductive period (> 2 years) and low seed germination

and seedling survival under natural conditions (Chen, personal observation), it was not feasible to conduct the reciprocal transplant studies exclusively with seed.

Seed Transplants.

Four categories of seeds were used in the reciprocal transplant experiment at the early life stage: the two parental species and two F1 hybrids derived from reciprocal crosses between the parents, with either *C. allenii* as the female and *C. villosissimus* as male ($F1_{allenii}$), or vice versa ($F1_{villo}$). These two parental species and two F1 hybrids are thereafter referred as the four categories of plants. These seeds were produced by hand-pollinating flowers in the natural populations on PLR in June 2007 and 2008. The flowers were bagged before and after being hand pollinated to prevent natural pollination. Pollen collected from a flower was applied to the stigma with a flat toothpick in the mornings to mimic the timing of natural pollinators. Fifty-five seeds from each of the four categories were transplanted into the *C. allenii* habitat (AP) and the *C. villosissimus* habitat (VP) on PLR in both October 2007 and 2008 for a total of 880 seeds. The seeds were planted in transects at approximately 50 cm spacing and randomly arranged along the forest edge in *C. villosissimus* habitat or along a forested ravine in *C. allenii* habitat. A plastic disposable cup (300 ml, 7 cm in diameter and 12 cm in depth) was used as a mini-pot for each seed. The bottom of the cup was removed in order to allow water to be transported in or out of the cup naturally. For each transplanted seed, I dug a round hole (approximately 7 cm in diameter and 8 cm in depth), inserted the bottomless cup, filled the cup with native soil to ground level, and placed the seed at the center of the cup, approximately 0.5 cm below the surface.

In the seed transplant experiments, two fitness components were measured: seed germination and seedling survival. The 2007 cohort was monitored on a monthly basis from the date of transplanting to May 2009, the end of the second dry season experienced by the

transplants. For the 2008 cohort, these fitness components were assessed on a monthly basis for approximately one year. Because the total number of seeds germinating and surviving was low and the overall pattern remained consistent in the two cohorts, to improve statistical power of fitness comparisons, the data of the two cohorts were averaged at a monthly basis for the first12 months since transplanting. In the 2007 cohort, the additional data which was collected after one year of transplanting were used to demonstrate how different categories may respond to the second dry season in their life history. To compare fitness differences among categories and between habitats, the proportion of seeds germinating and the proportion of germinated seedlings surviving in the one-year period was analyzed with logistic regression with the averaged dataset of the two cohorts. The proportion of seeds germinating and also surviving in the one-year period was calculated to represent absolute fitness of seed transplants. Absolute fitness was then compared among categories and between habitats using logistic regression with the averaged dataset. Within each site, the average absolute fitness of $F1_{allenii}$ and $F1_{villo}$ was compared to the absolute fitness of the parental species in its home habitat with a G test of goodness of fit.

Cutting Transplants.

Cuttings of *C. allenii*, *C. villosissimus*, $F1_{allenii}$, and $F1_{villo}$ were used in the reciprocal transplant experiment conducted for estimating the post-seedling phase of the life history. Plants used to produce the cuttings were derived from hand pollination of flowers of the two parental species. These seed parents were established from seed collected from natural populations in Central Panama and were grown to flowering in the greenhouse at Michigan State University. Clonal cuttings of 16 *C. allenii*, 36 *C. villosissimus*, and 5 $F1_{allenii}$, and 4 $F1_{villo}$ were made in the MSU greenhouse before being transported to Panama. A healthy stem was cut into several

small pieces (9 to 12 cm long) and grown in incubators. Each cutting contained at least one node, and most leaf tissue was removed from the cutting before being planted. A small section (~ 5 cm^2) of healthy, green tissue on the newest leaf was retained to allow growth during cultivation. Cuttings were placed individually in test tubes (Aquatube #53, Syndicate Sales, Inc., IN) with distilled water. Incubators were set for 12 hour light (6 am to 6 pm), at temperatures of 30°C daytime and 24°C nighttime. Cuttings were checked twice a week to remove damaged tissue and to change water.

After 2 months of growth in the tubes, cuttings that had produced new shoots were sealed with parafilm and shipped to Panama. These were then planted into 1-L plastic grow bags containing potting soil (manufactured by Do it Center, Panama), and grown in a greenhouse in Gamboa. To obtain enough healthy cuttings for all transplant sites, this cloning/shipping process was repeated three times in 2006 and 2007. Importation permits were obtained from Autoridad Nacional del Ambiente (SIM/P-3-06, No. SIM/P-3-07), and from Ministerio de Desarrollo Agropecuario Direccion (#560569, #560575, #561805, #580448, #580459, #580448, #607295, #607296, #607297).

Cuttings were randomly assigned to different transplant sites and planted in a random order along the forest edges at the *C. villosissimus* sites (VP and VG) and along ravines at the *C. allenii* sites (AP and AG). In June 2006, I planted 18 *C. allenii*, 18 *C. villosissimus*, 20 $F1_{allenii}$, and 26 $F1_{villo}$ cuttings at the AG site and 13 *C. allenii*, 18 *C. villosissimus*, 10 $F1_{allenii}$, and 13 $F1_{villo}$ cuttings at each of the *C. villosissimus* sites (VP and VG). In June 2007, I planted 13 *C. allenii*, 18 *C. villosissimus*, 10 $F1_{allenii}$, and 13 $F1_{villo}$ cuttings at the AP site. The number of

leaves present on each plant was counted at the time of transplanting. Across all sites and years, a total of 224 plants were transplanted.

Three fitness components, survival, growth, and reproduction, were measured in the cutting transplant experiment. The survival and size of each plant was monitored twice a year until 2010, in March to represent the dry season, and in June to represent the wet season. An index of plant growth was calculated as the number of leaves present on the stems at a given census divided by the number of leaves at the time of transplanting. Plants that had maintained the same number of leaves during a given period would have a growth index of 1, plants that had increased the number of leaves would have a growth index > 1, and plants that had lost all their leaves would have a growth index of 0. Plants without aboveground tissue were considered dead, and growth was calculated only for living plants.

Because plant survival varied among categories and between habitats, some categories had small sample sizes for the analyses of growth and reproduction. Given that the fitness patterns are consistent between sites of the same parental habitats, data from the two sites representing the same parental habitats were combined in all fitness comparisons to improve statistical power. Plant survival from the beginning of the experiment to June 2010 was compared among categories and between habitats using logistic regression with initial size, i.e., leaf number, as a covariate. To compare the fitness differences among surviving plants, plant growth from the date of transplanting to June 2010 was also compared among categories and between habitats using a two-way ANOVA. I compared the average survival and growth of F1 hybrids with that of the parental species in its home habitat with a G test of goodness of fit and an ANOVA, respectively.

To analyze reproduction, the number of plants which produced at least one inflorescence from 2007 to 2010 and the number of inflorescences produced by flowering plants was compared among plant categories and between habitats using G tests of goodness of fit. Because only a small proportion of cutting transplants flowered during the experimental period and some currently non-flowering plants may produce flowering beyond the experimental period, reproduction was omitted in the fitness calculation of cutting transplants. Absolute fitness of cutting transplants was calculated as the product of the first two fitness components, i.e., the proportion of cuttings surviving and the proportional change of plant size of the surviving plants.

Pest damage to parents and hybrids was measured in the cutting transplant experiment. The proportion of leaf damage was estimated on new leaves in June and July, 2008 and 2010. In June, a twist tie was loosely applied to each stem between the second and the third leaf from the apex. At this time, the third leaf is usually newly-expanded, and can support the weight of the twist tie. The position of the twist tie does not affect subsequent growth. Pest damage on the new leaves was estimated approximately 30 days after the plants were marked. Each new leaf produced in the 30-day period was assigned to a damage level of 0%, 0-25%, 25-50%, 50-75%, and 75-100%. A damage index was calculated as the average damage level on these new leaves. Only plants alive at the time of damage measurements were included in the analysis, and therefore, sample sizes among sites and among species and hybrids differed. Pest damage on new leaves was arcsine-transformed and then compared among categories and between habitats using a two-way ANOVA and Tukey's HSD. For plants which were alive in 2008 and 2010, the measurements of the two years were averaged to reduce heterogeneity of damage level due to fluctuation within individual plants. For plants which were alive in 2008 but not 2010, the one-time measurements were included in the analysis as these were the best data available.

Total Fitness.

The results of the seed and cutting transplants were combined into an estimate of total fitness for each category in each habitat. Seed data from the 2007 and 2008 cohorts were combined to generate one absolute fitness value, the proportion of seeds germinating and also surviving after one year of transplanting, for each category in each habitat. Absolute fitness of cuttings transplanted into sites AG and AP were combined for calculating their fitness in *C. allenii* habitats, while absolute fitness of cuttings transplanted into site VG and VP were combined for calculating their fitness in *C. villosissimus* habitats. As the fitness components measured in the two transplant experiments are independent of each other, absolute total fitness was the product of absolute fitness in seed transplants and absolute fitness in cutting transplants. Relative total fitness was determined by dividing absolute fitness of each category in each habitat by absolute fitness of the parental species in its home habitat.

Reproductive Isolation

Reproductive isolation due to microhabitat differentiation ($RI_{habitat}$) was calculated for each species using the distribution of the two species along PLR. The distribution was determined from the number of flowering plants located within each kilometer section because only flowering plants can contribute to gene flow. Following Sobel and Chen (in prep), $RI_{habitat}$ was calculated as

$$RI_{habitat} = 1 - 2\sum_i \left(\frac{A_i}{A_{total}} \times \frac{V_i}{A_i + V_i}\right)$$

for *C. allenii* and

$$RI_{habitat} = 1 - 2\sum_i \left(\frac{V_i}{V_{total}} \times \frac{A_i}{A_i + V_i}\right)$$

for *C. villosissimus*, with A_i and V_i representing the number of *C. allenii* and *C. villosissimus* individuals found in kilometer section *i*, respectively, while A_{total} and V_{total} representing the total number of *C. allenii* and *C. villosissimus* plants found along PLR, respectively. This approach assumes that the population sizes are similar for the two species (Sobel and Chen, in prep). If the isolation index equals zero, the distribution of the two species is completely random and there is no spatial isolation. If the isolation index equals one, there is no overlap in the distribution of the two species; thus, they are parapatric or allopatric. In such cases, heterospecific gene flow would be completely eliminated unless pollinators travel and transfer heterospecific pollen efficiently across the geographic barrier.

The performance of hybrids in the reciprocal transplant experiments in comparison with the performance of the transplanted parental species in their home habitats represents the strength of postzygotic isolation. The differences in fitness between hybrids and parental species are affected by both genetic and environmental factors, which contribute to intrinsic and extrinsic postzygotic isolation, respectively. When grown under greenhouse conditions, F1 hybrids between *C. allenii* and *C. villosissimus* germinate, survive, grow, and reproduce as well as the parental species (Chen, personal observation). In contrast, any differences in fitness between hybrids and parental species when grown in parental habitats in the field are mostly environment-dependent. Therefore, reproductive isolation caused by the reduced performance of hybrids in the reciprocal transplant experiments mostly represents extrinsic postzygotic isolation.

To estimate postzygotic isolation for each species, the fitness of hybrids and parental species were compared at two life stages. Seed germination and early seedling survival were used for calculating an index RI_{seed}, while cutting survival and growth was used for calculating

an index $RI_{cutting}$. Because only a few transplants flowered during the experimental period and the surviving plants may produce inflorescences beyond the experimental period, reproduction was not included in the calculation of $RI_{cutting}$. For both RI_{seed} and $RI_{cutting}$ of each species, the indices were calculated as

$$RI = 1 - 2 * \left(\frac{H}{C + H} \right)$$

(Sobel and Chen, in prep). The probability of conspecific gene flow (C) was estimated as the fitness of one parental species transplanted into its home habitats, and the probability of heterospecific gene flow (H) was estimated as the average fitness of $F1_{allenii}$ and $F1_{villo}$ transplanted into the same parental habitats. In the calculation of RI_{seed}, the fitness of each category was measured as the number of seeds that germinated and survived after one year. In the calculation of $RI_{cutting}$, the fitness of each category was measured as the multiplicative product of the proportion of surviving transplants and the growth of the surviving plants from the time of transplanting to June 2010.

RESULTS

Spatial and Seasonal Variation in Water Availability

The precipitation data showed that more rain falls in Frijolito (average monthly precipitation = 200.34 mm) in the north than in Gamboa in the south (183.16 mm; $t = 2.60$, $df = 115$, $p = 0.01$). The precipitation difference between locations was primarily due to a significant difference in the dry season. In the dry season, from January to April, more rain fell in Frijolito (92.59 mm) than in Gamboa (69.18 mm) ($t = 2.85$, $df = 44$, $p = 0.007$). In the wet season, from

May to December, similar amount of rainfall was recorded in Frijolito (268.62 mm) and Gamboa (255.41 mm) ($t = 2.85$, $df = 70$, $p = 0.17$) (Fig. 2.2).

Soil moisture increased northward along PLR (Fig. 2.3A). A positive correlation between gravimetric water content and road kilometer sections was found in both the wet ($R = 0.62$, $p = 0.046$) and the dry season ($R = 0.66$, $p = 0.03$).

Spatial Distribution

I located 1203 individual plants on PLR, including 254 plants which had flowered at least once during the four year period and 949 plants which did not flower in these four years. The flowering plants included 93 *C. allenii*, 156 *C. villosissimus*, and 5 putative hybrids, while the non-flowering plants included 557 *C. allenii*, 386 *C. villosissimus*, and 6 putative hybrids. There was a significant difference in the distribution of flowering *C. allenii* and *C. villosissimus* ($D = 0.65$, $p = 0.001$), and in the distribution of non-flowering plants ($D = 0.65$, $p = 0.003$) (Fig. 2.3). Among the flowering individuals, 95.5% of *C. villosissimus* were located toward the south side of PLR (between 0 km and 5.8 km), while 95.7% of *C. allenii* were found in the north side of the road (between 7.8 km and 17 km). For non-flowering plants, 91.2% of *C. villosissimus* were located from the south entrance to approximately 5.8 km northwards, while 90.5% of *C. allenii* were found beyond the 7.8 km point of PLR. The shortest distance between two heterospecific, flowering individuals was approximately 75 m, which occurred approximately at the 5.8 km point of PLR. More than 80% of the flowering individuals are at least 500 m away from a heterospecific flowering plant. Two flowering and five non-flowering putative hybrids were located approximately at the 5.8 km point, close to the northern border of *C. villosissimus*. Two flowering and one non-flowering putative hybrids were found approximately at the 13.0 km point, and another flowering putative hybrid was at the 13.7 km point, all of which were located

within the range of *C. allenii*. $RI_{habitat}$ of *C. allenii* was calculated as 0.886 and that of *C. villosissimus* was 0.932.

In the wet season, there was no significant correlation between gravimetric water content and species composition (= the proportion *C. allenii*) in each kilometer section, either for flowering plants ($R = 0.42$, $p = 0.20$) or for non-flowering plants ($R = 0.44$, $p = 0.18$). In contrast, in the dry season, gravimetric water content was positively correlated with species composition for flowering plants ($R = 0.82$, $p = 0.002$) and for non-flowering plants ($R = 0.79$, $p = 0.004$).

Reciprocal Transplant Experiments

Seed Transplants.

More transplanted seeds germinated at the *C. villosissimus* site (VP) than at the *C. allenii* site (AP) in the average dataset of the 2007 and 2008 cohorts ($p < 0.001$; Fig. 2.4A). The proportion of seeds germinating was significantly different among categories ($p < 0.001$; Fig. 2.4A). There was no significant interaction between transplant site and plant category ($p = 0.44$; Fig. 2.4A). Because the overall germination rate is low at the *C. allenii* site for all categories (Fig. 2.4A), the timing of germination will only be discussed at the *C. villosissimus* site (Fig. 2.5B). For *C. allenii*, $F1_{allenii}$, and $F1_{villo}$, seeds germinated before early January, when the dry season begins. In contrast, a sharp increase of seed germination in *C. villosissimus* occurred between April and May, which coincides with the end of the dry season and the beginning of the wet season.

The average seedling survival after one year of transplanting was significantly higher at the *C. allenii* site than at the *C. villosissimus* site ($p < 0.001$; Fig. 2.4B). The seedling survival across habitats was significantly different among the four categories ($p < 0.001$, Fig. 2.4B). There was a significant interaction between transplant site and category ($p < 0.001$), indicating

that plant lineages with high survival in one habitat had lower survival in the other habitat (Fig. 2.4B). For both cohorts, seedlings at the *C. allenii* site died at a relatively constant rate (Fig. 2.5C). However, heterogeneity in timing of seedling mortality was observed at the *C. villosissimus* sites. Most of the seedlings of *C. allenii* and $F1_{allenii}$ at the *C. villosissimus* site died between January and April, while the seedling mortality of $F1_{villo}$ and *C. villosissimus* was roughly linear during the one-year period (Fig. 2.5D).

Seed germination and seedling survival was monitored for another seven months after the one-year period in the 2007 cohort. During these seven months, none of the seeds germinated. Between October 2008 and January 2009, before the second dry season started, 9 seedlings (2 *C. allenii*, 4 $F1_{allenii}$, 3 $F1_{villo}$) in *C. allenii* habitat and 7 seedlings (2 $F1_{villo}$ and 5 *C. villosissimus*) in *C. villosissimus* habitat were dead. During the second dry season (between January and May 2009), 4 seedlings (2 *C. allenii*, 1 $F1_{allenii}$, and 1 $F1_{villo}$) in *C. allenii* habitat and 1 *C. villosissimus* seedling in *C. villosissimus* habitat were dead. Because the least favorable categories in each habitat had no survived seedlings at the end of one year, the additional seven months of observation in seed fitness could not be lower than zero in these categories. Although the overall pattern of seedling mortality was similar from the end of one year to the end of the second dry season, the increase of seed mortality in other categories reduced the statistical power to determine differences among categories and between habitats. Therefore, the measurements of fitness components were terminated at the end of one year in the 2008 cohort.

The absolute fitness of seed transplants, i.e., the number of transplanted seeds which germinated and survived for one year, was not different between transplant sites ($p = 0.55$), nor among categories ($p = 0.21$), but there was a significant interaction between transplanting site

and category ($p < 0.001$; Fig. 2.4C). At the end of one year, none of the germinated *C. allenii* seeds survived in *C. villosissimus* habitats, and vice versa. In contrast, few germinated *C. allenii* and *C. villosissimus* seeds survived in their home habitats. Therefore, both species had higher fitness in home habitats (Fig. 2.4C). F1 hybrid seeds had higher fitness in the habitats of their maternal parents than in the habitats of their paternal parents (Fig. 2.4C). The average fitness of $F1_{allenii}$ and $F1_{villo}$ (0.05) was lower than the fitness of *C. villosissimus* (0.14) in *C. villosissimus* habitat ($G = 4.58$, $p = 0.03$), but averaged hybrid fitness (0.07) was similar to the fitness of *C. allenii* (0.06) in *C. allenii* habitat ($G = 0.07$, $p = 0.80$). RI_{seed} of *C. allenii* was calculated as 0.067 and that of *C. villosissimus* was 0.463. The results of the G tests combined with RI_{seed} values indicate that extrinsic postzygotic isolation was significant in *C. villosissimus* habitat but not in *C. allenii*.

Cutting Transplants.

The overall transplant survival until June 2010 at the *C. allenii* sites (31.5%) was similar to that at the *C. villosissimus* sites (34.3%; $p = 0.44$; Fig. 2.6A). There was a significant difference in proportion surviving among categories (*C. allenii*: 19.3%, *C. villosissimus*: 18.3%, $F1_{allenii}$: 48.0%, and $F1_{villo}$: 50.8%; $p < 0.001$; Fig. 2.6A). The survival of both *C. allenii* and *C. villosissimus* was highest in their home habitats, and each species had higher survival than the other species in their home habitats, as indicated by the significant interaction between transplant site and category ($p < 0.001$; Fig. 2.6A). None of the *C. allenii* transplants survived in *C. villosissimus* sites, none of the *C. villosissimus* transplants survived at one of the *C. allenii* sites (AP), and only 10.7% of *C. villosissimus* survived at the other *C. allenii* site (AG) (Fig. 2.7). The survival of F1 hybrids was higher than that of parental species at the two *C. villosissimus* sites

and at one *C. allenii* site (AG), but lower than *C. allenii* at the other *C. allenii* site (AP) (Fig. 2.7). The mortality rates of *C. villosissimus* and the F1 hybrids were relatively consistent throughout the whole experiment and across transplant sites. However, heterogeneity in cutting mortality was observed in *C. allenii* transplants at the *C. villosissimus* sites. These plants had a sharp decrease in survival between March and June 2007, which was the end of the dry season and beginning of the following wet season (Fig. 2.7C and 2.7D). In *C. allenii* habitats, the proportion of F1 hybrids surviving was similar to that of the *C. allenii* transplants ($G = 0.61$, $p = 0.43$). In *C. villosissimus* habitats, the proportion of F1 hybrids surviving was also similar to that of the *C. villosissimus* transplants ($G = 2.03$, $p = 0.15$).

Of the 83 plants which survived through the experiment (until June 2010), 30.1% had fewer leaves than at the time of transplanting, 6.0% had the same number of leaves, and 63.9% had more leaves. Plant growth, measured as the proportional increase in number of leaves, was significantly lower in *C. allenii* habitats than in *C. villosissimus* habitats ($p < 0.001$, Fig. 2.6B). There was no significant difference in growth between the two species or between the two F1 hybrids ($p = 0.12$; Fig. 2.6B). In *C. allenii* habitats, the growth of F1 hybrids was similar to that of *C. allenii* ($p = 0.81$; Fig. 2.8A and 2.8B). In contrast, in *C. villosissimus* habitats, the growth of F1 hybrids was significantly greater than that of *C. villosissimus* ($p = 0.03$; Fig. 2.8C and 2.8D).

Combining the two fitness components, survival and growth, the absolute fitness of *C. allenii* was zero in *C. villosissimus* habitats and that of *C. villosissimus* was nearly zero in *C. allenii* habitats (Fig. 2.6C). The hybrids did not have reduced fitness in either of the parental habitats (Fig. 2.6C). $RI_{cutting}$ of *C. allenii* was calculated as -0.163 and that of *C. villosissimus*

was -0.689. The negative values of $RI_{cutting}$ indicate that hybrids have higher fitness than the parental species in the corresponding parental habitats.

None of the plants grown at *C. allenii* sites reproduced, while plants in each category reproduced in *C. villosissimus* sites (6 *C. villosissimus*, 3 $F1_{allenii}$, and 2 $F1_{villo}$ at VG; 1 *C. allenii*, 4 *C. villosissimus*, 6 $F1_{allenii}$, and 8 $F1_{villo}$ at the VP). There was a significant difference in the number of flowering plants between parental habitats ($p < 0.001$). There was also a difference in number of flowering plants among categories ($p = 0.002$) as there was only one *C. allenii* but more plants of the other three categories flowered. For flowering plants, there was a significant pattern for increasing number of inflorescences produced from *C. allenii* (1 inflorescence produced by 1 plant), $F1_{allenii}$ (4.33 ± 2.58), to $F1_{villo}$ (7.20 ± 4.06) and *C. villosissimus* (9.0 ± 5.37).

The proportion of leaf damage on new leaves (Fig. 2.9) was slightly, but significantly higher in *C. villosissimus* sites (0.08 ± 0.04) than in *C. allenii* sites (0.06 ± 0.03, $p = 0.046$). There was also a significant difference among categories ($p = 0.04$), with more damage on *C. villosissimus* (0.10 ± 0.05) than on *C. allenii* (0.01 ± 0.01, $p = 0.04$). There was no interaction between transplant site and category for leaf damage.

Total Fitness.

Combining the results of the two reciprocal transplant experiments, relative fitness of *C. allenii* was zero in *C. villosissimus* habitats, and vice versa (Fig. 2.10). Neither $F1_{allenii}$ and $F1_{villo}$ in *C. allenii* habitats had reduced fitness. $F1_{allenii}$ had zero fitness in *C. villosissimus* habitats, while $F1_{villo}$ had higher fitness than *C. villosissimus* in the home habitat (Fig. 2.10).

Discussion

Water Availability and Species' Distribution

The precipitation data revealed that more rain falls at Frijolito in the north than at Gamboa in the south. This between-location difference is parallel to the north-south pattern of more rainfall on the Atlantic side than on the Pacific side across the Isthmus of Panama. The same pattern is also found along PLR, with an increase in soil moisture as the road progresses northward. All these observations indicate higher water availability in the northern region. Because Frijolito and Gamboa represent the preferred habitats of *C. allenii* and *C. villosissimus*, respectively, the between-location difference in precipitation at these localities indicates that *C. allenii* habitats receive more rainfall than those of *C. villosissimus*. Interestingly, the precipitation difference was found only during the dry season. Moreover, the correlation between species composition and soil moisture was significant only in the dry season. These findings indicate that the spatial distributions of *C. allenii* and *C. villosissimus* are affected mainly by water availability in the dry season. Given the finding of higher drought tolerance in *C. villosissimus* (Ch. 3), it is suggested that *C. villosissimus* is adapted to drier habitats, and can thus survive in southern regions of the Isthmus of Panama that are beyond the range of *C. allenii*. Due to the marked differences in water availability, natural populations of *C. allenii* and *C. villosissimus* on PLR display a parapatric distribution, which correlates with the soil moisture gradient in the area.

Water availability has been shown to be a limiting factor of plant species' distribution in many systems (e.g., Whittaker, 1965; Gentry 1988; Duivenvoorden, 1995; Bongers et al., 1999; Pyke et al., 2001; Engelbretch et al., 2007; Giriraj et al., 2008). Across the Isthmus of Panama, correlations between drought sensitivity and tree species distribution suggested that soil water

availability is a direct determinant of species distribution (Engelbretch et al., 2007). While such correlations have been found among distantly-related tree species (Engelbretch et al., 2007), the results presented here demonstrate a correlation between soil moisture and the distribution of closely related species, and suggest that the underlying cause is divergent adaptation to different soil moisture. A greenhouse experiment of drought tolerance has shown that *C. villosissimus* has higher drought tolerance than *C. allenii*. This provides an explanation of why *C. villosissimus* is found in drier habitats (Ch. 3). A potential mechanism to explain the parapatric distribution of the two species is that limited water availability in the dry season prevents *C. allenii* from invading the habitat of *C. villosissimus*. This potential mechanism is supported by the results of high mortality of *C. allenii* transplanted in *C. villosissimus* habitats.

Local Adaptation

The reciprocal transplant experiment initiated with seeds showed that the parental species performed best in their home habitats. Although *C. allenii* had a high germination rate (58.2%) in *C. villosissimus* habitat, none of the seedlings survived (Fig. 2.4). The germination rate of *C. villosissimus* in *C. villosissimus* habitats (30.9%) was lower than that of *C. allenii* in *C. villosissimus* habitat (58.2%), but a few seedlings (44.5% of germinants) survived after one year of transplanting (Fig. 2.4). The difference in seedling survival between the two parental species in *C. villosissimus* habitats is probably a result of the difference between species in germination timing. Most *C. allenii* seeds germinated in the late wet season (October to December), while most of *C. villosissimus* germinated in the interval between the end of the dry season and the beginning of the wet season (April to May; Fig. 2.5B). Most *C. allenii* seedlings died in the early dry season (January to March), while mortality of *C. villosissimus* seedlings in the dry season is not different from that in the wet season (Fig. 2.5D). Therefore, I speculate that the delayed

timing of *C. villosissimus* seed germination is associated with seasonal drought in *C. villosissimus* habitats.

Drought-adapted plants usually cope with drought by escape, by avoiding the negative consequences of dehydration, or by tolerating low tissue water potential (reviewed in Chaves et al., 2003). A short life cycle or short growing season enables plants to reproduce before drought—such species are usually categorized as using drought-escape strategies (e.g., McKay et al., 2003; Wu et al., 2010; Franks, 2011). Delayed seed germination is another mechanism of escape from drought (Venable and Lowlor; 1980; Volis et al., 2009). Given the differences in the timing of seed germination and seedling mortality between species, I suggest that seeds of *C. villosissimus* germinate later than those of *C. allenii* to escape the severe drought condition that is typical of *C. villosissimus* habitats during the dry season.

Observations of seeds grown under uniform conditions with high water availability in the greenhouse and in incubators also indicate that seeds of *C. allenii* germinate earlier than those of *C. villosissimus* (Chen, unpublished data). The consistent difference in the timing of germination between species suggests that germination timing is a heritable trait, and that intrinsic factors, as opposed to extrinsic environmental cues, may play an important role in germination. Further investigation into the factors responsible for seed germination may help understand how *C. villosissimus* uses seed dormancy to adaptively escape from seasonal drought in its habitat.

In the reciprocal transplant experiment initiated with cuttings, the parental species also perform best in their home habitats. None of the *C. allenii* transplants survived in *C. villosissimus* habitats, while < 10 % of the *C. villosissimus* transplants survived in *C. allenii* habitats (Fig. 2.6A). The surviving *C. villosissimus* transplants in *C. allenii* habitats had fewer leaves (Fig. 2.6B). Taken together, both parental species had lower fitness when transplanting

into foreign habitats (Fig. 2.6C). Interestingly, most mortality of *C. allenii* in *C. villosissimus* habitats occurred in the dry season, while most *C. villosissimus* mortality in *C. allenii* habitats occurred in the wet season (Fig. 2.7). These results suggest a trade-off between being able to survive in a dry habitat and being able to survive and grow in shade. This is consistent with the hypothesis proposed by Smith and Huston (1989) that plants which are able to survive in dry habitats are likely to grow more slowly in shady habitats (also see empirical evidence in Brenes-Arguedas et al., 2011). Coley et al. (1985) hypothesized that when growing slower in shady habitats, plants with lower pest resistance may fail to produce new leaves fast enough to compensate the loss due to pest and thus have lower survival. The observations of higher pest damage, reduced growth and high mortality of *C. villosissimus* transplants in *C. allenii* habitats support both hypotheses. These observations also suggest that local adaption in *C. allenii* and *C. villosissimus* is a result of natural selection caused by interactions among pest, water, and light availability in their habitats.

Despite the fact that reproduction was low overall, with no plants flowering in *C. allenii* habitat, the proportion flowering of *C. villosissimus* was higher than that of *C. allenii* in *C. villosissimus* habitats. In fact, the only flowering *C. allenii* produced one inflorescence in 2009 and was dead in 2010, while flowering *C. villosissimus* produced an average of nine inflorescences during the experimental period and more than half of these *C. villosissimus* were alive at the end of the experiment. Although reproduction was not included in the calculation of cutting fitness, the results are also consistent with the prediction of local adaptation in *C. villosissimus*.

Both seed and cutting reciprocal transplants indicate local adaptation to different habitats in *C. allenii* and *C. villosissimus*, suggesting that the parapatric distribution of the two species is

largely due to local adaptation. As further evidenced, greenhouse measurements revealed that *C. allenii* has higher leaf mass per area, a physiological feature typical of shade-adapted species, and that *C. villosissimus* has higher drought tolerance (Ch. 3). These results are consistent with the hypothesis that the preferred habitats of these species, wet, shaded understory habitats for *C. allenii* and drier, open gaps and forest edges for *C. villosissimus*, are a product of local adaptation.

Local Adaptation and Reproductive Isolation

Reciprocal transplant experiments of the parental species demonstrated that *C. allenii* and *C. villosissimus* are locally adapted to their different home habitats. The parapatric distribution of the two species leads to prezygotic, habitat isolation. Habitat isolation was calculated by the distribution of the two species along PLR, and the index values were high in both species ($RI_{habitat}$ = 0.886 for *C. allenii* and $RI_{habitat}$ = 0.932 for *C. villosissimus*). Because habitat isolation acts early in the life history, its relative contribution to total isolation is greater than later acting, sexual , postmating, and postzygotic isolating barriers (Schemske, 2010). By calculating the relative contribution of the habitat isolating barrier to total isolation with comparisons among all barriers (as suggested by Coyne and Orr, 1989, 1997; Ramsey et al., 2003; see reviewed in Schemske, 2010), habitat isolation is a major contributor to total isolation between *C. allenii* and *C. villosissimus* (Ch. 4). As the two species diverged quite recently (Kay et al., 2005), local adaptation to different habitats may be the primary isolating mechanism at the time of speciation between *C. allenii* and *C. villosissimus* (Ch. 4).

The reciprocal transplant experiment initiated with seeds showed that the F1 hybrids performed as well as *C. allenii* in *C. allenii* habitats but worse than *C. villosissimus* in *C. villosissimus* habitats. Reproductive isolation measured in these transplants was low in *C. allenii*

(RI_{seed} = 0.067) and moderate in *C. villosissimus* (RI_{seed} = 0.463). The proportion of hybrid seeds that germinated and survived depends on transplant sites, suggesting extrinsic rather than intrinsic postzygotic isolation. Moreover, there was a higher probability of germinating and surviving when a hybrid seed was transplanted in the habitat of its maternal parent, i.e., $F1_{allenii}$ in *C. allenii* habitat and $F1_{villo}$ in *C. villosissimus* habitat, than in the habitat of its paternal parent. The fitness differences in hybrids transplanting in different environments may be due to maternal environmental effects and/or local adaptation of cytoplasmic genes (Kimball et al., 2008).

The reciprocal transplant experiment initiated with cuttings showed that both F1 hybrids performed better than the parental species in the corresponding parental habitats. This indicates that there is no extrinsic postzygotic isolation at the juvenile stage ($RI_{cutting}$ = -0.163 for *C. allenii* and $RI_{cutting}$ = -0.689 for *C. villosissimus*). The trade-off between surviving in drought and surviving in shade observed in the parental species was not observed in the hybrids, as the hybrids survive and grow well in both parental habitats. *Costus* plants may live for decades, yet the field experiments reported here are short in comparison. Thus, the fitness estimates obtained in the reciprocal transplant experiments must be viewed with caution. Because the number of inflorescences produced per flowering plant was significantly higher in *C. villosissimus* than in hybrids within the experimental period, one may expect that the hybrids have reduced lifetime fecundity and reduced fitness comparing to *C. villosissimus*. Hybrid transplants producing fewer inflorescences than *C. villosissimus* beyond the experimental period might result in extrinsic postzygotic isolation. In addition to differential reproduction, fitness in backcrosses and later generations of hybrids may also cause extrinsic postzygotic isolation beyond the F1 generation.

When F1 hybrids reproduce, they are more likely to backcross to a parental species because flowering hybrids are rare in nature. For backcross and later generations of hybrids, hybrid fitness usually decreases and the chance of the foreign alleles carried in the heterozygotic F1 hybrids to be passed through generations is reduced by one-half in each generation (Rhode and Cruzan, 2005). A more comprehensive assessment of postzygotic isolation in perennials requires long-term reciprocal transplant experiments through the life history of multiple generations of hybrids in comparison with parental species.

Despite the finding of extrinsic postzygotic isolation for seeds transplanted into *C. villosissimus* habitats, there was no evidence of extrinsic postzygotic isolation for seeds transplanted into *C. allenii* habitats or for cuttings transplanted into either parental habitat. Postzygotic isolation is, therefore, asymmetrical between species and between different life history stages. When fitness differences between different species are compared at multiple ages or life stages, variation in fitness differences among ages or life stages is commonly observed (Aston and Bradshaw, 1961; Gross, 1981; Platekamp, 1990, 1991; Raabová et al., 2007, 2011; Raabová et al., 2008). One explanation of this variation was proposed by Platekamp (1991) that younger plants may be affected more by environmental conditions than older, clonally derived transplants, as the latter expressed greater genetic difference among plant species than the former. For *C. allenii*, *C. villosissimus* and their F1 hybrids, higher overall survival was observed in cuttings than in seed transplants, indicating that seed transplants are affected more by environmental conditions. However, the fitness of hybrid seeds was lower than that for *C. villosissimus* seeds in *C. villosissimus* habitat, but survival of hybrid cuttings was not significantly different from that of cuttings of the parental species in its home habitat. This difference between plant life stages may be a result of stronger selection against younger plants.

When selection acts strongly on seeds and early seedlings, the genotypic differences between hybrids and the parental species in its home habitats may cause greater fitness differences between these plants than between older cuttings.

Although the reciprocal transplant experiment initiated with cuttings showed no postzygotic isolation, the actual likelihood of heterospecific gene flow may be greatly reduced by other barriers. This is because local adaptation leads to strong habitat isolation and eliminates the majority of heterospecific gene flow prior to hybridization. When the probability of hybrid formation is low, the relative contribution of postzygotic isolation is limited (Schemske, 2010). Although the proportion of hybrid offspring has not been measured in naturally-pollinated fruits of the two study species, it has been measured in a pollination array where flowering plants of both species were intermixed artificially. In the pollination array, a lower proportion of hybrid offspring than that of parental species was observed in the naturally pollinated fruits (Ch. 1), indicating a reduced likelihood of hybrid formation. The low frequency of hybrids found along PLR also suggests that heterospecific gene flow is limited although the edges of the natural distributions are in close proximity to each other.

Reciprocal transplant experiments demonstrated 1) that *C. allenii* and *C. villosissimus* are locally adapted to their home habitats, 2) that local adaptation leads to strong habitat isolation between the two species, and 3) that local adaptation leads to asymmetrical extrinsic postzygotic isolation only in seed transplants. Therefore, between the sister species *C. allenii* and *C. villosissimus*, local adaptation contributes mainly to prezygotic but not postzygotic isolating barriers.

Local adaptation has also been suggested as the primary isolating mechanism in other plant systems (see reviews in Lowry et al., 2008a, and Schemske, 2010). For example, the

closely related and naturally hybridizing species *Ipomopsis aggregata* and *I. tenuituba* were found to be adapted to different light intensities (Wu and Campbell, 2006; Campbell and Waser, 2007). Reciprocal transplant plant experiments conducted with these species demonstrated strong habitat isolation and weak extrinsic postzygotic isolation (Campbell and Waser, 2001, 2007), parallel to the findings presented here. Ramsey et al. (2003) found that habitat isolation is the primary mechanism of reproductive isolation between *Mimulus cardinalis* and *M. lewisii*. Because of almost complete prezygotic isolation, extrinsic postzygotic isolation, which was not measured in this study, may not be relevant to reducing heterospecific gene flow between the two species. Reciprocal transplant experiments and greenhouse experiments suggest that the two species are locally adapted to their different elevation levels (Angert and Schemske, 2005; Angert et al., 2008).

Local adaptation to drought has been shown to contribute to habitat isolation between inland and coast races of *Mimulus guttatus* (Lowry et al., 2008b). Reciprocal transplant experiments and corresponding greenhouse experiments in this species revealed that selection caused by seasonal drought reduced fitness of coast transplants in inland habitats while inland populations with low salt tolerance had low fitness when being transplanted to the coast (Lowry et al., 2008b). Extrinsic postzygotic isolation is weak in the inland race but negative in the coast race, (Lowry et al., 2008b), similar to the case of *C. allenii* and *C. villosissimus* presented here. In summary, local adaptation leading to habitat isolation, which is often the primary isolating barrier, is commonly found in plant systems.

Conclusion and Future Directions

Reciprocal transplant experiments conducted with seeds and cuttings indicate that local adaptation to different habitats is the cause of the parapatric distribution of *C. allenii* and *C.*

villosissimus. I conclude that local adaptation contributes to strong habitat isolation and weak extrinsic postzygotic isolation between these two recently diverged species. As the parapatric distribution of the two species is correlated with the soil moisture gradient across the Isthmus of Panama, further greenhouse experiments on how the two species and their hybrids respond to drought have been conducted (Ch. 3). The reciprocal transplant experiment in this chapter and the greenhouse experiment in Chapter 3 together suggest that *C. villosissimus* has higher drought tolerance. In contrast, *C. allenii* habitats have lower light availability, thus the higher leaf mass per area in *C. allenii* suggest that the leaf physiology of this species is due to local adaptation. By using the components-of-isolation method to measure the strength of multiple isolating barriers, the relative contribution of habitat isolation, extrinsic postzygotic isolation, and other isolating barriers has been compared to determine the primary barriers of reproductive isolation (see Ch. 4). Given that habitat isolation is the primary isolating barrier between *C. allenii* and *C. villosissimus* (Ch. 4) and that local adaptation contributes to habitat isolation, the results presented here support the idea of local adaptation being the primary mechanism of speciation. Taken together, this study enhances our understanding of how local adaptation and habitat isolation contribute to speciation.

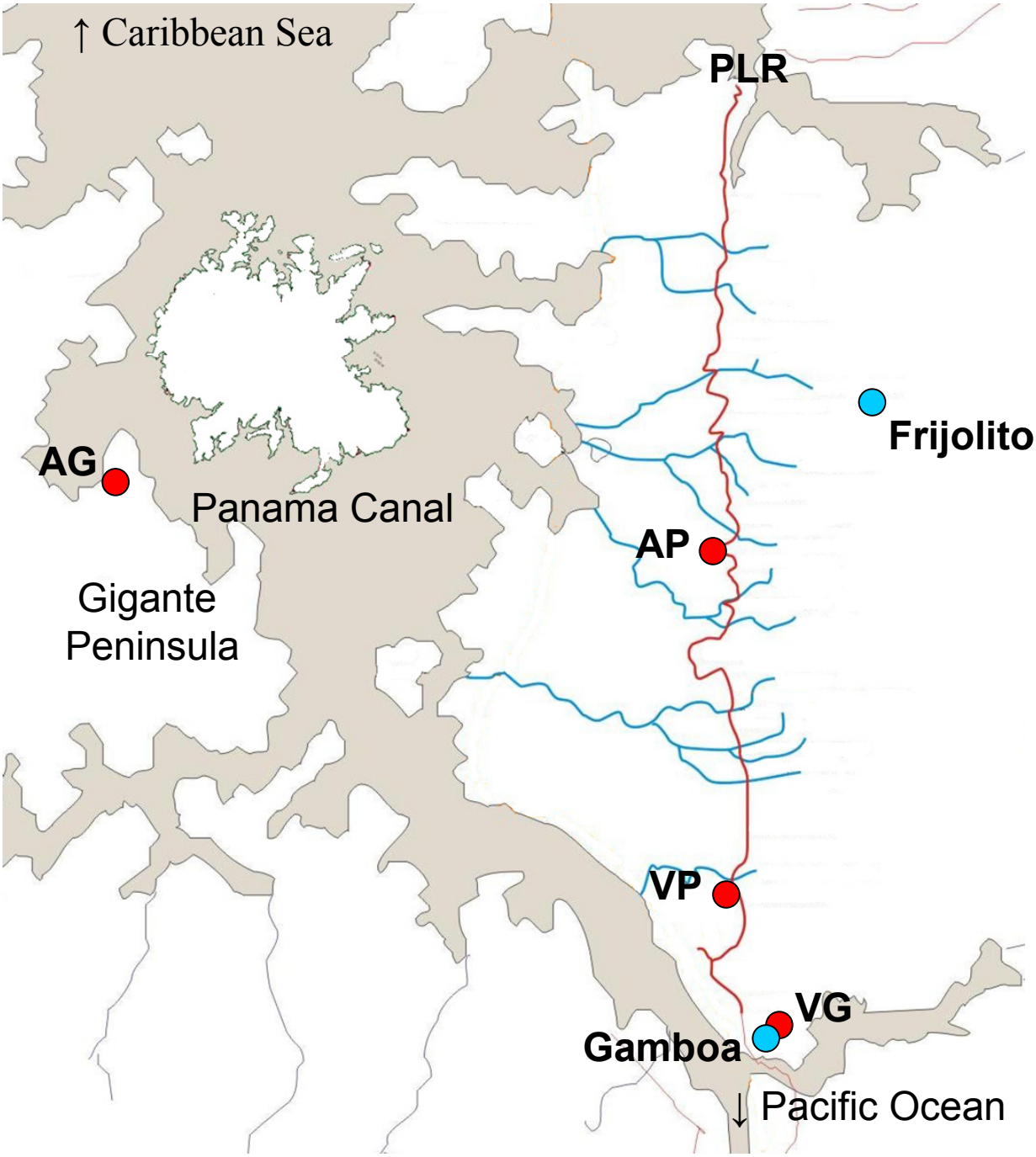

Figure 2.1. Map of the study sites in Central Panama (modified from http://www.stri.si.edu./images/Mapas/Pipeline_BarroColorado.jpg). Pipeline Road (PLR) is marked by the red solid line on the right side of the map. The precipitation data were collected at the Frijolito and Gamboa weather stations marked with blue dots. Red dots indicate the location of the reciprocal transplant sites: *C. allenii* sites on Pipeline Road (AP) and on the Gigante Peninsula (AG), and *C. villosissimus* sites on Pipeline Road (VP) and in Gamboa (VG).

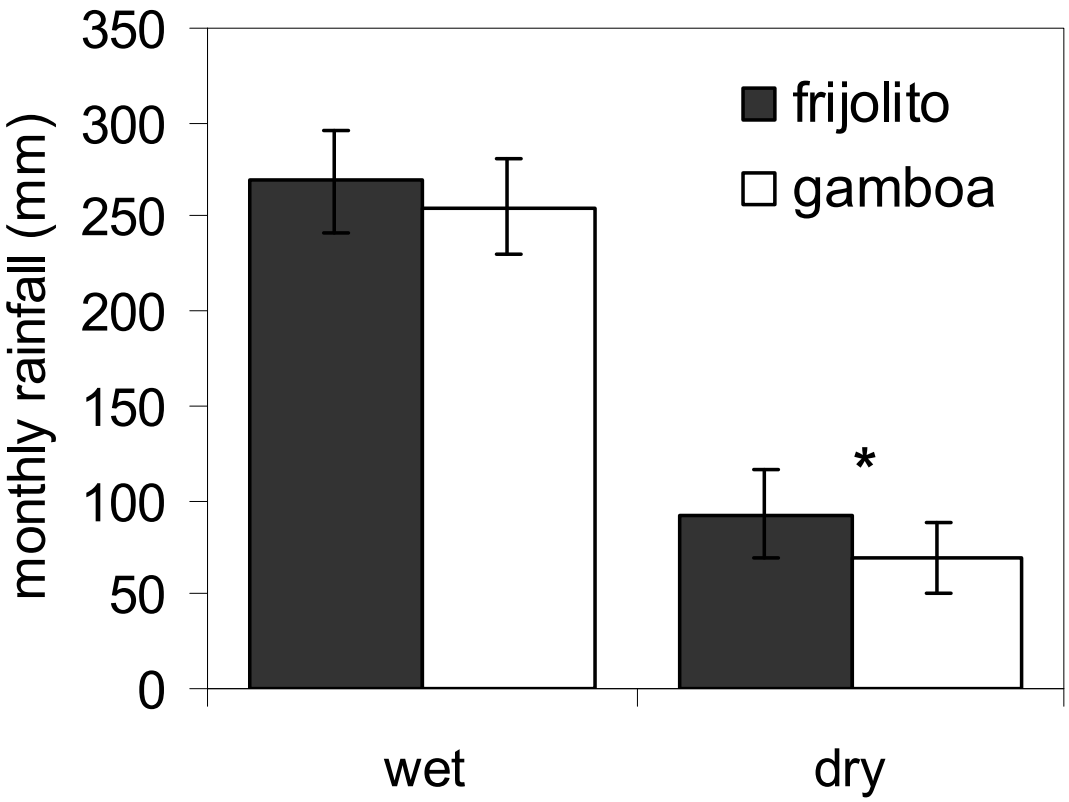

Figure 2.2. Comparisons of monthly precipitation at Frijolito and Gamboa in the wet and dry seasons. Error bars denote the mean ± 95% CI. *: $p < 0.05$ in a paired t-test.

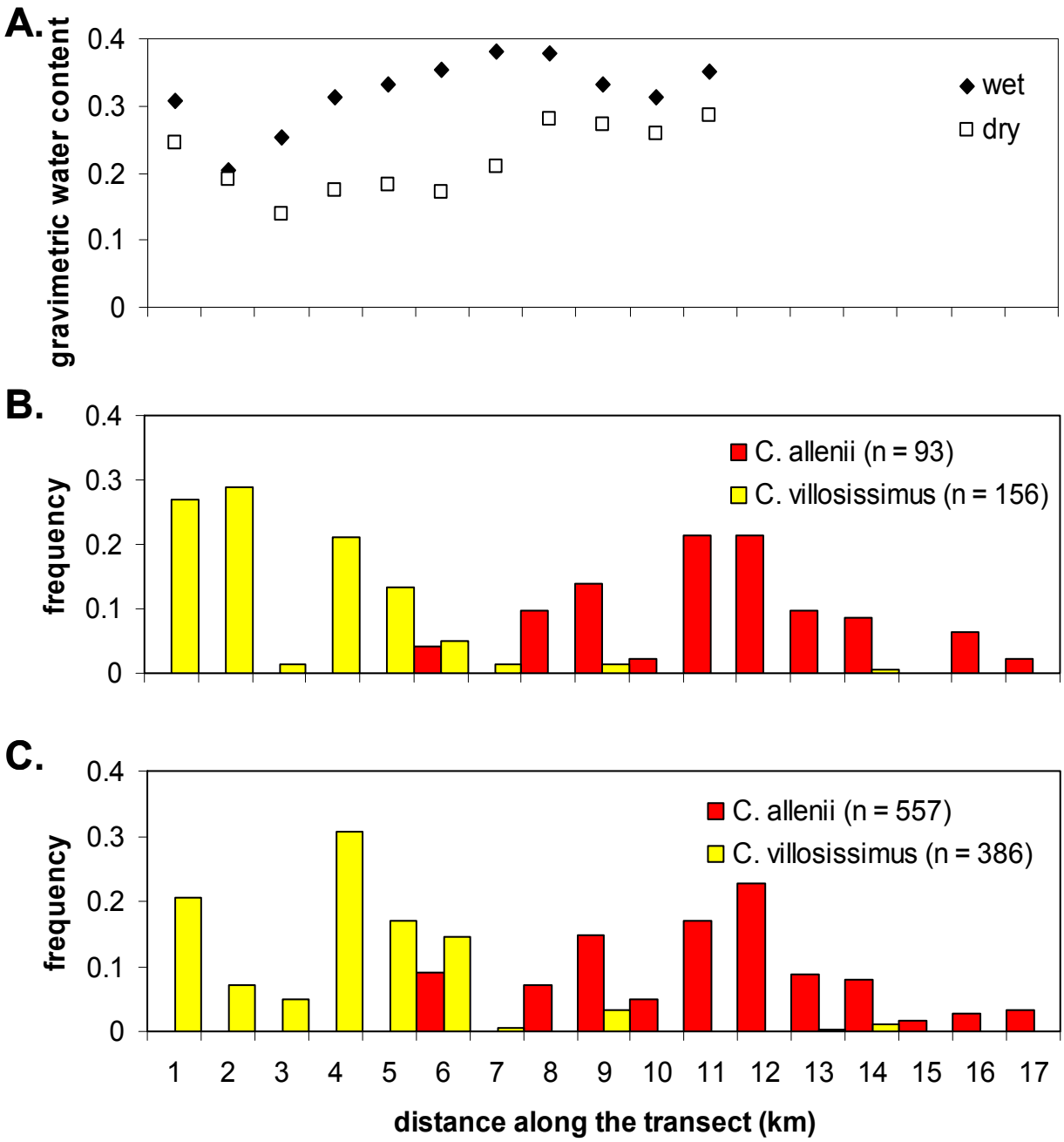

Figure 2.3. Soil moisture and species' distribution along Pipeline Road (PLR). (A) Soil moisture measured as gravimetric water content in each kilometer section in the wet and dry seasons. (B) Frequency distribution of flowering plants of *C. allenii* and *C. villosissimus* in each kilometer section on PLR. (C) Frequency distribution of non-flowering plants of *C. allenii* and *C. villosissimus* in each kilometer section on PLR.

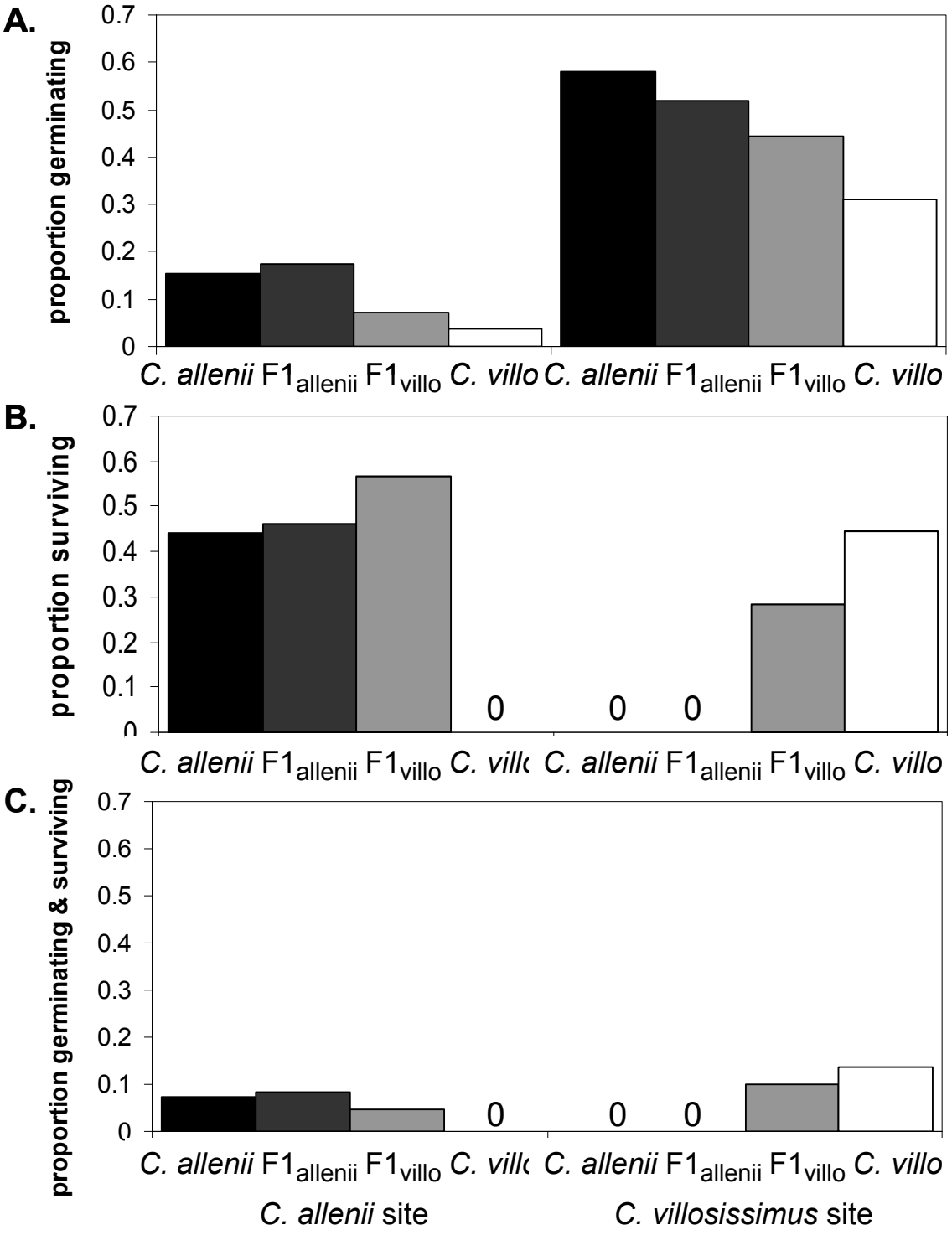

Figure 2.4. Fitness components of seed reciprocal transplant experiment for the 2007 and 2008 cohorts combined. Four categories are *C. allenii*, *C. villosissimus* (*C. villo*), F1 hybrids with *C. allenii* as the maternal parent ($F1_{allenii}$), and F1 hybrids with *C. villosissimus* as the maternal parent ($F1_{villo}$). (A) Seed germination; (B) seedling survival; (C) the proportion germinating and surviving.

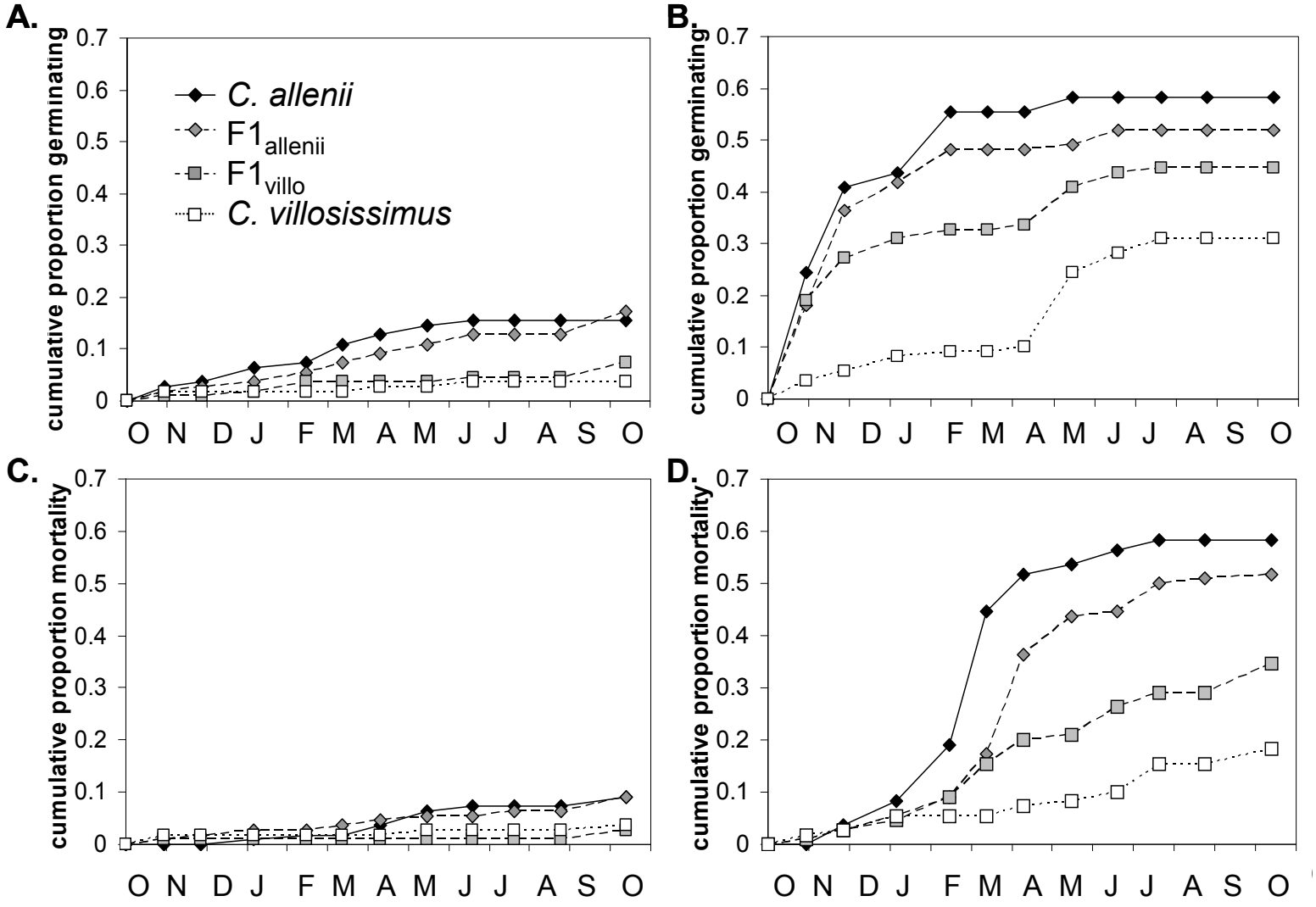

Figure 2.5. Comparisons of the changes in seed germination and seedling mortality through time between reciprocal transplanting sites and among *C. allenii*, $F1_{allenii}$, $F1_{villo}$, and *C. villosissimus* for the 2007 and 2008 cohorts combined. (A) Seed germination in *C. allenii* habitats; (B) seed germination in *C. villosissimus* habitats; (C) seedling mortality in *C. allenii* habitats; (D) seedling mortality in *C. villosissimus* habitats.

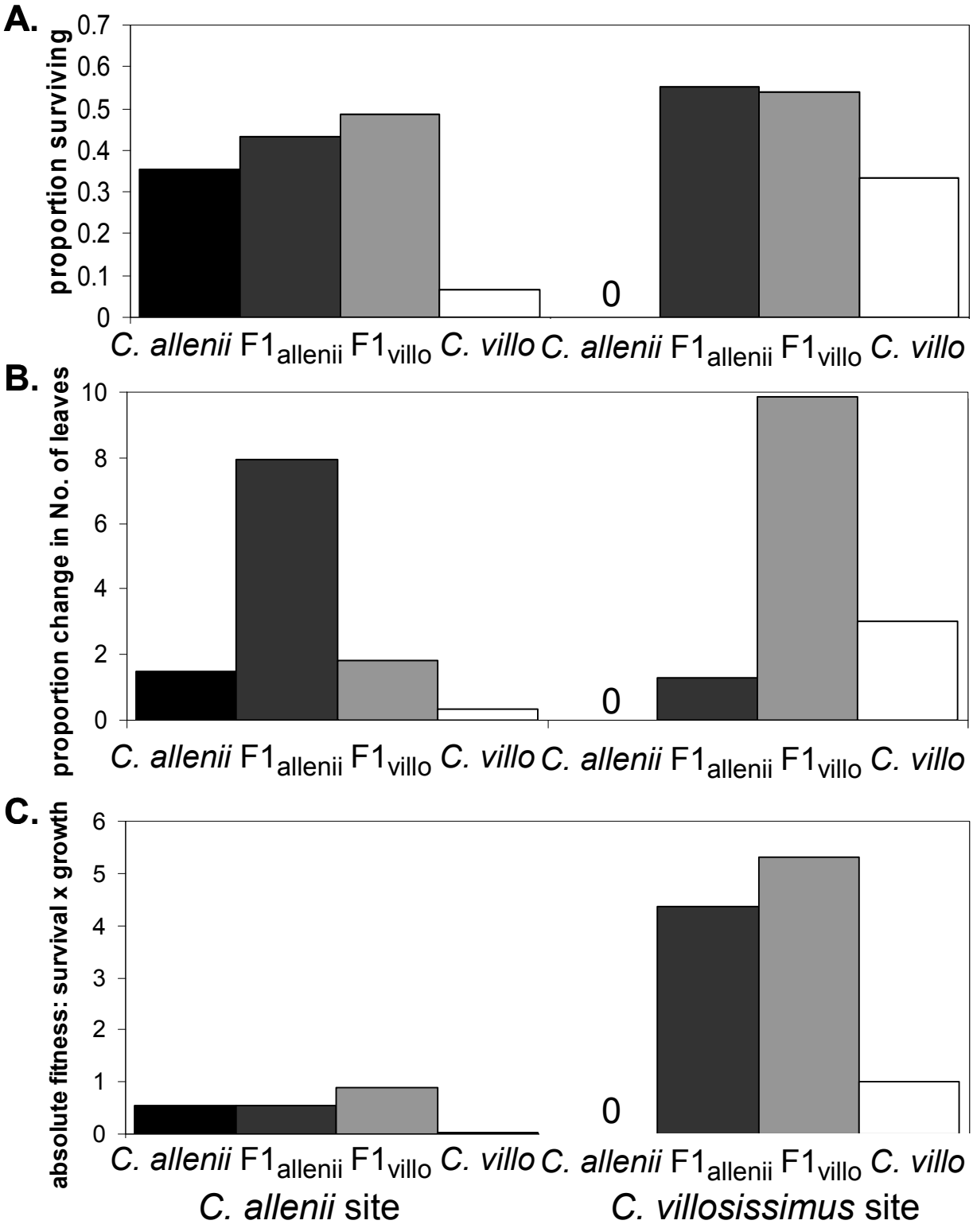

Figure 2.6. Fitness components of cutting reciprocal transplant experiment. Data from sites AG and AP are combined, and data from site VG and VP are combined. (A) Cutting survival; (B) cutting growth; (C) absolute fitness of cuttings.

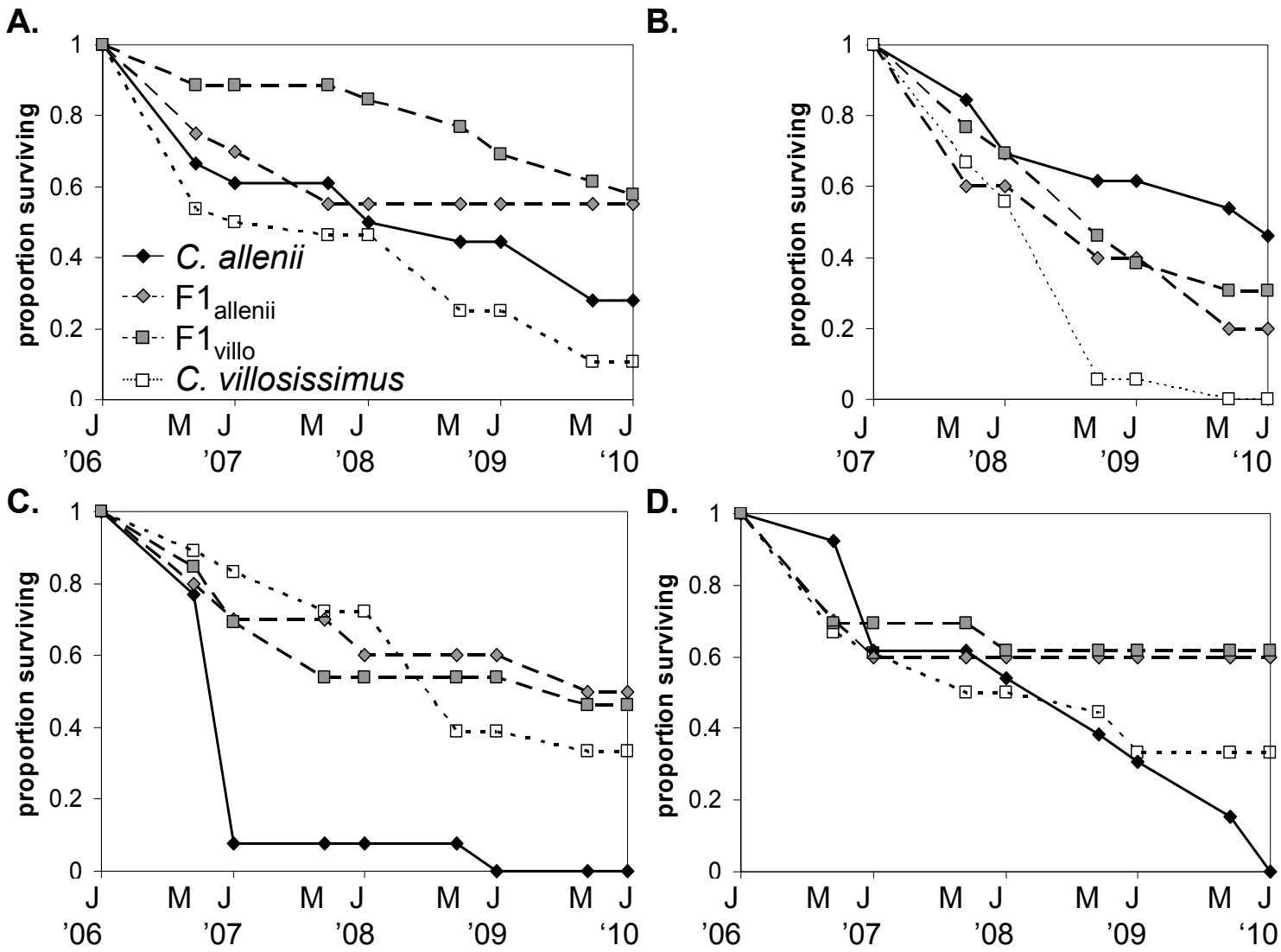

Figure 2.7. Comparisons of cutting survival among *C. allenii*, $F1_{allenii}$, $F1_{villo}$, and *C. villosissimus* and among transplanting sites AG (A), AP (B). VG (C), and VP (D). The x axes represent the time of each census. M denotes March, representing a census in the dry season; J denotes June, representing a census in the wet season.

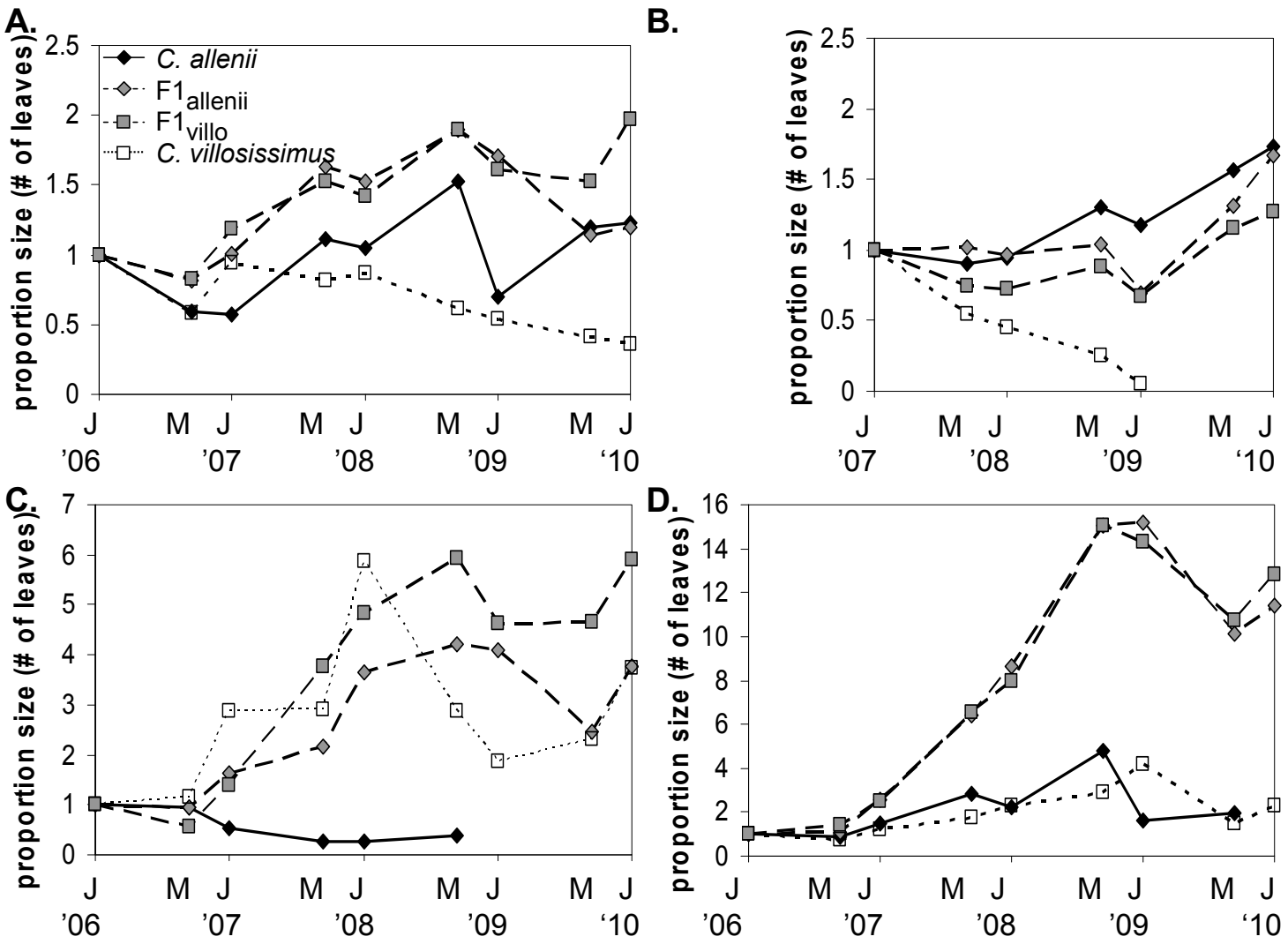

Figure 2.8. Comparisons of cutting growth among *C. allenii*, $F1_{allenii}$, $F1_{villo}$, and *C. villosissimus* and among transplanting sites AG (A), AP (B). VG (C), and VP (D). The y axes represent proportion change in the number of leaves from the time of transplanting to a given census. The x axes represent the time of each census. M denotes March, representing a census in the dry season; J denotes June, representing a census in the wet season.

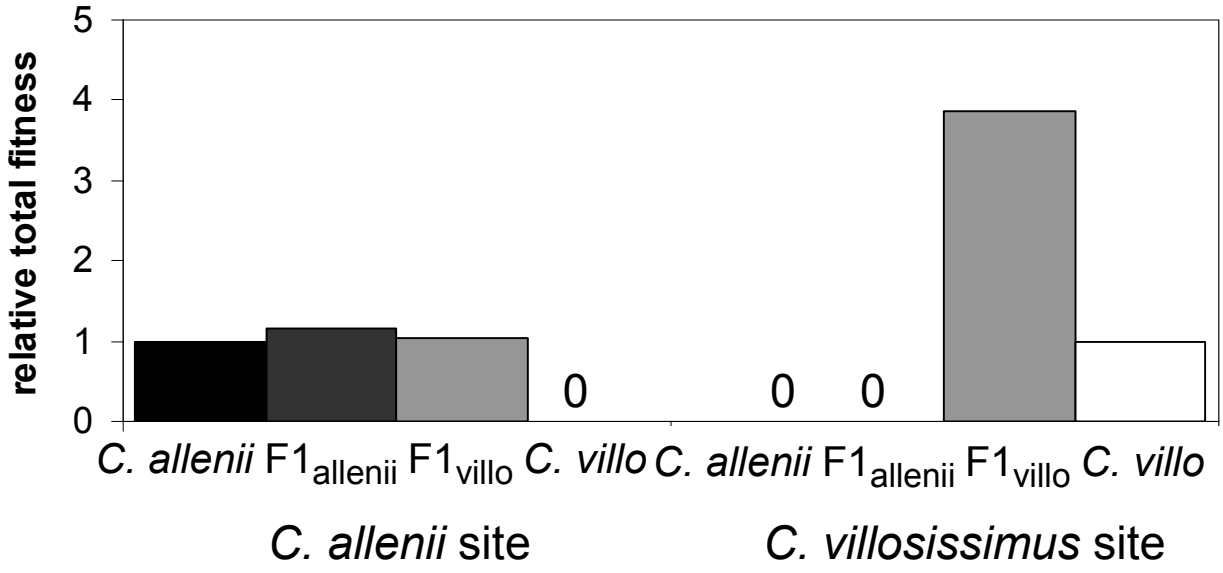

Figure 2.9. Comparison of relative total fitness among *C. allenii*, $F1_{allenii}$, $F1_{villo}$, and *C. villosissimus* and between parental habitats. The product of fitness components measured in seed and the cutting transplants of each category in each habitat was compared with that of the parental species transplanted in its home habitats.

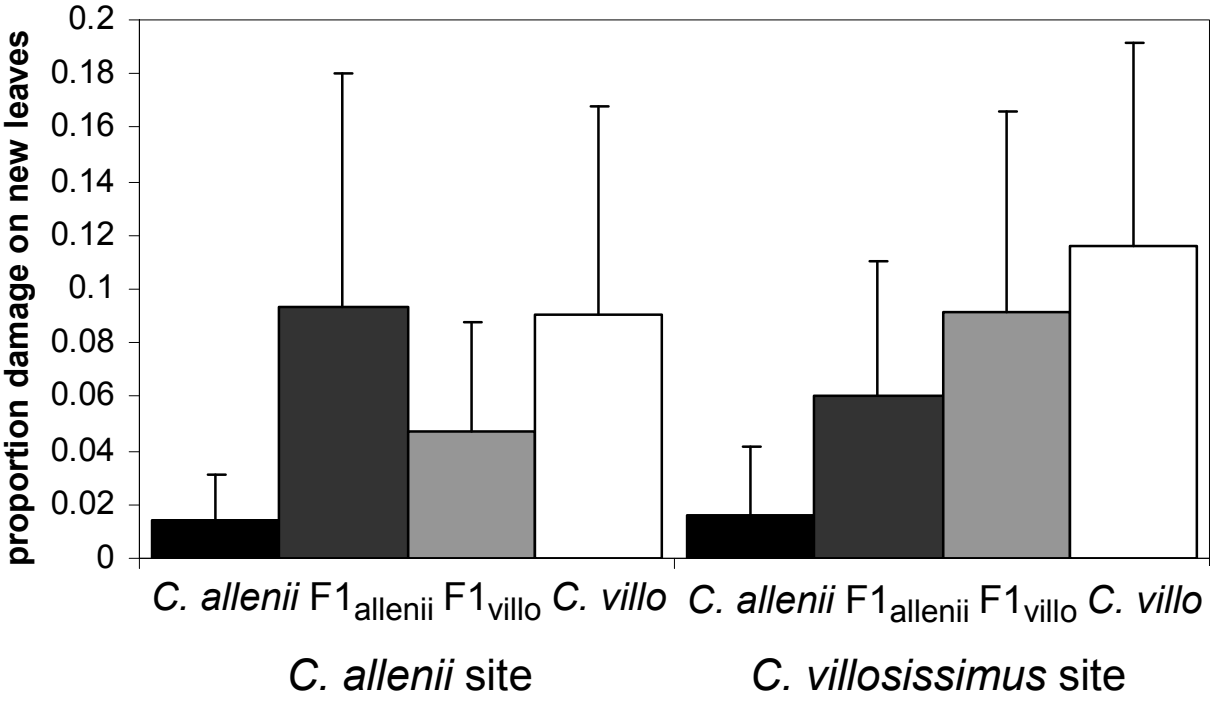

Figure 2.10. Proportion of pest damage on new leaves of *C. allenii*, $F1_{allenii}$, $F1_{villo}$, and *C. villosissimus* in the cutting reciprocal transplant experiment. Error bars denote the mean + 95% CI.

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Chapter 3

Ecological Factors Contributing to Local Adaptation in Two Neotropical *Costus*

ABSTRACT

Local adaptation has been proposed as the primary mechanism of speciation. As populations adapt to different habitats, prezygotic isolation evolves because locally adapted individuals are unable to colonize "foreign" habitats. Local adaptation may also contribute to extrinsic, postzygotic isolation if F1 hybrids experience reduced growth, survival or reproduction in either of the parental habitats. I compared the habitats of two recently diverged Neotropical plant species, *Costus allenii* and *C. villosissimus*, to identify the principal environmental factors that are responsible for local adaptation. Although both species are found in Central America and northern South America, they occupy different habitats and display parapatric distributions in the region where they co-occur. *Costus allenii* occurs along ravines in the understory of primary forests, while *C. villosissimus* is found along forest edges. Previous reciprocal transplant experiments conducted with these species in central Panama revealed strong local adaptation, with each species performing best in their "home" environment. These experiments also indicated that F1 hybrids had lower fitness at the early seedling stage in the *C. villosissimus* habitats but not in the *C. allenii* habitats, suggesting asymmetrical extrinsic postzygotic isolation. Light availability was lower and soil moisture was higher in *C. allenii* habitats. Two physiological traits, leaf mass per area (LMA) and drought tolerance, were measured in the parental species and their F1 hybrids to determine if these traits contribute to local adaptation and to extrinsic postzygotic isolation. LMA was larger in *C. allenii* than in the hybrids and *C. villosissimus*, suggesting that *C. allenii* produces thicker leaves with lower photosynthetic capacity and slower growth rate, which are typical features of shade-adapted plants. Higher drought tolerance was found in *C. villosissimus*, which coincides with the fact that *C. villosissimus* plants are found in drier habitats. The F1 hybrids had *C. villosissimus*-like LMA,

despite the fact that hybrids do not have lower, *C. villosissimus*-like, fitness in the *C. allenii* habitats. The F1 hybrids also had intermediate drought tolerance, which is consistent with the fact that hybrids had lower fitness than *C. villosissimus* in the *C. villosissimus* habitats. Because the recently diverged *C. allenii* and *C. villosissimus* are mainly isolated by their microhabitats, examining the mechanisms by which these species have adapted to their different habitats enhances our knowledge of the relationship between adaption and speciation.

INTRODUCTION

Since Darwin (1859) proposed that adaptation is the primary mechanism of speciation, the relationship between adaptation and speciation has been a subject of interest and debate. As populations adapt to different habitats, locally adapted individuals have higher fitness in their "home" habitats than in their "foreign" habitats (see review in Leimu and Fischer, 2008). Natural selection in the foreign habitats may cause the locally adapted populations to be unable to colonize foreign habitats (Mayr, 1963; Nosil et al., 2005). Local adaptation to divergent selection may reduce gene flow between populations and cause ecogeographic and/or microhabitat isolation because the absence of one population in the foreign habitat eliminates the probability of hybridization between populations (Coyne and Orr, 2004). Locally adapted populations become species when reproductive isolation is complete (Mayr, 1949). Therefore, adaptation to local environments can contribute to prezygotic isolation and lead to speciation between closely related species (reviewed in Schluter, 2001; Coyne and Orr, 2004; Rundle and Nosil, 2005; Hendry et al., 2007; Schemske, 2010; Sobel et al., 2010). Furthermore, when F1 hybrids experience reduced fitness in the parental habitats, extrinsic postzygotic isolation may restrict heterospecific gene flow as a result of local adaptation (reviewed in Coyne and Orr, 2004; Rundle and Nosil, 2005; McBride and Singer, 2011). As two species are locally adapted to different habitats, F1 hybrids of the two species may have intermediate traits which may not be suitable for either of the parental habitats (e.g., Hatfield and Schluter, 1999). As adaptive alleles of one parental species are contained in a heterozygotic F1 hybrid living in the other parental habitat, the hybrid may have lower fitness due to genotype-by-environment interactions (e.g., Campbell and Waser, 2007). Taken together, local adaptation can contribute to speciation through ecogeographic, microhabitat, and/or extrinsic postzygotic isolating barriers.

Empirical evidence of ecogeographic, microhabitat, and extrinsic postzygotic isolation has also been provided to support the theory of how adaptation contributes to speciation. In their review of plant speciation studies in which multiple isolating barriers were examined and compared, Lowry et al. (2008a) found that more than half of the cases provided evidence of isolation due to geography and/or immigrant inviability and that a few cases supported the importance of extrinsic postzygotic isolation. Schemske (2010) reviewed speciation studies of both plant and animal systems and concluded that habitat isolation makes a significant contribution to speciation. These speciation studies suggest the importance of ecogeographic, microhabitat, and extrinsic postzygotic isolating barriers, yet how local adaptation contributes to these isolating barriers is largely unknown. In order to understand the relationship between adaptation and speciation, I studied the mechanisms of local adaptation in recently diverged species by identifying the environmental factors and corresponding traits contributing to adaptation of closely related species which occupy different habitats. Although differences in adaptive traits may accumulate after two species have been completely speciated, differences between recently diverged species are more likely to be the contributing factor at the time of speciation (Schemske 2010). When heterospecific gene flow is largely reduced by habitat isolation between recently diverged species, the environmental factors and corresponding traits contributing to adaptation to different habitats are likely to play major roles in the process of speciation.

In plants, reciprocal transplant experiments are frequently used to determine if different species are locally adapted to their habitats (e.g., Clausen et al., 1940; Campbell and Waser, 2001; Angert and Schemske, 2005). Support for local adaptation comes from the finding that the fitness of each species is higher in its "home" habitat than in the "foreign" habitat. In statistical

terms, this is represented by an interaction between source and transplant sites (Leimu and Fischer, 2008). However, reciprocal transplant experiments alone cannot identify the environmental factors or traits responsible for local adaptation. To study the mechanisms of local adaptation and the relationship between adaptation and speciation, it is necessary to identify the environmental factors that cause selection and identify the traits that have adaptively diverged in response to these selective agents (Schluter, 2001). Environmental factors which differ between species' habitats are potentially the driving forces which select against the immigrants but favor the local species in a reciprocal transplant experiment. Traits that affect the performance of plants when exposed to different environmental factors are strong candidates for contributing to the local adaptation observed (Reeve and Sherman, 1993). Furthermore, fitness trade-offs for putative adaptive traits are implicated when a species cannot achieve high fitness in both the home and the foreign habitats, and if hybrids cannot perform as well as the parents in either habitat (Angert et al., 2008).

A handful of speciation studies have examined the environmental factors and traits responsible for local adaptation and contributing to speciation. In naturally hybridizing species of *Ipomopsis aggregata* and *I. tenuituba*, habitat isolation contributes most to reproductive isolation despite the existence of the hybrid zone (Lowry et al., 2008a; Schemske, 2010). Reciprocal transplant experiments showed that each species had higher fitness in its home habitats than in the habitats of the other species, suggesting local adaptation (Campbell and Waser, 2007). Differences in photosynthetic rate in response to different light intensity but not to different temperature have been identified as an adaptive difference that partially explains the distribution of the two *Ipomopsis* species and their hybrids (Wu and Campbell, 2006). The hybrids of the two species had ecophysiological traits that are intermediate or equivalent to the parental species (Wu

and Campbell, 2006) and they performed well in the hybrid zone (Campbell and Waser, 2001; Campbell and Waser, 2007), resulting in weak extrinsic postzygotic isolation (Lowry et al., 2008a). Studies in *Mimulus cardinalis* and *M. lewisii* provide another example of the relationship between adaptation and speciation. *Mimulus cardinalis* and *M. lewisii* are sister species which are isolated primarily by their ecogeographic distribution and their distinct pollinators (Ramsey et al., 2003). The hummingbird-pollinated *M. cardinalis* is found primarily at low elevations, while the bee-pollinated *M. lewisii* is found at high elevations. Reciprocal transplant experiments demonstrated that the two species are locally adaptive to their home habitats (Angert and Schemske, 2005). Species' differences in leaf physiology and flowering phenology under different temperatures suggest that the reduction in heterospecific gene flow is largely due to adaptive differentiation between these species (Angert, 2006; Angert et al., 2008). A recent study on *Ainsliaea faurieana* and *A. apiculata* also revealed that adaptation to different light availability and water currents contributes to speciation between these two riparian and nonriparian plant species (Mitsui et al., 2011). These studies demonstrated how local adaptation contributes to speciation of herbaceous plants in temperate regions, but no such studies have been conducted in the tropical region, where ecological and evolutionary explanations for the origin of high biodiversity are still a matter of debate (Mittelbach et al., 2007).

To investigate the relationship between adaptation and speciation, recently diverged species which occupy different habitats can be studied to compare the species' differences in habitats and in adaptive traits responsible for local adaptation and speciation. Environmental differences in understory and forest edge habitats between *Costus allenii* and *C. villosissimus*, a pair of perennial tropical rainforest herb species, present a good opportunity to study the mechanism of local adaptation and the relationship between adaptation and speciation.

Reproductive isolation between these two sister species is largely due to ecogeographic and microhabitat isolation (Ch. 4). Both species are native to Central America and northern South America, but they occupy different habitats and display parapatric distributions. *Costus allenii* is usually found in wet, shaded habitats along ravines in primary forests, while *C. villosissimus* is found in drier, open sites along forest edges. Hybrids are observed at low frequency, although there is no hybrid zone, suggesting the lack of suitable habitats for hybrids. Reciprocal transplant experiments have shown that the two species are locally adapted to their home habitats (Ch. 2). The objectives of this study were to examine possible environmental factors and putative adaptive traits that may contribute to local adaptation in *C. allenii* and *C. villosissimus*. Given the microhabitat differences observed between species, I hypothesized that spatial differences in the environmental factors, light availability and soil moisture, and in corresponding plant traits contribute to local adaptation and speciation of these two *Costus* species.

Light availability is a potential factor that influences habitat preference and contributes to local adaptation (Givnish et al., 2004 and papers cited therein; Mitsui et al., 2011), but a few studies suggested no correlation between light availability and species distribution of adult trees (reviewed by Théry, 2001). The forest understory is a resource-poor habitat due to its limited light availability. Shade-adapted plant species typically grow slower, produce leaves with a lower photosynthetic rate and slower turnover rate, and show a larger investment in herbivore defense than light-demanding species (Coley et al., 1985). To determine whether light availability is a determinant of habitat divergence and local adaptation in *C. allenii* and *C. villosissimus*, I compared the difference in photosynthetically active radiation (PAR) between the habitats of the two species and the adaptive difference in leaf physiology. In this study, a composite parameter, leaf mass per area (LMA), was measured as an indication of leaf

physiology. Slower growth rate and higher herbivore defense investments in shade-adapted plants result in a higher LMA (Sterck et al., 2006; Lusk and Warton, 2007), which represents thicker and denser leaves with longer lifespan and lower mass-based photosynthetic capacity (Wright et al., 2004; Hassiotou et al., 2010). I predicted that *C. allenii* would have higher LMA than *C. villosissimus* because the former is found in habitats with lower light availability.

Precipitation and soil moisture have been long recognized as important factors limiting the distribution of plant species (Whittaker, 1965; Duivenvoorden, 1995; Pyke et al., 2001; Engelbrecht et al., 2007). Despite high annual precipitation, seasonal variation in water availability is a common feature in tropical rainforests, and tropical plants display substantial differentiation for drought tolerance (Condit et al., 1995; Baltzer et al., 2008; Comita and Engelbrechi, 2009). Reciprocal transplant experiments found that *C. allenii* transplants had high mortality during the dry seasons when grown in *C. villosissimus* habitats (Ch. 2). To determine if local differences in water availability contribute to the differences in spatial distributions of *C. allenii* and *C. villosissimus*, I compare differences in soil moisture between the two species' habitats and examine how the two species respond to drought. Species with high drought tolerance are predicted to be found in habitats with lower soil moisture.

In order to understand the potential mechanisms of local adaptation, here I compare LMA and drought tolerance of *C. allenii* and *C. villosissimus*. To further understand how natural selection may act upon hybrids, these two traits were also examined in the F1 hybrids of the two parental species. Despite that hybrid fitness was similar to fitness of *C. allenii* transplants in the *C. allenii* sites, the F1 hybrid seeds transplanted to the *C. villosissimus* sites showed lower fitness than *C. villosissimus* (Ch. 2). Because no intrinsic postzygotic isolation caused by unfavorable interactions between divergently adaptive traits has been detected between the two species, the

reduction in hybrid fitness in the *C. villosissimus* habitats was an indication of extrinsic postzygotic isolation (Ch. 2; Ch. 4). The fitness differences between parental species and hybrids depend on how natural selection acts on the traits expressed in the hybrids (Burke and Arnold, 2001). The traits which are adaptive in the parental species usually are intermediate or equivalent to the parental species when expressed in the F1 hybrids (Wu and Campbell, 2006). Hybrids may have reduced fitness in habitats of one parental species when the adaptive traits are intermediate or equivalent to the traits of the other parental species. Hybrids may have similar fitness to one parental species in the corresponding parental habitats when the traits express equally in hybrids and the parental species. Hybrids may have superior fitness if the interactions between divergently adaptive traits produce novel, favorable phenotypes (Burke and Arnold, 2001). If hybrids are intermediate or equivalent to the unfavorable parental species in some traits while interactions of other traits are favorable, the counteracting effects among traits may result in no fitness reduction in hybrids. Examining LMA and drought tolerance in *C. allenii*, *C. villosissimus*, and their F1 hybrids enhances our knowledge of how two recently divergent species adapt to different environmental factors in their habitats, how hybrids respond to these environmental factors, and how adaptation contributes to reproductive isolation.

MATERIALS AND METHODS

Study System

The genus *Costus*, commonly known as spiral gingers, is a clade of perennial herbs which has recently undergone a rapid diversification in the Neotropics (Kay et al., 2005). The species occupy various habitats such as streamsides, tree-fall gaps, forest edges, are found on limestone, red clay and white sand soils, and from low to mid elevation (Maas, 1972; Schemske, 1983; Kay and Schemske, 2008). Individual plants usually consist of one to several upright stems with

leaves arranged spirally around the stems. *Costus* species typically produce terminal inflorescences bearing morphologically complex flowers which are pollinated by either hummingbirds or orchid bees (Kay and Schemske, 2003).

The two study species, *C. allenii* and *C. villosissimus*, are closely related and occur in the same geographic region (Maas, 1972), but occupy different habitats. A molecular phylogeny of Neotropical *Costus* places these two species as sister taxa (Kay et al., 2005). Both species flower in the wet season (see Ch. 4), and both are pollinated by euglossine bees (see Ch.1). The two species can be easily crossed to produce fully fertile F1 and F2 hybrids, yet hybrids between the two species are rarely found in nature (see Ch. 2).

Study Site

The field component of this study was conducted in Central Panama, the primary center of distribution of Neotropical *Costus* (Maas, 1972; Kay et al., 2005). Across the Isthmus of Panama, a distance of 80 km, more rain falls on the Atlantic side (mean annual precipitation = 3234 mm at Cristobal) than on the Pacific side (mean annual precipitation = 1798 mm at Balboa Heights) (Panama Canal Authority Meteorological and Hydrological Service weather station network). The study was conducted in the vicinity of Pipeline Road (Ch. 2), which runs 17 km from south to north through the primary tropical rainforest in Soberania National Park, parallel to the Panama Canal. The precipitation gradient observed across the Isthmus of Panama can also be detected along Pipeline Road: more rain falls to the north than to the south (Ch. 2). Forest canopy cover also differs, with a more open canopy in the drier, southern region. Natural populations of both species are found along Pipeline Road and in adjacent forest, with *C. allenii* predominating in the northern stretch and *C. villosissimus* to the south.

Light Availability

To determine light availability in natural habitats of *C. allenii* and *C. villosissimus*, photosynthetically available radiation (PAR) was measured at the apex of individual plants of both species (N = 27 for *C. allenii*, N = 42 for *C. villosissimus*). Measurements were taken over a 4-hour period around solar noon to minimize the influence of solar elevation angle. Because the plants in the field were too widely spaced to measure all individuals in a single 4-hour period, the measurements of the two species were taken on two overcast days: June 28, 2006 for *C. allenii* and July 12, 2006 for *C. villosissimus*. Because plants of both species generally grow along ravines or forest edges, light availability for individual plants is higher on the side away from the forest and lower on the side closer to the forest. Because *Costus* plants usually contain multiple stems, the stems within individual plants may receive different amount of light due to the different directions of stem growth. To measure light availability of each individual in consideration of this microsite variation among stems, an average of four adjacent measurements were obtained for each plant. An imaginary line was drawn perpendicular to the ravine or the forest edge to the base of each plant. In each direction away from the base of the plant, two points along the imaginary line were marked, one at 30 cm and one at 60 cm. A total of four measurements of PAR were measured at the height of the tallest stem by a LI-185B photometer and a quantum sensor LI-190SB (LI-COR Inc., Nebraska, USA) for each plant. These four measurements covered the range of an average plant sampled in this study. PAR was also measured in full sun at a nearby large gap or clearing every 30 minutes, and light availability of each sampling point of each plant was expressed as a percentage of full sun (Haig et al., 2000). A two-way ANOVA was performed for each species on the individual measurements of light availability to determine the differences among plant individuals and among sampling points

within each plant individual. A t-test was used to compare the average light availability of plant individuals between species.

Soil Moisture

To determine soil moisture in habitats occupied by *C. allenii* and *C. villosissimus*, soil samples were collected at the base of individual plants of both species (N = 22 for *C. allenii*, N = 38 for *C. villosissimus*). In central Panama, the wet season usually lasts about eight months, from May to December, and the dry season starts around mid-December and ends in late April. To capture this seasonal variation, samples were collected in both the wet season (July 2006, 2007) and the dry season (March 2007, 2008). For each plant, a soil core was extracted from the top 15 cm of soil, representing the rooting depth of *Costus*, and within 30 cm of an individual target plant. The samples were sealed in a plastic bag and weighed with a digital balance within 12 hours. Samples were then weighed after being oven dried at 60ºC for 7 days. The gravimetric water content in the soil sample was calculated as: (wet weight – dry weight) / wet weight. The effects of seasons, species, and their interactive effects on gravimetric water content in soil were analyzed with ANOVA and Tukey's HSD.

Leaf Mass per Area

Four categories of plants were used to evaluate leaf mass per area (LMA): the two parental species, *C. allenii* and *C. villosissimus*, and two F1 hybrids derived from reciprocal crosses between the parents, with either *C. allenii* as the female and *C. villosissimus* as male ($F1_{allenii}$), or vice versa ($F1_{villo}$). A total of 36 *C. allenii*, 35 *C. villosissimus*, 17 $F1_{allenii}$, and 25 $F1_{villo}$ were measured. These plants were produced by hand-pollinating *C. allenii* and *C. villosissimus* plants located in the natural populations along Pipeline Road, or from hand-pollination of greenhouse-raised parental plants germinated from seeds collected from the natural

populations on Barro Colorado Island, Panama. All the plants used in this experiment were germinated in potting soil (High Porosity Professional Mix, Baccto) and raised in the greenhouse at Michigan State University. The environmental conditions of the greenhouse were set to be near a maximum of 26°C during the day and a minimum of 15°C during the night, with the actual temperature being subject to change depending on the conditions outside of the greenhouse. The plants experienced the natural day length and light levels in Michigan. Despite their differences in ages at the time of sampling, all plants were well-hydrated, multi-stem adults of similar sizes.

Sample collection for estimating LMA was done at least 2-3 hours after sunrise and 3-4 hours before sunset, as suggested by Garnier et al. (2001). Samples were taken in June, 2009 on fully hydrated plants. Four leaf disks per plant were collected using a round squeeze punch (Fiskars Craft). To determine the area of the leaf disks, a preliminary collection of 39 fresh disks was sealed in a plastic bag with wet paper towels until the area was measured with a portable area meter (LI-3000A with a belt conveyer LI-3050A, LI-COR Inc., Nebraska, USA). Because all disks were obtained using the same squeeze punch, the average area of the fresh disks was used to represent the area of all the disks. All leaf disks were cut from the third to the fifth leaves from the apex of a stem. The leaves sampled were young, fully expanded, and free of serious herbivore or pathogen damage, as recommended by Garnier et al. (2001) and the literature reviewed therein. The disks were collected between the midridges and the edges of the leaves to prevent unequal thickness due to leaf architecture. The collected disks were dried at 60ºC for 7 days. The dry weight of the disks was measured with a digital balance. Following Ellsworth and Reich (1992), LMA of each plant was calculated as the average ratio of dry weight to area (g /

m^2). The values of LMA were compared among the two parental species and their F1 hybrids with ANOVA and Tukey's HSD.

Drought Tolerance

Plants of *C. allenii*, *C. villosissimus*, and their F1 hybrids derived from reciprocal crosses between the parental species were used to evaluate drought tolerance. To produce the seeds for this experiment, during the wet season in 2006, I hand-pollinated eight *C. allenii* and seven *C. villosissimus* located in natural populations along Pipeline Road. The flowers were bagged before and after being hand pollinated to prevent natural pollination. Pollen collected from a flower was applied to the stigma with a flat toothpick. Seeds from 33 *C. allenii*, 38 $F1_{allenii}$, 23 $F1_{villo}$, and 18 *C. villosissimus* fruits were collected in October 2006. Seeds of each fruit were immediately sowed in potting soil (High Porosity Professional Mix, Baccto) in a 4-L pot in the greenhouse at Michigan State University. The environmental conditions of the greenhouse were set as described above. Once a seed germinated and the first true leaf was fully expanded, the seedling was transplanted to a 5 cm x 5 cm rose pot. Seedlings were grown in these pots until May 2007, when for each species and each direction of F1 hybrids, 45 seedlings of similar size were transplanted to 4-L pots. These plants were randomly arranged and watered daily with fertilized water (18-9-18 pH Reducer Fertilizer, 100 ppm N, PLANTEX®) until the drought treatment began on July 2, 2007. The number of leaves and stems per plant was first counted on June 28 to July 1, before the drought treatment began. At the beginning of the drought treatment, all plants were watered weekly with progressively reduced volume: 1000 ml on July 9, 500 ml on July 16, 250 ml on July 23, and finally, 150 ml on July 30. The progressive reduction in water volume was designed to mimic the beginning of a dry season in the natural habitats of *C. villosissimus*, where gradually less precipitation is received, followed by a long dry period. To

make sure that all plants received an equal volume of water, each pot was placed on a tray that retained water. The plants were randomized approximately every other month over the course of the experiment (approximately 16 months).

To measure drought tolerance, I calculated a drought tolerance index (DTI), which gives the number of days from the day the drought treatment began (July 2) to the day a plant became stemless. To estimate DTI, plants were censused for the number of stems biweekly early in the drought treatment (August to November, 2007) and then weekly until the end of the experiment (December 2007 to October 2008). It is difficult to determine the exact time of death for plants and to measure the exact duration a plant can survive in a dry condition. Therefore, the number of days until a plant becomes stemless was used as an indicator of the date of death. This was based on the observation in preliminary studies that plants that had become stemless due to drought never recovered after being rehydrated, while plants that retained stems usually resprouted after rehydration (Chen unpublished data). To ensure that the absence of stem is a good indication of plant death, I rehydrated the plants in 15-plant subsets to test their viability. Because preliminary studies showed that *C. allenii* has a much lower drought tolerance than *C. villosissimus*, the rehydration treatment was first applied when all of the *C. allenii* plants were stemless. Fifteen plants per plant group were choose randomly among the experimental plants and rehydrated to test their viability after drought stress (Fig. 3.1). This rehydration procedure was repeated when the remaining F1 hybrids and *C. villosissimus* became stemless (Fig. 3.1).

Only plants which were stemless before being rehydrated were included in the analysis of DTI. To demonstrate the change in stem numbers during the experimental period, the average number of stems of each species and each direction of reciprocal crosses of F1 hybrids was graphed across time. To examine differences in drought tolerance among the two parental

species and their F1 hybrids, DTI was compared with ANOVA and Tukey's HSD. All statistical analyses in this study were done in R, version 2.12.2 (R Development Core Team, 2011).

RESULTS

Light Availability

Light availability to individual plants, measured as the average percentage PAR of full sun, varied from 0.3% to 65.7% in *C. allenii* habitats and from 1.2% to 87.9% in *C. villosissimus* habitats. There was a significant variation among plant individuals and among sampling points for both species ($p < 0.001$ for each factor in each species). Light availability of individual plants was significantly lower in the *C. allenii* plants (mean ± 95% CI = 11.3% ± 5.9) than in the *C. villosissimus* plants (26.1% ± 4.9) ($t = 3.93$, $p < 0.001$).

Soil Moisture

The ANOVA results for the analysis of gravimetric water content in soil are presented in Table 3.1. The *C. allenii* soil samples had significantly higher gravimetric water content (32.2% ± 1.6) than the *C. villosissimus* samples (23.2% ± 1.4), and samples collected in the wet seasons had a significantly higher gravimetric water content (31.9% ± 1.2) than those collected in the dry seasons (21.1% ± 1.6). Furthermore, the difference in soil moisture between species was greater in the dry seasons than in the wet seasons (Fig. 3.2), as is reflected in the significant season × species interaction (Table 3.1).

Leaf Mass per Area

There was significant variation in LMA among *C. allenii*, *C. villosissimus*, and their F1 hybrids ($F = 13.464$, $p < 0.001$). *Costus allenii* had significantly higher LMA than $F1_{allenii}$, $F1_{villo}$, or *C. villosissimus*, but there was no difference among these three latter categories (Fig. 3.3).

Drought Tolerance

Plants continued to grow for approximately one month after their last watering, maintained their sizes for a period, and then started to wilt (Fig. 3.4). Plants suffering from drought stress first displayed wilted lower leaves. The lower leaves gradually turned yellow and dried, while the tips of the stems remained green. Eventually all leaves wilted, became brown, and dropped off the stems. Leafless stems eventually dried and broke from the rhizomes at the soil surface.

Plants of *C. allenii* retained most of their stems through early October, and then lost the stems rapidly in late October and November. Plants of $F1_{allenii}$ and $F1_{villo}$ showed a similar pattern to each other: they retained most of their stems through November, and then lost their stems rapidly from December, 2007 to February, 2008. In contrast, plants of *C. villosissimus* remained most of their stems through February, and then gradually lost their stem until all plants were stemless in late September, 2008 (Fig. 3.4).

When all the *C. allenii*, all the hybrids, and all the *C. villosissimus* were stemless on January 14, April 21, and September 22, respectively, the three rehydration treatments were applied. Across all three rehydration treatments, none of the stemless plants resprouted after being rehydrated. Under these experimental conditions, all stemless plants were indeed dead. Among the 58 plants which retained stems before being rehydrated, 5 $F1_{allenii}$ and 5 $F1_{villo}$ did not recover from the severe drought stress. The hybrid plants that retained stems but did not recover indicated that these plants were dead before they were stemless. Therefore, a measurement of DTI may be an overestimate of the number of days the plant can survive under drought, but it is accurate enough to show the differences in drought tolerance among plant categories.

DTI was recorded for plants which were stemless before being rehydrated, including 45 *C. allenii*, 30 $F1_{allenii}$, 31 $F1_{villo}$, and 16 *C. villosissimus*. There were significant differences in DTI among *C. allenii*, *C. villosissimus* and their F1 hybrids ($F = 179.37$, $p < 0.001$). The DTI of *C. allenii* was significantly lower than that of *C. villosissimus*, and that of F1 hybrids was intermediate to the parents (Fig. 3.5).

DISCUSSION

The field studies demonstrate that *C. allenii* occupies habitats with lower light availability and produces leaves with higher LMA while *C. villosissimus* occupies habitats with lower soil moisture and has higher tolerance to drought. Given the findings of strong local adaptation of *C. allenii* and *C. villosissimus* obtained from reciprocal transplant experiments (Ch. 2), the results presented here provide evidence for major differences between species in their habitats. Moreover, the differences observed in LMA and drought tolerance strongly suggest that these traits contribute to local adaptation. The drought tolerance of F1 hybrids is intermediate to the parents, but LMA is similar to that of *C. villosissimus*. Although hybrids are rarely found in nature, intermediate drought tolerance in F1 hybrids may be related to the extrinsic postzygotic isolation observed to act on them in the *C. villosissimus* habitats. In contrast, the observation that hybrid LMA is *C. villosissimus*-like does not reflect on the unreduced hybrid fitness in the *C. allenii* habitats.

Adaptation to Low Light Availability in *C. allenii* Habitats

Average light availability was more than two-fold higher in *C. villosissimus* habitats than in *C. allenii* habitats. *Costus villosissimus* habitats are usually along forest edges, where canopy cover is lower than in the primary forest understory typical of *C. allenii* habitats. Because plants grow rapidly at the beginning of each wet season (Chen, personal observation), the measurement

was conducted for each plant only once in a wet season. Thus, species' differences in PAR measurements obtained in the wet season represent the species' differences in light availability when plants are actively producing new leaves. One caveat of the comparison of between species' habitats is that there may be seasonal variation in light availability, and I only sampled light in the wet season. Sampling in a dry season may result in a higher PAR measurement because many trees lose their leaves in the dry seasons and more light may penetrate the canopy during the dry season (Wirth et al., 2001; Lemos-Filho et al., 2010). The seasonal differences in light availability may be greater in the understory than along forest edges; nonetheless, the trend of higher light availability along edges and lower availability in understory would be consistent despite the seasonal changes (Oshima et al., 1997).

Costus allenii has higher LMA, indicating thicker and/or denser leaves. Higher LMA is usually correlated with lower photosynthetic capacity, and slower growth. These features are common in plants growing under low-resource conditions (Poorter et al., 2009), including shady habitats. Shade-adapted species tend to have tougher leaves per unit dry mass compared with co-occurring sun-adapted species (reviewed in Onoda et al., 2011) and are better protected against herbivores, with reduced leaf turnover and higher survival under low light conditions (Poorter, 2009). This is consistent with the hypothesis of higher anti-herbivory defense investments in plants living in habitats with lower resource availability (Coley et al., 1985) and with the observation of higher LMA leaves experiencing less herbivore damage (Poorter et al., 2009). The higher LMA found in *C. allenii* may indicate that it is adapted to the low light availability characteristic of their natural habitats. Lower LMA in *C. villosissimus* may prevent the species from invading *C. allenii* habitats, as suggested by the low survival of *C. villosissimus* transplants in *C. allenii* habitats observed in the reciprocal transplant experiments (Ch. 2). The underlying

mechanisms of local adaption to *C. allenii* habitats may also involve *C. villosissimus* having poor defense against herbivores as there was a higher proportion of pest damage on new leaves of *C. villosissimus* than on those of *C. allenii* (Ch.2).

I hypothesized that *C. allenii* has higher LMA because it inhabits environments with lower light availability under which plants generally have lower photosynthetic capacity and growth rates. However, LMA is not only a heritable trait but also a plastic characteristic which responds to many environmental factors, including light availability of the growing condition (Lusk et al., 2008; Poorter et al., 2009; Lusk et al., 2010). Plants within a species increase their LMA as the light availability of their growing environments increases (Poorter et al., 2009). The intraspecific correlation between LMA and light availability is contradictory to the interspecific correlation. The contradiction between intra- and interspecific LMA-light correlation results from the differences in leaf structure responses to different growing environments (Lusk et al., 2010). Leaves of shade-adapted species have higher cell wall mass per unit area, which are more resistant to fracture than those of light-demanding species. However, leaves of plants growing in shade have less cell content per unit area than those of plants growing in sun (Lusk et al., 2010). When growing in environments with high light availability, the magnitude of the plastic response to increasing environmental light is similar between shade-adapted and light-demanding species (Poorter et al., 2009; Lusk et al., 2010). Light availability in the greenhouse at Michigan State University around solar noon in June is higher than that in both parental habitats in Panama (Chen, personal observation). The LMA measurements presented in this study may be higher than those in the field, but the two species should respond to the field-greenhouse light difference similarly to each other. Therefore, the differences in LMA among *C. allenii*, *C. villosissimus*, and their hybrids are proper measurements of the inherited differences among these plants.

LMA for F1 hybrids produced from both directions of the cross between *C. allenii* and *C. villosissimus* was similar to that of *C. villosissimus*. This suggests that *C. villosissimus* has a dominant allele(s) for LMA and that the gene(s) are unaffected by the cytoplasm. However, unbalanced sample sizes across the four groups of parents and hybrids may reduce statistical power in the comparisons of LMA. Although F1 hybrids have low, *C. villosissimus*-like LMA, which should be unfavorable in the *C. allenii* habitats, the fitness of hybrid was not less than that of the *C. allenii* transplants in the *C. allenii* habitats in reciprocal transplant experiments (Ch. 2). The effects of the unfavorable LMA on hybrid fitness may be compensated by some beneficial interactions between other divergently adaptive traits. Future examination of other adaptive traits and interactions among traits may provide a better understanding of how adaptive traits affect hybrid fitness in the parental habitats.

Adaptation to Low Water Availability in *C. villosissimus* Habitats

Soil moisture differed substantially between seasons and by species, with a significant interaction between seasons and species. The rainfall data presented in Chapter 2 also revealed significant differences between seasons and species' habitats. The difference in rainfall between habitats of *C. allenii* and *C. villosissimus* was greater in the dry season than in the wet season. Because the level of soil moisture is a result of the balance between gain (precipitation) and loss (evaporation and plant transpiration), it is not surprising to find that the soil moisture data has a similar pattern to the rainfall data. Both rainfall data and soil moisture measurements indicate higher water availability in *C. allenii* habitats than in *C. villosissimus* habitats, higher water availability in both habitats in wet seasons than in dry seasons, and a greater difference between species habitats in the dry seasons. This temporal variation becomes important in determining species distribution when the ability to survive through the dry season in the drier habitats may

limit the potential for *C. allenii* to invade *C. villosissimus* habitats, as observed in the reciprocal transplant results (Ch. 2).

The greenhouse drought tolerance experiment showed significant differences between species and their hybrids in the number of days the plants retained stems under drought stress. The parental species and their F1 hybrids had the same sample sizes at the beginning of the drought treatment but the sample sizes reduced unequally in the analysis of DTI because of the rehydration treatments. Although all *C. allenii* but fewer F1 hybrids and fewer *C. villosissimus* remained in the analysis, highly significant differences in DTI were detected among the two parental species and their hybrids. The results provide clear evidence of divergence in drought tolerance between *C. allenii* and *C. villosissimus*.

Among the two parental species and their hybrids, *C. villosissimus* was surprisingly drought tolerant: the species was able to survive in the greenhouse an average of 381 days without water (Fig. 3.5). Humidity in the greenhouse was likely higher than in the field in the dry season, and light intensity in Michigan was not as strong as in central Panama. Therefore, plant transpiration in the greenhouse drought experiment was potentially much less than that in *C. villosissimus* habitats during the dry season. Nevertheless, it is surprising that a tropical rainforest herb is able to survive >1 year without water.

From assessments of drought sensitivity of 48 tree and shrub species conducted in central Panama, Engelbrecht et al. (2007) observed a correlation between soil moisture and species distribution patterns. They found that tree species' density was correlated with the precipitation pattern across the isthmus: wet on the north Atlantic side and dry on the south Pacific side. They also concluded that drought sensitivity is highly correlated to species distribution in the Panamanian tropical forests. The natural populations of *C. allenii* and *C. villosissimus*, although

on a relatively local scale (17 km on Pipeline Road), also demonstrate a remarkable difference in habitat soil moisture (Ch. 2). Soil moisture is identified as an environmental factor responsible for local adaptation in *C. villosissimus* habitats, and the higher drought tolerance of *C. villosissimus* is a putative adaptive trait which allows *C. villosissimus* to grow and reproduce in dry, seasonal environments. Similarly, the low drought tolerance of *C. allenii* probably prevents its expansion into *C. villosissimus* habitats.

The drought tolerance data not only showed a significant difference between species, but also that the F1 hybrids are intermediate to the two parental species, with no effect of the direction of the cross. Based upon the broad segregation of drought tolerance observed in an F2 population (Chen unpublished data), the intermediate phenotype of drought tolerance in the F1 hybrids implies that this is a quantitative trait probably controlled by multiple genes with additive effects. Hybrids that have intermediate drought tolerance may not be able to perform as well as *C. villosissimus* plants under drought stress. The intermediate phenotype in the hybrids may be responsible for the reduced hybrid fitness in the *C. villosissimus* habitats in the reciprocal transplant experiment (Ch. 2), suggesting extrinsic postzygotic isolation. Adaptation to drought may contribute to both pre- and postzygotic isolation in these recently diverged species.

However, drought tolerance may not be the only trait selected by drought stress in *C. villosissimus* habitats. Other traits such as late seed germination phenology may also contribute to the adaptation of *C. villosissimus* to dry habitats. When seeds of the two species are germinated in the field and greenhouse, *C. villosissimus* consistently displays longer seed dormancy (Ch.2; Chen unpublished data). Seeds of *C. allenii* begin germination in late October, approximately one month after seed maturation. In contrast, seeds of *C. villosissimus* do not germinate until late April of the following year, when the wet season begins. As a result, *C.*

villosissimus seedlings avoid the dry season in central Panama (January to April) and have lower mortality (Ch. 2). This delay of germination in *C. villosissimus* was observed under a range of light and soil moisture environments, suggesting innate control of germination timing rather than environmental cues (Ch. 2).

Interaction between Environmental Factors

Soil moisture and light availability were suggested to be important environmental factors which contribute to local adaptation in *C. allenii* and *C. villosissimus*. Although these factors were measured independently in this study, in natural populations high light availability is generally associated with low soil moisture, and vice versa. The correlation between soil moisture and light availability is an integrated consequence of precipitation and canopy coverage. In the Isthmus of Panama, Brenes-Arguedas et al. (2011) examined the interaction between light availability and water availability on the performance of seedlings from 24 species, including trees, shrubs, and lianas. They found that seedlings had better survival and growth under high light condition with ample water availability but worse performance under high light condition when water was limiting. Instead of a trade-off that species with lower drought tolerance are more shade-tolerated, Brenes-Arguedas et al. (2011) found that species with lower drought tolerance were better able to take advantage of small increases in light and grow faster. They suggested a trade-off between being tolerant to drought and growing fast in understory where light availability is low (Brenes-Arguedas et al., 2011). These findings across unrelated species are different from the findings of closely related *C. allenii* and *C. villosissimus*, as the fitness difference between species transplanted to *C. allenii* sites lies in the difference in plant survival instead of growth (Ch. 2). In the system of *C. allenii* and *C. villosissimus*, the results of

reciprocal transplant experiments and this study suggest a trade-off between surviving in drought and surviving in shady habitats.

Besides soil moisture and light availability, numerous biotic factors may be important in determining plant survival. In the tropics, higher pest (herbivore and pathogen) pressure is suggested to be associated with wetter sites with lower light availability (Brenes-Arguedas et al., 2009), e.g., the habitats of *C. allenii*. A general hypothesis of trade-offs between growth and defense states that a shade-adapted plant will evolve greater resistance to pests because any loss of photosynthetic area would be costly to replace (Coley and Barone, 1996). Light-adapted plants, such as *C. villosissimus*, usually grow fast, as implied by their low LMA, and tolerate leaf loss without allocating too much energy to pest resistance. When growing in shady habitats, *C. villosissimus* may fail to grow fast but is also poorly defended against pests. If *C. villosissimus* is less resistant to pests, it is possible that the top-down selection of pests would reduce *C. villosissimus* fitness in *C. allenii* habitats. This hypothesis is supported by the results from the reciprocal transplant experiments which indicate a lower fitness and a higher level of pest damage on the new leaves of *C. villosissimus* than those of *C. allenii* (Ch. 2).

Local Adaptation and Speciation

The significant differences in drought tolerance and LMA observed between the two parental species support the hypothesis that these traits cause habitat preference, and therefore habitat isolation between the two species. The microhabitat differences that largely isolated *C. allenii* and *C. villosissimus* suggest that local adaptation is the driving force of speciation. In this case, local adaptation directly causes spatial isolation due to different habitat preferences. A similar pattern has been reported in inland and coastal *Mimulus guttatus*, in which populations are adapted to either seasonal drought in inland habitats or to high salinity in coastal habitats

(Lowry et al., 2008b). The effect of light availability on habitat differentiation leading to speciation has been observed between *Ainsliaea faurieana* and *A. apiculata*, two naturally hybridizing riparian and nonriparian plants (Mitsui et al., 2011). Light availability is lower in the habitats of *A. apiculata* than those of *A. faurieana*, and survival rate of *A. faurieana* under a greenhouse low light condition was significantly reduced, suggesting maladaptation of *A. faurieana* in habitats of *A. apiculata* (Mitsui et al., 2011). Isolation between *Mimulus cardinalis* and *M. lewisii* is mainly due to their ecogeographic distribution, which is affected by divergent adaptation to different temperatures at the elevations where the plants occur (Ramsey et al., 2003; Angert and Schemske, 2005; Angert 2006). All these studies demonstrated the role of local adaptation as a primary mechanism of speciation. The study of *C. allenii* and *C. villosissimus* agrees with these studies and expands our understanding of the relationship between adaptation and speciation into the tropics.

In addition to habitat isolation, local adaptation may also contribute to speciation through extrinsic postzygotic isolation (reviewed in Coyne and Orr, 2004; Rundle and Nosil, 2005; McBride and Singer, 2011). The hybrids between *C. allenii* and *C. villosissimus* have lower LMA compared to *C. allenii* and lower drought tolerance compared to *C. villosissimus*, suggesting that the hybrids may be less fit than the parents in the corresponding parental habitats, resulting in extrinsic postzygotic isolation. Reciprocal transplant experiments demonstrated a reduction in hybrid fitness to inferior seed germination and/or seedling survival but not to survival or growth in juvenile cuttings in *C. villosissimus* habitats and no reduction in hybrid fitness was detected in *C. allenii* habitats (Ch. 2). Hybrid fitness reduction in *C. villosissimus* habitats may be a consequence of the intermediate phenotype of drought tolerance of the hybrids, suggesting that local adaptation to drought contributes to extrinsic postzygotic isolation. A

moderate level of extrinsic postzygotic isolation was found in the early stage of hybrid establishment in *C. villosissimus* habitats (Ch. 2; Ch. 4), but there is no evidence of how established hybrid juveniles may respond to drought and reduce their fitness in *C. villosissimus* habitats. In contrast, hybrids had similar fitness to *C. allenii* in *C. allenii* habitats, indicating no extrinsic postzygotic isolation (Ch. 2; Ch. 4). The relationship between the trait of LMA and hybrid fitness in *C. allenii* habitats and whether local adaptation to low light environments contributes to extrinsic postzygotic isolation are still largely unknown. Asymmetrical and incomplete extrinsic postzygotic isolation between the two species may be reflected by the occasional finding of natural hybrids located in the parental habitats because there may not be strong selection against hybrids once their seedlings are established. In *C. allenii* and *C. villosissimus*, most of the heterospecific gene flow is restricted by earlier acting, prezygotic barriers, mainly ecogeographic and microhabitat isolation, but not postzygotic barriers (Ch. 4). In fact, there is very little empirical evidence of extrinsic postzygotic isolation in plants (Widmer et al., 2009)--further investigation of this barrier is needed.

In addition to these direct contributions to speciation, local adaptation to different habitats may indirectly contribute to speciation through its interaction with other barriers (Sobel et al., 2010). For example, flowering time may depend on the abiotic conditions of the habitats and environmentally-mediated differences in flowering time can cause temporal isolation between populations located in different habitats (Stanton et al., 1997). Geographic separation caused by local adaptation may have allowed adaptation to different pollinators and resulted in pollinator isolation between closely related species (Kay and Sargent, 2009). Different edaphic conditions in different habitats may also cause pollen-pistil incompatibility and lead to gametic isolation (Searcy and MacNair, 1990). All these studies suggests that local adaption, directly or

indirectly, contribute to speciation. However, in the system of *C. allenii* and *C. villosissimus*, flowering time of the two species overlap greatly (Ch. 4), the two species share the same pollinators (Ch. 1), and gametic isolation does not contribute significantly to total isolation (Ch. 1; Ch. 4). Weak isolation due to phenology, pollinator, and gametic barriers suggests that local adaptation of *C. allenii* and C. *villosissimus* contribute to speciation mainly through habitat isolation instead of indirectly through interactions with other isolating barriers.

Conclusion

This study shows that high drought tolerance in *C. villosissimus* is associated with its drier habitats and high LMA in *C. allenii* is associated with its shady habitats. Local adaptation to different habitats causes divergent habitat preferences and thus microhabitat isolation between the two species. Intermediate drought tolerance in F1 hybrids may cause extrinsic postzygotic isolation. To confirm whether drought tolerance and LMA are the adaptive traits contributing to habitat isolation and speciation, studies of the causality between these putative adaptive traits and natural selection in the parental habitats are necessary. To this end, reciprocal transplant experiments using F2 populations that display wide segregation for these putative adaptive traits in the parental habitats may provide insight into whether these traits are currently under selection. Such experiments may also shed light on whether and how trade-offs between drought tolerance and shade tolerance contribute to local adaptation and speciation. Preliminary data for a F2 population grown in *C. villosissimus* habitats show that drought tolerance is significantly correlated with higher survival through the first dry season after transplanting, suggesting drought tolerance being an important adaptive trait contributing to habitat isolation. In addition to the F2 reciprocal transplant experiments, quantitative trait locus mapping on the adaptive traits may further help us to understand the genetic basis of local adaptation and speciation.

Table 3.1. Summary of ANOVA results for soil moisture measured in habitats of *Costus allenii* and *C. villosissimus* in two dry and wet seasons. The numbers in bold indicate a p value of less than 0.05.

	Df	Sum of squares	Mean square	F value	**p value**
Season	1	6920.0	6920.0	177.35	**< 0.001**
Species	1	4580.1	4580.1	117.38	**< 0.001**
Season × Species	1	977.2	977.2	25.04	**< 0.001**
Residuals	236	9208.4	39.0		

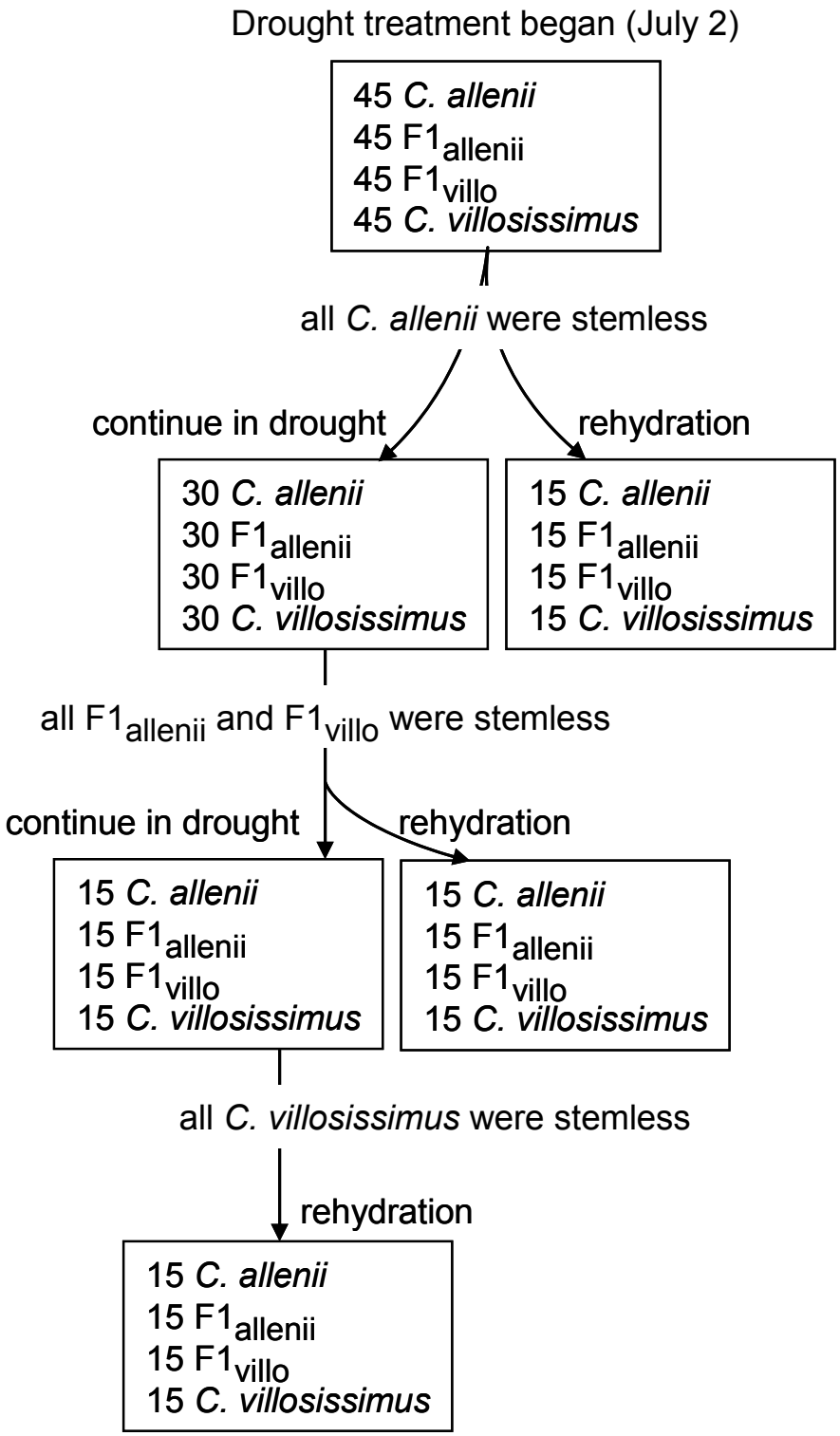

Figure 3.1. Experimental design of the rehydration treatments in the greenhouse drought tolerance experiment.

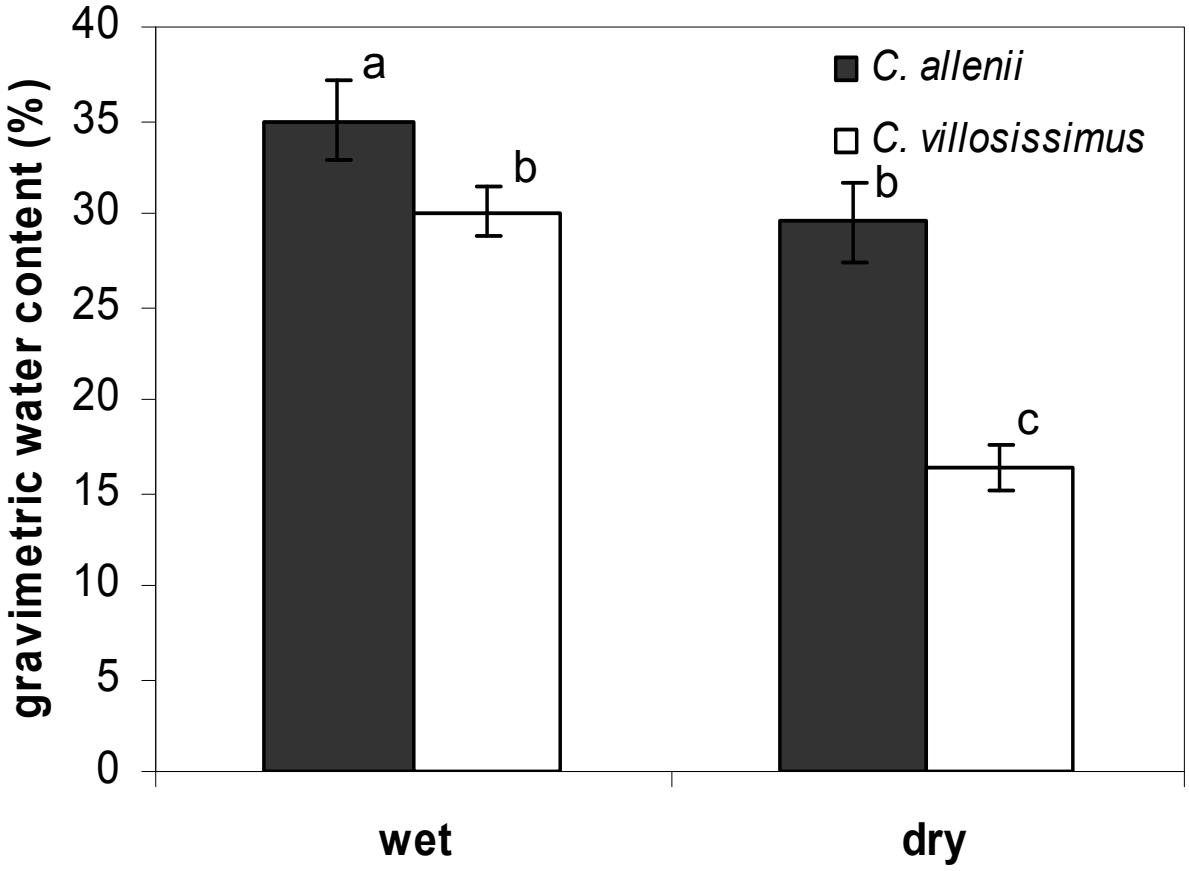

Figure 3.2. Soil moisture measured in habitats of *C. allenii* and *C. villosissimus* in wet and dry seasons. Error bars denote the mean ± 95% CI. Bars with different letters represent means that are significantly different at the level of $\alpha = 0.05$ based on the Tukey's HSD test.

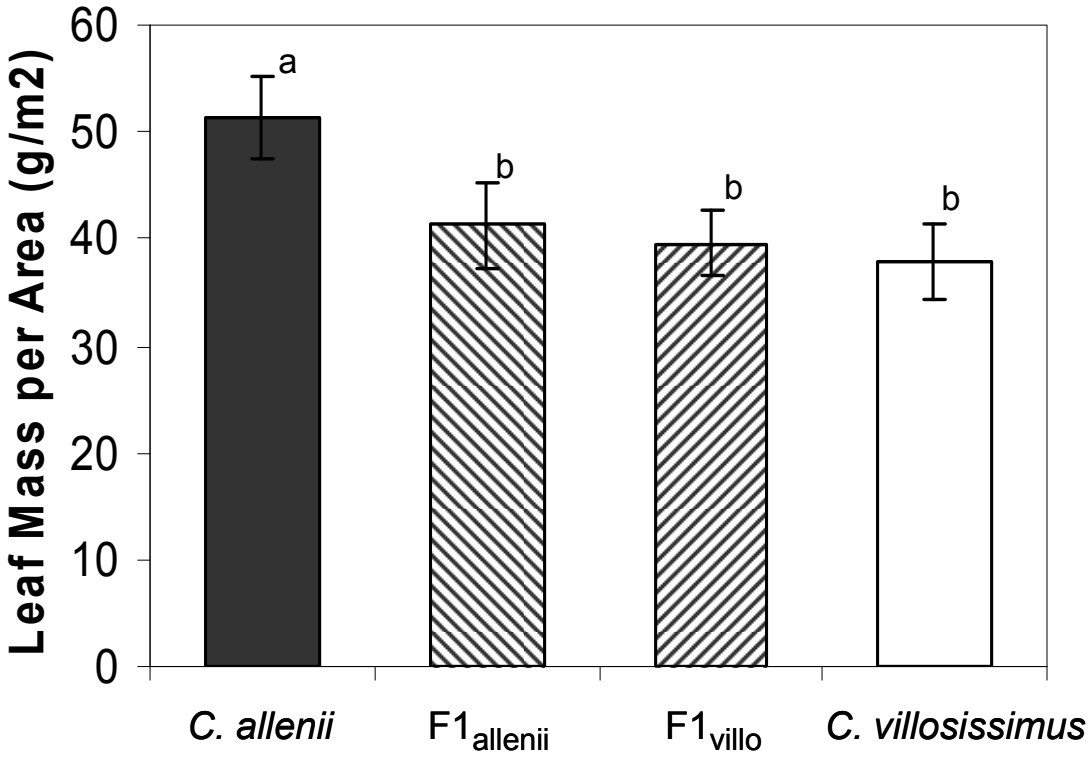

Figure 3.3. Comparisons among *C. allenii*, *C. villosissimus* and their F1 hybrids in Leaf Mass per Area (LMA). The sample sizes were different among species and hybrids: *C. allenii* = 36, $F1_{allenii}$ = 17, $F1_{villo}$ = 25, and *C. villosissimus* = 35. Error bars represent mean ± 95% CI. Bars with different letters represent means that are significantly different at the level of $\alpha = 0.05$ based on Tukey's HSD tests.

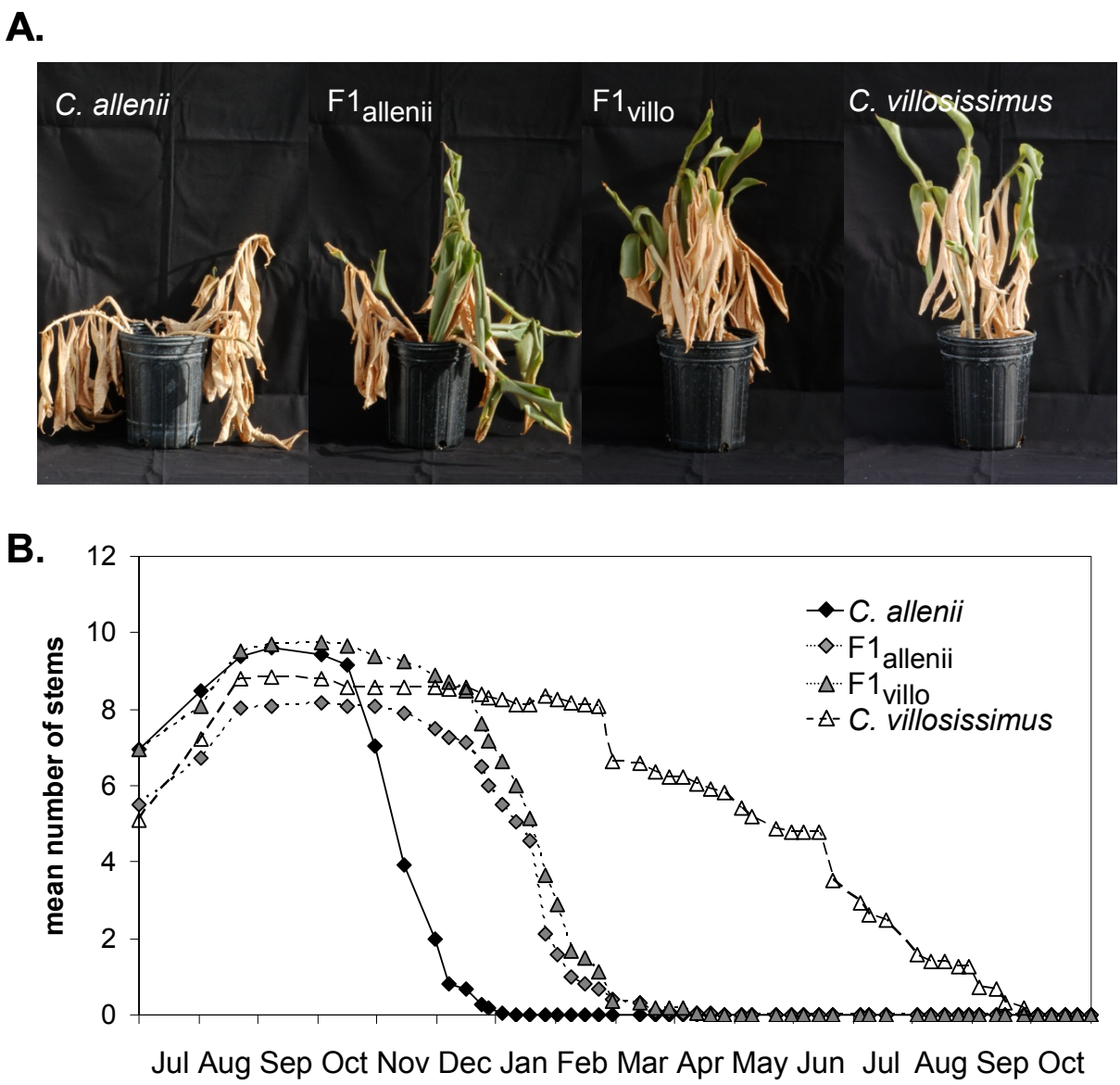

Figure 3.4. Changes in plant appearance and size in the drought tolerance experiment. (A) Pictures of *C. allenii*, $F1_{allenii}$, $F1_{villo}$, and *C. villosissimus* taken on September 18, 2007. The four plants were the same size (number of leaves) at the beginning of the experiment but showed different level of wilting under drought stress. (B) Changes in the number of stems per plant among *C. allenii*, *C. villosissimus*, and the F1 hybrids through the experiment. For each group, n = 45 before January 14, n = 30 between January 14 and April 22, and n = 15 after April 22.

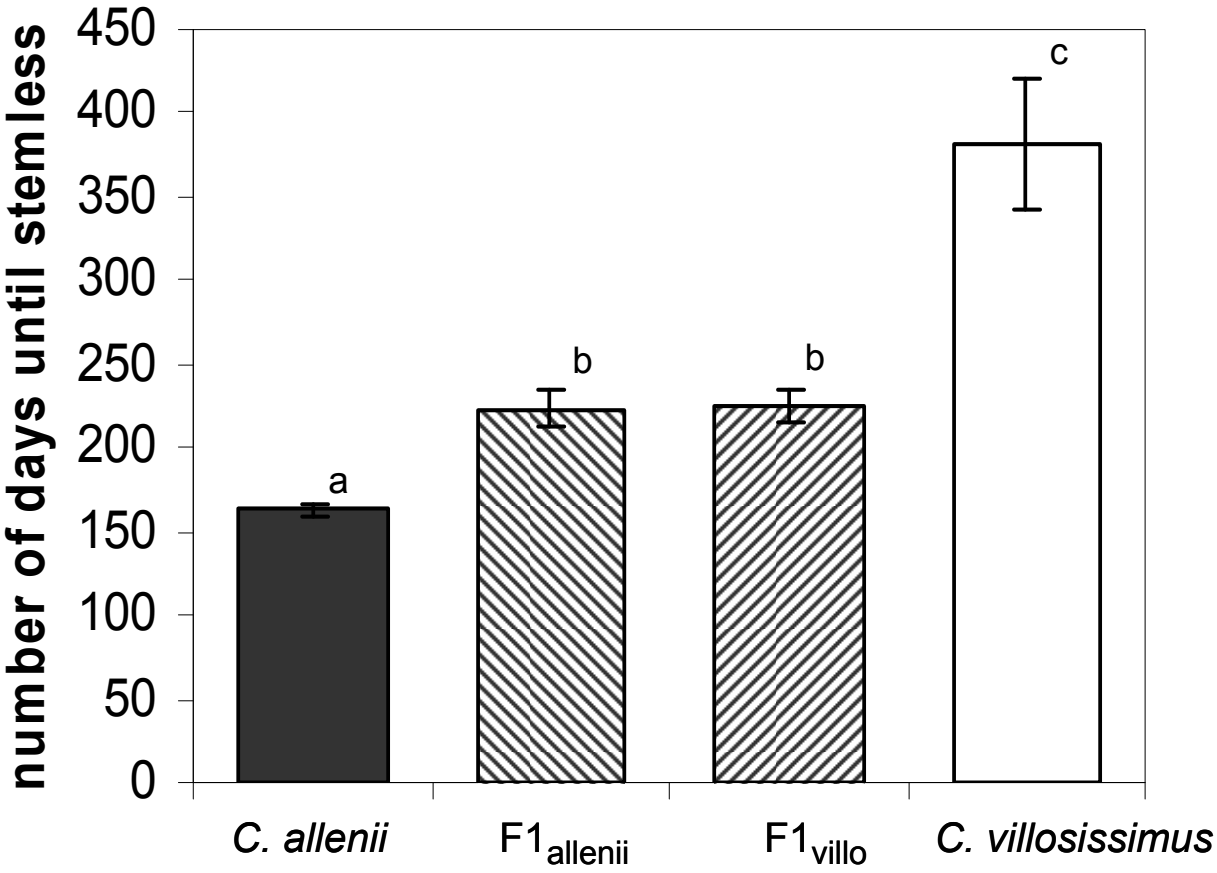

Figure 3.5. Comparisons among *C. allenii*, *C. villosissimus* and their F1 hybrids in drought tolerance (DTI) measured as the number of days until the plant is stemless. Because of the rehydration treatments, sample sizes were different among species and hybrids: *C. allenii* = 45, $F1_{allenii}$ = 35, $F1_{villo}$ = 36, and *C. villosissimus* = 16. The error bars represent means ± 95% CI. The bars with different letters represent means that are significantly different at the level of α = 0.05 based on Tukey's HSD tests.

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Chapter 4

Reproductive Isolation between Two Closely Related Neotropical Herbs

ABSTRACT

Comprehensive studies of the mechanisms by which populations diverge to form different species are required to understand the origins of biodiversity. Speciation research demands an examination of the full spectrum of isolating barriers and their relative contribution in gene flow reduction. I investigated and compared nine sequential reproductive isolating barriers between two closely related, bee-pollinated Neotropical herbs, *Costus allenii* and *C. villosissimus*. Four of the barriers were examined in this chapter: ecogeographic isolation, temporal isolation, interspecific seed set, and F1 hybrid pollen viability. The other five barriers, microhabitat isolation, sexual isolation, F1 hybrid seed germination and survival in parental habitats, F1 juvenile survival in parental habitats, and F1 juvenile growth in parental habitats, were presented in previous chapters. In both species, ecogeographic and microhabitat barriers were the most important factors contributing to total isolation. Both of these barriers represent spatial isolation due to local adaptation to different habitats, but at different spatial scales. Prezygotic isolation, mainly due to ecogeographic and microhabitat isolation, was found to be stronger than postzygotic isolation. I also estimated total isolation with barriers of ecogeographic isolation, the frequency of hybrids naturally found in the suitable habitats for the two species, and F1 pollen viability. The average total isolation of the two species was 0.988, suggesting that the two species are highly isolated. Taken together, with the findings of the previous chapters, the results of this chapter suggest that ecological factors result in local adaptation and contribute to reproductive isolation. I conclude that local adaptation is the primary mechanism of speciation between *C. allenii* and *C. villosissimus*.

INTRODUCTION

As the foundation of biodiversity, speciation is one of the most important topics in the field of evolutionary biology. How to study speciation largely depends on how species and species boundaries are defined. As most evolutionists agree that species are reproductively isolated from each other (biological species concept; Mayr, 1942), analyzing reproductive isolation helps us understand the mechanism of species formation and maintenance in nature. When reproductive isolation accumulates, inter-population gene flow decreases, and populations may diverge to form different species. A number of barriers have been identified as components of reproductive isolation (Dobzhansky, 1937; Mayr, 1947, 1963; Schluter, 2001; Coyne and Orr, 2004; Nosil et al., 2005), and their relative contributions have been compared in several systems (e.g., Coyne and Orr, 1989, 1997; Husband and Sabara, 2003; Ramsey et al., 2003; Kay, 2006; Matsubayashi and Katakura, 2009; Dopman et al., 2010).

Reproductive isolating barriers can be classified according to the timing of their action throughout the life history (Dobzhansky, 1937; Mayr, 1942; Coyne and Orr, 2004). Prezygotic isolating barriers include habitat, temporal, and sexual isolation which act before the formation of hybrids by preventing hybridization. Habitat differentiation caused by local adaptation to different ecological environmental factors can reduce the probability of hybridization (Mayr, 1963; Coyne and Orr, 2004; Nosil et al., 2005). The genetic-based differences in species' distribution may lead to ecogeographic at a large scale and microhabitat isolation at a small scale (reviewed in Schluter, 2001; Coyne and Orr, 2004; Rundle and Nosil, 2005; Hendry et al., 2007; Schemske, 2010; Sobel et al., 2010). Temporal isolation restricts heterospecific gene flow by differences in the timing of reproduction (e.g., Cruzan and Arnold, 1994; Martin et al., 2007). When diverging taxa reproduce at the same location and at the same time, sexual isolation

reduces hybridization by having differences in mating preference (e.g., Nagel and Schluter, 1998; Ramsey et al., 2003) and efficiency (e.g., McCartney and Lessios, 2004; Kay 2006). Postzygotic barriers act after the formation of hybrids by reducing hybrid fitness. Environment-independent hybrid unfitness may result in intrinsic postzygotic isolation that occurs as a consequence of genetic incompatibilities between the parental genomes. Extrinsic postzygotic isolation may occur when hybrids experience lower fitness dependent upon their environments.

Among all reproductive isolating barriers, intrinsic postzygotic isolation, i.e., the reduction in fertility and/or viability of hybrids, has traditionally received more attention. This is in part because it is easier to study genetic incompatibility in the laboratory than to estimate the importance of multiple ecological isolating barriers in the field (Mallet, 2006). Although strong intrinsic postzygotic isolation may restrict gene flow between species with large genetic divergence, it is unclear whether these isolating barriers evolve early or late in speciation (Schemske, 2000; Coyne and Orr, 2004). In Centrarchid fish, Bolnick and Near (2005) concluded that hybrid inviability evolved long after reproductive isolation was essentially complete, suggesting that prezygotic barriers are of primary importance. While studies of individual barriers show how the focal barriers reduce heterospecific gene flow, comprehensive comparisons among barriers between closely related species are required to determine which isolating barrier contributes most at the time of speciation.

To compare the relative importance of isolating barriers acting sequentially throughout the life history, Coyne and Orr (1989; 1997) developed the components-of-isolation method (CIM). By using this method, they concluded that prezygotic isolation evolves more rapidly than postzygotic isolation in *Drosophila*. Ramsey et al. (2003) generalized the application of the CIM and compared multiple barriers simultaneously between *Mimulus cardinalis* and *M. lewisii*. They

suggested that ecogeographic isolation and pollination fidelity are the major isolating mechanisms. Other studies that employed CIM also indicated that the postzygotic barriers play a relatively small role in speciation (e.g., Husband and Sabara, 2004; Kay, 2006; Matsubayashi and Katakura, 2009; Dopman et al., 2010). In his review of seven plant speciation studies (including the case presented here), Schemske (2010) suggested that prezygotic barriers often play the major role in speciation while intrinsic postzygotic isolation typically evolves after speciation is complete. Unfortunately, comparisons of the relative contribution among multiple isolating barriers have only been conducted in a few species pairs. Here I used CIM in two recently diverged species to identify the primary isolating barriers and to determine the primary mechanism of speciation.

Although first articulated by Mayr (1947), the relative importance of ecological isolating barriers has recently been revisited by evolutionists (reviewed by Schluter, 2001; Nosil et al., 2005; Lowry et al., 2008; Schemske, 2010; Sobel et al., 2010). By observing recently diverged species, ecological factors contribute to speciation directly and/or indirectly (Sobel et al., 2010). The direct contribution results from ecogeographic (e.g., Nagel and Schluter, 1998), microhabitat (e.g., Matsubayashi and Katakura, 2009), temporal (e.g., Dopman et al., 2010), sexual (e.g., Mendelson, 2003), or extrinsic postzygotic isolation (e.g., Hatfield and Schluter, 1999). In addition, ecological factors promote divergence indirectly through pleiotropy or linkage disequilibrium with other isolating barriers, for instance, environment-dependent postmating prezygotic isolation (Searcy and Macnair, 1990) and Dobzhansky-Muller incompatibilities (e.g., Fishman and Willis, 2001). Ecological factors generating divergent selection and resulting in local adaptation in allopatric populations were suggested to be one of the common mechanisms of speciation.

Although many studies have demonstrated how ecological factors contribute to individual isolating barriers, the relative contribution of these barriers to total isolation is not always clear. In studies of which relative contributions of multiple barriers were compared by using CIM, ecogeographic isolation is often neglected (Sobel et al., 2010). One major impediment to incorporating ecogeographic isolation in estimates of reproductive isolation comes from the difficulties in distinguishing ecogeographic isolation from effective geographic isolation (Sobel et al., 2010). While both ecological and historical factors may determine species' distribution and result in effective geographic isolation, measuring ecogeographic isolation decouples the ecological factors from the historical ones and provides a potential geographic isolation due to local adaptation (Sobel et al., 2010). As ecogeographic isolation acts as the first potential barrier in the life history (Ramsey et al., 2003), calculating contributions of other barriers without considering the effects of ecogeographic isolation could be misleading. In a review of 19 plant species pairs in which multiple barriers were measured (Lowry et al., 2008), only 2 studies (Ramsey et al., 2003; Kay, 2006) estimated effective geographic isolation; in both cases, its contribution was substantial. In a species pair examined in one of these two studies, a strong degree of ecogeographic isolation was proved in a reciprocal transplant experiment (Angert and Schemske, 2005). Having ecogeographic isolation in the comparisons of multiple isolating barriers is urged to properly estimate the role of ecology in reproductive isolation.

Although a few studies compared relative contribution of multiple isolating barriers and showed the importance of ecology in speciation, whether these studies demonstrated general patterns of speciation requires more investigation. Comparisons of multiple isolating barriers in recently diverged taxa allow us to determine the importance of different barriers at the time of speciation (Schemske, 2010). Such comparisons including barriers which are consequences of

local adaptation to ecological factors may lead to determination of whether ecology is the primary mechanism of speciation (Schemske, 2010; Sobel et al., 2010). To conduct comprehensive comparisons of isolating barriers, ecogeographic isolation must be included (Schemske, 2010; Sobel et al., 2010). Further examinations of the identification and the genetic basis of adaptive traits that are responsible for reproductive isolation rely on studies of these comprehensive comparisons (Schemske, 2010; Sobel et al., 2010). To this end, here I studied the relative contribution of multiple isolating barriers, from ecogeographic isolation to intrinsic and extrinsic postzygotic isolation, between two closely related plant species. The main questions are: 1) What are the primary isolating barriers? 2) Are prezygotic or postzygotic isolating barriers more important? 3) How much of the total isolation is due to ecological factors? To answer these questions, I studied two closely related Neotropical herbs, *Costus allenii* and *C. villosissimus*. *Costus allenii* is found along moist ravines in dense forests, while *C. villosissimus* lives in more open, drier areas. These sister taxa (Kay et al., 2005) have similar flowering times, utilize the same pollinators, and are interfertile. Therefore, I hypothesized that ecogeographic and microhabitat isolation, which act prezygotically and are driven by ecological factors, are important components of reproductive isolation between these species.

MATERIALS AND METHODS

Study System

Costus allenii and *C. villosissimus* are perennial herbs found in Neotropical rainforests (Maas, 1972). The two species are segregated by their habitats: *C. allenii* occupies along ravines in rainforest understory while *C. villosissimus* is found along the forest edges. Reciprocal transplant experiments and greenhouse assessment of leaf physiology and drought tolerance revealed that the *C. allenii* is adapted to low light availability and *C. villosissimus* is adapted to

low water availability in their home habitats (Ch. 2; Ch. 3). The two species are morphologically distinct (Maas, 1972). *Costus allenii* produces broad leaves and has brownish trichomes on stems and leaves, while *C. villosissimus* produces narrow leaves and the trichomes on stems and leaves are usually white. The inflorescences of *C. allenii* are comprised of flowers with rounded bracts and large extrafloral nectaries, while those of *C. villosissimus* are comprised of flowers with leafy bracts and small extrafloral nectaries. *Costus allenii* has cream-colored flowers with a small red-striped labellum, and *C. villosissimus* has yellow flowers with a large labellum. In spite of their floral morphological differences, both species are pollinated by euglossine bees (Ch. 1). The two species are easily crossed to generate fully fertile F1 and F2 hybrids, but only 2% of the natural populations were found to be hybrids in the study region (Ch. 2). Phylogenetic analyses of the internal and external transcribed spacer (ITS and ETS) regions of nuclear ribosomal DNA suggest that *C. allenii* and *C. villosissimus* are sister taxa (Kay et al., 2005). As the subgenus *Costus*, which comprises the majority of species in the genus *Costus*, has undergone a rapid diversification and given rise to more than 50 species in the Neotropics in just three to five million years (Kay et al., 2005), the divergence between this pair of sister taxa is very recent.

Study Sites

The field components of this study were conducted in a number of sites across the Isthmus of Panama, where both natural populations of *C. allenii* and *C. villosissimus* are found (Maas, 1972). A steep rainfall gradient has been observed in this region, from the wet Atlantic side (mean annual precipitation = 3234 mm at Cristobal) to the dry Pacific side (mean annual precipitation = 1798 mm at Balboa Heights) (Panama Canal Authority Meteorological and Hydrological Service weather station network).

Specific study sites included Pipeline Road (PLR), Gamboa, and Gigante Peninsula. PLR runs 17 km from south to north through the primary forest of Soberania National Park, parallel to the Panama Canal. The precipitation gradient observed across the Isthmus of Panama is also reflected in the soil moisture gradient along PLR (Ch.2). The southern stretch of PLR is drier with more open canopy, and, as the road progresses northward, both soil moisture and canopy cover increase. The gradient of soil moisture and light availability along PLR represent typical habitats of *C. allenii* and *C. villosissimus*, which are found in the northern and southern stretch of the road, respectively. Natural populations of both species were used to measure microhabitat, phenology, sexual isolation, and postzygotic isolation. To measure extrinsic postzygotic isolation, reciprocal transplant experiments were conducted with two sites representing habitats of each parental species: Gigante Peninsula and the northern stretch of PLR for *C. allenii*, and Gamboa and the southern stretch of PLR for *C. villosissimus* (Ch. 2).

Studied Barriers

Nine reproductive isolating barriers were included in sequential order of their life-history stages in the estimate of total isolation. Ecogeographic, microhabitat, temporal and sexual isolation are prezygotic barriers. The studied postzygotic barriers include interspecific seed set, F1 hybrid seed germination and early seedling survival in parental habitats, F1 juvenile survival in parental habitats, F1 juvenile growth in parental habitat, and F1 hybrid pollen viability. Detailed examination of microhabitat isolation, sexual isolation, F1 hybrid seed and juvenile performance in parental habitats were reported in Chapter 1 (sexual isolation) and Chapter 2 (microhabitat and F1 hybrids in parental habitats). Therefore, only summaries of the methods, findings, and interpretations of these barriers are included here.

The strength (*RI*) of each individual isolating barrier was measured independently. Because reproductive isolation may evolve asymmetrically between species, all barriers were estimated separately for *C. allenii* and *C. villosissimus*, as suggested by Kay (2006) and Martin and Willis (2007). While several studies (e.g., Coyne and Orr, 1989, 1997; Ramsey et al., 2003) used CIM to measure and to compared insolating barriers, there was no consistency in calculating the strength of isolation (Martin and Willis, 2007; Sobel and Chen, in prep.). Martin and Willis (2007) employed calculations that are comparable between both pre- and postzygotic barriers. Sobel and Chen (in prep.) further adjusted these calculations and proposed a set of equations which are comparable across barriers with a linear relationship with the frequency of heterospecific gene flow. The general format of the equations proposed by Sobel and Chen (in prep.) is

$$RI = 1 - 2 * \left(\frac{H}{C + H} \right) \quad (1)$$

where *H* represents the proportion of heterospecific gene flow and *C* represents the proportion of conspecific gene flow. This equation generates *RI* indices which have a biologically and mathematically meaningful range of -1 (complete heterospecific mating or heterosis) to 0 (no isolation) to 1 (complete isolation). The index values stand for the relative under-representation of heterospecific gene flow and the relative over-representation of conspecific gene flow in respect to expectations under random mating or equal fitness in parental species and hybrids. Therefore, all *RI* indices in this study were calculated using the procedures of Sobel and Chen (in prep.).

Ecogeographic Isolation

I determined the geographic distribution of *C. allenii* and *C. villosissimus* using ecological niche modeling (Phillips et al., 2006) with georeferenced locality data of herbarium

specimens at the Missouri Botanical Garden. Following Sobel (2010), a predicted map of suitable environmental conditions from spatial data on abiotic variables and species occurrence was built for each species using ArcGIS (version 9.2, ESRI, Redlands, CA). The map was restricted to the area of Latin America between 24°N and 8°S, which are the northern and southern distributional limits, respectively, of the two species (Maas, 1972; www.Tropicos.org, Missouri Botanical Garden). Twelve climatic variables, a mix of both temperature and precipitation variables, were selected from the dataset WORLDCLIM (www.worldclim.org) (Table 4.1). These variables, consisting of a grid of 1 km^2 resolution pixels, were used to build a ecological niche model for each species in the mapped area using Maxent software (Maxent version 3.3.3e; http://cs.princeton.edu/~sharire/maxent) (Phillips et al., 2006; Sobel, 2010).

Specimens of 31 *C. allenii* and 72 *C. villosissimus* were used to establish the Maxent models. For each species, georeferenced locality data for 75% of the specimens were randomly selected and used for training the model while the remaining 25% were used for testing. This training/testing process was run for 500 iterations. Using a threshold of 10 percentile training presence, a value of 1 was assigned to every pixel of suitable habitat and 0 was assigned to pixels of unsuitable habitats for each species. The predicted maps of suitable habitats of the two species were then superimposed. The number of pixels predicted as suitable only for *C. allenii* was used as the unshared area for *C. allenii*, the number of pixels suitable only for *C. villosissimus* was used as the unshared area for *C. villosissimus*, and the number of pixels predicted as suitable for both species was used as the shared area. Ecogeographic isolation index (RI_{ecogeo}) was computed as:

$$RI_{ecogeo} = 1 - \frac{\text{shared area}}{\text{shared area} + \text{unshared area}} \quad (2)$$

for each species (Sobel and Chen, in prep).

The relative contribution of each climatic variable to the predicted distribution for each species was determined by Maxent software (Maxent version 3.3.3e; http://cs.princeton.edu/~sharire/maxent). For the three variables that contributed most to the Maxent model of each species, their differences were compared between species to determine whether these variables also contributed to the differences of the distribution between the two species. Values of these variables at the location of each specimen were compared between species with t-test.

Microhabitat Isolation

The estimate of microhabitat isolation was presented in Chapter 2, and here I summarize the approach. In central Panama, where the two species co-occur, microhabitat isolation was measured from species distribution data collected along a 17 km transect on PLR. The spatial distributions of both species were determined by censusing individual flowering plants of the two species in the vicinity of PLR from March 2006 to July 2009. The numbers of flowering *C. allenii* and *C. villosissimus* individuals located within each kilometer section of PLR were counted and the distributions of the two species were compared using a Kolmogorov-Smirnov test.

Following Sobel and Chen (in prep), $RI_{habitat}$ was calculated as

$$RI_{habitat} = 1 - 2\sum_i \left(\frac{Sp1_i}{Sp1_{total}} \times \frac{Sp2_i}{Sp1_i + Sp2_i}\right) \quad (3)$$

for each species, with $Sp1_i$ and $Sp2_i$ representing the number of individuals of the focal species and the other species found in kilometer section i, respectively, while $Sp1_{total}$ and $Sp2_{total}$ are,

respectively, the total number of plants of the focal species and the other species found along PLR. See detailed methods of measuring microhabitat isolation in Chapter 2.

Temporal Isolation

Each *Costus* terminal inflorescence typically produces one flower per day which opens at dawn and wilts by mid-afternoon. This one-flower-per-day pattern was used to estimate the flowering span of individual plants. I marked the bract which had the current day's flower and recorded the date. The plants were examined approximately two weeks later to count bracts between the current flower and the marked bract. The flowering rate of each inflorescence was thus estimated. This measurement was repeated every two weeks for the whole flowering season. The number of bracts below the first marked flower and above the last examined flower was counted to determine the start and end dates of flowering for the inflorescence by multiplying the number of flowers by the flowering rate. Flowering periods of 10 *C. allenii* and 19 *C. villosissimus* inflorescences from natural populations on PLR were estimated in 2007, and those of 26 *C. allenii* and 56 *C. villosissimus* inflorescences were estimated in 2008. For each species, the data were plotted as the proportion of flowers that opened on each day across the whole flowering season. The Julian dates representing the midpoint of individual inflorescence flowering periods were compared between species. This comparison in flowering phenology between species was conducted yearly with a Wilcoxon rank sum test.

The temporal isolation index ($RI_{temporal}$) of each species was computed yearly as

$$RI_{temporal} = 1 - \frac{2 \times \frac{\sum_i \left(\frac{Sp1_i}{Sp1_{total}} \times \frac{Sp2_i}{Sp1_i + Sp2_i} \right)}{Sp2_{total} / (Sp1_{total} + Sp2_{total})}}{\frac{\sum_i \left(\frac{Sp1_i}{Sp1_{total}} \times \frac{Sp1_i}{Sp1_i + Sp2_i} \right)}{Sp1_{total} / (Sp1_{total} + Sp2_{total})} + \frac{\sum_i \left(\frac{Sp1_i}{Sp1_{total}} \times \frac{Sp2_i}{Sp1_i + Sp2_i} \right)}{Sp2_{total} / (Sp1_{total} + Sp2_{total})}} \quad (4)$$

for each species (Sobel and Chen, in prep). In this equation, $Sp1_i$ and $Sp2_i$ represent the number of flowers that opened on day *i* for the focal species and the other species, respectively, while $Sp1_{total}$ and $SP2_{total}$ represent the total number of flowers of the focal species and the other species produced throughout each season, respectively. This approach was originally developed by Martin and Willis (2007) in their studies of reproductive isolation between *Mimulus guttatus* and *M. nasutus*. While their equations represent reproductive isolation as 1 – (proportion of heterospecific gene flow / proportion of conspecific gene flow), Sobel and Chen (in prep.) modified the equations to fit a unified calculation of reproductive isolation, i.e., equation (1) in this chapter. This equation gives an accurate estimate of temporal isolation, which considered both the duration of the flowering period and the frequency distribution of the flowers during this period for each species. $RI_{temporal}$ of 2007 and 2008 were averaged to generate a single estimate of the strength of temporal isolation for each species.

Sexual Isolation

The estimate of sexual isolation was presented in Chapter 1. In summary, sexual isolation was measured by the proportion of hybrid formation in naturally-pollinated fruits in a pollination array in June and July 2008. This estimate includes isolation due to pollinator isolation, floral mechanical isolation, and gametic isolation (Ch. 1). The pollination array was located on PLR close to the edges of the natural distributions of the two species (approximately 6.9 km north of the road entrance near Gamboa). The pollination array consisted of multiple plants of each species and a total of two flowers per species per day. The flowers were naturally pollinated. Mature fruits were collected in late September, and the seeds were sown in potting soil (High Porosity Professional Mix, Baccto) shortly thereafter in the greenhouse at Michigan State

University. Eighty-one seedlings from 15 *C. allenii* fruits and 131 seedlings from 19 *C. villosissimus* fruits were genotyped with species-specific AFLP markers to determine the proportion of hybrid seedlings in each fruit. The strength of sexual isolation (RI_{sexual}) was calculated with Equation (1) (Sobel and Chen, in prep.), in which the proportion of hetero- (H) and conspecific gene flow (C) were represented by the proportion of hybrid and parental seedings. See detailed descriptions of the pollination array, greenhouse setting, and the methods of analysis of hybrid seedling formation in Chapter 1.

Postzygotic Isolation

Five sequential barriers of postzygotic isolation were measured in this study: interspecific seed set, F1 hybrid seed germination and survival in parental habitats, F1 hybrid juvenile survival in parental habitats, F1 hybrid juvenile growth in parental habitats, and F1 hybrid pollen viability. Hybrid inviability was measured in the first four barriers, while hybrid sterility was measured as the reduction in pollen viability. In reciprocal transplant experiments with both parents and hybrids, seed germination and survival, juvenile survival, and juvenile growth of hybrids were compared to those of parental species growing in the home habitats. These fitness components are thereafter referred to as transplant performance in parental habitats. Reduction in hybrid fecundity (number of inflorescences, flowers, or seeds) was not included as a barrier here. Assessing plant fecundity was not applicable in the time frame of this study because *Costus* plants are perennials which take at least two years to mature and flower once a year for decades. For the same reason, hybrid unfitness was not measured in backcrosses or later generations, either. Among the five studied postzygotic barriers, seed set and pollen viability represent intrinsic barriers while transplant performance in parental habitats represent extrinsic barriers.

Interspecific Seed Set

I compared seed set of heterospecific crosses with that of conspecific crosses. Four categories of crosses were made: the two intraspecific crosses, *C. allenii* pollinated with *C. allenii* and *C. villosissimus* pollinated with *C. villosissimus*, and two reciprocal interspecific crosses between the parents, with either *C. allenii* as the female and *C. villosissimus* as male, or vice versa. The progeny produced by these two intraspecific crosses and two interspecific crosses are thereafter referred to as "the four categories" or as *C. allenii*, *C. villosissimus*, $F1_{allenii}$, and $F1_{villo}$, respectively. The crosses were made using plants from natural populations along PLR. The parental plants included 9 *C. allenii* and 7 *C. villosissimus* in 2006, 5 *C. allenii* and 6 *C. villosissimus* in 2007, and 7 *C. allenii* and 3 *C. villosissimus* in 2008. A total of 59 *C. allenii* fruits, 66 $F1_{allenii}$ fruits, 45 $F1_{villo}$ fruits, and 50 *C. villosissimus* fruits were produced across 3 years by hand pollination. The flowers were bagged before and after being hand pollinated to prevent natural pollination. Pollen collected from a flower was applied to the stigma with a flat toothpick.

Mature fruits were collected in October and the seeds in each fruit were counted. For each parental plant, the number of seeds per fruit was averaged separately for intraspecific cross and for interspecific cross. These averages are referred to as the seed sets of a given cross for each parental plant. The seed sets were compared among years, between species of the maternal parent, and between species of the paternal parent using a multi-factorial ANOVA (seed set = μ + year + maternal species + paternal species + maternal species*paternal species + year*maternal species*paternal species + ε) and Tukey's HSD. In the ANOVA model, the interactive term of maternal species and paternal species represented interactions between parental species' genomes (Kay, 2006).

Because there was no interaction between year and the interactive term of maternal and paternal species (see results below), I combined data of three years to estimate the strength of postzygotic isolation at the seed production stage. Equation (1) was used to calculate the strength of isolation ($RI_{seedset}$) due to differences in seed set between parental and hybrid fruits (Sobel and Chen, in prep.). For *C. allenii*, the proportion of hetero- (*H*) and conspecific gene flow (*C*) in the calculation of $RI_{seedset}$ were represented by the mean seed set of $F1_{allenii}$ and *C. allenii* fruits, respectively. For *C. villosissimus*, *H* and *C* were represented by the mean seed set of $F1_{villo}$ and *C. villosissimus* fruits, respectively.

For sexual isolation and isolation at the seed set stage, interspecific seed set and the proportion of hybrid offspring were compared to intraspecific seed set and the proportion of parental offspring, i.e., comparisons between $F1_{allenii}$ and *C. allenii* or between $F1_{villo}$ and *C. villosissimus*). These comparisons estimate the differences between inter- and intraspecific crosses within the same maternal plants.

F1 Hybrid Seed Germination and Early Seedling Survival in Parental Habitats

The estimate of F1 hybrid seed germination and early seedling survival in parental habitats was described in Chapter 2-- a summary of the approach is presented here. A reciprocal transplant experiment with seeds of *C. allenii*, *C. villosissimus*, $F1_{allenii}$, and $F1_{villo}$ was conducted in *C. allenii* (AP) and *C. villosissimus* habitats (VP) on PLR in both 2007 and 2008. Fifty-five seeds per category per site were transplanted each year. Because the proportion of germinated seeds surviving was low and the patterns were similar between years, data of the two years were combined to increase statistical power. The average number of F1 hybrid seeds that germinated and survived (after one year) was compared to that of the parental species

transplanted into its home habitat using a G-test of goodness of fit. Equation (1) was used to calculate the strength of isolation (RI_{seed}) due to differences in germination and early survival between parental and hybrid seeds (Sobel and Chen, in prep.). The frequency of conspecific gene flow (C) was estimated as the proportion of seeds of parental species that germinated and survived after one year in its home habitats, and the frequency of heterospecific gene flow (H) was estimated as the average proportion of hybrid seeds that germinated and survived after one year in the same parental habitat. See detailed description of the reciprocal transplant experiment and the calculation of RI_{seed} in Chapter 2.

F1 Hybrid Juvenile Survival in Parental Habitats

The estimate of F1 hybrid survival for the juvenile stage was presented in Chapter 2, and is summarized here. A reciprocal transplant experiment initiated with cuttings of mature plants of the four categories was conducted in *C. allenii* (AP and AG) and *C. villosissimus* habitats (VP and VG) from 2006 to 2010. These cuttings were at juvenile stages when they were transplanted into parental habitats. Because survival was low in each category at each site, data from the two sites representing the same parental habitats were combined to increase statistical power. Survival from the time of transplanting to June 2010 was calculated for each category in each habitat. Within each parental habitat, the average number of $F1_{allenii}$ and $F1_{villo}$ transplants survived was compared to that of the parental transplants in its home habitat using a G-test of goodness of fit. Equation (1) was used to calculate the strength of isolation (RI_{juvsur}) due to differences in juvenile survival between parents and hybrids (Sobel and Chen, in prep.). The frequency of conspecific gene flow (C) was estimated as the proportion of parental species surviving in its home habitat, and the frequency of heterospecific gene flow (H) was estimated as

the average proportion of $F1_{allenii}$ and $F1_{villo}$ transplants surviving in the same parental habitat. See detailed description of the reciprocal transplant experiment and the estimate of transplant survival in Chapter 2.

F1 Hybrid Juvenile Growth in Parental Habitats

The estimate of F1 hybrid growth for the juvenile stage was presented in Chapter 2, and is summarized here. In the reciprocal transplant experiment of juveniles, growth of the surviving transplants was measured for each category in each habitat. Plant growth was calculated as the proportional change in the number of leaves from the time of transplanting to June 2010 for the surviving plants. Because survival was low in each category at each site, data from the two sites representing the same parental habitats were combined to increase statistical power. Within each parental habitat, the average growth of $F1_{allenii}$ and $F1_{villo}$ transplants was compared to that of the parental transplants in its home habitat using an ANOVA. Equation (1) was used to calculate the strength of isolation ($RI_{juvgrow}$) due to differences in juvenile growth between parents and hybrids (Sobel and Chen, in prep.). The frequency of conspecific gene flow (C) was estimated as the growth of parental transplants in its home habitat, and the frequency of heterospecific gene flow (H) was estimated as the average growth of $F1_{allenii}$ and $F1_{villo}$ transplants in the same parental habitat. See detailed description of the reciprocal transplant experiment and the estimate of transplant growth in Chapter 2.

F1 Hybrid Pollen Viability

To compare male fertility between hybrids and parental species, I examined pollen tube germination, a common measure of pollen viability, for the four categories of plants. The plants were grown from seed in the greenhouse at Michigan State University. See descriptions of

greenhouse setting in Chapter 3. From May to September 2009, 8 *C. allenii*, 12 $F1_{allenii}$, 17 $F1_{villo}$, and 13 *C. villosissimus* plants flowered in the greenhouse. Fresh pollen was collected from three to five flowers of each plant in the early morning. Pollen of each flower was then immediately placed in a microcentrifuge tube with 200 µl of pollen tube growth medium (15% sucrose, 0.03% calcium nitrate, and 0.02% boric acid in distilled water, modified from the formula used in Schemske and Fenster, 1983). After incubation at room temperature in the growth medium for at least 2 hours, approximately 80µl of the pollen/growth medium mix was placed on a microscope slide with a cover slip. For each flower, 200 pollen grains were counted and the proportion of germinating pollen was recorded. Pollen was scored as viable when a pollen tube had elongated outside of the circular pollen grain and when the tail was longer than the diameter of the grain itself.

The proportion of viable pollen was averaged among flowers within a plant. Averages were compared among the four categories using an ANOVA. Equation (1) was used to calculate the strength of isolation (RI_{pollen}) due to differences in pollen viability between parental and hybrid flowers (Sobel and Chen, in prep.). The frequency of conspecific gene flow (*C*) was estimated as the average proportion of viable pollen in flowers of the parental species, and the frequency of heterospecific gene flow (*H*) was estimated as the mean of the average for both $F1_{allenii}$ and $F1_{villo}$ flowers.

The fitness components of $F1_{allenii}$ and $F1_{villo}$ were averaged and then compared to those of a parental species for the last four postzygotic barriers, F1 seed germination and survival in parental habitats, F1 juvenile survival in parental habitats, F1 juvenile growth in parental habitats, and F1 hybrid pollen viability. Because progenies of both directions of reciprocal crosses

potentially occur in the parental habitats and mate with the parental species, these comparisons represent the differences between parental species and hybrids regardless the direction of reciprocal crosses.

Hybrid Frequency in Suitable Habitats for Both Species

The frequency of flowering hybrids in the suitable habitats for both species predicted in the ecological niche models was examined in Chapter 2. In summary, plants with large yellow flowers with red stripes were identified as hybrids. These hybrids also had phenotypes of leafy bract and extrafloral nectary that were intermediates of the phenotypes of the two parental species. These intermediate phenotypes observed in natural hybrids were consistent with those observed in greenhouse-bred F1 hybrids. I located all flowering plants, including parental species and hybrids, in the vicinity of PLR from 2006 to 2009. I estimated the frequency of hybrids as the number of flowering hybrids divided by the sum of the number of flowering parental species and hybrids in this region.

Total Reproductive Isolation

Total reproductive isolation was estimated using a multiplicative function of the individual isolating barriers for each species as suggested by Sobel and Chen (in prep.). The product of the proportion of hetero- (H) and conspecific gene flow (C) in each isolating barrier was calculated to represent the overall proportion of hetero- and conspecific gene flow through sequential stages in the life history. Total reproductive isolation (T), which also varies from -1 to 0 to 1, is:

$$T = 1 - \frac{2 \times \prod H_i}{\prod C_i + \prod H_i} \qquad (5)$$

(Sobel and Chen, in prep.), where the subscripted *i* denotes the order of an individual barrier: 1 for ecogeographic isolation, 2 for microhabitat isolation, 3 for temporal isolation, 4 for sexual isolation, 5 for seed set, 6 for seed fitness in parental habitats, 7 for juvenile survival in parental habitats, 8 for juvenile growth in parental habitats, and 9 for pollen viability.

For isolating barriers affecting co-occurrence of the two species, i.e., ecogeographic isolation, microhabitat isolation, and temporal isolation, the proportion of hetero- (*H*) and conspecific gene flow (*C*) was not directly assessed in the methods described above. To include these barriers in the calculation of total isolation, *H* and *C* of these barriers were calculated from their estimated *RI* values:

$$H = \frac{1 - RI}{2} \tag{6}$$

and

$$C = 1 - H \tag{7}.$$

To dissect the individual contribution of each isolating barrier to total isolation (*T*), the absolute contribution (*AC*) of each barrier was calculated as

$$AC_i = \frac{2 \times \prod H_{i-1}}{\prod C_{i-1} + \prod H_{i-1}} - \frac{2 \times \prod H_i}{\prod C_i + \prod H_i} \tag{8}$$

(Sobel and Chen, in prep.), where the subscripted *i* denotes the order of an individual barrier as described above. Because ecogeographic isolation was the first-acting barrier measured in this study, AC_{ecogeo} was set to be the same as RI_{ecogeo} as suggested by Ramsey et al. (2003).

The relative contribution (*RC*) of an isolating barrier presents the relative influence of the given barrier to total isolation. For barrier *i*, the relative contribution is:

$$RC_i = \frac{AC_i}{T} \tag{9}$$

(Ramsey et al., 2003; Sobel and Chen, in prep). A greater *RC* value indicates a greater contribution to total isolation, and the barrier with the greatest *RC* was identified as the primary isolating barrier. When total isolation is almost complete, a *RC* value is almost identical to the *AC* value of a given barrier. When total isolation is not complete, a *RC* value represents the proportion of total isolation that is due to a given barrier.

I also estimated reproductive isolation from hybrid frequency in suitable habitats for both species, substituting hybrid frequency for the multiplicative effects of microhabitat isolation, temporal isolation, sexual isolation, interspecific seed set, F1 seed germination and survival, and F1 juvenile survival and growth. This was based on the assumption that all hybrids found in nature were F1s. The values of *H*, *C*, and *RI* for ecogeographic isolation and F1 pollen viability were averaged between the two species. Estimates of total reproductive isolation (*T*) and the corresponding *AC* and *RC* were computed with the averaged ecogeographic isolation, hybrid frequency in suitable habitats for both species, and F1 pollen viability.

RESULTS

Ecogeographic Isolation

The distribution of *C. villosissimus* is different from that of *C. allenii*. The herbarium specimens of *C. allenii* indicated that this species is found in Costa Rica, Panama, and Columbia while those of *C. villosissimus* indicated that this species is found in Costa Rica, Panama, Columbia, Mexico, Ecuador, Nicaragua, and Venezuela. Ecological niche models of the two species predicted that the suitable habitats of *C. allenii* and *C. villosissimus* included 68,596 and 1,136,490 pixels, respectively, which were slightly larger than the distribution of the herbarium specimens (Fig. 4.1). The shared area which was suitable for both species included 58,827 pixels (Fig. 4.1). Thus, RI_{ecogeo} was 0.142 for *C. allenii* and 0.948 for *C. villosissimus*.

Climatic variables contributing to the niche models were similar in the two species. The three variables contributing the most to the model for the distribution of *C. allenii* were mean temperature diurnal range (BIO2, 42%), precipitation of warmest quarter (BIO18, 13%), and precipitation of the wettest quarter (BIO16, 12%). The three climatic variables contributing the most to the model of *C. villosissimus* were mean temperature diurnal range (BIO2, 34%), precipitation of driest quarter (BIO17, 17%), and precipitation of the warmest quarter (BIO18, 16%). The mean temperature diurnal range (BIO2) of *C. villosissimus* (mean ± 95% CI = 84.69 ± 3.39) was significantly higher than that of *C. allenii* (77.65 ± 3.69; $t = 2.83$, $p = 0.006$). The precipitation of the wettest quarter (BIO16) of *C. allenii* (1300.03 ± 106.92) was significantly higher than that of *C. villosissimus* (1052.19 ± 73.07; $t = 3.86$, $p < 0.001$). There was no differences in precipitation of the driest quarter (BIO17) between *C. allenii* (194.73 ± 97.53) and *C. villosissimus* (125.92 ± 29.45; $t = 1.37$, $p = 0.18$). There was no difference in precipitation of the warmest quarter (BIO18) between *C. allenii* (514.38 ± 87.84) and *C. villosissimus* (489.09 ± 54.45; $t = 0.49$, $p = 0.62$).

Microhabitat Isolation

As described in the results of Chapter 2, there was a significant difference in the distribution of flowering *C. allenii* and *C. villosissimus* ($D = 0.65$, $p = 0.001$). I located 93 flowering *C. allenii* and 156 flowering *C. villosissimus* in the vicinity of PLR. Most *C. allenii* plants were found on the northern stretch of the road, where soil moisture is relatively high and the canopy is closed. Most *C. villosissimus* plants were found on the southern stretch of the road where soil moisture is lower and the canopy is more open. $RI_{habitat}$ of *C. allenii* was 0.886 and that of *C. villosissimus* was 0.932 (Ch. 2).

Temporal Isolation

Both *C. allenii* and *C. villosissimus* flower in the early wet season. In 2007, the mean estimated flowering period for *C. allenii* was from June 20 to July 27, while it for *C. villosissimus* was from June 22 to July 19. The Julian dates of the midpoint of the flowering periods showed no difference between species, indicating that the two species essentially flowered at the same time (W = 106.5, p = 0.61). $RI_{temporal}$ was 0.028 for *C. allenii* and 0.019 for *C. villosissimus* (Fig. 4.2A). In 2008, the mean estimated flowering period for *C. allenii* was from June 28 to August 6, while it for *C. villosissimus* was from June 30 to August 1. As was the case in 2007, the Julian dates of the midpoint of the 2008 flowering periods were not different (W = 803.5, p = 0.4547, Fig. 4.2B). In 2008, $RI_{temporal}$ was 0.061 for *C. allenii* and 0.035 for *C. villosissimus*. The average $RI_{temporal}$ of 2007 and 2008 was 0.045 for *C. allenii* and 0.027 for *C. villosissimus*.

Sexual Isolation

Sexual isolation was observed in *C. villosissimus*, but not in *C. allenii*. As described in Chapter 1, the average proportion (± 95% CI) of hybrid formation following natural pollination in the array was 0.44 ± 0.22 for *C. allenii* (n = 15 fruits), which was not significantly lower than the null expectation of 0.50. Thus, sexual isolating barriers did not reduce heterospecific gene flow from *C. villosissimus* to *C. allenii*. In contrast, there was a significant reduction in the proportion of hybrid seedling formation observed for *C. villosissimus*, with an average proportion of hybrid formation in the array of 0.21 ± 0.14 (n = 19 fruits). RI_{sexual} was estimated as 0.121 for *C. allenii* and 0.581 for *C. villosissimus*.

Postzygotic Isolation

Interspecific Seed Set

A total of 9,571 seeds from the experimental crosses were collected and counted from 2006 to 2008. Seed sets differed among years ($p < 0.001$): more seeds were produced per fruit in 2007 (58.44 ± 9.40) than in 2008 (43.10 ± 8.35) and 2006 (33.63 ± 6.06). Seed sets were higher in fruits produced by *C. villosissimus* plants (58.22 ± 7.76) than in fruits produced by *C. allenii* plants (32.40 ± 3.71; $p < 0.001$). There was no significant difference in seed set between fruits with different paternal parents ($p = 0.21$), and no significant interaction between year and category ($p = 0.11$).

For all fruit categories taken together, i.e., intra-and interspecific crosses, seed set differed significantly among categories ($p = 0.01$; Fig. 4.3), indicating incompatibility between species. For *C. villosissimus* as the maternal parent, seed set was lower in interspecific crosses (51.67 ± 10.85) than in intraspecific crosses (64.76 ± 11.32; $p = 0.03$). For *C. allenii* as the maternal parent, seed set of interspecific crosses (30.78 ± 4.94) was similar to seed set of intraspecific crosses (34.01 ± 5.90). Therefore, there was significant isolation in *C. villosissimus* ($RI_{seedset} = 0.112$) but an insignificant isolation of *C. allenii* ($RI_{seedset} = 0.050$).

F1 Hybrid Seed Germination and Early Seedling Survival in Parental Habitats

As described in Chapter 2, reduction in hybrid seed germination and survival was found in *C. villosissimus* habitats. The average number of $F1_{allenii}$ and $F1_{villo}$ seeds (5.5) that germinated and also survived was lower than the number of *C. villosissimus* seeds (15) that germinated and also survived in *C. villosissimus* habitat ($G = 4.58$, $p = 0.03$). However, the averaged germination and survival of hybrid seeds (7) was similar to that of *C. allenii* (8) in *C. allenii* habitat ($G = 0.07$, $p = 0.80$). RI_{seed} of *C. allenii* was calculated as 0.067 and that of *C.*

villosissimus was 0.463. The results of the G tests combined with RI_{seed} values indicated that postzygotic isolation at the seed stage in F1 hybrids was significant in *C. villosissimus* habitat but not in *C. allenii* (Ch.2).

F1 Hybrid Juvenile Survival in Parental Habitats

As described in Chapter 2, there was no reduction in hybrid juvenile survival in parental habitats. The average proportion of F1 hybrids surviving was similar to the proportion of the *C. allenii* transplants surviving in *C. allenii* habitats ($G = 0.61$, $p = 0.43$), and was also similar to that of the *C. villosissimus* transplants in *C. villosissimus* habitats ($G = 2.03$, $p = 0.15$; Ch. 2). RI_{juvsur} of *C. allenii* was -0.129 and that of *C. villosissimus* was -0.240.

F1 Hybrid Juvenile Growth in Parental Habitats

As described in Chapter 2, there was no reduction in hybrid juvenile growth in parental habitats. In *C. allenii* habitats, the growth of the surviving F1 hybrids was similar to that of surviving *C. allenii* ($p = 0.81$). In contrast, the growth of surviving F1 hybrids was significantly greater than that of *C. villosissimus* in *C. villosissimus* habitats ($p = 0.03$). Therefore, $RI_{juvgrow}$ of *C. allenii* was -0.014 and that of *C. villosissimus* was -0.493.

F1 Hybrid Pollen Viability

The average proportion of viable pollen was 0.35 ± 0.10 for *C. allenii*, 0.42 ± 0.10 for $F1_{allenii}$, 0.38 ± 0.09 for $F1_{villo}$, and 0.47 ± 0.09 for *C. villosissimus* (Fig. 4.4). There was no significant difference among categories ($p = 0.39$). Isolation was not significant for either *C. allenii* ($RI_{pollen} = -0.069$) or *C. villosissimus* ($RI_{pollen} = 0.074$).

Hybrid Frequency in Suitable Habitats for Both Species

Five flowering hybrids were found in the vicinity of PLR (Ch. 2). Given that a total of 249 flowering plants were observed in this region, the hybrid frequency was estimated as 2%.

Total Reproductive Isolation

Total isolation was higher in *C. villosissimus* (T = 0.999; Table 4.2) than in *C. allenii* (T = 0.924). The high value of total isolation in *C .villosissimus* indicates that it was almost completely isolated from *C. allenii*. In contrast, there remains a reasonably high probability of gene flow from *C. villosissimus* to *C. allenii*. For each species, prezygotic barriers contributed much more than postzygotic barriers (Fig. 4.5A). Specifically, ecogeographic and microhabitat isolation are the two strongest barriers in both species (Table 4.2), contributing 15.37% and 83.46%, respectively, to the total isolation in *C. allenii*, and 94.85% and 5.02%, respectively to the total isolation in *C. villosissimus* (Table 4.2; Fig. 4.5A). Because ecogeographic isolation and microhabitat isolation represented spatial isolation due to local adaptation to different habitats, the strong isolation due to these barriers indicates that habitat differentiation was is the most important isolating mechanism between *C. allenii* and *C. villosissimus*.

The observed frequency of flowering hybrids in the natural populations of the two species (2%) was similar to the expected frequency calculated multiplicatively with the estimates of individual barriers from microhabitat isolation to F1 hybrid juvenile growth in parental habitats (2.55%). When the barriers from microhabitat isolation to F1 juvenile growth were replaced with hybrid frequency in suitable habitats for both species, the average total isolation was estimated to be 0.988. In this estimate, ecogeographic isolation and hybrid frequency in suitable habitats for both species contributed 55.2% and 44.8% to total isolation (Fig. 4.5B). Therefore, the two species are highly isolated, mainly due to their ecogeographic differences.

DISCUSSION

Ecogeographic Isolation

Local adaptation to different environments often causes differences in species' distributions and thus leads to spatial isolation (Mayr, 1947). Most speciation studies have been conducted in regions of sympatry, without considering the effects of geographic isolation. By using ecological niche modeling, ecogeographic isolation can be distinguished from effective geographic isolation caused by historical factors, and can be estimated and incorporated into reproductive isolating measures (Sobel et al., 2010). I found that ecogeographic isolation for *C. villosissimus* (RI_{ecogeo} = 0.948) was considerably stronger than that for *C. allenii* (RI_{ecogeo} = 0.142). Because suitable habitats for *C. villosissimus* are more widespread than those of *C. allenii*, most suitable habitats for *C. villosissimus* are not suitable for *C. allenii*. Therefore, the likelihood of *C. villosissimus* being in proximity to *C. allenii* is low, as reflected in the high RI_{ecogeo} value. In contrast, most suitable habitats for *C. allenii* are also suitable for *C. villosissimus*, suggesting a higher likelihood of *C. allenii* being in proximity to *C. villosissimus*. From results of this experiment, I conclude that ecogeographic isolation is strong and asymmetrical between *C. villosissimus* and *C. allenii*.

Mean diurnal temperature was the best predictor of the ecogeographic distribution of both species, and was significantly different between the two species. Large diurnal temperature range is associated with low humidity and high daily solar radiation (Bristow and Campbell, 1984). A larger diurnal temperature range associating with a larger diurnal humidity range and higher solar radiation were found along forest edges in comparison to adjacent forests (Chen et al., 1993). Therefore, diurnal temperature range may be a determinant of the distribution of *C. allenii*

and *C. villosissimus* because these species are found in moist forest and along forest edges, respectively.

In addition to diurnal temperature range, precipitation in the warmest and wettest quarter contributes to the niche model of *C. allenii*, while precipitation in the driest and warmest quarter plays an important role in predicting the distribution of *C. villosissimus*. Precipitation differs significantly in the wettest quarter, but not in the driest nor in the warmest quarters between the locations of herbarium specimens of *C. allenii* and those of *C. villosissimus*. However, this is not consistent with the comparisons with precipitation and soil moisture directly measured in the habitats of *C. allenii* and *C. villosissimus* (Ch. 2). Specifically, *C. allenii* habitats have significantly higher precipitation and higher soil moisture than *C. villosissimus* habitats in the dry season (Ch. 2; Ch. 3). Along PLR, dry season soil moisture is highly correlated with the distribution of the two species (Ch. 2). Although the resolution of the variables available in dataset WORLDCLIM was 1 km^2, I suspect that the measurements of these variables were not as accurate as the direct measurements conducted in the natural populations of the two species. Nevertheless, ecogeographic isolation is associated with different water and light availability in the habitats of *C. allenii* and *C. villosissimus*. Therefore, I suggest that adaptation to different water and light availabilities causes habitat differentiation and leads to ecogeographic isolation between *C. allenii* and *C. villosissimus*.

There are two potential caveats of using ecological niche modeling to assess ecogeographic isolation. One big limitation is the availability of environmental variables. Although the WORLDCLIM database consists of 19 climatic variables of temperature and precipitation, other environmental variables that may be important, such as edaphic composition and biotic factors, are not available. Edaphic composition has been suggested as one major

determinant of distribution of Neotropical tree species (Fine et al., 2005). Plant distribution may also be affected by the distribution of their pollinators, seed dispersers, and symbiotic microorganisms. The lack of edaphic layers and biotic layers may reduce the accuracy of the prediction of species distribution.

Another potential problem is the level of resolution of environmental variables. I used a resolution of 1 km^2 to examine the distribution of the two study species, yet environmental variables may vary within a grid of 1 km^2. As a consequence, species may be spatially differentiated over a small spatial scale and show little ecogeographic isolation. Compared to a finer resolution, a grid of 1 km^2 cells in the model may overestimate the range of suitable habitats for each species alone and for both species. Therefore, the strength of ecogeographic isolation may be underestimated due to the limitation of the resolution of environmental variables. Detailed studies of microhabitat isolation conducted at a finer scale are needed to improve the predictions of ecological niche modeling. Nevertheless, for *C. allenii* and *C. villosissimus*, the resolution of 1 km^2 may be sufficient because their shared pollinators, euglossine bees, are able to fly long distances (Janzen, 1971; Dressler, 1982; Wikelski et al., 2010). The distance of pollen dispersal, in which the frequency of hybrid formation is quantified in natural populations, is required to determine the proper resolution of environmental variables to establish ecological niche models.

Microhabitat Isolation

Costus allenii and *C. villosissimus* display a parapatric distribution along PLR. The high values of $RI_{habitat}$ ($RI_{habitat}$ = 0.886 for *C. allenii*; $RI_{habitat}$ = 0.932 for *C. villosissimus*) indicate that microhabitat isolation is strong. The reciprocal transplant experiments conducted on these species provided evidence of local adaptation (Ch. 2). Therefore, the high values of

$RI_{habitat}$ are caused by local adaptation to different environmental factors in the natural habitats of the two species. As soil moisture and light availability were significantly different between parental habitats, these environmental factors are suggested to be responsible for local adaptation (Ch. 3). In addition, two traits which are associated with light and water availability were different between species. Leaf mass per area, a composite physiological trait, was larger in *C. allenii*, suggesting that *C. allenii* is adapted to low light environment. Higher drought tolerance was found in *C. villosissimus*, suggesting that *C. villosissimus* is adapted to low soil moisture (Ch. 3). Therefore, leaf mass per area and drought tolerance are putative adaptive traits that contribute to the evolution microhabitat isolation. Further studies of natural selection experiments and QTL mapping will be required to determine the effects of these traits on reproductive isolation and to investigate their genetic basis.

Temporal Isolation

Both *C. allenii* and *C. villosissimus* flower in the early wet season, mostly in June and July. In comparison with the flowering phenology in 2008, the length of the flowering period seems to be shorter and the frequency distribution does not present as a normal distribution in 2007. This may be due to the smaller sample size examined in 2007. By averaging the results of the two years, flowering phenology was not different between *C. allenii* and *C. villosissimus*. In addition, reproductive isolation due to this temporal barrier was not significant.

Temporal isolation was estimated by incorporating the number of flowers that opened on each day for each species ($Sp1_i$ and $Sp2_i$) and the total number of flowers of each species($Sp1_{total}$ and $Sp2_{total}$), as suggested by Sobel and Chen (in prep.). This approach was originally developed by Martin and Willis (2007), who found strong temporal isolation between

M*imulus guttatus* and *M. nasutus* (Martin and Willis, 2007). In contrast, there was no evidence of temporal isolation between *C. allenii* and *C. villosissimus*. Of note, flowering phenology of the two species was only measured along PLR. It is likely that different environmental factors in other localities may change flowering phenology and result in variation in the estimate of temporal isolation (Sobel, 2010). However, given the insignificant isolation in the highly contrasting environments of *C. allenii* and *C. villosissimus* along PLR, I suspect that temporal isolation between the two species is low across their geographic ranges.

Sexual Isolation

While both *C. allenii* and *C. villosissimus* are pollinated by euglossine bees, there is substantial sexual isolation in *C. villosissimus* (RI_{sexual} = 0.581) but not in *C. allenii* (RI_{sexual} = 0.121; Ch1). In the pollination array, pollinators preferred *C. villosissimus* but displayed low floral constancy (Ch. 1). Significant sexual isolation in *C. villosissimus* was largely due to pollinator and gametic isolation but not floral mechanical isolation (Ch. 1). The strong preference for *C. villosissimus* increased the likelihood of intraspecific crosses among *C. villosissimus* flowers. Despite that floral mechanical and gametic isolation decreased heterospecific gene flow, sexual isolation was not significant in *C. allenii* (Ch. 1).

I estimated sexual isolation by the proportion of hybrid formation. This measurement not only represented the effects of pollinators, floral mechanical structures, and pollen-pistil interactions on hybrid formation, it also included the potential differences between parents and hybrids in seed set, seed germination and seedling survival. It is difficult to measure sexual isolation independent of these early-acting intrinsic postzygotic barriers. While intrinsic postzygotic isolation was weak between *C. allenii* and *C. villosissimus* (see results above), sexual isolation measured in Chapter 1 was largely due to prezygotic but not postzygotic barriers.

Interspecific Seed Set

Reduction in Interspecific seed set can be viewed as an intrinsic postmating, mostly postzygotic, barrier which acts during hybrid formation (e.g., Kay, 2006) and during the development of hybrid embryo (e.g., Runions and Owens, 1999; Yasumoto and Yahara, 2008). Pollen-pistil interaction may reduce the frequency of heterospecific pollen fertilizing the ovules (e.g., Kay 2006; Escobar-Restrepo et al., 2007), while genetic incompatibility causes hybrid seed abortion (e.g., Runions and Owens, 1999). Among the four categories of seeds, it is clear that seed production is higher in the fruits that had *C. villosissimus* as the maternal parent than those that had *C. allenii* as the maternal plant. This is presumably due to species-level differences in the number of ovules per fruit. For fruits produced with *C. allenii* as the maternal parent, there was no difference in seed set between fruits of intra- and interspecific crosses, and there was no evidence of intrinsic postzygotic isolation during the formation of the hybrids or the development of hybrid embryo. For fruits produced with *C. villosissimus* as the maternal parent, *C. villosissimus* fruits produced more seeds than $F1_{villo}$ fruits, resulting in significant, but weak isolation. The results clearly indicate the possibility of making viable F1 hybrid seeds. Isolation observed in interspecific seed set was absent or weak, allowing me to use the proportion of hybrid progeny for estimating gametic isolation (Ch. 1). Gametic isolation, which acts prior to fertilization but after mating, was estimated with the proportion of hybrid progeny in fruits produced by hand pollination with a 50 interspecific: 50 intraspecific pollen mixture (Ch. 1). Theoretically, both gametic and early stage intrinsic postzygotic isolation is included in this estimate. Given that intrinsic postzygotic isolation in seed set is absent or weak, isolation observed in the estimate of gametic isolation is mainly due to gametic isolation per se. While

gametic isolation was included as a component of sexual isolation, using proportion of hybrid offspring to estimate sexual isolation was also appropriate.

In *Costus pulverulentus* and *C. scaber*, Kay (2006) demonstrated severe reduction in seed set of interspecific crosses. For fruits produced with *C. pulverulentus* as the maternal parent, the reduction in hybrid seed set is because the pollen tubes of *C. scaber* do not grow long enough to fertilize the ovules of *C. pulverulentus*. For hybrid fruits with C. *scaber* as maternal parent, pollen of *C. pulverulentus* fail to adhere and germinate on the stigma of *C. scaber*. In both species, the reduced hybrid seed formation is a consequence of gametic isolation. However, the degree of isolation varies across populations: sympatric heterospecific populations are more isolated from each other than allopatric heterospecific populations (Kay and Schemske, 2008). The stronger gametic isolation in sympatry suggests that direct selection on pollen-pistil interactions reinforces speciation in these species. This is very different from the findings in *C. allenii* and *C. villosissimus* since there is no sympatric population of *C. allenii* and *C. villosissimus* for such reinforcement to take place.

F1 Hybrid Transplant Performance

Reciprocal transplant experiments with parents and hybrids demonstrated that F1 hybrids had reduced fitness at the seed stage in *C. villosissimus* habitats, but not at other stages or in *C. allenii* habitats (Ch. 2). Therefore, extrinsic postzygotic isolation was only significant for *C. villosissimus* at the seed stage. Although the importance of local adaption in ecogeographic and microhabitat isolation was recognized in this study system, local adaptation did not contribute to extrinsic postzygotic isolation. Unfortunately, plant fecundity and hybrid performance in later generations were not included in this study. The lack of these potentially important barriers may result in an underestimation of extrinsic postzygotic isolation.

F1 Hybrid Pollen Viability

Hybrid male sterility is an intrinsic postzygotic barrier which has been commonly examined in speciation studies of animals (e.g., Coyne and Orr, 1989, 1997; Price and Bouvier, 2002). Hybrid pollen inviability, the equivalent measure in plants, is also frequently examined (e.g.. Fishman and Willis, 2001; Moyle, et al., 2004; Scopece et al., 2008). In a guild of food-deceptive orchids with weak sexual isolation, significant male sterility reduced hybrid fitness and caused intrinsic postzygotic isolation (Scopece et al., 2008). However, in *C. allenii* and *C. villosissimus*, there was no evidence of hybrid male sterility. In this study, the average proportion of viable pollen was only 41%, which was lower than expected. This reduction of pollen viability across categories may be due to the unnatural humidity conditions in the greenhouse, where the sampled plants were grown. Hybrid male sterility was also irrelevant to reproductive isolation between *C. pulverulentus* and *C. scaber* (Kay, 2006). This is potentially because *C. pulverulentus* and *C. scaber* are recently diverged and have not had time to accumulate genetic incompatibility that causes intrinsic postzygotic isolation (Kay and Schemske, 2008).

Total Isolation

The estimates of total isolation demonstrate that *C. allenii* is highly, but not completely isolated from *C. villosissimus* ($T = 0.924$), which is in turn almost completely isolated from *C. allenii* ($T = 0.999$). Total isolation was also estimated by replacing the barriers from microhabitat isolation to F1 juvenile growth in parental habitats with observed hybrid frequency in suitable habitats for both species. In this estimate, the average total isolation ($T = 0.988$) improved, but was still not complete. As *C. allenii* and *C. villosissimus* are good species, some barriers might have been underestimated, which lead to the estimate of incomplete total isolation. One barrier which might have been underestimated is sexual isolation, which was measured as the proportion

of hybrid offspring produced by naturally pollinated flowers in a pollination array (Ch. 1). During this process, the amount of pollen deposited on the stigma may be lower than natural pollen loads. The source of pollen was limited as there were only two flowers per species in the pollination array and as one of them had its pollen stained with florescent dye and the staining process caused pollen loss. If few pollen grains were deposited on stigmas, the pollen germination rate could be reduced (Schemske and Fenster, 1983), and the proportion of heterospecific fertilization might be underestimated. After seeds were collected, it was difficult to distinguish the hybrids from the parental seeds and seedlings by their morphology. Instead, hybrid seedlings were identified with genetic markers. It was assumed that the proportion of hybrid offspring remained consistent from seed formation, seed viability, seed germination, to early seedling survival (Ch. 1). However, it is certainly possible that hybrids and parental seeds differ in these fitness components, which were not measured in this study, and cause isolation.

A second possible explanation of the estimated incomplete total isolation in *C. allenii* may be that F1 hybrid transplant performance did not properly represent hybrid fitness in parental habitats. Because none of the transplants in *C. allenii* habitats flowered during the experimental period (Ch. 2), the likelihood of hybrid reproduction was not included as a postzygotic barrier. In addition, hybrid fitness was studied only in F1s. Previous studies have shown that F1 hybrids may have better fitness than parental habitats but fitness of hybrids may be reduced in later generations (Rhode and Cruzan, 2005). Including hybrid reproduction and hybrid fitness in later generations in the calculation of total isolation may result in a higher value of total isolation observed in *C. allenii*, yet measuring these barriers requires a much longer period of time, which was beyond the scope of this research.

Relative Importance of Isolating Mechanisms

Reproductive isolation between *C. allenii* and *C. villosissimus* is mainly due to the difference between their spatial distributions. Ecogeographic isolation was the primary isolating barrier in *C. villosissimus*, and this barrier also contribute substantially to total isolation in *C. allenii*. While heterospecific gene flow was not completely eliminated by ecogeographic isolation, microhabitat isolation reduced most of the remaining heterospecific gene flow (Fig. 4.5A). Microhabitat isolation between *C. allenii* and *C. villosissimus* was shown to be a direct consequence of local adaptation based on the finding of zero fitness of parental species transplanted into foreign habitats (Ch. 2). As local adaptation contributes 100% to ecogeographic and microhabitat isolation and these two barriers together contribute > 99% to total isolation, I conclude that local adaptation is the primary mechanism of speciation in *C. allenii* and *C. villosissimus*.

Furthermore, both ecogeographic and microhabitat isolation are estimates of spatial isolation, which are caused by habitat differences between species. The habitats of *C. allenii* and *C. villosissimus* differ in water and light availability (Ch. 3). Plant physiological response (leaf mass per area) to low light availability in *C. allenii* habitats and high drought tolerance to cope with low water availability in *C. villosissimus* habitats are putative adaptive traits in these species (Ch. 3). Given that ecogeographic and microhabitat isolation are the major contributor to total isolation, reproductive isolation between *C. allenii* and *C. villosissimus* is almost all due to prezygotic barriers. The large contribution of prezygotic isolation found in this study agrees with the statement that prezygotic barriers play a major role in speciation and that postzygotic isolation evolves mainly after speciation is complete (Schemske, 2010).

Speciation in *Costus*

Reproductive isolation has been examined in two species pairs of *Costus,* and in both cases, prezygotic barriers are more important than postzygotic barriers. *Costus pulverulentus* and *C. scaber* (Kay, 2006) are hummingbird-pollinated species which are partially isolated by its habitats. As small patches of habitats of *C. pulverulentus* and *C. scaber* are often intermingled with each other, isolating barriers, other than habitat isolation, play important roles in speciation of these two species. *Costus pulverulentus* and *C. scaber* are mostly isolated by the combined effects of their spatial distribution, floral mechanical isolation, and gametic isolation. This is different from the case of *C. allenii* and *C. villosissimus*, of which habitat isolation contributes the most to total isolation. While extrinsic postzygotic isolation does not contribute to total isolation between *C. allenii* and *C. villosissimus*, this barrier was not evaluated in *C. pulverulentus* and *C. scaber*. Although both studies recognized that ecological factors contribute to speciation, the mechanisms were different between species pairs. Natural selection against parental species in foreign habitats results in a parapatric distribution of *C. allenii* and *C. villosissimus* (Ch. 2), while reinforcement through natural selection of hybrid pollen-pistil incompatibility strengthen reproductive isolation in sympatric populations of *C. pulverulentus* and *C. scaber* (Kay and Schemske, 2008). Studies of these two species pairs suggest that ecological factors contribute to reproductive isolation, while the details and adaptive mechanisms differ between species pairs.

Conclusion

Overall, I found that strong ecogeographic and microhabitat isolation are the major isolating barriers between *C. allenii* and *C. villosissimus*. With the findings of the previous chapters, I demonstrated how ecological factors contribute to adaptive divergence and ultimately

lead to reproductive isolation. Here I conclude that local adaptation is the primary mechanism of speciation in this system. Future studies of taxa with varying genetic distance are needed to compare the relative importance of pre- and postzygotic barriers and to determine whether local adaptation is the general mechanism of reproductive isolation at the time of speciation.

Table 4.1. Climatic variables used in ecological niche modeling. All variables were from the publicly available dataset WORLDCLIM (www.worldclim.org). A quarter is a three-month period.

Code	Description
BIO1	Annual mean temperature (°C)
BIO2	Mean diurnal range (mean of monthly (maximum temp – minimum temp))
BIO4	Temperature seasonality (standard deviation * 100)
BIO5	Maximum temperature of the warmest month
BIO6	Minimum temperature of the coldest month
BIO7	Temperature annual range (BIO5 – BIO6)
BIO12	Annual precipitation
BIO15	Precipitation seasonality (coefficient of variation)
BIO16	Precipitation of wettest quarter
BIO17	Precipitation of driest quarter
BIO18	Precipitation of warmest quarter
BIO19	Precipitation of coldest quarter

Table 4.2. Estimates of the studied reproductive isolating barriers between *C. allenii* and *C. villosissimus*. The proportion of hetero- (*H*) and conspecific gene flow (*C*) causing the strength (*RI*) of individual barriers and the absolute contribution (*AC*) to total isolation (*T*) are listed for each species. *: *H* and *C* of the isolating barrier were calculated from *RI* values.

Isolating barriers	*C. allenii*				*C. villosissimus*			
	H	*C*	*RI*	*AC*	*H*	*C*	*RI*	*AC*
Ecogeographic isolation*	0.43	0.57	0.142	0.142	0.03	0.97	0.948	0.948
Microhabitat isolation*	0.06	0.94	0.886	0.771	0.03	0.97	0.932	0.050
Temporal isolation*	0.48	0.52	0.045	0.007	0.49	0.51	0.027	<0.001
Sexual isolation	0.44	0.56	0.121	0.017	0.21	0.79	0.581	0.001
Interspecific seed set	0.48	0.52	0.050	0.006	0.44	0.56	0.112	<0.001
F1 seed in parental habitats	0.47	0.53	0.067	0.007	0.27	0.73	0.463	<0.001
F1 juvenile survival in parental habitats	0.46	0.54	-0.129	-0.014	0.62	0.38	-0.240	-0.000
F1 juvenile growth in parental habitats	0.51	0.49	-0.014	-0.002	0.75	0.25	-0.493	-0.000
F1 pollen viability	0.53	0.47	-0.069	-0.009	0.46	0.54	0.074	<0.001
Total	0.00017	0.00441	*T* = 0.924		0.000002	0.00804	*T* = 0.999	

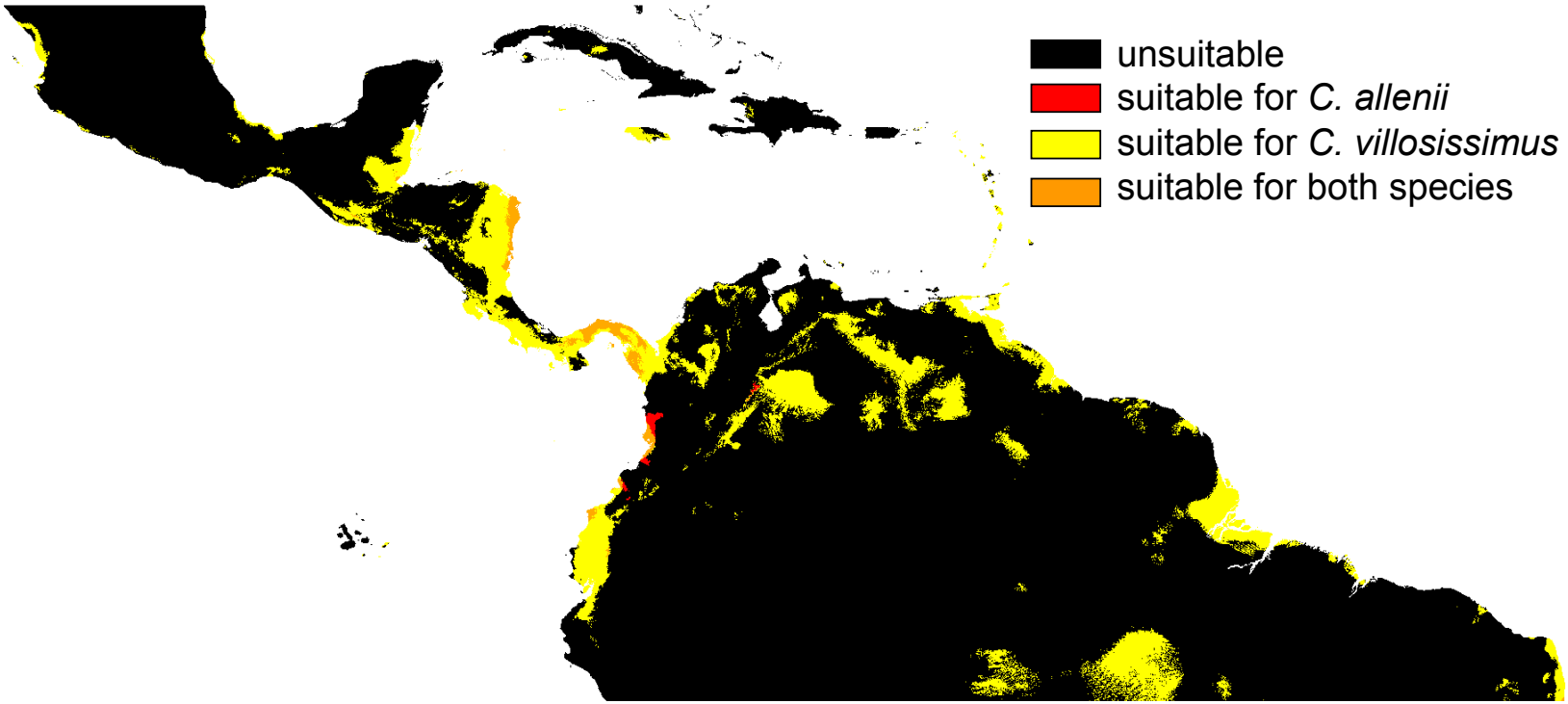

Figure 4.1. Overlay map showing ecogeographic isolation between *C. allenii* and *C. villosissimus*. The ecological niche models show that 9,769 pixels are suitable for *C. allenii* alone, 1,077,663 pixels were suitable for *C. villosissimus* alone, and 58,827 pixels are suitable for both species. The sizes of the shared and unshared suitable habitats results in $RI_{ecogeo} = 0.142$ for *C. allenii* and $RI_{ecogeo} = 0.948$ for *C. villosissimus*.

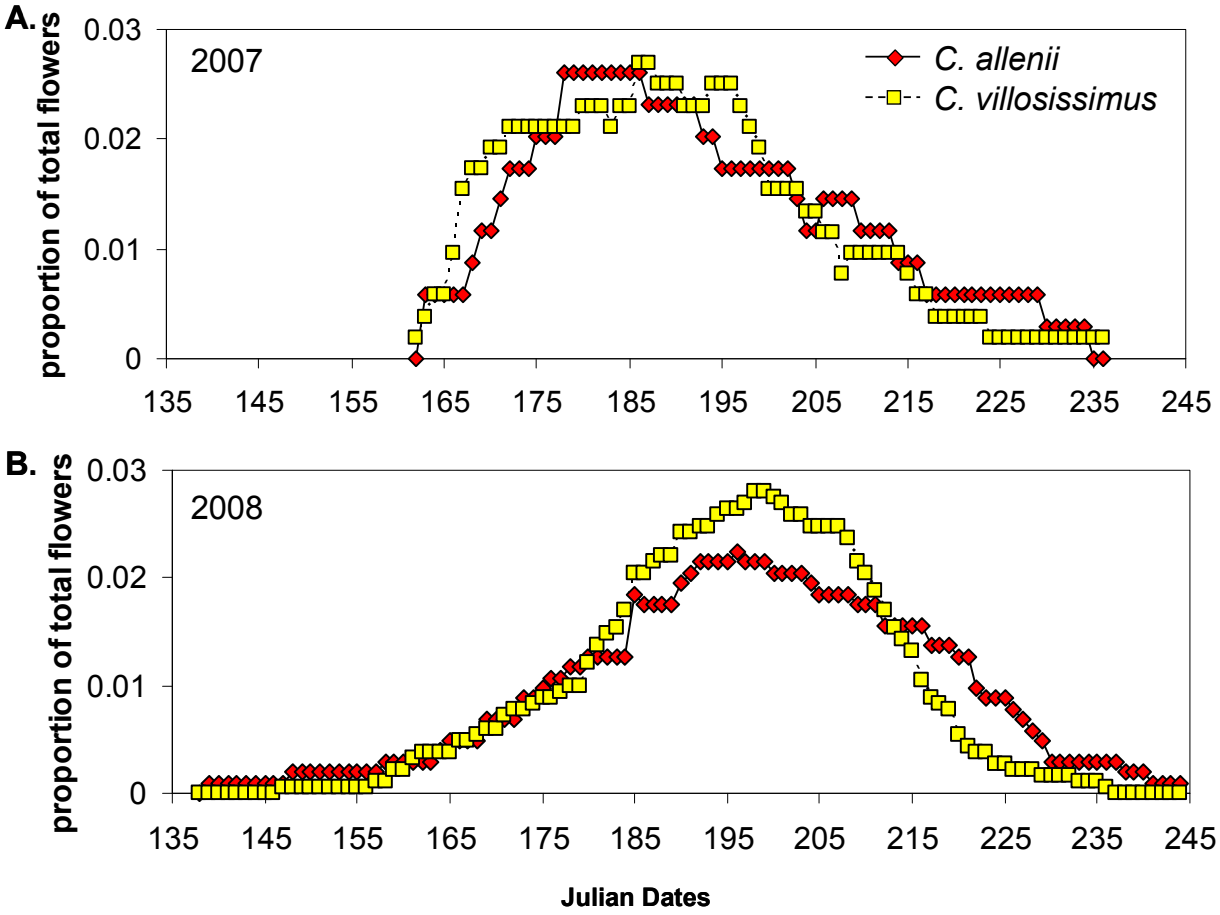

Figure 4.2. Flowering phenology of *C. allenii* and *C. villosissimus* in 2007 (A) and 2008 (B). There was no significant difference between the flowering phenology in neither year ($p > 0.05$). $RI_{temporal}$ = 0.028 for *C. allenii* and 0.019 for *C. villosissimus* in 2007; $RI_{temporal}$ = 0.061 for *C. allenii* and 0.035 for *C. villosissimus* in 2008. The average $RI_{temporal}$ of 2007 and 2008 was 0.045 for *C. allenii* and 0.027 for *C. villosissimus.*

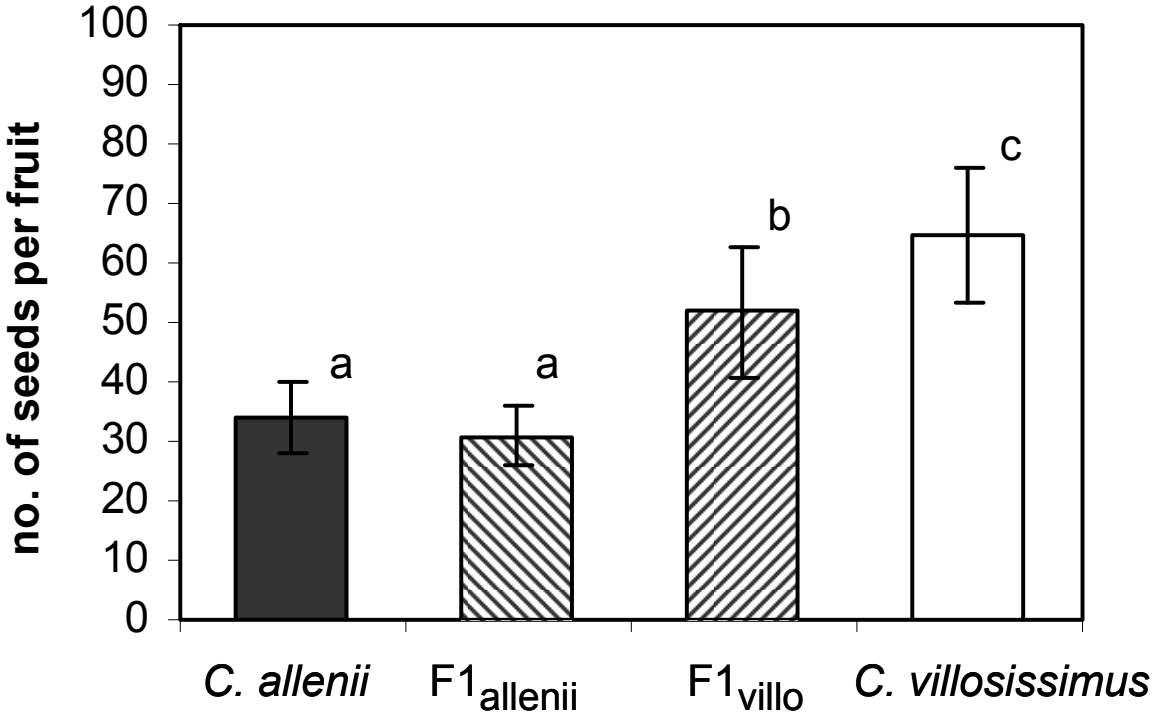

Figure 4.3. Comparisons of seed set among *C. allenii*, $F1_{allenii}$, $F1_{villo}$, and *C. villosissimus*. Error bars represent mean ± 95% CI. Bars with different letters represent means that are significantly different at the level of $\alpha = 0.05$ based on Tukey's HSD tests.

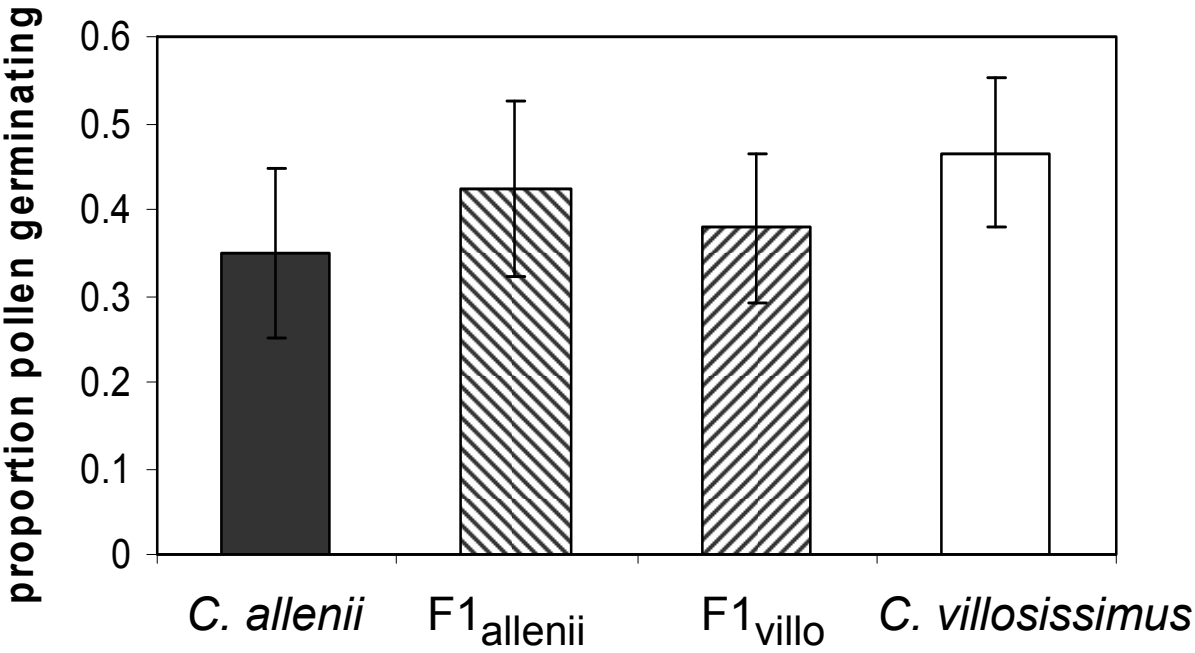

Figure 4.4. Comparisons of proportion of pollen germinating, representing male fertility, among *C. allenii*, $F1_{allenii}$, $F1_{villo}$, and *C. villosissimus*. Error bars represent mean ± 95% CI. There was no significant difference among categories ($p > 0.05$).

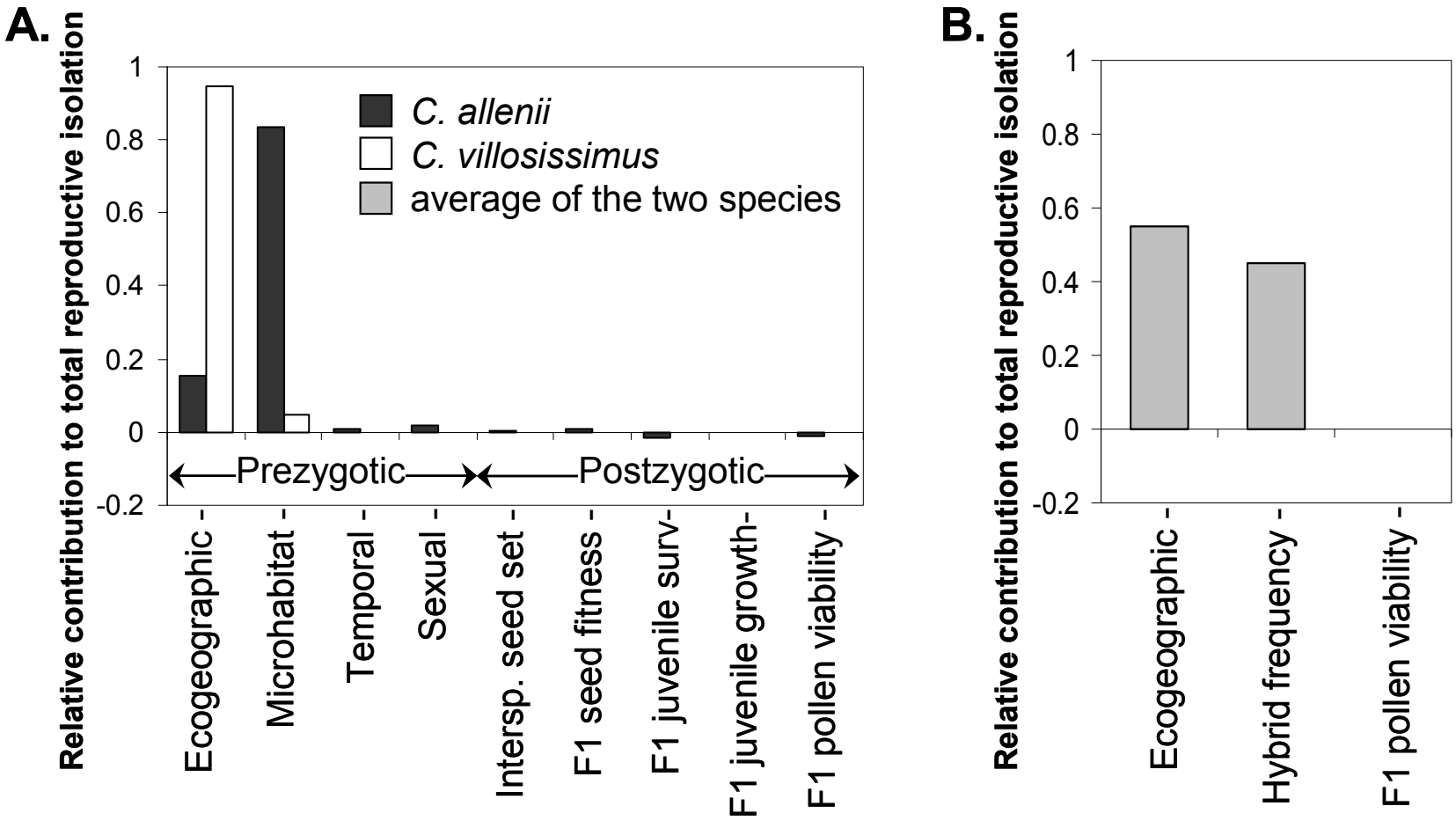

Figure 4.5. Relative contribution of isolating barriers to total isolation in *C. allenii* and *C. villosissimus*. (A) Relative contribution of each barrier. (B) Relative contribution of ecogeographic isolation, hybrid frequency in suitable habitats for both species, and F1 hybrid pollen viability to total isolation, based on species averages.

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