

## The evolution of postpollination reproductive isolation in *Costus*

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**Abstract** Reproductive isolation is critical to the diversification of species. Postpollination barriers may be important in limiting gene flow between closely related species, but they are relatively cryptic and their evolution is poorly understood. Here, we review the role of postpollination reproductive isolation in plants, including the various stages at which it operates and the hypotheses for how it may evolve. We then review empirical studies in the plant genus *Costus*, evaluating documented postpollination barriers in light of these hypotheses. We summarize isolation due to parental style length differences and present evidence supporting the hypothesis that the differences are in part a by-product of selection on floral morphology. Additionally, we show that reduced pollen adhesion, germination, and tube growth contribute to reproductive isolation between two closely related sympatric species of *Costus*. Geographic variation in the strength of these crossing barriers supports the hypothesis that they evolved under reinforcement, or direct natural selection to strengthen isolation.

**Keywords** Postpollination · Reproductive isolation · *Costus* · Reinforcement

“The simplest and best known case of prepotent action in pollen... is that of a plant’s own pollen over that from a distinct species.” – Charles Darwin 1876 p. 391

A highly coordinated set of mechanisms determine successful fertilization. In plants, these mechanisms include pollen adhesion, hydration, tube elongation, and tube navigation of the pollen tube to the ovule (reviewed in Swanson et al. 2004). Multiple genes, in both the pollen and pistil affect the success of these stages. These molecular mechanisms can evolve to be species specific (Zinkl et al. 1999) and thus can contribute to reproductive isolation between lineages and play an important role in speciation. Pollen–pistil interactions therefore have evolutionary implications for the maintenance and diversification of species (Williams 2008).

Reproductive isolation is a key component of speciation, and it is a central goal of evolutionary biology to understand the types of isolating mechanisms functioning in nature and the processes by which they evolve. When species are geographically isolated, evolutionary divergence should lead to reproductive isolation over time (Mayr 1947), but the identity and strength of reproductive barriers varies (Coyne and Orr 1989, 1997). Reproductive barriers function at different stages. Premating barriers include spatial, temporal, behavioral, and mechanical barriers, whereas postmating barriers, or postpollination barriers in plants, include interactions between sperm or pollen and the female reproductive tract (Mayr 1963; Grant 1981). Throughout this paper, we use the terms postmating and postpollination to refer to barriers that occur prior to fertilization. Finally, there are postzygotic barriers to gene flow that include zygote abortion, hybrid inviability, infertility, and reduced competitive ability (Dobzhansky

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1937; Schluter 2000). Much speciation research has focused on understanding the evolution of postzygotic hybrid inviability and infertility, or alternatively, the evolution of striking pre mating barriers such as divergent mating signals in male animals or pollination syndromes in plants (Coyne and Orr 2004). In contrast, postmating barriers may be relatively cryptic and underappreciated as significant factors in speciation.

In plants, numerous postpollination reproductive barriers have been described that result in varying degrees of reproductive isolation (reviewed in Grant 1981; Howard 1999). Darwin was one of the first to describe these barriers. He conducted experiments in which he pollinated plants with heterospecific pollen and quantified fertilization success. In many instances, he found that heterospecific pollen set fewer seed than conspecific pollen, and that heterospecific pollen was even less successful when mixed pollen loads were applied to the stigma (Darwin 1876). More recently, postmating isolation through conspecific sperm or pollen precedence has been found to contribute to overall reproductive isolation in both animal and plant systems, including ladybirds in northern Japan (Nakano 1985), grasshoppers in the Pyrenees (Bella et al. 1992), irises in Louisiana (Carney et al. 1996), and sunflowers in the central United States (Rieseberg et al. 1995).

Conspecific pollen precedence in mixed pollinations can result from greater pollen adherence to the stigma, greater germination rates, or faster pollen tube growth rates of conspecific pollen (reviewed in Howard 1999). The growth rate of heterospecific pollen tubes may be hindered by the foreign pistil or may be inherently slower, causing them to reach the ovary only after the faster growing conspecific pollen has already achieved fertilization. In the Louisiana irises, conspecific pollen precedence has been attributed to faster pollen tube growth rates; however, if heterospecific pollen is given a head start to overcome the slow pollen tube growth rate, fertilization success increases (Arnold et al. 1993; Carney et al. 1996). The lack of hybrid formation in artificial crosses of *Hibiscus* can be explained in large part by differences in pollen tube growth rate (Klips 1999).

Pollen attrition, defined as the cessation of tube growth prior to reaching the ovule, can also function to isolate taxa (Perez and Moore 1985; Lee et al. 2008) and has been most frequently documented in crosses where there is a mismatch in parental style length. Pollen from long-styled plants can often pollinate a shorter-styled plant, yet pollen from short-styled plants often lacks the ability to grow the required distance on a long-styled plant. Attrition has been shown to be a significant cause of reduced artificial hybridization success in *Prunus* (Perez and Moore 1985), *Rhododendron* (Williams and Rouse 1988), and *Nicotiana* (Lee et al. 2008). Yet these examples do not address the

importance of pollen attrition as an isolating mechanism in natural populations.

Generalized predictions about the relative importance of postpollination barriers are difficult to make. If pre mating barriers are weak and pollen transfer regularly occurs between closely related species, postpollination barriers may be critical in preventing hybridization and allowing coexistence. For example, Wolf et al. (2001) found asymmetrical pollen transfer based on pollen placement on the shared hummingbird pollinator in the *Ipomopsis aggregata* species complex. In this case, birds move pollen from *I. arizonica* to the stigma of *I. aggregata*, but the pollen performs poorly and has low fertilization success. Artificial pollinations in the opposite direction show that *I. aggregata* pollen performs well on *I. arizonica*, but this transfer is rare in nature (Wolf et al. 2001). Scopece et al. (2007) tested the relative importance of postpollination and postzygotic barriers using two different categories of deceptive orchids, those with highly specific pollination systems and those with more generalized pollination systems. They hypothesized that orchids with generalized pollination are more likely to experience pollen transfer in nature and should therefore have stronger postpollination barriers than orchids with specialized pollination systems for which heterospecific pollen transfer is less likely. While this pattern was supported by their analysis, there was no assessment made of actual pollen transfer, and the clades of generalists and specialists were of vastly different ages, making comparisons tenuous (Sobel and Randle 2009).

Understanding the ways in which postpollination reproductive isolation evolves is critical in understanding the contribution of this mode of isolation to speciation in nature. There are three major hypotheses for the evolution of postpollination isolation. Postpollination reproductive isolation can evolve as (1) an incidental consequence of divergence in other traits such as floral morphology, adaptations to environmental conditions, or pathogen defense, (2) an incidental consequence of evolution of the mate recognition system within populations, or (3) the result of reinforcement. Here, we briefly review these hypotheses and discuss how they might be distinguished from one another.

Postpollination reproductive isolation may evolve as a consequence, through pleiotropy or linkage, of selection on other morphological or physiological traits. For example, selection for traits associated with pollinators may actively drive divergence between the pistils of different populations. Grant (1966) proposed that postpollination isolation has evolved between coastal and desert races of *Gilia ochroleuca* as a by-product of adaptation for insect pollination and autogamy, resulting in style length divergence. Traits that diverge as the result of character displacement may result in a similar pattern. While there is currently

little evidence to suggest that defenses against pathogens have contributed to reproductive isolation in plants, pathogen defense has been implicated in the hostility of the female reproductive tract in birds (Sheldon 1993). If the evolution of gametes and the female reproductive tract is being driven by pathogen defense, extensive intraspecific variation in defense proteins is expected (Figueroa et al. 1988). Searcy and MacNair (1990) have shown that edaphic adaptation can affect the pistil in such a way as to prevent hybridization. They demonstrated that *Mimulus guttatus* grown under high copper concentrations have inhospitable pistils that prevent the germination of pollen from copper-sensitive populations but not from copper-tolerant populations.

Postpollination isolation may also evolve as the result of intraspecific evolution of pollen–pistil recognition. Differences in the pollen and pistil can result from sexual conflict, sexual selection, or facilitative interactions between the male gametophyte and pistil. Sexual conflict arises when a beneficial trait evolves in one sex that has a harmful effect on the other (Linder and Rice 2005). Each sex evolves counter adaptations over time and a coevolutionary arms race ensues between the sexes resulting in rapid divergence between lineages (Gavrilets 2000; Swanson and Vacquier 2002a, b). As an example of sexual conflict, manipulative pollen traits can change the timing of stigma receptivity in *Collinsia heterophylla*, which ultimately resulted in lower seed set (Lankinen and Kiboi 2007). Indications of strong positive selection on reproductive proteins in both plants and animals suggest that sexual conflict and sexual selection may be driving rapid divergence and species specificity in postmating mechanisms, yet direct tests of these hypotheses remain difficult (Clark et al. 2006). Isolation can occur whenever populations adapt to local pollen–pistil conditions regardless of whether they are antagonistic or facilitative. These three processes may lead to rapid evolution within a lineage and, incidentally, isolation between lineages.

Finally, there may be direct natural selection for postpollination reproductive barriers if pollen transfer results in the production of less fit hybrids, a process known as reinforcement (Dobzhansky 1940; Grant 1965). Under reinforcement, selection favors those individuals that do not waste gametes on inferior hybrid offspring. Reinforcement remains a highly controversial concept in evolutionary biology (Coyne and Orr 2004) because of the theoretical difficulty of evolving isolation in the face of gene flow and the paucity of strong empirical examples (reviewed in Butlin 1987; Noor 1999; Servedio and Noor 2003). As a precondition for reinforcement to act on postpollination barriers, taxa must be sympatric, pollen transfer must occur, and hybrids must have reduced fitness. If reinforcement is responsible for the evolution of

postpollination barriers, one should observe a difference in barrier strength between sympatric and allopatric populations of a species pair.

These three hypotheses are not mutually exclusive of one another and may function in concert to produce significant isolation in nature. It is possible to imagine a situation in which selection pressures act on floral morphology, soil relations, male–female interactions, and hybrid fitness, all of which may contribute to the evolution of postpollination isolation. While many studies have documented postpollination isolation in natural systems, little work has been done to identify or distinguish between the underlying evolutionary causes of these barriers.

The neotropical genus *Costus* presents a unique study system for identifying the causes and consequences of postpollination barriers between closely related taxa. There are multiple cases of floral adaptation to different pollinators in the genus, allowing us to address the hypothesis that postpollination reproductive barriers may evolve as an incidental consequence of selection on morphological or physiological traits. We test for correlations between changes in floral morphology and changes in style length, which have been found to contribute to isolation between taxa, while controlling for phylogenetic relatedness. Additionally, for one species pair that experiences pollen transfer in nature, we review crossing results from multiple geographically dispersed populations in order to evaluate the importance of reinforcement in driving the evolution of postpollination barriers (Maas 1972, 1977). We therefore use *Costus* to explore the following questions: Does adaptation to different pollinator assemblages confer postpollination isolation as a by-product of selection? What form do postpollination barriers take between closely related species in nature? Do postpollination barriers show evidence of reinforcement?

### The study system

*Costus* is a genus of herbaceous understory monocots that represents a recent radiation in Central and South America (>50 species in ~5 million years; Kay et al. 2005). Through monographic work (Maas 1972, 1977) and field studies (Schemske 1981; Kay and Schemske 2003; Kay 2006), we have a good understanding of whether pollen transfer is possible or actively occurring between pairs of species in nature. Each species is specialized for pollination by either hummingbirds or orchid bees, and these differences serve as effective premating barriers for sympatric species (Kay and Schemske 2003). Flowers of bee-pollinated *Costus* have a large, pale labellar tube with a distinct white or yellow limb, the lateral lobes of which are often striped with red or purple. Hummingbird-pollinated

species have flowers with a narrow, tubular, and often short labellum without an attached limb, and the labellum and floral bracts are yellow, orange, or red. We use repeated evolutionary shifts between these floral syndromes to explore the role of floral adaptation to pollen–pistil mismatch in interspecific crosses.

In addition to exploring the contribution of pollination-syndrome shifts to the evolution of postpollination barriers, we evaluate the hypothesis of reinforcement between two closely related species, *C. pulverulentus* and *C. scaber*, which are sympatric throughout much of Central and northwestern South America. These species share their primary pollinator (*Phaethornis longirostris*) and experience some pollen transfer, but hybrids are rarely found in nature (Kay 2006).

### Postpollination barriers as a by-product of selection

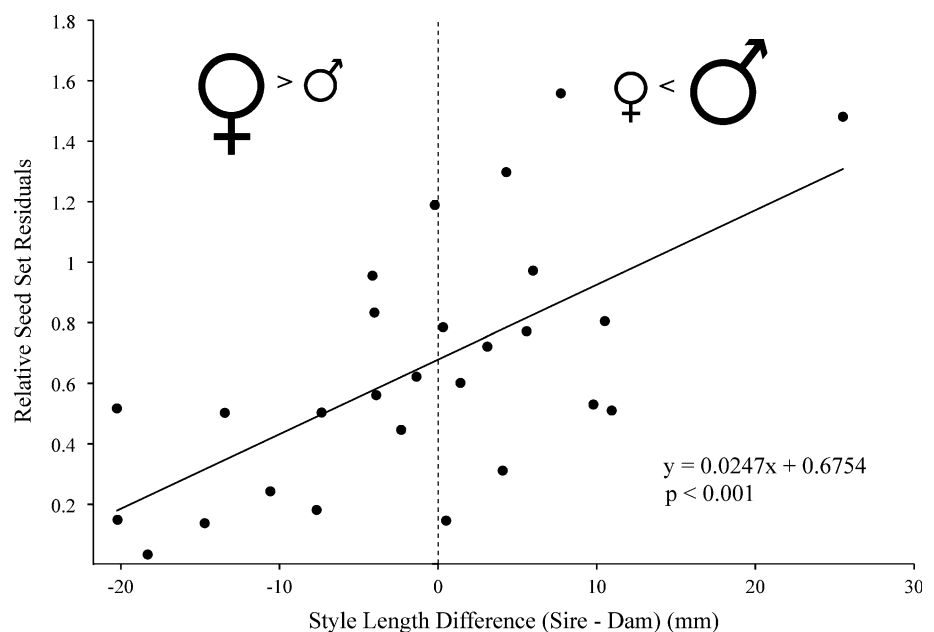
We used varying floral syndromes in *Costus* to examine whether postpollination isolation may have evolved as an incidental consequence of direct selection on floral morphology due to pollinator shifts. Crosses between 26 pairings of 10 different species from the genus (Kay and Schemske 2008) show that style length differences contribute significantly to crossing success when controlling for genetic distance and putative cases of reinforcement (Fig. 1). As seen in other plant systems, when the maternal style is much longer than the paternal style, seed set relative to intraspecific crosses is lower (Kay and Schemske 2008). Genetic distance between crossed taxa, based on estimated substitutions per site for two loci used in a

phylogenetic study (Kay et al. 2005), did not significantly explain relative seed set (Kay and Schemske 2008).

What drives the evolution of style length? One possibility is that transitions to new pollinators involve adaptive changes in floral length. These changes could then contribute to postpollination isolation through parental style length mismatch. Across the genus, there have been numerous transitions to bird pollination from bee-pollinated ancestors (Kay et al. 2005). The orchid bees that visit *Costus* flowers typically land on the limb of the labellum and crawl inside a large floral chamber, whereas the hummingbirds hover in front of the flower and insert their bills into a narrow floral tube (Kay and Schemske 2003). We therefore asked whether these transitions from bee to bird pollination are associated with consistent shortening in flower length.

We first estimated phylogenetic relationships in the genus using DNA sequence data (ITS and ETS), and then reconstructed the history of both pollination syndromes and floral length measures. To estimate the phylogeny, we used the data from Kay et al. (2005) and added six taxa that became available subsequently (GenBank accession numbers AY972877–AY973004, AY994750, AY994752, AY994730, AY994731, AY994746, GQ294458–GQ294467). All methods for sequencing and phylogeny estimation were as detailed in Kay et al. (2005), except here we used MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). We searched for 10 million generations, sampled results every 10,000 generations, and discarded 25% as burn-in. Pollination-syndrome classifications, and corolla length were taken from Maas (1972, 1977) or when possible, were measured on live plants in the greenhouse. Although style length was not

**Fig. 1** Relative seed set residuals plotted against style length differences for 26 interspecific pairings of *Costus* species. This graph shows the relationship between style length difference and crossing success while controlling for genetic distance and putative cases of reinforcement of pollen–stigma incompatibility. Style length significantly affects relative seed set in this combined model ( $P < 0.001$ ). Relative seed set values (heterospecific seed set/ conspecific seed set) were truncated at 1.0 and arcsine square root transformed before statistical tests. See Kay and Schemske (2008) for crossing details



recorded in monographic descriptions, measures of corolla length are strongly correlated with style length for the plants measured in the greenhouse ( $N = 14$  species,  $R^2 = 0.66$ ,  $P < 0.001$ ).

Since differences in style length can effectively isolate some species, we assessed whether shorter flowers are associated consistently with shifts in pollination syndrome from bee to bird pollination. A majority rule consensus tree was constructed from the posterior distribution of trees, and we randomly resolved polytomies in this tree to produce 10 fully resolved consensus trees using Mesquite v.2.6 (Maddison and Maddison 2009). For each of these 10 trees, we used parsimony to reconstruct the history of changes in both pollination syndrome and corolla length and then recorded whether each transition in pollination syndrome was associated with an increase or decrease in corolla length. The consensus trees showed between six and nine independent shifts to hummingbird pollination. In one-tailed sign tests, nine out of the ten consensus trees showed that shifts to bird pollination were significantly associated with decreases in corolla length ( $0.0352 > P > 0.0039$ ). The tenth tree was marginally significant ( $P = 0.0625$ ). Only one shift to bird pollination, in the lineage leading to *C. stenophyllus*, showed an increase in corolla length. Figure 2 depicts the pollination-syndrome shifts and corresponding corolla lengths. In cases of pollination-syndrome shifts, pre-mating barriers substantially isolate species pairs and postpollination barriers may be secondarily important for conferring reproductive isolation. For example, at La Selva Biological Station in Costa Rica, there is an average proportional similarity in pollinator use of 10% between species differing in pollination syndrome (Kay and Schemske 2003), but many of these pairings show substantial style length differences and reduced interspecific seed set (Kay and Schemske 2008).

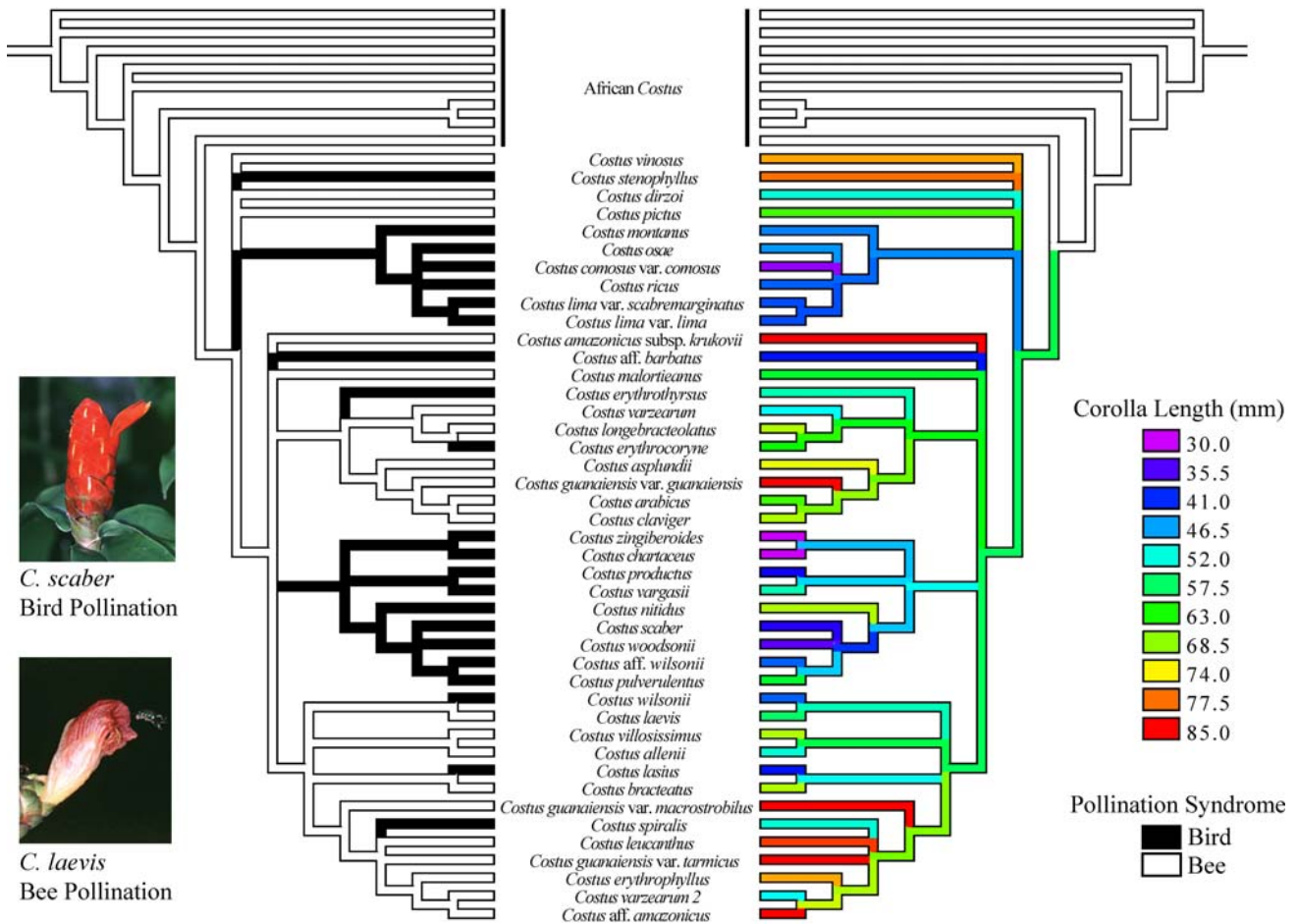
### Postpollination barriers as the result of reinforcement

*Costus pulverulentus* and *C. scaber* are two partially sympatric close relatives that share their primary pollinator, and therefore provide an excellent system to quantify the importance of postpollination isolation and to explore the role of reinforcement. There is substantial pollen transfer from *C. pulverulentus* to *C. scaber* by their shared pollinator, but differences in flower shape and size prevent pollen transfer in the other direction (Kay 2006). Despite heterospecific pollen deposition on *C. scaber*, hybrids are rarely found in nature. In hand pollinations, both crossing directions resulted in low seed set due to low fertilization success. In order to determine the causes of reduced fertilization, pollen adhesion, germination, and pollen tube growth rates were examined (Kay 2006). The mechanisms

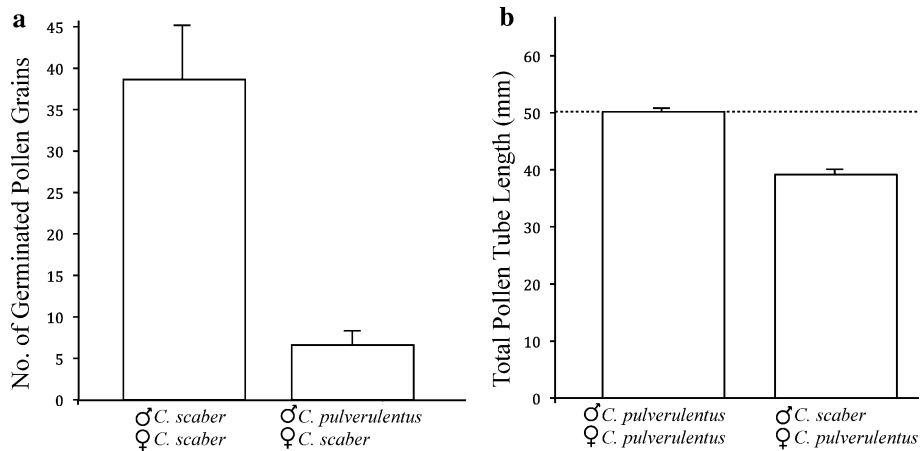
conferring postpollination isolation differed between the crosses depending on the pollen donor. When *C. scaber* was used as the maternal parent in interspecific crosses, low seed set resulted from low pollen adhesion, low pollen germination, and slower pollen tube growth rates. The adhesion and germination results are summarized in Fig. 3a. Since this is the direction pollen is transferred in nature, these postpollination barriers play a direct role in reproductive isolation. Interspecific crosses in the other direction, using *C. pulverulentus* as the maternal parent found no difference in pollen adhesion and germination but showed that pollen tubes did not grow the distance required to reach the ovules (Fig. 3b). Since pollen does not naturally move in this direction, attrition is likely unimportant for reproductive isolation.

The strong pollen–stigma incompatibility in the pollinations of *C. scaber* by *C. pulverulentus* was qualitatively different than style length mismatch and was striking in light of their close phylogenetic relationship, thus motivating the hypothesis that it evolved by reinforcement. If the incompatibility evolved under direct selection to prevent hybridization, it should be strongest between sympatric populations, whereas other hypotheses for the evolution of the incompatibility predict consistent barrier strength across sympatric and allopatric populations. To test for a pattern consistent with reinforcement, hand pollinations were performed between populations of *C. pulverulentus* and *C. scaber* from three sites across the species geographic ranges—two sites where the species occur sympatrically and one where *C. pulverulentus* occurs in isolation (Kay and Schemske 2008). Attempted crosses and origin of plants are diagrammed in Fig. 4. Pollen from sympatric populations of *C. pulverulentus* resulted in low seed set on *C. scaber* maternal plants, whereas pollen from geographically distant populations within the region of sympatry and allopatric populations of *C. pulverulentus* yielded significantly higher seed set. These crossing results provide strong support for the process of reinforcement driving pollen–stigma incompatibility between locally sympatric populations with incomplete pre-mating isolation. In contrast, similar cross-site pollinations in the other direction, using *C. pulverulentus* as the maternal parent, did not show a pattern consistent with reinforcement, which was expected because pollinators do not transfer pollen in this direction (Kay and Schemske 2008). Greenhouse studies have identified decreases in hybrid seed germination and pollen fertility in both the F1 and first backcross generation (Kay 2006), and this postzygotic isolation may provide the selection for pollen–pistil incompatibility.

It is interesting that both species have slower pollen tube growth rates on heterospecific pistils (Fig. 5). This may be an indication that species-specific divergence is occurring, possibly as a result of intraspecific sexual conflict or sexual

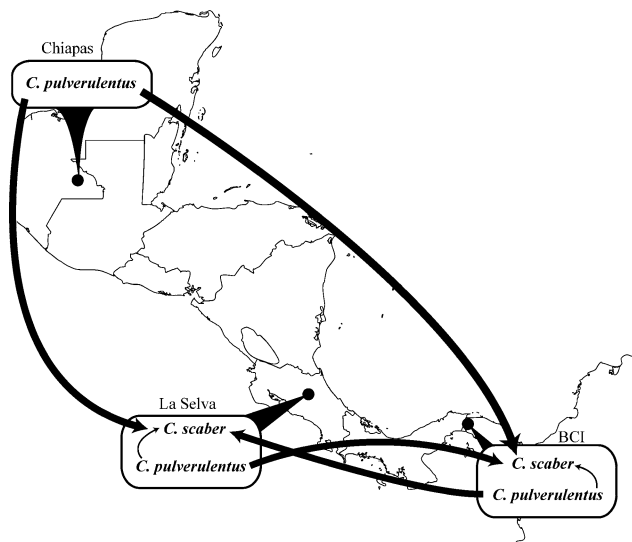


**Fig. 2** The majority rule consensus tree constructed from the posterior distribution of trees. Pollination syndrome and corolla length were reconstructed using parsimony. In lineages with hummingbird pollination, corolla length decreases (with the exception of *C. stenophyllus*). Photographs by Kay and Schemske



**Fig. 3** Postpollination isolation between sympatric *C. pulverulentus* and *C. scaber*, adapted from Kay (2006). **a** The number of germinated pollen grains for *C. scaber* as the maternal parent with *C. scaber* and *C. pulverulentus* as pollen donors. Differences in pollen adhesion and percent germination combine to give an overall difference in the number of germinated pollen grains per pollination. **b** For

*C. pulverulentus* as the maternal parent, differences in the final length of the pollen tubes contribute to a large difference in the number of pollen tubes reaching the ovary. The dashed line represents the average style length of *C. pulverulentus*, and therefore the total distance pollen tubes need to grow. *Costus scaber* pollen tubes do not grow the length necessary to reach the *C. pulverulentus* ovary

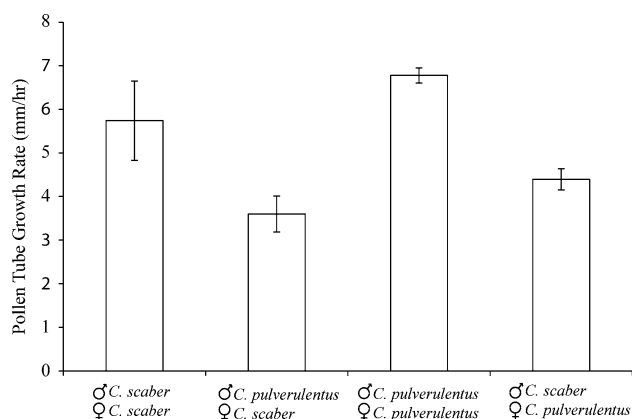


**Fig. 4** Map depicting the locations of *C. pulverulentus* and *C. scaber* and the relative success of the interspecific crosses, adapted from Kay and Schemske (2008). Arrows point in the direction of pollen transfer. Heavy arrows indicate higher crossing success (>60% relative seed set), and light arrows indicate poor crossing success (<30% relative seed set)

selection. However, we did not directly address this hypothesis.

## Discussion

In certain situations, postpollination reproductive barriers play an important role in isolating taxa. Known mechanisms of postpollination isolation include lower adhesion and germination rates, slower pollen tube growth rates, and pollen tube attrition. While many studies have documented these mechanisms operating in experimental crosses, few have documented the role they play in nature when other



**Fig. 5** Pollen tube growth rates (mm/h) for intraspecific and interspecific crosses of *C. pulverulentus* and *C. scaber* plants from La Selva

reproductive barriers may be functioning at different stages. Even fewer studies, especially in plant systems, have attempted to address the evolutionary origins of postmating isolating mechanisms.

Using the genus *Costus*, we have documented numerous postpollination barriers that result in low interspecific seed set. In 26 crosses of known style length, there is a marked decrease in seed set when paternal style length is shorter than maternal style length, suggesting pollen tube attrition. A detailed examination of crosses between *C. pulverulentus* and *C. scaber* reveals that other postpollination barriers are functioning. In this case, the disadvantage of heterospecific pollen is due to differences in adhesion, germination rates, and slower pollen tube growth rate, all of which contribute to the lack of hybrids observed in nature. Alternatively, when we hand-pollinate *C. pulverulentus* with *C. scaber* pollen, a transfer that does not happen in nature, we find no difference in adhesion or germination success, but pollen tube attrition occurs in the longer style of *C. pulverulentus*. This study contributes to a body of evidence indicating that postpollination barriers may be important in inhibiting hybridization between taxa experiencing pollen transfer in nature.

Using this knowledge of postpollination barriers, we have attempted to address two of the three hypotheses for how they might evolve. We find evidence throughout the genus that postpollination barriers may arise as an incidental consequence of adaptive changes in floral morphology, an idea originally proposed by Grant (1966). Grant (1965) also proposed that postpollination barriers in plants might evolve as the result of reinforcement, or direct natural selection for reproductive isolation. Within the leafy-stemmed gillias, Grant found strong incompatibility barriers between sympatric taxa, whereas allopatric taxa crossed easily. However, Grant lacked information on phylogenetic relatedness and the prevalence of interspecific pollen transfer in nature. In contrast, with *Costus* we are able to show that strong postpollination barriers function between closely related species in the face of substantial sympatric pollen transfer by shared pollinators. With crosses from across a geographic range, we find evidence that reinforcement has led to the evolution of postpollination barriers between locally sympatric populations of *C. pulverulentus* and *C. scaber*, and we are able to detail the mechanisms responsible for isolation. The results summarized here suggest that reinforcement may indeed play an important role in causing postpollination barriers.

As postpollination barriers are studied in more plant taxa, we likely will find more cases in which they are critical in the isolation and coexistence of species. This area of research is exciting because it reveals a whole suite of traits in both male and female plant structures that can function to isolate species in the face of pollen transfer.

Currently, with so few well-documented examples of postpollination isolation in nature, it is difficult to make predictions regarding when postpollination barriers are likely to function and how they may arise. In order to increase our understanding of postpollination reproductive isolation, we must continue to characterize the mechanisms conferring isolation. Molecular biologists are quickly elucidating the molecular mechanisms responsible for species-specific fertilization (Howard 1999; reviewed in Swanson et al. 2004) allowing a greater understanding of the genetic basis of these barriers. When we fuse our understanding of the molecular mechanisms with the evolutionary implications of reduced gene flow, we will have a greater understanding of how species are formed and maintained.

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