

## SELF-POLLINATION BY SLIDING POLLEN IN *CAULOKAEMPFERIA COENOBIALIS* (ZINGIBERACEAE)

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*Caulokaempferia coenobialis* (Zingiberaceae) forms dense populations on steep cliffs in shady, humid monsoon forests in south China. It produces few consecutively opening bright yellow flowers that are 3 cm long and oriented parallel to the ground. Upon anther dehiscence at about 0600 hours, each pollen sac releases a drop of pollen onto the horizontally oriented style, and the two drops then merge to form an oily film that slowly flows toward the stigma, carrying out self-pollination between about 1500 and 0730 hours the next day. The distance covered by the pollen film is ca. 3 mm. There is no significant difference in fruit set between experimentally cross- and self-pollinated flowers or between naturally pollinated and bagged flowers. The low pollen/ovule ratio of  $6 \pm 4$  probably relates to the pollen grains being held together by pollen-connecting threads. The latter ensure that pollen grains always arrive as multiples, and this is the first report of such threads in the Zingiberaceae. During 35 h of observation at several locations and during three flowering periods, only three individual bees, five flies, and two butterflies visited single flowers. It remained unclear whether they affected pollination because no return visits were observed. The automatic selfing by pollen that reaches the stigma ca. 9 h after the onset of anthesis apparently constitutes a case of delayed selfing, providing reproductive reassurance in situations of low pollinator visitation.

**Keywords:** *Caulokaempferia*, delayed selfing, floral morphology, pollenkitt, self-pollination, pollinator limitation.

### Introduction

Vogler and Kalisz (2001) suggest that 20% of angiosperms may be self-fertilized and another 33% intermediate between selfing and outcrossing (so-called mixed mating). This high frequency of self-pollination in angiosperms has to be placed in an ecological context, the foremost factor being seasonal and interannual variation in pollinators. Where pollinators occur in low numbers or are absent, individuals that can self-pollinate when they have not been outcrossed early during anthesis are at a selective advantage (Lloyd 1979, 1992; Kalisz et al. 1999, 2004 and references therein). This hypothesis is supported by theoretical studies (Schoen and Brown 1991; Morgan et al. 1997) and empirical observations (Kalisz et al. 2004). Both show that reproductive assurance via delayed selfing can selectively maintain a mixed-mating system. However, even though it is now well established that self-fertilization characterizes as many as a fifth of all flowering plants (Vogler and Kalisz 2001; Barrett 2002), no data exist on the frequency of accidental deposition of self-pollen on stigmas (by animals, wind, or water) compared with active self-pollination by mechanisms that deposit a flower's pollen on its own stigma. In animal-pollinated flowers, which are adapted for placing pollen grains onto vectors, it is often morphologically difficult or impossible for an anther to place pollen onto its

own stigma when it is receptive, precluding facultative self-fertilization. Only a few flowers are capable of bending stigmatic lobes toward pollen-carrying surfaces late during anthesis (Campanulaceae), bending stamens toward stigmas (*Collinsia*; Kalisz and Vogler 2003), bending pollinaria onto stigmas (Orchidaceae; Dressler 1981), or even selfing in bud (Anderson 1980). However, many more mechanisms probably remain to be discovered because more attention has been paid to mechanisms for outcrossing than to mechanisms for automatic selfing. During fieldwork in subtropical China, we recently discovered a new such mechanism, namely, the sliding of self-pollen along the style and into a relatively large stigmatic cavity (Wang et al. 2004b). Here we provide details of this form of selfing, which so far is known only from *Caulokaempferia coenobialis* (Hance) K. Larsen in the Zingiberaceae.

*Caulokaempferia* is a genus of ca. 10 species in India, Burma, China, Thailand, Laos, and Vietnam (Larsen 1964, 2002; Larsen and Smith 1972; Larsen and Jenjittikul 2004). Its phylogenetic affinities within Zingiberaceae are unresolved, and there is some indication that it may be polyphyletic (Kress et al. 2002; Larsen 2002; Williams et al. 2004). *Caulokaempferia coenobialis* is the only species in China (Wu and Larsen 2000), where it occurs on humid cliffs in monsoon forests. In general, the biology and mating systems of Zingiberaceae, a family of an estimated 1200 species (Williams et al. 2004), are still poorly known. Field studies so far have found butterflies, moths, bees, and rarely birds as legitimate pollinators (Classen 1987; Stone and Willmer 1989;

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Ippolito and Armstrong 1993; Cui et al. 1996; Larsen et al. 1999; Sakai et al. 1999; Li et al. 2001, 2002; Zhang et al. 2003). Seed dispersal appears to be by rain splash (Larsen et al. 1999). Andromonoecy, polygamoecy, and other forms of flower dimorphisms have been reported from *Amomum* and *Alpinia* (Cui et al. 1996; Sakai and Nagamasu 1998; Li et al. 2001; Zhang et al. 2003). On the basis of its diurnal anthesis; relatively long, narrow nectar tube; vivid yellow color; and size, exposure, and orientation of the flowers, we expected *C. coenobialis* to be butterfly pollinated. Initial observations in the natural habitat, however, revealed absolutely no pollinator activity. We therefore began to suspect that selfing and/or clonal propagation played roles in the reproduction of *C. coenobialis*. Given the flower morphology of Zingiberaceae (see "Results"), it was difficult to imagine how self-pollen might reach the stigmatic area. To investigate possible adaptations for outcrossing or selfing, we observed six populations of *C. coenobialis* at sites up to 150 km apart, measured nectar production, undertook experimental selfing and outcrossing, and determined the position and size of the stigmatic area and the pollen/ovule (P/O) ratio, both rough indicators of a plant's predominant mode of pollination.

## Material and Methods

### *Study Sites, Flowering Phenology, and Flower Visitors*

Three populations of *Caulokaempferia coenobialis* were studied in the nature reserve Din-hu Shan (approximately lat. 112°32'E, long. 23°10'N, 477–483 m above sea level [a.s.l.]) and three in the nature reserve Nan-kun Shan (approximately lat. 113°51'E, long. 23°38'N, 316 m a.s.l.), all in Guangdong Province. Populations comprised between ca. 2000 and 10,000 plants. A voucher specimen (Y. Wang 29) has been deposited in the herbarium of the South China Botanical Garden. Multiannual observations of Zingiberaceae flowering in Guangdong have shown that *C. coenobialis* flowers between May and August. Six natural populations of this species were therefore visited repeatedly between May and August 2002, 2003, and 2004, with observation frequency depending on whether populations were flowering. Anther dehiscence was monitored every 2 h between 0630 and 1830 hours, and flowers were monitored for insect visitors during a total of 35 h on 20 d, distributed as follows: June 15–18, 2002, at Din-hu Shan: 0730–0830, 0930–1030, 1130–1230, 1330–1430, 1630–1730 hours; July 8–10, 2002, at Nan-kun Shan: 0730–0830, 1000–1100, 1300–1400, 1530–1630, 1730–1830 hours. In addition to these observation periods, occasional checks for flower visitors were made at Din-hu Shan on June 12–13 and July 4, 2003, and at Nan-kun Shan on May 27–28, 2003; June 2–4, 2003; June 23–25, 2003; May 10, 2004; and July 11 and July 20, 2004. At any one time, up to 20 inflorescences could be monitored. All flower visitors and their visiting times were recorded, and insects were then captured for identification.

### *Measurement of Nectar Volume, Sugar, and Amino Acid Concentration*

We randomly selected 10 freshly opened flowers and with 1- or 2- $\mu$ L capillaries extracted their nectar every 2 h be-

tween 0700 and 1700 hours. Nectar sugar concentration was measured with a handheld temperature-compensated refractometer. Amino acid concentration was measured following Dafni (1992) by dissolving 3.9 mg/mL of histidine in a 20% sucrose solution, and from this stock, we prepared a series of 50% dilutions, of which 2- $\mu$ L drops were placed on several filter paper strips. After these calibration scales had dried, we added 2  $\mu$ L of ninhydrin to nectar spotted onto filter paper and to the diluted histidine reference drops. Paper strips were left at room temperature for 24 h, and the color intensity of the nectar samples was then compared with that of the calibration drops to estimate amino acid concentrations.

### *Experiments to Determine the Mating System*

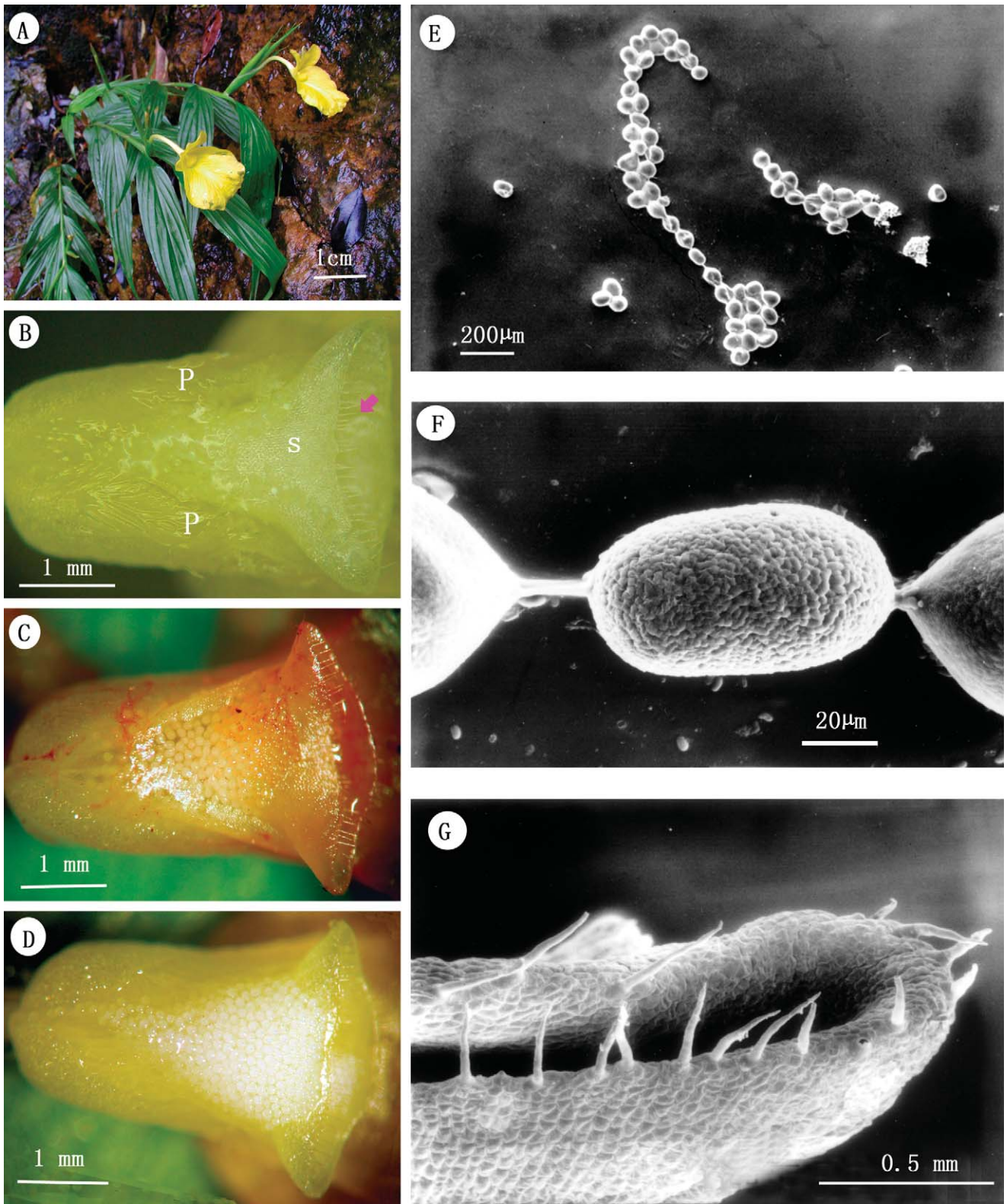
From May to August 2002 and 2003, we performed the following pollination treatments in two populations at Din-hu Shan and in three populations at Nan-kun Shan: (1) open pollination: flowers were marked before anthesis and then left exposed; (2) agamospermy A: stamens were removed just before flower anthesis, and flowers were then enclosed in bags; (3) agamospermy B: stigmas were removed just before anthesis, and flowers were then enclosed in bags; (4) automatic selfing: flowers about to open were enclosed in bags; (5) experimental selfing: freshly opened flowers were hand-pollinated with self-pollen and then enclosed in bags; (6) experimental outcrossing: pollen from plants at least 5–10 m away was placed directly onto the stigmas of freshly opened flowers.

### *Pollen and Stigma Morphology, Pollen Fat Content, and P/O Ratios*

Flower morphology was studied under a stereomicroscope that had been brought to the field. Details of pollen and stigmas were later also observed under a scanning electron microscope (the stigmas after sputter-coating them). Fresh pollen was stained (in a makeshift laboratory in the field) with dilute Sudan III and Sudan IV solutions to test for fatty pollenkit (Dafni 1992), and P/O ratios were obtained by dissecting fresh flowers as described by Wang et al. (2004a). To determine the speed of the sliding pollen film, we randomly selected flowers on 20 plants and measured the distances between the pollen film's advancing front and the stigma rim at different times during anthesis under a magnifying glass (directly in the field). To assess the possible influence of ambient humidity on pollen sliding, a few plants with almost mature flower buds were potted and observed indoors at 25%–65% relative humidity.

### *Pollen Viability and Stigma Receptivity*

We used dimethylthiazol-diphenyl-tetrazolium bromide (MTT) to test for the presence of dehydrogenase in the pollen (Rodríguez-Riaño and Dafni 2000). Following these authors' protocol, we dissolved MTT in a sucrose solution and strained the solution through a filter paper. Pollen samples were placed in a drop of this reagent, mixed, and set to dry on a microscope slide. After adding a droplet of glycerine to the stained dry samples, they were studied under a microscope. Dark purple-brown staining indicates the presence of dehydrogenase, which is a sign of pollen viability. The same test was used to assess stigma receptivity and the viability of



**Fig. 1** Pollen sliding in *Caulokaempferia coenobialis*. *A*, Plant growing on a rock outcrop. *B*, Stigma (*S*) and pollen sacs (*P*) in the natural horizontal position; the stigma is ca. 3 mm in diameter. The arrow points to the hair fringe that surrounds the stigmatic cup. *C*, Pollen films coming from the two pollen sacs. *D*, Pollen film on the style surface; individual grains visible in the oily film. *E*, Chain of pollen grains held together by pollen-connecting threads (scanning electron micrograph [SEM]). *F*, Detail of a pollen-connecting thread (SEM). *G*, Stigma surface with its surrounding fringe of hairs (SEM).

pollen grains already on the stigma (Rodríguez-Riaño and Dafni 2000).

## Results

### *Plant Habit, Flower Morphology, and Flowering Phenology*

*Caulokaempferia coenobialis* is a deciduous perennial herb that is endemic in Guangdong and Guangxi in South China. It grows semipendant on steep rocks or cliffs in monsoon forests, always in localities with high humidity (>97%). In its natural habitats, *C. coenobialis* forms monospecific patches of several square meters, and the populations we studied comprised many hundreds of flowering individuals. The plants are 16–53 cm tall and slender, with a main stem 8–36 cm long and ca. 3 mm wide. The five to 11 glabrous leaves are sessile or shortly petiolate, with lanceolate blades ca. 5–20.6 × 1–2.5 cm in size. The terminal inflorescences consist of one to three (occasionally four to five) conspicuous bracts, each enclosing one to three flowers, with one or rarely two flowers open at any one time (fig. 1A). The flower calyx is tubular and ca. 1–1.5 cm long, and the corolla tube is 2.9–4.9 cm long and narrow but with a widened mouth. Flowers are yellow and have two short lateral lobes (staminodes) and one large central lobe (labellum) up to 3 cm long (fig. 1A). The androecium morphology of the genus is typical of the family, with a single anther that has two elongated, lengthwise-dehiscing pollen sacs that enclose the style. The anther is ca. 3 mm long. The anther connective is crest shaped to oblong and ca. 4 mm long. The concave stigma lies almost exactly at the end of the pollen sacs (fig. 1B), and the receptive surface (as assessed by MTT staining) is the concave surface surrounded by the hair fringe visible in figure 1D.

Petals unfolded at about 0600–0630 hours and stayed spread for 2 d (ca. 36 h). They began to fade after 1500 hours on the second day and had withered by 1800 hours. The flowering period of a single inflorescence lasted 9–22 d. Capsules matured and dehisced lengthways within another 22 d. Mature capsules are ovoid-oblong and measure 1.2–1.9 × 0.3–0.5 cm.

At our study sites, *C. coenobialis* flowered from early May to early August. Flowering at sites with only 92%–97% rela-

tive humidity occurred earlier than that at sites with >97% humidity (table 1). The lag time between the earliest- and the latest-flowering plants in a population was 2 mo.

### *Pollen Morphology and Viability and Stigma Receptivity*

Pollen grains of *C. coenobialis* are elongate with a psilate testa (fig. 1F). Pollen and stigmas tested with the MTT test 1 d before anthesis showed neither viability nor receptivity. In newly opened flowers in which the labellum had barely spread and the pollen sacs had just dehisced, pollen viability was 98%, and stigma receptivity was 0. After the labellum had completely unfolded, pollen viability and stigma receptivity were 99.5% and 100%, respectively. During anthesis, stigma receptivity stayed at 100% and returned to 0 on the morning of the third day only after flowers had faded. Pollen viability also remained at a high level (>86%) up to 1600 hours but went down to 66.20% after 1800 hours on the second day and to 50% by 0830 hours on the third day, when flowers had wilted.

### *Nectar Volume, Sugars, and Amino Acid Concentration*

Mean nectar volume over the entire period of anthesis was  $1.33 \pm 0.42 \mu\text{L}$  ( $n = 10$ ), with a sugar concentration of  $17.25\% \pm 0.83\%$  and an amino acid concentration of ca. 0.008 mg/mL. The peak nectar volume of  $1.92 \pm 0.643 \mu\text{L}$  was reached at about 1500 hours, thereafter descending to  $1.76 \pm 0.57 \mu\text{L}$  at about 1700 hours (fig. 2).

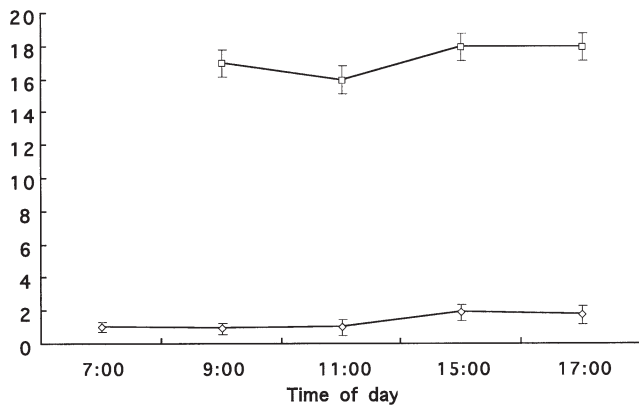
### *Flower Visitors*

During the 35 h of observation, five flies (Syrphidae), three bees (one Mellitidae, two Apidae), and two butterflies were recorded visiting the flowers. The flies were abundant in the habitat and occasionally landed on a labellum but would not proceed to nectar foraging, nor did we see them visit several flowers in a row. The single mellitid bee also landed on a labellum and then left. The apid bees and the butterflies were seen visiting single flowers, but it was not clear whether they affected pollination; no return visits were seen.

**Table 1**

**Flowering Phenology of *Caulokaempferia coenobialis* in Six Populations at the Nature Reserves Din-Hu Shan and Nan-Kun Shan in Guangdong Province, China**

	Humidity (%)	Onset of flowering	Peak of flowering	End of flowering
Din-hu Shan:				
Population 1	>97	June 14, 2002	June 20, 2002	July 3, 2002
Population 2	>97	June 20, 2002	July 3, 2002	August 3, 2002
		June 10, 2003	June 20, 2003	July 4, 2003
Population 3	>97	June 10, 2002	June 20, 2002	July 3, 2002
		June 10, 2003	June 20, 2003	July 4, 2003
Nan-kun Shan:				
Population 1	>97	June 20, 2002	July 6, 2002	August 3, 2002
		May 27, 2003	June 23, 2003	July 16, 2003
		June 10, 2004	July 20, 2004	August 10, 2004
Population 2	92–97	May 10, 2003	May 23, 2003	June 1, 2003
		May 10, 2004	June 10, 2004	July 11, 2004



**Fig. 2** Mean nectar volume ( $\mu\text{L}/\text{flower}$ ) (lower line) and mean sugar concentration (%) (upper line) in *Caulokaempferia coenobialis* ( $\pm\text{SD}$ ,  $n = 10$ ).

### Breeding System

Fruit set following self-pollination and cross-pollination did not differ significantly ( $t = 0.6622$ ,  $P > 0.05$ ), nor was there a significant difference between fruit set in control flowers and flowers bagged before opening ( $t = 0.1717$ ,  $P > 0.05$ ; table 2). The species is incapable of asexual seed set as shown by the absence of seed set in flowers that had their stamens or stigmas cut off before they could self-pollinate.

### Automatic Self-Pollination by Sliding Pollen

Pollen grains are held together by pollen-connecting threads (fig. 1E, 1F) and have an abundance of an oily matrix (pollenkitt?) on their surface as confirmed by staining with Sudan III and Sudan IV. The oily matrix is clear and apparently rich in unsaturated lipids, judging from its low viscosity, suggesting short fatty acids. It forms an oily film in which the pollen grains are suspended. Soon after anther dehiscence in the morning, pollen oozes from each pollen sac onto the style. The pollen drops then merge (fig. 1C) to form a film that quickly spreads over the style surface and slides toward the stigma (fig. 1D). The minute adnate hairs along the style (fig. 1B, 1G) may help guide it toward the stigma. The speed of the advancing pollen film is ca. 3 mm/13.5 h, and it did not change in plants brought into the laboratory at

25%–65% relative humidity. At about 1500 hours, 9 h after anther dehiscence, pollen arrives at the stigma margin and passes between the row of fine hairs (fig. 1D), at which point self-pollination begins and continues until the next morning. Pollen sliding in potted plants brought indoors occurred just as it had in the natural habitat, but anthesis of flowers lasted only 1 d instead of 2 d.

## Discussion

### The Mechanism of Pollen Sliding

Pollen grains in *Caulokaempferia coenobialis* are surrounded by an abundant oily matrix, probably pollenkitt. Pollenkitt is a mixture of hydrophobic components produced by the inner lining of the anther (tapetum) and saturated and unsaturated lipids. In addition, it contains a species-specific admixture of carotenoids, proteins, and carboxylated polysaccharides (Dobson 1988). The pollenkitt in *C. coenobialis* is clear and rich in unsaturated lipids so that it forms a film in which the pollen chains appear suspended. Abundant oily pollenkitt is also found in other angiosperms, especially bee-pollinated ones (Dobson 1988), but sliding of pollenkitt as a means of transporting pollen grains into a flower's own stigma had not been reported before our study of *C. coenobialis*. Because the flowers are oriented horizontally, the pollen film's active flowing along the style toward the stigma must be guided mostly by the minute adnate hairs along the style surface that point toward the stigma rim (fig. 1B, 1G). Like other Zingiberaceae, *C. coenobialis* has a secretory, papillose stigma surface (Heslop-Harrison and Shivanna 1977), and stigma exudates may play a role in the final stage when the pollen film enters the stigma cup by flowing between the setae that mark the stigma rim. These setae may originally have functioned in cross-pollination, perhaps to dislodge pollen grains from visitors, or they may help keep pollen inside the stigmatic cup. The 2-d flowers of *C. coenobialis* allow considerable time for the pollen film to reach the stigma and carry out self-pollination between the late afternoon of the first day of flowering and 1800 hours the next day, when flowers wither.

Pollen grains in *C. coenobialis* are held together by pollen-connecting threads. The occurrence of pollen-connecting threads that do not contain sporopollenin is restricted to a few genera in 12 families of angiosperms (Hesse et al. 2000), including *Strelitzia reginae* and possibly species of

**Table 2**

**Fruit Set in *Caulokaempferia coenobialis* Following Experimental Treatments**

Treatment	No. flowers	Fruit set (%)		
		Mean	SD	CV
Open pollination	95 (50)	78.89	12.63	16.01
Automatic selfing (bagged flowers)	54 (54)	75.93	22.37	29.46
Experimentally outcrossed	49 (49)	97.96	4.97	5.07
Experimentally selfed	49 (49)	95.92	4.77	4.97
Agamospermy A: anthers removed in bud	50 (50)	0	0	0
Agamospermy B: stigma removed in bud	50 (50)	0	0	0

Note. CV = coefficient of variation.

*Heliconia* (Kronstedt and Bystedt 1981; Rose and Barthlott 1995). This study adds the Zingiberaceae to the list of families possessing pollen-connecting threads.

#### *Insect Pollination Compared with Automatic Selfing in Caulokaempferia*

Despite its autogamy, *C. coenobialis* retains characteristics of a bee- or butterfly-pollinated flower, such as relatively large bright yellow flowers with a landing platform, nectar offered in a narrow tube, and sticky pollen produced in small amounts. This indicates that autogamy in this species evolved from allogamy, perhaps in response to pollinator limitation. (Pollination in the other nine species of *Caulokaempferia* has not been studied, and no comparative assessment is possible.) Autogamy occurs some 9 h after the onset of anthesis, making *C. coenobialis* a clear case of delayed selfing, a strategy in which self-pollination is delayed until after the opportunity for outcrossing has passed. Such a system incurs no costs under conditions of high insect abundance but is selectively advantageous when pollinators or pollen are limiting (Kalisz et al. 2004). If this interpretation is correct, it would explain why nectar production is maintained. Nothing is known about occasional gene flow via insect pollination, and a study of the genetic variability within and among different populations of *C. coenobialis* would be interesting. Prolonged autogamy is often accompanied by a reduction in the number of pollen grains per flower, and *C. coenobialis* with an average P/O ratio of  $6 \pm 4$  (Wang et al. 2004a) may be an extreme

example of this (for compilation of P/O ratios, see Cruden 1977). However, the P/O ratios and possible possession of pollen-connecting threads in closely related species are not known, and it therefore cannot be decided whether the low P/O ratio in *C. coenobialis* relates to the chainlike pollen strings and pollen film or to selfing per se. Other species with pollen grains connected via threads or transported as polyads also often have low P/O ratios (e.g., Cruden and Jensen 1979; Schlising et al. 1980; Vasek and Weng 1988; Wyatt et al. 2000). It has been proposed that agglutination of pollen evolves to minimize the likelihood of receiving mixed pollen loads (Wyatt et al. 2000). However, in our populations, mixing of pollen loads by insect visitors seems an implausible selective factor behind the evolution of pollen-connecting threads, and it appears more likely that they serve to enhance the efficient sliding of an unbroken film of pollen.

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