

Mating system and stigmatic behaviour during flowering of *Alpinia kwangsiensis* (Zingiberaceae)

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Abstract. Little is known about the breeding systems of tropical perennial ginger species. In this paper, we provide information about the breeding system of *Alpinia kwangsiensis* in Yunnan, Southwest China, specifically self-compatibility, mechanisms promoting outcrossing, and the visitation characteristics of pollinators. Populations of *A. kwangsiensis* have two specific phenotypes that differ in flowering behaviour: 1) “cataflexistyle” individuals in which the stigma is held erect above the dehiscent anther when anthesis begins in the morning and becomes decurved under the anther at noon and 2) “anaflexistyle” individuals in which the receptive stigma is decurved under the indehiscent anther first and moves into a reflexed superior position above the anther as it begins to shed pollen at mid-day. The stigmatic movements in the two floral phenotypes, which occur in a ratio of 1:1 in natural populations, are synchronous and correlate with the foraging behaviour of floral visitors; pollination is effected only between floral forms. Field experiments indicate that *A. kwangsiensis* is self-compatible and dependent upon insects for fertilization. This newly reported floral mechanism, which we have named “flexistyle,” adds to the repertoire of devices that have evolved in flowering plants to insure outcrossing.

Key words: *Alpinia kwangsiensis*, anaflexistyle, cataflexistyle, flexistyle, pollination, stigma behaviour, tropics, Zingiberaceae.

Inbreeding depression, a common phenomenon in plants, causes a reduction in fitness in the progeny of closely related individuals (Darwin 1876, Charlesworth and Charlesworth 1987) and is an important selective pressure in the evolution of plant mating systems (Charlesworth and Charlesworth 1987, 1990; Lande and Schemske 1985; Holsinger 1988, 1991). Selfing has many benefits, such as the ability to colonize new habitats (Baker 1955, Stebbins 1957), the avoidance of unreliable pollinators (Darwin 1876, Lloyd 1979, Piper et al. 1984), the maintenance of local adaptations in plant populations (Antonovics 1968), and the automatic genetic transmission advantage to offspring (Jain 1976). However, the resultant inbreeding depression that follows repeated selfing (Darwin 1876, Antonovics 1968) has led to strong selection for diverse mechanisms that promote outcrossing, including mechanisms that separate pistillate and staminate structures in time (dichogamy, heterodichogamy), space

(herkogamy, enantiostyly, heterostyly), and physiology (self-incompatibility [SI]) (Wyatt 1983, Endress 1994, Richards 1997).

Dichogamy, the separation of pistillate and staminate phases in hermaphroditic (bisexual) flowers or monoecious individuals, decreases the probability of self-fertilization in self-compatible (SC) species (Stout 1928). "Heterodichogamy differs from normal dichogamy in the presence of two genetic morphs in a population that occur in a 1:1 ratio: one floral morph starts with the female phase followed by the male phase, the other floral morph has the opposite sequence" (Renner 2001). The phenomenon in some plants in which various floral parts move during anthesis has been recognized by several researchers (Stout 1927, Endress 1984, Fetscher and Kohn 1999), but it has been either ignored as an outcrossing mechanism (Richards 1997), considered as a type of dichogamy or herkogamy (Fægri and van der Pijl 1979, Lloyd and Webb 1986, Webb and Lloyd 1986, Endress 1994), or interpreted as a mechanism to favor pollen retention on the stigma (Fetscher and Kohn 1999). Here, we present an unique floral mechanism involving extreme stigmatic movement, which we called flexistyly, that enhances the outcrossing rate in plants (Li et al. 2001a).

Alpinia, a member of the ginger family (Zingiberaceae), comprises more than 250 species found in Southeast Asia extending from Japan in the north to Australia in the south and into the Western Pacific (Smith 1990). Members of the genus are perennial herbs, usually 1.5–4 m tall with leafy, many-bladed and ginger-like shoots. The terminal inflorescences on the leafy-shoots are made up of congested bracts with each bract subtending a single hermaphroditic flower or a cincinnus with two to several flowers. The flowers (Figs. 1–2, 4–5) are composed of relatively inconspicuous sepals and petals, but have a petaloid, showy labellum (derived from two fused staminodes), which is often united with the single fertile stamen. The free part of the labellum is expanded as a landing platform for visiting insects. Each inflorescence produces

two to ten open flowers per day, each flower lasting only one day, with the blooming season extending over a two-month period.

As is true of other genera in the ginger family, but puzzling considering their diverse and conspicuous flowers, relatively few studies have been conducted on the pollination ecology of *Alpinia* (Endress 1994, Sakai et al. 1999). A limited number of investigations have documented visitors to the flowers of *Alpinia* (Müller 1888, Porsch 1924, van der Pijl 1954), and their mating systems (Porsch 1924, Endress 1994). In addition, movement of styles in this genus has been observed (Roxburgh 1820, Smith 1990), but there are no complete studies of mating systems or pollination biology. Here we report on details of the pollination biology and the mating systems of *A. kwangsiensis* T. L. Wu & S. J. Chen, one of the most common ginger species in tropical areas of south China and the neighbouring Southeastern countries. This species as well as other congeners, have a specific stigmatic behaviour that appears to promote outcrossing in local populations. The objectives of our study were: (1) to document the behavioural details of the flowers during anthesis; (2) to test the levels of self-compatibility and the probability of selfing; (3) to determine if stigmatic behaviour is a mechanism that prevents selfing and promotes outcrossing; and (4) to ascertain if the behaviour of floral visitors is correlated with floral behaviour.

Materials and methods

Study site. The study was conducted from March to July, 2000, in a protected forest area of Xishuangbanna Tropical Botanical Garden (XTBG) in southern Yunnan (21°45'N, 101°02'E; 580 m in altitude). The vegetation of the research site is tropical seasonal rainforest dominated by *Pometia tomentosa* and *Terminalia myriocarpa* with an average canopy height of 30 m (Zhang and Cao 1995).

Plant description. *Alpinia kwangsiensis* is a perennial herb, that inhabits tropical forest gaps and roadsides. Plants are usually 1.5–3 m tall with many-bladed leafy shoots. Inflorescences are ter-

minal on leafy-shoots and composed of congested bracts, with each bract subtending a single flower. The petaloid, red and yellow showy labellum is fused with the single stamen to form a tube, the free part of which is expanded and forms a landing platform for visitors. During blooming, each inflorescence produces 2 to 10 open flowers per day, each flower lasts one day, and the entire blooming season extends about 2 months.

Observations of floral behaviour. Observations of floral behaviour were conducted in the protected forest and a cultivated ginger collection at XTBG. Eighteen inflorescences were observed over three days (April 3–5); every day, three individuals of each floral form (see below) were observed from early morning (ca. 05:30) to late evening (ca. 22:00). For each flower, the stigmatic position (measured as the angle with the anther's ventral face) was observed, recorded, and photographed every thirty minutes during the morning and afternoon, and every ten minutes at mid-day (11:30–15:00, during flower movement). The time of anther dehiscence in both flower forms was also recorded. In addition to these three days of intensive observation and one month of experimental manipulations, the flowering behaviour of both phenotypes was observed under different pollination and weather conditions.

Observations of flower visitors. Visitors and visitation frequencies to *Alpinia kwangsiensis* were observed each day from 08:00 to 18:00. All visitors and their visiting times were recorded. After observations were completed, at least three individuals of each visitor morphospecies were captured and identified.

Pollination experiments. To examine the breeding system of *Alpinia kwangsiensis* and the contribution of insect visitors to pollination, we performed seven experimental treatments on 86 individual plants. Treatments were: (1) Natural pollination: flowers on 17 anaflexistyle inflorescences (seven of them broke before mature) and 10 cataflexistyle inflorescences were left exposed, permitting visitation by insects naturally. (2) Bagged: flowers on 16 inflorescences (eight of each phenotype) were bagged before anthesis. (3) Partial-bagged: flowers on 6 inflorescences (three of each phenotype) were bagged for part of the time during anthesis when the stigma was not in a receptive position and pollen was being presented; cataflexistyle flowers were released in the morning and bagged in the afternoon; anaflexistyle

flowers were bagged in the morning and opened in the afternoon. To determine the receptivity of the stigma and the germination abilities of pollen at different gender phases with self- and cross-pollination, four artificial pollination treatments were included, (4) AM-selfing (selfing in the morning): flowers of 3 inflorescences of anaflexistyle individuals and 6 inflorescences of cataflexistyle individuals were hand-pollinated with geitonogamous or xenogamous pollen of the same floral phenotype in the morning. (5) AM-crossing (crossing in the morning): flowers of 6 inflorescences of anaflexistyle individuals were hand-pollinated with pollen of the opposite phenotype; in this treatment, cataflexistyle flowers as pollen receptors were omitted in the morning because the anaflexistyle flowers did not release pollen at that time. (6) PM-selfing (selfing in the afternoon): flowers of 7 inflorescences of anaflexistyle individuals and 5 inflorescences of cataflexistyle individuals were hand-pollinated with geitonogamous or xenogamous pollen of the same floral phenotype in the afternoon. (7) PM-crossing (crossing in the afternoon): flowers of 4 inflorescences (3 of them broke before mature) of anaflexistyle individuals and 6 inflorescences of cataflexistyle individuals were hand-pollinated with pollen of the opposite phenotype in the afternoon.

Randomly selected inflorescences were covered with nylon tricot bags before their first flower emerged on March 28, 2000. On subsequent days, hand pollinations were performed in the morning (08:00–10:00) and afternoon (15:00–17:00) for cataflexistyle and anaflexistyle flowers, respectively. For self-pollination of anaflexistyle flowers in the morning, we opened the indehiscent anthers with forceps and transferred the pollen to the same flower's downward stigma. These experimental manipulations lasted 28 days until all flowers had bloomed; in total, 86 individual plants and 7510 flowers were treated (Li et al. 2001b). Four months after blooming, when the fruits had matured, fruits on each treated inflorescence were counted and fruit set per inflorescence for each treatment were calculated (Table 1).

Statistical analysis. Floral phenotype ratios in the populations were compared using chi-square analysis (Zar 1984). Data on fruit set rate were compared among the treatments and phenotypes using a two-way analysis of variance (ANOVA; Norusis 1999). To determine which treatments differ from each other, we performed a multiple

Table 1. The fruit set of different pollination treatments of *Alpinia kwangsiensis*. To determine the compatibility of different phenotypes and the receptivity of the stigmas at different stages of anthesis, seven different pollination treatments within each morph (phenotype) were conducted on 86 inflorescences (individuals) including 7510 flowers. However, four months after blooming, only 76 inflorescences (N) within 6445 flowers remained due to natural and human activities. Morning (or afternoon)-cross: pollinations made between two phenotypes during the morning (or afternoon); morning (or afternoon)-selfing: pollinations made with self pollen during the morning (or afternoon); natural: pollination by natural visitors; bagged: inflorescences bagged and all visitors excluded during anthesis; morning (or afternoon)-bagged: inflorescences bagged during the morning (or afternoon). See text for additional description of each treatment. The fruit set were calculated from mature fruit numbers four months after anthesis divided by the total number of flowers within an individual inflorescence; values in parentheses are total numbers of flowers examined

Morph	Treatment	Fruit set per inflorescence			
		Mean (%)	Standard deviation	Covariance	N
Anaflexistyle (stigma goes up)	1a) Natural	33.52	12.78	38.13	10 (756)
	2a) Bagged	0.84	0.46	54.76	8 (770)
	3a) Partial-bagged	0.00	0.00	0.00	3 (149)
	4a) AM-selfing	37.50	22.77	60.72	3 (174)
	5a) AM-crossing	57.81	24.49	42.36	6 (524)
	6a) PM-selfing	52.31	19.25	36.80	7 (541)
	7a) PM-crossing	67.35	–	–	1 (49)
Cataflexistyle (stigma goes down)	1b) Natural	34.8	9.76	28.05	10 (938)
	2b) Bagged	8.04	7.33	91.17	8 (859)
	3b) PM-bagged	0	0	0	3 (121)
	4b) AM-selfing	40.64	15.83	38.95	6 (630)
	5b) AM-crossing	–	–	–	–
	6b) PM-selfing	41.07	10.40	25.32	5 (459)
	7b) PM-crossing	52.45	15.22	29.02	6 (475)

comparison of means with the statistic program of Biomstat (Applied Biostatistics, Inc., USA 1996); to test specifically whether cross-pollinations between the two phenotypes are different, we compared the means of the cross-pollination of the two morphs (phenotypes); to detect whether the fruit set of cross-pollination between the morphs differ from that of self-pollination within the same morph, or are there any inbreeding depression effects within the morph, we compared the fruit sets of selfing and cross treatments for each morph.

Results

Two flowering behaviours in the same species. All populations of *Alpinia kwangsiensis* studied have two phenotypes that differ in floral

behaviour. We have named the two phenotypes “anaflexistyle” and “cataflexistyle” (*ana*-meaning upwards; *cata*- meaning downwards; *flexi*- meaning bending). In the widely distributed natural populations of *Alpinia kwangsiensis* the ratio of individuals of the two phenotypes is 1:1 (86:78) ($\chi^2 = 0.39$, $P > 0.5$, $n = 1$), suggesting that these two phenotypes are under simple genetic control. The duration of anthesis of both phenotypes is 24 hours and begins before dawn. When the flowers are fully open (6:00–6:30), cataflexistyle plants have the stigma exerted above the anther, and their pollen sacs are dehiscent (Figs. 1, 7). At the same time, the receptive stigma of anaflexistyle flowers is curved downward below the undehiscent anther sacs from which pollen has not yet been shed

(Figs. 4, 8). Flowers of both types retain these respective positions until about mid-day (11:00–12:00). At that time the stigmas of anaflexistyle flowers begin to bend upward and become erect above the anther (Figs. 5, 7). As the stigma moves out of direct contact with any floral visitor ($a_1 > 170^\circ$, Fig. 7; 11:45–13:30), the anther dehisces and pollen is presented (14:30–15:00). The movement of the stigma of the cataflexistyle flower is slower: here the stigma begins to move downward and enters the receptive position ($a_2 < 170^\circ$, Fig. 8) between 14:40 and 15:00 (several minutes after anther dehiscence in anaflexistyle flowers; Figs. 3, 8). Anthesis in both forms ends after dusk (20:30–21:00) when the anthers collapse and the corolla hangs down limply. The speed of stigmatic movement varies depending on weather conditions, but all flowers of a given phenotype that open on the same day are strictly synchronous. The anaflexistyle anthers never dehisce before all of the same phenotype's stigmas have moved out of the receptive position.

Non-fertilized flowers abscise in two or three days, while the ovaries of fertilized flowers enlarge over the next several days; bracts and corollas of fertilized flowers remain attached almost until fruit maturity. In artificial pollinations, the behaviour of pollinated anaflexistyle individuals does not differ from that of unpollinated individuals. However, our preliminary observations indicate that the stigma movement in hand-pollinated cataflexistyle flowers was slightly delayed and less pronounced. This observation suggests that in cataflexistyle flowers, early pollination success requires no further movement of the stigma to effect fertilization. On the other hand, in anaflexistyle flowers, even if the stigma has been pollinated, natural selection may favor movement of the stigma to decrease the interference between stigma and anther during subsequent pollinator visits (Lloyd and Webb 1986).

Floral visitors and visitation frequency. The large flowers of *Alpinia kwangsiensis* attract many kinds of insects searching for nectar. However, successful pollination is only affected by large, solitary bees that contact both the

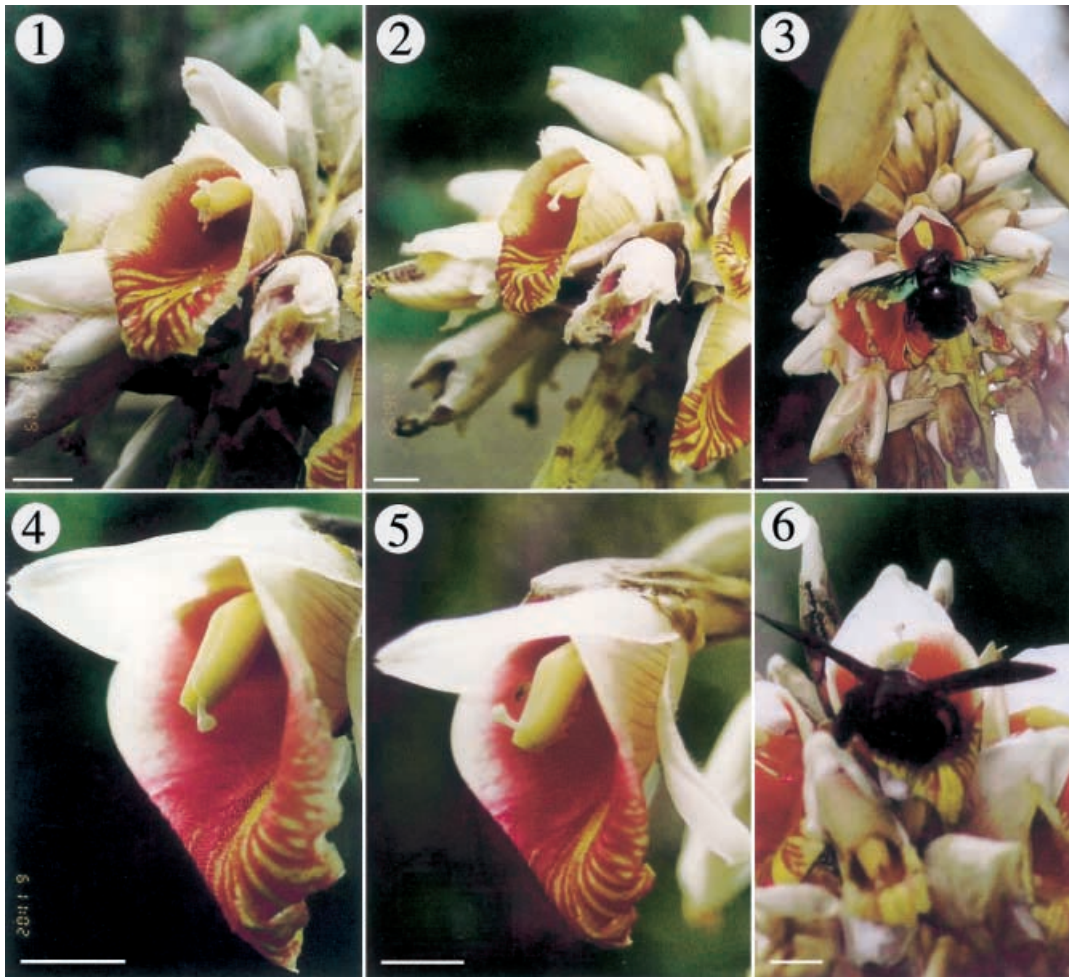
anthers and stigmas. These bees, such as the carpenter bee *Xylocopa magnifica* (Figs. 3, 6) and *X. tenuiscapa*, apparently forage over long distances and visit the same plants repeatedly along a feeding route (i.e. trapline). Small bees, such as *Nomia yunnanensis*, and various types of flies frequently visit flowers, but are not successful pollinators due to their small body size.

Pollination treatments. The results (Table 1) of the different pollination treatments within and between phenotypes of *Alpinia kwangsiensis* indicate that there are no differences between the two morphs (phenotypes) in fruit set ($F_{[\text{Morph}]} = 0$, $df = 1$, $P = 0.999$; Table 2), but that the treatment performed strongly affects the fruit set ($F_{[\text{Treat}]} = 24.724$, $df = 6$, $P < 0.001$; Table 2), there is a significant difference between different treatments within the same phenotype. Furthermore, within a specific treatment (self-pollination, cross-pollination; bagged; natural; in the mornings and afternoons), fruit set did not differ significantly between phenotypes ($F_{[\text{Interaction}]} = 0.772$, $df = 4$, $P = 0.548$; Table 2). Our result suggested that stigmas of cataflexistyle and anaflexistyle flowers are receptive throughout anthesis regardless of their position and that pollen is viable in both flower forms in both dehisced and undehisced anthers.

The results of multiple comparison of means of different treatments indicate (Table 3) that the fruit set after cross-pollination between the morphs is not significantly different (treatment 5a compare with 7b), suggesting inter-compatibility between the two morphs. However, in both morphs, and during both gender phases, cross-pollination always results in significantly higher fruit set than self-pollination (treatment 4a versus 5a; treatment 6b versus 7b), suggesting self-compatibility with a possible inbreeding depression effect.

Discussion

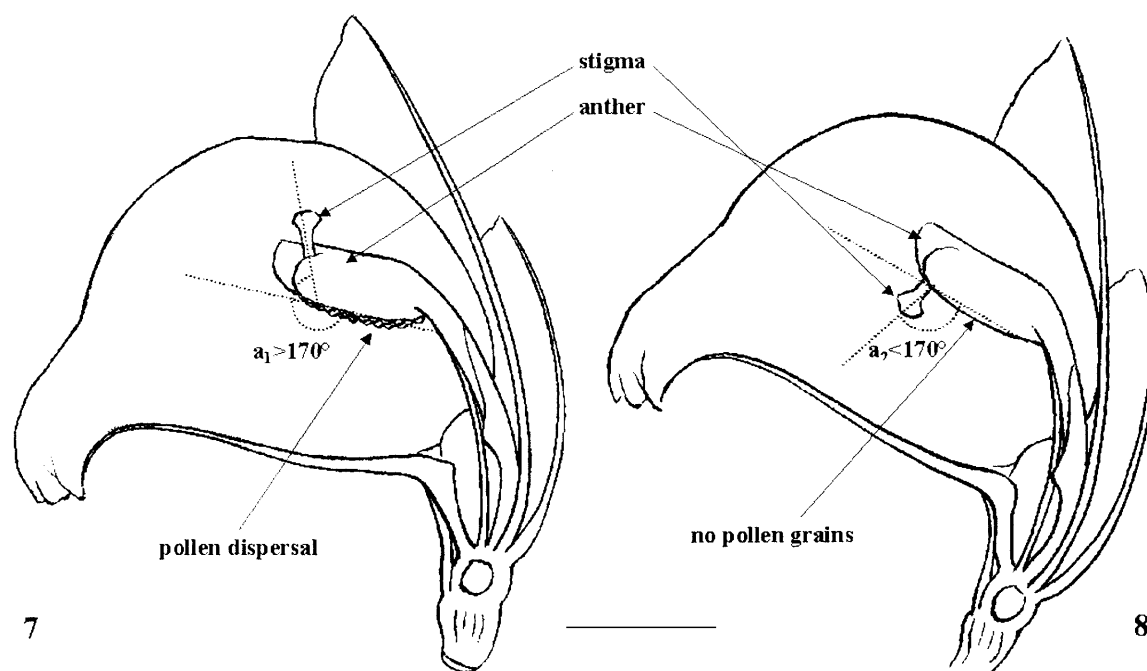
Because high levels of selfing in a species could lead to inbreeding depression, any floral trait that promotes outcrossing is likely to be advantageous (Lande and Schemske 1985).



Figs. 1–6. Two flower phenotypes of *Alpinia kwangsiensis* and their insect visitors. **1** Cataflexistyle flower during the “staminate” stage in the morning when the stigma is curved above the dehisced anther with exposed pollen. **2** The same cataflexistyle flower as in Fig. 1 during its “pistillate” stage during the afternoon when the stigma is decurved below the anther; most pollen has been removed from the anther due to earlier visits by nectar-collecting bees. **3** *Xylocopa magnifica* visiting a cataflexistyle flower during the afternoon. **4** Anaflexistyle flower during the “pistillate” stage in the morning when the stigma is decurved below the undehisced anther from which the pollen has not yet been shed. **5** The same anaflexistyle flower as in Fig. 4 during its “staminate” stage in the afternoon when the stigma has curved above the now dehisced anther. **6** *Xylocopa magnifica* visiting a anaflexistyle flower during the afternoon; the stigma positioned above the anther prevents pollination, while the bee picks up pollen from the open anther. Bars = 1 cm

However, if pollen is proximal to the stigma and the stigma is receptive when the pollen is viable, autonomous selfing (Lloyd and Schoen 1992) may occur frequently. Additionally, selfing may be common in plants with many inflorescences and homogamous flowers (i.e. in which both female and male phases are mature at the same time) that allow pollinators to

forage among flowers of the same plant (Handel 1985, Harder and Barrett 1995). The proportion of outcrossing and selfing in a population depends on a combination of physiological self-incompatibility mechanisms, phenological patterns, and pollinator behaviour (Lloyd and Schoen 1992). Among the diverse outcrossing mechanisms, dioecy and



Figs. 7, 8. Longitudinal section of a flower of *Alpinia kwangsiensis* illustrating the different positions of the stigma at different flowering stages. **7** The “staminate” stage of a cataflexistyle flower in the morning or of an anaflexistyle flower in the afternoon. **8** The “pistillate” stage of a cataflexistyle flower in the afternoon or of an anaflexistyle flower in the morning. Bars = 1 cm

self-incompatibility strongly promote or enforce outcrossing while herkogamy and dichogamy do so less strongly (Wyatt 1983, Richards 1997).

Since the publication of Darwin (1877) on the fertilization process in flowering plants, the phenomenon of floral dimorphism has received much attention. However, all of the floral dimorphisms described so far, including heterostyly, monoecy, dioecy, heterodichogamy, etc., are “passive” mechanisms that promote outcrossing without the radical movement of floral parts. In the ginger family, we have discovered in the genus *Alpinia*, a form of “active” dimorphism that has two different phenotypes in which the styles actually move within a flower. It is a kind of behaviour defined as “flexible change in response to temporal change” (Silvertown and Gordon 1989). We believe this is the first demonstration of the short-term alteration of style position in dimorphic flowers in order to enhance outcrossing.

The floral mechanism found in *Alpinia* prevents not only self-pollination within a flower (autogamy) and within the same individual (geitonogamy), but also among individuals of the same phenotype in a population. We conclude that this elaborate floral mechanism results in a low level of inbreeding despite the presence of self-compatibility in the populations.

It has been suggested that species of plants that are distributed in forested habitats and produce only a few flowers per day are adapted to pollination by traplining animals (Janzen 1971, Linhart 1973, Kress and Beach 1994). Pollination by trapliners is an effective means of outcrossing, and traplined plants show a ‘steady-state’ flowering pattern (Gentry 1974, Endress 1994). The flowering traits of *Alpinia kwangsiensis* and the behaviour of its visitors appear to be an example of traplining pollination that in association with floral movement may promote out-crossing in this species.

Table 2. Tests of between-subject effects of the different pollination treatments (TREAT) within and between phenotypes (MORPH) of *Alpinia kwangsiensis*. The dependent variable was fruit set per inflorescence

Source	Type III Sum of squares	df	Mean square	F	P
MORPH	1.506E-04	1	1.506E-04	0.000	0.999
TREAT	27858.595	6	4643.099	24.724	0.000**
INTERACTION (MORPH * TREAT)	579.620	4	144.905	0.772	0.548
Error	11831.213	63	187.797		
Total	113940.160	75			
Corrected Total	40875.251	74			

Table 3. The multiple comparison of means of the fruit set with different pollination treatments within and between phenotypes of *Alpinia kwangsiensis*

	1a	2a	3a	4a	5a	6a	7a ^a	1b	2b	3b	4b	5b ^b	6b	7b
1a	—													
2a	*	—												
3a	*	NS	—											
4a	NS	*	*	—										
5a	*	*	*	*	—									
6a	*	*	*	*	NS	—								
7a ^a	—	—	—	—	—	—	—							
1b	NS	*	*	NS	*	*	—	—						
2b	*	*	*	*	*	*	—	*	—					
3b	*	NS	NS	*	*	*	—	*	*	—				
4b	*	*	*	NS	*	*	—	NS	*	*	—			
5b ^b	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6b	*	*	*	NS	*	*	—	NS	*	*	NS	—	—	
7b	*	*	*	*	NS	NS	—	*	*	*	*	—	*	—

* Indicates $P \leq 0.05$ for at least one method.

^a Didn't compare due to the only one sample left.

^b No result because of lacking results of PM-cross pollination.

Fruit set was higher in controlled pollinations than in flowers visited by natural pollinators (Table 1), suggesting that pollination in *Alpinia kwangsiensis* is pollinator-limited (Holsinger 2000). In the controlled pollination treatments, fruit set in bagged plants was moderate, with fruit set in cataflexistyle plants higher than in anaflexistyle individuals. No fruit was set in inflorescences that had been bagged when the stigmas were at their receptive positions. Similar results were obtained in controlled experiments with a second flexistyle

species *Alpinia galanga* (Li et al., unpublished data). Our results suggest that at least two species of the genus *Alpinia* that we have studied, and maybe others, are self-compatible and insect-pollinated (e.g. *Xylocopa magnifica* and *X. tenuiscapa*), and that selfing appears to be prevented by flexistyle.

Flexistyle is present in all nine species of *Alpinia* that we have studied so far (Li et al. 2001b; *Alpinia kwangsiensis* T. L. Wu & S. J. Chen, *A. galanga* (L.) Willd., *A. blepharocalyx* K. Schum., *A. hainanensis* K. Schum., *A. conchi-*

gera Griff., *A. blepharocalyx* var. *glabrior* (Handel-Mazzetti) T. L. Wu, *A. zerumbet* (Pers.) B. L. Burtt & R. M. Smith, *A. maclurei* Merr., *A. platytilus* K. Schum.). In a molecular analysis of phylogenetic relationships within Zingiberaceae (Kress, Prince and Williams, unpubl.), the nine species we studied are found in three different clades of *Alpinia*. This observation suggests that flexistylly either evolved independently several times in the family or it is widespread, but as yet unrecorded for many taxa in the Zingiberaceae (e.g. *Amomum*, *Boesenbergia*). We are currently surveying additional species of *Alpinia* and genera in the Zingiberaceae to determine the distribution of this floral mechanism. These investigations will provide further insight into the systematic implications of flexistylly within Zingiberaceae as well as the evolution of pollination mechanisms in angiosperms as a whole.

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