

An example of flexistyly in a wild cardamom species (*Amomum maximum* (Zingiberaceae))

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Abstract. We studied the floral and pollination biology of *Amomum maximum*. Our observations suggest that flexistyly is present in this species, and stigmatic behavior is similar as reported for *Alpinia* species. Two phenotypes occur in a ratio of 1:1 in natural populations. No fruit set occurred in the unpollinated bagged plants. Significant differences in seed set between selfing and crossing manipulations demonstrate that inbreeding depression or partial self-incompatibility is apparent in this species. Despite the resemblance in floral behavior, there are still some differences between the previous studies and ours: with special floral design, the body size of legitimate visitors ranged wider in *Amomum maximum* than in *Alpinia* species; the presence of anther appendage is suggested to prevent competing and delayed selfing. This is the first report of flexistyly in a wild cardamom species, which is the second largest group in ginger family (Zingiberaceae).

Key words: *Amomum maximum*, flexistyly, stigma behavior, pollination biology, anther appendage.

Introduction

Flexistyly as a special form of heterodichogamy promoting outcrossing, is a unique and

motile sexual dimorphism first found in *Amomum tsao-ko* Crevost & Lemarie (Cui et al. 1995, 1996) and then in *Alpinia* Roxb. species (Li et al. 2001a, b, 2002; Zhang et al. 2003; Takano et al. 2005). There are two floral morphs in a flexistylous species, anaflexistylous (protogynous) and cataflexistylous (protandrous) individuals with styles behaving oppositely during anthesis. Both floral types change almost synchronously from one sexual stage to another without gender overlap.

Amomum Roxb. is a large genus in Zingiberaceae, including over 150 species (Wu and Larsen 2000), which produces hermaphroditic flowers with exception of the andromonoecious species *Am. polycarpum* (K. Schum.) R.M. Smith (Sakai et al. 1999) (to distinguish *Amomum* from *Alpinia*, we abbreviate them as *Am.* and *Al.*, respectively thereafter). This genus is a close relative to *Alpinia* (Kress et al. 2002, Pedersen 2004). Flexistylous mechanism has been only reported on a cultivated species *Am. tsao-ko* (Cui et al. 1995, 1996). But whether flexistyly is common in this genus, especially in wild species, has not been reported yet. Furthermore, to fully understand the origin and evolution of this unique floral

mechanism, studies of flexistyly in various ginger groups are needed.

In this research, we reported the pollination biology of *Am. maximum* Roxb., a wild cardamom species, which is widely distributed in tropical areas of South China and Southeast Asia (Wu and Larsen 2000). The main goal of this study was to address the following questions: (1) Are floral behaviors of *Am. maximum* the same as those of *Am. tsao-ko* and *Alpinia* species in prior reports? (2) How does flexistyly affect the fruit and seed sets of this species? (3) Does the anther appendage of the flower play any role during the flexistylous movement of the stigma?

Materials and method

Study site. The study was conducted from April 26 to May 6, 2003, in a rubber plantation in Man'e village (101°25' E, 21°41' N, 580 m in altitude), Mengla, Yunnan province, Southwest China. The annual average precipitation is 1557 mm, and annual mean temperature reaches 21.5°C in the study site (Feng et al. 1998). *Am. maximum* inhabits understory of tropical rainforest margin. In the study site, primary forest was cleared for rubber planting 10 years ago, but *Am. maximum* were left for fruits collection by local villagers.

Research species. *Amomum maximum* Roxb., is a perennial herb of Zingiberaceae, usually 2–3 m in height. Inflorescence consists of a densely flowered panicle that arises from rhizomes. The petaloid, pale labellum with showy yellow and red nectar guide is fused with the single stamen to form a tube, the free part of which is expanded and forms a landing platform for visitors. An apparent anther appendage (0.4 × 1 cm) surrounds the stigma and forms an arch at the top of the anther. Flowering occurs from April to June, and by late August–September the capsules are ripe (Wu and Larsen 2000).

Floral biology. Detailed flowering phenology was monitored and recorded in the ginger collection of Xishuangbanna Tropical Botanical Garden (XTBG, 2 km from the study site) during the entire blooming season from 2000 to 2003. Ten inflorescences with 230 open flowers were observed over three days (May 1–3, 2003) in the study site; every day, three inflorescences were

observed from late morning (ca. 10:00) to the next day until flower wilt. We randomly counted 30 inflorescences of each morph to get the number of flowers on an inflorescence. For each flower, the opening of the labellum, the stigmatic position and the time of pollen sac dehiscence were observed and recorded. Morph ratio was also investigated within a 1-ha population in the study site.

Pollinator observations. Flower visitors and their behavior were observed from April 27–30, 2003. We randomly selected 6 marked shoots of each floral morph. Observations were conducted continuously from 08:00 to 19:00, to determine the visiting frequencies and behaviors. To identify whether stigmas or anthers were touched by visitors, visiting behaviors were also recorded by a video-recorder. At the end of the observations, at least 3 individuals of each visitor morphospecies were captured for identification.

Pollination treatments. To examine the breeding system of *Am. maximum* and the contribution of visitors to pollination, we conducted several experimental manipulations on both phenotypes. Ten individuals of each morph and four inflorescences from each of these individuals were selected at random, all selected inflorescences of the same plant were subjected to one of the following treatments respectively: (1) natural: inflorescences were marked and left exposed, permitting insects to visit naturally; (2) bagged: inflorescences were bagged to prevent insects visiting during the whole blooming season; (3) selfing: flowers were hand-pollinated with self-pollen in the afternoon (ca. 16:00); (4) crossing: flowers were hand-pollinated with pollen from the opposite when their stigmas were in their receptive position (ca. 08:00 of ana-morph flowers, ca. 16:00 of cata-morph flowers). All hand-manipulated inflorescences bagged before anthesis, were rebagged following pollination. Those treatments were performed from May 1–6, 2003; then floral buds were removed from each inflorescence. The fruit set (number of fruits/treated flowers per inflorescence) and seed set (number of seeds / seeds and aborted ovules per fruits) were counted on August 10.

Statistic analyses. Data on visiting frequencies of different gender stages between two floral morphs were square root-transformed, then compared by one-way analysis of variance (ANOVA) in SPSS (10.0 version). The data of fruit and seed

set ratio were arcsine transformed, then compared by one-way ANOVA. Tukey's Honestly Significant Difference Test was used to analyze the variances among different pollination treatments.

Results

Floral biology. Every inflorescence had a total number of 61.4 ± 11.0 (Mean \pm SD, range from 44 to 85) flowers, with 7.8 ± 3.1 (5 to 12) flowers blossoming each day at the observation site. The longevity of a single flower of both phenotypes was one day, it opened at 01:00 and wilted at 24:00. Anthesis in each inflorescence lasted 10–15 days.

At the beginning of blooming (ca. 01:00), the stigma of both floral morphs was located at the tip of the anther. In the process of flower opening, the style of both morphs was elongated. When the flowers were fully open (ca. 04:00), the stigmas of anaflexistylous flowers still closed moved down below the anthers (Fig. 1), and the stigmas of cataflexistylous flower still closed curved above the anther (Fig. 2). The pollen sacs of cataflexistylous flowers began to dehisce at 6:00. The stigmas of the two phenotypes remained in their position until noon, then the styles of anaflexistylous flower began to bend upward (12:00 to 12:30), around 14:00 to 14:30, the stigma was already located above the anther, far away from the insect visiting channel (Fig. 3), then its pollen sacs began to dehisce about 15:00; the stigmas of cataflexistylous flowers decurved below the anthers at 14:30 to 15:00 (Figs. 4 and 5).

The two morphs occurred in a ratio of 1:1 (49:38, $\chi^2 = 1.1494$, $P > 0.2$) within our research population.

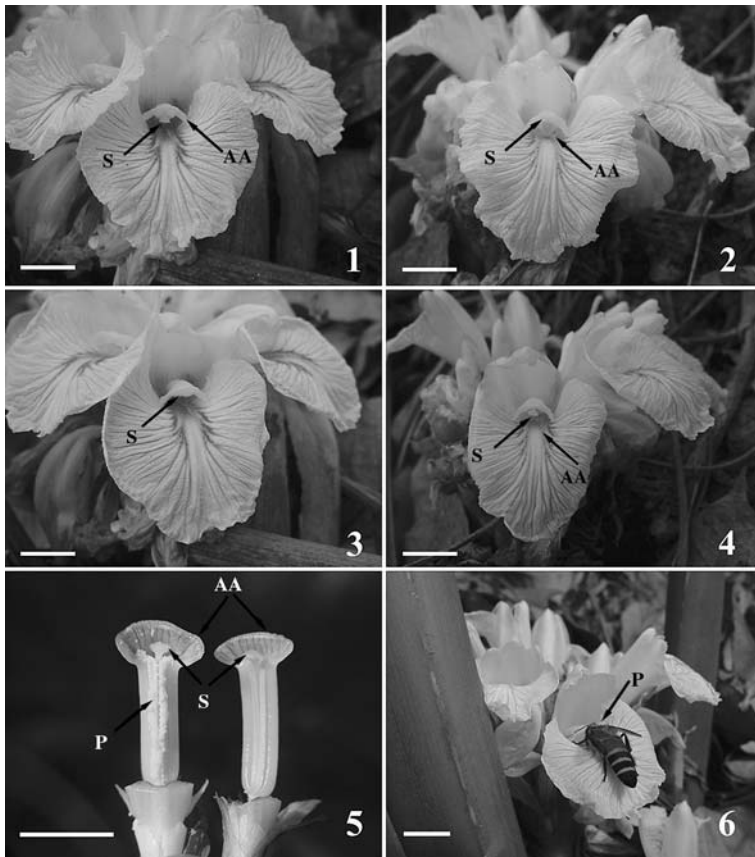
Pollinator observations. Among the seven species of visitors we observed, different insects came for various rewards and behaved diversely when visiting. *Apis (Megapis) dorsata* (Fig. 6) and *A. (Sigmatapis) cerana cerana*, were the most frequent and effective visitors of *Am. maximum* in the study site. Visiting frequencies of *A. dorsata* on anaflexistylous and cataflexistylous flowers were 119.1 ± 53.1

(mean \pm SD) and 115.2 ± 60.9 times/ flower/ day respectively, without significant difference ($F_{1,18} = 0.0491$, $P > 0.5$). The visiting frequencies of *A. cerana* showed two peaks a day (10:00–11:00 in the morning and 15:00–17:00 in the afternoon); they preferred cata- to ana-morph in the morning but without significant difference ($F_{1,18} = 0.0406$, $P > 0.5$). However, in the afternoon, *A. cerana* visited anaflexistylous flowers more frequently than cataflexistylous ones ($F_{1,18} = 4.7138$, $P < 0.05$), and they visited the flowers mainly for collecting pollen grains and nectar (Fig. 7).

Pollination treatments. All the bagged inflorescences without any pollination treatment yielded no fruit, while flowers visited by natural pollinators and hand-pollinated treatments had higher fruit and seed set (Fig. 8). The results in fruit set of different pollination treatments indicate that significant differences ($F_{5,51} = 4.609$, $P = 0.0015$) occurred between ana-selfing ($45.60 \pm 7.13\%$, mean \pm SE) and other manipulations, viz. ana-crossing ($70.89 \pm 3.76\%$), ana-natural ($72.40 \pm 2.67\%$), cata-selfing ($74.18 \pm 4.12\%$), cata-crossing ($72.22 \pm 8.30\%$), and cata-natural ($64.28 \pm 4.64\%$), no significant differences of fruit set could be detected among the latter 5 treatments. However, the treatment performed strongly affected the seed set, since there are significant differences among different pollination manipulations ($F_{5,180} = 10.2391$, $P < 0.0001$). Seed set of crossing treatments in both morphs were significantly higher than that of selfing treatments (ana-crossing vs. -selfing ($72.73 \pm 3.10\%$ vs. $46.58 \pm 4.53\%$), and by cata-crossing vs. -selfing ($73.64 \pm 2.41\%$ vs. $47.34 \pm 3.30\%$)). Seed set between two floral morphs with same treatment (natural, selfing and crossing) did not differ significantly.

Discussion

Although self-fertilization has many advantages in execrable habitat, it often leads to inbreeding depression due to expression of deleterious recessive genes (Darwin 1876; Charlesworth and Charlesworth 1987, 1999).



Figs. 1–6. Two flower phenotypes of *Amomum maximum* and their insect pollinator. **1** Anaflexistylous flower in its female stage (before noon), the stigma is decurved below the undeheisced anther from which the pollen has not yet been shed. **2** Cataflexistylous flower in its male stage (before noon), the stigma is curved above the deheisced anther. **3** The same flower as in Fig. 1 during its male stage (afternoon) when stigma is curved above the deheisced anther. **4** The same flower as in Fig. 2 during its female stage (afternoon) when the stigma is decurved below the anther; most pollen has been removed from the anther by earlier visitors. **5** The anthers and stigmas of the two floral morphs with labellum peeled off in the afternoon, anaflexistylous is on the left, cataflexistylous is on the right. **6** *Apis (Megapis) dorsata* visiting a flower. *S*: stigma, *P*: pollen grains, *AA*: anther appendage, Bars = 1 cm

Plants are famously diverse in reproductive strategies (Barrett 2002), displaying a broad range of floral forms across species and presenting numerous examples of floral or reproductive polymorphisms maintained within a population (Pannell et al. 2005). Flexistylous is a genetic polymorphism in which plant populations are composed of two morphs that differ reciprocally in the time of stigma receptivity and pollen release (Li et al. 2001a).

Our research indicates that flexistylous outcrossing mechanism existed in *Am. maximum*, a wild species of cardamom. The popu-

lation has two morphs with the ratio approximately 1:1. The rhythm of styler behavior is similar to the previously reported species, such as *Am. tsao-ko*, *Al. kwangsiensis* T. L. Wu & S. J. Chen, *Al. galanga* (L.) Willd., *Al. blepharocalyx* K. Schum. and *Al. nieuwenhuizii* Val. etc. (Cui et al. 1995, 1996; Li et al. 2001a, b, 2002; Zhang et al. 2003; Takano et al. 2005). With the existence of flexistylous strategy, those species exhibit temporal dioecy, that prevents not only self-pollination within a flower (autogamy) and within the same inflorescence (geitonogamy),

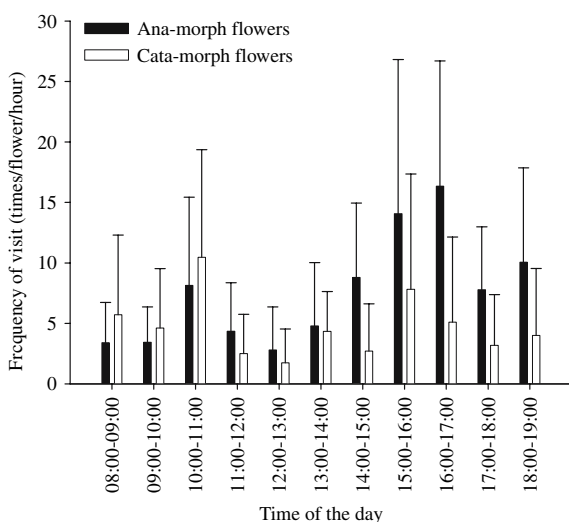


Fig. 7. The visiting frequency of *Apis* (*Sigmatapis*) *cerana cerana* to *Amomum maximum*

but also among individuals of the same floral morph in a population, thus in natural condition, pollination can only occur between different morphs. With this floral polymorphism, the sexual organs are separated temporarily and spatially, thus interference of male and female functions may reduce to small extent (Li et al. 2001a, b; Barrett 2002; Zhang and Li 2002; Zhang et al. 2003; Zhang 2004).

The distance between anther and labellum was long in *Alpinia* species, as a result, only large insect species and sunbirds can touch the anther or stigma completely (Li et al. 2001b, 2002). However, in *Am. maximum*, the anther with anther appendage could touch the labellum during anthesis, and the space could be changed by the visitors' body size and their positions in flowers. When the insects are more than 0.2 cm in height, and visit the flower through the channel between the anther and the labellum the male or female organ would be touched by visitors according to different gender stage. Due to the weaker limitation of effective pollinators' body size, the range of legitimate visitors of *Am. maximum* was considered much wider than for *Alpinia* species.

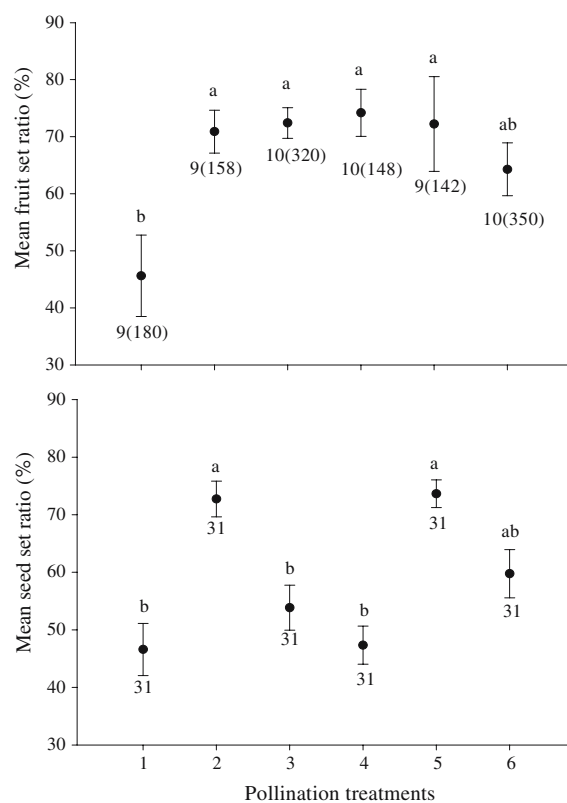


Fig. 8. The effect of pollination treatments on fruit and seed set in the floral morphs of *Amomum maximum*. Plotted are the means and standard errors of the following groups: **1** Anaflexistylous selfing; **2** Crossing (*Anaflexistylous*♀ × *Cataflexistylous*♂); **3** *Anaflexistylous* natural; **4** *Cataflexistylous* selfing; **5** Crossing (*Cataflexistylous*♀ × *Anaflexistylous*♂); **6** *Cataflexistylous* natural. All bagged inflorescences yielded no fruit, and this treatment is not shown in the figure. Group sample sizes, inflorescence number (flower number), fruit number are given below the bars, and statistically homogenous groupings based on one-way ANOVA and Tukey's HSD Test are indicated by same letter above the bars

Two visiting peaks of *A. cerana* indicate that pollinator foraging behavior matched with functional sex stages through 1 d anthesis. Reduction in visiting frequency in gender transition stage suggests that pollinators may adjust to avoid visiting 'asexual' flowers for pollen rewards. This bimodal pattern was also reported in flexistylous *Alpinia* species (i.e. Li et al. 2001b, Takano et al. 2005). Therefore,

disassortative mating could be maintained by precise pollination.

P/O ratio of *Am. maximum* was 320.82 ± 54.56 (mean \pm SD, Wang et al. 2004) suggesting an obligate xenogamy breeding system according to Cruden (1977). Bagged treatments (excluded insects from visiting) had no fruit set, showing that *Am. maximum* is an insect-dependent species given that insects are the pollinators of the species. Selfing treatments in both floral morphs had significantly lower seed set than crossing treatments, this result maybe due to inbreeding depression or partial self-incompatibility.

In the genus *Amomum*, anther appendage is an important morphological structure, some of the genus' classification was even based on the presence of the anther appendage (Schumann 1904), but the ecological function of this structure has not been known. According to our primary observation, we assumed that competing and delayed self-pollination might be reduced due to the existence of the anther appendage. In the male stage of *Amomum* species with anther appendage, e.g. *Am. maximum*, the stigma curved upward and the funneled stigma chamber was covered by the anther appendage (Fig. 5 left stage), preventing pollination. Besides, the visiting route was restricted by the anther appendage; so that, in the course of foraging for rewards, flower visitors could reduce autogamy. Thereby, competing self-pollination seldom takes place in *Am. maximum*. Because of the anther appendage, the stigma was kept separated distant from the labellum and they could not touch each other directly even until wilt, thus the stigma could not collect the pollen grains previously fallen on the labellum. This is probably the reason why the delayed selfing mechanism could be found in *Alpinia* species without anther appendage during senescence (Li et al. 2001b, 2002), but have not been detected in *Am. maximum*.

As a specific heterodichogamy (Renner 2001), flexistylous mechanism was observed in several genera of Zingiberaceae, such as *Alpinia*, *Amomum*, *Etilingera*, and *Paramomum*. In the genus *Alpinia*, more than ten

flexistylous species were demonstrated in details (Li et al. 2001a, b, 2002; Zhang et al. 2003; Takano et al. 2005). Moreover, this mechanism was found in all *Alpinia* species which have been observed so far (unpublished observation). In *Amomum*, besides *Am. maximum* and *Am. tsao-ko*, flexistylous mechanism was also observed in *Am. purpureorubrum* S. Q. Tong & Y. M. Xia, *Am. coriandriodorum* S. Q. Tong & Y. M. Xia, *Am. glabrum* S. Q. Tong, *Am. sericeum* Roxb., *Am. menglaensis* S. Q. Tong, *Am. putrescens* D. Fang and *Am. koenigii* J. F. Gmelin. However, not all species in this genus have this mating strategy, such as *Am. villosum* Loureiro, *Am. villosum* var. *xanthioides* Wallich ex Baker, *Am. aurantiacum* H. T. Tsai et S. W. Zhao, *Am. quadratolaminar* S. Q. Tong, *Am. kravanh* Pierre ex Gagnep. and *Am. compactum* Solander ex Maton (Li et al. unpublished data). Therefore, with both flexistylous and non-flexistylous species in this genus, *Amomum* is an ideal model for discussing flexistylous phylogeny. All species in *Amomum* with flexistylous except *Am. koenigii* were from the *Am. maximum* group and *Am. tsao-ko* group, and the species without flexistylous were from the *Am. villosum* group according to a recent classification system (Xia et al. 2004). This suggests that the mechanism of flexistylous may have evolved from a common ancestor of *Amomum*, but lost in some species of the *Am. villosum* group, or evolved independently at least one or two times in this genus. Furthermore, according to recent studies of the phylogeny of Zingiberaceae, these genera of Alpinieae are paraphyletic (Kress et al. 2002, 2005; Xia et al. 2004), and more than one clade includes both species of *Amomum* and *Alpinia*. So, we suggested that flexistylous might have evolved from a common ancestor of the tribe Alpinieae or independently at least three to five times in the tribe (Kress et al. 2005). To better understand the origin and evolution of flexistylous, groups which have both flexistylous and non-flexistylous species will be ideal targets. In this sense, flexistylous in *Amomum* is worth to be studied in details.

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