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海滨锦葵花内五柱头裂片弯曲的独自调节

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摘 要：海滨锦葵花柱有5柱头裂片，若传粉失败，柱头裂片向下弯曲使柱头与自身花粉接触，发生延迟自交。传粉不足条件下发生的延迟自交可能提供繁殖保障。该研究定量分析了花粉搁置和授精胚珠数对花内未授粉柱头裂片运动的影响，并测定了花粉－胚珠比及柱头可授性和花粉生活力。结果表明，花内未授粉柱头裂片的弯曲不受其他柱头接受花粉量及授精胚珠数的影响，仅响应于其自身是否接受到花粉、花粉－胚珠比值显示海滨锦葵交配系统属兼性异交；未授粉柱头经弯曲与自身花粉授触时的强柱头可授粉和高花粉生活力为自交授粉的发生提供了可能。包括柱头裂片运动在内的多个花性状有机地展示了一种新花内混合交配系统，且花内柱头裂片弯曲的独自调节为从花水平验证被广泛接受的自交进化解释——繁殖保障假说提供了可能。

关键词：海滨锦葵；独自调节；柱头裂片弯曲；花粉－胚珠比；混合交配系统；繁殖保障

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Independent regulation of curvature of five stigma lobes within *Kosteletzkya virginica* flowers

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Abstract: *Kosteletzkya virginica* has a pistil with five stigma lobes. If un-pollinated, individual stigma lobes downward curve to bring them contact with a flower's own anthers, possibly resulting in delayed selfing, which might provide reproductive assurance when pollinators are scarce. It was observed, but not experimentally tested, that each of the five lobes within flowers acts independently. Here, we quantitatively tested the effects of pollen loads and seed set on the curvature of un-pollinated lobes within flowers, pollen-ovule ratio and stigma receptivity and pollen viability. Results showed that the curvature of un-pollinated lobes within flowers did not appear to be a response to the amount of pollen grains deposited on the adjacent stigmas and seed set, depending on if each received pollen. The value of pollen-ovule ratio indicated that mating system belonged to facultative xenogamy. When un-pollinated stigmas were in contacting with its own anthers via style movements, high stigma receptivity and pollen viability provided a possible for resulting in delayed selfing. Multiple floral traits including independent curvature of stigma lobes harmoniously exhibit a mixed mating system within flowers. Our data provided the first empirical demonstration that the curvature of the five stigma lobes within flowers regulates independently, which provides incentive for testing at the flower level the reproductive assurance widely accepted explanation for the evolution of selfing.

Key words: *Kosteletzkya virginica*; independent regulation; stigma lobe curvature; pollen-ovule ratio; mixed mating system; reproductive assurance

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1 Introduction

Flowers exhibit a wide array of anatomical specializations and behaviors which are strongly linked to plant pollination and mating system. These consist of pistil (style) movements such as stigma closure (Fetscher & Kohn, 1999; Yang *et al.*, 2004), late curling of stigma lobe (Buttrose *et al.*, 1977; Klips & Snow, 1997; Ruan *et al.*, 2004), flexistyly (Li *et al.*, 2001) and style curvature (Culley, 2002). They also include stamen (pollen) movement, such as stamen curvature (Rathcke & Real, 1993), stamen elongation (Eckert & Schaefer, 1998), pollen sliding (Wang *et al.*, 2004), pollen catapult (Edwards *et al.*, 2005) and anther rotation (Liu *et al.*, 2006). Other floral activities include corolla abscission (Dole, 1990) and wilting (Sun *et al.*, 2005). The putative adaptive significance of floral behavior includes reduction in intrafloral male-female interference (Edwards *et al.*, 2005), promotion of outcrossing (Verma *et al.*, 2004; Peter & Johnson, 2006), achievement of delayed autonomous selfing (Culley, 2002; Etcheverry *et al.*, 2003) and response to harsh environments (Bynum & Smith, 2001; Huang *et al.*, 2002).

The facultative nature of autonomous pollination through curvature of un-pollinated styles towards its anthers in Malvaceae is intriguing. It has been reported in *Hibiscus laevis* (Klips & Snow, 1997), *H. trionum* (Buttrose *et al.*, 1977; Seed *et al.* 2006) and *Kosteletzkya virginica* (Ruan *et al.*, 2005a). Various hypotheses addressing the adaptive significance of this curvature have been proposed, such as promotion of outcrossing (Stephens, 1948), achievement of delayed selfing (Blanchard, 1976; Klips & Snow, 1997; Ruan *et al.*, 2004) and first preference to outcrossing but facilitating delayed selfing if outcrossing fails (Buttrose *et al.*, 1977). The lobed curvature facilitates un-pollinated stigmas to receive cross-pollen by positioning the stigmas in the flight path of pollinators subsequent to a predominantly male phase of anthesis when the stigmas are held together, projecting forward. After this, it can lead to delayed self-pollination if they are in contacting with the anthers when the opportunity of outcrossing has passed.

In *H. trionum* var. *trionum*, curvature is reversible, with partially curved styles regaining an erect position after receiving pollen; and the effect of pollination is broadly inhibitory such that application of pollen to a single stigma lobe is sufficient to prevent curvature of adjacent lobes (Buttrose *et al.*, 1977). In contrast, the five styles within *H. trionum* var. *vesicarius* flowers act independently (Seed *et al.*, 2006), and if un-reversible curvature ceases depend on the amount of pollination that each receives. Pollinated styles of *H. laevis* still curve (moved angle less than 135°), although to a lesser extent than un-pollinated styles (moved angles more than 135°) (Klips & Snow, 1997). In contrast, the curvature of un-pollinated stigma lobes in *K. virginica* permanently ceases once pollination takes place, and it is un-reversible (Ruan *et al.*, 2004). It was observed, but not experimentally tested, that the curvature of each of the five stigma lobes within *K. virginica* flowers act independently; namely pollination of one to four stigmas does not prevent the curvature of the adjacent un-pollinated lobes.

Kosteletzkya virginica (Malvaceae), a perennial herbaceous plant, is native to salt marsh in American (Gallagher, 1985). In 1993, it was introduced into China as a potential species for improving soil and developing ecologically sound saline agriculture. The fruit capsule has five ventricles, each containing a single ovule. Flowers of *K. virginica* have a monadelphous column (18-57 stamens are fused into a column), surrounding pistil with five stigma lobes (Ruan *et al.*, 2005b). Each plant produces one to twenty-five flowers everyday, and each flower only lasts one day. Pollination of *K. virginica* includes insect pollinators (e. g. *Bombus specious*, *Colias hyale* and *Manduea florestan*) and delayed self-pollination via stylar movements (Ruan *et al.*, 2005b). If newly-opened flowers are pollinated, stigma lobes remain erect; but if un-pollinated, the stigma lobes begin curving and will touch the anthers when completing the flowering lifespan.

This study addresses two main questions. First, if the curvature of un-pollinated stigma lobes within flowers responds to seed set and different number pollen loads on the adjacent stigmas? We applied different numbers of pollen grains to the stigmas from one to four to observe curvature responses of the adjacent un-pollinated stigma lobes and pollinated lobes within flowers. This was used to test the effects of pollen loads and seed set on the curvature of un-pollinated lobes within flowers. Second, what is mating system the curvature of un-pollinated lobes within flowers combining with other floral traits exhibit? We tested pollen-ovule ratio, stigma receptivity, pollen viability and pollination limitation to analyze the mating system influenced by multiple floral traits.

2 Materials and Methods

Seeds of *K. virginica* from the Halophyte Biotechnology Center (University of Delaware, USA) were sown at the Dafeng site for controlled experiments and assessment in 1993. The Dafeng site is located on the tideland of Yancheng City (longitude 119°27′—120°54′ E, latitude 32°34′—34°28′ N), Jiangsu, China. The continuous field experiments from 1994 to 2004 demonstrated that *K. virginica* is a good halophytic species with a great potential industrially and ecologically for new marshlands in China. The Dafeng naturalized populations of *K. virginica* grow more than 100 000 individuals in 2004. Seeds, collected from the Dafeng naturalized population in 2004, were sown in Dalian site in the spring of 2005. Individuals growing in Dalian site were over 20 000 in the autumn of 2005. The Dalian site is located on the tideland of Dalian city (longitude 121°39′ E, latitude 38°55′ N), Liaoning, China.

2.1 Effects of pollen loads and seed set on curvature of un-pollinated lobes

In spring 2007, two thousands mixed seeds from Dalian naturalized population were germinated on a compost substrate (humus : pearlstone = 3 : 1) in polystyrene plates flowerpots (100 seeds per plate) in the culture room, with 45 μmol · s^{-1} · m^{-2} light, a 14-h photoperiod, 70% — 80% relative humidity and at 28 ℃. After 10—15 days, when grew to about 2 — 3 cm in length, seedlings were transplanted into the compost substate (ash and slay with sand, or fluvial heavy sand) in an insect-free greenhouse (Dalian Nationalities University). Individuals were kept in a controlled growing condition at 20—25 ℃ (25—30 ℃ for flowering period) with a 16h/8h light/dark regime and under a relative humidity of 70%±5%. We gained 1493 individuals that normal grew to over 1 m tall in May, 2007.

To determine the effects of different amount of pollen loads and seed set on the curvature of un-pollinated stigma lobes, eight flowers were randomly selected from eight different individuals (one flower per individual) randomly selected daily, and randomly variable amounts of pollen grains from the un-selected individuals were applied to one to four stigmas within a flower (two flowers per treatment) at 7:00, at that time all stigma lobes are just curving. After pollination, the flowers were continuously observed (4—5 h) to determine if pollination prevented the curvature of pollinated stigmas and if pollination was sufficient to inhibit the curvature of the adjacent un-pollinated lobes within flowers. Once un-pollinated lobes continued to curve and their location relative to the monadelphous column was clearly different from the lobes that ceased curvature after pollination, these was cut using sharp, sterile scissors to prevent delayed autonomous selfing. This phenomenon is easy to observe because it is the same as the angle between each of un-pollinated stigma lobe within a flower and the line along the entire length of the monadelphous column. The angle of the stigma lobe will not change once it ceases curving after pollination takes place(see Fig. 1); in contrast, since the curvature of un-pollinated lobes will last until occurrence of pollination, the angle continues to decrease gradually(see Fig. 1). The top of hand-pollinated lobes(2 mm length)in each treated flower were removed and collected six hours after pollination at about 13:00. This cut did not influence the flowers to set fruit, because pollen tubes required 1.0—2.5 h to pass style and reach ovules and only required less than 0.5 h to pass the lobes(Ruan *et al.*, unpublished data). The number of pollen grains per treated flower was estimated using a dissecting microscope (Olympus SZ2-ILST). Mature capsules were harvested and the number of seeds in each capsule was counted. For each treatment (one to four stigmas), fifty flowers from fifty different individuals(one flower per individual) were treated in a total of 25 days.

We determined whether cessation of the curvature of un-pollinated lobes corresponded to the amount of pollen grains deposited on the stigmas from one to four and seed set from pollination of stigmas from one to four or not. We also examined the effect of the number of pollen grains deposited on a single stigma on itself curvature by the data of one stigma treatment.

Our previous experiments on a deleterious effect of cut stigma lobes(about 2 mm) on seed set by physical damage to the flowers indicated that it did not produce negative effects on seed set, this was because there was no significant difference in the mean number of seeds per capsule between the hand-pollinated flowers that four stigmas was cut and the intact flowers that only one stigma was hand-pollinated(mean ± s. e., 4.29 ± 0.08 versus 4.31 ± 0.07, a two-sample t-test: $t = -0.541$, $df = 98$, $P = 0.589$).

2.2 Pollination limitation and pollen-ovule ratio

We randomly selected and tagged 20 pairs of individuals at the Dalian field population in 2005, with the members of each pair located within 3 m of each other and different pairs located over 10 m apart from each other. In each day across the flowering season, for each individual each pair, all flowers exposed to open pollination as the control group (Control); where all flowers on the second individual were also exposed to natural pollinators, but we supplemented the number of pollen grains arriving to the stigmas by hand-pollinating all flowers with pollen from at least 5 donor plants(Pollen-supplementation treatment). Artificial pollination was repeated in each flower at least twice(one at 9:00, and another at 14:00) to ensure successful pollination. Mature fruits were harvested for counting fruit sets and seeds per capsule, and the comparison between pollen-supplementation treatment and control flowers was used to assess pollen limitation in *K. virginica* by the method of Rathcke (2003). We tested the differences in fruit set and in the number of seeds per capsule between control and pollen-supplemented individuals, using a two-sample t-test test (SPSS 11.0).

In addition, we measured pollen-ovule ratio(P/O) by the method of Cruden(1977). Five flowers were randomly selected from five different individuals(one flower per individual) randomly selected from the Dalian field site. All stamens on the monadelphous column per flower before anthers dehisce were collected, and the number of pollen grains per stamen was estimated using the dissecting microscope(Olympus SZ2-ILST). The number of ovules per flower was counted under the dissecting microscope(Olympus SZ2-ILST). A total of 120 flowers were treated in 24 days. Breeding system of *K. virginica* was estimated by the data of Cruden (1977): cleistogamous with pollen-ovule ration ranging from 2.7 to 6.7, 18.1—38.9 for autogamous-obligate, 31.9—396.0 for autogamous-facultative, 245.0—1 894.0 for xenogamous-facultative and 2 108.0—195 525.0 for xenogamous-obligate.

2.3 Stigma receptivity and pollen viability

To test receptivity in the Dalian field population in 2005, we used an indirect assay modified from Kalisz *et al.* (1999) and Pu *et al.* (2008) that detects the presence of stigma peroxidase. When receptive stigmas are placed in a 3% solution of hydrogen peroxide, vigorous bubbling occurs on the stigmatic surface. Non receptive stigmas do not produce bubbles. In the field, gynoecia were dissected from flowers on plants loosely covered with muslin bags before flowers open, at intervals of 2 h from 6:00 to 18:00 on the day of anthesis. Individual stigma lobes were immediately sandwiched between two cover slips with a drop of hydrogen peroxide. The stigmatic area was examined under a dissecting microscope in the field. Stigmas were scored as positive for peroxidase activity only if we observed vigorous bubbling across the entire surface of the stigma. For each time interval, three-hundred fifty(350)lobes of 70 flowers from 70 different individuals(one flower per individual) were tested for peroxidase activity.

We applied TTC(2,3,5-triphenyl tetrazolium chloride)to examine pollen viability during a flower's lifetime in the field populations by the method of Huang *et al.* (2004). At interval of 2 h from 6:00 to 18:00 on the day of anthesis, 30 flowers were collected from thirty flowering plants, and pollen viability was assessed by the percentage of pollen stained red. Freshly harvested pollen was dusted onto a microscope slide with a brush to which four or five drops of stain were added. Then the slide was immediately covered with a cover slip and the edges sealed with nail varnish. The percentage of pollen of 200—300 grains per slide(one to three slides for each treated flower) that exhibited the appropriate staining reaction was determined using an Olympus IX71 microscope at× 100 magnification.

3 Results

3.1 Effects of pollen loads and seed set on curvature of un-pollinated lobes

The un-pollinated stigma lobes within flowers still curved after 1—4 stigmas received different amount of pollen grains(Table 1, Plate Ⅰ), and this curvature was independent of the number of seeds per capsule(Table 1). For one stigma, pollination of different amount of pollen grains ranging from 1 to 162 immediately and irreversibly prevented itself curvature.

3.2 Pollen-ovule ratio and pollination limitation

The number of pollen grains per flower was 2831.56±43.14(mean±s.e., n=120). The number of ovules per flower was five. The pollen-ovule ratio was 566.31±18.63, ranging from 296.64 to 939.36. This indicated that breeding system belongs to facultative xenogamy by the criterion of Cruden(1977).

Table 1 Effects of pollen loads and seed set on curvature of un-pollinated stigma lobes

No. polli-nated stigma	Amount of pollen grains deposited on the pollinated stigma (mean s. e.)	No. seeds per capsule (mean s. e.)	If curvature of pollinated lobes ceases	If curvature of un-pollinated lobes ceases
1	47.06±4.02	4.10±0.20	Yes	No
2 a	59.02±8.82	3.68±0.22	Yes	No
3 a	155.08±8.88	4.02±0.19	Yes	No
4 a	170.54±13.04	4.06±0.20	Yes	No

Note: a: For treatments of stigmas from two to four, mean was the average of total numbers of pollen grains deposited on the all stigmas per flower.

Pollen limitation did not occur in *K. virginica*, because there were no significant differences in fruit set and in the number of seeds per capsule between control and pollen-supplemented individuals(fruit set: mean±s.e., 81.32%±2.64% *vs.* 82.41%±1.86%, a two-sample t-test: $t=-1.837$, $df=38$, $P=0.134$; seeds per capsule: 4.43±0.07 *vs.* 4.51±0.07, $t=-1.632$, $df=28$, $P=0.079$).

3.3 Stigma receptivity and pollen viability

Stigmatic peroxidase tests indicated that stigma receptivity was 56.0%±10.2% at 06:00, and optimum stigma receptivity was about 10:00(Fig. 3A). At 16:00, 82.86%±6.91% of the stigma lobes collected in the field had receptive stigmas. Pollen viability at 6:00 was 53.79%±1.97%, and it was higher from 8:00 to 12:00 (Fig. 3B).

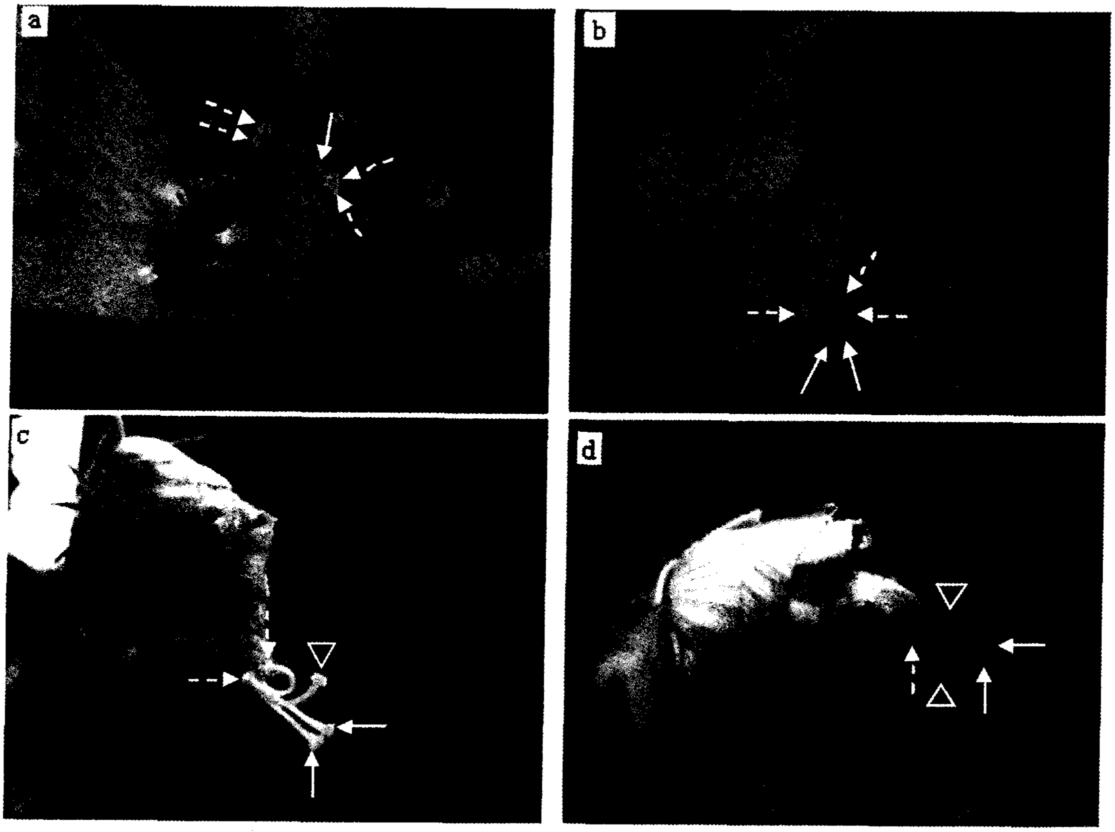

Plate Ⅰ　Independent regulation of curvature of the five stigma lobes within *Kosteletzkya virginica* flowers　**a**: one stigma lobe(solid arrowhead) ceased curving after pollination took place, the remaining four un-pollinated lobes(dashed arrowheads) continued to curve; **b**: two stigma lobes(solid arrowheads) ceased curving after pollination took place, the remaining three un-pollinated lobes(dashed arrowheads) continued to curve; **c**: two stigma lobes(solid arrowheads) ceased curving after pollination took place, the remaining three un-pollinated lobes continued to curve. One of them(triangle) ceased curving after pollination occurred in the progress of curvature, the last two stigma lobes(dashed arrowheads) continued to curve until contacting the anthers to self-pollinate; **d**: two stigma lobes(solid arrowheads) ceased curving after pollination occurred at the curved primary stage. Other two stigma lobes(triangles) ceased curving after pollination occurred at the curved middle stage. The last stigma lobe(dashed arrowhead) was not hand-pollinated, and continued to curve until contacting the anthers to self-pollinate.

4　Discussion

The present work provided a first empirical demonstration that independent regulation of curvature of un-pollinated stigma lobes within *K. virginica* flowers does not appear to be a response to the amount of pollen grains deposited on the adjacent stigmas or seed set, depending on if each received pollen. In other words, each of the five lobes with a flower acts independently, where pollinated lobes do not curve back, while adjacent lobes will still curve if un-pollinated. Even if the pollen loads of 1—4 stigmas is enough to set 5 seeds in a single capsule, un-pollinated stigma lobes still curve. When an un-pollinated stigma lobe continues to curve until it enters into contact with the anthers, it takes longer(about 5—7 h) to do so than it takes the pollen tube of other pollinated stigmas to reach ovules(< 3 h, Ruan *et al.*, unpublished data), providing addition support for the suggestion that independent curvature is not a response to the number of fertilized ovules. This mechanism is not consistent with from reported bi-lobed stigma behavior: reopening of some stigmas after pollination in *Mimulus aurantiacus* appears to be a response to low seed set(fewer than one-third of the ovules are fertilized) rather than to low pollen load(Fetscher

& Kohn,1999),and the pollinated stigma secondary open in *Campsis radicans may* be due to the limited pollen grains deposited on the stigmas (Yang *et al.*, 2004). Style curvature in *H. trionum* var. *vesicarius* is prevented when 50 or more grains are deposited per stigma(Seed *et al.*, 2006). Some reopen stigmas in *C. radicans* close permanently if a sufficient amount of pollen(>350 grains)is received(Yang *et al.*, 2004). In contrast, in *K. virginica* if the curvature of un-pollinated lobe ceases only responds to if stigma is pollinated but not to pollen grains number deposited on the stigmatic surface. Even one pollen grain is sufficient to halt curvature if it germinated. This is consistent with the results of(Fetscher & Kohn, 1999), upon receipt of pollen, most stigmata in *M. aurantiacus* remain closed for the remaining lifetime of the flower, even if less pollen is received than is needed for full seed set.

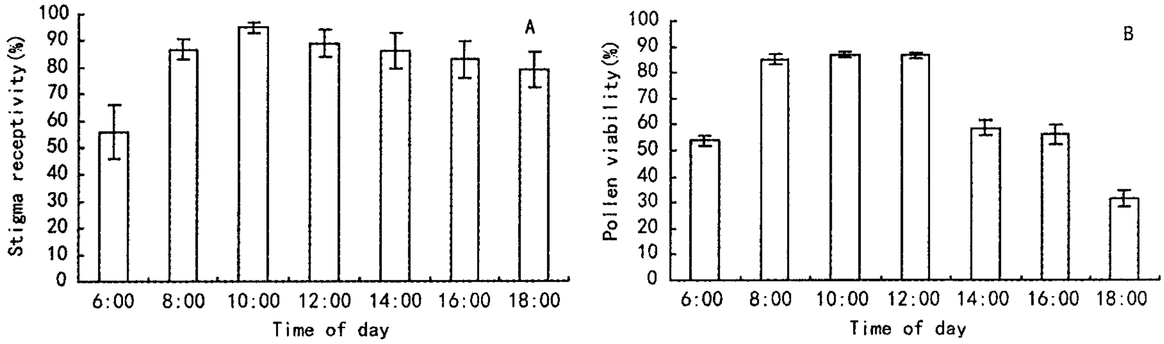

Fig. 2 Stigmatic receptivity(A)and pollen viability(B)in different times on the day of anthesis in *Kosteletzkya virginica*

Independent regulation of the curvature of unpollinated lobes within *K. virginica* flowers, combining with other floral traits, harmoniously exhibited a mixed mating system within flowers. This could be supported by the following three conditions: (1)high stigma receptivity and pollen viability when stigmas are in contacting with the anther by the curvature; (2)floral traits adapting for outcrossing and selfing resulting from un-pollinated lobes curvature; (3)delayed self-pollination via curvature of un-pollinated stigma lobes desponding on pollinator environments.

First, stigma lobes, if pollen is not received, curve to ensure contact with anthers at the end of the day (about 14: 00 — 16: 00) (Ruan *et al.*, 2005a), providing a mechanism for delayed selfing in *K. virginica*. This is because at that time stigma receptivity and pollen viability were over 82% and 56%, respectively(Fig. 3). If pollen is proximal to the stigma and the stigma is receptive when the pollen is viable, autonomous selfing may occur frequently(Lloyd & Schoen, 1992). Flowers of *K. virginica* fully open at about 05:00, un-pollinated stigma lobes begin to curve at about 7:00. Corolla close before 16: 00, at that time delayed autonomous selfing via un-pollinated stigma curvature occurs. Most pollinators of *K. virginica*(observation by CJ Ruan)act at 7:00—16:00, at this times the flowers have good stigma receptivity and pollen viability(Fig. 2).

Second, *K. virginica* exhibits floral traits adapting to outcrossing and selfing. On the one hand, flowers of *K. virginica* display some characters of outcrossed species, such as large corolla size (5.27±0.70 cm), showy color(common pink, purple and white) attractive to pollinators, nectar and pollen rewards. Pollen-ovule ratio provides a useful insight into the breeding system of species(Cruden, 1977; Jürgens *et al.*, 2002; Jacquemart, 2003). There is a substantial decrease in pollen grain numbers and in pollen-ovule ratio from xenogamy to autogamy(Cruden, 1977). The value of pollen-ovule ratio indicated breeding system of *K. virginica* belongs to facultative xenogamy. On the other hand, *K. virginica* flowers appear special traits that change as the flower matures and may be associated with selfing. When pollinators are scarce or absent, stigma lobes curve to overcome herkogamy and achieve self-pollinate. If the opportunity of outcrossing has passed, stylar movements as a means to effect selfing has also been reported in some plants, as in many *Campanula* species (Faegri & van der Pijl, 1979), *H. laevis* (Klips & Snow, 1997), *Viola pubescens* (Culley, 2002) and many orchids etc.

Finally, pollination modes in *K. virginica* including insect pollination and context-dependent autonomous self-pollination can occur in different stigma lobes within a flower, providing a mixed mating system within flowers and optimal mating system plasticity. Pollinators' activity of *K. virginica* directly responds to pollination conditions. There are more kinds and amounts of pollinators and higher pollination frequency in sunny days than in rainy / cloudy days, especially in late flowering lower pollinator abundance and / or activity under the low temperature conditions display a variable pollinator environment (Ruan *et al.*, 2005b). Percentage of stigma lobes that curve to contact the anthers on sunny days was significant lower than on cloudy / rainy days (Ruan & Jin, 2007).

The potential value of curvature of un-pollinated stigma lobes within flowers as a mechanism for reproductive assurance depends highly on the fitness of selfed relative to outcrossed seeds. Though inbreeding depression of *K. virginica* was 0.54 (Shan *et al.*, 2007), delayed autonomous selfing in *K. virginica* directly responds to the abiotic environment, as the percentage of flowers displaying delayed selfing was significantly lower on sunny days than on inclement days (Ruan *et al.*, 2005b, Ruan & Jin, 2007). This indicates that delayed autonomous selfing in *K. virginica* is adaptive for promoting reproductive success under unpredictable pollinator environments, because emasculated flowers (there is no seed set from delayed selfing via the curvature of un-pollinated stigma lobes) set fewer seeds than intact flowers (there is seed set from delayed selfing via stigma curvature) when open-pollinated (Ruan *et al.*, 2008) and there are no pollen limitation. Fisher (1941) showed that selfing is advantageous from the "automatic selection hypothesis"; an allele for selfing will spread if selfed progeny are at least half as fit as outcrossed progeny (Lloyd, 1979; Nagylaki, 1976). In contrast, Darwin (1876) proposed that an adaptive benefit of self-pollination is to provide reproductive assurance when the opportunity of outcrossing has passed (Baker, 1955; Lloyd, 1992; Kalisz *et al.*, 2004). In addition, self-pollination under variable pollinator environments can be advantageous (Kalisz & Vogler, 2003) despite strong inbreeding depression (0.64 for *H. trionum*) (Goodwillie *et al.*, 2005, Seed *et al.*, 2006).

Independent regulation of curvature of un-pollinated stigma lobes within flowers of *K. virginica* ensures that if outcross-pollination was insufficient to fertilize all ovules and the opportunity of outcross-pollination has passed, delayed autonomous selfing from this curvature would augment overall seed set. This provides incentive for testing the reproductive assurance hypothesis at the flower level in future studies. Reproductive assurance is one of the most longstanding and widely accepted explanations for the evolution of selfing. This theory predicts that self-pollination is advantageous in environments or conditions where mates or pollinators are rare. Cases of selfing leading to reproductive assurance have been reported in several species (Donnelly *et al.*, 1998; Nagy *et al.*, 1999; Anderson *et al.*, 2003; Elle & Carney, 2003). More comprehensive analyses were conducted by Kalisz *et al.* (2004) and Herlihy & Eckert (2002) to test population and time effects, and alternatives to the reproductive assurance hypothesis, such as pollen discounting (Holsinger, 1996), seed discounting (Lloyd, 1992), selfing rate, and inbreeding depression. Kalisz *et al.* (2004) showed that delayed selfing of *Collinsia verna*, responding to unpredictable pollinator environments, supports reproductive assurance. Herlihy and Eckert (2002) indicated that auto-pollination in *Aquilegia canadensis* increases seed production; however, this benefit was outweighed by the loss of high quality seed as a result of seed discounting and inbreeding depression. The above cases were conducted at the level of populations, but this has not yet been addressed at the level of individuals or flowers (Zhang, 2004).

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