

Floral morphology and mating system of *Alcea rosea* (Malvaceae)

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Background and aims – Variations in floral traits and floral structures influence mating systems. The flowers of *Alcea rosea* are protandrous and herkogamous, with style curvature at anthesis.

Methods – We measured: (1) floral morphology, including style curvature, (2) stigma receptivity and pollen viability, (3) breeding system, and (4) outcrossing rate estimated by ISSR markers.

Key results – Results indicate that when stigmas were in contact with anthers via style curvature, stigma receptivity and pollen viability was 89.7 and 42.33%, respectively, indicating the potential of delayed selfing. Delayed selfing in *A. rosea* did not provide obvious contribution to seed production, because there was no significant difference in seed set between intact and emasculated open flowers ($P = 0.765$). However, successful reproduction of 23.98% flowers in the absence of pollinators suggested that selfing provides reproductive assurance during a year, in which pollinators were limiting. The multilocus outcrossing rates in different populations varied from 0.972 to 0.981, with a mean of 0.977.

Conclusion – Our data provide an empirical demonstration of a predominantly outcrossing species with potential delayed selfing when pollinators are absent or scarce.

Key words – *Alcea rosea*, style curvature, outcrossing rate, ISSR markers, delayed selfing, reproductive assurance.

INTRODUCTION

The evolutionary and functional significances of sexual diversity in flowering plants have long intrigued evolutionary botanists (Barrett 2002a), especially the separation of sexual structures in hermaphroditic plants. It includes dichogamy and the temporal separation of male and female function within hermaphroditic flowers, which can be (i) protandry, in which the male function precedes the female function, or (ii) protogyny, in which the female function precedes the male function (Lloyd & Webb 1986, Bertin & Newman 1993). Herkogamy, the spatial separation of male and female within a flower, occurs in many animal-pollinated plants (Webb & Lloyd 1986, Barrett 2002a, 2002b). Based on the position of pollen and stigmas, Webb & Lloyd (1986) described two types of herkogamy: approach herkogamy (stigmas positioned above anthers) and reverse herkogamy (stigmas positioned below anthers). Although dichogamy and herkogamy have been interpreted as mechanisms for reducing self-fertilization (Darwin 1862, 1876, Holtsford & Ellstrand 1992, Belaussoff & Shore 1995, Karron et al. 1997, Klips & Snow 1997, Brunet & Eckert 1998, Motten & Stone 2000, Miyajima 2001, Elle & Hare 2002), increasing evidence suggests that they may act as mechanisms to reduce intrafloral male-

female interference, namely, the interference between pollen receipt by stigmas and pollen export from anthers within a flower (Lloyd & Webb 1986, Bertin & Newman 1993, Barrett 2002a, Ruan et al. 2010).

In many angiosperms, floral structures can undergo dramatic movements over relatively short periods of time and can influence plant pollination and mating systems, including pistil (style) movement (Schlessman 1986, Fetscher & Kohn 1999, Li et al. 2001, Yu & Huang 2006, Zhang & Li 2008, Ruan et al. 2010), stamen (filament, anther, pollen) movement (Darwin 1862, Schlessman 1986, Kalisz et al. 1999, Edwards et al. 2005, Liu et al. 2006), and corolla closure (Juncosa & Webster 1989, Bynum & Smith 2001, Ruan et al. 2005, Sun et al. 2005, Carrió et al. 2008). Since Darwin (1862) noted that reconfiguration of pollinia in orchids avoids self-pollination (Peter & Johnson 2006), various hypotheses have been proposed to explain the adaptive significance of floral movements. These include reduction in intrafloral male-female interference (Fetscher & Kohn 1999, Fetscher 2001, Sun et al. 2007), avoidance of self-pollination and promotion of outcrossing (Armbruster et al. 2002, Verma et al. 2004, Freitas & Sazima 2009), delayed autonomous pollination (Wang et al. 2004, Ruan et al. 2004, Liu

et al. 2006) and tolerance to harsh environments (Bynum & Smith 2001, Huang et al. 2002). However, the adaptive significance of floral movement in some species has yet to be fully explained, for example style curvature in *Kosteletzkya virginica* (L.) C.Presl ex Gray, which may involve three advantages: reducing intrafloral male-female interference, promoting outcrossing and delaying selfing (Ruan et al. 2010).

Species with floral movement that promotes outcrossing when pollinators are present but ensures self-pollination when pollinators are scarce (Lloyd & Schoen 1992, Kalisz et al. 2004), may present a mixed mating system (Goodwillie et al. 2005). Despite selection against self-pollination, the transition from outcrossing to self-fertilization is one of the most common evolutionary trends in plants (Stebbins 1957, 1974, Holsinger 2000, Barrett 2002b, Herlihy & Eckert 2002). Outcrossing rates in plant populations have traditionally been estimated from polymorphism data via isozyme loci (Adams 1983). Recently, the application of hypervariable genetic markers provides opportunities to accurately determine the mating system, such as random amplified polymorphic DNA (RAPD) markers (Fritsch & Rieseberg 1992, Gaiotto et al. 1997), amplified fragment length polymorphism (AFLP) marker (Ruan et al. 2009), inter-simple sequence repeat (ISSR) markers (Ge & Sun 1999, Han et al. 2009), and simple sequence repeat (SSR) markers (Barnaud et al. 2008, Zhao et al. 2009, Barner et al. 2011).

Alcea rosea L. (Malvaceae) is a biennial or perennial, polycarpic dicotyledonous herb. Flowers are hermaphroditic and protandrous, with a monadelphous androecium in which filaments are united to form a single tube with the anthers inserted on it. The stigmas are exerted beyond the anthers in the female stage, forming approach herkogamy (electronic appendix). Previous observations indicate that style branches in *A. rosea* flowers can curve at anthesis, and eventually bring some stigmas down or in contact with the anthers, potentially resulting in delayed selfing. This raises the question: what mating system is applicable to *A. rosea* with protandrous and approach herkogamous flowers? This main question was addressed using the following measures: (1) flower opening process, especially style curvature, (2) pollen viability and stigma receptivity to test if delayed selfing occurs in this species, (3) breeding system, autofertility and reproductive assurance potentially provided by delayed selfing under different floral manipulations, and (4) outcrossing rate estimated by ISSR markers.

MATERIALS AND METHODS

Species

Alcea rosea L. has flowers displaying multiple colours such as pink, white and red, etc. It is widely grown worldwide as an ornamental plant. It originated from China but in this country too is only known as cultivated or naturalized (Tang et al. 2007). Plants are 200–300 cm tall, and each plant produces 0–40 new flowers daily. Fully opened flowers have five free imbricate petals with an adnate base to the stamen. The coloured corolla and floral nectar attract bee species, of which *Apis mellifera* is the most efficient pollinator (Li, pers. obs.). To investigate floral morphology, stigma receptivity

and pollen viability, and breeding system, we used a naturalized population of *A. rosea* growing in Dalian city located in southern of Liaoning Province, China. There are 50–60 individuals per hectare (ha) in a field investigation of twelve ha from June to October 2010. Dalian city (39°01'–39°04'N 121°44'–121°49'E) has a maritime climate with an annual mean temperature of 10.2°C and an annual mean rainfall of 658.7 mm. It has 191 frost-free days and a total annual insolation of 2764 h.

We estimated outcrossing rates in three naturalized populations: Dalian city (DL), Anshan (AS) and Shenyang (SY) in Liaoning Province. AS and SY are located in centre of Liaoning province, where *A. rosea* grows widely. There were in average 30–40 and 90–100 individuals per ha in the AS and SY populations, respectively. In each population, we randomly selected sixty individuals at least 10 m apart from each other, and all seeds per plant were collected from August to October 2009. Two months after collection, the seeds were germinated in growth chambers, with 45 $\mu\text{mol s}^{-1} \text{m}^{-2}$ light, a 12 h photoperiod, 70–80% relative humidity, at 28°C. Ten days after germination, at least fifteen seedlings per individual were randomly chosen, and between nineteen and 21 individuals were used for each population.

Floral morphology and style curvature

We observed flower development and style curvature during June to October 2010 in DL population. Eighty flowers (one flower per randomly selected individual) were chosen from eighty individuals, and the following floral traits were measured: corolla width (diameter of the circle surrounding the five petal tips), petal length and width (three per flower), stamen column length and the number of style branches. In addition, we randomly selected ten individuals to record the date of the first and the last flower and the total number of flowers produced by each plant (flowers were counted every day during anthesis).

To observe the flowering process, we randomly selected 100 flowers from 100 individuals (one flower per individual) in the DL population during twenty days in 2010. At a 2 h interval from 06:00 to 19:00 every day, we measured (1) the length of the portion of the style exerted out of the monadelphous androecium (three styles per flower), and (2) the time style branches began to curve and brought stigmas into contact with its own anthers.

Stigma receptivity, pollen viability and P/O ratio

To test receptivity in the DL population in 2010, an indirect procedure modified from Kalisz et al. (1999) was used that detects the presence of stigma peroxidases (E.C.1.11.1.7). When receptive stigmas were placed in a 3% solution of hydrogen peroxide, vigorous bubbling occurred on the stigmatic surface. Non-receptive stigmas did not produce bubbles. At 4 h intervals from 07:00 to 19:00 during the female phase, intact styles of emasculated flowers were immediately sandwiched between two cover slips with a drop of 3% hydrogen peroxide. The stigmatic area was examined under a dissecting microscope in the field. Stigmas were scored as positive for peroxidase activity by observing vigorous bub-

Table 1 – Sequences of the selected ISSR primers used for the analyses of outcrossing rate, annealing temperature and the number of bands they produced in three *A. rosea* populations.

Primers	Sequences (5'-3')	Total number of bands			Annealing temperature (°C)
		DL	AS	SY	
UBC 807	(AG)8T	18	23	19	53
UBC 808	(AG)8C	17	23	22	53
UBC 809	(AG)8G	20	18	22	53
UBC 810	(GA)8T	16	21	11	53
UBC 811	(GA)8C	17	20	18	53
UBC 812	(GA)8A	19	16	22	53
UBC 813	(CT)8T	9	20	16	53
UBC 814	(CT)8A	14	17	18	53
UBC 822	(TC)8A	15	23	17	53
UBC 823	(TC)8C	13	17	17	53
UBC 834	(AG)8YT	20	26	25	53
UBC 835	(AG)8YC	21	14	14	53
UBC 836	(AG)8YA	13	22	18	53
UBC 840	(GA)8YT	15	26	13	53
UBC 881	(GGGTG)3	19	28	18	53
UBC 887	DVD (TC)7	19	17	12	53

bling across their entire surface. For each time interval, about 140 lobes of ten flowers from ten different individuals (one flower per individual) were tested for peroxidase activity. A total of 400 flowers were tested over ten days.

TTC (2, 3, 5-triphenyl tetrazolium chloride) was applied to examine pollen viability during a flower's lifetime in the field populations by following the method of Huang et al. (2004). At 4 h intervals from 07:00 to 19:00 on the day of anthesis, ten flowers were collected from ten flowering plants (one flower per individual). Pollen viability was assessed by the percentage of pollen that 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 (1–3 slides for each treated flower) that exhibited an appropriate staining reaction was determined using an Olympus IX71 microscope at 100 × magnification. A total of 400 flowers were tested over ten days.

In addition, the pollen-ovule ratio (P/O) was measured by the method of Cruden (1977). Five flowers were randomly selected from five different individuals (one flower per individual) randomly selected from DL population. For each flower, all stamens on the monadelphous column were collected before anthers dehisced, and the number of pollen grains per stamen was estimated while the number of ovules per flower was counted using a dissecting microscope (Olympus SZ2-ILST). A total of 120 flowers were collected in 24 days. The *A. rosea* breeding system was estimated by the data of Cruden (1977, 2000) and Cruden & Hermann-Parker (1997): cleistogamous if the P/O ranged from 2.7 to 5.4, 18.1 to 39.0 for autogamous-obligate, 31.9 to 396.0 for autogamous-facultative, 244.7 to 2588.0 for xenogamous-facultative, and 2108.0 to 195525.0 for xenogamous-obligate.

Breeding system, delayed selfing and reproductive assurance

To assess the breeding system, we conducted an experiment in DL population from June to October 2010. Six treatments were designed, in which each treatment consisted of five individuals, and eighty flowers were randomly chosen from each individual. These included: (1) intact open flowers (Io): buds were marked before anthesis and then left intact; (2) agamospermy: flowers were emasculated before the style branches exerted from the monadelphous column and bagged; (3) autonomous self-pollination (As): flowers were bagged at anthesis; (4) emasculated flowers with open pollination (Eo): flowers were emasculated before the style branches exerted from the monadelphous column and were then left without bagging; these flowers could only receive pollen delivered by pollinators, either from other flowers on the same plant (geitonogamy) or from other plants (outcrossing); (5) intact flowers with hand self-pollination (Is): flowers were bagged at anthesis, and when style branches stretched out of the monadelphous column, self-pollen was placed on the stigmas by hand at 8:00 and at 12:00, and flowers were bagged again; and (6) emasculated flowers with hand cross-pollination (Ec): flowers were emasculated and then bagged, and when the style branches stretched out of the monadelphous column, pollen from plants at least 10 m away was placed directly on stigmas between 8:00 and 12:00. After 28 days, the fruits were collected and the number of mature seeds per fruit was counted. Differences in fruit set and the number of seeds per fruit among different treatments were analyzed using one-way ANOVA (SPSS 11.5) followed by a contrast test.

The capacity for autonomous self-pollination, or autofertility (AF), was calculated as F_{As}/F_{Io} , where F_{As} is the mean seed production of the As treatment, while F_{Io} is the mean seed production of the Io treatment (including the flowers that did not produce any fruit at all) (Lloyd & Schoen 1992).

To test the contribution of AF to reproductive assurance (RA), a measure of RA was calculated as $(F_{Io} - F_{Eo})$, where F_{Io} and F_{Eo} are the seed set of Io (which can produce seeds from either autonomous selfing or vector-assisted pollen movement) and Eo (which can only produce seeds via vector-assisted pollen movement) (Eckert et al. 2006).

Mating system

A total of 1015 seedlings were used for genomic DNA extraction. Total genomic DNA was extracted from fresh ten-days old seedlings using the protocol of Doyle & Doyle (1987). A total number of 60 ISSR primers that were from the Biotechnology Laboratory, the University of British Columbia (UBC set No. 9), were screened using four DNA samples. Based on the number and quality of polymorphic fragments, sixteen ISSR primers (table 1) were selected. PCR amplification was performed in 20 μ l reaction volume containing 10 mM Tris-HCl pH 8.0, 50 mM KCl, 2.0 mM $MgCl_2$, 0.5 mM of each dNTP, 0.5 μ M of primer, 2% formamide, 1 unit of *Taq* Polymerase and 30 ng of genomic DNA. Initial denaturation was for 3 min at 94°C, followed by 45 cycles of 45 s at 94°C, 45 s at 53°C, 90 s at 72°C, and a 7 min final extension step at 72°C. PCR products were analyzed on 2% agarose gels at a constant voltage of 80 V for 1.5 h, then stained with ethidium bromide, visualized with ultraviolet light and photographed.

ISSR markers are typically dominant. Bands were scored by considering only two possible alleles: band presence (1), band absence (2). The program MLTR version 3.2 (Ritland 2002) was used to estimate population multilocus (t_m) and single-locus (t_s) outcrossing rates, and the difference between the two ($t_m - t_s$), which is often used to characterize the level of biparental inbreeding, the inbreeding resulting from matings between closely related individuals. It also calculated the multilocus correlation of paternity within progeny arrays (r_{pm}), which is the fraction of siblings that share the same father, and the inbreeding coefficient (F) of the maternal parents (Ritland 2002). The expected inbreeding coefficient at equilibrium (F_e) was calculated using $F_e = (1 - t_m) /$

$(1 + t_m)$ (Wright 1965, Hedrick 1985). By comparing parental F with F_e , it is possible to assess whether inbreeding is having a negative effect (inbreeding depression) on the survivorship of selfed progeny in the populations. Under significant inbreeding depression, survivorship of selfed progeny should be lower than survivorship of outcrossed progeny, and therefore parental F should be lower than F_e . Standard errors of these estimates were obtained based on 1000 bootstraps.

RESULTS

Floral morphology and style curvature

In the DL population, the first flower of *A. rosea* appeared on 20 Jun. 2010; the flowering period lasted 103 days, with the peak flowering dates (50% of flowering individuals) being from June 30 to July 14. Each individual produced 0–40 new flowers daily. A maximum of 108 opening flowers was observed in one plant on 3 July. The flowering period for each individual was 91.51 ± 10.28 days. The flowers have a wide corolla (9.13 ± 0.62 cm in width and 3.52 ± 0.05 cm in length), and the petals are 4.50 ± 0.05 cm in length and 6.70 ± 0.05 cm in width. The length of the monadelphous column was 1.88 ± 0.04 cm, with approach herkogamy of 5.18 ± 0.03 mm after style branches were exerted from the monadelphous column but before they began to curve. The number of style branches was 30.16 ± 0.64 .

The life-time of a single flower was about 3 to 4 days. Anthers dehiscence one day before anthesis (electronic appendix A). Style branches were still hidden inside the monadelphous column when the flowers fully opened (electronic appendix B). Most pollen had been shed 1 to 2 days after flowers opened. After flowering for 2 to 3 days, erect style branches exerted out of the monadelphous column (electronic appendix C). Independent of pollination, the stigma branches began to curve when the length reached 3–4 mm at 9:00–11:00 (electronic appendix C₂, C₃). Some stigmas were brought downwards or were in contact with the anthers at approx. 17:00–19:00 (electronic appendix C₄), at this time,

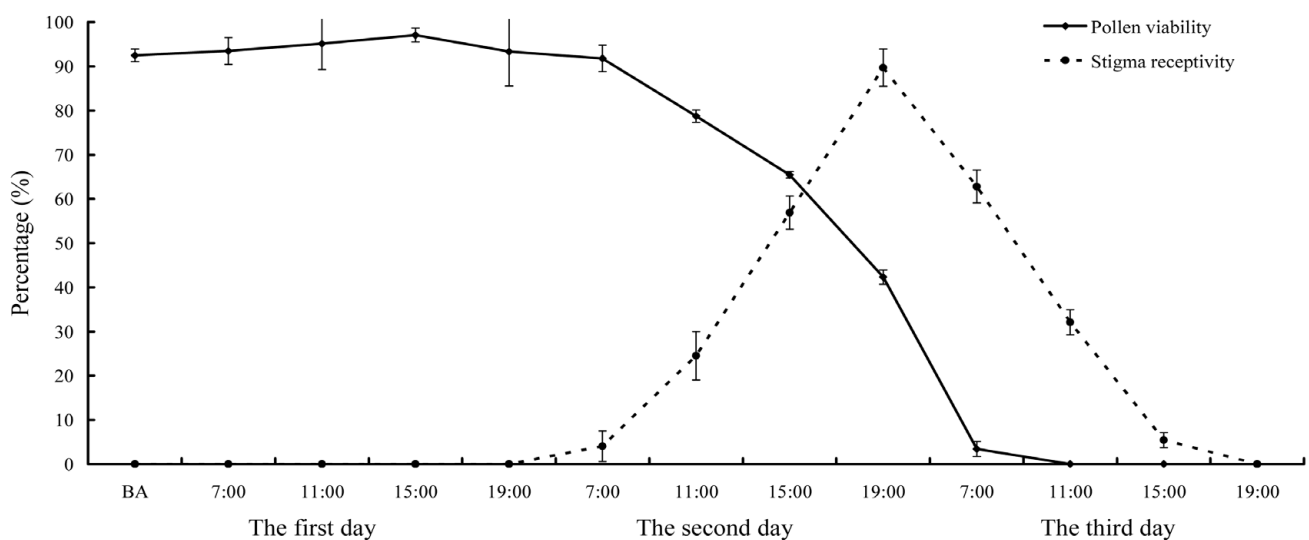


Figure 1 – Pollen viability and stigma receptivity of *Alcea rosea*. Error bars are ± 1 SE. BA: before anthesis.

Table 2 – Mating system parameters in three populations of *Althaea rosea*.

The parameters examined included the multilocus (t_m) and average single-locus (t_s) outcrossing rates, biparental inbreeding rate ($t_m - t_s$), multilocus correlation of paternity (r_{pm}), and the inbreeding coefficient among maternal parents (F). Standard errors in parentheses.

Population	t_m	t_s	$t_m - t_s$	r_{pm}	F
DL	0.979 (0.003)	0.595 (0.006)	0.384 (0.005)	0.258 (0.018)	0.590 (0.008)
AS	0.972 (0.000)	0.507 (0.004)	0.465 (0.004)	0.553 (0.012)	0.507 (0.004)
SY	0.981 (0.007)	0.700 (0.008)	0.281 (0.011)	0.156 (0.035)	0.498 (0.006)

the length of style branches was 4~9 mm and most pollen of flowers had shed.

Stigma receptivity, pollen viability and P/O ratio

Pollen viability of *A. rosea* was 92.5% before flowering, and maintained this maximum level at the first day of anthesis; but it reduced to less than 50% at the second day of anthesis. In contrast, stigma receptivity was only 4.03% when the style branches grew out of the monadelphous column (about

7:00). The maximum stigma receptivity (89.7%) occurred at 19:00 at the second day of anthesis, at which time, some stigmas touched their own anthers via curvature of style branches (fig. 1).

The numbers of pollen grains and ovules per flower were 31606.67 ± 474.08 and 42.25 ± 4.48 (mean \pm s.e., $n = 120$), respectively. The P/O was 744.55 ± 59.47 , ranging from 633.74 to 888.67, suggesting that the breeding system of *A. rosea* is facultative xenogamy according to the criteria of Cruden (1977).

Breeding system

There is no agamosperous seed set in *A. rosea*, because fruit set of the emasculated and bagged flowers was zero. *A. rosea* is capable of autonomous selfing (AF = 0.292), both fruit and seed set in this treatment were significantly lower than in the other treatments (fruit set $F_{4,10} = 2642.4$, $P < 0.0001$; seed set $F_{4,599} = 11.68$, $P < 0.0001$; fig. 2). There were no significant differences in fruit and seed set between intact and hand-pollinated flowers with either self- or cross-pollen, indicating that *A. rosea* has a high level of self-compatibility. There was no significant difference in seed set between intact and emasculated open flowers (Io and Eo; fig. 2B), indicating that the pollinators (mostly *Apis mellifera*, Li et al., unpublished data) were abundantly available in our study population in 2010. Delayed selfing in *A. rosea* did not provide a large contribution to seed production, since RA was only 0.026.

Mating system

The multilocus outcrossing rate of *A. rosea* in three naturalized populations ranged from 0.972 to 0.981, with a mean of 0.977. This suggests that this species is highly outcrossing. Interestingly, the rate of biparental inbreeding ($t_m - t_s$) ranged from 0.281 to 0.465, indicating that a considerable amounts of crossings occurred between closely related siblings in all three populations. The fraction of siblings that share the same father (r_{pm}) was relatively high (> 0.5) in the AS population, indicating that only 1--2 male parents provided pollen and contributed to the outcrossing for the individual family. In addition, the values for F in the three populations were 0.011 (DL), 0.014 (AS) and 0.010 (SY), lower than the estimated F (table 2).

DISCUSSION

Our data showed that *A. rosea* is predominantly an outcrossing species with potential delayed selfing when pollinators are absent or scarce. It displays floral traits that may be cor-

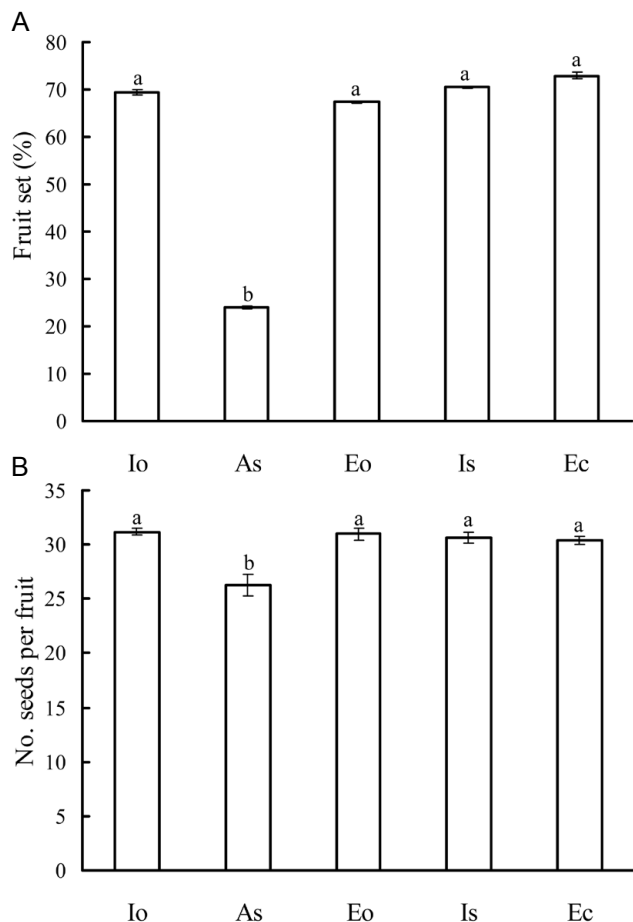


Figure 2 – Effects of five pollination treatments on fruit set (A) and the number of seeds per fruit (excluding fruits with no seeds) (B) in *A. rosea*. Error bars are ± 1 SE. Io: intact open flowers; As: autonomous self-pollination; Eo: emasculatation with open pollination; Is: supplemental pollination with self-pollen on intact flowers; Ec: supplemental pollination with outcross-pollen on emasculated flowers.

related with outcrossing (protandrous and approach herkogamous), but style curvature potentially results in delayed selfing. First, the flowers have a large corolla size, showy colours, nectar and pollen rewards, which are attractive to pollinators. Second, approach herkogamy in *A. rosea* obviously segregated male and female reproductive organs, decreasing contact between them, which is expected to enhance outcrossing (Lloyd & Webb 1986, Webb & Lloyd 1986, Holtsford & Ellstrand 1992, Belaoussoff & Shore 1995, Karron et al. 1997, Brunet & Eckert 1998, Motten & Stone 2000). Third, obvious protandry in *A. rosea* is also adaptive to outcrossing, which has been interpreted as a mechanism for reducing self-fertilization (Darwin 1862, Webb & Bawa 1983, Li et al. 2001, 2002). In *A. rosea*, anthers dehisce before the flowers open; at the second day of flowering, pollen viability begins to decline, but at that time the style branches still do not protrude from the monadelphous column. Most pollen has already been shed when some stigmas come into contact with the anthers via the curvature of style branches. Finally, the curvature of style branches positions the stigmas in the path of the visiting pollinator and this takes 10 to 12 h, which offers more opportunity to receive outcrossing pollen (Buttrose et al. 1997, Li et al. 2001, Ruan et al. 2010).

On the other hand, the curvature of style branches in *A. rosea* eventually brings some un-pollinated stigmas down to touches unshed pollen, potentially resulting in delayed selfing. At that time, stigma receptivity and pollen viability were 89.7 and 42.33%, respectively. Successful reproduction of 24% of flowers in the absence of pollinators suggested that selfing provides reproductive assurance when pollinators were scarce.

Reproductive assurance from delayed autonomous self-pollination in *A. rosea* is low ($RA = 0.026$) in our populations where pollinators (native *Apis mellifera*) were abundant. This is consistent with the results of Klips & Snow (1997) who observed that autonomous self-pollination in *Hibiscus laevis* did not contribute significantly to fruit production of open-pollinated flowers. Nagy et al. (1999) observed a similar result in *Kalmia latifolia*. In contrast, an increase in delayed selfing rates in *Collinsia verna* supports reproductive assurance when the pollination environment in wild populations necessitates reproductive assurance (Kalisz et al. 2004). Delayed selfing in *Kosteletzkya virginica* also provides reproductive assurance, because emasculated open flowers set fewer seeds than intact open flowers (Ruan et al. 2008).

Biparental inbreeding may reflect (i) the spatial genetic substructure of the population caused by the limited dispersal of pollen and/or seeds (Ennos & Clegg 1982, Turner et al. 1982), and (ii) the variation in flowering synchrony among individuals (Hall et al. 1996). There was no significant difference in total outcrossing rates (t_m) among the three populations, but significant variation was found in biparental inbreeding. Biparental inbreeding in this species is possibly associated with plant density and the product of flower size and number (Goodwillie et al. 2010). In the three studied *A. rosea* populations, the highest biparental inbreeding appeared in the AS population, which has a relative small size, compared with the other two populations.

Protandry may encourage cross-fertilization, but it still allows pollination between flowers on the same individual (geitonogamous self-pollination) to occur (Lloyd & Webb 1986, Bertin & Newman 1993). In the studied *A. rosea* populations, the pollinators are native bee species, especially *Apis mellifera* (electronic appendix D & E), which often travel predominantly among neighbouring flowers or individuals. These neighbouring individuals may be close relatives if seed dispersal is limited. In addition, many flowers in different phases are open simultaneously on a plant (up to 108), which could lead to high levels of geitonogamy. However, the outcrossing rates of three *A. rosea* natural populations were high (0.977). There was no inbreeding depression observed in *A. rosea*, because the expected inbreeding coefficient F_e was lower than our estimated inbreeding coefficient F . The high maternal inbreeding coefficient in *A. rosea* can be explained by the high level of biparental inbreeding with many full sibs produced per fruit and a subsequent limited seed dispersal. In contrast, in *Kalmia latifolia*, which has numerous flowers per individual, selfing is more prevalent within a flower than among flowers in the same plant (Levri 2000). Thus, protandry in *A. rosea* may act as a mechanism to reduce intrafloral male-female interference.

In conclusion, floral traits in *A. rosea* contribute to outcrossing and reduce intrafloral male-female interference, but can still provide some reproductive assurance by autonomous selfing if pollination levels are low.

SUPPLEMENTARY DATA

Supplementary data are available at *Plant Ecology and Evolution*, Supplementary Data Site (<http://www.ingentaconnect.com/content/botbel/plecevo/supp-data>), and consist of photographs of the flower opening process, style curvature and pollination of *Alcea rosea* (pdf format).

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REFERENCES

- Adams W.T. (1983) Applications of isozymes in tree breeding. In: Tanksley S.D., Orton T.J. (eds) *Isozymes in plant genetics and breeding*, Part A: 60–64. Amsterdam, Elsevier Science Publishers.
- Armbruster W.S., Mulder C.P.H., Baldwin B.G., Kalisz S., Wessa B., Nute H. (2002) Comparative analysis of late floral development and mating-system evolution in tribe Collinsieae (Scrophulariaceae s.l.). *American Journal of Botany* 89: 37–49. <http://dx.doi.org/10.3732/ajb.89.1.37>
- Barnaud A., Trigueros G., McKey D., Joly H.I. (2008) High outcrossing rates in fields with mixed sorghum landraces: how are landraces maintained? *Heredity* 101: 445–452. <http://dx.doi.org/10.1038/hdy.2008.77>

- Barner A.K., Pfister C.A., Wootton J.T. (2011) The mixed mating system of the sea palm kelp *Postelsia palmaeformis*: few costs to selfing. *Proceedings of the Royal Society B: Biological Sciences* 278: 1347–1355. <http://dx.doi.org/10.1098/rspb.2010.1928>
- Barrett S.C.H. (2002a) Sexual interference of the floral kind. *Heredity* 88: 154–159. <http://dx.doi.org/10.1038/sj.hdy.6800020>
- Barrett S.C.H. (2002b) The evolution of plant sexual diversity. *Nature Reviews Genetics* 3: 274–284. <http://dx.doi.org/10.1038/nrg776>
- Belaousoff S., Shore J.S. (1995) Floral correlates and fitness consequences of mating-system variation in *Turnera ulmifolia*. *Evolution* 49: 545–556. <http://dx.doi.org/10.2307/2410278>
- Bertin R.I., Newman C.M. (1993) Dichogamy in angiosperms. *Botanical Review* 59: 112–152. <http://dx.doi.org/10.1007/BF02856676>
- Brunet J., Eckert C.G. (1998) Effects of floral morphology and display on outcrossing in blue columbine, *Aquilegia caerulea* (Ranunculaceae). *Functional Ecology* 12: 596–606. <http://dx.doi.org/10.1046/j.1365-2435.1998.00231.x>
- Buttrose M.S., Grant W.J.R., Lott J.N.A. (1997) Reversible curvature of style branches of *Hibiscus trionum* L., a pollination mechanism. *Australian Journal of Botany* 25: 567–570. <http://dx.doi.org/10.1071/BT9770567>
- Bynum M.R., Smith W.K. (2001) Floral movements in response to thunderstorms improve reproductive effort in the alpine species *Gentiana algida* (Gentianaceae). *American Journal of Botany* 88: 1088–1095. <http://dx.doi.org/10.2307/2657092>
- Carrió E., Herreros R., Bacchetta G., Güemes J. (2008) Evidence of delayed selfing in *Fumana juniperina* (Cistaceae). *International Journal of Plant Sciences* 169: 761–767. <http://dx.doi.org/10.1086/588070>
- Cruden R.W. (1977) Pollen-ovule ratios: A conservative indicator of breeding systems in flowering plants. *Evolution* 31: 32–36. <http://dx.doi.org/10.2307/2407542>
- Cruden R.W. (2000) Pollen grains: why so many? *Plant Systematics and Evolution* 222: 143–165. <http://dx.doi.org/10.1007/BF00984100>
- Cruden R.W., Hermann-Parker S.M. (1997) Temporal dioecism: An alternative to dioecism? *Evolution* 31: 863–866. <http://dx.doi.org/10.2307/2407448>
- Darwin C. (1862) On the various contrivances by which British and foreign orchids are fertilised by insects. London, Murray.
- Darwin C. (1876) The effects of cross and self-fertilization in vegetable kingdom. London, Murray.
- Doyle J.J., Doyle J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.
- Eckert C.G., Samis K.E., Dart S. (2006) Reproductive assurance and the evolution of uniparental reproduction in flowering plants. In: Harder D.L., Barrett S.C.H. (eds) *Ecology and evolution of flowers*: 183–203. Oxford, Oxford University Press.
- Edwards J., Whitaker D., Klionsky S., Laskowski M.J. (2005) A record-breaking pollen catapult. *Nature* 435: 164. <http://dx.doi.org/10.1038/435164a>
- Elle E., Hare J.D. (2002) Environmentally induced variation in floral traits affects the mating system in *Datura wrightii*. *Functional Ecology* 16: 79–88. <http://dx.doi.org/10.1046/j.0269-8463.2001.00599.x>
- Ennos R.A., Clegg M.T. (1982) Effect of population substructuring on estimates of outcrossing rate in plant population. *Heredity* 48: 283–292. <http://dx.doi.org/10.1038/hdy.1982.33>
- Fetscher A.E. (2001) Resolution of male-female conflict in an hermaphroditic flower. *Proceedings of the Royal Society B: Biological Sciences* 268: 525–529. <http://dx.doi.org/10.1098/rspb.2000.1395>
- Fetscher A.E., Kohn J.R. (1999) Stigma behavior in *Mimulus aurantiacus* (Scrophulariaceae). *American Journal of Botany* 86: 1130–1135. <http://dx.doi.org/10.2307/2656976>
- Freitas L., Sazima S. (2009) Floral biology and mechanisms of spontaneous self-pollination in five neotropical species of Gentianaceae. *Biological Journal of the Linnean Society* 160: 357–368. <http://dx.doi.org/10.1111/j.1095-8339.2009.00989.x>
- Fritsch P., Rieseberg L.H. (1992) High outcrossing rates maintain male and hermaphrodite individuals in populations of the flowering plant *Datisca glomerata*. *Nature* 359: 633–636. <http://dx.doi.org/10.1038/359633a0>
- Gaiotto F.A., Bramucci M., Grattapaglia D. (1997) Estimation of outcrossing rate in a breeding population of *Eucalyptus urophylla* with dominant RAPD and AFLP markers. *Theoretical and Applied Genetics* 95: 842–849. <http://dx.doi.org/10.1007/s001220050634>
- Ge X.J., Sun M. (1999) Reproductive biology and genetic diversity of a crypto viviparous mangrove *Aegiceras corniculatum* (Myrsinaceae) using allozyme and intersimple sequence repeat (ISSR) analysis. *Molecular Ecology* 8: 2061–2069. <http://dx.doi.org/10.1046/j.1365-294x.1999.00821.x>
- Goodwillie C., Kalisz S., Eckert C.G. (2005) The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annual Review of Ecology, Evolution, and Systematics* 36: 47–79. <http://dx.doi.org/10.1146/annurev.ecolsys.36.091704.175539>
- Goodwillie C., Sargent R.E., Eckert C.G., Elle E., Geber M.A., Johnston M.O., Kalisz S., Moeller D.A., Ree R.H., Marin M.V., Winn A.A. (2010) Correlated evolution of mating system and floral display traits in flowering plants and its implications for the distribution of mating system variation. *New Phytologist* 185: 311–321. <http://dx.doi.org/10.1111/j.1469-8137.2009.03043.x>
- Hall P., Walker S., Bawa K.S. (1996) Effect of forest fragmentation on genetic diversity and mating system in a tropical tree, *Pithecellobium elegans*. *Conservation Biology* 10: 757–768. <http://dx.doi.org/10.1046/j.1523-1739.1996.10030757.x>
- Han Y.C., Teng C.Z., Gituru R.W., Zhou M.Q., Hu Z.L., Song Y.C. (2009) Mating system and genetic diversity in natural populations of *Nelumbo nucifera* (Nelumbonaceae) detected by ISSR markers. *Plant Systematics and Evolution* 277: 13–20. <http://dx.doi.org/10.1007/s00606-008-0096-x>
- Hedrick, P.W. (1985) *Genetics of populations*. Boston, Jones & Bartlett.
- Herlihy C.R., Eckert C.G. (2002) Genetic cost of reproductive assurance in a self-fertilizing plant. *Nature* 416: 320–323. <http://dx.doi.org/10.1038/416320a>
- Holsinger K.E. (2000) Reproductive systems and evolution in vascular plants. *Proceedings of the National Academy of Sciences* 97: 7037–7042. <http://dx.doi.org/10.1073/pnas.97.13.7037>
- Holtsford T.P., Ellstrand N.C. (1992) Genetic and environmental variation in floral traits affecting outcrossing rate in *Clarkia tembloriensis* (Onagraceae). *Evolution* 46: 216–225.
- Huang S.Q., Takahashi Y., Dafni A. (2002) Why does the flower stalk of *Pulsatilla cernua* (Ranunculaceae) bend during anthesis? *American Journal of Botany* 89: 1599–1603. <http://dx.doi.org/10.3732/ajb.89.10.1599>

- Huang Z. H., Zhu J. M., Mu X. J. (2004) Pollen dispersion, pollen viability and pistil receptivity in *Leymus chinensis*. *Annals of Botany* 93: 295–301. <http://dx.doi.org/10.1093/aob/mch044>
- Juncosa A.M., Webster B.D. (1989) Pollination in *Lupinus nanus* (Leguminosae). *American Journal of Botany* 76: 59–66. <http://dx.doi.org/10.2307/2444774>
- Kalisz S., Vogler D., Fails B., Finer M., Shepard E., Herman T., Gonzales R. (1999) The mechanism of delayed selfing in *Collinsia verna* (Scrophulariaceae). *American Journal of Botany* 86: 1239–1247. <http://dx.doi.org/10.2307/2656771>
- Kalisz S., Vogler D.W., Hanley K.M. (2004) Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature* 430: 884–887. <http://dx.doi.org/10.1038/nature02776>
- Karron J.D., Jackson R.T., Thumser N.N., Schlicht S.L. (1997) Outcrossing rates of individual *Mimulus ringens* genets are correlated with anther-stigma separation. *Heredity* 79: 365–370. <http://dx.doi.org/10.1038/hdy.1997.169>
- Klips R.A., Snow A.A. (1997) Delayed autonomous self-pollination in *Hibiscus laevis* (Malvaceae). *American Journal of Botany* 84: 48–53. <http://dx.doi.org/10.2307/2445882>
- Levri M.A. (2000) A measure of the various modes of inbreeding in *Kalmia latifolia*. *Annals of Botany* 80: 415–420. <http://dx.doi.org/10.1006/anbo.2000.1202>
- Li Q.J., Kress W.J., Xu Z.F., Xia Y., Zhang L., Deng X.B., Gao J., Bai Z. (2001) Pollination: flexible style that encourages outcrossing. *Nature* 410: 432. <http://dx.doi.org/10.1038/35068635>
- Li Q.J., Xu Z.F., Kress W.J., Xia Y., Zhang L., Deng X.B., Gao J. (2002) Mating system and stigmatic behaviour during flowering of *Alpinia kwangsiensis* (Zingiberaceae). *Plant Systematics and Evolution* 232: 123–132. <http://dx.doi.org/10.1007/s006060200031>
- Liu K.W., Liu Z.J., Huang L.Q., Li L.Q., Chen L.J., Tang G.D. (2006) Self-fertilization strategy in an orchid. *Nature* 441: 945–946. <http://dx.doi.org/10.1038/441945a>
- Lloyd D.G., Schoen D.J. (1992) Self- and cross-fertilization in plants. I. functional dimensions. *International Journal of Plant Sciences* 153: 358–369. <http://dx.doi.org/10.1086/297040>
- Lloyd D.G., Webb C.J. (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. *New Zealand Journal of Botany* 24: 135–162. <http://dx.doi.org/10.1080/0028825X.1986.10409725>
- Miyajima D. (2001) Floral variation and its effect on self-pollination in *Salvia splendens*. *Journal of Horticultural Science & Biotechnology* 76: 187–194.
- Motten A.F., Stone J.L. (2000) Heritability of stigma position and the effect of stigma-anther separation on outcrossing in a predominantly self-fertilizing weed, *Datura stramonium* (Solanaceae). *American Journal of Botany* 87: 339–347. <http://dx.doi.org/10.2307/2656629>
- Nagy E. S., Strong L., Galloway L.F. (1999) Contribution of delayed autonomous selfing to reproductive success in Mountain Laurel, *Kalmia latifolia* (Ericaceae). *The American Midland Naturalist* 142: 39–46. [http://dx.doi.org/10.1674/0003-0031\(1999\)142%5B0039:CODAST%5D2.0.CO;2](http://dx.doi.org/10.1674/0003-0031(1999)142%5B0039:CODAST%5D2.0.CO;2)
- Peter C.I., Johnson S.D. (2006) Doing the twist: a test of Darwin's cross-pollination hypothesis for pollinium reconfiguration. *Biology Letters* 2: 65–68.
- Ritland K. (2002) Extensions of models for the estimation of mating systems using n independent loci. *Heredity* 88: 221–228. <http://dx.doi.org/10.1038/sj.hdy.6800029>
- Ruan C.J., Li H., Susan M. (2009) *Kosteletzkya virginica* displays mixed mating in response to the pollinator environment despite strong inbreeding depression. *Plant Ecology* 203: 183–193. <http://dx.doi.org/10.1007/s11258-008-9525-8>
- Ruan C.J., Qin P., Han R.M. (2005) Floral morphology and stigma-anther separation in gynomonoeious-gynodioecious *Kosteletzkya virginica* (Malvaceae). *South African Journal of Botany* 71: 367–373.
- Ruan C.J., Qin P., He Z.X. (2004) Delayed autonomous selfing in *Kosteletzkya virginica* (Malvaceae). *South African Journal of Botany* 70: 640–645.
- Ruan C.J., Teixeira da Silva J.A., Qin P. (2010) Style curvature and its adaptive significance in the Malvaceae. *Plant Systematics and Evolution* 288: 13–23. <http://dx.doi.org/10.1007/s00606-010-0305-2>
- Ruan C.J., Zhou L., Zeng F., Han R., Qin P., Lutts S., Sadd L., Mahy G. (2008) Contribution of delayed autonomous selfing to reproductive success in *Kosteletzkya virginica*. *Belgian Journal of Botany* 141: 3–31.
- Schlessman M.A. (1986) Floral protogyny, self-compatibility and the pollination of *Ourisia macrocarpa* (Scrophulariaceae). *New Zealand Journal of Botany* 24: 651–656. <http://dx.doi.org/10.1080/0028825X.1986.10409948>
- Stebbins L.G. (1957) Self fertilization and population variability in higher plants. *American Naturalist* 91: 337–354. <http://dx.doi.org/10.1086/281999>
- Stebbins G.L. (1974) Flowering plants: evolution above the species level. Cambridge, USA, Belknap Press of Harvard University Press.
- Sun S., Gao J.Y., Liao W.J., Li Q.J., Zhang D.Y. (2007) Adaptive significance of flexistylis in *Alpinia blepharocalyx* (Zingiberaceae): a hand-pollination experiment. *Annals of Botany* 99: 660–661. <http://dx.doi.org/10.1093/aob/mcl292>
- Sun S.G., Guo Y.H., Gituru R.W., Huang S.Q. (2005) Corolla wilting facilitates delayed autonomous self-pollination in *Pedicularis dunniiana* (Orobanchaceae). *Plant Systematics and Evolution* 251: 229–237. <http://dx.doi.org/10.1007/s00606-004-0260-x>
- Tang Y., Gilbert M.G., Dorr L.J. (2007) Malvaceae. *Flora of China* 12.
- Turner M.E., Stephens J.C., Anderson W.W. (1982) Homozygosity and patch structure in plant populations as a result of nearest neighbor pollination. *Proceedings of the National Academy of Sciences* 79: 203–207. <http://dx.doi.org/10.1073/pnas.79.1.203>
- Verma S., Magotra R., Koul A.K. (2004) Stylar movement avoids self pollination and promotes cross-pollination in *Eremurus himalaicus*. *Current Science* 87: 872–873.
- Wang Y., Zhang D., Renner S.S., Chen Z. (2004) A new self-pollination mechanism. *Nature* 431: 39–40. <http://dx.doi.org/10.1038/431039b>
- Webb C.J., Bawa K.S. (1983) Pollen dispersal by hummingbirds and butterflies: a comparative study of two lowland tropical plants. *Evolution* 36: 1258–1270. <http://dx.doi.org/10.2307/2408846>
- Webb C.J., Lloyd D.G. (1986) The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *New Zealand Journal of Botany* 24: 163–178. <http://dx.doi.org/10.1080/0028825X.1986.10409726>
- Wright S. (1965) The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution* 19: 395–420. <http://dx.doi.org/10.2307/2406450>
- Yu Q., Huang S.Q. (2006) Flexible stigma presentation assists context-dependent pollination in a wild columbine. *New*

- Phytologists 169: 237–242. <http://dx.doi.org/10.1111/j.1469-8137.2005.01620.x>
- Zhang Z.Q., Li Q.J. (2008) Autonomous selfing provides reproductive assurance in an Alpine ginger *Roscoea schneideriana* (Zingiberaceae). *Annals of Botany* 102: 531–538. <http://dx.doi.org/10.1093/aob/mcn136>
- Zhao R., Xia H.B., Lu B.R. (2009) Fine-scale genetic structure enhances biparental inbreeding by promoting mating events between more related individuals in wild soybean (*Glycine soja*; Fabaceae) populations. *American Journal of Botany* 96: 1138–1147. <http://dx.doi.org/10.3732/ajb.0800173>
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