

## Research Article

Relationship of stigma behaviors and breeding system in three *Mazus* (Phrymaceae) species with bilobed stigmaXiao-Fang Jin<sup>1,2</sup>, Zhong-Ming Ye<sup>1,2</sup>, Qing-Feng Wang<sup>1\*</sup>, and Chun-Feng Yang<sup>1\*</sup><sup>1</sup>Key Laboratory of Aquatic Botany and Watershed Ecology, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074, China<sup>2</sup>University of Chinese Academy of Sciences, Beijing 100049, China

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**Abstract** Sensitive stigma has been recognized to facilitate outcrossing. We hypothesized that species with different levels of sensitivity might have corresponding differences in components of their breeding system. In this study, three *Mazus* species with bilobed stigmas were used to test the hypothesis. We explored stigma behaviors of the species in reaction time, recovery time, permanent closing time, and the minimum pollen load causing permanent closure. We investigated floral traits, pollinator type and behavior, pollination intensity, and natural schedule of pollen deposition on stigma. Moreover, we evaluated the mating system of the species by checking seed set after controlled pollination treatments, namely, natural flowers with open pollination, enclosed flowers without pollination, and enclosed flowers with self and outcross hand pollination. Results indicated that stigma of *M. pumilus* (N. L. Burman) Steenis was not sensitive, whereas stigmas of *M. miquelii* Makino and *M. stachydifolius* (Turcz.) Maxim. closed and reopened quickly in response to pollination. Accordingly, hand pollination treatments revealed that seed set of self-spontaneous pollination in *M. pumilus* was similar to the other treatments. For *M. miquelii*, outcross pollen resulted in significantly higher seed set than self-pollen. *Mazus stachydifolius* was self-incompatible. Additionally, the corresponding characteristics in other components of the breeding system for each species were found. Our study indicated that the sensitivity of bilobed stigma might be linked with floral traits and the mating system in a given species. Sensitive stigma should be regarded as an evolutionary mechanism for enhancement of outcrossing.

**Key words:** bilobed stigma, breeding system, mating system, *Mazus*, pollination, self-compatible, stigma sensitivity.

Bilobed stigma is widely spread in the high core Lamiales and the stigma close after contact by pollinators (Newcombe, 1922, 1924; Angiosperm Phylogeny Group, 2009; Schaferhoff et al., 2010). The significance of sensitive stigma has been intensely explored with regard to its two stages, namely, first closure and permanent closure (Fetscher & Kohn, 1999; Barrett, 2002; Fishman et al., 2002; Yang et al., 2004; Milet-Pinheiro et al., 2009; Rana, 2009; Sweet Singh, 2009; Sritongchuay et al., 2010). The first closure of a sensitive stigma may avoid self-pollen deposition because the stigma closes after being deposited with outcross pollen when a pollinator visits the flower, and stays closed until the pollinator flies away. The stigma therefore has no chance to receive self-pollen (Newcombe, 1922; Sharma et al., 2008). Stigma usually closes permanently if an adequate amount of pollen for full reproduction is deposited and is assumed to result in increased cross-pollination (Richardson, 2004; Yang et al., 2004; Milet-Pinheiro et al., 2009). Additionally, for species with bilobed stigma, the closure of stigma was also suggested to enhance pollen export (Fetscher et al., 2002).

The level of sensitivity in bilobed stigmas differs widely among plants of the same family (Milet-Pinheiro et al., 2009) and of the same genus (Kimata, 1978; Macnair et al., 1989;

Ritland & Ritland, 1989; Dole, 1992). For example, the primary closing time after contact by pollinators varies from 2 to 90 s in different plants (Lloyd & Schoen, 1992; Milet-Pinheiro et al., 2009; Rana, 2009). The reopening time also differs among plants and under different treatments such as mechanical touch, or touch with self or outcross pollen grains (Fetscher & Kohn, 1999; Richardson, 2004; Qu et al., 2007). In some species, permanent closure may also happen even though there is insufficient pollen load for seed set (Fetscher & Kohn, 1999).

Studies have indicated that behavior of bilobed stigma may correlate with many components of the reproductive system of a given species, such as corolla height and width (Fetscher et al., 2002), floral longevity (Jorgensen & Arathi, 2013), pollinator species (Richardson, 2004), visit duration (Milet-Pinheiro et al., 2009), pollination intensity (Bertin, 1990), and extent of self-compatibility (Sharma et al., 2008; Milet-Pinheiro et al., 2009; Ruan & da Silva, 2012). In this study, we hypothesize that plants with bilobed stigma may have a corresponding level of stigma sensitivity according to differences in their breeding system; therefore, they take different strategies to achieve reproductive success. To test this hypothesis, we compared the stigma behaviors, floral traits,

and pollination processes as well as mating systems in three *Mazus* species with bilobed stigma. Additionally, the three *Mazus* species have different breeding systems and different levels of stigma sensitivity. We aim to ascertain the linkage between stigma sensitivity and breeding system in the three *Mazus* species.

## Material and Methods

### Study species and site

Three *Mazus* species, *M. pumilus* (N.L. Burman) Steenis, *M. miquelii* Makino, and *M. stachydifolius* (Turcz.) Maxim. (Phrymaceae), were used in this study. *Mazus pumilus* is an annual species that grows in dry habitats. *Mazus miquelii* and *M. stachydifolius* are perennial, grow in grassland and wet places at the forest edges, and use short stolon for asexual reproduction (Hong et al., 1998). Generally, in Wuhan (Hubei Province, China), these three species flower from late March to early June. *Mazus miquelii* and *M. stachydifolius* offer both pollen and nectar to pollinators, whereas *M. pumilus* only pollen (Hong et al., 1998). *Mazus pumilus* has 1–3 inflorescences each with 6–13 whitish flowers, *M. miquelii* grows 1–16 inflorescences each with 13–20 whitish to blue flowers, and individuals of *M. stachydifolius* produce 1–4 inflorescences each with 7–23 blue flowers (Hong et al., 1998).

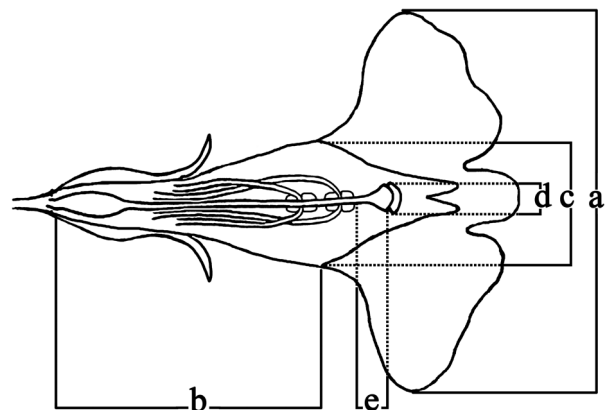
Natural populations of the three species from Wuhan Botanic Garden, Hubei Province, China were used in this study. The population of each species consisted of at least 100 individuals. The studied populations were separated by more than 50 m. Field investigation was carried out from March to May in 2012 and 2013. In addition, we carried out hand pollination treatments in 2013 to detect differences in mating systems and stigma behavior of the studied species. Thirty individuals of each species were enclosed with fine bridal veil netting before the flowers opened in order to exclude pollinators.

### Floral traits

To compare the floral morphology of the three species, we measured corolla width, corolla-tube length, corolla-opening width, stigma width, and anther–stigma separation (Fig. 1). At least 10 randomly selected flowers, each from different individuals of each species, were used for measurements with a digital caliper. For each species, we also counted pollen and ovule production per flower ( $n=10$ ) as well as the longevity of a single enclosed and open flower (*M. pumilus*,  $n=10$ , 33; *M. miquelii*,  $n=27$ , 41; *M. stachydifolius*,  $n=12$ , 14, respectively). Each flower was randomly selected from different individuals. Pollen grains were detached from non-dehiscent anthers and counted in five 20- $\mu$ L subsamples from each flower under a light microscope (80i; Nikon, Tokyo, Japan). Ovules were dissected from an ovary and counted under a stereo microscope (SMZ 800; Nikon). Flower longevity indicated the days from corolla opening to wilting.

### Stigma behavior

To investigate the differences in stigma behavior, we compared the first closure time, recovery time, and permanent closure time after hand-pollinating with outcross pollen for the three species. We carried out hand pollination from 09:00 to



**Fig. 1.** Top view of a *Mazus* flower (drawn by Xiao-Fang Jin) shows the proportions of (a) corolla width, (b) corolla-tube length, (c) corolla-opening width, (d) stigma (lower lobe) width, and (e) anther–stigma separation.

10:30 on sunny days in randomly selected new flowers, which were enclosed in advance to exclude pollinators. Reaction time (in seconds) was determined from the moment of touching the lower stigma lobe to the time when the two lobes closed entirely. Recovery time (in minutes) was recorded as the time from stigma closure to reopening of the two lobes. Permanent closure time (in hours) was estimated by the time from the first touch to the point that lobes closed and would not open again. Stigmas for measurement of permanent closure time were checked every hour after pollination until 19:00. To detect the amount of pollen load for permanent stigma closure, we deposited different pollen loads on the stigma by using different sizes of fine brush for each of the species. Enclosed flowers with permanent closure stigmas were fixed in FAA solution (formalin : acetic acid : 70% ethanol at a ratio of 5:6:89 by volume) for count of stigmatic pollen load. Pollen grains were detached from a squashed stigma and counted in five 20-mL subsamples from each flower under a light microscope (80i; Nikon). In addition, for *M. miquelii* and *M. stachydifolius*, pollination with self and outcross pollen was carried out in order to test the potential effects of pollen source on stigma behavior. Furthermore, to link the relationship of pollen tube growth and stigma permanent closure for each species, we also observed growth of pollen tube under a fluorescence microscope (E-600; Nikon) after treatment with 8 mol/L NaOH for 24 h followed by 0.1% aniline blue dye. For each treatment, 20 flowers from different individuals of each species were used.

### Frequency and behavior of pollinators

For all the studied species, we carried out pollination observation on sunny days from 09:00 to 16:00 when pollinator frequency was relatively high. We noted different pollinator types and recorded the duration of each pollinator during foraging on a single flower of each species ( $n > 30$ ). Pollinator frequency was compared among the three species and was estimated by the total number of pollinators recorded during foraging on an inflorescence within the total pollination observation time, where 15 min was used as an individual observation period.

### Stigmatic pollen load

For each species, to detect the effects of differences in stigma behavior on the pattern of pollen deposition on stigma, we collected stigmas from flowers on the first day of anthesis three times on a sunny day, namely 09:00, 14:00, and 19:00. Both enclosed and open flowers were harvested to estimate the potential pollen load without pollinators. To evaluate the pollen removal rate of the three species under open pollination, we counted the pollen remains in anthers when stigmas of *M. miquelii* and *M. stachydifolius* closed permanently in nature. And for *M. pumilus*, we counted pollen remains in anthers after the first day. For each treatment, more than 12 randomly selected flowers were picked up at the exact time and fixed immediately into FAA solution. The stigmas were then used for pollen load count under a fluorescence microscope (E-600; Nikon) after treatment with 8 mol/L NaOH for 24 h followed by 0.1% aniline blue dye. The anthers were squashed to dislodge pollen grains, and the number of pollen grains in five 20  $\mu$ L subsamples from each flower was counted under a light microscope.

### Mating system

To test the differences in mating systems among the three species, we carried out hand pollination with self and outcross pollen grains on stigmas of intact flowers. The flowers were then enclosed for setting fruits. The self-pollen was from the same flower whereas the outcross pollen was collected from other individuals in the population. For *M. pumilus*, outcrossed pollination was carried out on emasculated flowers, although there might be self-pollen grains deposited on stigmas. All hand-pollinations were carried out using a fine brush twice a day to deposit enough pollen grains on stigma. Seed set as well as fruit set after selfed and outcrossed pollination were used to detect the differences in mating systems among species. Moreover, to detect the potential autonomous pollination and pollen limitation, these data were also used to compare with seed set for enclosed flowers and for flowers under open pollination. In this study, seed set meant the percentage of seeds per fruit to the average ovules per flower while fruit set indicated numbers of fruit divided by total numbers of flowers used in the treatment.

### Data analysis

Differences in floral traits and stigma reaction time among studied species were compared by one-way ANOVA, which was also used to detect the differences in stigmatic pollen load and seed set among different treatments within each species. Multiple comparisons with Tukey's honest significant differ-

ence test (homogeneous variances) or Games–Howell test (non-homogeneous variances) were carried out when one-way ANOVA revealed significance. Differences in pollinator frequency among the three species were detected by Pearson's  $\chi^2$ -test. To fulfil requirements of each test, all data were analyzed for normality of variance prior to analysis. All analyses were carried out in SPSS version 16.0 (SPSS, Chicago, IL, USA).

## Results

### Floral traits

Floral traits of the three *Mazus* species are significantly different in several aspects (Table 1). Corolla width, corolla-tube length, and corolla-opening width of *M. pumilus* were much smaller than those of *M. miquelii* and *M. stachydifolius*. Stigma width of *M. miquelii* was the largest. Anther–stigma separation of *M. stachydifolius* was the largest, while *M. pumilus* was absolutely 0. Anther–stigma separation did not change with time in *M. miquelii* and *M. stachydifolius*, whereas anthers of *M. pumilus* extended and wrapped up the stigma. Pollen number and pollen/ovule ratio of *M. pumilus* were much smaller than those of *M. miquelii* and *M. stachydifolius*.

Floral longevity of *M. pumilus* ( $1.80 \pm 0.20$  days for enclosed flowers,  $1.45 \pm 0.11$  days for open flowers;  $P = 0.131$ ) was shorter than *M. miquelii* ( $4.41 \pm 0.28$  days for enclosed flowers;  $3.07 \pm 0.18$  days for open flower;  $P < 0.001$ ) and *M. stachydifolius* ( $4.42 \pm 0.34$  days for enclosed flowers;  $3.14 \pm 0.25$  days for open flowers;  $P < 0.001$ ). Corolla of a fresh flower for the three *Mazus* species opened at approximately 09:00 and closed at approximately 19:00, while a closed flower reopened corolla at approximately 08:00. The stigmas of all the three *Mazus* species were opened and the anthers dehisced upon the flowers' opening.

### Stigma behavior

The stigmas' behaviors differed among the three *Mazus* species (Table 2). *Mazus pumilus* stigmas curved into the long anthers, which allowed pollen deposition before opening of the flowers. Moreover, the stigmas of the species showed very low levels of sensitivity and changed inconspicuously after touch. In contrast, the stigmas of *M. miquelii* and *M. stachydifolius* closed quickly, within 5 s, but showed no differences between the closed time of two species ( $F_{1,29} = 0.085$ ,  $P = 0.773$ ). In addition, *M. miquelii* stigmas reopened more rapidly than *M. stachydifolius* ( $F_{1,76} = 82.746$ ,  $P < 0.001$ ). Our results indicated that stigmas closed

**Table 1** Comparison of floral traits (mean  $\pm$  SE,  $n = 10$  for each parameter) in three *Mazus* species. Values with the same superscripted letters are not significantly different

Floral traits	<i>M. pumilus</i>	<i>M. miquelii</i>	<i>M. stachydifolius</i>	F	P-value
Corolla width (mm)	$7.15 \pm 0.36^c$	$18.56 \pm 0.14^a$	$16.08 \pm 0.82^b$	131.87	<0.001
Corolla-tube length (mm)	$3.89 \pm 0.14^b$	$7.94 \pm 0.11^a$	$8.72 \pm 0.24^a$	229.08	<0.001
Corolla-opening width (mm)	$4.42 \pm 0.30^b$	$5.88 \pm 0.08^a$	$5.81 \pm 0.17^a$	15.90	<0.001
Stigma width (mm)	$0.84 \pm 0.03^b$	$1.12 \pm 0.08^a$	$0.93 \pm 0.03^b$	24.04	<0.001
Anther–stigma separation (mm)	$0.00 \pm 0.00^c$	$0.27 \pm 0.04^b$	$1.32 \pm 0.12^a$	96.02	<0.001
Ovule number per flower	$366.90 \pm 13.55^b$	$407.20 \pm 9.12^{ab}$	$440.40 \pm 16.38^a$	7.60	<0.001
Pollen number per flower	$8109.70 \pm 419.59^c$	$17718.70 \pm 1250.20^b$	$20590.43 \pm 1149.14^a$	25.11	0.002
Pollen/ovule ratio	$22.07 \pm 0.66^c$	$43.22 \pm 2.45^b$	$55.37 \pm 5.24^a$	25.16	<0.001

**Table 2** Comparison of stigma behaviors (mean  $\pm$  SE,  $n = 20$  for each treatment) in three *Mazus* species

Stigma behavior	<i>M. pumilus</i> <sup>†</sup>	<i>M. miquelii</i>	<i>M. stachydifolius</i>	P-value
Reaction time (s)	–	5.19 $\pm$ 0.35	5.00 $\pm$ 0.53	0.773
Recovery time (min)	–	11.87 $\pm$ 1.92	25.16 $\pm$ 8.91	<0.001
Permanent closure time	–	Approx. 8 h	Approx. 6 h	–
Pollen loads needed for permanent closure of stigma	–	>600	>300	–
Pollen tube position when stigma permanently closed	–	In the style	Reached the ovules	–

<sup>†</sup>Stigma of *M. pumilus* was not sensitive. Student's *t*-test was applied to compare the differences in reaction time and recovery time between *M. miquelii* and *M. stachydifolius*. –, No data.

permanently when provided with more than 600 and 300 pollen grains for *M. miquelii* and *M. stachydifolius*, respectively (Table 2). Otherwise, the stigmas would reopen until adequate pollen deposition. However, even though pollen load was more than necessary, the stigmas would still be sensitive for several hours and then close permanently.

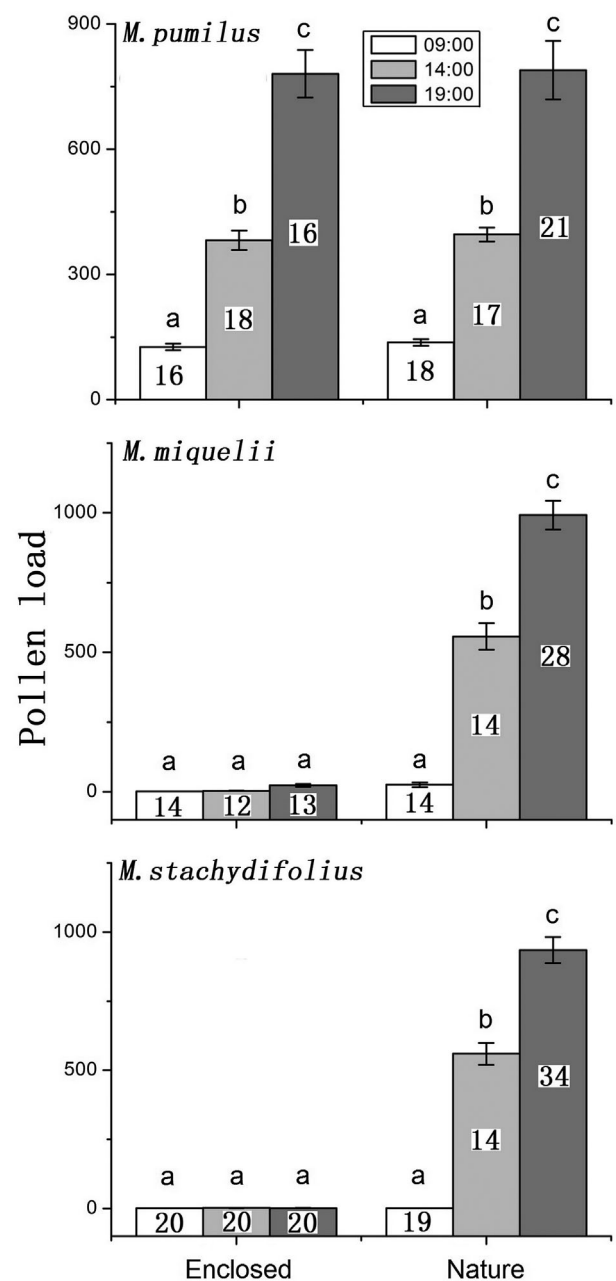
Under open pollination, stigmas of *M. miquelii* and *M. stachydifolius* opened the first day and closed permanently in the afternoon. In enclosed flowers, stigmas stayed open even after corolla wilting. However, *M. pumilus* stigmas did not close whether the flowers were enclosed or opened. For *M. miquelii* and *M. stachydifolius*, natural pollen load was more than necessary for triggering permanent closure of the stigma (Fig. 2). Pollen tubes of *M. stachydifolius* grew more quickly than those of *M. miquelii* (Table 2), which reached the ovules when the stigma had permanently closed, taking approximately 6 h after pollination. For *M. miquelii*, pollen tubes were still in the style when the stigma had permanently closed 8 h after pollination. No differences were detected in stigma behavior after hand pollination with self or outcross pollen grains. Both *M. miquelii* and *M. stachydifolius* stigmas closed permanently following reception of enough self-pollen.

#### Frequency and behavior of pollinators

Bees were the main pollinators for the three *Mazus* species. For *M. pumilus*, *Halictidae*, consisting of *Halictus calceatus*, *Halictoides* sp., and *Lasioglossum* sp. were observed. *Mazus miquelii* and *M. stachydifolius* shared the pollinators, which consisted of *Apidae* (*Tetralonia jacoti*, *Tetralonia chinensis*, *Habropoda* sp., and *Habropoda tainanica*) and *Meganomiidae* (*Osmia rufina*, *Osmia caerulescens*, *Osmia jacoti*, and *Megachile ligniseca*). Pollinator frequency of *M. miquelii* and *M. stachydifolius* was much higher than *M. pumilus*. During an observation period (15 min) within an inflorescence, *M. miquelii* received 21.8 visits, *M. stachydifolius* attracted 9.2 visits, and *M. pumilus* was observed with only 1.8 visits. Each type of pollinator showed no differences in pollination duration when foraging on a single flower among the three species but did differ among each other. The pollination duration was  $29.25 \pm 3.29$  s for *Halictidae*,  $1.89 \pm 0.07$  s for *Apidae*, and  $3.86 \pm 0.29$  s for *Meganomiidae*. *Halictidae*, pollinators with small body size, mainly collected pollen whereas the larger pollinators *Apidae* and *Meganomiidae* mainly sought nectar. Stigmas of *M. miquelii* and *M. stachydifolius* closed as soon as *Apidae* and *Meganomiidae* entered the flower.

#### Stigmatic pollen load

Amount of pollen deposited in the stigmas increased significantly along the day in open pollinated flowers. For *M. pumilus*, enclosed and open flowers did not show



**Fig. 2.** Stigmatic pollen load of enclosed and open flowers from *Mazus pumilus*, *M. miquelii*, and *M. stachydifolius* at three times in the first day of each flower. Within each species, sites with different lowercase letters are significantly different ( $P < 0.05$ ). Bar = 1 SE. Sample sizes are shown within each bar.

differences in pollen load. However, enclosed flowers of both *M. miquelii* and *M. stachydifolius* could not achieve pollen deposition on stigmas (Fig. 2). Generally, under conditions of open pollination, pollen load at the end of the first day of anthesis was greater than the number of ovules in all three *Mazus* species. However, it was more pollen than was needed for stigmas to close permanently in *M. miquelii* and *M. stachydifolius*. Pollen remaining in anthers after permanent closure of stigma in *M. miquelii* was  $7557.19 \pm 708.94$  ( $n = 26$ ), which was 42.6% of total pollen production per flower. In *M. stachydifolius*, pollen remaining was 30.8% of total pollen production per flower ( $6333.67 \pm 4484.75$ ,  $n = 34$ ), whereas in *M. pumilus* pollen remaining in anthers after the first day was  $7370.21 \pm 710.44$  ( $n = 21$ , 90.9% of total pollen production per flower).

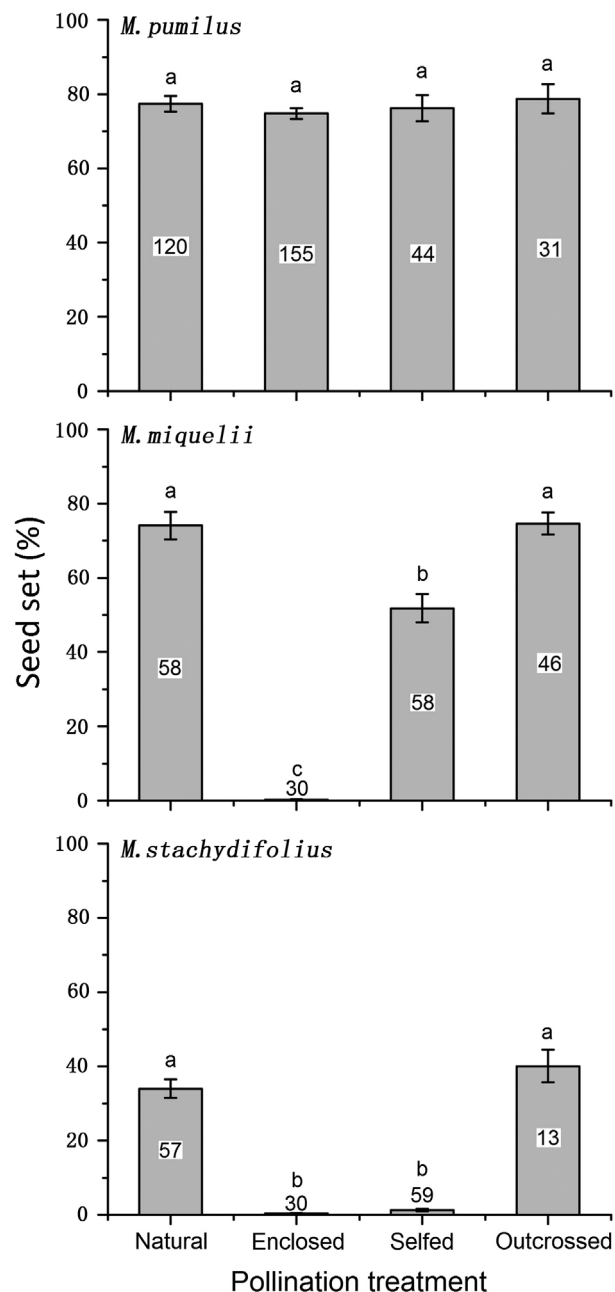
### Mating system

*Mazus pumilus* showed no significant differences in seed set under conditions of excluding pollination, open pollination, and hand pollination with self and outcross pollen grains ( $F_{3,349} = 0.530$ ,  $P = 0.662$ ). *Mazus miquelii* and *M. stachydifolius* showed significant differences in seed set under different pollination treatments ( $F_{3,191} = 73.044$ ,  $P < 0.001$  and  $F_{3,155} = 99.130$ ,  $P < 0.001$ , respectively). However, they could not set seeds when the flowers were enclosed (Fig. 3). For *M. miquelii*, seed set was significantly lower in self-pollination than in outcross pollination; for *M. stachydifolius*, pollination with self-pollen yielded almost no seeds. Seed set under open pollination and outcrossed hand pollination did not differ significantly in both *M. miquelii* and *M. stachydifolius* (Fig. 3). Fruit set for flowers under the four pollination treatments, namely natural, enclosed, self, and outcross pollinated, were 100%, 100%, 100%, and 100% for *M. pumilus*, 100%, 30%, 89.6%, and 100% for *M. miquelii* and 100%, 27%, 30.5%, and 100% for *M. stachydifolius*, respectively. Moreover, fruits of enclosed and self-pollinated flowers in *M. miquelii* and *M. stachydifolius* were smaller in size than others and contained very few seeds (Fig. 3).

### Discussion

Results of pollination treatments indicated that *Mazus pumilus* was completely self-compatible and mainly depended on autonomous selfing for seed production. *Mazus miquelii* was partially self-compatible as self-pollination reduced fruit set and yielded lower seed production than outcrossed pollination. In *M. stachydifolius*, self-pollination resulted in low fruit set whereby the fruits produced almost no seeds, indicating that *M. stachydifolius* might be self-incompatible. The significant and gradual differences in mating systems of the three *Mazus* species provide ideal materials to establish the linkage between the levels of stigma sensitivity and other components of their breeding systems.

One of the important aspects of sensitive stigma, in terms of evolutionary significance, is considered to be the reduction of self-interference (Newcombe, 1922, 1924; Sharma et al., 2008). Our results fit well with this statement. The species whose stigma has a higher level of sensitivity has a more obvious tendency to outcross mate instead of self mate. *Mazus pumilus* is mainly selfing because the stigma contacted with the



**Fig. 3.** Seed production per fruit under different pollination treatments in three *Mazus* species. Within each species, sites with different lowercase letters are significantly different ( $P < 0.05$ ). Bar = 1 SE. Sample sizes are shown within each bar.

dehiscing anther before anthesis, whereby the stigmatic pollen load in the enclosed flower was sufficient for seed set. It is therefore suggested that stigma sensitivity seems not to be necessary in either reducing the selfing rate or facilitating pollination. The fact that its stigma is always open during anthesis well supports the argument. For the other two species, *M. miquelii* is partially self-compatible and *M. stachydifolius* is self-incompatible. It is important to prevent self-pollen deposition on the stigma as self-pollen can germinate on the stigma, grow into the style and, moreover,

cause permanent closure of the stigmas. The self-pollen may clog the stigma and style, preventing deposition of further alien pollen, thereby compromising reproductive success (Richardson, 2004). Stigmas of *M. miquelii* and *M. stachydifolius* began to close quickly in response to pollinators' entry in the flowers. The stigmas curve backwards and place the receptive surface away from the path of the exiting insects, thus preventing autogamy and interference in self-pollen transfer (see also Sharma et al., 2008). The mechanism of avoiding self-pollen deposition has been widely reported in species with sensitive stigma (Newcombe, 1922, 1924; Ritland & Ritland, 1989; Fetscher & Kohn, 1999; Milet-Pinheiro et al., 2009).

Although there is no significant difference in reaction time of stigma between *M. miquelii* and *M. stachydifolius*, the latter has a significantly longer reopening time (recovery time). This may help to reduce the possibility of geitonogamous mating and contribute to its high tendency of outcross mating. Additionally, pollen growth rate of *M. stachydifolius* is more rapid than that of *M. miquelii*, which infers a more intense pollen competition. Studies have indicated that pollen competition can reduce inbreeding depression and favor outcross mate (Lankinen & Armbruster, 2007; Lankinen et al., 2009). For self-incompatible *M. stachydifolius*, elimination of the possibility of ovules disabled by self-pollen through pollen competition may be helpful in enhancing reproductive success.

Sensitive stigma has also been shown to play important roles in facilitating pollination (Bertin, 1982, 1990; Fetscher & Kohn, 1999; Yang et al., 2004). Open stigma is beneficial for pollen import but impedes pollen export and vice versa (Fetscher et al., 2002). In this aspect, the three *Mazus* species also showed differences in stigma behaviors. *Mazus pumilus* uses all means necessary to deposit more pollen (regardless of self or outcross) on stigma as this species mainly depends on self-pollination for seed production. It is reasonable that the stigma is always open during anthesis. For *M. miquelii* and *M. stachydifolius*, a balance between pollen import and export through alternating stigma closure and opening is essential.

Studies have indicated that sensitive stigmas will keep open until the pollen load is adequate for all ovules to be fertilized in some species, for example, *Campsis radicans* (L.) Seem. and *Mimulus aurantiacus* Curtis (Bertin, 1982, 1990; Fetscher & Kohn, 1999; Yang et al., 2004). However, the stigmas of *M. miquelii* and *M. stachydifolius* do not close permanently upon deposition of sufficient pollen grains on the stigmas. They remain sensitive for several hours, which provides opportunities for more outcross pollen to be deposited. Milet-Pinheiro et al. (2009) have also shown that earlier permanent stigma closure may result in reduced seed set in *Tabebuia impetiginosa* (Mart. ex DC.) Standl. and *Jacaranda rugosa* A. H. Gentry. Prolonged stigma opening time may lead to more pollen deposition on stigma, which may intensify pollen competition and, therefore, enhance the female function (Niesenbaum, 1999; Marshall et al., 2007). However, Fetscher et al. (2002) indicated that, in *Mimulus aurantiacus*, flowers with closed stigmas export twice as many pollen grains than flowers with open stigmas. The rapid reaction response to pollination of stigmas in *M. miquelii* and *M. stachydifolius* reduces the risk of impeding pollen export. Moreover, they close stigmas permanently after the first day and only present male function for pollen export as pollen remains are relatively high at that time (Arathi et al., 2002). *Mazus miquelii* and *M. stachydifolius*

temporally segregate male and female stages to improve both pollen import and export (see also Muchhala, 2006).

Stigma characteristics should evolve together with other components of the breeding system in given taxa (Yang et al., 2002). For these three species, differences in anther behavior have been found correlating with floral syndrome as well as pollination system (see also Ritland & Ritland, 1989; Fishman et al., 2002; Ivey & Carr, 2012; Kalisz et al., 2012). First, they differ in pollinator type. *Mazus pumilus*, the selfing species with small and nectarless flowers, mainly attracted *Halictus*, a pollen-collecting insect that cannot contact stigmas when entering or exiting the stigma due to its small body size. *Mazus miquelii* and *M. stachydifolius*, however, were pollinated by nectar-seeking insects, *Meganomiidae* and *Apidae*. The pollinators have larger body size and cause a rapid stigma reaction when entering flowers of these species. Second, they also have different strategies in sex allocation. According to Cruden's prediction (Cruden, 1977), the pollen-ovule ratio of the three species is increasing with the decreasing self mate choice. Finally, the mating pattern is also correlated with anther-stigma separation, which is positively correlated with outcrossing capability (see also Karron et al., 1997; Kelly & Arathi, 2003).

In conclusion, the three *Mazus* species represent different levels of sensitivity in stigma behaviors, which are shown to correspond with respective floral traits as well as the mating system. *Mazus pumilus* mainly depends on autonomous selfing for sexual reproduction and its stigma is not sensitive. *Mazus miquelii* is partially self-compatible because selfing reduced reproductive output in comparison with outcrossing. Correspondingly, sensitive stigma of the species is beneficial for receiving outcross pollen but reduces deposition of self-pollen. Self-incompatible *M. stachydifolius* also has a sensitive stigma and a large anther-stigma separation, which effectively reduces the risk of self-pollen transfer. In addition, the sensitive stigma may also avoid interfering with pollen export. Our results suggest that sensitive stigma is an evolutionary mechanism for enhancement of outcrossing, which should act together with other floral traits.

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