

# Pollination ecology of *Arnebia szechenyi* (Boraginaceae), a Chinese endemic perennial characterized by distyly and heteromorphic self-incompatibility

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Heterostyly, a genetically controlled floral polymorphism, includes both distyly and tristily. In Boraginaceae, distyly was reported in several genera but it was rarely studied quantitatively in the genus *Arnebia*. We experimentally studied the pollination ecology of *Arnebia szechenyi*, a perennial herb native to China. It exhibited precisely reciprocal herkogamy and marked between-morph pollen number and pollen shape dimorphism, and the ratio between the two morphs in each of the four populations studied was nearly equal to 1. This indicates an equilibrium ratio in distylous species with heteromorphic self-incompatibility, which was further supported by the fact that no fruit was produced in flowers subjected to self-pollination or intra-morph pollination. In comparison with naturally pollinated flowers, hand inter-morph pollination increased both fruit set and seed set significantly, indicating pollen limitation in both morphs under natural conditions. *Bombylius* sp. and *Nomia femoralis* were the main visitors and could efficiently pollinate between the morphs of *A. szechenyi*. Overall, our results indicate that *A. szechenyi* is a typically distylous species with heteromorphic self-incompatibility, and the pollen limitation of seed production might suggest that distyly in *A. szechenyi* could be selected via male fitness.

## Introduction

Heterostyly is a genetically controlled floral polymorphism in which plant populations are composed of two (distyly) or three (tristyly) floral

morphs (Barrett 1992), with distyly being much more common than tristily (Ganders 1979a). Distylous flowers differ in reciprocal stigma and anther position (Lloyd & Webb 1992a), the long-styled (LS) and short-styled (SS) morphs being

similar in morphology to approach herkogamy or reverse herkogamy (Barrett *et al.* 2000). In distylous plants, the supergene determining floral morphology also controls a diallelic sporophytic self-incompatibility system (Ganders 1979a). Therefore, a common feature of heterostyly is the presence of a self- and intra-morph incompatibility system which allows only legitimate (between anthers and stigmas of the same level) pollination to set fruit (Darwin 1877, Barrett 1990) and thus distyly provides precise pollen transfer without the costs that are associated with sexual interference and self-pollination (Kohn & Barrett 1992, Paillet *et al.* 2002). However, morphological traits associated to distyly may vary among species, populations and individuals (Ganders 1979a, Dulberger 1992, Rossi *et al.* 2005), and heteromorphic self-incompatibility is not always or partially expressed in heterostylous species (Dulberger 1992).

Distyly is known to have evolved independently in approximately 31 angiosperm families (Zhou & Wang 2009). Boraginaceae, with nine distylous genera, is one of the most widespread such families (Ganders 1979a). Most previous studies concerning distyly focused on few species in *Amsinckia*, *Anchusa*, *Cryptantha*, *Lithospermum* and *Pulmonaria*, but few studies were carried out in the other genera.

*Arnebia* is a small genus containing more than 20 species in Boraginaceae. However, almost no experimental study has been performed on the floral biology and breeding system in this genus, although variations in seed production were reported in *A. euchroma* (Feng & Tan 2006). Accordingly, we studied *A. szechenyi* to understand the floral biology and breeding system of *Arnebia* comprehensively. We aimed to (1) quantitatively investigate the floral traits of *A. szechenyi*, (2) examine a self and/or heteromorphic incompatibility system (3) observe pollinators and their corresponding behavior towards *A. szechenyi*.

## Material and methods

### Study species and site

*Arnebia szechenyi*, a herbaceous perennial with

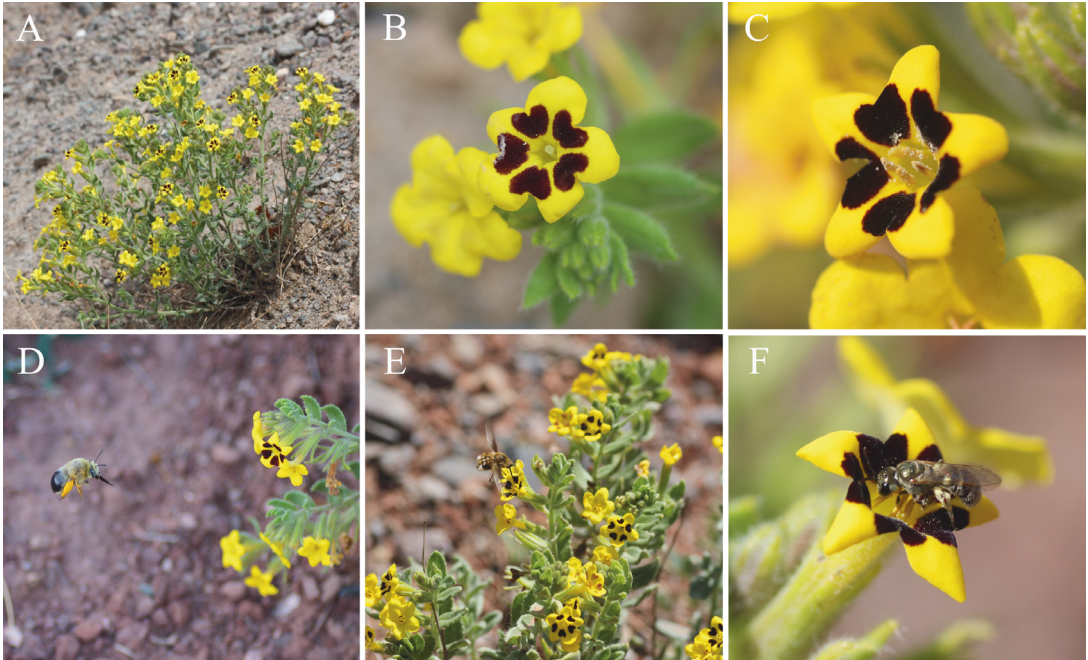
individual plants bearing either long- (Fig. 1B) or short-styled (Fig. 1C) flowers is endemic to China and usually inhabits sunny mountain slopes along the Yellow River. The stems are 20–30 cm tall and sparsely branched. The cymes are 1.5–5 cm, producing several small, tightly closed, tubular and yellowish flowers (Fig. 1A). The anthesis lasts from May to July and the flower longevity is 3–4 days. The freshly opened flowers of *A. szechenyi* have five contrasting black patterns on the corolla, which are considered as nectar guides (Fig. 1B). However, on the second day after flower opening, the nectar guides begin to fade, and then disappear completely by the afternoon (Fig. 1B). Furthermore, flowers without nectar guides were maintained for about two days before wilting. The nutlets are light brown, triangular-ovate and usually contain one to four seeds when ripe. The experiments were performed in Xunhua Salar Autonomous County (35°50′38.6″N, 102°35′39.9″E; 1800–1900 m), Qinghai province, from May to June 2012.

### Floral traits

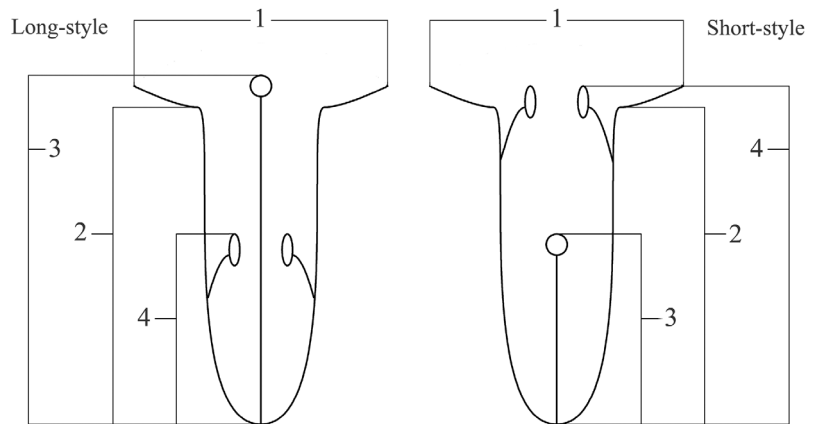
To test the floral dimorphism, 40 flowers from different plants including 20 long-styled and 20 short-styled morphs were selected to measure the corolla diameter, and the heights of corolla tube, stigma and anther (Fig. 2). All floral measurements were made to the nearest 0.01 mm using digital callipers.

### Pollen and ovule

The number of pollen grains and ovules was quantified on 20 plants per morph. In the field, a single almost mature floral bud per individual was collected and fixed in FAA solution (formalin: acetic acid: anhydrous ethanol at a ratio of 5:5:90 by volume). In the laboratory, each bud was dissected and examined under a stereoscope to count the ovule number; then each anther was split and all the pollen grains were suspended in 5 ml of water solution with a drop of eradicator for full suspension (Dafni 1992). The number of pollen grains in each flower was determined by counting the number of pollen grains in ten



**Fig. 1.** Flowers and pollinators of *Arnebia szechenyi*. — **A:** Habitat and plant. — **B:** Long-styled flower. — **C:** Short-styled flower. — **D and E:** *Bombylius* sp. — **F:** *Nomia femoralis*.



**Fig. 2.** Floral measurements of the two morphs of *Arnebia szechenyi*. — 1 = corolla diameter, 2 = corolla tube height, 3 = stigma height, 4 = anther height.

replicates of pollen solution of 10  $\mu$ l. In addition, the pollen morphology was observed under the stereoscope.

### Morph ratios

Four populations (S1–S4) of *A. szechenyi* in eastern Qinghai province (locations: 35°46′41.3″N, 102°03′43.2″E; 35°43′21.1″N, 102°03′34.2″E; 35°50′38.6″N, 102°35′39.9″E;

and 35°50′38.6″N, 102°35′39.9″E; respectively) were sampled to quantify morph ratios in natural conditions. Every plant (1154 in total) was sampled from each population.

### Artificial pollination

To determine the mating system of *A. szechenyi*, the following treatments were carried out: (1) floral buds were emasculated and netted (to test

apomixes), (2) intact floral buds were netted (to test spontaneous self-pollination), (3) hand self-pollination (flowers were pollinated with pollen from the other flowers of the same individual), (4) hand intra-morph pollination (flowers were pollinated with pollen from flowers of the same morph), (5) hand inter-morph pollination (flowers were pollinated with pollen from flowers of the opposite morph), including LS pollen on SS stigma and SS pollen on LS stigma, and (6) control (flowers were naturally pollinated). Emasculation was not done until the flowers were fully open; then the treated flowers were netted to exclude floral visitors. Hand-pollination was conducted one day after the flowers were fully open. Only one treatment was represented on each plant and in each treatment, 40 flower buds on 40 separate plants with equal long- and short-styled morphs (1:1) were treated and harvested after 20 days to evaluate the fruit set and seed set of different pollination treatments.

### Observation of flower visitors

Preliminary observations of flower visitors were carried out at 30-min intervals between 8 a.m. and 8 p.m. on sunny and windless days. During each interval, one or two plants were selected randomly. The flower morph, numbers of flowers with and without nectar guides as well as visitation times of different flower visitors of each plant were recorded. The corresponding behaviors of different flower visitors were also recorded to determine whether they could efficiently pollinate between the two morphs of *A. szechenyi*. Based on our observations, in most cases, flower visitors just visited flowers with nectar guides. Consequently, the numbers of flowers with nectar guides rather than total opened flowers were recorded to calculate visitation rate per flower per hour of different flower visitors.

### Statistical analysis

Differences between morphs in terms of quantitative floral traits, amount of pollen grains

and pollen/ovule ratio were evaluated by *t*-test. Morph ratios were analyzed using a  $\chi^2$ -test to verify whether there was inequality of frequencies. Two-way ANOVA was employed first to evaluate effects of flower morph (long-styled and short-styled) and treatment (hand inter-morph pollination and control) on seed set, and then to evaluate effects of flower morph (long-styled and short-styled) and pollinator (*Bombus* sp. and *Nomia femoralis*) on visitation rate per flower per hour (square-root arccosine transformation). Binary Logistic Regression was employed to evaluate effects of flower morph (long-styled and short-styled) and treatment (hand inter-morph pollination and control) on fruit set. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean  $\pm$  SE.

## Results

### Floral dimorphism

The studied population of *A. szechenyi* showed morphological distyly and reciprocal herkogamy (Table 1). No between-morph differences in corolla diameter ( $p = 0.67$ ) and corolla tube height ( $p = 0.17$ ) were detected, whereas the stigma height and anther height differed significantly between morphs (Table 1). There was no significant difference between long-styled stigma height and short-styled anther height ( $t_{38} = 0.40$ ,  $p = 0.69$ ), whereas long-styled anthers were significantly higher than short-styled stigmas ( $t_{38} = 8.56$ ,  $p < 0.0001$ ) (Table 1).

### Pollen and ovule counts

*Arnebia szechenyi* showed a marked between-morph pollen number and pollen shape dimorphism. Long-styled flowers produced significantly more pollen grains than short-styled flowers and the statistics were identical for P/O ratio (Table 1) because four ovules were contained in the ovary in both morphs. Moreover, the high P/O ratios of both two morphs indicated xenogamy in *A. szechenyi* (Cruden 1977). Besides, long-styled pollen grains were dumbbell-shaped

while short-styled pollen grains were dumbbell- or triangle-shaped.

### Morph ratios

No significant differences in the relative abundance of long- and short-styled morphs were found in any of the four studied populations (Table 2).

### Artificial pollination

The flowers that were netted and emasculated as well as the flowers that were netted but not otherwise manipulated produced no fruits,

suggesting absence of apomixes and spontaneous self-pollination (Table 3). Additionally, *A. szechenyi* was self- and intra-morph incompatible, manually pollinated flowers produced fruits solely through inter-morph crosses (Table 3). Under hand inter-morph pollination and open pollination, flower morph ( $F_{1,41} = 2.64, p = 0.11$ ), pollination treatment ( $F_{1,41} = 0.20, p = 0.66$ ) and flower morph  $\times$  pollination treatment interaction ( $F_{1,41} = 0.17, p = 0.69$ ) had no significant effects on seed set. According to the results of Binary Logistic Regression, there was no significant difference between morphs in fruit set ( $p = 0.19$ ); however, in comparison with naturally pollinated flowers, hand inter-morph pollination increased fruit set ( $p < 0.0001$ ) significantly.

**Table 1.** Floral and pollen attributes (mean  $\pm$  SE) of the two morphs of *Arnebia szechenyi*.

Floral and pollen attributes	Long-styled ( $n = 20$ )	Short-styled ( $n = 20$ )	$t$
Corolla diameter (mm)	10.63 $\pm$ 0.27	10.48 $\pm$ 0.19	0.44 <sup>ns</sup>
Corolla tube height (mm)	12.55 $\pm$ 0.25	12.05 $\pm$ 0.25	1.40 <sup>ns</sup>
Stigma height (mm)	13.72 $\pm$ 0.29	7.17 $\pm$ 0.12	20.66 <sup>***</sup>
Anther height (mm)	8.91 $\pm$ 0.16	13.57 $\pm$ 0.23	-16.58 <sup>***</sup>
Number of pollen grains per flower	83157.89 $\pm$ 2243.62	37507.5 $\pm$ 1062.81	18.39 <sup>***</sup>
Number of ovules per flower	4	4	
P/O ratio	20789.47 $\pm$ 560.90	9376.88 $\pm$ 265.70	18.39 <sup>***</sup>

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ns = no significant difference.

**Table 2.** Frequencies of plants of the two morphs of *Arnebia szechenyi* in different sample plots. LS = long-styled, SS = short-styled.

Sample plots	Total plant number	LS number	SS number	LS/SS	$\chi^2$	$p$
S1	210	100	110	0.91	0.48	0.49
S2	324	179	145	1.23	3.57	0.06
S3	162	75	87	0.86	0.89	0.35
S4	458	228	230	0.99	0.01	0.93

**Table 3.** Fruit set and seed set (mean  $\pm$  SE) of the two morphs of *Arnebia szechenyi* under different pollination treatments.

Treatments	Long-style ( $n = 20$ )		Short-style ( $n = 20$ )	
	Fruit set	Seed set	Fruit set	Seed set
Netting with emasculation	0	NA	0	NA
Netting without emasculation	0	NA	0	NA
Self-pollination	0	NA	0	NA
Intra-morph pollination	0	NA	0	NA
Inter-morph pollination	90%	2.28 $\pm$ 0.16 ( $n = 18$ )	75%	1.80 $\pm$ 0.18 ( $n = 15$ )
Open pollination	35%	2.29 $\pm$ 0.29 ( $n = 7$ )	25%	2.00 $\pm$ 0.32 ( $n = 5$ )



## Pollinating insects

Based on a 60-hour observation, it could be concluded that an as yet undescribed long-tongue fly (*Bombylius* sp.) (Fig. 1D and E) and a small solitary bee (*Nomia femoralis*) (Fig. 1F) were the main visitors and could efficiently pollinate between the morphs in *A. szechenyi*. *Bombylius* sp. inserted their long proboscids into the bottom of the corolla tube to get the nectar, while *Nomia femoralis* crawled around the corolla entrance of short-styled flowers and crawled into the corolla tube of long-styled flowers to collect pollen, during which processes different parts of the proboscids of *Bombylius* sp. and the body of *Nomia femoralis* could contact with corresponding anther and stigma in the opposite morphs of *A. szechenyi*. The visitation rate per flower per hour of *Bombylius* sp. in long- and short-styled morphs was  $0.009 \pm 0.002$  and  $0.013 \pm 0.006$ , respectively, while the visitation rate per flower per hour of *Nomia femoralis* in long- and short-styled morphs was  $0.008 \pm 0.002$  and  $0.012 \pm 0.004$ , respectively. In addition, no differences of visitation rate per flower per hour (square-root arcsine transformation) were found between the two morphs ( $F_{1,92} = 0.22, p = 0.64$ ) or the two main visitors ( $F_{1,92} = 0.00, p = 0.98$ ), and the flower morph  $\times$  visitor type interaction also had no significant effects on visitation rate per flower per hour ( $F_{1,92} = 0.10, p = 0.75$ ).

## Discussion

*Arnebia szechenyi* exhibits precisely reciprocal correspondence in stigma and anther position, which is the typical morphological syndrome in distylous species. On the adaptive significance of style-stamen length polymorphism, the traditional viewpoint, first suggested by Darwin (1877), considered it a mechanism to enforce outcrossing. It was proposed that the reciprocal position was a mechanical device to promote precise, insect-mediated pollination between anthers and stigmas located at the same relative level in the two morphs (see also Lloyd & Webb 1992a, 1992b). However, it is generally accepted that the function of reciprocal herkogamy is mainly to reduce physical interference

between anthers and stigma, improving male fertility (Barrett 2003). Collectively, the reciprocal style-stamen length polymorphism of heterostylous plants may first appear and evolve as a mechanism to avoid interference between male and female functions, and subsequently be modified and strengthened under selective pressures for promoting outcrossing and avoiding selfing (Darwin 1877, Lloyd & Webb 1992a, 1992b, Barrett et al. 2000, Zhou & Wang 2009).

Our controlled pollinations indicated that in *A. szechenyi* self-pollination and pollination between plants of the same morph were incompatible and flowers only developed fruits when their stigmas were pollinated with pollen from the opposite morph. This heteromorphic self-incompatible system and morphological heterostyly do enhance the phenotypic disassortative mating, i.e., only pollination between anthers and stigmas located at the same relative level in the two morphs is possible. This is thought to have evolved as a means of avoiding inbreeding and ensuring the effective exchange of pollen between different mating types (long-styled and short-styled) (Barrett 1992, Brys et al. 2004). In Boraginaceae, most of the distylous species exhibit this strict disassortative mating, such as *Anchusa hybrida* (Ganders 1979b) and *Lithospermum cobrense* (Dulberger 1970). Heuch (1979) suggested that if there was no between-morph fitness difference, equal proportions of different morphs will be the only possibility in a phenotypic disassortative mating population large enough. In this work, as expected, the two morphs of *A. szechenyi*, which is fully heteromorphic and self-incompatible, were fairly equal in abundance and randomly distributed within all of the four populations studied.

Fruit set and seed set of hand inter-morph pollinated flowers increased dramatically in comparison with that of naturally pollinated flowers in *A. szechenyi*. For distylous taxa which are self- and heteromorphic incompatible, the reasons leading to low fruit set are various, such as imbalanced ratio of floral morphs (Zhou & Wang 2009), lack of pollinators, or inefficient pollinating (Kéry et al. 2003). Since the current morph ratios of *A. szechenyi* populations are approximately 1:1, we speculated that the low fruit set under natural conditions is due to

limitation of pollen transfer. According to our observations, pollinator visiting rates of both two morphs of *A. szechenyi* were at relatively low level. Besides, among the visitors, some insects such as *Syrphus corolla*, *Andrena parvula* and *Heriades sauteri* could not fulfill pollen transfer between two morphs because their tongues were too short to touch the anther and/or stigma located deeply in the corolla tube. This indicates that the fruit set of *A. szechenyi* under natural conditions is limited by the frequency and efficiency of legitimate pollen transfer, which is consistent with observations on other species in Boraginaceae studied previously, such as *Amsinckia spectabilis* (Ganders 1975), *Pulmonaria obscura* (Olesen 1979) and *Lithospermum caroliniense* (Weller 1980).

In many distylous species, the two morphs also differ in terms of pollen production, pollen size, stigmatic papillae and many other ancillary features (Dulberger 1992). Like in a large number of species such as *Amsinckia douglasiana* (Ganders 1976), *Lithospermum californicum* (Dulberger 1992) and *Pulmonaria obscura* (Olesen 1979) in Boraginaceae, long-styled flowers produce significantly more pollen grains than short-styled flowers in *A. szechenyi*. In addition, although there was no significant difference between the height of long-styled stigmas and short-stalked anthers, long-stalked anthers were significantly higher than short-styled stigmas in *A. szechenyi*. Queller (1997) suggested that mating frequency had comparatively little effect on female fitness, whereas male success was limited seriously by access to females. In long-styled flowers, stigmas are at the mouth of the corolla, so the female fitness can be well achieved. However, anthers are located deeply in the corolla tube which means it is difficult for the pollen grains to disperse. As a result, only appropriate increase of the pollen grain number and anther height can make the male and female functions both well realized. By contrast, anthers of short-styled flowers are at the corolla mouth, which can benefit the male fitness. Although stigmas are deeply in the corolla tube, they still have a chance to acquire enough pollen grains and the female function is not greatly limited. In conclusion, the differences in pollen production and anther position between morphs are likely

attributable to selection on male function in *A. szechenyi*.

To conclude, as a typical distylous species, *A. szechenyi* exhibits precisely reciprocal herkogamy and heteromorphic self-incompatibility which ensures legitimate between-morph pollination. Moreover, other morphological dimorphisms further promote the efficiency of disassortative mating. Since the origin, evolution and breakdown of distyly are still unsolved issues, more multi-disciplinary studies, such as phylogenetic reconstruction, quantitative evaluation of between-morph pollen flow and genetic research about the heteromorphic incompatibility system, are needed to obtain a more comprehensive understanding.

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