Nectarless flowers with deep corolla tubes in *Pedicularis*: does long pistil length provide an arena for male competition?

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Received 18 January 2015; revised 13 July 2015; accepted for publication 27 July 2015

Pollinator-mediated selection does not seem to have a direct influence on the evolution of a long corolla tube in a nectarless flower. We hypothesized that the long pistil length of the nectarless flower with a deep corolla tube provided an opportunity for male competition. *Pedicularis siphonantha*, a nectarless and partially self-incompatible lousewort with substantial variation in corolla tube length, was used to test the hypothesis. We tested whether and how corolla tube length affected seed production per capsule and seed germination rate with different pollination treatments. Flowers were hand-pollinated with pollen from one self donor and one outcross donor and mixed pollen grains consisting of equal amounts from the two donor types, respectively. Additionally, seeds from open-pollinated flowers with different corolla tube lengths were collected separately for measurement of germination rate. Pollination treatment and corolla tube length significantly affected number of seeds per capsule. Moreover, a significant positive relationship between seeds per capsule and corolla tube length was found when mixed hand pollination was conducted. Seeds of self hand-pollinated flowers had a lower germination rate than those from outcross-pollinated flowers. Under open pollination, seeds from flowers with longer corolla tubes tended to have higher germination rate. In *P. siphonantha*, outcross pollen may have a higher probability of contributing to the next generation when transferred to flowers with longer corolla tubes. The pistil length, therefore, should be seen as a female choice mechanism, which provides an arena for male-to-male competition. © 2015 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2015, 179, 526–532.


INTRODUCTION

Studies on the evolution of flowers with extraordinary corolla depths provide perfect cases for understanding pollinator-mediated selection (Nilsson, 1988). Experimental and theoretical studies have demonstrated that directional selection imposed by long-proboscid pollinators is responsible for the evolution of long corolla tubes (Nilsson, 1988; Rodriguez-Girones & Llandres, 2008; Pauw, Stofberg & Waterman, 2009). In the evolutionary view of accomplishment of pollination for a flower, the long corolla tube might be a result of pollinator shift (Whittall & Hodges, 2007) and/or a Darwinian race; a reciprocal selection between flower and pollinator (Pauw *et al*., 2009). Obviously, one prerequisite of such a co-evolutionary relationship is that the plant provides nectar at the bottom of the corolla. In the case of a nectarless flower with a deep corolla tube, the size of the proboscis of the pollinator does not seem to influence corolla depth because there is no offered reward. Therefore, the evolutionary mechanism and the significance of a nectarless flower with an elongated corolla tube may have no relationship with the pollinator tongue, but instead with another selective process.

*Pedicularis* L. (lousewort, Orobanchaceae) is a genus with species that have corolla tubes ranging from short...
to unusually long. Plants with long corolla tubes (longer than twice the length of the calyx tube) are endemic to the Himalayas (Li, 1951; Tsoong, 1963). The stigmas protrude slightly from the galea or corolla beak (upper part of the corolla) and the styles go through the corolla tube from the stigma to the ovary located at the bottom of the flower. Thus, plants with deeper corolla tubes always have longer styles. Evolution of the deep corolla tube cannot be directly selected by long-proboscid pollinators because louseworts with deep corolla tubes do not produce nectar although at least some long-tubed *Pedicularis* spp. do have a rudimentary nectary structure (Liu et al., 2015) and are exclusively pollinated by pollen-collecting bumble bees (Macior & Sood, 1991; Macior & Tang, 1997; Yang et al., 2002; Ree, 2005; Huang & Fenster, 2007; Eaton et al., 2012). Because most long-tubed louseworts are rosette plants or have lax stems that bear flowers close to the ground, a long corolla tube was suggested to function in elevating the corolla away from vegetative parts obstructing pollinator attraction (Macior & Sood, 1991; Macior & Tang, 1997; Huang & Fenster, 2007). In addition, Ree (2005) and Eaton et al. (2012) suggested that the long styles might potentially serve an adaptive purpose as a prezygotic barrier facilitating conspecific pollen outperforming foreign pollen deposited on stigmas. However, no empirical studies based on field investigations directly supported these hypotheses. Using molecular data, studies have demonstrated that the long-tubed corolla derives from a short-tubed corolla (Ree, 2005; Eaton & Ree, 2013). For short-tubed louseworts, short-tongued insects such as *Bombus* might impose a constraint on corolla tube length because a historical association was found between nectar and short corolla tube (Ree, 2005; Yu et al., 2013). However, for species with long corolla tubes, the selective factors that have driven increased tube length remain unknown.

The stigmas of animal-pollinated flowers often capture more pollen than is needed to fertilize all available ovules, and mixed-donor conspecific pollen loads are probably common (Snow, 1994). In addition, pollen competitive ability is potentially affected by different donor–recipient interactions, variation in genetic similarity, and environmental factors such as herbivore damage and nutrient deficiency (Snow & Spira, 1996). Therefore, it is assumed that male-male competition may be common among pollen grains of these plants. Theoretical models have deduced that plants with high stigmatic pollen load should intensify pollen competition and increase mate choice, which may tend to elongate their pistil length (Mulcahy, 1983; Lankinen & Skogsmyr, 2001). However, experimental studies are needed to directly support the argument.

In *Pedicularis*, our previous studies showed that capability of pollen retention might increase along with elongation of corolla tubes due to the more expanded gully-like stigmatic surface (Yang et al., 2002). Intense pollen competition should be common on stigmas and in styles of long-corolla-tubed lousewort species (Yang et al., 2002). Based on these assertions, we hypothesized that the long pistil length might be a result of mate choice due to intense pollen competition. Flowers with long corolla tubes may have advantages in selection of high-quality offspring compared to those with short corolla tubes. *Pedicularis siphonantha* D. Don provides an ideal opportunity to test this hypothesis because of its substantial variation in the length of corolla tubes (Fig. 1). We tested whether and how corolla tube length affected seed
production per capsule and seed germination rate under different pollination treatments.

MATERIALS AND METHODS

STUDY SPECIES AND SITE

Pedicularis siphonantha is one of the most widespread louseworts in the Himalayas. The plant always grows in wet meadows and co-flowers with other lousewort species. Its red flower consists of a beak-like upper lip and a lower lip with three spread petals supported by a long corolla tube. Four anthers are contained in the hood-like portion of the galea. The stigma protrudes slightly from the corolla beak and the style goes through the corolla tube (Fig. 1). On sunny days, anthers dehisce as soon as the corolla spreads out. At our study site, populations of P. siphonantha always occupied a relatively large area with abundant humus from wet meadow to forest edge. Generally, individual plant had one to ten inflorescences and each inflorescence had 12–25 flowers (Tsoong, 1963). Floral longevity of a single flower was about 4 days and two to three flowers bloomed every day within an inflorescence (Yang et al., 2002). Flowering of inflorescences on a plant and plants in a population was quite synchronous, whereas that among populations varied substantially. The flowering period of a population always lasted 1.5 months. Relatively large floral display sizes could always be observed in individual plants and populations of the species (Yang, 2004; Yang, Sun & Guo, 2005; Yang, Gituru & Guo, 2007).

Our previous studies demonstrated that plants of P. siphonantha has a mixed mating system and that outcross pollen results in more seeds than self pollen (Yang et al., 2005). Moreover, in a large population, we demonstrated that seed production of this lousewort is not limited by pollen quantity but by available resources and pollen source (Yang et al., 2005). Stigmas of the plant have the greatest pollen retention ability in the genus due to the fully expanding gully-like surface (Yang et al., 2002). Bumble bees are the exclusive pollinators and are necessary for seed production in this nectarless lousewort (Yang et al., 2005; Huang & Fenster, 2007). The plants usually attract substantial numbers of bumble bees and have no autonomous mating mechanism (Yang et al., 2005). Observations of natural pollination indicated a single visit by a bumblebee resulted in a deposition of 35.07 ± 8.05 [standard error (SE)] pollen grains, which is higher than the number of ovules (18.7 ± 2.5 SE) to be fertilized (Yang et al., 2002, 2005).

Field investigations were conducted in a natural population from Sijiacun valley (27°37′59″N and 99°47′20″E), Shangeri-La County, Yunnan Province, China from July to August 2012 and 2013. The population is located near a forest edge and extends to a wet meadow. It consists of >1000 individual plants. In this habitat, at least four other louseworts co-flower with P. siphonantha, namely, P. densisipica Franch, ex Maxim., P. gruina Franch, ex Maxim, P. longiflora Rudolph, and P. rhinanthoides Schrenk ex Fisch. & C.A. Mey. The pollinators (mainly Bombus richardi and B. yunnanicola) visited flowers of P. siphonantha with a high frequency. A pilot observation revealed that average stigmatic pollen load for an individual flower was >48 (48.8 ± 7.90 SE, N = 18).

POLLINATION TREATMENTS

In 2012, we conducted hand-pollination treatments to determine whether and how corolla tube length affected seed production per capsule. Hand-pollinated flowers were divided into three groups following three pollination treatments: (i) pollination with pollen grains from a single flower within the same inflorescence (self); (ii) pollination with pollen grains from a single flower of another individual (outcross); and (iii) pollination with mixed pollen grains with equal contributions from one self donor and one outcross donor. For each treatment, seed yield of each flower with different corolla tube length were counted when the fruits were fully mature.

We manipulated flowers in a similar situation as much as possible to reduce influences of environmental factors on seed production. The recipient plants (N = 120) were from a meadow and individuals with only three inflorescences were used. These plants were bagged with fine bridal veil netting before the flowers opened in order to exclude pollinators. For each plant, three flowers were pollinated with self, outcross and mixed pollen grains, respectively. To minimize the influence of resource limitation on seed production, only the three hand-pollinated flowers were left to set fruit and all other flowers of the plant were removed at the bud stage after hand pollination. Flowers used as pollen recipient and donor opened in the morning of the same day. Plant individuals providing outcross pollen were c. 50 m away from the recipient individuals. In addition, pollen recipient and donor were carefully checked to exclude damage by florivores. Length of corolla tube (distance between base of the corolla beak and floral receptacle) for each recipient flower was measured by a Vernier caliper before hand pollination and marked with a tag until fruit matured. All pollination treatments were conducted from 10:00 h to 13:00 h on a fine day. For each pollination treatment, c. 100 pollen grains were deposited on a stigma. Pollen grains were deposited on a glass slide and counted under a portable microscope. For mixed pollination,
500 self and 500 outcross pollen grains were mixed and about 100 from the mixture were used. Pollen grains were transferred to the stigma using a soft brush. The glass slide was checked under the microscope to ensure full removal of the pollen grains. A pilot experiment had revealed that pollen grains of *P. siphonantha* begin to germinate in 20% solution of sucrose 12 h after removal from dehisced flowers. This indicates that pollen maintains viability throughout the processes of collection and mixing.

We also investigated the relationship between corolla tube length and seeds per capsule for flowers under open pollination in 2013. At least 200 flowers (*N* > 200) each from a single randomly selected individual were used. The flowers were marked with a tag by their corolla tube length (mm) from anthesis until the fruits were fully mature. The numbers of seed from each fruit were counted separately.

**SEED GERMINATION**

To detect whether seeds from open-pollinated flowers with different corolla tube length differed in germination ability, seeds from different flowers were collected separately according to their corolla tube length. Seeds from individual flowers were too few to conduct germination experiment. Therefore, we divided the seeds into three groups according to the corolla tube length of the flowers: short group (seeds from flower with a corolla tube < 32 mm long); medium group (seeds from flower with a corolla tube 32–45 mm long); and long group (seeds from flower with a corolla tube > 45 mm long). Germination rates of seeds from self- and outcross-pollinated flowers were set as references for comparisons of potential differences in germination rate among seeds from the three groups.

To conduct measurement of seed germination rate, we set ten seeds as a batch (each sample). In total, we used 20 batches for self-pollinated seeds (*N* = 20) and 30 batches for outcross-pollinated seeds (*N* = 30). For seeds from open-pollinated flowers with different corolla tube length, 30 batches of seeds (*N* = 30) were used(4,4),(994,995) for each group of short, medium and long groups, respectively. Seeds were stored at −18 °C for 2 months to break dormancy, then placed on filter papers in Petri dishes and kept in a growth chamber at 14 h light/15 °C and 10 h dark/4 °C. The number of germinated seeds was determined daily for c. 8 weeks until no more seeds germinated.

**DATA ANALYSES**

For treatments of self, outcross, and mixed hand pollination, we sought to find whether seed production per capsule was affected by pollination treatment, corolla tube length, and their interactions. We used a general linear mixed-effect model (LME) to detect factors affecting seed production. In this model, we set plant ID as a random factor, pollination treatment as a fixed-effect categorical predictor and corolla tube length as a fixed-effect continuous predictor. If a significant interaction was revealed, we used Pearson correlation coefficients to detect the relationship between number of seeds per capsule and length of the corolla tube for each pollination treatment. Such an analysis was also used to find the relationship between corolla tube length and seeds per capsule under open pollination. A one-way analysis of variance (ANOVA) was used to detect the differences in percentage of seed germination rate among different treatments after the data were arcsine transformed. Tukey honest significant difference (HSD) multiple comparisons tests were conducted if significant differences were revealed among different treatments and groups. All statistical analyses were carried out in SPSS 18.0 and at a 5% significance level.

**RESULTS**

Results of LME indicated that number of seeds per capsule was significantly affected by pollination treatment (*F*$_{2, 217}$ = 46.08, *P* < 0.001), corolla tube length (*F*$_{1, 217}$ = 31.57, *P* < 0.001), and their interaction (*F*$_{2, 217}$ = 33.69, *P* < 0.001) but not by plant identity (*F*$_{119, 217}$ = 1.08, *P* > 0.5). Pearson correlations revealed that both self and outcross hand pollination did not affect seeds per capsule among flowers with different corolla tube lengths (Fig. 2A, B). Under open pollination, we did not find a significant relationship between seeds per capsule and length of the corolla tube (Fig. 2C). However, a significant positive correlation was found between the number of seeds per capsule and the length of the corolla tube under the hand pollination treatment (Fig. 2D), which indicates that mixed pollen affected seed production as a function of pistil length.

One-way ANOVA revealed that seeds from open-pollinated flowers with different corolla tube lengths and self- and outcross-pollinated flowers differed significantly in percentage of germination rate (*F*$_{4, 135}$ = 16.111, *P* < 0.001). Multiple comparisons indicated that outcross-pollinated seeds displayed significantly higher germination rates than all other seeds (Fig. 3). Moreover, for open-pollinated flowers, the germination rate of seeds from flowers of the long group was significantly higher than those from flowers of medium and short groups and close to that of outcross-pollinated seeds (Fig. 3).

**DISCUSSION**

Our previous study based on hand pollination revealed that outcross pollen could result in higher seed pro-
duction than self pollen in *P. siphonantha* because self pollen caused a higher level of seed abortion (Yang *et al*., 2005). In the present study, although there were no data that provided direct evidence that outcross pollen outperformed self pollen in the mixed pollination, we found that number of seeds per capsule increased with increased length of the corolla tube (Fig. 2D). Although we failed to find a significant relationship between corolla tube length and seed production for open-pollinated flowers, it may be ascribed to resource limitation and complex composition of stigmatic pollen load since seed production was affected by both resource availability and pollen source (Yang *et al*., 2005). Moreover, seeds produced by open-pollinated flowers with longer corolla tubes displayed a higher viability than those with shorter corolla tube. Considering the seed germination pattern (Fig. 3), ovules of flowers with long corolla tubes were probably fertilized by outcross pollen rather than self pollen. These results indicate that more outcross pollen should be selected by long pistils. According to sexual selection theory, long pistils should be an evolutionary consequence of mate choice mechanism as such a female trait favours certain pollen donors and thus provides an arena for male to male competition (Skogsmyr & Lankinen, 2002).

A long pistil may evolve in a species in which pollination is effective so that there is intense selection for male quality (Mulcahy, 1983; Lankinen & Skogsmyr, 2001). Our former study indicated that among 17 *Pedicularis* spp. those with long corolla tubes have a higher capability of pollen retention due to their more expanded gully-like stigmatic surface compared to those with short corolla tubes (Yang *et al*., 2002). Among those species, *P. siphonantha* had the highest capability of pollen retention and resulted in

**Figure 2.** Relationship between length of corolla tube (mm) and number of seeds per capsule under different pollination treatments: self pollination (A); outcross pollination (B); open pollination (C); and pollination with mixed pollen grains (D).
spp. co-occur frequently, flower synchronously and long corolla tubes are mainly distributed, yas) of south-central China, where louseworts with 2012). In the Hengduan Mountains (eastern Himala-
tubes from reaching the ovules (Ree, 2005; Eaton precluded larger or slower growing foreign pollen
potentially serve an adaptive purpose as a prezygotic barrier (Huang & Fenster, 2007). It has been
nectaries may play as a key innovation by releasing 
short-tongued insects having a large floral display may result in a resource cost in increasing pollination frequency. However, maintaining a large floral display and the synchronous flowering within a population, may have a collective role in increasing pollination frequency. However, maintaining a large floral display may result in a resource cost for the plant since flower removal can increase seed yield (Yang et al., 2005), which may partly explain the substantial variation in corolla tube lengths. Moreover, a large floral display also has a genetic cost due to the high possibility of geitonogamous mating because pollen-collecting bumble bees always pollinate most flowers within an inflorescence in a single foraging trip, which might result in mixed stigmatic pollen loads with a high proportion of self pollen. In these circumstances, a deep corolla tube (long pistil length) should be beneficial for the plants by providing an arena for male to male competition and offering opportunities for outcross mating (see also Malti & Shivanna, 1985) since self pollen caused higher possibility of seed abortion in this partially self-incompatible lousewort (Yang et al., 2005).

ACKNOWLEDGEMENTS
We thank Professor David Inouye for improvements on an earlier manuscript, Dr Jakub Těšitel and two anonymous reviewers for helpful comments, Kuo Liao, Xiao-Fang Jin, and Zhong-Ming Ye for their assistance in field work, Dr Jakub Těšitel, Gituru Robert, and Kadiori Edwin Luguba for language improvement, and Yan-Wen Zhang for helpful discussions. This work was supported by grants from the National Natural Science Foundation of China to C.-F.Y. (30970209).

highest stigmatic pollen load per visit by a bumble bee (Yang et al., 2002), which may intensify pollen competition on the stigma and in the style (see also Holm, 1994; Niesenbaum, 1999; Yang et al., 2002; Marshall, Shaner & Oliva, 2007). For this species, frequent visits by bumble bees resulted in the deposition of a high pollen load on the stigma, which was more than twice the numbers of ovules in a flower (see also Yang et al., 2002, 2005). It can therefore be inferred that with the increase of pollination efficiency, increasing mate choice due to intense pollen competition may play important role in the evolution of long corolla tubes. Compared with short corolla tubes, a longer corolla tube (longer pistil) might provide more space to ensure that outcross pollen outperforms self or heterospecific pollen deposited synchronously on stigmas or even earlier than outcross pollen.

In Pedicularis spp. with short corolla tubes, the historical association between short corolla tubes and nectar production indicating short-tongued insects may impose a constraint on corolla size (Ree, 2005; Yu et al., 2013). For long-tubed louseworts, the loss of nectaries may play as a key innovation by releasing this constraint (Huang & Fenster, 2007). It has been postulated that long styles in Pedicularis might potentially serve an adaptive purpose as a prezygotic barrier preventing smaller or slower growing foreign pollen tubes from reaching the ovules (Ree, 2005; Eaton et al., 2012). In the Hengduan Mountains (eastern Himalayas) of south-central China, where louseworts with long corolla tubes are mainly distributed, Pedicularis spp. co-occur frequently, flower synchronously and

Seeds from different pollination treatment

Figure 3. Differences in germination rate of seeds from flowers with self and outcross pollination and open-pollinated flowers with short, medium or long corolla tubes (means ± SE). a,b,c Different letters indicate significant differences between the means under Tukey HSD multiple comparisons (P < 0.05).
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