Plant Ecology and Evolution 150 (1): 59–66, 2017
https://doi.org/10.5091/plecevo.2017.1274

REGULAR PAPER

High autonomous selfing capacity and low flower visitation rates in a subalpine population of *Prunella vulgaris* (Lamiaceae)

Tial C. Ling[1,2,3], Lin-Lin Wang[1,2], Zhi-Qiang Zhang[1,2], Amots Dafni[4], Yuan-Wen Duan[1,2] & Yong-Ping Yang[1,2,*]

[1]Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, Yunnan, P.R. China
[2]Institute of Tibetan Plateau Research at Kunming, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, Yunnan, P.R. China
[3]University of the Chinese Academy of Sciences, Beijing 100049, P.R. China
[4]Department of Evolutionary and Environmental Biology, Institute of Evolution, Haifa University, Haifa, Israel
*Author for correspondence: yangyp@mail.kib.ac.cn

Background and aims – Autonomous selfing is a potential strategy to overcome pollinator scarcity or unreliability, but the specific mechanisms of autonomous selfing need further study to clarify our understanding of this phenomenon. This study investigated the mechanism of autonomous selfing in a subalpine population of *Prunella vulgaris* (Lamiaceae), a species with didynamous stamens.
Methods – We studied the floral biology, pollinator activity, and breeding system of *Prunella vulgaris* in a natural population. The short stamens and long stamens were removed separately to examine their contribution to self-pollinated seed production. In addition, the stamens were removed at different developmental stages to determine the timing of autonomous selfing.
Key results – The short stamens were closer to the stigma than the long stamens. Seed production after removal of the long stamens was higher than following removal of the short stamens, suggesting that spatial separation of the anthers from the stigma is crucial and pollen from the short stamens contributes most frequently to self-pollination. Self-fertilization started at the final bud stage of flower development, which may be consistent with the prior selfing mode, but seed production gradually increased during the flower life-span, suggesting that competing selfing and delayed selfing may also contribute to autonomous selfing. Bagged flowers set as many seeds as flowers hand-pollinated with outcross pollen or self-pollen, and thus the population shows a high capacity for autonomous selfing. Emasculated flowers open to visitation by pollinators produced almost no seed, showing that the pollinator-visitation rate was low, and that the majority of the seeds in intact flowers resulted from self-fertilization.
Conclusions – The findings show that under low pollinator availability, preanthesis autogamy is likely to be selectively advantageous as a reproductive assurance mechanism in a subalpine population of *Prunella vulgaris*.

Key words – Outcrossing, pollinator scarcity, reproductive assurance, self-fertilization, didynamous stamens.

INTRODUCTION

Approximately 80% of flowering plant species may be pollinated by biotic and abiotic vectors, whereas about 20% are self-pollinated species (Barrett 2002, Ollerton et al. 2011). Vogler & Kalisz (2001) proposed that 33% out of 80% of outcrossing species have a mixed reproductive strategy incorporating both selfing and outcrossing. To fully understand the high frequency of autonomous selfing among flowering plant species, the benefits and costs of selfing under natural conditions should be considered (Eckert & Herlihy 2004). Two main hypotheses have been proposed to account for the benefits of selfing. One hypothesis is that a gene for selfing has a transmission advantage over those causing outcrossing (Busch & Delph 2012). The second hypothesis is that selfing evolves when pollinator availability is low, because it

All rights reserved. © 2017 Botanic Garden Meise and Royal Botanical Society of Belgium
ISSN: 2032-3913 (print) – 2032-3921 (online)

provides reproductive assurance (Darwin 1876, Baker 1955, Brys & Jacquemyn 2011, Delmas et al. 2014). The latter hypothesis can be tested by emasculating the flowers before the pollen grains are released from the pollen sacs and allowing pollinator visits (Schoen & Lloyd 1992, Fenster & Martén-Rodríguez 2007). If emasculated flowers set fewer seeds than intact flowers, autonomous selfing would provide a degree of reproductive assurance (Lloyd & Schoen 1992). However, the benefit of reproductive assurance alone cannot be determined without accounting for fitness costs associated with pollen/seed discounting and inbreeding depression (Herlihy & Eckert 2002). The benefits of selfing will be eroded when pollen used in selfing reduces pollen availability for outcrossing as pollen discounting (Nagylaki 1976, Wells 1979, Charlesworth 1980) or when seed fitness of selfers is lower than that of outcrossers owing to inbreeding depression (Charlesworth & Charlesworth 1987, Lloyd 1992, Jacquemyn & Brys 2015). Hence, the benefits and costs of selfing that rely on the timing of self-fertilization and functional pollinator effectiveness must be investigated (Lloyd 1992, Kalisz et al. 2004, Elle et al. 2010).

Autonomous selfing has been classified into three types, termed prior (before anthesis), competing (during anthesis), and delayed selfing (at late anthesis), on the basis of the timing of self-fertilization during the floral life-span (Lloyd 1979, Lloyd & Schoen 1992). Prior selfing occurs when anthers dehisce and stigmas are receptive before the flower opens (Lloyd & Schoen 1992). Both prior selfing and competing selfing can cause pollen discounting compared with delayed selfing, because pollen or ovules that participate in self-fertilization in early in anthesis are no longer available for outcrossing (Elle et al. 2010). However, when the pollinator visitation rate is low, pollen or ovule discounting does not act as a barrier to the evolution of prior selfing (Lloyd 1992, Kennedy & Elle 2008).

Autonomous selfing may also evolve as a derivative of floral structure (e.g. cleistogamy or variation in floral size) (Lloyd 1992, Fenster et al. 1995, Elle et al. 2010). Cleistogamous flowers are self-fertilized in the bud stage without opening of the flowers (Anderson 1980), whereas chasmogamous flowers can be cross-pollinated with pollen from another individual (Darwin 1877, but also see Forrest & Thomson 2008). An additional category of cleistogamy is "preanthesis cleistogamy", through which self-fertilization occurs in the bud, followed by anthesis and the opportunity for outcrossing (Culley & Klooster 2007). Several genera (*Ajuga* L., *Lamium* L. and *Salvia* L.) in the Lamiaceae display a mixed reproductive strategy in which a plant can produce both cleistogamous and chasmogamous flowers (Culley & Klooster 2007). Within the same population of *Prunella vulgaris* L., smaller flowers show preanthesis cleistogamy, whereas larger flowers are chasmogamous (Nelson 1964, 1967). Other studies suggest that in *P. vulgaris*, selfing is favoured under pollinator limitation, whereas outcrossing is favoured when pollinator visitation rate is high (Müller 1883, Aluri 1995). *Prunella vulgaris* is frequently visited by bumblebees, butterflies and honey bees and exhibits a wide elevational range up to 2,050 m a.s.l. (Aluri 1995, Qu & Widrlechner 2011, Kuriya et al. 2015). Thus, this species is likely to have a mixed reproductive strategy of both self- and cross-pollination within the same population depending on floral size and pollinator availability.

Qu & Widrlechner (2011) showed that, although *P. vulgaris* flowers release pollen grains before the flower opens, selfing in the bud did not result in effective seed production in some flowers. In this species, it is not clear how many pollen grains are deposited on the stigma and effect self-fertilization in the bud stage, but pollen deposition is indicated to gradually increase with the flower age. Kuriya et al. (2015) stated that in all manipulated flowers of different sizes, no pollen grains were deposited on the stigma during the bud stage in *P. vulgaris*. Qu & Widrlechner (2011) documented that some *P. vulgaris* flowers bear exerted styles and show extremely low seed set, including accessions from a population in Sichuan Province, China. Thus, rather than floral size, in this species spatial separation between the anthers and stigma and also the amount of pollen grain deposition with floral age may determine seed production by selfing, because the capacity or mode of selfing is suggested to depend on the spatial separation or movement of the anthers and stigma during anthesis (Kalisz et al. 1999, Duan et al. 2010, Fan & Li 2012). In each flower of *P. vulgaris* the filaments are of unequal length: two are short and two long. It remains unclear whether the relative contribution of the two pairs of stamens to selfing is different in species with a mixed reproductive strategy. Thus, additional detailed studies on the roles of the breeding system, potential differences in seed contribution between the two pairs of anthers, and the mode of autonomous selfing in *P. vulgaris* are needed. In the present study, we investigated the mechanism of selfing in a subalpine population of *P. vulgaris*. Three questions were addressed: (1) is the study population capable of autonomous selfing? (2) If so, when and how does autonomous selfing take place? (3) Does autonomous selfing provide a degree of reproductive assurance?

MATERIAL AND METHODS

Study site and species

Observations on a natural population of *Prunella vulgaris* were conducted at the Lijiang Alpine Botanical Garden (27˚00′N, 100˚01′E; 2,830 m a.s.l.) from July to September 2015. The site is located on the south-facing slopes of the snow-capped Yulong (Jade Dragon) Mountains, approximately 24 km from Lijiang, Yunnan Province. The mean annual precipitation at Lijiang City (2,393 m a.s.l.) is 934.9 mm, and the peak rainy season is from July to August (Zhang & Li 2008, Fan & Li 2012).

Prunella vulgaris (Lamiaceae) is widely distributed throughout the Northern Hemisphere. It is a small, herbaceous perennial inhabiting lowlands, gravel streams, moist pasturelands and subalpine meadows. The flowers of this species are protandrous and have didynamous stamens, two short and two long stamens (Aluri 1995). The study population grows in a subalpine meadow and contains more than 1,000 flowering individuals. The four stamens and the style of mature flowers adhere to the upper lobe of the corolla. The corolla is purple and flowers are borne from June to Septem-

ber, a period that includes the peak rainy season at the study site.

Floral biology

To investigate floral morphology in the study population, we selected one flower per inflorescence and one inflorescence per plant from thirty individual plants. We counted the flower number per inflorescence and recorded the floral longevity for a single flower and floral longevity for all flowers per inflorescence. We selected an additional thirty plants to measure plant height, floral length, corolla tube length, style length, long stamen length, and short stamen length from a single flower per individual plant with a Vernier calliper (to 0.01 mm accuracy). A single flower from a single inflorescence was selected from an additional fifty plants to calculate the percentage of exerted styles. Styles that were longer than all of the stamens or exerted beyond the upper lobe of the corolla were considered to be exerted, whereas styles that were shorter or the same length as the long stamens were classified as inserted. One-way ANOVA was performed using a *post-hoc* least significant difference (LSD) test to reveal the significance of differences in length among the two pairs of stamens and the style.

One flower bud per plant ($n = 15$) was collected and fixed in FAA solution (formalin: acetic acid: ethanol at a ratio of 5:5:90 by volume) in an individual vial for determination of the total number of pollen grains. Each pair of anthers was dissected from the stamens and the pollen sacs were opened and the pollen grains were dispersed in 1.5 ml micro-centrifuge tubes containing 0.5 ml FAA solution. For each pair of anthers, ten subsamples of 10 µl each were placed on a glass microscope slide, the total number of pollen grains on the slide was counted under a light microscope (XSZ-0900, Wuzhou Oka Optical Instrument Co., Ltd., Wuzhou, Guangxi, China) and the mean was calculated. We used an independent-sample *t*-test to determine the significance of differences in pollen number between short stamens and long stamens. The pollen-to-ovule (P/O) ratio was calculated (Cruden 1977, Dafni 1992) as the total number of pollen grains per flower divided by four, the number of ovules per flower in the Lamiaceae.

Breeding system and the role of autonomous selfing

To examine the breeding system and the capacity for autonomous selfing, 180 flower buds were randomly selected and subjected to the following six pollination treatments. Two buds from the middle position (the second whorl from the inflorescence base) per inflorescence per plant ($n = 15$) were chosen for each treatment: (I) buds (before dehiscence of the pollen sacs) were carefully emasculated without harming floral development and the whole inflorescence was covered with a fine nylon mesh bag before anthesis to test the potential for apomixis; (II) buds were emasculated and left uncovered to examine the contribution of pollinators (open pollination) to seed production; (III) buds were covered with a fine nylon mesh bag to examine the potential for autonomous selfing; (IV) buds were emasculated and covered with a fine nylon mesh bag before the anthers dehisced, and then hand-pollinated after the corolla had opened with fresh pollen collected from the same plant (geitonogamy) to test for self-compatibility; (V) buds were emasculated and covered with a fine nylon mesh bag before the anthers dehisced, and then manually pollinated with fresh pollen collected from multiple individuals located at least 5 m from the pollinated plant (cross-pollination); and (VI) flowers were left undisturbed for open pollination, as a control. In each treatment, we carefully emasculated all anthers of non-manipulated flowers without harming floral development to prevent self-pollination resulting from pollen transfer from other flowers on the same inflorescence and then covered the whole inflorescence with a fine nylon mesh bag. We excluded potential manipulated flowers that were damaged after emasculation. Therefore, the results presented are an accurate indication of the final seed production from all treatments applied.

We removed the short and long stamens separately to examine their possible relative contribution to seed production as a result of autonomous selfing. We manually performed emasculations as follows: (I) removal of the short stamens of flower buds and bagging of the buds before anther dehiscence; (II) removal of the long stamens of flower buds and bagging of the buds before anther dehiscence; and (III) control: flowers were not subjected to any treatment and bagged. In each treatment, at least two flower buds from the middle position (the second whorl from the inflorescence base) per inflorescence per plant ($n = 15$) were selected and the whole inflorescence was covered with a fine nylon mesh bag. For the emasculation, we chose buds that were almost open but in which the pollen sacs had not dehisced. After 45 days, seed number per flower was counted.

Data were analysed using generalized linear mixed models (GLMMs) to test for significant differences in seed production among treatments using treatment as a fixed factor, plant as a random factor, and considering treatment × plant interaction. *Post-hoc* LSD tests were used to explore all possible pairwise comparisons between treatments. The data for seed production among the treatments were not normally distributed; we therefore selected a Poisson distribution for the errors with a log-link function to test the statistical significance of paired-comparisons between treatments. All statistical analyses were conducted with IBM SPSS version 19.0 for Windows (2010, IBM Corp., Armonk, NY, USA).

Timing of autonomous self-pollination

To determine the timing of autonomous selfing, two flower buds per plant ($n = 15$) were selected and bagged for each treatment. The two buds on each plant were subjected to one of the following treatments and then re-bagged: (I) emasculation at the final bud stage before the corolla was fully open, in which the upper lobe of the corolla was still in contact with the lower lobe (1 day before full opening); (II) emasculation on the first day of corolla opening; (III) emasculation on the third day of corolla opening. Non-treated flowers were used as a control. For treatments (I) and (II), emasculation and bagging were performed as described for the treatments in the breeding system experiment. A GLMM analysis was used to test for significant differences in seed production among treatments using treatment as a fixed factor, plant as a random factor, and considering treatment × plant interac-

tion. A *post-hoc* LSD test was used to explore all possible pairwise comparisons between treatments. The data for seed production were not normally distributed; therefore, a Poisson distribution for the errors with a log-link function was used to determine which pairs of means were significantly different.

Pollinator activity

We observed floral visitors to *Prunella vulgaris* during the peak of the flowering season on three consecutive clear, sunny days from 09:00 to 18:30 h. Before we started the observation on each day, we carefully counted the numbers of flowers and selected a total of 4,312 flowers for each observed day within an area of 30 m × 30 m in the study population. Given that the frequency of pollinator visitation was low, we were able to observe when each pollinator visited each flower, and thus all visits to flowers by individual pollinators within the observation period were recorded. The visitation rate per flower per hour was calculated for the different pollinators. All insect visitors were collected using a butterfly net and were killed in jars filled with ethyl acetate fumes. All pinned and labelled specimens were preserved at the Kunming Institute of Botany, Chinese Academy of Sciences.

RESULTS

Floral biology

Plants of *P. vulgaris* began to bloom in late June, with peak flowering in late July and early August, and completion of flowering in mid-September. The mean plant height was 119.48 ± 3.83 mm (mean ± s.e., $n = 30$), with flowers 10.80 ± 0.29 mm long and corolla tubes 7.78 ± 0.26 mm long (fig.1A & B). Each inflorescence produced 20.10 ± 1.26 flowers ($n = 30$) and the longevity of an individual flower was 3–5 days (mean = 4.20 ± 0.14, $n = 30$), in which up to twelve flower buds opened simultaneously. All flowers in each inflorescence completed blooming after 13–25 days (17.53 ± 0.67, $n = 30$). Only 10% ($n = 50$) of the styles were exerted. The short stamens were closer to the stigma than were the

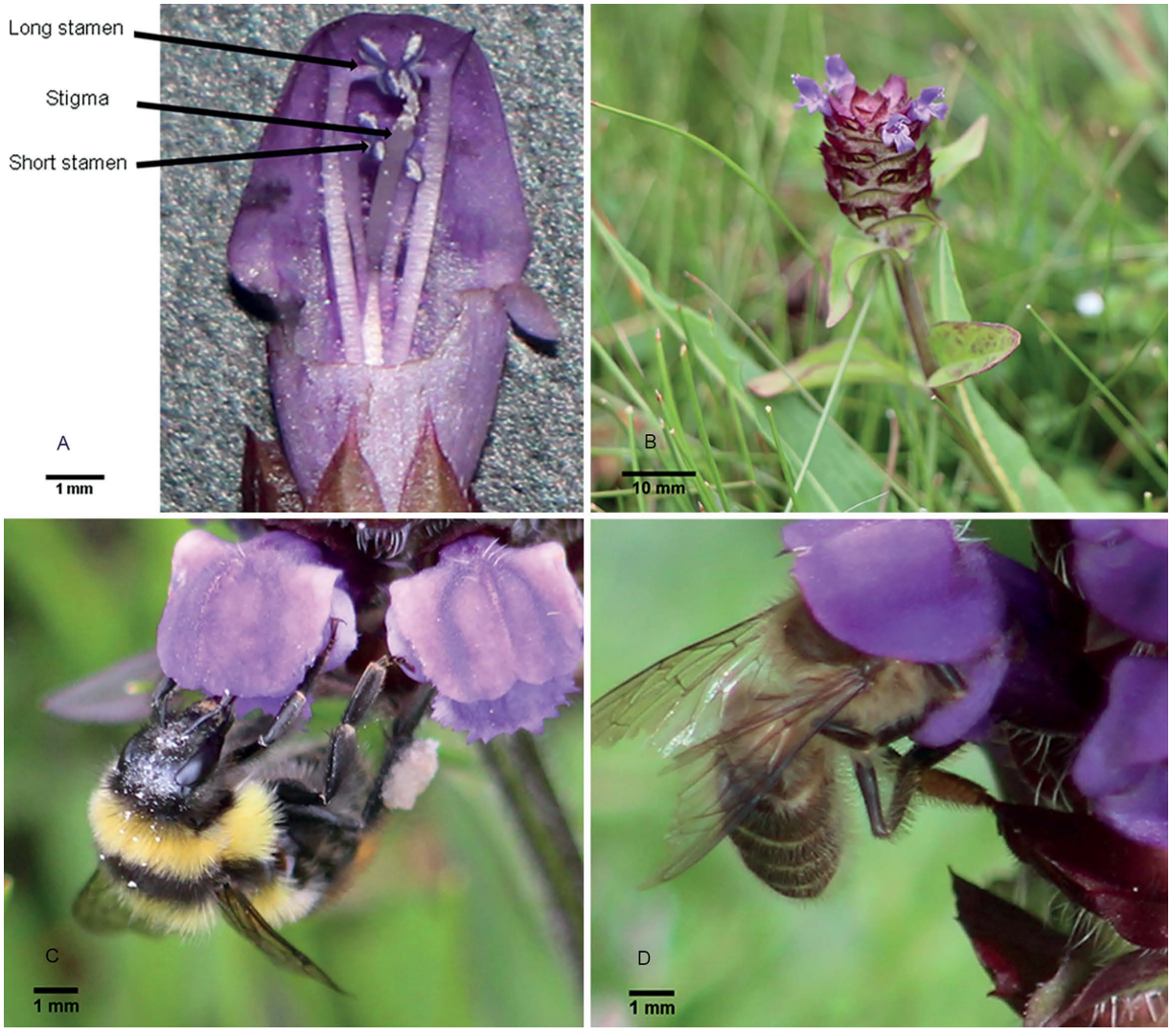


Figure 1 – *Prunella vulgaris* flowers and their pollinators: A, mature flower; B, *P. vulgaris* plant; C, bumblebee (*Bombus* sp.); D, honey bee (*Apis* sp.). The arrows point to a bifid stigma and the stamens.

long stamens. A significant length difference was observed between the short stamens, long stamens, and style (one-way ANOVA, $F_{2,87} = 11.38$, $P < 0.001$). The length of the long stamens (9.64 ± 0.32 mm, $n = 30$) was significantly longer than that of the short stamens (7.70 ± 0.29 mm, $n = 30$) and the style (8.49 ± 0.25 mm, $n = 30$), whereas no significant difference between the lengths of the short stamens and the style was observed (fig. 1). The numbers of pollen grains produced in the short stamens and the long stamens per flower were 643.33 ± 99.17 and 592.00 ± 89.58, respectively. The difference in pollen production between the short stamens and long stamens was non-significant (*t*-test, $t = -0.38$, $P = 0.70$). The total number of pollen grains per flower was 1235.33 ± 153.31 ($n = 15$). Thus, the P/O ratio was 308.83 ± 38.33. Some of the pollen grains were released before the flower was fully open (the final bud stage), but no pollen grains were released in flowers 2–3 days before opening.

Breeding system and capacity for autonomous selfing

Emasculated, bagged flower buds did not produce any seeds. Bagged-intact flowers, emasculated hand-selfed flowers and hand-outcrossed flowers did not show any significant difference in seed production (fig. 2), which also demonstrates that emasculation did not have a negative effect on seed set. However, bagged emasculated flowers showed greatly reduced seed production compared to bagged intact flowers (GLMM: $F_{5,167} = 14.352$, $P < 0.001$). Intact flowers produced as many seeds as did hand cross-pollinated flowers. A significant difference in seed production among plants was observed, although the treatment × plant interaction was also significant ($F_{14,86} = 2.78$, $P < 0.01$ for plant; $F_{50,86} = 2.29$, $P < 0.001$ for treatment × plant interaction).

Compared with intact flowers as a control, removal of the long stamens reduced seed production non-significantly, whereas removal of the short stamens reduced seed number significantly (GLMM: $F_{2,86} = 4.414$, $P = 0.015$) (fig. 3). No significant difference in seed production among plants was observed and the treatment × plant interaction was non-significant ($F_{14,44} = 1.74$, $P = 0.82$ for plant; $F_{29,44} = 1.44$, $P = 0.14$ treatment × plant interaction).

Timing of autonomous selfing

Flowers emasculated at the final bud stage produced as many seeds as did flowers emasculated on the first day of anthesis. However, compared with flowers emasculated on the third day of anthesis and the intact (control) flowers, emasculation at the final bud stage resulted in significantly decreased seed production, whereas seed number after anther removal on the first day after anthesis was not significantly different from all other treatments (GLMM: $F_{3,110} = 3.441$, $P = 0.019$) (fig. 4). No significant difference in seed production among plants was observed and the treatment × plant interaction was non-

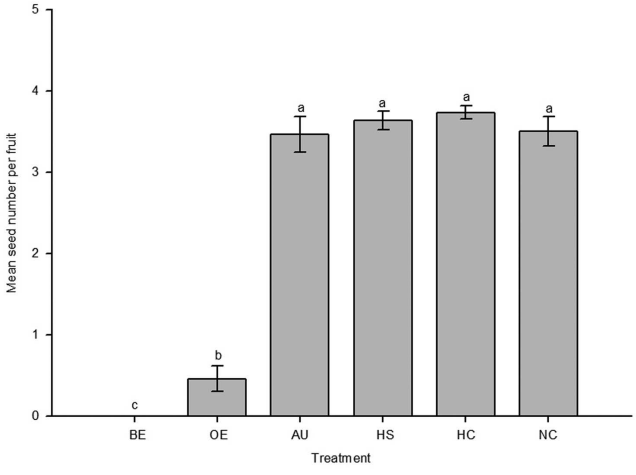


Figure 2 – Seed number of flowers subjected to different pollination treatments. BE, bagged and emasculated flowers (apomixis); OE, emasculated and open flowers; AU, bagged without manipulation (autogamy); HS, hand self-pollination; HC, hand cross-pollination; NC, natural control. Different letters indicate significant differences (GLMM: $F_{5,167} = 14.352$, $P < 0.001$). Bars indicate means (± s.e.).

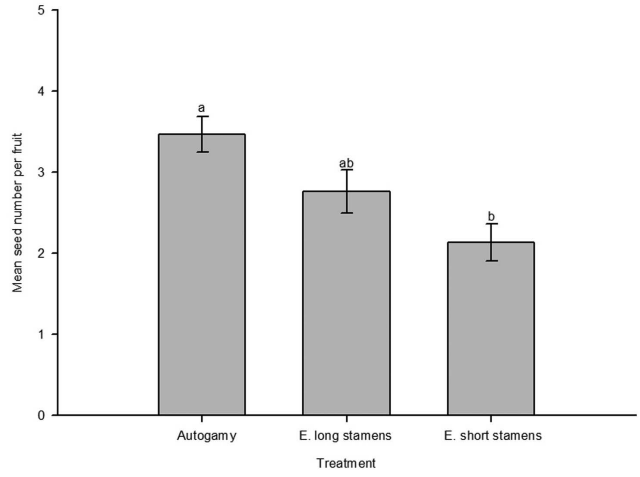


Figure 3 – Seed number of flowers subjected to emasculation (E) by removal of the long or the short stamens. Different letters indicate significant differences (GLMM: $F_{2,86} = 4.414$, $P = 0.015$). Bars indicate means (± s.e.).

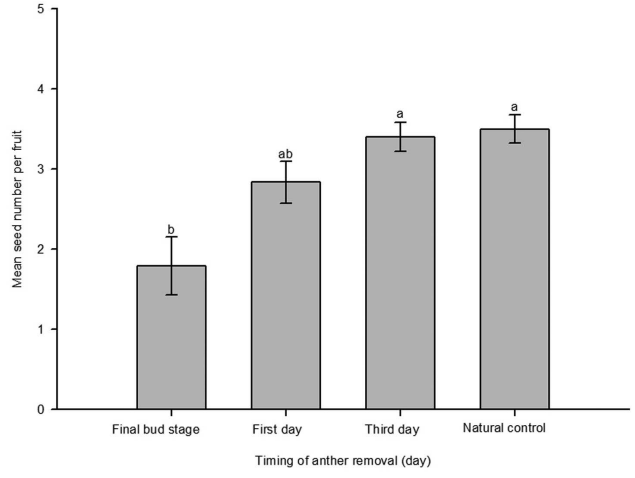


Figure 4 – Seed number of flowers subjected to emasculation at different stages of the floral life span. Different letters indicate significant differences (GLMM: $F_{3,110} = 3.441$, $P = 0.019$). Bars indicate means (± s.e.).

significant ($F_{14,57}$ = 1.17, P = 0.323 for plant; $F_{37,57}$ = 0.85, P = 0.695 for treatment × plant interaction).

Pollinator visitation rate

During the course of three consecutive observation days, two types of pollinators visited flowers of *P. vulgaris*. Bumblebees (*Bombus* sp.) showed a mean of 0.06 ± 0.02 visits per flower per hour (n = 53), whereas the mean rate of honey bee (*Apis* sp.) visitation was 0.02 ± 0.02 visits per flower per hour (n = 28) (fig. 1C & D). No significant difference in visitation rate between these two pollinators types was observed (t = 1.63, P = 0.155).

DISCUSSION

Our data revealed that *Prunella vulgaris* is not apomictic, because emasculated and bagged flower buds (2–3 days before flowering) did not produce any seed. However, as a result of the short distance between the stigma and the anthers, bagged intact flowers produced as many seeds as did flowers subjected to hand pollination. This finding indicates that *P. vulgaris* has a high capacity for autonomous selfing, which is consistent with the results of bagging experiments conducted on multiple populations of *P. vulgaris* with inserted styles (Qu & Widrlechner 2011). Selfing begins in the final bud stage (1 day before full corolla opening) and can be classified as facultative autogamy following the definition of Cruden (1977). The visitation rate of bumblebees in the present study (0.06 ± 0.02) was less than previously reported rates (0.64 ± 0.10) (as reviewed by Duffy & Stout 2008, see also Hegland & Totland 2005). These results support the hypothesis that reproductive assurance developed in this species as an adaptation under low pollinator availability, as also suggested for two delayed autonomous self-pollinated species in the Zingiberaceae, *Roscoea schneideriana* (Loes.) Cowley (Zhang & Li 2008) and *Roscoea debilis* Gagnep. (Fan & Li 2012), which grow under similar conditions near to the present study area.

Mechanism and mode of autonomous selfing

Most previous studies on the spatial separation or the movement of anthers and stigmas have documented how selfing occurs under poor pollinator availability (e.g. Fan & Li 2012, Ma et al. 2012). For instance, in the prior selfing stage of *Silene noctiflora* L. (Caryophyllaceae), some of the anthers dehisce and come into contact with the stigmas before anthesis, thereby effecting self-pollination (Davis & Delph 2005). Zhang & Li (2008) stated that delayed selfing in *Roscoea schneideriana* is characterized by a progressive downward curling of the stigmatic lobes towards the pollen-carrying anther surfaces. In the present study, we observed that in a subalpine population of *Prunella vulgaris*, spatial separation between the stigma and the two pairs of stamens contributes to the frequency of self-pollination. Pollen from the short stamens only, which are of similar length to the style, is sufficient to produce as many seeds as observed for intact flowers, whereas pollen from the long stamens contributes to seed production at a much lower frequency. This finding is in stark contrast to Qu & Widrlechner's (2011) report of a population of *P. vulgaris* from Sichuan Province, China, which was originally collected from an alpine meadow at 3,030 m a.s.l. but observed when cultivated in Iowa, USA under low-elevation conditions (c. 300 m a.s.l.), displayed styles exerted beyond the corolla lobes and anthers, which represents an adaptation that favours outcrossing.

For flowers capable of autonomous selfing, the role of the timing of self-pollination should be determined. According to our results, self-pollination of *P. vulgaris* flowers started from the final bud stage, which may be consistent with the category of prior selfing. Thus, this finding supports previous studies on this species, in which Qu & Widrlechner (2011) observed that pollen grains were deposited on the stigma and self-pollination occurred in the final bud stage. This phenomenon of self-fertilization has been termed "bud-autogamy" (Noormets & Olson 2006) or "preanthesis cleistogamy", in which "self-fertilization occurs in the bud, followed by anthesis and opportunities for outcrossing" (Culley & Klooster 2007). Furthermore, we showed that seed production resulting from selfing gradually increased over the life-span of the flower in *P. vulgaris*, which has not been previously reported in this species. This phenomenon was found in a study on the flowers of *Tillandsia multicaulis* Steud. (Bromeliaceae), in which self-fertilization occurred prior to flower opening, but seed production increased with advancing stage of floral development (Bush 2009), suggesting that some of the autonomous seed set observed in *P. vulgaris* may also be caused by modes of competing selfing and delayed selfing, as defined by Lloyd & Schoen (1992). The timing of autonomous selfing does not always fit into the "prior", "competing" or "delayed" categories (Zhang & Li 2008, Fan & Li 2012). Prior selfing is not always considered to provide reproductive assurance in the classic sense, because of the costs associated with pollen and seed discounting (Lloyd 1992). Under low pollinator availability, these costs are reduced and prior selfing can evolve more easily (Morgan & Wilson 2005).

Autonomous selfing is commonly observed in smaller flowers (e.g. Fenster et al. 1995, Runions & Geber 2000, Elle & Carney 2003, Elle et al. 2010). Smith-Huerta & Huerta (2015) also stated that smaller flowers within the same species show a greater capacity for self-pollen deposition when compared with larger flowers that are outcrossing. Flower sizes of *P. vulgaris* are reported to vary along different altitudinal gradients (Kuriya et al. 2015), indicating that floral size is closely related to environmental factors. The present results showed that floral size (as presented by corolla tube length) in our study population, at 7.78 ± 0.26 mm (mean ± s.e., n = 30), was less than the shortest floral tube reported by Kuriya et al. (2015), at 11.33 ± 0.14 mm (mean ± s.e, n = 50). Thus, it is assumed that *P. vulgaris* is capable of autonomous selfing, as was observed in smaller flowers of the species by Nelson (1964). However, Kuriya et al. (2015) did not test experimentally whether the breeding system associated with floral size variation differed along altitudinal gradients. In addition, these authors mentioned that no pollen was deposited on the stigma in early floral developmental stages of *P. vulgaris*. Qu & Widrlechner (2011) reported variation in reproductive success of this species between two types of flowers differing in style length, with only 6% seed set recorded for bagged flowers with an exerted style, whereas populations

with an inserted style showed up to 94% seed set. The exerted style was only present in accessions from China (Sichuan), Japan and one population from the USA (Oregon), whereas inserted styles were exclusively observed in 27 populations of *P. vulgaris* from the USA and the Republic of Georgia. In the present study, 90% ($n = 50$) of the styles were inserted and shorter than the long stamens, and up to 93% ($n = 30$) self-pollinated seed set was observed in bagged-intact flowers. Thus, the frequency of selfing in this species likely depends on relative stamen length rather than floral size.

Reproductive assurance and opportunities for outcrossing

Although *P. vulgaris* flowers are visited by a variety of insects, including bumblebees (Kuriya et al. 2015), butterflies (Aluri 1995), and honey bees (Qu & Widrlechner 2011), we observed bumblebees and honey bees to be the most frequent visitors in the subalpine population of the current study, but at a very low visitation rate. This is reflected in our observation that emasculated flowers exposed to potential pollinators produced significantly fewer seeds than did intact flowers, indicating that the majority of seeds produced in intact flowers were a result of autonomous selfing. For seed production among plants as well as the treatment × plant interaction, significant differences were only observed among treatments of the breeding system experiment, whereas no significant differences were observed among the emasculation treatments and among the timing of autonomous selfing. This may be because some of the plants, particularly those in which flowers were emasculated and exposed to pollinators to examine the contribution of open pollination to seed production, were probably not visited by some of the pollinators, and thus would influence reducing seed production more in those plants than in other visited plants.

Considering the present results collectively, this subalpine population of *P. vulgaris* shows a high rate of autonomous selfing (a trait common to many other populations of this species), and the additional contribution of pollinator-mediated outcrossing to seed production seems to be negligible. These results support the hypothesis that autonomous self-pollination serves as a means of reproductive assurance, which likely developed under scarce pollinator availability, in the present case associated with subalpine conditions.

ACKNOWLEDGEMENTS

We sincerely thank M. P. Widrlechner and two anonymous reviewers for their invaluable suggestions and comments on earlier versions of the manuscript. Tial C. Ling acknowledges the Chinese Academy of Sciences (CAS) and the World Academy of Sciences (TWAS) for receiving the CAS-TWAS President's Fellowship to carry out the research. We are grateful to Mr Kun Xu and Dr Zhi-Kun for assistance with field experiments. This work was financially supported by grants from the 973 Program (2014CB954100) and the National Natural Science Foundation of China (NSFC) (31590820, 31590820, 31570385).

REFERENCES

Aluri R.J.S. (1995) Floral biology and pollination in Prunella vulgaris L. (Lamiaceae) in Kashmir Himalaya. Journal for Nature Conservation 7: 71–74.

Anderson W.R. (1980) Cryptic self-fertilization in the Malpighiaceae. Science 207: 892–893. https://doi.org/10.1126/science.207.4433.892

Baker H.G. (1955) Self-compatibility and establishment after "long distance" dispersal. Evolution 9: 347–349. https://doi.org/10.2307/2405656

Barrett S.C.H. (2002) The evolution of plant sexual diversity. Nature Reviews Genetics 3: 274–284. https://doi.org/10.1038/nrg776

Brys R., Jacquemyn H. (2011) Variation in the functioning of autonomous self-pollination, pollinator services and floral traits in three Centaurium species. Annals of Botany 107: 917–925. https://doi.org/10.1093/aob/mcr032

Bush S.P. (2009) Prior autonomous selfing in the hummingbird-pollinated epiphyte Tillandsia multicaulis (Bromeliaceae). Selbyana 30: 114–121. Available from https://www.jstor.org/stable/41760355 [accessed 10 Oct. 2016].

Busch J.W., Delph L.F. (2012) The relative importance of reproductive assurance and automatic selection as hypotheses for the evolution of self-fertilization. Annals of Botany 109: 553–562. https://doi.org/10.1093/aob/mcr219

Charlesworth B. (1980) The cost of sex in relation to mating system. Journal of Theoretical Biology 84: 655–671. https://doi.org/10.1016/S0022-5193(80)80026-9

Charlesworth D., Charlesworth B. (1987) Inbreeding depression and its evolutionary consequences. Annual Review of Ecology and Systematics 18: 237–268. https://doi.org/10.1146/annurev.es.18.110187.001321

Cruden R.W. (1977) Pollen-ovule ratios: a conservative indicator of breeding systems in flowering plants. Evolution 31: 32–46. https://doi.org/10.2307/2407542

Culley T.M., Klooster M.R. (2007) The cleistogamous breeding system: a review of its frequency, evolution, and ecology in angiosperms. The Botanical Review 73: 1–30. https://doi.org/10.1663/0006-8101(2007)73[1:TCBSAR]2.0.CO;2

Dafni A. (1992) Pollination ecology: a practical approach. Oxford, Oxford University Press. New York.

Darwin C.R. (1876) The effects of cross- and self-fertilization in the vegetable kingdom. London, John Murray. https://doi.org/10.5962/bhl.title.110800

Darwin C.R. (1877) The different forms of flowers on plants of the same species. John Murray, London. Reprint (1986). Chicago, University of Chicago Press.

Davis S.L., Delph L.F. (2005) Prior selfing and gynomonoecy in Silene noctiflora L. (Caryophyllaceae): opportunities for enhanced outcrossing and reproductive assurance. International Journal of Plant Sciences 166: 475–480. https://doi.org/10.1086/428630

Delmas C.E., Cheptou P.-O., Escaravage N., Pornon A. (2014) High lifetime inbreeding depression counteracts the reproductive assurance benefit of selfing in a mass-flowering shrub. BMC Evolutionary Biology 14: 243. https://doi.org/10.1186/s12862-014-0243-7

Duan Y.-W., Dafni A., Hou Q.-Z., He Y.-P., Liu J.-Q. (2010) Delayed selfing in an alpine biennial Gentianopsis paludosa (Gentianaceae) in the Qinghai-Tibetan Plateau. Journal of Integrative Plant Biology 52: 593–599. https://doi.org/10.1111/j.1744-7909.2010.00951.x

Duffy K.J., Stout J.C. (2008) The effects of plant density and nectar reward on bee visitation to the endangered orchid Spiranthes romanzoffiana. Acta Oecologica 34: 131–138. https://doi.org/10.1016/j.actao.2008.04.007

Eckert C.G., Herlihy C.R. (2004) Using a cost-benefit approach to understand the evolution of self-fertilization in plants: the perplexing case of Aquilegia canadensis (Ranunculaceae). Plant Species Biology19: 159–173. https://doi.org/10.1111/j.1442-1984.2004.00112.x

Elle E., Carney R. (2003) Reproductive assurance varies with flower size in Collinsia parviflora (Scrophulariaceae). American Journal of Botany 90: 888–896. https://doi.org/10.3732/ajb.90.6.888

Elle E., Gillespie S., Guindre-Parker S., Parachnowitsch A.L. (2010) Variation in the timing of autonomous selfing among populations that differ in flower size, time to reproductive maturity and climate. American Journal of Botany 97: 1894–1902. https://doi.org/10.3732/ajb.1000223

Fan Y.-L., Li Q.-J. (2012) Stigmatic fluid aids self-pollination in Roscoea debilis (Zingiberaceae): a new delayed selfing mechanism. Annals of Botany 110: 969–975. https://doi.org/10.1093/aob/mcs169

Fenster C.B., Diggle P.K., Barrett S.C.H, Ritland K. (1995) The genetics of floral development differentiating two species of Mimulus (Scrophulariaceae). Heredity 74: 258–266. https://doi.org/10.1038/hdy.1995.39

Fenster C.B., Martén-Rodríguez S. (2007) Reproductive assurance and the evolution of pollination specialization. International Journal of Plant Sciences 168: 215–228. https://doi.org/10.1086/509647

Forrest J., Thomson J.D. (2008) Pollen limitation and cleistogamy in subalpine Viola praemorsa. Botany 86: 511–519. https://doi.org/10.1139/B08-020

Hegland S.J., Totland Ø. (2005) Relationship between species' floral traits and pollinator visitation in a temperate grassland. Oecologia 145: 586–594. https://doi.org/10.1007/s00442-005-0165-6

Herlihy C.R., Eckert C.G. (2002) Genetic cost of reproductive assurance in a self-fertilizing plant. Nature 416: 320–323. https://doi.org/10.1038/416320a

Jacquemyn H., Brys R. (2015) Pollen limitation and the contribution of autonomous selfing to fruit and seed set in a rewarding orchid. American Journal of Botany 102: 67–72. https://doi.org/10.3732/ajb.1400449

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. https://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. https://doi.org/10.1038/nature02776

Kennedy B.F., Elle E. (2008) The reproductive assurance benefit of selfing: importance of flower size and population size. Oecologia 155: 469–477. https://doi.org/10.1007/s00442-007-0924-7

Kuriya S., Hattori M., Nagano Y., Itino T. (2015) Altitudinal flower size variation correlates with local pollinator size in a bumblebee-pollinated herb, Prunella vulgaris L. (Lamiaceae). Journal of Evolutionary Biology 28: 1761–1769. https://doi.org/10.1111/jeb.12693

Lloyd D.G. (1979) Some reproductive factors affecting the selection of self-fertilization in plants. The American Naturalist 113: 67–79. https://doi.org/10.1086/283365

Lloyd D.G. (1992) Self- and cross-fertilization in plants. II. The selection of self-fertilization. International Journal of Plant Sciences 153: 370–380. https://doi.org/10.1086/297041

Lloyd D.G., Schoen D.J. (1992) Self- and cross-fertilization in plants. I. Functional dimensions. International Journal of Plant Sciences 153: 358–369. https://doi.org/10.1086/297040

Ma Y., Wu Z., Tian X., Zhang C., Sun W. (2012) Growth discrepancy between filament and style facilitates autonomous self-fertilization in Hedychium yunnanense (Zingiberaceae). Plant Ecology and Evolution 145: 185–189. https://doi.org/10.5091/plecevo.2012.652

Morgan M.T., Wilson W.G. (2005) Self-fertilization and the escape from pollen limitation in variable pollination environments. Evolution 59: 1143–1148. https://doi.org/10.1111/j.0014-3820.2005.tb01050.x

Müller H. (1883) The fertilization of flowers. Translated by D'Arcy Thompson. London, Macmillan. https://doi.org/10.5962/bhl.title.21000

Nagylaki T. (1976) A model for the evolution of self-fertilization and vegetative reproduction. Journal of Theoretical Biology 58: 55–58. https://doi.org/10.1016/0022-5193(76)90138-7

Nelson A.P. (1964) Relationships between two subspecies in a population of Prunella vulgaris L. Evolution 18: 43–51. https://doi.org/10.2307/2406418

Nelson A.P. (1967) Racial diversity in Californian Prunella vulgaris. New Phytologist 66: 707–746. https://doi.org/10.1111/j.1469-8137.1967.tb05441.x

Noormets M., Olson A.R. (2006) Bud-autogamy in the velvet-leaf blueberry, Vaccinium myrtilloides Michx. Canadian Journal of Plant Science 86: 245–250. https://doi.org/10.4141/P04-118

Ollerton J., Winfree R., Tarrant S. (2011) How many flowering plants are pollinated by animals? Oikos 120: 321–326. https://doi.org/10.1111/j.1600-0706.2010.18644.x

Qu L., Widrlechner M.P. (2011) Variation in the breeding system of Prunella vulgaris L. HortScience 46: 688–692.

Runions C.J., Geber M.A. (2000) Evolution of the self-pollinating flower in Clarkia xantiana (Onagraceae). I. Size and development of floral organs. American Journal of Botany 87: 1439–1451. https://doi.org/10.2307/2656870

Schoen D.J., Lloyd D.G. (1992) Self- and cross-fertilization in plants. III. Methods for studying modes and functional aspects of self-fertilization. International Journal of Plant Sciences 153: 381–393. https://doi.org/10.1086/297042

Smith-Huerta N.L., Huerta A.J. (2015) Floral biology and the evolution of selfing in natural populations of Clarkia tembloriensis Vasek (Onagraceae). The Journal of the Torrey Botanical Society 142: 240–248. https://doi.org/10.3159/TORREY-D-14-00087.1

Vogler D.W., Kalisz S. (2001) Sex among the flowers: the distribution of plant mating systems. Evolution 55: 202–204. https://doi.org/10.1111/j.0014-3820.2001.tb01285.x

Wells H. (1979) Self-fertilization: advantageous or deleterious? Evolution 33: 252–255. https://doi.org/10.2307/2407381

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. https://doi.org/10.1093/aob/mcn136

Manuscript received 18 Jul. 2016; accepted in revised version 23 Jan. 2017.

Communicating Editor: Renate Wesselingh.