Identification of effective pollinators of Primula sieboldii E. Morren in a wild habitat in Hiroshima, Japan

Yoshida, Yasuko
Nikkeshi, Aoi
Chishiki, Akane

(Citation)
Plant Species Biology,36(2):157-169

(Issue Date)
2021-04

(Resource Type)
journal article

(Version)
Accepted Manuscript

Original article

Identification of effective pollinators of *Primula sieboldii* E. Morren in a wild habitat in Hiroshima, Japan

Yoshida Yasuko[1,*], Aoi Nikkeshi[2], Akane Chishiki[3]

[1] Food Resources Education and Research Center, Graduate School of Agricultural Science, Kobe University
[2] Division of Biodiversity, Institute for Agro-Environmental Science, National Agriculture and Food Research Organization
[3] Faculty of Agriculture, Kobe University

*Corresponding author
Yasuko Yoshida
Food Resources Education and Research Center, Graduate School of Agricultural Science,
Kobe University, 1348 Uzurano, Kasai, Hyogo 675-2103, Japan
Phone: +81-790-49-3121
Fax: +81-790-49-0343
E-mail: yasukoy@landscape.kobe-u.ac.jp

Short running title: Effective pollinators of *P. sieboldii* in Hiroshima

Abstract

Primula sieboldii E. Morren is a heterostylous clonal herb that is widely distributed in Japan but in danger of extinction in the wild. The existence of pollinators in each habitat is imperative for its long-term survival, because seeds can be produced only by insect cross-pollination between different flower morphs. In this study, we identified the pollinators of *P. sieboldii* in a wild habitat in Hiroshima as those insects that we observed to (1) put the proboscis into a corolla tube, (2) deposit pollen grains on the proboscis, and (3) have a proboscis of appropriate length and width. Effective pollinators were identified from their contribution to pollination. In 2015 and 2016, the flower visitations of 232 and 558 insects, respectively, were recorded and 85 and 13 insects were captured. Two *Bombylius* species, *B. major* L. and *B. shibakawae* Matsumura, accounted for 90% of flower-visiting insects in both years. All 14 species that we captured were considered pollinators of *P. sieboldii*, because they had proboscises that were long enough to reach pollen and that had pollen grains deposited on them. The total visitation rate of “Bombyliidae” was the highest among all pollinator categories. The results of potential pollen transport per flower per hour, which was based on total pollen number and total visitation rate of each pollinator category, indicated that “Bombyliidae” species were the most effective pollinators of *P. sieboldii* in this habitat.

Keywords

Bombyliidae, effective pollinators, endangered species, heterostyly, *Primula sieboldii*

Introduction

Genetic diversity that is maintained among and within remnant wild populations plays a key role in the adaptive evolutionary potential of a species in response to fluctuating environments (Frankham et al., 2002). Because sexual reproduction increases the genetic diversity of wild populations, the existence of pollinators locally is one of the most important factors for the long-term survival of endangered insect-pollinated plants. Thus, understanding the ecology of pollinators is critical for the conservation of endangered plant species. Moreover, it is also essential to identify which species make large contributions to pollination, because these effective pollinators should be preferentially preserved in habitats with a high extinction risk.

Primula sieboldii E. Morren is a perennial clonal herb that occupies a range of moist habitats, from the understory of deciduous forests to well-managed grasslands. The species is distributed in Japan, on the Korean Peninsula, in northern China, and in eastern Siberia (Yamazaki, 1993). *P. sieboldii* has also been bred as a traditional garden herb for about 300 years, beginning in the Edo era in Japan (Torii, 1985), and there are more than 300 cultivars with various petal colors and shapes that originated by crossbreeding between wild *P. sieboldii* individuals in Japan (Honjo et al., 2008). However, overexploitation, destruction of the species' habitats, and horticultural collection are threatening wild populations with extinction in Japan. This species was originally classified in the lower rank of vulnerable, but it was reclassified as near threatened in 2007 because conservation activities increased the number of individuals (Iwatsuki, 2008). However, 19 Japanese prefectures regard *P. sieboldii* as a threatened species at high risk of extinction and it has already been extirpated in four prefectures (Association of Wildlife Research and EnVision, 2017).

P. sieboldii is a heterostylous species with two different flower forms (morphs): short-styled (thrum; T) and long-styled (pin; P). Because plants produce seeds only by insect cross-pollination between different morphs, usually called "legitimate pollination," the existence of pollinators in each habitat is essential for long-term survival of wild populations. Under severe pollinator limitation, however, studies revealed that shortages in the supply of compatible pollen caused low seed production (Washitani et al., 1994), and model simulations indicated that the short-styled genets would disappear from the populations, even with high inbreeding depression (Washitani, 1996). These particular outcomes in the *P. sieboldii* population in Japan's Tajimagahara Nature Reserve were due to the absence of a suitable pollinator, whose habitat was limited by urban areas and golf courses where insecticides are heavily applied (Washitani et al., 1991).

Washitani et al. (1995) reported that queens of bumble bees, such as *Bombus diversus* *tersatus* Smith, *Bombus schrencki albidopleuralis* Skorikov, and *Bombus deuteronymus deuteronymus* Schulz, were pollinators of *P. sieboldii* in Hokkaido, northern Japan. However, seed production has been observed in some habitats with no bumble bee queens, where flower visitors included *Bombylius major* L., *Rhingia laevigata* Loew, *Daimio tethys* Ménétlès, and *Parnara guttata guttata* Bremer et Gret (Takahashi et al., 1992; Yoshida et al., 2012). These studies did not reveal whether these insects played an important role as pollinators, but suggested that many pollinators besides bumble bee queens likely contribute to legitimate pollination in *P. sieboldii*. In addition, *B. diversus tersatus* is distributed only in Hokkaido and so is not common in wild habitats across Japan, so suitable pollinators differ among habitats. Thus, we need to examine the pollinators in each habitat where *P. sieboldii* faces extirpation for the long-term conservation of the species.

This study was carried out in a wild habitat (Geihoku population) in Kita-Hiroshima town, Hiroshima prefecture, Japan (132°23′E, 34°44′N). *P. sieboldii* is classified as a special natural monument and as threatened I (critically endangered or endangered species) in Kita-Hiroshima town and in Hiroshima prefecture, respectively. Honjo et al. (2005) reported 35 *P. sieboldii* genets (T, 17; P, 15; unknown, 3) in the population in 2003, and the morph ratio, which is important for effective seed production, was equal. Gene diversity of the Geihoku population was 0.701, relatively high compared with values in 32 wild populations (0.420–0.786) evaluated by Honjo et al. (2009). There is no information about the pollinators of this population, but seed production has been observed every year, indicating that there are some suitable pollinators in this population.

In Kita-Hiroshima town, there is also a cultivated area of *P. sieboldii* in addition to ramets growing in wild habitat. This cultivated area is about 30 km, as the crow flies, from the Geihoku population, and the ramets’ flowers look like those of wild plants, not cultivars. On the basis of chloroplast DNA variation, Honjo et al. (2005) suggested that these cultivated ramets originated from other areas such as the Kanto region, not from the Geihoku population. Many *P. sieboldii* ramets grow in the cultivated area, and some insects were visiting their flowers. Therefore, we also caught flower visitors in this area to examine whether they could be candidate pollinators.

In this study, we aimed at identifying the pollinators of *P. sieboldii* in the Geihoku population, which grows in a wild habitat in Japan. We regarded as pollinators those insects that we observed to: (1) put the proboscis into a corolla tube, (2) deposit pollen grains on the proboscis, and (3) have a proboscis of appropriate length and width. Thus, we examined the visiting insects, counted the number of pollen grains deposited on the proboscises of visiting insects, and compared the length and width of each flower visitor's proboscis to the floral morphological traits. The contribution of each species to pollination was estimated from the number of pollen grains and the visitation frequency. From these contributions, we identified the effective pollinators in this habitat. Exhaustive studies have been conducted on *P. sieboldii* as a model species for plant conservation (Washitani et al., 2005). Although genetic analyses of the species (e.g., QTL analysis and gene identification) have progressed, practical field research within its local habitats is also important. Our findings will be valuable for the conservation of habitats where *P. sieboldii* is at high risk of extinction.

Materials and Methods

Flower-visiting insects

Four quadrats (2 m × 2 m, quadrats A to D) were placed in the Geihoku population in 2015 and 2016. Although the quadrats were not in the same place in both years, they were placed as close to the original position as possible. The number of *P. sieboldii* flowers of each morph was counted in each quadrat every half day. We regarded those insects inserting their proboscises into a corolla tube as flower-visiting insects of *P. sieboldii*. We monitored insect visits to flowers for 7 days in 2015 and 3 days in 2016, from the end of April to mid-May. Flower-visiting species were recorded every 15 min per quadrat on non-rainy days from 08:00 to 16:00 h, which corresponds to the activity period of insects in this habitat. Continuous flower visitations to different flowers within the same quadrat by a single insect were not recorded. We did not record details of insect visits in the cultivated area, but instead captured the flower-visiting insects that the number of captured were small or zero in this habitat on 2 days in 2015, in order to increase the sample size of pollen grains deposited on proboscis for analysis.

In both places, the insects were captured with a nylon net when possible. All captured insects were stored in a 50-mL tube or paraffin paper (for lepidopterans) marked with an identification number and kept in a freezer. For those species that carry pollen loads on their legs, immediately after capture we used carbon dioxide to knock them out, and then cut the legs from the body with scissors to prevent contamination of the pollen grains on the proboscis.

Number of pollen grains on the proboscis

We counted the number of pollen grains on the proboscis of all flower-visiting insects that we captured in 2015 and 2016. Each proboscis was cut using tweezers and scissors and placed into 100 µL of 0.4 mol/L sucrose solution in a 1.5-ml tube, and the pollen grains were washed in this solution as described by Nikkeshi et al. (2016). Then, 0.5 µL of Safranin O was added to each tube to stain pollen grains. Because the pollen grains of short-styled flowers (14–15 × 17–18 µm) are larger than those of long-styled flowers (11.5–12 × 13–13.5 µm) in *P. sieboldii* (Ikuse, 2001), it is easy to distinguish between morphs. The number of pollen grains from each morph was counted under an optical microscope (DM2500, Leica, Wetzlar, Germany). To minimize counting error, we counted the pollen grains included in 10 µL twice and then calculated the average; this procedure was performed 10 times on each 100-µL sample. We summed the 10 average values to calculate the total pollen grains on the proboscis of each flower-visiting insect.

Comparison between proboscis of flower-visiting insects and floral morphological traits

To investigate whether flower-visiting insects have appropriate proboscises for pollination of *P. sieboldii*, we measured morphological traits of both the captured insects and flower morphs and compared them with each other. Because *P. sieboldii* has a long corolla tube and nectar is located at the bottom of the tube, only flower-visiting insects with a long proboscis can reach the nectar. However, those flower-visiting insects with a proboscis that is long enough to reach pollen can be pollinators. Moreover, previous studies revealed that proboscis width is also important for pollination in some plants with tubular flowers (Barrios et al., 2016; Moré et al., 2007). Therefore, we measured the proboscis length and width using a digital caliper as morphological traits of the flower-visiting insects; the maximum width was regarded as the proboscis width.

Petal traits in *P. sieboldii* are largely determined by genetic factors. We could identify genets visually from the differences among floral characters (Yoshida et al., 2008), so we selected 11 individuals (T, 4; P, 7) in 2015 and 32 individuals (T, 17; P, 15) in 2016 with different petal traits as far as possible, and collected two flowers from each individuals (i.e., total 86 flowers). Seven floral morphological traits were measured (Fig. 1a): (1) corolla tube length, (2) corolla tube width, (3) stigma height, (4) anther height (maximum height from the base of the ovary), (5) nectar distance, (6) pollen distance (minimum distance from the top of the anther to the corolla tube entrance), and (7) petal area. In this species, nectar is pooled on the top of the ovary (Fig. 1b), and the actual nectar distance spans from the corolla tube entrance to the top nectar level. However, it was difficult to measure actual nectar distance of each flower, because the amount of nectar differed depending on flower-visitation frequency. Therefore, we defined the nectar distance as the maximum distance from the corolla tube entrance to the point where the ovary and corolla tube meet. When some nectar was available in a corolla tube, the actual distance was shorter than the nectar distance. Each flower was photographed alongside a scale marker (9 mm × 9 mm) with a digital camera (OM-D E-M5, Olympus), and these morphological traits were measured in the image processing program ImageJ v. 1.48 (Schneider et al., 2012). Petal area was regarded as a parameter of display size.

Estimation of the contribution to pollination

Two species of *Bombylius*, *B. major* L. and *B. shibakawae* Matsumura, were observed in this habitat. Although these species have a different wing pattern, it is difficult to distinguish between them when they are flying, so they were analyzed together as the category "Bombyliidae." Likewise, it was sometimes difficult to identify lepidopterans to species by visual observation, so we analyzed them as the category "Lepidopterans." For the pollinators that fulfilled the three conditions, visitation rate per flower per hour was calculated by dividing the number of flower visitations by the number of flowers and observation time for each pollinator species or category in each year. The visitation rate was calculated by using weighted averages to account for the different observation time between years, such that the total visitation rate indicated the weighted average of pooled data for 2 years. We calculated potential pollen transport per flower per hour (PPT; Nikkeshi et al., 2019) by multiplying the number of pollen grains by visitation rate, as a parameter of the contribution to pollination of *P. sieboldii* in the Geihoku population. From these calculations, the species with the highest value of PPT was regarded as the most effective pollinator in this habitat.

Statistical analysis

To examine the effects of proboscis length and width on pollination efficiency in *P. sieboldii*, we used the generalized linear mixed models (GLMMs) function in the glmmADMB package (Skaug et al., 2014), in which we used $\log_{10}$-transformed number of pollen grains deposited on the proboscis of all captured insects as the response variable (family = Gaussian), the proboscis length and width as explanatory variables, and observation day and quadrat ID as a random effect. Because quadrats with the same ID (e.g., 2015A and 2016A) were not placed at exactly the same position each year, eight quadrat IDs (2015A, 2015B, …, 2016C, 2016D) were used for the analysis. In addition, we analyzed the $\log_{10}$-transformed number of deposited pollen grains of two categories ("Bombyliidae" and "Lepidopterans") for which we captured more than 20 individuals as the response variable and the proboscis length and width as explanatory variables (family = Gaussian, random effect = observation day and quadrat

ID). The significance of differences in floral morphological traits between morphs was tested by one-way ANOVA. We also tested the correlation between morph (short-styled or long-styled) and the number of pollen grains on the proboscis of flower visitors. All statistical analyses were performed in the free R v. 3.6.1 software (R Development Core Team, 2019).

Results

Quadrat A had only long-styled flowers in, but the other three had both morphs (Table S1). In 2015, our observations began at the peak of flowering on 5 May. No insects visited the flowers on 17 May, so we finished our observations, even though many insects were observed in the habitat. At that time, there were many plants that were taller than *P. sieboldii*, such as *Equisetum arvense* L. and *Cardiocrinum cordatum* (Thunb.) Makino, and they covered *P. sieboldii* in this habitat. In 2016, we started the observation at the beginning of flowering to examine the pollinators through the flowering period of *P. sieboldii* for 2 years.

Flower-visiting insects

In the Geihoku population, total observation times were 46.5 h in 2015 and 68.0 h in 2016 (Table 1). We recorded the flower visitations of 232 and 558 insects and captured 85 and 13 insects in 2015 and 2016, respectively. All captured insects were identified to the species level, and they represented 10 species. Two *Bombylius* species accounted for 90% of flower-visiting insects in both years. Because we had captured a sufficient number of *Bombylius* individuals in 2015, we did not capture them in 2016. Twelve lepidopteran insects that could not be captured were classified as “unknown” in the category “Lepidopterans” (Table 1).

In the cultivated area, we captured 16 insects belonging to six species (Table 2). Because the number and density of flowers in the cultivated area were greater than those in the habitat, many insects visited the flowers. *Bombylius* individuals also accounted for the largest number of observations in the cultivated area, but none were captured there because the number of individuals captured in the habitat was sufficient. The lepidopterans *Lycaena phlaeas*, *Pieris melete*, and *Papilio maackii* and the bumble bee *Bombus ardens ardens* were observed only in the cultivated area.

Number of pollen grains deposited on the proboscis

All captured insects had pollen grains deposited on the proboscis (Table 2). *Bombus diversus diversus* had the largest total number of pollen grains (mean = 19829.5), but the number differed greatly between the two specimens (SD = 16916.1). On the other hand, the smallest numbers of total pollen grains were observed on *Pterodecta felderi* and *L. phlaeas*. The total number of pollen grains on *P. maackii* (mean = 964.0), with a longer and wider proboscis, was larger than those of other "Lepidopterans," but all values in this category were <1000 and were smaller than those in other categories except "Campsomeris prismatica." Furthermore, the number of deposited pollen grains of the long-styled morph was more than that of the short-styled morph in all categories except "Campsomeris prismatica." There was a significant positive correlation between the $\log_{10}$-transformed number of deposited pollen grains of the two morphs ($P < 0.01$; Fig. 2). Pollen grains from plants other than *P. sieboldii* were also observed in this study. Some of them could be identified to the species level, such as *Ixeris japonica*, *Lamium purpureum*, *Smilax china*, *Tristagma uniflorum*, and *Veronica persica*. The pollen sizes of these species, ranging from 24.5–27 × 24.5–27 µm in *S. china* to 33–35 × 35–37 µm in *V. persica* (Ikuse, 1956, 2001), were larger than that of *P. sieboldii*. The number of total pollen grains deposited on each pollinators in these species was not counted.

Comparison between proboscis of flower-visiting insects and floral morphological traits

Six floral traits differed significantly between morphs (Table S2). The corolla tube length and width of the short-styled morph were greater than those of the long-styled morph, indicating that the flower of the short-styled morph was slightly larger. The pollen distance in the short-styled morph (0.79 mm) was much shorter than that in the long-styled morph (3.46 mm), indicating that short-styled flowers have pollen that is more easily accessible to pollinators. On the other hand, there was no difference in petal area between morphs (Table S2). Yoshioka et al. (2007) reported that bumble bees could discriminate only an extreme difference in petal area (~3.4 cm^2), suggesting that petal area has no effect on the visitation rate of flower-visiting insects in this habitat.

Papilio maackii had the longest proboscis (21.01 mm) and *B. diversus diversus* and *Anthophora plumipes* also had proboscises >15 mm in length, whereas *P. felderi* and *L. phlaeas* had very short proboscises (4.13 and 4.37 mm, respectively; Table 2). This result indicates that there were large differences in proboscis length among flower-visiting insects of *P. sieboldii*.

When comparing proboscis length with nectar distance, *B. diversus diversus*, *A. plumipes* (except one individual), and *P. maackii* had longer proboscises than nectar distance in all flowers (Fig. 3a). Half of the captured individuals of *B. major* and *B. shibakawae*, *B. ardens*

ardens, and *Campsomeris prismatica* Smith and most lepidopteran species had shorter proboscises than nectar distance in some flowers, and *Eucera nipponensis* Pérez and the lepidopterans *Anthocharis scolymus* (Butler), *L. phlaeas*, *P. melete*, and *P. felderi* had shorter proboscises than nectar distance in all flowers. In addition, the proboscises of most insects were sufficiently long to reach pollen of all flowers in both morphs (Fig. 3a). Proboscises of *P. felderi* (4.13 mm) and *L. phlaeas* (4.37 mm) were longer than pollen distance in all short-styled flowers, but were of insufficient length to reach pollen in some long-styled flowers.

Bombus ardens ardens, *A. plumipes*, *E. nipponensis*, and *C. prismatica* had thicker proboscises than those of the other 10 species (Table 2; Fig. 3b). Their proboscises were thicker than the corolla tube width in some long-styled flowers, but pollen grains of the long-styled morph were deposited on their proboscises, indicating that the thickness of a proboscis does not prevent insertion into a corolla tube.

Relationship between proboscis traits and total pollen grains on the proboscis

From the results of the GLMM analysis of all insect categories, proboscis length and width had significantly positive effects on the number of deposited pollen grains (Table 3, Figure S1). Likewise, we noted significant positive effects of proboscis length and width within each morph. In the categories "Bombyliidae" and "Lepidopterans," only proboscis length had a significantly positive effect on total pollen grains and that of each morph.

Potential pollen transport per flower per hour

All captured insects fulfilled the three conditions, indicating that 14 species were pollinators of *P. sieboldii* in the two places. The total visitation rate of "Bombyliidae" was the highest among all categories (Table 1) and was nearly 16 times that of "Lepidopterans," which had the second highest rate. PPT, which was based on total pollen number and total visitation rate, was highest in "Bombyliidae" (66.8916 grains/flower/h), whereas the values of the other six categories were <6 grains/flower/h (Table 4).

Discussion

Pollinators of P. sieboldii *in the Geihoku population*

All insects that we captured had proboscises that were long enough to reach pollen, and pollen grains were deposited on their proboscises. Thus, we revealed that 14 species in eight families—Bombyliidae, Apidae, Scoliidae, Pieridae, Callidulidae, Papilionidae, Lycaenidae, and Hesperiidae—were pollinators of *P. sieboldii* in the Geihoku population of Hiroshima. In addition to *B. major* and some lepidopteran species for which flower visitations have been previously reported, *A. plumipes*, *B. shibakawae*, *B. diversus diversus*, *B. ardens ardens*, *E. nipponensis*, and *C. prismatica* were also identified as pollinators of *P. sieboldii.*

The 14 pollinator species could be roughly divided into five functional groups: Bombyliidae (bee-flies), bumble bees, medium-sized bees, scolid wasps, and lepidopterans. Hoehn et al. (2008) and Albrecht et al. (2012) indicated that pollinator diversity (species richness and functional group) was positively related to seed set, and Albrecht et al. (2012) also indicated that flower visitation by specific taxa was important for seed set. These studies indicated that flower visitation by multiple pollinators is more effective for seed production than by one pollinator, even if the number of flower visitations is the same. Moreover, although "Lepidopteran" species carry fewer pollen grains than the other categories, pollination by these species is essential for effective seed production in *P. sieboldii*, because butterflies tend to contribute to cross-pollination because of longer flights between individual flower visits (Willmer, 2011). Thus, for seed production in the Geihoku population, it is necessary to maintain habitats suitable for *Bombylius* species, with the highest flower visitation rate, as well as for the other four functional groups.

Most effective pollinators in the Geihoku population

Our data indicate that the two *Bombylius* species were the most effective pollinators of *P. sieboldii* in the Geihoku population. Considering the numbers of *B. major* and *B. shibakawae* captured, *B. major* may be the more effective pollinator in this habitat. Kastinger & Weber (2001) reported that *B. major* preferred tubular or hypocrateriform type flowers to disc flowers and lip flowers; and *P. sieboldii* has the tubular flowers that *B. major* prefers. Shimono & Washitani (2007) showed that *B. major* was also one of two effective pollinators of *Primula modesta* in Nagano, central Japan. *P. modesta* was pollinated complementarily by two species depending on the different seasonality and daily activities, whereas we observed consecutive visits by both *Bombylius* species to *P. sieboldii* during the flowering period in the Geihoku population, suggesting that steady visitation by *B. major* and *B. shibakawae* is essential for the pollination of *P. sieboldii*.

Bombylius major is found across the whole northern temperate zone, from Europe to Japan and in North America (Kastinger & Weber, 2001). It is distributed throughout Japan, from Hokkaido to the Kyushu region (Masunaga, 2014), and is not listed in the Red List of Japan. On the other hand, *B. shibakawae* is distributed in Japan and Korea (Lee & Han, 2017) and has a limited geographic range in Japan (Masunaga, 2014). *Bombylius shibakawae* is classified as an endangered species in four prefectures (Association of Wildlife Research and EnVision, 2017), as threatened I (critically endangered or endangered) in Yamaguchi, as vulnerable in Kyoto, and as near threatened in Osaka and Nara. In 1916, Masunaga (2014) reported the distribution of this species as only Osaka and Kyoto. Since then, however, it has been found in other prefectures, including Hiroshima, and it is expected that *B. shibakawae* has a wider geographic range than previously reported. Currently, there is no report that *B. shibakawae* has become endangered in Hiroshima, and the possibility that the number of *B. major* will decline in Hiroshima is low.

Bumble bee queens have been considered to be important pollinators of *P. sieboldii* (Washitani et al., 2005). In this study, however, the PPT value of "Bombus diversus" in the Geihoku population was considerably lower than that of "Bombyliidae." Washitani et al. (1995) and Matsumura & Washitani (2002), respectively, reported that the average total numbers of pollen grains deposited on queen bumble bee proboscises were 19,642 ($n = 4$) and 10,031 ($n = 13$) from the short-styled morph of *P. sieboldii* and 18,758 ($n = 7$) and 13,475 ($n = 12$) from the long-styled morph. These reported numbers of pollen grains were greater than the numbers measured in this study (Table 2). One reason may be that the number of pollen grains within each flower decreases with multiple pollinator visits in the Geihoku population. However, even if sufficient pollen grains were deposited on the proboscis of *B. diversus diversus*, the PPT of "Bombyliidae" (66.8916 grains/flower/h) was larger than that of "Bombus diversus," based on average total pollen grains of those previous studies (11.5200 and 7.0518 grains/flower/h, respectively). Although we should consider that visiting the same flowers is not effective for seed set in *P. sieboldii* and that *Bombylius* species tend to visit flowers of the same ramets (Yoshida, personal observation), we did not observe continuous flower visitations to different flowers of the same ramets in this study. Therefore, our results indicate that "Bombyliidae" bee-flies, with high frequency of flower visitation, contribute to the pollination of *P. sieboldii* in this habitat.

Effects of proboscis length and width on pollination

Pollinators had three types of proboscis length: (1) longer than the nectar distance in all flowers, (2) longer than the nectar distance in some flowers, and (3) shorter than the nectar distance in all flowers (Fig. 3a). It is difficult to measure nectar distance accurately in the field, however, because the nectar amount depends on the frequency of flower visitation by pollinators. Therefore, we standardized nectar distance as the maximum distance to nectar. This meant that some pollinators could not suck nectar when visiting flowers. On the other hand, two insects had a shorter proboscis than pollen distance in some long-styled morphs (Fig. 3a), but pollen of the long-styled morph was still deposited on their proboscis. Thus, pollinators with a short proboscis sometimes could not reach nectar in long-styled flowers, but they could still pollinate flowers of *P. sieboldii*. Similar results were reported by Darrault & Schlindwein (2005), who showed that one-third of the flower-visiting species have mouth parts of insufficient length to reach the nectar in *Hancornia speciosa*. However, these species could still pollinate flowers because they touch the pollen and the receptive portion of the style head. These results show that having a proboscis that is long enough to reach pollen is an important condition for pollinators of *P. sieboldii*. The only insect that did not have pollen of the short-styled morph deposited on the proboscis was *P. felderi* (Table 2), whose proboscis length, at 4.13 mm, was sufficient to reach pollen in short-styled flowers. Therefore, we consider that this lack of short-styled pollen on the single *P. felderi* individual captured occurred by chance.

In all species except *C. prismatica*, the number of pollen grains deposited on the proboscis from long-styled flowers was greater than that from short-styled flowers (Table 2). Matsumura & Washitani (2006) reported that the number of pollen grains carried after one visitation of *B. diversus tersatus* was 10,000 ± 4800 ($n = 13$) to a short-styled morph and 13,500 ± 4800 ($n = 12$) to a long-styled morph. They also reported that the number of pollen grains produced was 150,000 ± 28,000 in the short-styled morph and 300,000 ± 60,000 in the long-styled morph. Our result also indicate that pollinators more easily transfer the pollen of long-styled flowers than that of short-styled flowers in *P. sieboldii*.

Proboscis length and width had significant positive effects on the number of pollen grains on the proboscis in all categories (Table 3, Figure S1). This result indicates that a longer and thicker proboscis of pollinators could transfer more pollen grains of *P. sieboldii*. However, having this type of proboscis does not mean the insect is a more effective pollinator, because the number of total pollen grains on *B. diversus diversus* was more than that on *P. maackii*, with the longest proboscis, and *A. plumipes*, with the thickest proboscis. The results of this and previous studies indicate that the proboscis length and width of *B. diversus diversus* might be suitable for transferring the most pollen grains of *P. sieboldii*. Moreover, the results of GLMM analysis also revealed that the effects of proboscis length and width on pollination efficiency were similar between flower morphs. In terms of the category, proboscis width had no significant effect on total pollen grains and only proboscis length had a significant positive effect in “Bombyliidae” and “Lepidopterans” (Table 3). The range of proboscis lengths was quite different between “Bombyliidae” (6.86–10.06 mm) and “Lepidopterans” (3.55–21.54

mm), but proboscis length is important for transferring many pollen grains in both insect categories. This study reveals that both proboscis length and width were related to pollination efficiency in *P. sieboldii*, but the effect on pollination efficiency of each trait depends on the species or category of insect.

Conclusion

A large number of genets within a population by sexual reproduction is very important for long-term survival of heterostylous *P. sieboldii*. For that, the existence of pollinators as well as equal morph ratio within a population are essential. Of the 14 species identified as pollinators in the Geihoku population, 13 (except *B. shibakawae*) also have the potential to serve as pollinators of *P. sieboldii* in other habitats, because they are widely distributed throughout Japan (Inomata et al., 2013; Masunaga, 2014; Yamane, 2008). Furthermore, most insects with a long proboscis that we observed in this habitat visited the flowers of *P. sieboldii*, suggesting that any species with a long proboscis could be a pollinator of *P. sieboldii* if it lives in the *P. sieboldii* habitats.

Corolla tube length differed significantly among five wild populations of *P. sieboldii* used in previous studies (Yoshida et al., 2008, 2009) in a common-garden experiment (Yoshida et al., unpublished), suggesting that these populations have become differentiated as a result of directional natural selection because of different pollinators in each habitat. Thus, we expect that there are not only common insect species, but also unique species that serve as effective pollinators in each habitat. Although it would be ideal to identify the species' pollinators in all habitats, conservation studies should first focus on identifying pollinators in *P. sieboldii* populations showing a decreasing number of genets and seed production

Acknowledgments

We thank Dr. Katsunobu Shirakawa and Takashi Shimosugi for their support in this experiment; Kyoko Ohtsubo, Hiroki Sawada, and Yumiko Dan for the examination of flower-visiting insects; Dr. Kazuyuki Itoh for the examination of vegetation in the Geihoku population; and Dr. Masayoshi K. Hiraiwa for statistical analysis.

Author contributions

Y.Y designed the experiments. Y.Y, A.N and A.C performed experiments and analyzed data. Y.Y wrote the manuscript. All authors read and approved this manuscript.

References

Albrecht, M., Schmid, B., Hautier, Y., Müller, C. B. (2012) Diverse pollinator communities enhance plant reproductive success. *Proceedings of the Royal Society B*, 279, 4845–4852.

Association of Wildlife Research and EnVision (2017) Search system of Japanese red data. http://jpnrdb.com/index.html. Accessed 9 October 2019

Barrios, B., Pena, S. R., Salas, A., Koptur, S. (2016) Butterflies visit more frequently, but bees are better pollinators: the importance of mouthpart dimensions in effective pollen removal and deposition. *AoB Plants*, 8, plw001; doi:10.1093/aobpla/plw001

Darrault, R. O & Schlindwein, C. (2005) Limited fruit production in *Hancornia speciosa* (Apocynaceae) and pollination by nocturnal and diurnal insects. *Biotropica*, 37, 381–388.

Frankham, R., Ballou, J. D., Briscoe, D.A. (2002) Introduction to conservation genetics. Cambridge Univ. Press, Cambridge, pp. 617.

Hoehn, P., Tscharntke, T., Tylianakis, J.M., Steffan-Dewenter, I. (2008) Functional group diversity of bee pollinators increases crop yield. *Proceedings of the Royal Society B*, 275, 2283–2291.

Honjo, M., Tsumura, Y., Washitani, I., Ohsawa, R. (2005) The chloroplast DNA variation of *Primula sieboldii* populations in Geihoku, Hiroshima prefecture. *Natural History of Nishi-Chugoku Mountains*, 10/11, 81–90. (in Japanese)

Honjo, M., Handa, T., Tsumura, Y., Washitani, I., Ohsawa, R. (2008) Origins of traditional cultivars of *Primula sieboldii* revealed by nuclear microsatellite and chloroplast DNA variations. *Breeding Science*, 28, 347–354.

Honjo, M., Kitamoto, N., Ueno, S., Tsumura, Y., Washitani, I., Ohsawa, R. (2009) Management units of the endangered herb *Primula sieboldii* based on microsatellite variation among and within populations throughout Japan. *Conservation Genetics* 10, 257–267.

Ikuse, M. (1956) Pollen Grains of Japan. Hirokawa Pub. Co., Tokyo, pp. 303. (in Japanese)

Ikuse, M. (2001) Pollen Grains of Japan, 2nd ed. Hirokawa Pub. Co., Tokyo, pp. 369. (in Japanese)

Inomata, T., Uemura, Y., Yago, M., Jinbo, U., Ueda, K. (2013) Catalogue of the insects of Japan. Volume 7, Lepidoptera. Part 1. Hesperioidea–Papilionoidea. Touka Shobo, Fukuoka, pp. 119. (in Japanese)

Iwatsuki, K. (2008) Endangered vascular plants in Japan. – Present status and a proposal for conservation. *Proceedings of the Japan Academy Series B: Physical and Biological Sciences*, 84, 275–286.

Kastinger, C. and Weber, A. (2001) Bee-flies (*Bombylius* spp., Bombyliidae, Diptera) and the pollination of flowers. *Flora*, 196, 3–25.

Lee, YB. and Han, HY. (2017) Taxonomic review of the genera *Bombylius* and *Bombylella* (Diptera: Bombyliidae) in Korea. *Journal of Asia-Pacific Entomology*, 20, 1278–1286.

Masunaga, K. (2014) Family Bombyliidae. In: Editorial Committee of Catalogue of the Insect of Japan (Ed.), Catalogue of the Insects of Japan. Volume 8, Diptera. Part 1. Nematocera–Brachcera Aschiza. Touka Shobo, Fukuoka, p. 393. (in Japanese)

Matsumura, C. & Washitani, I. (2002) Heterostylous morph differences in pollen transfer and deposition patterns in *Primula sieboldii* on a visitation by a queen bumblebee, measured with a semi-natural experimental system. *Plant Species Biology*, 17, 1–12.

Matsumura, C. & Washitani, I. (2006) Experimental measurements of the pollen carried by *Bombus diversus tersatus* queen. In: Washitani I (ed), Conservation molecular genetic ecology on *Primula sieboldii*. University of Tokyo Press, Tokyo, pp 240–258. (in Japanese)

Moré, M., Sérsic A. N., Cocucci, A. A. (2007) Restriction of pollinator assemblage through flower length and width in three long-tongued hawkmoth-pollinated species of *Mandevilla* (Apocynaceae, Apocynoideae). *Annals of the Missouri Botanical Garden*, 94, 485–504.

Nikkeshi, A., Hiraiwa, M. K., Ushimaru, A., Hoshizaki, K., Makita, A., Inoue. M. (2016) Established method of deposited pollen grains on the surface of pollinated insect body. Japanese Journal of Palynology, 62, 3–7. (in Japanese)

Nikkeshi, A., Inoue, H., Arai, T., Kishi, S., Kamo, T. (2019). The bumblebee *Bombus ardens ardens* (Hymenoptera: Apidae) is the most important pollinator of Oriental persimmon, *Diospyros kaki* (Ericales: Ebenaceae), in Hiroshima, Japan. *Applied Entomology and Zoology*, 54, 409–419.

R Development Core Team (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org. Accessed 24 December 2019.

Schneider, C. A., Rasband, W. S., Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9, 671–675.

Shimono, A. & Washitani, I. (2007) Factors affecting variation in seed production in the heterostylous herb Primula modesta. *Plant Species Biology*, 22, 65–76.

Skaug, H., Fournier, D., Bolker, B., Magnusson, A., Nielsen, A. (2014) Generalized linear mixed models using AD Model Builder. R package version 0. 8. 0.

Takahashi, H., Washitani, I., Namai, H., Kuroda, Y. (1992) Conservation biology of *Primula* *sieboldii* in the Yatsugatake University Forest. I. Distribution, habitat characteristics and phenology. Bulletin of Tsukuba University Forests, 8, 275–286.

Torii, T. (1985) Sakurasou. Nippon Television Network Corporation, Tokyo. p. 114–123. (in Japanese).

Washitani, I. (1996) Predicted genetic consequences of strong fertility selection due to pollinator loss in an isolated population of *Primula sieboldii*. *Conservation Biology* 10, 59–64.

Washitani, I., Namai, H., Osawa, R., Niwa, M. (1991) Species biology of *Primula sieboldii* for the conservation of its lowland-habitat population: I. Inter-clonal variations in the flowering phenology, pollen load and female fertility components. *Plant Species Biology*, 6, 27–37.

Washitani, I., Osawa, R., Namai, H., Niwa, M. (1994) Patterns of female fertility in heterostylous *Primula sieboldii* under severe pollinator limitation. *Journal of Ecology* 82, 571–579.

Washitani, I., Kato, M., Nishihiro, J., Suzuki, K. (1995) Importance of queen bumble bees as pollinators facilitating inter-morph crossing in *Primula sieboldii*. *Plant Species Biology* 9, 169–176.

Washitani, I., Ishihama, F., Matsumura, C., Nagai, M., Nishihiro, J., Nishihiro, A. M. (2005) Conservation ecology of *Primula sieboldii*: Synthesis of information toward the prediction of the genetic/demographic fate of a population. *Plant Species Biology* 20, 3–15.

Willmer, P. (2011) Chapter 14: Pollination by butterflies and moths. In Pollination and floral ecology. Princeton University Press, Princeton, pp. 322–336.

Yamane, S. (2008) *Campsomeris prismatica* Smith. In Hirashima, Y. & Morimoto, K. (Eds.) Iconographia Insectorum Japonicorum Colore Naturali Edita, Vol. III. Revised Edition. Hokuryukan, Tokyo, p. 544. (in Japanese)

Yamazaki, T. (1993) Primula. In: Iwatsuki, K., Yamazaki, T., Boufford, D. E., Ohba, H. (eds), Flora of Japan IIIa. Kodansha, Tokyo, pp. 87–94.

Yoshida, Y., Honjo, M., Kitamoto, N., Ohsawa, R. (2008) Genetic variation and differentiation of floral morphology in wild *Primula sieboldii* evaluated by image analysis and SSR markers. *Breeding Science*, 58, 301–307.

Yoshida, Y., Honjo, M., Kitamoto, N., Ohsawa, R. (2009) Reconsideration for conservation units of wild *Primula sieboldii* in Japan based on adaptive diversity and molecular genetic diversity. *Genetics Research*, 91, 225–235.

Yoshida, Y., Kodama, M., Honjo, M., Ohsawa, R. (2012) Maintaining and restoring genetic diversity in a wild *Primula sieboldii* population found in the Arakawa Basin, Saitama. *Japanese Journal of Conservation Ecology*, 17, 211–219. (in Japanese)

Yoshioka, Y., Ohashi, K., Konuma, A., Iwata, H., Ohsawa, R., Ninomiya, S. (2007) Ability of bumblebees to discriminate differences in the shape of artificial flowers of *Primula sieboldii* (Primulaceae). *Annals of Botany*, 99, 1175–1182.

Table 1. Number of visits and insects captured and visitation rates of each category and species in 2015 and 2016

		2015			2016			
	Total observation time	46.5 h			68.0 h			
	Total no. of flowers	5928 flowers			2179 flowers			
Category	species	No. of visits	No. of insects captured	Visitaion rate	No. of visits	No. of insects captured	Visitaion rate	Total visitation rate
Bombyliidae	*Bombylius major*	207	68	0.01644	518	-	0.04534	0.03360
	Bombylius shibakawae		12			-		
Bombus diversus	*Bombus diversus diversus*	11	2	0.00056	2	-	0.00013	0.00030
Bombus ardens	*Bombus ardens ardens*	2	-	0.00013	1	-	0.00006	0.00009
Anthophora plumipes	*Anthophora plumipes*	-	-	-	7	2	0.00080	0.00048
Eucera nipponensis	*Eucera nipponensis*	-	-	-	2	1	0.00019	0.00011
Campsomeris prismatica	*Campsomeris prismatica*	1	1	0.00004	1	-	0.00013	0.00009
Lepidopterans	*Anthocharis scolymus*	1	1	0.00094	9	5	0.00284	0.00207
	Erynnis montanus	-	-		8	2		
	Lycaena phlaeas	-	-		-	-		
	Papilio maackii	1	-		-	-		
	Parnassius citrinarius	-	-		-	-		
	Pieris melete	-	-		6	3		
	Pterodecta felderi	1	1		-	-		
	Unknown	8	-		4	-		
Total		232	85		558	13		

Table 2. Morphological traits and number of pollen grains deposited on the proboscis of all captured insects

Category	Species	No. of insects captured[a]	Proboscis length (mm)		Proboscis width (mm)		Total no. of pollen grains		No. of T pollen grains[b]		No. of P pollen grains[b]	
			Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*
Bombyliidae	*Bombylius major*	68 (0)	8.51	0.78	0.50	0.09	2757.6	2796.8	183.1	218.0	2574.5	2759.4
	Bombylius shibakawae	12 (0)	8.95	0.62	0.51	0.10	1223.5	989.6	36.6	45.3	1186.9	966.2
Bombus diversus	*Bombus diversus diversus*	2 (0)	17.90	0.38	0.64	0.01	19829.5	16916.1	4415.0	2911.9	15414.5	14004.2
Bombus ardens	*Bombus ardens ardens*	1 (1)	9.71	-	0.83	-	1373.0	-	499.0	-	874.0	-
Anthophora plumipes	*Anthophora plumipes*	6 (4)	15.49	2.07	0.95	0.28	3328.5	891.3	1339.5	956.0	1989.0	664.3
Eucera nipponensis	*Eucera nipponensis*	1 (0)	6.04	-	0.91	-	1177.0	-	360.0	-	817.0	-
Campsomeris prismatica	*Campsomeris prismatica*	1 (0)	8.48	-	0.86	-	202.0	-	118.0	-	84.0	-
Lepidopterans	*Anthocharis scolymus*	8 (2)	10.16	2.16	0.26	0.07	276.3	239.7	54.6	59.6	221.6	192.8
	Erynnis montanus	2 (0)	11.68	1.02	0.26	0.04	264.5	2.1	82.5	71.4	182.0	73.5
	Lycaena phlaeas	2 (2)	4.37	1.15	0.22	0.07	13.0	4.2	1.0	1.4	12.0	5.7
	Parnassius citrinarius	3 (0)	10.64	0.21	0.29	0.02	186.0	256.6	21.7	20.1	164.3	237.6
	Papilio maackii	2 (2)	21.01	0.75	0.60	0.02	964.0	1032.4	387.5	507.0	576.5	525.4
	Pieris melete	5 (5)	9.32	0.70	0.43	0.13	140.8	174.2	30.8	65.6	110.0	128.6
	Pterodecta felderi	1 (0)	4.13	-	0.27	-	30.0	-	0.0	-	30.0	-

SD, standard deviation.

[a] The numbers in parentheses indicate the number of insects captured in the cultivated area in 2015.

[b] T, short-styled; P, long-styled morph.

Table 3. Results of the generalized linear mixed model (GLMM) analysis of the effects of proboscis length and width on pollination efficiency in *Primula sieboldii*

Category	Explanatory variable	Total pollen grains				Short-styled (T)				Long-styled (P)			
		Coefficient	*SE*	*Z* value	*p* value	Coefficient	*SE*	*Z* value	*p* value	Coefficient	*SE*	*Z* value	*p* value
All insect	(intercept)	0.257	0.324	0.79	0.427	-0.659	0.330	-2.00	0.0454	1.012	0.337	3.00	0.0027
	Proboscis length	0.129	0.027	4.89	<0.001	0.164	0.025	6.48	<0.001	0.107	0.027	4.01	<0.001
	Proboscis width	1.169	0.356	3.28	0.001	1.313	0.348	3.78	0.0002	1.014	0.372	2.73	0.0064
Bombyliidae	(intercept)	0.452	0.807	0.56	0.575	-0.401	0.662	-0.60	0.5453	1.201	0.674	1.78	0.0750
	Proboscis length	0.233	0.094	2.48	0.013	0.249	0.076	3.27	0.0011	0.207	0.077	2.70	0.0070
	Proboscis width	-0.237	0.747	-0.32	0.751	-0.006	0.609	-0.01	0.9921	-0.229	0.607	-0.38	0.7070
Lepidopterans	(intercept)	0.732	0.363	2.02	0.044	0.163	0.438	0.37	0.7090	1.300	0.462	2.81	0.0049
	Proboscis length	0.118	0.034	3.43	0.001	0.158	0.042	3.81	0.0001	0.078	0.044	1.78	0.0750
	Proboscis width	-1.533	1.099	-1.39	0.163	-2.158	1.326	-1.63	0.1036	-0.908	1.400	-0.65	0.5165

SE, standard error of mean.

Table 4. Potential pollen transport per flower per hour for each insect category

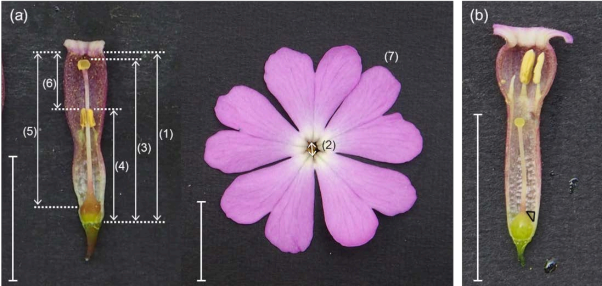
Category	Potential pollen transport (/flower/hour)
Bombyliidae	66.8916
Bombus diversus	5.9805
Bombus ardens	0.1233
Anthophora plumipes	1.5840
Eucera nipponensis	0.1353
Campsomeris prismatica	0.0186
Lepidopterans	0.5547

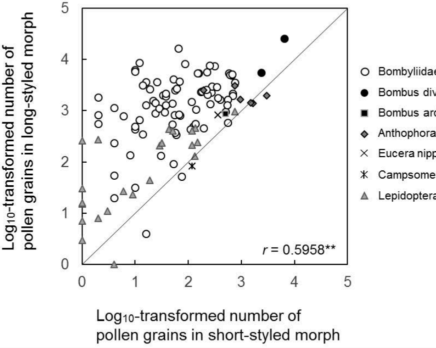
Figure legends

Figure 1. Flower morphology in *Primula sieboldii*. (a) Schematic diagram of the seven floral morphological traits measured in this study: (1) corolla tube length, (2) corolla tube width, (3) stigma height, (4) anther height, (5) nectar distance, (6) pollen distance, and (7) petal area. (b) The position of nectar (enclosed in black triangle) in a *P. sieboldii* flower of the Geihoku population. Each scale-bar indicates 9 mm.

Figure 2. Relationship between the $\log_{10}$-transformed numbers of pollen grains of the short-styled and long-styled morphs of *Primula sieboldii* deposited on captured insects' proboscises.

Figure 3. (a) Comparison between proboscis length of the seven categories of captured pollinators and nectar distance and pollen distance in the short-styled (T) and long-styled (P) flowers of *Primula sieboldii*, and (b) comparison between proboscis width and corolla tube width.


(a)
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(b)



Log₁₀-transformed number of pollen grains in long-styled morph
5
4
3
2
1
0
0
1
2
3
4
5
Log₁₀-transformed number of pollen grains in short-styled morph
r = 0.5958**
Bombyliidae
Bombus diversus
Bombus ardens
Anthophora plumipes
Eucera nipponensis
Campsomeris prismatica
Lepidopterans

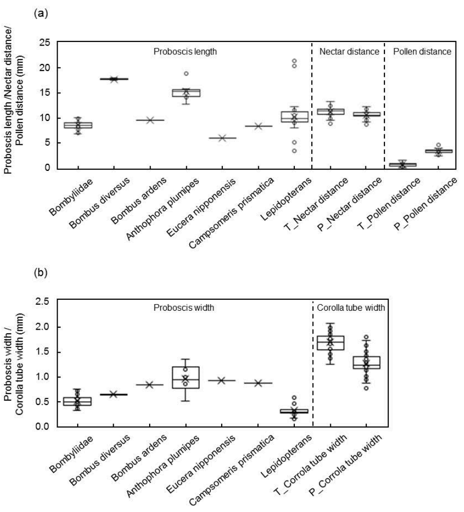

(a)
Proboscis length /Nectar distance/ Pollen distance (mm)
25
20
15
10
5
0
Proboscis length
Nectar distance
Pollen distance
Bombyliidae
Bombus diversus
Bombus ardens
Anthophora plumipes
Eucera nipponensis
Campsomeris prismatica
Lepidopterans
T_Nectar distance
P_Nectar distance
T_Pollen distance
P_Pollen distance
(b)
Proboscis width / Corolla tube width (mm)
2.5
2.0
1.5
1.0
0.5
0.0
Proboscis width
Corolla tube width
Bombyliidae
Bombus diversus
Bombus ardens
Anthophora plumipes
Eucera nipponensis
Campsomeris prismatica
Lepidopterans
T_Corrola tube width
P_Corrola tube width

Table S1. Number of total flowers and of the short-styled (T) and long-styled (P) morphs in four quadrats (A–D) in 2015 and 2016

Year	Date	A			B			C			D		
		Total	T	P	Total	T	P	Total	T	P	Total	T	P
2015	6-May	316	0	316	123	85	38	228	201	27	662	465	197
	7-May	312	0	312	133	85	48	208	171	37	624	466	158
	8-May	306	0	306	138	83	55	236	189	47	629	480	150
	9-May	202	0	202	103	50	53	215	169	46	422	285	137
	10-May	217	0	217	111	54	57	220	175	45	526	388	138
	17-May	10	0	10	21	5	16	32	17	15	88	61	27
	18-May	6	0	6	17	4	13	26	15	11	52	29	23
2016	29-Apr	116	0	116	87	12	75	169	54	115	226	215	11
	30-Apr	152	0	152	93	17	76	228	61	167	246	235	11
	1-May	178	0	178	118	11	107	238	75	163	328	299	29

T, short-styled; P, long-styled morph.

Table S2. Mean values and standard deviations of seven floral morphological traits in the short-styled (T) and long-styled (P) morphs of *Primula sieboldii*

Trait	T (n=42)		P (n=44)		t value
	Mean	*SD*	Mean	*SD*	
(1) Corolla tube length (mm)	12.55	0.15	11.90	0.17	7.8 **
(2) Corolla tube width (mm)	1.68	0.03	1.26	0.03	89.0 **
(3) Stigma height (mm)	7.57	0.12	10.98	0.14	345.7 **
(4) Anther height (mm)	11.62	0.13	8.32	0.15	279.2 **
(5) Nectar distance (mm)	11.44	0.15	10.74	0.16	10.0 **
(6) Pollen distance (mm)	0.79	0.06	3.46	0.07	893.5 **
(7) Petal area (cm^2)	3.81	0.13	4.05	0.16	1.4

SD, standard deviation.

**$p < 0.01$

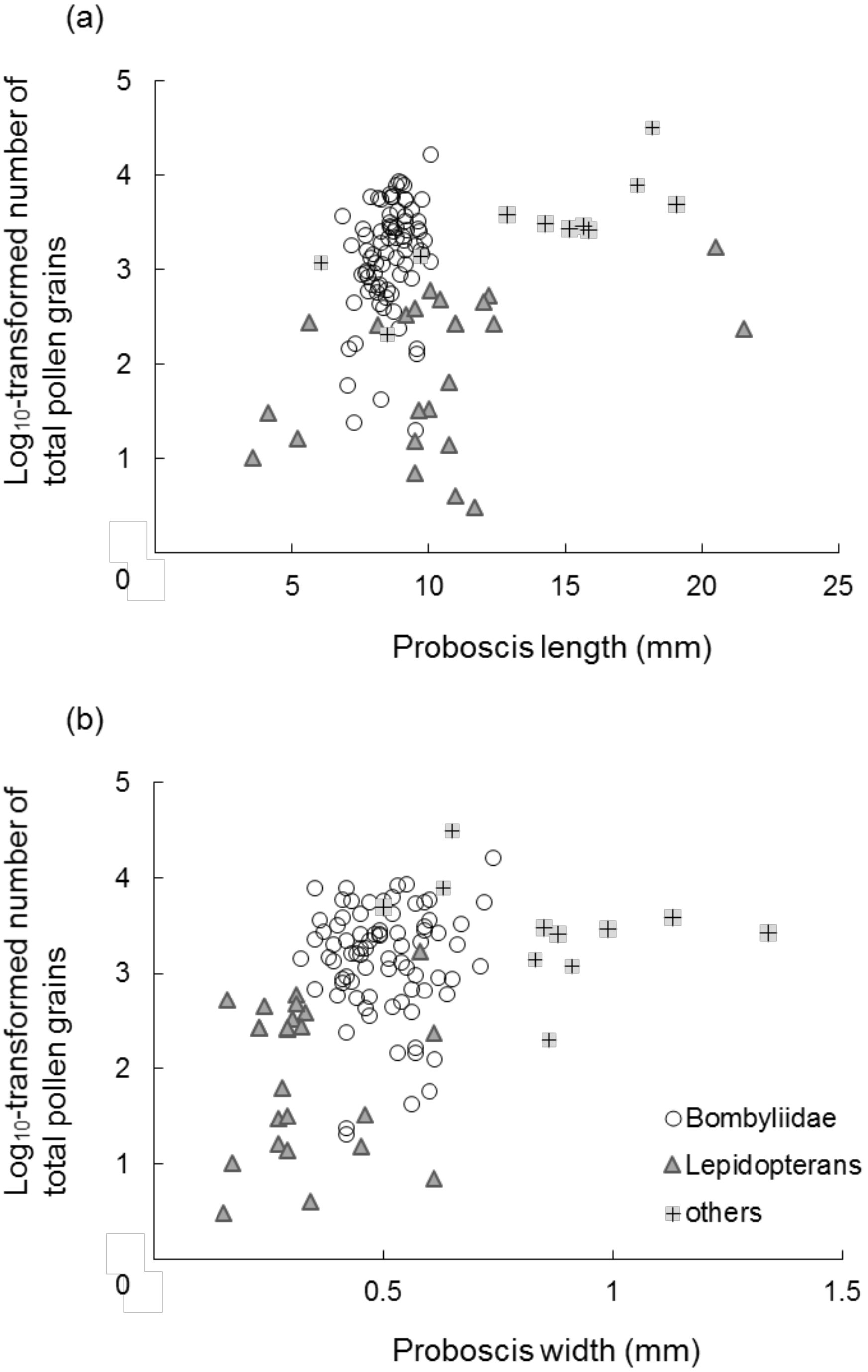


Figure S1. Relationships between the $\log_{10}$-transformed number of total pollen grains deposited on the proboscises of three categories of pollinators of *Primula sieboldii* and proboscis length (a) and width (b).