Altitudinal flower size variation correlates with local pollinator size in a bumblebee-pollinated herb, *Prunella vulgaris* L. (Lamiaceae)

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Abstract

The influence of locally different species interactions on trait evolution is a focus of recent evolutionary studies. However, few studies have demonstrated that geographically different pollinator-mediated selection influences geographic variation in floral traits, especially across a narrow geographic range. Here, we hypothesized that floral size variation in the Japanese herb *Prunella vulgaris* L. (Lamiaceae) is affected by geographically different pollinator sizes reflecting different pollinator assemblages. To evaluate this hypothesis, we posed two questions. (1) Is there a positive correlation between floral length and the proboscis length of pollinators (bumblebees) across altitude in a mountain range? (2) Does the flower–pollinator size match influence female and male plant fitness? We found geographic variation in the assemblage of pollinators of *P. vulgaris* along an altitudinal gradient, and, as a consequence, the mean pollinator proboscis length also changed altitudinally. The floral corolla length of *P. vulgaris* also varied along an altitudinal gradient, and this variation strongly correlated with the local pollinator size but did not correlate with altitude itself. Furthermore, we found that the size match between the floral corolla length and bee proboscis length affected female and male plant fitness and the optimal size match (associated with peak fitness) was similar for the female and male fitness. Collectively, these results suggest that pollinator-mediated selection influences spatial variation in the size of *P. vulgaris* flowers at a fine spatial scale.

Introduction

Trait variations across a narrow geographic range (e.g. along altitude) are often caused by locally different selection regimes (Endler, 1977; Olsson & Agren, 2002; Herrera *et al.*, 2006; Anderson & Johnson, 2008; Toju, 2008). A number of evolutionary studies have recently focused on the influence of locally different species interactions on trait evolution (Anderson & Johnson, 2008; Pauw *et al.*, 2009; Zhang *et al.*, 2013). In angiosperms, interspecific floral trait evolution is often affected by interactions with pollinators (Chittka & Thomson, 2001; Pellmyr, 2002; Gómez *et al.*, 2006; Herrera *et al.*, 2006; Waser & Ollerton, 2006; Whittall & Hodges, 2007). Recently, a number of studies have examined whether geographically different pollinator-mediated selection influences geographic intraspecific variation in floral traits (Gómez *et al.*, 2009a, b; Pauw *et al.*, 2009; Zhang *et al.*, 2013; Boberg *et al.*, 2014; Nagano *et al.*, 2014). To show that floral size, for example, is driven by spatially variable selection by pollinators, we first need to demonstrate a geographic correlation between local floral size and pollinator size, and then we should examine further whether floral size is subject to selection by pollinators that differ locally in size.

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Although many studies have surveyed spatial variation in pollinator assemblages (e.g. Hattori *et al.*, 2014), among those that have examined the correlation of pollinator size with flower size (Galen, 1996; Scobell & Scott, 2002; Cosacov *et al.*, 2014; Newman *et al.*, 2014; Sun *et al.*, 2014), less have investigated the effect of the size match on plant fitness (reviewed by Herrera *et al.*, 2006), especially by assessing both female and male fitness (but see Sahli & Conner, 2011; Kulbaba & Worley, 2012).

In this study, we first examined the pattern of covariance between flower and pollinator sizes in a Japanese herb, *Prunella vulgaris* L. (Lamiaceae), and its bumblebee pollinators (Nelson, 1967; Hayashi, 1989, 2009). Then, we examined whether the match between local flower and pollinator sizes affected female and male plant fitness.

Prunella vulgaris is pollinated by several bumblebee species (Nelson, 1967), and in central Japan, where this study was conducted, the bumblebee assemblage differs at different altitudes (Tomono & Sota, 1997). The existence of variation in the bumblebee assemblage suggests that plant floral traits might also vary in ways that reflect adaptive shifts to different bumblebee assemblages along altitude.

We hypothesized that the observed floral size variation in *P. vulgaris* has resulted from adaptation to local bumblebee pollinator assemblages. To evaluate this hypothesis, we posed two questions: (1) Is there a positive relationship between floral length and the proboscis length of the pollinators (bumblebees) across altitude in a mountain range? (2) Does the flower–pollinator size match influence female and male plant fitness?

Materials and methods

Plant species

Prunella vulgaris L. (Lamiaceae) is a protandrous, perennial herb broadly distributed in north temperate regions (Nelson, 1967; Hayashi, 1989, 2009). It has two-lipped purple flowers that open during June–August and are pollinated primarily by bumblebees. Flowering individuals produce erect stems, each bearing a number of inflorescences. Each inflorescence usually consists of a series of whorls with up to six partially self-compatible flowers per whorl (Fig. 1a) (Winn & Werner, 1987). When a bumblebee enters the corolla of *P. vulgaris* to collect nectar, the dorsal surface of its head just touches the stamens or pistil, provided that there is a good fit between the sizes of the pollinator and flower (Fig. 1b, Laverty, 1994).

Study site

We studied seven populations of *P. vulgaris* (1150–2050 m a.s.l.) on Mt. Norikura with 3026 m a.s.l., central Japan (Table 1, Fig. 2). In 2010, we studied altitudinal changes in floral size and pollinator assemblages in seven of these populations (Table 1, Fig. 2). In 2011, we measured pollinator size and examined the effect of the flower–pollinator size match on plant female and male fitness in one population (Table 1). The surveys were conducted during the peak flowering season of each population. The geographic distance between the populations ranged from 215 m to 8 km (Fig. 2).

Variations in local pollinator assemblages

To examine the assemblages of pollinators of *P. vulgaris* along the altitudinal gradient, we selected the largest plant patch (size range, 20–50 m^2) in each of seven populations and haphazardly established a 1 m × 1 m quadrate within the patch each established for each census day. We then conducted dozens censuses per day, taking 3 min for each census, between 06:00 and 16:00. During each census, we observed the bumblebees flying into the quadrate and counted the number
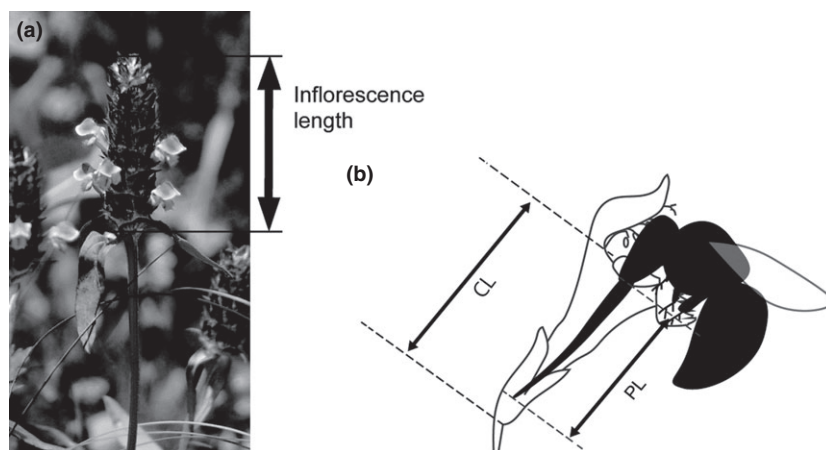

Fig. 1 (a) Inflorescences of *Prunella vulgaris*, each with several open flowers. The double-headed arrow shows the inflorescence length. (b) Illustrate of a *P. vulgaris* flower and a visiting bumblebee. CL and PL are corolla length and proboscis length, respectively.

of flowers each bumblebee individual visited. We conducted 27–148 censuses in each population during the peak flowering season ranging for 2–4 weeks each population in summer 2010. To avoid identifying and quantifying the pollinators associated with a given period of the day with each population, we alternated the censuses between populations along the time when they were carried out. Then we calculated the relative abundance of each bumblebee species that visited each population as the ratio of the number of flowers that a bumblebee species visited to the total number of flowers visited by all of the bumblebee species.

To evaluate the average size of the bumblebee pollinators visiting each population, we calculated the average pollinator proboscis length (PPL):

$$PPL = \sum_{i=1}^{n} Pi(Ni/Nt)$$

where n = the total number of bumblebee species visiting a *P. vulgaris* population, Pi = mean proboscis length (mm) of the ith bumblebee species (see Fig. 1b), Ni = the number of flowers that the ith bumblebee species visited, and Nt = the total number of flowers that all bumblebee species visited (thus, Ni/Nt is the relative abundance of ith bumblebee species visiting the population). We observed six bumblebee species (*Bombus hypocrita, B. beaticola, B. honshuensis, B. ussuriensis, B. diversus, B. consobrinus*) visiting the populations (Fig. 3). As for *B. hypocrite,* we could not collect it in the field (due to its rarity) so that we could not measure its proboscis length. To

Table 1 Site information of the seven studied populations of *Prunella vulgaris*. A + (−) sign means that the indicated survey was conducted (not conducted) in that population.

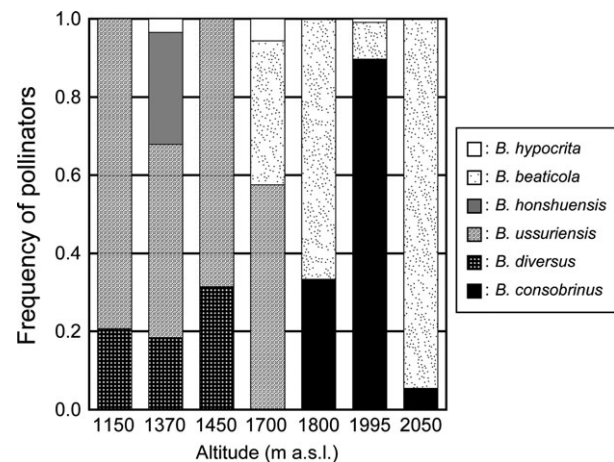| Altitude (m)	Latitude (N)	Longitude (E)	Survey		Plant fitness
			Floral traits	Pollinator assemblages	
1150	36°8′15″	137°40′17″	+ (n = 50)	+ (207 min, 3 days)	−
1370	36°7′9″	137°38′6″	+ (n = 44)	+ (81 min, 2 days)	−
1450	36°7′9″	137°37′46″	+ (n = 49)	+ (198 min, 3 days)	+
1700	36°6′4″	137°36′11″	+ (n = 50)	+ (444 min, 8 days)	−
1800	36°6′47″	137°35′54″	+ (n = 50)	+ (195 min, 5 days)	−
1995	36°7′17″	137°35′17″	+ (n = 50)	+ (297 min, 4 days)	−
2050	36°7′22″	137°35′11″	+ (n = 50)	+ (132 min, 5 days)	−

Fig. 3 Altitudinal differences in assemblages of pollinators of *Prunella vulgaris*. In the key, the bumblebee species are arranged from the species with the shortest proboscis (top) to the one with the largest proboscis (bottom): *Bombus hypocrita* Pérez, *B. beaticola* Takalcu, *B. honshuensis* Takalcu, *B. ussuriensis* Morawitz, *B. diversus* Smith, *B. consobrinus* Dahlbom.

Fig. 2 A relief map (contour interval, 100 m) showing the locations of studied populations in central Japan.
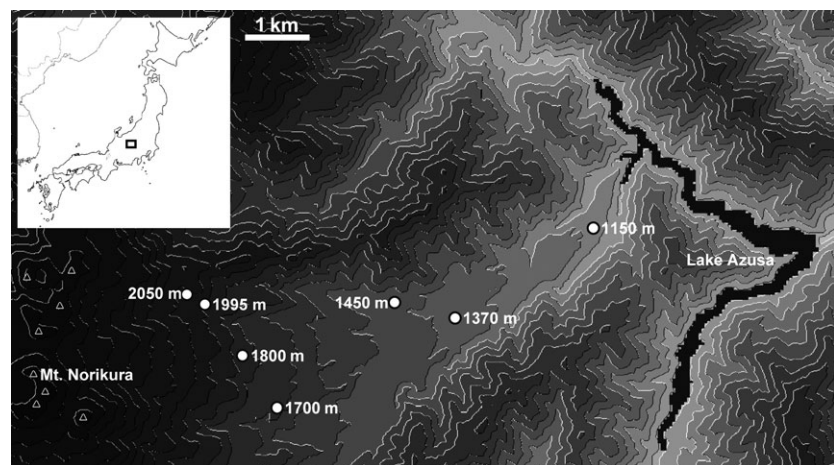
evaluate the mean proboscis length of the *i*th bumblebee species (*Pi*), we haphazardly collected 3–29 workers of each of five bumblebee species and measured the proboscis length with a digital callipers (precision, 0.01 mm) (*B. beaticola*, *n* = 3; *B. honshuensis*, *n* = 7; *B. ussuriensis*, *n* = 5; *B. diversus*, *n* = 7; *B. consobrinus*, *n* = 29). We defined proboscis length as glossa length + prementum length. As the measure for matching between bee and flower size, we used absolute value of (proboscis length – flower corolla length + 2). This is based on our observation that the flower-visiting bumblebees received pollen grains on their heads and the quantity of pollen grains on the bee's head appeared to be maximized when the proboscis length was 1–3 mm shorter than the flower corolla length (see Fig. 1b). We hypothesized that the plant male and female fitness increases as the matching measure approaches to zero.

Floral size variation

From each population, we haphazardly selected 44–50 individual plants, and then haphazardly selected an inflorescence from each plant for morphological measurement. We measured the length of each inflorescence (mm) and collected an open flower from the lowermost part of the inflorescence (Fig. 1a). The collected flowers were preserved in 70% EtOH, and 2 months after collection, the corolla length (mm) was measured with a digital callipers (precision 0.01 mm) (Fig. 1b). We compared corolla length among populations using one-way analysis of variance (ANOVA) and Tukey's HSD tests. For these analyses, we used JMP ver. 9.0 (SAS institute, Tokyo, Japan).

Factors influencing local floral size

To examine factors possibly influencing floral corolla length, we used a generalized linear model (GLM) for the mean corolla length in each population (*n* = 7) with a Gaussian error distribution, in which the pollinator proboscis length, inflorescence length and the altitude of each population were included as predictive variables. We considered the inflorescence length in each population to be a proxy for flower number, which can be considered an index of resource availability, because Winn & Gross (1993) reported that soil nutrient conditions and the illumination levels positively affected the number of flowers of *P. vulgaris*. We used altitude as a proxy for clinal abiotic environmental changes (e.g. meteorological changes). This analysis was performed with JMP ver. 9.0 (SAS Institute).

Effect of the flower–pollinator size match on pollen deposition and pollen removal

We evaluated the effects of the flower–pollinator size match on pollen deposition from a bee onto the stigma (an estimate of female flower fitness) and pollen removal from the anthers onto a bee's body (an estimate of male flower fitness). To evaluate the effects of the size match on female and male fitness, we pooled data from three different bumblebee species (*B. honshuensis, B. diversus* and *B. consobrinus*) because in these species, body shape was relatively constant both within species and among species (Nagano *et al.*, 2014).

Pollen deposition

To measure pollen deposition on the stigma per bee visit, we haphazardly selected 11 flower buds in the population at 1450 m a.s.l. and bagged them until they had opened and entered the female phase. In a preliminary observation, little or no pollens were deposited on stigmas in the male-stage flowers. We then removed the bag from each female-phase flower to allow bumblebees to visit the flower. For each flower, after a single visit, we caught the bumblebee, identified its species and measured its proboscis length. We also measured the flower's corolla length. Then, we collected the stigma, placed it on a slide and sealed it with clear nail polish. We counted the *P. vulgaris* pollen grains on the stigma under a light microscope (×100). We identified the pollen of *P. vulgaris* using explicit criteria based on pollen morph and size (Nakamura, 1980). These 11 flowers were visited by workers of *B. honshuensis* (*n* = 5), *B. diversus* (*n* = 5) and *B. consobrinus* (*n* = 1).

Initial number of pollen grains on the anthers

To calculate the number of pollen grains that were removed per bee visit, we first estimated the initial number of pollen grains (before any pollinator visit) on the anthers. We haphazardly selected 18 flower buds in the 1450 m a.s.l. population and placed a nylon mesh bag around each bud until it opened. As each flower opened and entered the male phase, we measured the floral corolla length and collected a sample of pollen, which we dislodged by sonication of the anthers for 5 min in a solution of 150 μL 95% ethanol. Each sample was vortexed for 10 s, and then the number of pollen grains in three subsamples of 5 μL each was counted under a light microscope (×100). To estimate the total number of grains on the anthers, we summed those counted in the three subsamples and multiplied the total by 10 (c.f. Johnson *et al.*, 1995). We then performed a linear regression of the total number of pollen grains on the flower anthers against corolla length ($F_{1, 16}$ = 4.64, R^2 = 0.23, P = 0.047, n = 18) and used the regression equation [No. pollen grains on the flower anthers = $2.59 \times 10^3 \times$ corolla length – (2.58×10^4)] to estimate the initial number of pollen grains on the anthers of a flower from the corolla length.

Pollen removal

We calculated the number of pollen grains that were removed from the anthers per bee visit as the difference

between the initial pollen number (determined as described above) and the sum of the pollen grains remaining on the anthers after the bee visit. We haphazardly selected 17 flower buds in the 1450 m a.s.l. population and placed a nylon mesh bag around each bud until it opened. As each flower opened, we removed the bag to allow a bumblebee to visit the flower. For each flower, after a single visit, we caught the bumblebee, identified the species and measured its proboscis length. We also measured the floral corolla length so that we can estimate the initial number of pollen grains. Then, we collected the anthers and counted the pollen grains on the anthers as described in the previous subsection. We calculated the number of pollen grains removed from the anthers by the bee visit as follows:

$$\text{No. removed grains} = \text{No. initial grains}$$
$$- \text{No. remaining grains.}$$

The 17 flowers were visited by workers of *B. honshuensis* ($n = 6$), *B. diversus* ($n = 7$) and *B. consobrinus* ($n = 4$).

Effect of the flower–pollinator size match on pollen deposition and pollen removal

To determine the effect of the flower–pollinator size match on pollen deposition and removal, we calculated a measure for matching between bee and flower size: |proboscis length – flower corolla length + 2|. The measure indicates the position of the bee's head in relation to the anthers and stigma of the flower. The best match is supposed to be attained when the proboscis length was 1–3 mm shorter than the flower corolla length (Fig. 1b) so that we hypothesized that the plant male and female fitness increases as the matching value approaches to zero. We collected these data from each visited flower and visiting bumblebee as described in the previous subsections.

We used a GLM with a Poisson error distribution for pollen deposition analysis and a GLM with Gaussian error for pollen removal analysis. In these analyses, the flower–pollinator size match index was used as the predictive variable. These analyses were performed with JMP ver. 9.0 (SAS Institute).

Results

Variations in local pollinator assemblages

The bumblebee pollinator assemblage changed along the altitudinal gradient (Fig. 3, Table 3). Although other insect visitors (butterflies, hoverflies, small bees) were sporadically observed on *P. vulgaris* flowers, it is doubtful that they functioned as pollinators because they were rarely observed, and if they did visit any flowers, their body size was too small to touch the stigma or anthers. Flowers in the low-altitude (1150—1450 m a.s.l.) populations were visited mainly by the medium-sized species *B. ussuriensis*, and the flowers in high-altitude populations (1800–2050 m a.s.l.) were visited mainly by the smallest bumblebee species, *B. beaticola* (excepting *B. hypocrita*, as explained in the Methods) and the largest species, *B. consobrinus*. Visitations by *B. consobrinus* were exceptionally frequent in the 1995 m a.s.l. population, and *B. beaticola* was the most frequent visitor in the 1800 m a.s.l. and 2050 m a.s.l. populations.

The proboscis length of the bumblebees varied among the frequently observed five species (Table 3). Thus, because the pollinator assemblage differed notably among the seven populations, the calculated mean proboscis length varied greatly among the populations (Fig. 4b, Table 3).

Floral size variation

We detected significant variations in the *P. vulgaris* corolla length among populations (Fig. 4a, $F_{6,\ 336} = 18.30$, $P < 0.001$). In the low-altitude populations (1150–1450 m a.s.l.), corolla lengths were relatively long and fairly uniform among populations. Among the high-altitude populations (1800–2050 m a.s.l.), they varied over a wide range of values (e.g. longer in the 1995 m a.s.l. population and shorter in the 1800 m a.s.l. population).

Factors influencing local floral size:

The GLM analysis results showed that, among the predictive variables, pollinator size (i.e. proboscis length) influenced floral corolla length but altitude and inflorescence length had no effect on corolla length (Table 2).

Effect of the flower–pollinator size match on pollen deposition and pollen removal

The GLM analysis results indicated that the flower–pollinator size match |proboscis length – flower corolla length + 2| affected both female plant fitness (pollen deposition on the stigma, Coeff. = −0.26, SE = 0.06, x^2 = 20.57, $P < 0.001$, Fig. 5a) and male plant fitness (pollen removal by bees, Coeff. = −595.18, SE = 228.47, x^2 = 5.71, $P = 0.017$, Fig. 5b). The pollens deposited on stigma included only those of *P. vulgaris*.

Discussion

We found geographic variation in the assemblage of bumblebee pollinators of *P. vulgaris* along an altitudinal gradient (Fig. 3). As a consequence, the mean pollinator size (i.e. proboscis length) changed altitudinally (Fig. 4b). The floral size of *P. vulgaris* also varied along
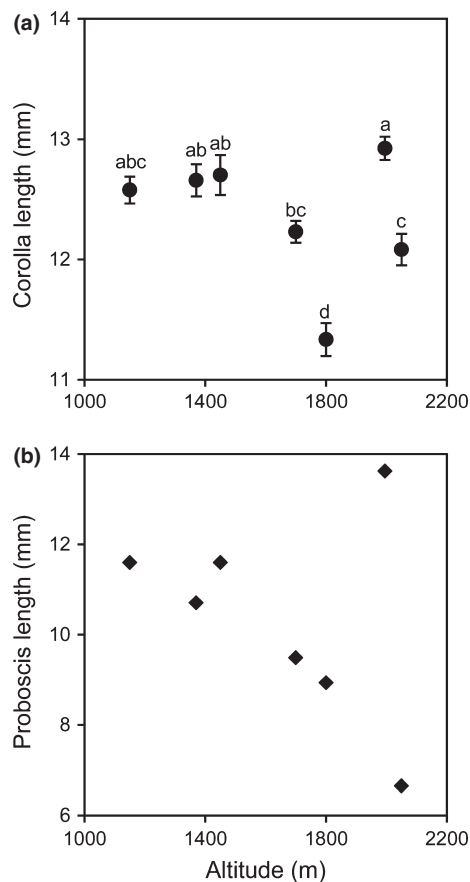
Fig. 4 Altitudinal variation in (a) floral size (corolla length, mean ± SE) of *Prunella vulgaris* and (b) average pollinator proboscis length (PPL) among seven populations. For the calculation of the average PPL, see text. For the flower and pollinator dimensions, see Fig. 1b. Symbols with different letters are significantly different at *P* < 0.05 (Tukey's HSD test).

Table 2 Outcome of the generalized linear model analysis testing the effect of pollinator proboscis length (see Fig. 1), altitude and inflorescence length on the mean floral corolla length of each population.

Factor	Coeff. (SE)	χ^2	*P*-value
Proboscis length	1.72×10^{-1} (6.31×10^{-2})	5.06	0.03
Altitude (AL)	6.01×10^{-5} (4.71×10^{-4})	0.32	0.90
Inflorescence length (IL)	9.84×10^{-3} (1.24×10^{-2})	0.59	0.44

an altitudinal gradient (Fig. 4a). Moreover, this variation was not influenced by altitude itself, but it was strongly influenced by the local pollinator size (Table 2). Furthermore, the size match between the flowers and the bees influenced both female and male plant fitness (Fig. 5). These results suggest that *P. vulgaris* floral size is under a pollinator-mediated selection
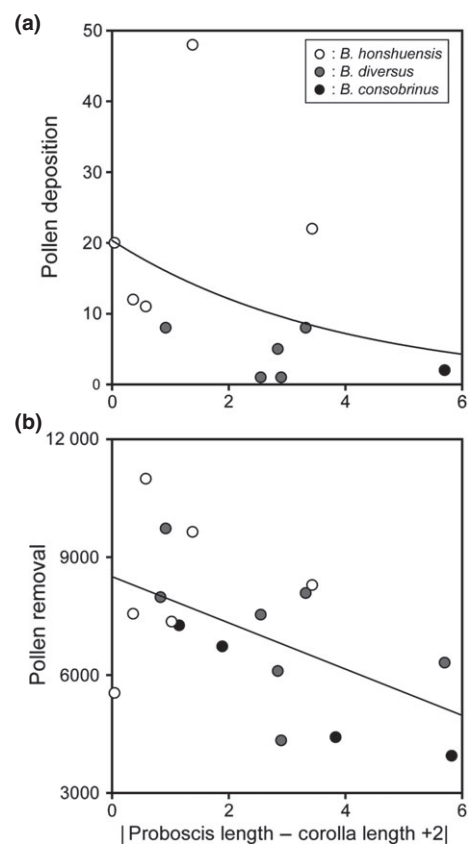

Fig. 5 (a) Female fitness (as indicated by the amount of pollen deposition onto the stigma per pollinator visit) and (b) male fitness (as indicated by the amount of pollen removed from the flower anthers per pollinator visit) as a function of the flower–pollinator size match as indicated by the absolute value of (proboscis length – flower corolla length + 2). Lines represent the predicted values of the GLMs.

regime and that local adaptation of flower size in *P. vulgaris* occurs at a fine spatial scale.

Variations in the local pollinator assemblage

The pollinator assemblage changed along altitude. At higher altitudes, the smallest and largest of five bumblebee species, *B. beaticola* and *B. consobrinus,* respectively, were the main visitors to *P. vulgaris* flowers, whereas at low altitudes, the medium-sized species *B. ussuriensis* was the most frequent visitor (Fig. 3). This bumblebee distribution pattern largely reflects the altitudinal ranges of these bumblebees species in central Japan (Kato, 1993; Tomono & Sota, 1997), although the altitudinal distribution of *B. ussuriensis* is not clear in the literatures. The pollinator assemblages changed drastically beyond 1700 m a.s.l. in this study (Fig. 3). This change may reflect altitudinal zonation of plant vegetation. In Mt. Norikura, the study area, tree

Table 3 Observed frequencies of bumblebee pollinators to *Prunella vulgaris*, mean proboscis length of each bumblebee species and mean proboscis length in each population.

Altitude (m)	B. consobrinus	B. diversus	B. ussuriensis	B. honshuensis	B. beaticola	B. hypocrita	Average pollinator proboscis length (PPL, mm)
1150	0	166	636	0	0	0	11.6
1370	0	16	43	25	0	3	10.7
1450	0	132	288	0	0	0	11.6
1700	0	0	92	0	59	9	9.5
1800	43	0	0	0	86	0	8.9
1995	497	0	0	0	52	5	13.6
2050	5	0	0	0	87	0	6.6
Proboscis length (mean ± SE, mm)	14.4 ± 0.31	11.6 ± 0.62	11.6 ± 0.54	8.6 ± 0.38	6.2 ± 0.23	N/A	

N/A, not applicable.

vegetation zones include montane deciduous broad-leaved forests (800–1600 m a.s.l.) and subalpine coniferous forests (1600–2500 m a.s.l.) (Miyajima *et al.*, 2007). On the other hand, even within the subalpine zone, the relative abundances of the smallest and largest bumblebee species, *B. beaticola* and *B. consobrinus*, were different among the populations: *B. consobrinus* was most abundant at 1995 m a.s.l., whereas *B. beaticola* was observed most often at 1800 and 2050 m a.s.l. (Fig. 3). Although the size measurements of bumblebee species in this study (Table 3) were based on rather small and unbalanced sampling, the proboscis lengths of the bee species shown here are consistent with those reported in the literatures (e.g. Dohzono *et al.*, 2011).

Factors influencing local floral size and the effect of the flower–pollinator size match on plant fitness

We showed that the local floral size of *P. vulgaris* correlated with the average proboscis length of the local pollinators (Table 3). Besides pollinator size, abiotic factors reflecting clinal differences in meteorological factors along altitude can potentially affect local floral size (Galen, 1996; Strauss & Whittall, 2006). However, our GLM analysis results indicated no effect of altitude itself on floral size (Table 2). Therefore, we concluded that changes in clinal abiotic factors along altitude did not strongly affect floral size in *P. vulgaris*.

As for intraspecific floral variation, not a few studies have shown that morphology of pollinator can exert strong selection on floral size (Herrera *et al.*, 2006; Anderson & Johnson, 2008; Pauw *et al.*, 2009; Peter & Johnson, 2014). However, less is known about the selection mosaic across a narrow geographic range (e.g. along altitude, Gómez *et al.*, 2009a, b; Nagano *et al.*, 2014). In this study, our results indicated that fine scale geographic variation of floral size reflects the local pollinator size. Furthermore, pollen deposition onto the flower stigma (female fitness) and pollen removal from the anthers onto bees (male fitness) were both strongly influenced by the size match between corolla length and the pollinator size (Fig. 5b). Considering that the bumblebee pollinators can fly several hundred metres (Goulson, 1999), a comparable distance among the study populations, and may thus accelerate gene flow of *P. vulgaris*, the results suggest that the effect of natural selection exerted by bumblebee pollinators on *P. vulgaris* floral size is strong. Indeed, although gene flow can potentially uniform the trait variations among populations (Kawecki & Ebert, 2004), the variation of floral size between the nearest (215 m) populations (1995 m a.s.l. and 2050 m a.s.l.) was very large (Fig. 4a) probably reflecting the drastic difference of pollinator size (Fig. 4b).

The scatter plots of fitness (i.e. pollen deposition or pollen removal) against the size-match measure show that female and male fitness peaks when the proboscis length was around 2 mm shorter than the flower corolla length (Fig. 5). This result suggests the existence of 'ideal' size match. While a bee is collecting nectar, pollen grains stick to the head. As illustrated in Fig. 1b, the 'best' size match appears to occur when the nectar-sucking bee's head just touches the anther–stigma complex (concealed beneath the top of the upper petal). As a result, when the size difference between the corolla length and the pollinator proboscis length was larger or smaller than the ideal value, pollen deposition and removal by the bumblebees drastically decreased (Fig. 5). In this context, *B. consobrinus* that has by far the longest proboscis is supposed to exert intensive selection pressure on *P. vulgaris* with small flowers.

In conclusion, we demonstrated that the pollinator assemblage and, consequently, the average size of pollinators of *P. vulgaris* both changed altitudinally at fine spatial scale, and we showed that spatial variation of the *P. vulgaris* floral size changed in parallel with the pollinator size, but not with altitude itself. Further, we documented that the size match between flowers and bumblebees affected both female and male plant fitness and that the optimal size match (associated with peak

fitness) was similar in the female and male fitness. Collectively, our results strongly indicate that pollinator-mediated selection influences spatial variation in the size of *P. vulgaris* flowers at fine spatial scale.

Acknowledgments

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