### ORIGINAL ARTICLE



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# Geographic changes in pollinator species composition affect the corolla tube length of self-heal (*Prunella vulgaris* L.): Evidence from three elevational gradients

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### Abstract

Geographic difference in pollinator fauna and variation in average pollinator size may lead to local adaptations of flower size that enhance pollen transfer efficiency. Here, we show that flower size (corolla tube length) of Prunella vulgaris varies both laterally and along elevational gradients, in parallel with local pollinator proboscis length. We measured geographic variation in corolla tube length and leaf length of *P. vulgaris*, and in proboscis length of its bumblebee pollinators, in 12 populations on three different mountains. In estimating pollinator proboscis length, we considered both the average proboscis length of each bumblebee species and the species composition of bumblebees visiting P. vulgaris flowers. We then ascertained whether pollinator proboscis length, elevation itself, or resource availability (indicated by leaf length) correlated with corolla tube length. We found that the local pollinator species composition varied along elevation and among mountain areas, and this variation corresponded to geographic variation in the average pollinator proboscis length. Similarly, corolla tube length of P. vulgaris varied along elevation and among mountain areas. We found that the corolla tube length variation was not associated with elevation itself or local resource availability but was strongly associated with local pollinator average proboscis length. Although corolla tube length was generally shorter at higher elevation, it was relatively longer in some high-elevation populations, where bumblebees having long proboscis length (Bombus consobrinus and B. diversus) visited the flowers. Therefore, parallel changes in P. vulgaris corolla tube length and pollinator proboscis length occur across a wide geographic region in central Japan.

#### K E Y W O R D S

elevational variation, floral morphology, insect-plant interaction, mountain biodiversity, pollination mutualism

# **1** | INTRODUCTION

Animal-pollinated plants display wide variation in flower shape and size, which are regarded as having evolved through plant-pollinator interactions (Darwin, 1862; Grant & Grant, 1965). Selective pressure exerted by pollinators leads to adaptations of flower shape and size to enhance pollen transfer efficiency (Herrera, Castellanos, & Medrano, 2006; Johnson, 2010; Pauw, Stofberg, & Waterman, 2009; Peter & Johnson, 2014); thus, geographic variation in pollinator fauna is associated with local adaptation in flower shape and size WILEY- ECOLOGICAL

(Harder & Johnson, 2009; Nagano et al., 2014; Valente, Manning, Goldblatt, & Vargas, 2012; Van der Niet, Peakall, & Johnson, 2014).

In this study, we show that corolla tube length of Prunella vulgaris L. varies at broad geographic scale, both laterally and along elevational gradients, in parallel with the size of local pollinators. The P. vulgaris system is particularly well suited for studying pollinator-mediated local adaptation of corolla tube length for two reasons. First, P. vulgaris is pollinated by several different-sized bumblebee species, and the bumblebee species composition varies along elevational gradients. Consequently, average local pollinator proboscis length differs at different elevations and, thus, the selective pressure exerted on P. vulgaris corolla tube length also varies (Egawa & Itino, 2020; Kuriya, Hattori, Nagano, & Itino, 2015). Second, Kuriya et al. (2015) have already shown that the size match between P. vulgaris flowers and their pollinator bees affects both the male and female fitness of P. vulgaris.

Kuriya et al. (2015) showed that elevational variation in the P. vulgaris corolla tube length was correlated with local pollinator proboscis length along an elevational gradient on Mount Norikura in central Japan; both bees and flowers were generally smaller at higher elevations, and an appropriate size relationship between bee proboscis and flower corolla tube increased both male and female fitness of the plant. Although Kuriya et al. (2015) conducted their study in only a single geographical area, their results suggest that pollinator-mediated selection influences the elevational variation in P. vulgaris corolla tube length. It is not yet known, however, whether a similar size relationship prevails more broadly, for example, along elevational gradients on different mountains. Moreover, Kuriya et al. (2015)'s conclusion that P. vulgaris corolla tube length correlated not with elevation itself but with local pollinator proboscis length relied mainly on data from just one population (among seven), where exceptionally large pollinator species visited the P. vulgaris flowers.

Egawa, Hattori, and Itino (2015), who studied 23 *P. vulgaris* populations on four different mountains in central Japan, reported that although corolla tube length was generally smaller at higher elevations, corolla width and calyx length, which do not affect pollination efficiency, did not differ along elevational gradients. Their results suggest that elevational variation in *P. vulgaris* corolla tube length may correlate with local pollinator proboscis length at a wide spatial scale, but they did not investigate proboscis length differences in the bumblebee pollinators among the studied populations. Therefore, the specific factor controlling the elevational variation in corolla tube length is unclear; candidate factors include

pollinator proboscis length, elevation-related abiotic environmental factors such as temperature and light intensity, and elevation-unrelated abiotic environmental factors such as resource availability.

In this study, we measured the geographic size variation in P. vulgaris flowers, leaves and bumblebee pollinator mouthparts in 12 populations on three different mountains. Most of these populations were different from those studied by Kuriya et al. (2015) and Egawa et al. (2015). We hypothesized that in these populations, elevational variations in corolla tube length would be correlated with local pollinator proboscis length, but not with elevation itself or with leaf size (used as an index of local resource availability). We also hypothesized that the pattern of elevational variation in bumblebee species composition as well as that in corolla tube length would differ across the three mountains. Our specific objectives were to (a) estimate geographic differences in the average P. vulgaris pollinator proboscis length, considering both the average proboscis length of each bumblebee species and the species composition of bumblebees visiting P. vulgaris flowers; (b) assess elevational changes in the P. vulgaris corolla tube length; and (c) ascertain which of the three factors (pollinator proboscis length, elevation itself, or resource availability) correlated with corolla tube length of P. vulgaris.

### 2 | MATERIALS AND METHODS

### 2.1 | Plant species

*Prunella vulgaris* L. (Lamiaceae) is a protandrous, perennial herb broadly distributed in northern temperate regions (Hayashi, 1989, 2009; Nelson, 1967). Its flowers are purple and have a lipped tubular shape; they bloom from June to August and are mainly pollinated by bumblebees (Hayashi, 1989, 2009; Nelson, 1967). Flowering individuals have straight stems terminating in a spike bearing one or several inflorescences. Each inflorescence consists of several rows of six flowers, which are partially self-compatible (Winn & Werner, 1987). When a bumblebee visits a *P. vulgaris* flower for nectar, the dorsal surface of its head or thorax touches the stamens or pistil of the flower (Figure 1), if the fit between bumblebee and corolla tube lengths is good (Laverty, 1994).

### 2.2 | Study sites

We studied 12 populations of *P. vulgaris* on three mountains in central Japan: Mt. Norikura, the Utsukushi-gahara Highland and Mt. Ontake (Figure 2, Table 1).

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**FIGURE 1** (a) An inflorescence of *Prunella vulgaris* with several open flowers. (b) Diagram of a *P. vulgaris* flower with a visiting bumblebee. CTL, corolla tube length; PL, proboscis length



**FIGURE 2** Map showing the locations of the studied populations in central Japan

During each population's peak flowering season in 2016, we measured corolla tube length and leaf length of *P. vulgaris* and estimated the species composition of bumblebee pollinators visiting the flowers. Although we observed other insects (butterflies, hoverflies, and small solitary bees) intermittently visiting *P. vulgaris* flowers, we inferred that they did not act as pollinators because of their rare visitation frequency and morphological mismatch with *P. vulgaris* flowers (small body size of hoverflies and small solitary bees; long, slender proboscis of butterflies).

## 2.3 | Variations in local pollinator species composition and pollinator proboscis length

To ascertain the local pollinator (bumblebee) species composition of *P. vulgaris* for each of the 12 populations, we first selected the largest *P. vulgaris* patch (size range,  $20-50 \text{ m}^2$ ) in each population and arbitrarily established

a 2 m  $\times$  2 m quadrat within the patch. On a fine day during the peak flowering season of each population, we first counted the number of flowers in the quadrat (Table 1). Then, we conducted three pollinator observation censuses between 09:00 and 14:00 local time, taking 20 min for each census (thus, total observation time for a site was 60 min). During each census, we observed the bumblebees flying into the quadrat and counted the number of flowers visited by each bumblebee individual and recorded the bumblebee species. Then, we calculated the relative abundance of each bumblebees species as the ratio of the number flowers that bumblebees of that species visited to the total number of flowers visited by all bumblebee species.

To evaluate the average proboscis length of the bumblebee pollinators visiting *P. vulgaris* flowers in each population, we calculated the average pollinator proboscis length (PPL):

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

where *n*, total number of bumblebee species visiting a P. vulgaris population, Pi, average proboscis length (mm) of the *i*th bumblebee species (see Figure 1, Table 1), Ni, the number of flowers that the *i*th bumblebee species visited, and Nt, the total number of flowers that all bumblebee species visited (thus, Ni/Nt is the relative abundance of the *i*th bumblebee species visiting the *P. vulgaris* flowers). We observed six bumblebee species visiting P. vulgaris, that were Bombus hypocrita hypocrita, B. beaticola beaticola, B. honshuensis, B. ussurensis, B. diversus diversus and В. consobrinus wittenburgi (according to Kinota, Takamizawa, & Ito, 2013). To evaluate average proboscis length of the *i*th bumblebee species (Pi), we arbitrarily collected 5-57 workers of each of the six bumblebee species (B. hypocrita, n = 10; B. beaticola, n = 57; B. honshuensis, n = 16, B. ussurensis, n = 5, B. diversus, n = 5, B. consobrinus, n = 32) in Kamikochi, Nagano (1,500–2,500 m a.s.l.) in August and September 2011, and measured the proboscis length with digital calipers (precision, 0.01 mm). We defined proboscis length as glossa length + prementum length.

### 2.4 | Corolla tube length variation

From each *P. vulgaris* population, we arbitrarily selected 15–34 individual plants, and then arbitrarily selected one inflorescence from each plant for morphological measurement. From each inflorescence, we collected three open flowers from the lowermost part of the inflorescence and the second leaf below the inflorescence

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**TABLE 1** The 12 surveyed populations of *Prunella vulgaris* (N, Mt. Norikura; U, Utsukushigahara Highland; O, Mt. Ontake; see Figure 2 for the locations of the populations), observed bumblebee visits per hour to flowers of *P. vulgaris* in a quadrat, total number of flowers visited in the quadrat, and average pollinator proboscis length of pollinators of each population (estimated from the average proboscis length of each bumblebee species and the relative frequencies of the visiting bumblebee species)

	Elevation	No. of flower visits per hour by each bumblebee species						Number of opened flowers in the observed quadrat (2 m × 2 m)	Average pollinator proboscis length (mm)	
Population	(m)	B. hy.	<i>B. b.</i>	B. ho.	В. и.	<i>B</i> . <i>d</i> .	В. с.	. ,		
N1	1,252				7	22		79	14.1	
U1	1,446					128	56	273	15.5	
N2	1,501			66		75	66	162	14.2	
U2	1,611			4			422	198	16.8	
U3	1,651			1		824	280	544	15.4	
N3	1,665		3	534		117		540	11.5	
U4	1,793			61		29	7	313	12.4	
N4	1,801		7	111			35	483	12.0	
01	1,934	2		1			1,259	341	16.8	
N5	2,019	3	81	10			393	778	15.3	
O2	2,064		12	6		360	435	288	15.8	
N6	2,112		31	23				670	9.5	

*Note: B. hy., Bombus hypocrita* (average proboscis length  $\pm$  *SD*, 8.4  $\pm$  0.3 mm); *B. b., B. beaticola* (8.6  $\pm$  0.3 mm); *B. ho., B. honshuensis* (10.7  $\pm$  0.3 mm); *B. u., B. ussurensis* (11.6  $\pm$  0.5 mm); *B. d., B. diversus* (14.9  $\pm$  0.5 mm); *B. c., B. consobrinus* (16.8  $\pm$  0.4 mm).

(Figure 1). Since the flowers bloom from the bottom of the inflorescence, the three flowers at the lowermost part would have opened recently and would have bloomed at almost the same time. The second leaf below the inflorescence was chosen because it was well spread and was not yet damaged by insect herbivores. The collected flowers and leaves were pressed between two clear plastic boards (210 mm  $\times$  294 mm  $\times$  0.5 mm thick), and they were scanned immediately after the field sampling (on the same day) with a Canon 8000 scanner. Photo Measure software (Kenis, Japan) was used to measure the corolla tube length (CTL) and leaf length (LL) on the digital images. We defined corolla tube and the tip of the lower petal (Figure 1).

We used analysis of covariance (ANCOVA) to compare CTL among the three mountain areas. The predictive variable was mountain area, and the covariate was elevation. We used Tukey's HSD to compare CTL among the three mountains. For these analyses, we used R version 2.13.0 software (R Development Core Team, 2018).

# 2.5 | Factors influencing local corolla tube length

To examine factors influencing CTL in *P. vulgaris*, we used a general linear model (GLM) with a Gaussian error

distribution. The predictive variables were pollinator proboscis length (PPL), LL and elevation (EL), and the response variable was CTL. We considered LL to be an indicator of resource availability, and we used elevation as a proxy for abiotic clinal environmental changes (e.g., meteorological changes). The analyses were performed with R version 2.13.0 software (R Development Core Team, 2018).

### 3 | RESULTS

# 3.1 | Variations in local pollinator species composition and pollinator proboscis length

The species composition of the bumblebee pollinators changed across populations of *P. vulgaris* (Figure 3, Table 1). Among the bumblebee visitors to *P. vulgaris* flowers, large bumblebee species (*B. consobrinus* and *B. diversus*) accounted for 82.4% of all bumblebee visits in all populations (Figure 3, Table 1). However, the visiting frequencies of smaller species, in particular, the middle-sized bumblebee species *B. honshuensis* and the smallest species, *B. beaticola*, tended to increase at higher elevations (but not at O1, N5 or O2). At N3, U4 and N4 (1,665–1,801 m a.s.l.), *P. vulgaris* flowers were mainly visited by *B. honshuensis* (Figure 3, Table 1), and at N6



**FIGURE 3** Interpopulation variation in the species composition of bumblebees visiting *Prunella vulgaris* flowers. In order from shorter to longer proboscises: *B. hy., Bombus hypocrita; B. b., B. beaticola; B. ho., B. honshuensis; B. u., B. ussurensis; B. d., B. diversus; B. c., B. consobrinus.* See Table 1 for the average proboscis length of each species. Population code shows the 12 surveyed population of *P. vulgaris* (N, Mt. Norikura; U, Utsukushigahara Highland; O, Mt. Ontake; see Figure 2 for the locations of the populations). The elevation of each population is shown in parentheses under the Population code and the unit is meter above sea level (m a. s. l.)



**FIGURE 4** Variation in corolla tube length (CTL, mean ± *SD*) of *Prunella vulgaris* in populations located along elevational gradients in three mountain areas

(2,112 m a.s.l.), *P. vulgaris* flowers were visited by both *B. beaticola* and *B. honshuensis* (Figure 3, Table 1).

Average proboscis length differed among the six bumblebee species, and the calculated average proboscis length of pollinators visiting the flowers differed among the 12 populations because of the variation in the species composition of the visitors (Figure 3, Table 1).

### 3.2 | Corolla tube length variation

CTL was significantly different among the three mountain areas (ANCOVA, df = 2, F = 13.17, p < .001). On



**FIGURE 5** Relationship between *Prunella vulgaris* corolla tube length (mean  $\pm$  *SD*) and pollinator proboscis length (PPL)

Mt. Ontake, CTL was significantly longer than on Mt. Norikura and the Utsukushigahara Highland (Figure 4, Tukey's HSD, p < .001). CTL did not differ significantly between Mt. Norikura and the Utsukushigahara Highland (Tukey's HSD, p = .37).

# 3.3 | Factors influencing local corolla tube length

The GLM analysis showed that PPL influenced flower CTL (Figure 5), whereas EL and LL had no effect on CTL (Table 2).

### 4 | DISCUSSION

# 4.1 | Geographic changes in bumblebee species composition

The local pollinator species composition varied geographically, both along elevation and among mountain areas (Table 1, Figure 3). Consequently, the average pollinator proboscis length (PPL) varied across a wide geographic range, both laterally and along elevational gradients (Table 1). We measured PPL of only those bumblebees visiting *P. vulgaris*, but, ideally, the pollinator landscape should be sampled independent of the focal study plant. Egawa (2018) and Egawa and Itino (2020) investigated the elevational and seasonal species compositions of bumblebees visiting the flowers of herbaceous plants on Mt. Norikura (one of the study areas of the present study). They showed a coarse-grained elevational change in the bumblebee species composition (at 200 or 600 m elevational intervals). Comparison of the species composition of

Factor	Coeff	SE	Z value	р
Pollinator proboscis length (PPL)	$1.33 \times 10^{-1}$	$5.20 \times 10^{-2}$	2.55	.034
Leaf length (LL)	$3.75 \times 10^{-2}$	$2.10\times10^{-2}$	1.78	.113
Elevation (EL)	$-3.65 \times 10^{-4}$	$5.89 \times 10^{-4}$	-0.62	.553

\*p < .05

bumblebees visiting *Prunella* in each elevational range in this study (Figure 3) with that of bumblebees visiting many different herbaceous plant species (Egawa & Itino, 2020) showed that they are roughly similar; that is, in both cases, smaller *B. beaticola* and *B. honshuensis* are abundant at high elevations and larger *B. diversus* and *B. consobrinus* prevail at low elevations. However, they are not in agreement at some "special" high-elevation sites in this study (O1, N5 and O2, Table 1, Figures 3 and 4), where large bumblebees having long proboscis length, *B. consobrinus* and *B. diversus*, visited *P. vulgaris* flowers.

Why were individuals of large bumblebee species abundant at these "special" high-elevation sites? Usually, differentsized bumblebee species prefer and are dependent on different-sized plant flowers (Heinrich, 1976). Thus, the "special" high-elevation sites, where the larger bumblebee species were abundant may have large-sized flowers throughout the year. Indeed, at sites N5, O1 and O2, plants with long flower tubes were observed from spring to autumn (Egawa, personal observation). Egawa (2018) found that around site N5, plants with long flower tubes such as aconites and columbines are seen in all seasons, and the bumblebee B. consobrinus, which has the longest proboscis among the local species, occurs particularly around the site (ca. 2,000-2,100 m a.s.l. on Mt. Norikura). This finding suggests that geographic difference in bumblebee species composition are affected not only by elevational gradients of abiotic environmental factors such as temperature but also by geographic mosaics of biotic environmental factors such as local flora.

# 4.2 | Correlation between corolla tube length and pollinator proboscis length

Although corolla tube length (CTL) of *P. vulgaris* was generally shorter at higher elevations (Figure 4), it was relatively long at the three high-elevation sites (O1, N5 and O2), where large bumblebees having long proboscis length (*B. consobrinus* and *B. diversus*) were predominant (Figures 3 and 4, Table 1). Thus, CTL variation was not influenced by the elevation itself but by the local pollinator average proboscis length (Figure 5, Table 2).

However, it is possible to consider that CTL is actually determined by abiotic factors (e.g., soil nutrient conditions) and that only bumblebees having a proboscis **TABLE 2**Outcome of thegeneralized linear model analysis of theeffect of pollinator proboscis length, leaflength and elevation on *Prunella*vulgaris corolla tube length in eachpopulation

length matching the *Prunella* CTL visit the flowers. Vogler et al. (Vogler, Peretz, & Stephenson, 1999), for example, reported that the number of flowers produced by *Campanula rapunculoides* correlates with soil nutrient conditions. But if CTL correlates with soil nutrient conditions, other parts of the plant should also correlate. Thus, in this study, if floral size at each site is affected by the soil nutrient conditions there, then we would expect CTL to correlate with leaf size at that site. Contrary to this prediction, we found no association of leaf length (LL) with CTL (Table 2). This result suggests that soil nutrients cannot fully explain the floral size variation, and that the selective pressures acting on CTL and LL differ.

Another possibility is that proboscis length of the visitors represents an evolutionary adaptation to the tube length of the local flowers (as opposed to tube length being an adaptation to proboscis length). However, this adaptive response may not occur in the bumblebee–plant system because each bumblebee species ranges widely, visiting a variety of plant species in each season; as a result, specialized adaptation of proboscis length to specific, local conditions is unlikely. Indeed, Inoue and Yokoyama (2006) reported that intraspecific size variation in Japanese bumblebee species among geographic areas is limited.

Last, the major species composition of bumblebees varies from year to year, reflecting the population dynamics of individual species in the study area. For this reason, we examined interannual changes in bumblebee species composition by comparing our data at certain sites with data at nearby sites reported by Kuriya et al. (2015). Kuriva et al. (2015) described the bumblebee species composition at sites near sites N4, N5 and N6 of this study (here, "near" means the elevational difference smaller than 40 m). At sites N4, N5 and N6, bumblebee species compositions censused in 2016 were not very different from those reported by Kuriya et al. (2015). For example, site N5 of this study was near site "1995," censused by Kuriya et al. (2015) in 2013. At both sites, B. consobrinus was particularly abundant, whereas that species was not usual at other high-elevation sites on Mt. Norikura (1,700–2,600 m a.s.l.), where B. beaticola or B. honshuensis were predominant.

Taken together, these results suggest that the geographic variation in *P. vulgaris* corolla tube length is due

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to selective pressures from the average proboscis length of local pollinators not just along the one narrow mountain transect studied by Kuriya et al. (2015) but at wide spatial scale (among three mountains).

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