

Geographic Variation in Flower Size and Flower-Visitor Composition of Two Bumblebee-Pollinated, Spring-Flowering Herbs, *Lamium album* L. var. *barbatum* (Lamiaceae) and *Meehania urticifolia* (Lamiaceae)

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Abstract

Geographic variation of flower size is potentially affected by various biotic and abiotic factors such as pollinator size, abundance of nectar robbers, and temperature. To elucidate the effect of geographic variation of flower-visitor fauna on flower size, we investigated two spring-flowering, bumblebee-pollinated herbs, *Lamium album* L. var. *barbatum* (seven populations) and *Meehania urticifolia* (five populations), in central Japan. Flower-size variations were not correlated with altitude (an index of abiotic environmental change) in either species, but in both species they were related to the local species composition of flower visitors. In *L. album*, flowers were bigger in populations visited by long-tongued *Bombus consobrinus* and *B. diversus* queens, whereas they were smaller in populations visited by short-tongued bees such as *Apis mellifera*. Flower size did not vary much among populations of *M. urticifolia* visited by long-tongued bumblebees, but it was uniquely small in a population whose flowers were predominantly visited by the nectar-robbing bee *Megachile japonica*. In conclusion, both the size and nectar-robbing behavior of bees affect local flower-size adaptation in these two long-tubed, spring-flowering herbs.

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Keywords

Bumblebee, Floral Character, Geographic Trait Variation, Nectar Robbing, Pollination

1. Introduction

Pollination mutualism is a major biotic interaction between plants and animals. As the pollinator animal greatly affects the fitness of flowering plants [1]-[3], various floral traits (e.g., flower color and shape) have evolved to attract suitable pollinators [4] [5] (reviewed by [6]). For example, traits of long-tubed irises (*Lapeirousia anceps*) and extremely long-proboscid flies (*Moegistorhynchus longirostris*) have been reciprocally selected and have coevolved as a result of their interaction [7]. On the other hand, Strauss and Whittall [8] have argued that the impact of non-pollinators on flower shape is frequently overlooked. Actually, many studies have presented evidence that abiotic agents or non-pollinator biotic agents affect flower size and shape [9]-[12]. For example, nectar robbing affects flower traits by directly or indirectly influencing the reproductive fitness of plants (reviewed by [9]). Therefore, flower trait evolution should be examined from the perspectives of both legitimate pollination and nectar robbing.

Bumblebees (*Bombus* spp., Apidae) are among the most important pollinator insects in temperate regions, and they have played an important role in the floral diversification of plants [13]-[15]. Many morphological traits of bumblebees are related to foraging (especially proboscis length), and these traits differ greatly between species and among castes (queens, workers, and males) [16]-[20]. Therefore, we can predict that the selection pressure exerted on floral traits by these traits is species- or caste-specific. Because the species composition of bumblebee populations varies among regions [17]-[19] [21], the floral traits of bumblebee-pollinated plants are expected to vary among regions. In fact, a number of studies have presented evidence from several plant species supporting this expectation [14] [22]-[24]. These studies focus on the interaction between summer-flowering plants and bumblebee workers in summer [25]; the influence of bumblebee queens, which are active in spring, on floral trait variation in spring-flowering plants has less been studied (but see [26]).

Nine bumblebee species are known to occur in central Japan, and they all differ in their altitudinal distributions; as a result, the species composition of bumblebees varies altitudinally [24]. In this study, we focused on two perennial herbs of the Lamiaceae: *Lamium album* L. var. *barbatum* (Siebold et Zucc.) Franch. et Sav. and *Meehania urticifolia* (Miq.) Makino. These species have long-tubed flowers and they are in full bloom in late spring. Moreover, bumblebees have been reported to visit these flowers (*L. album* [17] [27]; *M. urticifolia* [28] [29]). Many studies have shown that in long-tubed flower species, flower dimensions such as corolla length and corolla tube length are correlated with pollinator size [22] [30] [31]. However, nectar robbers sometimes seriously decrease the fitness of long-tubed flower species [32], and adaptive changes in flower traits to prevent nectar robbing have been reported in some plants [9] [11].

In this study, we measured corolla and calyx size in seven populations of *L. album* and five populations of *M. urticifolia*. In this paper, we present evidence that both species are visited by bumblebee queens and other smaller bees, and that the flower sizes of both species vary among populations. On the basis of our findings, we suggest that in these species, flower size variation is influenced by interpopulational variations in the species composition of the flower-visiting bees.

2. Materials and Methods

2.1. Study Sites

We studied altitudinal changes in flower size and visiting bumblebee species in seven populations of *L. album* and five populations of *M. urticifolia* in the mountainous region of central Japan (Table 1, Figure 1). We conducted our study during the peak flowering season in each population during April-July 2013.

2.2. Study Plants

Lamium album L. var. *barbatum* is a perennial herb growing along forest edges. Its white-pink flowers are characterized by a long-tubed labiate corolla and are open from April to July (Figure 2). Reported visitors include

Table 1. The studied populations of *Lamium album* L. var. *barbatum* and *Meehania urticifolia*.

Plant species	Population	Altitude (m)	No. of plants	No. of measured plants (flowers)	Habitat
<i>L. album</i>	Obokedani (OBO)	634	<200	25 (29)	Wet forest floor
	Nozawa (NOZ)	740	<500	17 (28)	Wet forest edge
	Onosawa (ONO) [†]	910	<200	20 (37)	Wet forest floor along stream
	Ohmizusawa (OHM) [†]	993	<1000	22 (35)	Dry forest edge
	Mitsumata (MIT) [§]	1346	<500	24 (49)	Wet forest edge
	Ougisawa (OUG)	1520	<50	24 (46)	Wet forest edge
	Super-Rindou (SUR)	1670	<50	13 (18)	Dry and slope grass
<i>M. urticifolia</i>	Shimashimadani_1 (SH1)	825	<50	15 (19)	Wet forest floor
	Shimashimadani_2 (SH2)	898	<30	12 (16)	Wet forest floor
	Onosawa (ONO) [†]	900	<30	12 (27)	Wet forest edge
	Ohmizusawa (OHM) [†]	993	<200	15 (25)	Dry forest edge
	Mitsumata (MIT) [§]	1361	<100	13 (13)	Wet forest floor

Each of the symbols ^{†§} represents the geographically close sites (within 500 m).

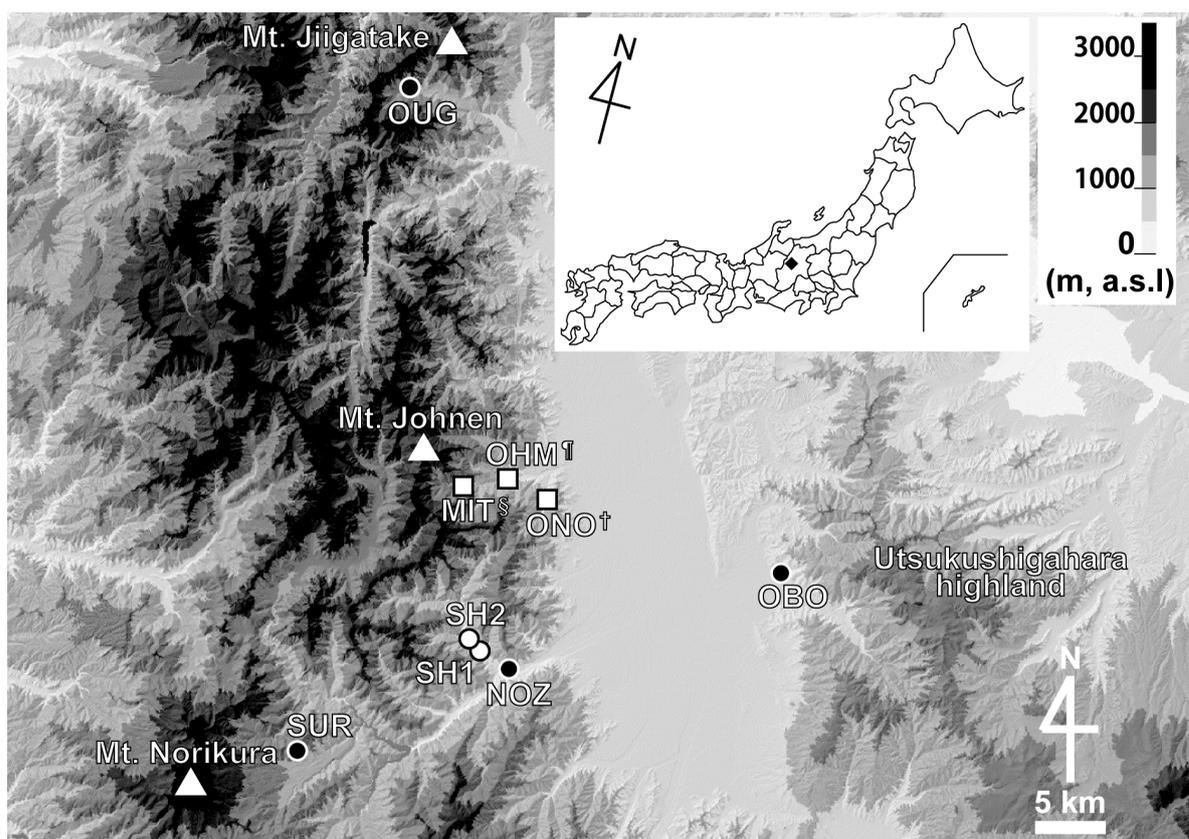


Figure 1. Locations of study sites. Locations of populations of *Lamium album* L. var. *barbatum* (○) and of *Meehania urticifolia* (●), and of populations including both species (□) in the mountainous region of central Japan (◆). See [Table 1](#) for details.

Apoidea bees, especially bumblebees (in central Japan, *Bombus consobrinus*, *B. diversus*, *B. usuriensis*, *B. honshuensis*, and *B. hypocrita* [17]). *Meehania urticifolia* is a perennial herb that grows on the forest floor. Its purple flowers have a long-tubed labiate corolla and are in full bloom from April to June ([Figure 2](#)). They are visited mainly by bumblebees (in central Japan, *B. consobrinus*, *B. diversus* and *B. hypocrita* [16] [29]).

2.3. Bumblebees

Nine bumblebee species are distributed in central Japan. Except in *B. norvegicus*, which is a social parasite of *B.*

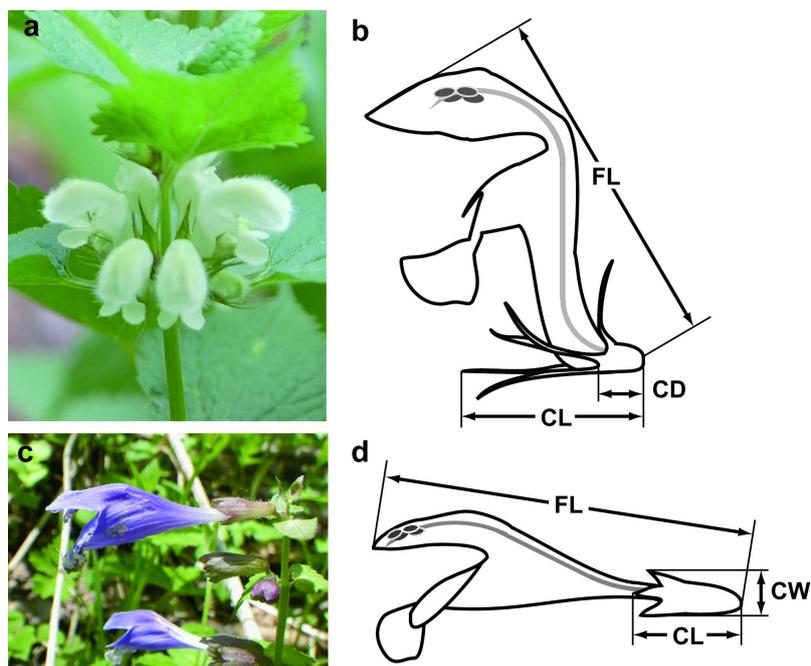


Figure 2. (a) A flowering *Lamium album* L. var. *barbatum* plant; (b) Measured flower traits: flower length (FL), calyx length (CL), and calyx depth (CD); (c) A flowering *Meehaniania urticifolia* plant; (d) Measured flower traits: flower length (FL), calyx length (CL), and calyx width (CW).

beaticola, a queen initiates the bumblebee colony in spring [17]. After awaking from hibernation, the queen finds a suitable place to nest (e.g., an abandoned rodent hole). She builds a little wax cup inside the nest in which she collects pollen and nectar, and then she incubates her eggs. After the young female worker bees emerge, the queen dedicates herself to laying eggs and no longer collects pollen and nectar [25].

2.4. Species Composition of Visiting Bumblebees

We observed the bumblebee species visiting each population (Table 1 and Table 2). We conducted our observations for at least 1 hour between 07:00 Japan Standard Time (JST) and 14:00 JST in the peak flowering season of each population. During each observation period, we counted the number of individuals of each bumblebee or other bee species that visited the population.

2.5. Flower Size Variation

From each population of *L. album* and *M. urticifolia*, we haphazardly selected 12 - 25 individuals, and then haphazardly selected 1 - 3 flowers from each individual plant for morphological measurement (Table 1, Figure 1 and Figure 2). We measured several dimensions of each flower with a digital caliper (precision, 0.01 mm). In *L. album*, we measured flower length (FL), calyx length (CL), and calyx depth (CD); in *M. urticifolia*, we measured flower length (FL), calyx length (CL), and calyx width (CW). When more than one flower from an individual plant was measured, we used the mean values as the data for that plant. We compared the flower dimensions of each species among populations by one-way analysis of variance followed by pairwise comparison with Tukey's HSD post hoc test. Additionally, we looked for damage caused by nectar robbers (bite marks on the calyx or at the base of the corolla tube). Because we frequently observed nectar robbing behavior of *Megachile japonica* in the Ohmizusawa population (OHM) of *Meehaniania urticifolia* flowers, we also examined whether calyx size and the presence of bite marks were correlated in that population by using a generalized linear model with binomial distribution and logistic link in which the calyx dimensions (CL and CW) and their interaction (CL \times CW) were included as a fixed effect. The statistical analyses were performed with JMP ver. 9.0 software (SAS Institute Japan).

Table 2. Pollinator assemblage of *Lamium album* L. var. *barbatum* and *Meehanian urticifolia*.

Plant species	Population	Visitors observed (<i>Bombus</i>) ^a	Visitors observed (other bees) ^a	Nectar robbing [‡]	Date and time of observation
<i>L. album</i>	OBO	Ba (W), 1; Bd (Q), 1	Am (W), 2; Tn, 8; Xa, 11	-/+	May 19, 11:40-12:40
	NOZ	–	Am (W), 2	+/+	Jun 9, 7:20-8:20
	ONO [†]	Bc (Q), 5; Bd (Q), 1; Bh (Q), 1	Xa, 1	-/-	Jun 2, 8:55-9:55
	OHM [¶]	Bc (Q), 1; Bd (Q), 1	Mj, < 10	+++	May 18, 12:30-13:30
	MIT [§]	Bc (Q), 5	–	-/-	Jun 2, 11:51-12:51
	OUG	–	–	-/+	Jul 1, 11:30-12:30
	SUR	Bc (Q), 4	–	+/+	Jun 9, 9:30-10:30
<i>M. urticifolia</i>	SH1	Bd (Q), 1	–	-/-	May 25, 8:45-9:45
	SH2	–	–	-/-	May 25, 10:20-11:20
	ONO [†]	–	–	-/-	Jun 2, 12:10-13:10
	OHM [¶]	Bc (Q), 1; Bd (Q), 1	N.sp., 10<; Mj, 10<	+++	May 13, 8:30-9:30; May 18, 12:30-13:30
	MIT [§]	–	–	-/-	Jun 2, 11:51-12:51

Each of the symbols ^{†§¶} represents the geographically close sites (within 500 m). ^a: species, caste, and number of bees observed in each population during an observation period of at least 1 h. Ba, *Bombus ardens*; Bc, *B. consobrinus*; Bd, *B. diversus*; Bh, *B. honshuensis*; Am, *Apis mellifera*; Mj, *Megachile japonica*; N.sp., *Nomada* sp.; Tn, *Tetralonia nipponensis*; Xa, *Xylocopa appendiculata*; –, not observed. Q, queen; M, male; W, worker. [‡]: frequency of bite marks on the calyx or at the base of the corolla in sampled flowers (– = 0; 0 < + < 0.2; 0.2 < ++ < 0.5; 0.5 < +++)/incidence of nectar robbing (–, not observed; +, observed).

3. Results

3.1. Geographic Variation in Pollinator Assemblage and Flower Size in *L. album*

The flower visitor assemblage of *L. album* differed among populations (Table 2). Although queens of the long-tongued species *B. consobrinus* and *B. diversus* were the main visitors at many populations, short-tongued bee species frequently visited several populations of *L. album* flowers, especially the low-altitude populations (Table 2). The short-tongued bees included Anthophorinae species (*Eucera spurcatipes* or *Tetralonia nipponensis*), *B. ardens*, *Xylocopa appendiculata*, and *Apis mellifera*. *Bombus consobrinus* queens, *B. diversus* queens, and Anthophorinae species were observed to insert their proboscis into the corolla in the proper way. In contrast, *B. ardens*, *Xylocopa appendiculata*, and *Apis mellifera* were observed trying to rob the flower of its nectar by biting a hole at the base of corolla or by sucking nectar from already existing holes, although bite marks were infrequently observed on the calyx or corolla base of the sampled flowers (Table 2) because flowers that had been bitten easily snapped off from the stem.

We found significant variations in the three flower dimensions of *L. album* among populations (FL: $F = 29.09$, $P < 0.001$; CL: $F = 11.59$, $P < 0.001$; CD: $F = 3.19$, $P = 0.006$; Table 3, Figure 3(a)). In OBO, the population at the lowest altitude, FL was the shortest among the populations, and FL tended to be longer in high-altitude populations (Figure 3(a)). Variations in CL and CD were relatively small among populations, although CD was smaller in OBO than in the other populations (Table 3).

3.2. Geographic Variation in Pollinator Assemblage and Flower Sizes in *M. urticifolia*

Although the visitation frequencies of pollinators to *M. urticifolia* were low compared with visits to *L. album*, long-tongued queens of *B. consobrinus* and *B. diversus* also visited *M. urticifolia* flowers (Table 2), and they were observed to insert their proboscis into the corolla in the proper way. However, many *Megachile japonica* individuals also visited the flowers in the OHM population, and they did not insert their proboscis into the corolla but instead tried to rob the nectar by biting through the base of the corolla (Table 2).

There were significant variations in the three flower dimensions (FL: $F = 26.42$, $P < 0.001$; CL: $F = 21.07$, $P < 0.001$; CW: $F = 31.74$, $P < 0.001$; Table 3, Figure 3(b)). In the OHM population, where we observed severe nectar robbing by *M. japonica* (Table 2), FL and CL were smaller than in the other populations (Table 3, Figure 3(b)). In contrast, variations in the three flower dimensions were relatively small among the other four populations of *Meehanian urticifolia* (Table 3, Figure 3(b)). In the OHM population, variations in the calyx dimensions (CW and CL \times CW) significantly correlated with the presence of bite marks (CL: $\chi^2 = 0.28$, $P = 0.60$; CW: $\chi^2 = 5.00$, $P = 0.03$; CL \times CW: $\chi^2 = 5.68$, $P = 0.02$).

Table 3. Geographic variation in calyx length and depth in *Lamium album* L. var. *barbatum* and in calyx length and width in *Meehanian urticifolia*.

Plant species	Population	Calyx length (mean ± SE)*	Calyx depth (mean ± SE)*
<i>L. album</i>	OBO	14.53 ± 0.34 ^b	6.10 ± 0.13 ^c
	NOZ	16.00 ± 0.37 ^{a,b}	7.11 ± 0.14 ^{a,b}
	ONO [†]	15.52 ± 0.34 ^{a,b}	6.95 ± 0.13 ^b
	OHM [†]	14.76 ± 0.33 ^{a,b}	6.83 ± 0.12 ^b
	MIT [‡]	15.50 ± 0.31 ^{a,b}	6.92 ± 0.12 ^b
	OUG	15.88 ± 0.31 ^{a,b}	7.12 ± 0.12 ^{a,b}
	SUR	16.21 ± 0.43 ^a	7.67 ± 0.16 ^a
Plant species	Population	Calyx length (mean ± SE)*	Calyx width (mean ± SE)*
<i>M. urticifolia</i>	SH1	13.22 ± 0.50 ^{a,b}	8.14 ± 0.25 ^a
	SH2	13.55 ± 0.28 ^{a,b}	7.87 ± 0.23 ^a
	ONO [†]	15.19 ± 0.45 ^c	9.64 ± 0.37 ^b
	OHM [†]	12.10 ± 0.25 ^b	6.81 ± 0.17 ^c
	MIT [‡]	12.87 ± 0.36 ^a	8.86 ± 0.21 ^{a,b}

*: Different lowercase letters indicate significant differences ($P < 0.05$; Tukey's HSD post hoc test); †‡: Each symbol shows the geographically close sites (within 500 m).

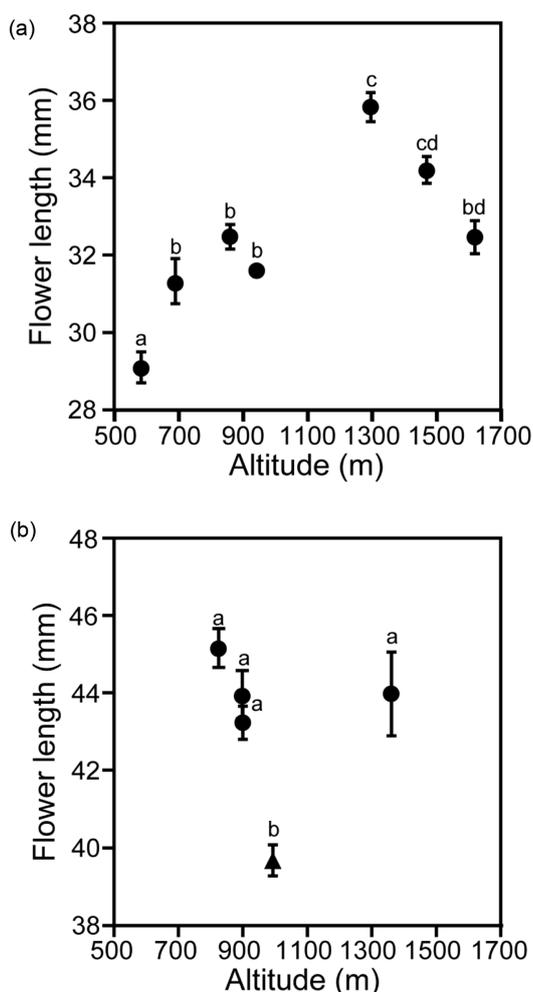


Figure 3. Variation of flower length among populations of (a) *Lamium album* L. var. *barbatum* and (b) *Meehanian urticifolia*. The average values (black dots) and standard errors (error bars) in each population are plotted (a black triangle symbol shows OHM population where flower size was smallest in *M. urticifolia*). Different lowercase letters indicate significant differences ($P < 0.05$; Tukey's HSD post hoc test).

4. Discussion

In both *L. album* and *M. urticifolia*, flower dimensions differed among populations (Table 3, Figure 3). However, the geographic pattern of the variation was different between these two species. Although FL in *L. album* varied greatly among populations, FL variation among *M. urticifolia* populations was relatively small, except in population OHM (Table 3, Figure 3).

In *L. album*, trait size variations did not correlate with the altitudinal gradient, which is an index of environmental change (Table 3, Figure 3). Therefore, these variations most likely resulted from biological interactions such as geographic variation in the pollinator species composition. The low-altitude population (OBO) of *L. album* was visited by short-tongued insects (Anthophorinae species, *Apis mellifera*, *B. ardens*, *Xylocopa appendiculata*), but the other populations of *L. album* were visited mainly by long-tongued visitors (*B. consobrinus* and *B. diversus* queens, Table 2). Moreover, the longest-tongued *B. consobrinus* queens frequently visited the *L. album* population with the greatest FL (MIT). However, not only *B. consobrinus* queens but also other bumblebee queens (*B. diversus* and *B. honshuensis*) visited the other populations of *L. album*. These results suggest that the FL variation reflects a local adaptation to the geographic variation in flower visitor size. Furthermore, some flower visitors wounded the base of the corolla where it was enclosed by the calyx when they tried to rob the flower of its nectar. This observation may indicate that the calyx can protect the flower against nectar robbing. However, the frequency of nectar robbing was low in all populations, and CL and CD did not show large variation (Table 2 and Table 3). Therefore, nectar robbing may not influence calyx dimensions in *L. album*.

In *M. urticifolia*, flower dimensions differed only between population OHM and the other four populations (Table 3, Figure 3(b)), perhaps because population OHM suffered from severe nectar robbing (Table 2).

Because nectar robbing often reduces plant fitness [33] [34], plants protect themselves against nectar robbing in many ways, including physical protection of the corolla tube by the calyx and chemical protection by producing nectar with a high pH (reviewed by [9]). Nectar-robbed *M. urticifolia* flowers were broken at the base of corolla and appeared not to have received legitimate pollination (Nagano personal observation). Therefore, nectar robbing may have a strong negative effect on this species. In *Pavonia dasypetala*, the physical structure of the calyx is reported to prevent nectar robbing [35]. In *M. urticifolia*, also, the calyx structure may help prevent nectar robbing. Typically, the nectar robber (*Megachile japonica*) enters the gap between the calyx and corolla and bites the corolla tube; thus, it is advantageous for plants to have a tight calyx-corolla gap so that nectar robbers cannot enter (Nagano personal observation). In fact, in the population that suffered from nectar robbing (OHM), calyxes were shorter and narrower than in other populations (Table 3). The smaller calyx may limit the size of the bud size and, consequently, flower size (Figure 3).

5. Conclusion

In summary, correlations of geographic variations in flower dimensions with flower visitor composition in two spring-flowering herb species suggest that flower-visiting bumblebee queens and nectar robbing bees lead to local adaptation of flower traits. Furthermore, our results strongly suggest that to understand the evolution of plant traits, it is important to adopt a community ecology perspective. This study showed that trait evolution, even in plants mainly pollinated by bumblebees, can be significantly affected by other pollinators, such as small bees, and nectar robbers, although further study is needed to reveal the impact of nectar robbers on flower trait evolution. Thus, by focusing on pollination mutualism among various community members (*i.e.*, including both legitimate pollinators and nectar robbers), we can obtain new insights into plant diversification.

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Abbreviations

OBO, Obokedani; NOZ, Nozawa; ONO, Onosawa; OHM, Ohmizusawa; MIT, Mitsumata; OUG, Ougisawa, SUR, Super-Rindou; SH1, Shimashimadani_1; SH2, Shimashimadani_2; CL, Corolla Length; CL, Calyx Length; CD, Calyx Depth; CW, Calyx Width; *L. lalbum*, *Lamium album* L. var. *barbatum*; *M. urticifolia*, *Meehanian urticifolia*; Ba, *Bombus ardens*; Bc, *Bombus consoburinus*; Bd, *Bombus diversus*; Bh, *Bombus honshuensis*; Am, *Apis mellifera*; Mj, *Megachile japonica*; N. sp., *Nomada* sp.; Tn, *Tetralonia nipponensis*; Xa, *Xylocopa appendiculate*; Q, Queen; M, Male; W, Worker.