Doctoral Dissertation (Shinshu University)

The effects of flower visitors on intraspecific trait variation in some entomophilous plants

虫媒植物の種内形質変異に対して訪花者がもたらす影響

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Summary

One of the largest goals in evolutionary biology is to elucidate mechanisms of diversification of various traits. Focusing on intraspecific variation, where speciation is still incomplete, can contribute to elucidation of mechanisms of trait diversification and early stages of speciation. It has been pointed out that occurrence of intraspecific trait differences can lead to reproductive isolation and speciation. This is because when trait differences occur between different populations within the same species, even in the case of secondary contact, both of which have undergone local adaptation, reproductive isolation may be established due to reduced fitness of hybrids and/or morphological mismatch.

Pollination is one of the major biotic interactions between insects and plants. It has contributed greatly to the diversification of plant traits. For example, flower-visitor trait-matching has been well studied, as the classic example of the relationship between orchid and hawkmoth by Darwin. Since 25% of the diversification events in angiosperms has been caused by flower visitor shifts, focusing on plant traits related to pollination will be useful for understanding the mechanisms of trait diversification and speciation. Therefore, I focused on morphological differences at the level of intraspecific variation and between ecotypes and examined how pollination faunas relate to and influence these differences, using some plant species, as described below.

In Chapter 1, I used *Cimicifuga simplex* (Ranunculaceae) as a material to detect the differentiation of reproductive systems among the three pollination morphs within the species. Morph I was distributed at high altitude (1350–2370 m) and was pollinated mainly by bumblebees, and morph II was found at middle altitudes (920–1500 m) and was pollinated mainly by butterflies. Morph III occurred at low altitude (650–1350 m)

and was pollinated mainly by dipteran insects although the visitation rates were low. In addition, each morph had a different reproductive system. Morph I, which had a high outcrossing rate, produced mainly gynodioecious ramets (i.e., they produced hermaphroditic and unisexual female ramets), along with a few andromonoecious ramets (i.e., ramets with a hermaphroditic primary raceme and lateral racemes with unisexual male flowers). Morph II, which had a high outcrossing rate, produced hermaphroditic and andromonoecious ramets. Morph III, which had a low outcrossing rate, produced mainly hermaphroditic ramets, along with a few andromonoecious ramets.

Based on these results, in Chapter 2, I clarified the seasonal changes in the quantity and quality of the flower visitors for three morphs. There were marked differences in the flower visitor environment among the three morphs, and these differences are related to the reproductive systems of each morph. As few examples of different reproductive systems at the ecotype level within a species have been reported thus far, *C. simplex* is shown to be a good material for examining the relationship between the flower visitor faunas and reproductive systems.

In Chapters 3 and 4, I investigated the flower-visitors trait-matching across multiple mountain regions using *Lamium album* var. *barbatum* and *Aquilegia buergeriana* var. *buergeriana* as materials. I conducted population genetic analysis to clarify the evolutionary history of the geographic variation in floral size. First, for both species, geographic variations of floral size and flower visitor size consistently matched. In other words, in all mountain regions, plant populations visited by large visitors had larger floral sizes, while those visited by small visitors had smaller floral sizes. Second, there was no relationship between the similarity of floral size between populations and their genetic similarity. Thus, populations within the same mountain region were genetically close to

each other, but populations in different mountain regions were genetically differentiated. These results suggest that the floral size evolved independently among different mountain regions adapting to the flower visitor size of each plant population.

In Chapter 5, I investigated the floral size bimodality in a population of *L. album* var. *barbatum*. In this population, the flower visitors (small and large bees) tended to visit and pollinate flowers of similar size. As a result of this flower visitor preference, the fitness of ramets with floral size of intermediate length was lower than that of ramets with long or short floral size. Microsatellite DNA analysis revealed a slight genetic differentiation between ramets with long or short floral size. Additional genetic analysis showed no evidence of secondary contact with allopatric populations with long or short floral size. These results strongly suggest that, in the population, the bimodal distribution of floral size has sympatric origin and is maintained by disruptive selection resulting from the flower visitor preferences to floral size.

These results elucidate some aspects of plant trait evolution in response to the local flower visitor fauna. The results of Chapters 1 and 2 suggest that even among closely related plant ecotypes, the flower visitor faunas can differ greatly, and that the reproductive system of each ecotype evolved to its own flower visitor fauna through adaptation. In Chapters 3 and 4, I found that the evolution of floral size among populations occurs independently among mountain regions. This indicates that floral size can evolve rapidly in response to differences in visitor size among local populations, and that such evolution has repeatedly occurred in different mountain regions. The results in Chapter 5 suggest that intraspecific floral size bimodality within a population may be maintained by differences in the behavior of the two types of flower visitors. This series of studies has provided new insights into the effects of flower visitors on intraspecific trait variation

in entomophilous plants from three perspectives that have not been previously focused on: differences in reproductive systems between ecotypes, the relationship between local adaptation of floral traits and population genetic structure, and the maintenance mechanism of trait bimodality within a single population.

Summary in Japanese

進化生物学における最大の課題の一つは様々な形質の多様化機構を明らかにすることである。特にまだ種としての分化が不完全な、種内変異のレベルに着目することで形質多様化ひいては種分化の初期段階のメカニズムの解明に貢献できる。同種内に形質の違いが生じると、これをもとに生殖的隔離が成立し、種分化につながる場合が指摘されている。なぜなら、同種内の異なる集団間に形質の違いが生じた場合、二次的に接触した場合でも局所適応を遂げた両者の間では雑種の適応度の低下や形態的不一致による生殖隔離が成立するためである。

特に昆虫と植物の主な生物間相互作用の一つである送粉は、植物の形質の多様化に大きく貢献している。例えば、ダーウィンによるランとスズメガの古典的な例に代表されるように、花-訪花者の形態的な対応関係はよく研究されてきた。被子植物の多様化イベントの 25%が訪花者シフトによって引き起こされたと報告されていることからも、送粉と関係する植物形質に着目することは形質多様化や種分化の機構を解明するのに役立つはずである。以上のことから、本研究では数種の虫媒植物の種内変異レベルあるいはエコタイプ間レベルの形質の違いに着目し、これに訪花者種構成の違いがどのように関係しているかを調べた。

第一章ではサラシナショウマを材料として、エコタイプ間での繁殖様式の分化を調査した。サラシナショウマには、異なる生態的特徴を持ち、遺伝的にも分

化した 3 つの送粉型（タイプI・II・III ）が存在する。高標高に生育するタイプIの訪花者は比較的マルハナバチ類が多く、中標高に生育するタイプIIにはチョウ類が主に訪れ、低標高に生育するタイプIIIでは訪花頻度が低く、アブ・ハエ類が主に訪れる。さらにサラシナショウマではそれぞれのタイプごとに繁殖様式が異なっていた。タイプIは他殖率が高く、花の性表現は両性株の他に雌性株が高い頻度で見られる雌性両全性異株であった。また、少数の両性＋雄性個体（雄性両全性同株）も存在した。タイプIIは他殖率が高く、花の性表現は両性株と雄性両全性同株であった。タイプIIIは自殖率が高く、ほとんどが両性株であり、少数の雄性両全性同株が見られた。

第二章ではこの結果を踏まえ、3 タイプそれぞれについて訪花者群集の量と質の季節的な変化を明らかにした。その結果、3 タイプ間で訪花者相には顕著な違いがあり、それが各タイプの繁殖様式と関連していることが示された。このように、種内のエコタイプレベルで繁殖様式が異なっている例はこれまでほとんど知られておらず、訪花者相と繁殖様式の関連を考察する上でサラシナショウマが有効な材料であることが明らかになった。

第三章と第四章ではオドリコソウとキバナノヤマオダマキを材料に日本の中央アルプスの複数の山域にわたって花-訪花者の形態的な対応関係を調査した。さらに、集団遺伝学的解析を併せて行うことで、どのような進化的な歴史を経て

花サイズの地理的変異が生じたのかを検討した。まず、どちらの植物種においても花サイズと訪花者サイズの地理的な対応関係が見られた。すなわち、どの山域でも大型の訪花者が訪れる植物集団は花サイズが大型化しており、小型の訪花者が訪れる植物集団は花サイズが小型化していた。次に、集団間の花サイズの類似度と遺伝的な類似度には関連性がなかった。すなわち、同じ山域内の集団同士は遺伝的に近かったが、異なる山域の集団同士は遺伝的により離れていた。これらの結果から、花サイズは、地域ごとの平均的な訪花者サイズに適応して山域間で独立して進化してきたことが示唆された。

第五章ではオドリコソウの一集団において花サイズの二峰性を調査した。まず、この集団では、訪花者である小型および大型のハチ類が、自らのサイズに合ったサイズの花に対してのみ有効な送粉行動をとっていることを明らかにした。この結果、花サイズが中間的な植物個体の適応度は、花サイズが小型および大型の個体よりも低くなっていた。次に、遺伝解析の結果、花サイズが大きい個体と小さい個体の間でわずかな遺伝的分化が見られた。さらに花サイズの二峰性が見られる集団と同じ山域に属する他の集団を含めて遺伝解析を行ったところ、この集団の大型花をもつ個体が周辺の大型花集団の個体と遺伝的に近縁である証拠は検出されなかった。これらの結果から、この集団の花サイズの二峰性は同所的に生じたものであり、花サイズに対する訪花者の選好性に起因する分断化

淘汰によって二峰性が維持されていることが示唆された。

以上の結果から、訪花者相に応じた植物の形質進化の一端を解明することができた。第一章、第二章の結果から、種内のエコタイプ間であっても、訪花者相が大きく異なることがあり、各エコタイプの繁殖様式はそれぞれの訪花者相に適応していることが示唆された。第三章、第四章では、花サイズの集団間での適応進化が山域間で独立して生じていることを明らかにした。このことは、花サイズが局所集団ごとの訪花者サイズの違いに応じて急速に進化しうること、またこのような進化が繰り返し山域毎に生じてきたことを示している。第五章の結果は、植物の一集団内で花サイズの二型が 2 タイプの訪花者の行動の違いによって維持されている可能性を示した。以上の一連の研究により、虫媒植物の種内形質変異に対して訪花者がもたらす影響について、これまで着目されてこなかったエコタイプ間の繁殖様式の違い、花形質の局所適応と集団遺伝構造との関係、一集団内における二型の維持機構の 3 つの観点から新たな知見を得ることができた。

Contents

General introduction

One of the biggest challenges in evolutionary biology is to elucidate the mechanisms of diversification of various traits (Naghiloo et al., 2021). Focusing on the level of intraspecific variation, where genetic differentiation is still incomplete, can contribute to the elucidation of the mechanisms of trait diversification and early stages of speciation (Good et al., 2008). It has been pointed out that reproductive isolation can be established when trait differences within the same species occur, and it leads to speciation (Grant-Stebbins model; Grant and Grant, 1965; Stebbins, 1970; Johnson and Anderson, 2010; Anderson et al., 2014). This is because trait differences between different populations within the same species can lead to reduced adaptation of hybrids (Dobzhansky, 1937; Servedio and Noor, 2003) and/or reproductive isolation due to morphological mismatch (Coyne and Orr, 2004) in the case of secondary contact.

Many traits of angiosperms diversify very much, including flower color (Campbell et al., 1997; Newman et al., 2012), floral shape (Hodges, 1997; Fenster et al., 2004; Gómez et al., 2006; Nagano et al., 2014), floral scent (Pellmyr, 1986; Majetic et al., 2009), leaf shape (Usukura et al., 1994; Setoguchi and Kajimura 2004), selfing or outcrossing (Pettengill et al., 2016), and sexual expression of flowers (Torices et al., 2011). Many of these evolutionarily diversified due to adaptations to specific environments. Pollination is one of the major biological interactions between insects and plants. It has contributed significantly to the diversification of plant traits. Since 25% of the diversification events in angiosperms has been caused by pollinator shifts (Van der Niet and Johnson, 2012), focusing on plant traits related to pollination will be useful for understanding the mechanisms of trait diversification and speciation. Therefore, I focused on morphological differences at the level of intraspecific variation and between ecotypes, and examined

how pollination environments relate to and influence these differences, using a variety of materials, as described below.

In Chapter 1, I used *Cimicifuga simplex* (Ranunculaceae) as a material to detect the differentiation of reproductive systems among the three pollination morphs within the species. In Chapter 2, based on these results, I compared the quantity and quality of flower visitors among the three morphs. I found marked differences in the floral visitor environments surrounding the three morphs and discussed their relationship to the reproductive systems of the three morphs. Such differences in reproductive systems at the ecotype level within a species have been rarely studied (but see Pettengill et al., 2012), and *C. simplex* is a very useful material for examining it.

In Chapters 3 and 4, I found the floral size of *Lamium album* var. *barbatum* and *Aquilegia buergeriana* var. *buergeriana* evolved independently in several mountain regions in accordance with local flower visitor size. Although there are a large number of studies showing flower-visitor size matching (Alexandersson and Johnson, 2002; Herrera et al., 2006; Anderson and Johnson, 2008; Johnson and Anderson, 2010; Boberg et al, 2014; Nagano et al., 2014; Kuriya et al., 2015), few studies have related it to population genetic structure (but see Anderson et al., 2014). In these two chapters, I focused on this point and considered genetic relationships among populations in different mountain regions in addition to the classical flower-visitor size matching.

In Chapter 5, I detected trait bimodality in floral size in a population of *L. album* var. *barbatum*. In this chapter, I found the possibility that trait bimodality is maintained by the flower visitor's behavior with very different sizes, and weak genetic differentiation between plants with small and large flowers. Studies on the sympatric trait dimorphism are very limited in both plants and animals. Although there have been several reports of

floral trait bimodality being maintained sympatrically after secondary contact (Campbell et al., 1997; Rymer et al., 2010), this is the first study to suggest the occurrence of sympatric trait bimodality without the trace of secondary contact. In addition to this, the present study is the first to report that sympatric large and small flower visitors vary their flower visiting behavior according to flower size.

Throughout the five chapters, the intraspecific traits differences among plant ecotypes and/or populations were confirmed by intensive field surveys. In addition, I used genetic markers to determine the selfing rates of the plants and the genetic structure of plant populations. By combining these methods, I have tried to answer some of the important questions that have been raised in the previous studies of plant-pollinator interaction.

Chapter 1

Differences in sex expression and mating systems in three pollination morphs of *Cimicifuga simplex*

1-1 Abstract

Cimicifuga simplex (Ranunculaceae) has three genetically distinct pollination morphs. Here, I report that each of the three pollination morphs of *C. simplex* differs from the others with regard to sex expression and mating system: morph I consists mostly of ramets with hermaphroditic flowers and ramets with only female flowers, morph II consists of ramets with hermaphroditic flowers and ramets with hermaphroditic and male flowers, and morph III consists mostly of ramets with hermaphroditic flowers. Microsatellite analysis of seed DNA showed that morph III has a high self-fertilization rate. Flowering season and flower visitor assemblages, which also differ among the three morphs, may influence the evolution and maintenance of the differences in sex expression and mating systems in the morphs.

1-2 Introduction

Angiosperms have various sexual systems from hermaphroditism to dioecy, and mating systems from outcrossing to predominant self-fertilization (Culley and Klooster, 2007; Wright et al., 2013; Renner, 2014). An important question in evolutionary biology is, "Why have angiosperm reproductive systems become so diversified?" (Barrett, 2002). In gynodioecy, female individuals have only pistils and can automatically outcross. It is suggested that dioecy evolves when hermaphroditic individuals have high selfing rates and suffer inbreeding depression (Lloyd 1975; Charlesworth and Charlesworth, 1978)

although there is still much debate about the evolutionary background of plant reproductive systems (Willson, 1983).

Phylogenetic studies have explored the evolutionary pathways of plant reproductive systems. For example, in *Silene* dioecy originated multiple times via gynodioecy and gynodioecy–gynomonoecy (Desfeux et al., 1996; Casimiro-Soriguer et al., 2015). In the genera *Collinsia* and *Arabidopsis*, directional evolution of the reproductive system (such as outcrossing to selfing) occurred several times (Shimizu et al., 2008; Wright et al., 2013). Because sexual systems evolve within a genus in this way, sexual systems can be compared among closely related species to elucidate the mechanisms of evolutionary diversification of plant reproductive systems. Still better would be to study intraspecific variation of reproductive systems. In general, however, flower sex expression is often consistent within species (Desfeux et al., 1996) although the selfing rate is often variable within species (e.g., Wirth et al., 2010).

In *Cimicifuga simplex* (Ranunculaceae), Pellmyr (1987) identified four types of ramets that could be differentiated by their sex expression: ramets with hermaphroditic flowers, ramets with hermaphroditic and male flowers (andromonoecious), ramets with only male flowers, and ramets with only female flowers. He also reported that the hermaphroditic flowers are in the male state in the early part of the flowering period, and in the female state in the later part of the flowering period. Furthermore, he showed that ramets with only female flowers bloom in the early part of the hermaphrodite flowering period (when male state flowers are in bloom), whereas ramets with only male flowers bloom in the later part of the hermaphrodite flowering period (when female state flowers of hermaphrodites are in bloom). Pellmyr (1987) suggested that this diversity of sexual expression is maintained because it is advantageous for flowers with different sex (i.e.,

male or female flowers) to bloom during different parts of the flowering period in a frequency dependent manner, in other words, the sexual minorities are favored by frequency dependent selection and can be maintained in the population.

C. simplex also comprises three, genetically differentiated pollination morphs (Pellmyr, 1986; Kuzume and Itino, 2013), here designated morphs I, II, and III, that differ in their altitudinal distribution and pollinator fauna. Morph I is distributed at high altitude and is pollinated mainly by bumblebees, and morph II is found at middle altitudes and is pollinated mainly by butterflies. Morph III occurs at low altitude and is pollinated mainly by bumblebees or dipteran insects (Pellmyr, 1986; Kuzume and Itino, 2013) although the visitation rates are low.

In this study, I examined the relationship between sex expression and the pollination morphs of *C. simplex.* I found that sex expression and outcrossing rate differed among the three pollination morphs. Morph I that has high outcrossing rates comprised hermaphroditic ramets and only female ramets. Morph II that has high outcrossing rates comprised hermaphroditic ramets and andromonoecious ramets. Morph III that has low outcrossing rates comprised hermaphroditic ramets. Such intraspecific variation of sex expressions has scarcely been investigated so that further research on this system would shade lights on the evolutionary study of plant sex expressions. In addition, as shown in many studies, it was suggested that mating limitation due to the lack of pollinators is related to the acquisition of selfing in morph III.

1-3 Materials and Methods

1-3-1 *Cimicifuga simplex* and the study sites

C. simplex is a perennial herb distributed in eastern and northeastern Asia (Nakai,

1916; Emura, 1970). Each ramet has many small self-incompatible flowers arranged in a simple raceme; some shorter lateral racemes may occur in lower positions on the ramet. Flowering is synchronous within a raceme, and all flowers on the raceme have the same sex state. The lateral racemes simultaneously flower after the primary raceme (Pellmyr, 1987). In the case of andromonoecious ramets, the primary racemes have hermaphroditic flowers and the secondary racemes have only male flowers (Pellmyr, 1987).

The three pollination morphs differ not only in their altitudinal distribution, but also with respect to their habitat, flowering season, and nuclear internal transcribed spacer gene sequences (Kuzume and Itino, 2013). Morph I is distributed in sunny highland habitats and blooms between late July and early September. Morph II is found in sunny midland habitats and has strongly fragrant flowers that bloom between early September and early October. Morph III is distributed in shaded lowland habitats and blooms between early October and early November (Figure 1-1).

In 2016, I studied 10 *C. simplex* populations in Nagano, central Japan (Figure 1-2): I studied five populations of morph I between late July and early September, two populations of morph II between early September and early October, and three populations of morph III between early October and early November (Table 1). The populations were selected because they had a large number of *C. simplex* ramets. This is because small populations are susceptible to genetic drift and accidental sex ratio bias.

1-3-2 Sex expression and inflorescence size

To determine population composition, during the 2016 flowering season, I marked 11–118 flowering ramets of *C. simplex* at the study sites (Table 1), and counted the numbers of hermaphroditic, female, and andromonoecious ramets. The area of each population

ranged up to about 100×200 m, and all ramets within the range were counted.

During the peak flowering period of each population, I measured the length of the primary inflorescence (the inflorescence at the top of the ramet) of each marked ramet.

1-3-3 Flower visitation rate of insects

To assess pollinator composition of each morph, during the peak flowering period of each population, I observed flower visitors from 9:00 a.m. to 12:00 noon local time on a fine day. I recorded the insects that visited the inflorescences and caught some for identification. The visitation rate of the insect visitors was recorded for 5 min at each of 24 inflorescences in each population (24 replicates).

1-3-4 Evaluation of outcrossing rate

To estimate the multilocus outcrossing rate (t_m), eight microsatellite loci (Cisi 1 to Cisi 8; Toji et al., 2018) were used. Sixteen ramets with each morph were haphazardly selected from each of two populations (selected populations: morph I, Norikura_1 and Norikura_5; morph II, Fukashi and Sakura; and morph III, Misuzu and Hora), and 5–6 seeds per plant were haphazardly collected for analysis. Genomic DNA was extracted from ovules with a DNeasy Plant Mini Kit (QIAGEN), and a polymerase chain reaction analysis for genotyping was conducted following the method of Toji et al. (2018).

1-3-5 Statistical analyses

A chi-square test was used to compare differences in sex expression between morphs. Tukey's HSD was used to compare inflorescence size among the populations. Tukey's HSD was also used to compare the flower visitation rates of insects among populations.

To estimate the outcrossing rate (t_m), I used MLTR software ver. 3.4 (Ritland, 2002) and Tukey's HSD to compare average t_m values among populations. All statistical analyses were performed with R ver. 3.2.4 software (R Core Team, 2013).

1-4 Results

1-4-1 Sex expression and inflorescence size

Each of the three pollination morphs of *C. simplex* differed with respect to sex expression and mating system (Figure 1-3): morph I consisted mainly of ramets with hermaphroditic flowers and ramets with only female flowers, morph II comprised ramets with hermaphroditic flowers and ramets with hermaphroditic and male flowers, and morph III consisted mainly of ramets with hermaphroditic flowers. Significant differences were found between all pairs of morphs (chi-square tests: morph I versus morph II, $\chi^2 = 120.8$, $P < 0.01$; morph I versus morph III, $\chi^2 = 42.2$, $P < 0.01$; and morph II versus morph III, $\chi^2 = 46.4$, $P < 0.01$).

Inflorescence size range was on average 18.9–25.5 cm in morph I (five populations), 26.3–29.6 cm in morph II (two populations), and 9.7–14.0 cm in morph III (two populations, Figure 1-4a). The inflorescence size of morph III was significantly smaller than that of the other morphs (Tukey's HSD, $P < 0.05$).

1-4-2 Flower visitation rates of insects

The visitation rate (number of insects per inflorescence per minute) was 0.97–2.73 on average for morph I, 1.68–1.84 for morph II, and 0.00–0.21 for morph III (Figure 1-4b). Morph III was visited significantly less frequently than the other morphs (Tukey's HSD, $P < 0.05$). Many dipteran insects were recorded as visiting morphs I–III, most of which

were Syrphidae (Table 2). Hymenopteran insects, including *Bombus beaticola beaticola* and, with lesser frequency, *Vespula flaviceps*, were frequent visitors to morph I flowers. Most coleopteran visitors belonged to *Ceresium*. Most lepidopteran insects visiting morph II flowers were *Parantica sita* or *Argynnis paphia*.

1-4-3 Outcrossing rate

Estimated t_m was 0.70–0.82 for morph I, 0.83–0.99 for morph II, and 0.37 for morph III (Figure 1-4c). The outcrossing rate was significantly lower in morph III than in the other morphs (Tukey's HSD, $P < 0.05$).

1-5 Discussion

I found that sex expression differed among the three pollination morphs of *C. simplex*: morph I comprised hermaphrodite and female ramets; morph II comprised hermaphrodite and andromonoecious ramets; and morph III comprised hermaphrodite ramets. Although Pellmyr (1987) reported that *C. simplex* includes male ramets, I did not find this sex expression.

Bumblebees, which are excellent pollinators of herbaceous plants in general (Mayfield et al., 2001; Schulke and Waser, 2001), visited morph I flowers frequently (Figure 1-4b), and they may be the main pollinator of that morph. The high visitation rate of bumblebees and their high pollination efficiency may lead to excessive pollen transport to morph I ramets and promote the maintenance of female (rather than male) ramets in morph I populations (Figure 1-3).

Dipteran insects (mostly syrphid flies) were frequent visitors to morph II flowers (Figure 1-4b, Table 2), but in general have lower pollination efficiency per flower visit

than bees (Rader et al., 2016). In addition, the pollination efficiency of butterflies, which Pellmyr (1986) reported to be the main pollinators of *C. simplex*, is also low (Herrera, 1987; Stone, 1996). In this study, the visitation rate of butterflies to morph II flowers was low (Figure 1-4b, Table 2). These results raise two questions: Why does morph II consist not only of ramets with hermaphroditic flowers but also ones with hermaphroditic and male flowers? And why were no ramets with only female flowers recorded?

In general, male flowers are decorative; their role is to attract pollinators (Wilson, 1983). I hypothesize that the role of the male flowers of morph II is to counter the pollen limitation caused by the low quality and quantity of morph II pollinators. I also hypothesize that ramets with only female flowers cannot survive in the morph II populations because of that pollen limitation. To verify this hypothesis, it will be necessary to quantify the pollination efficiency of each insect pollinator group and the degree of the pollen limitation (e.g., evaluate fruit set per single pollinator visit).

The mating system of morph III was different from that of the other morphs in that it had a higher selfing rate, which is consistent with the low insect visitation rates (Figure 1-4b) and the small size of its inflorescences (Figure 1-4a). A small flower display size is regarded as a selfing characteristic (Ornduff, 1969). The high selfing rate of morph III can be explained by the reproductive assurance hypothesis, which posits that where pollinators are scarce selection favors self-pollination in flowering plants (Darwin, 1876). In morph III, the transition from male phase to female phase occurred quickly (Toji, personal observation) so that self-pollination may occur. Pellmyr (1987) insists that the male phase and female phase overlap of hermaphroditic flowers sometimes occurs for 1 day. However, I confirmed that the overlap of male phase and female phase of morph III lasts more than 4 days. I suggested that the rapid sexual phase change of Morph III is

responsible for the high selfing rate. And, if *C. simplex* has self-incompatibility as mentioned in Pellmyr (1987), self-incompatibility may have lost in morph III. Loss of self-incompatibility is a major evolutionary trend in selfing plant species (Shimizu et al., 2008). Under the mating limitation (i.e. pollinator limitation), self-incompatibility tends to be disable by natural selection (Busch and Schoen, 2008).

Contrary to Pellmyr (1986), who reported that morph III is pollinated by bumblebees, only dipteran insects visited the morph III flowers in this study. This study was conducted in Nagano, about 180 km away from Nikko, where Pellmyr (1986) conducted his studies. Geographic variation in pollinator fauna may be responsible for geographic variation in the sex expression and mating system of morph III as well. Additional studies in different regions would be fruitful for clarifying this issue.

It is intriguing that three different sex expressions (hermaphrodite, female, and andromonoecy) occurred within a species, and their occurrence rates are different among the three (basically allopatric) pollination morphs (Figure 1-3). As mentioned in the introduction, such intraspecific variation of sex expressions has scarcely been investigated. Intraspecific variation in sex expressions can be viewed as a difference in the sex ratio between morphs or populations. It is simulated that the optimal resource allocation strategy changes due to exposure to different pollinator environments (Ezoe and Washizu, 2009; Harder and Aizen, 2010). The difference in sex ratio between morphs or populations may be caused by differences in the pollinator environment that result in different optimal resource allocation strategies. On the other hand, intraspecific variation of mating systems (i.e., outcrossing vs self-fertilization) has been well studied. Gervasi and Schiestl (2017) showed experimentally that *Brassica rapa* plants with hoverfly pollination increased fitness through augmented autonomous self-pollination,

demonstrating that changes in pollinator communities can have rapid consequences on the evolution of plant mating systems. Similar to Gervasi and Schiestl (2017), I suggested that different pollinator environments affect selfing rate of *C. simplex*; but more importantly, I suggested that different pollinator environments also affect flower sex expressions of *C. simplex*.

1-2 Tables

Table 1-1. Details of the study sites. Location, population size and sex ratio

Pollination morph	Population name	Altitude (m)	Latitude (N)	Longitude (E)	Number of ramets in the population	Frequency of plant ramets		
						with hermaphroditic flowers	with only female flowers	with hermaphroditic and male flowers
I	Norikura_1	2050	36°12'29"	137°58'48"	24	0.92	-	0.08
I	Norikura_2	2120	36°12'19"	137°57'98"	24	0.54	0.46	-
I	Norikura_3	2200	36°12'05"	137°57'40"	19	0.74	0.26	-
I	Norikura_4	2300	36°11'97"	137°57'25"	81	0.95	0.05	-
I	Norikura_5	2340	36°12'18"	137°57'19"	118	0.64	0.32	0.04
II	Fukashi	1350	36°25'13"	138°04'04"	108	0.77	-	0.23
II	Sakura	1300	36°21'60"	138°08'38"	92	0.49	-	0.51
III	Misuzu	1000	36°26'32"	138°01'26"	41	0.98	-	0.02
III	Hora	700	36°28'03"	137°98'47"	11	1.00	-	-
III	Gake	920	36°15'64"	138°01'08"	111	0.93	-	0.07

Table 1-2. Flower visitors to the three pollination morphs of *C. simplex* in 10 populations

	Percentage of flower visitors									
	Morph I populations					Morph II populations		Morph III populations		
	Norikura_1	Norikura_2	Norikura_3	Norikura_4	Norikura_5	Fukashi	Sakura	Misuzu	Hora	Gake
HYMENOPTERA:										
Bombus beaticola beaticola (Apidae)	13.8%	0.6%	20.8%	3.4%	17.5%					
Bombus hypocrita hypocrita (Apidae)						4.2%				
Tenthredinidae spp.				0.5%	0.3%					
Vespula flaviceps (Vespidae)	13.8%	0.6%	3.2%	9.7%	2.5%					
Paratrechina flavipes	5.2%	3.2%		0.6%	0.6%					
DIPTERA:										
Syrphidae spp.	11.2%	19.6%	19.5%	25.6%	20.0%	83.5%	92.1%		87.0%	100.0%
Others (Muscidae spp., Anthomyiidae spp., Tachinidae spp., Tipulidae spp.)	46.6%	48.7%	56.4%	59.7%	58.9%	9.9%	6.9%		13.0%	
LEPIDOPTERA:										
Parantica sita							1.0%			
Argynnis paphia						1.9%				
Macroglossum saga						0.5%				
COLEOPTERA:										
Lepturinae spp.	9.5%	27.2%		0.6%	0.3%					

1-7 Figures

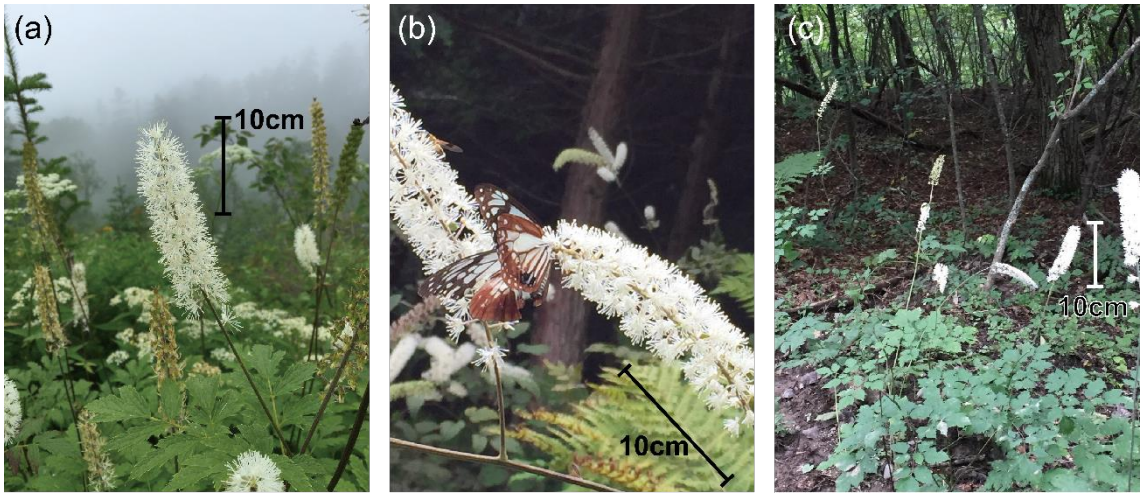

Figure 1-1. Three pollination morphs of *C. simplex*. (a) Morph I distributes in high alpine zone (at Norikura_5 population, 2340 m, a.s.l.). (b) Morph II distributes in midland forest edge (visited by the butterfly *Parantica sita* at Fukashi population, 1350 m, a.s.l.). (c) Morph III distributes in shaded lowland (at Gake population, 920m, a.s.l.).

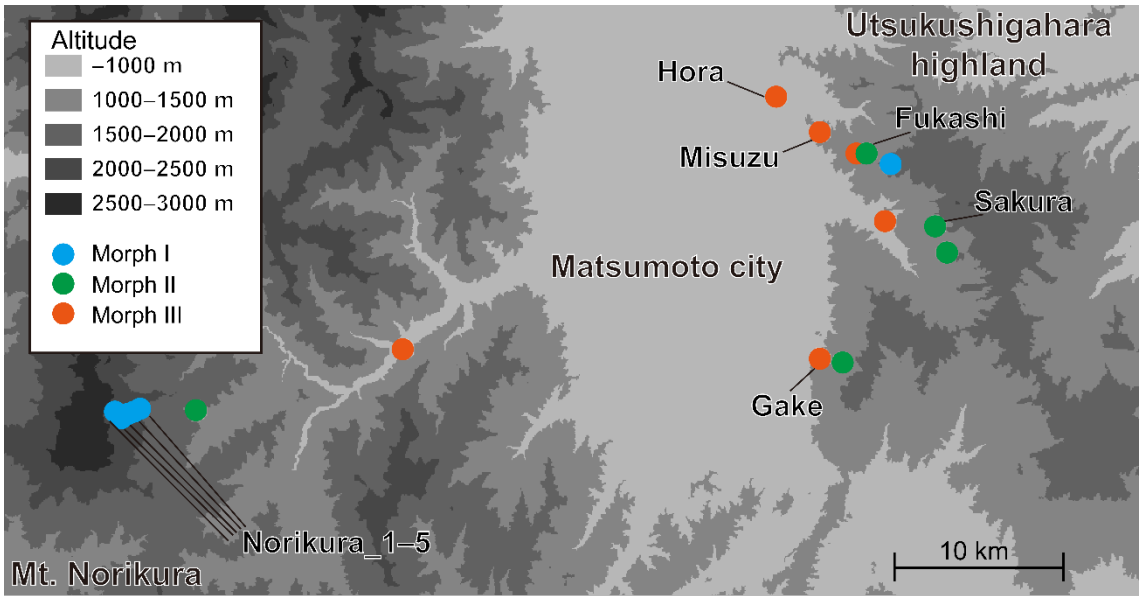

Figure 1-2. Distribution sites of three pollination morphs of *C. simplex* in Matsumoto, Nagano Japan. The studies sites are accompanied by the population names (see Table 1). The not-studied but only-distribution-checked sites are shown without names.

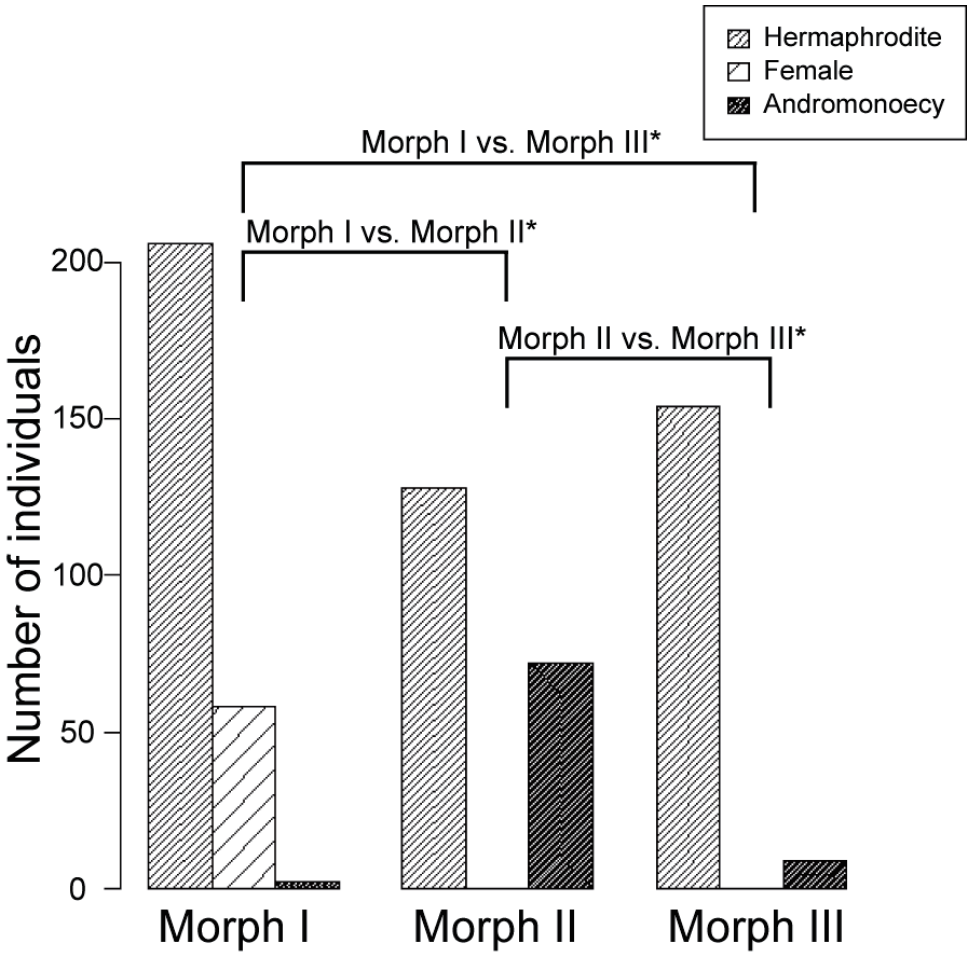

Figure 1-3. Observed sex expressions in the three pollination morphs of *C. simplex* (sum up all population). An asterisk indicates that the combination of flower sex expression forms differs between the morph pair (chi-square test, $P < 0.01$).

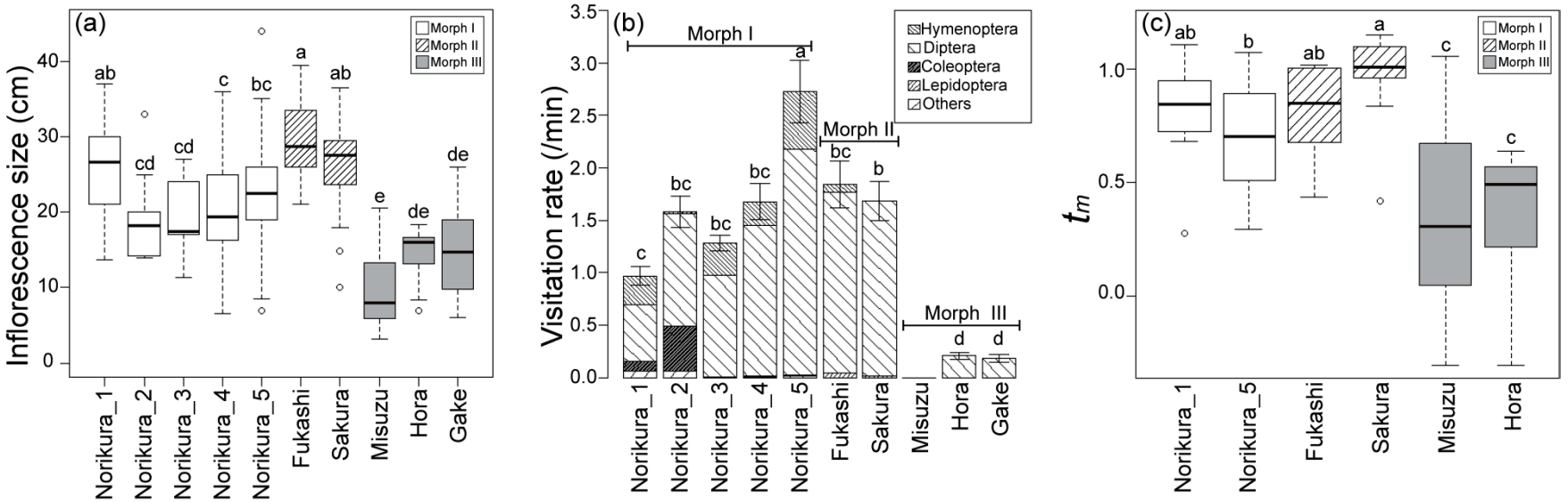

Figure 1-4. (a) Inflorescence size, (b) visitation rates of each insect order (number of insect visits per inflorescence per minute, mean ± SE), and (c) multilocus outcrossing rate (t_m) in the three pollination morphs of *C. simplex*. The box plots in (a) and (c) show the median (bar), the lower and upper quartiles (box ends), lower and upper quartile ± 1.5 × interquartile range (whiskers) and outliers (circles). Different lowercase letters indicate significant differences between the populations (Tukey's HSD, $P < 0.05$).

Chapter 2

Seasonal change of flower sex ratio and pollinator dynamics in three reproductive ecotypes of protandrous plant

2-1 Abstract

Cimicifuga simplex has three genetically and ecologically distinct pollination morphs with different flowering phenology, flower sex expressions, and selfing rates. A previous study showed that strong protandry in hermaphroditic flowers of *C. simplex* causes there to be seasonal minority sexes; for example, unisexual female ramets are advantageous in the first half of the flowering season and bloom then ('minority sex' hypothesis). That study, however, did not distinguish among the three pollination morphs of *C. simplex*. I investigated seasonal sex ratio changes and pollinator environments of the three morphs to verify and expand the minority sex hypothesis. I investigated flowering phenology, pollinator quantity and quality, seasonal population sex ratio dynamics, and stamen/ovule ratios in hermaphroditic flowers. I also examined the seasonal female reproductive success of hermaphroditic flowers, and the effect of male flower excision on fruit set by morph II hermaphroditic flowers. Morph I (mainly hermaphroditic and female ramets) had high pollinator quality and quantity. Fruit set of hermaphroditic ramets was high throughout the flowering season. Morph II (hermaphroditic and andromonoecious ramets) had low pollinator quality, and few pollinators visited near the beginning and end of the season. Removal of male flowers led to a pollen limitation. Morph III (hermaphroditic ramets and a high selfing rate) had very low pollinator abundance throughout the flowering season, and male and female phases largely overlapped. The scarcity of male flowers in morph I is likely a result of the high pollinator quantity and

quality. The high fruit set suggests that no pollen limitation existed. The low pollinator quality and quantity of the morph II population caused it to suffer from pollen limitation and may explain the absence of unisexual female ramets in this morph. The high selfing rate of morph III may be due to the extremely low pollinator abundance. Theoretical studies have indicated that the optimal investment allocation to male and female functions depends on whether a pollen limitation exists. In *C. simplex*, the observed relationships between pollinator environment and reproductive systems are consistent with these theoretical models.

2-2 Introduction

A question of great interest to ecologists is "Why have angiosperms become so diverse?" The modern answer to this question is the Grant–Stebbins model (Grant and Grant, 1965; Stebbins, 1970; Johnson, 2006). According to this model, the geographical mosaic of pollinator fauna acting on intraspecific plant diversity caused adaptive radiation. Indeed, many studies of geographically distinct intraspecific pollination ecotypes have revealed not only the existence of geographic variations in flower traits such as floral tube size but also that they are related to geographic differences in pollinators (Herrera et al., 2006; Anderson and Johnson, 2008; Johnson and Anderson, 2010; Anderson et al., 2014; Boberg et al., 2014; Nagano et al., 2014; Kuriya et al., 2015). Many studies of plant reproductive systems have also suggested that differences in pollinators affect geographic variation in selfing rates (Darwin, 1876; Baker, 1955; Faust et al., 2001; Kalisz and Vogler, 2003). For example, Gervasi and Schiestl (2017) have shown experimentally that *Brassica rapa*, when exposed to different pollinator environments, develops different reproductive systems; in a bumblebee-abundant environment, *B. rapa* evolves flower

traits that attract pollinators, whereas in a hoverfly-abundant environment, the rate of automatic self-pollination increases. Thus, the formation of the pollinator ecotype is not only a driving force for morphological diversification of flowers but also for the diversification of reproductive systems. Theoretical studies have shown that a pollen limitation resulting from pollinator scarcity alters the optimal allocation of investment in floral attractors (Ezoe and Washizu, 2009; Harder and Aizen, 2010). Attractive floral organs are often interpreted as indicating allocation to the male function (Bell, 1985; Johnson et al., 1995), so that I can expect that in plant populations visited by flower visitors of low quality and quantity, plant individuals with more investment in male function would be abundant.

In this study, I focused on *Cimicifuga simplex* Wormsk. (Ranunculaceae), a protandrous plant with three morphs, each with a different reproduction system and a different pollinator assemblage. First, I hypothesize that different pollination morphs are visited by different pollinators (of low or high quality and quantity), and thus, use different sexual allocation strategies. Second, as the sex ratio of each morph seasonally changes, I hypothesize that males are dominant in the seasons with low quality- and quantity pollinators. Although many studies have investigated spatial aspects of sex ratio changes (Alonso 2005; Ueno et al., 2007; Timerman and Barrett, 2019), little attention has been paid to seasonal sex ratio changes (Pellmyr, 1987). Furthermore, although theoretical research on the relationship between pollinator environments and sex ratios has advanced, empirical research has lagged far behind.

On the other hand, there is ‘minority sex’ hypothesis. If hermaphroditic flowers are strongly protandrous, a seasonal shift from male to female in the functional sex ratio of a population is expected (Pellmyr, 1987; Aizen, 2001). Thus, hermaphroditic flowers in the

male phase are dominant in the first half of the flowering season, whereas in the second half of the flowering season, when many individuals are approaching the end of their flowering season, so female hermaphroditic flowers in the female phase are dominant in the population. In a population that undergoes such a seasonal shift in the sex ratio, there is a period of time during which the minority sex has a fitness advantage compared with the majority sex (Thomson and Barrett, 1981; Devlin and Stephenson, 1987; Wells and Lloyd, 1991; Brunet and Charlesworth, 1995; Spencer and Rieseberg, 1995; Brunet, 1996; Morgan and Schoen, 1997; Medan and Bartoloni, 1998; Aizen, 2001). For example, in the case of protandry, the first half of the flowering season is dominated by hermaphroditic flowers in male phase, so individual plants with female phase flowers have an advantage. On the other hand, in the second half of the flowering season, many hermaphroditic plants are in the female phase, so individuals with flowers in male phase have an advantage. In other words, expression of the minority sex is advantageous and can be maintained in the population because selection for the minority sex is frequency-dependent. This is the 'minority sex' hypothesis.

Pellmyr (1987) examined seasonal shifts in the sex ratio of *C. simplex* (here, he did not distinguish the three morphs) and suggested why this species has four sex expressions (hermaphroditic, female only, andromonoecious, male only). He demonstrated that hermaphroditic flowers of *C. simplex* are strongly protandrous, and that in a population with hermaphroditic sex expression, the sex ratio changes from male-dominant to female-dominant over the course of the flowering season. He suggested that plants with unisexual flowers can invade and be maintained in hermaphroditic populations in a frequency-dependent manner. Indeed, Pellmyr (1987) supported this hypothesis by pointing out that many dichogamous species have multiple sex expressions, including unisexual flowers.

On the other hand, Kuzume and Itino (2013) identified three genetically differentiated morphs of *C. simplex*, each morph with a different reproductive system (Toji and Itino, 2020). Pellmyr (1987) did not mention reproductive system differences among of *C. simplex* morphs; thus, more explanation than that provided by Pellmyr (1987) is necessary to understand the diversity of sexual expression among *C. simplex* morphs. According to Toji and Itino (2020), morph I, which has a high outcrossing rate, is gynodioecious; it mainly produces hermaphroditic and unisexual female ramets, along with a few andromonoecious ramets. Morph II, which has a high outcrossing rate, produces hermaphroditic and andromonoecious ramets (i.e., ramets with a hermaphroditic primary raceme and lateral racemes with unisexual male flowers). Morph III, which has a low outcrossing rate, produces mainly hermaphroditic ramets, but also a few andromonoecious ramets (Table 2-1). The simple hypothesis of Pellmyr (1987), that strong protandry would allow plants with unisexual minority flowers to invade a population, cannot fully explain these reproductive system differences among the morphs. As the theoretical model shows, the optimal sexual investment strategy may differ depending on the flower visitor quality and quantity. For example, morph II plants with low quality flower visitors are expected to have a high investment ratio in males. I hypothesize that differences in pollinator quality and quantity among populations of *C. simplex* morphs are responsible for these inter-ecotype differences in reproductive systems. Thus, I propose expanding the ‘minority sex’ hypothesis.

In this study, I examined the influence of pollinator quality and quantity on seasonal sex ratio changes both among the three pollination morphs of *C. simplex* and within each morph. I conducted field surveys to determine the quality and quantity of pollinators of each morph. Theoretical studies have shown that when a highly active pollinator is

present, plants reduce their investment in floral attractors. Thus, in morph II, the dominance of andromonoecious ramets suggests that the quality and quantity of pollinators should be low. Conversely, in morph I populations, which are dominated by unisexual female individuals, both the quality and quantity of pollinators are predicted to be high. I also examined seasonal aspects of sex expression by measuring the seasonal change in the sex ratio of populations of each morph throughout the flowering season. In particular, I addressed two questions: (1) Does the sex ratio of each morph change over time? And (2) Does the quality and quantity of pollinators also change over time? The pollinator quality of each morph was assessed by evaluating fruit set per single pollinator visit and how many flowers an insect visited during a single visit to a raceme (Ne'eman et al., 2010). To evaluate pollinator quantity, I measured the frequency of flower visits by insect pollinators per minute. I monitored these parameters in populations of the three morphs for two years.

2-3 Materials and Methods

2-3-1 Plant species and the survey area

Cimicifuga simplex is a perennial herb distributed in eastern and north-eastern Asia (Nakai, 1916; Emura, 1970). In this species, a hermaphroditic ramet has many flowers arranged in a simple raceme (Figure 2-1); some shorter lateral racemes may occur in lower positions on the ramet. Morph II has more lateral racemes than morphs 1 and 3. Flowering on a raceme is synchronous, and all flowers on the raceme have the same sex state. The sexual phase of the flowers can be clearly distinguished visually (Figure 2-1). The male and female phases each last for 3–4 days. The lateral racemes flower simultaneously after the primary raceme has finished blooming. In the case of

andromonoecious ramets, the primary raceme has hermaphroditic flowers and the lateral racemes have unisexual male flowers (Figure 2-2; Pellmyr, 1987; Toji and Itino, 2020).

The three pollination morphs differ not only in their altitudinal distribution but also with respect to their habitat, flowering season, and genetics (e.g., nuclear internal transcribed spacer gene sequences and microsatellite genetic structure) (Kuzume and Itino, 2013; Toji et al., 2018). Morph I is distributed in sunny, open highland habitats and blooms between late July and early September. Morph II is found in sunny forest-edge middle-elevation habitats and has strongly fragrant flowers that bloom between early September and early October. The strong fragrance helps attract butterflies (Pellmyr, 1986). Morph III is distributed in shaded forest floor lowland habitats and blooms between early October and early November (Table 2-1; Pellmyr, 1986; Kuzume and Itino, 2013; Toji and Itino, 2020).

In each population, I examined ramets on plants growing within a 100 m × 200 m quadrat at intervals of 2–7 days (Table 2-2). I carried out periodic surveys of the insects visiting flowers, flowering phenology, number of stamens and ovules, and fruit set in populations in Matsumoto, Nagano, Japan, for 2 years (2017 and 2018). Morph I was surveyed at Norikura (2340 m a.s.l., 137°34’19” E, 36°7’18” N), Morph II was surveyed at Utsukushigahara (1350 m a.s.l., 138°2’28” E, 36°15’7” N), and Morph III was surveyed at Gakenoyu (1000 m a.s.l, 138°0’39” E, 36°9’22” N). In previous study, I examined reproductive systems at five sites for morph I, two sites for morph II, and three sites for morph III (Toji and Itino, 2020). Because each morph on multiple sites consistently had a similar reproductive system, I selected one site for each morph as study site.

2-3-2 Pollinator quality and quantity

To assess pollinator quality, a single-visit experiment was conducted in 2017 at two locations; each location was about 300 m away from the periodic survey site of morph I or II. I excluded morph III from this analysis because the number of visitors to morph III flowers was too small for pollinators of this morph to be tested. At each experimental location, I first covered a primary raceme in bud phase with a mesh nylon bag to prevent insects from visiting the flowers. After the hermaphroditic raceme attained the female phase, the bags were removed and any insect was allowed to visit the flower, but only once. Immediately following the visit, the base of each flower on the raceme that had been touched by the insect was marked with a colored pen. Then the raceme was again covered with a bag until the fruit had matured. Pollinator quality was assessed by comparing fruit set among flower visitors. The fruit set of a primary raceme on another plant that was bagged from the bud phase to the wither phase was used as a control. Fruit set was defined as the ratio of the number of mature fruits to the number of pistils in the flowers that had been touched by an insect visitor (experimental treatment) or to the number of pistils in all flowers on the raceme (control).

In addition to the above single-visit experiment, I counted the number of individual insects that touched flowers of a hermaphroditic primary raceme of each morph in its male phase during a single visit. I hid in front of each hermaphroditic primary raceme and counted how many flowers on the raceme were touched in a single visit by visiting insects.

To assess pollinator quantity, I counted the flower visitation rate at each periodic survey site. I observed the flower visitation rate at each site 6–38 times per day, each time for 5 min, and counted the number of insects that visited each raceme per minute. I carried out these observations for a total of 50 h 40 min in 2017 and 51 h in 2018 (about 105 min

per day on average). When an insect visited a raceme, it was counted as one visit, and visits by insects of different taxa were counted separately. Visits by each species of Hymenoptera and Lepidoptera were recorded separately. For Diptera, I recorded visits by Syrphidae spp. and Anthomyiidae spp. separately, because it was difficult to distinguish individuals at the species level during the observation. If an insect left the raceme and then immediately returned, the second visit was not counted as a separate visit. I observed flower visitors from 9:00 a.m. to 14:00 p.m. local time on a sunny day.

2-3-3 Flowering phenology and sex ratio changes

Flowering phenology was examined at each periodic survey site to assess seasonal changes in the sex ratio. The sex phases of hermaphroditic flowers could be visually distinguished, and all flowers on a raceme were in the same sex phase (Figure 2-1). Hermaphroditic racemes (male or female phase), unisexual female racemes, unisexual male racemes, and primary and lateral racemes were counted separately. In the case of andromonoecious ramets, the primary raceme was counted as hermaphroditic and the lateral racemes were counted as unisexual male racemes. From these counts, the seasonal dynamics of the sex ratio in the population were calculated. The male sex ratio was determined for each population as follows:

$$\frac{N_{hm} + N_{um}}{N_{hm} + N_{um} + N_{hf} + N_{uf}} = \frac{N_{hm} + N_{um}}{N_t}$$

where N_{hm} is the number of hermaphroditic ramets in the male phase, N_{um} is the number of unisexual ramets with male flowers in bloom, N_{hf} is the number of hermaphroditic ramets in the female phase, N_{uf} is the number of unisexual ramets with female flowers in bloom, and N_t is the total number of flowering ramets at the survey site. The male sex

ratio was calculated for each survey day, and the change in the ratio over time was examined. In addition, to focus on hermaphroditic ramets only, I calculated the male sex ratio without considering unisexual ramets as follows:

$$\frac{N_{hm}}{N_{hm} + N_{hf}}$$

In this case, the male sex ratio is expected to decrease from the first half to the second half of the flower season because of the protandry of hermaphroditic ramets.

2-3-4 Seasonal change in the stamen/ovule ratio and fruit set

When a hermaphroditic plant is protandrous, flowers in the first half flowering season invest more in the production of ovules, and flowers in the second half invest more in the production of pollen (Ishii and Harder, 2012). The reason for this is that in the first half of the flowering season, the sex ratio has a male bias, and in the second half it has a female bias, and allocation to the minority sex is advantageous. Therefore, I examined how allocation of investment to each sex by hermaphroditic flowers changed according to their first flowering date in 2017. In each flower, the number of stamens and the number of ovules vary; therefore, I considered the number of stamens as an indicator of male function and the number of ovules as an indicator of female function. Because the number of pollen grains did not differ among stamens (Table 2-3), I considered the number of stamens to be an effective measure of allocation to male function by hermaphroditic flowers of *C. simplex*. Furthermore, because seed size did not differ among the morphs (Table 2-3), I considered the number of ovules to be an effective measure of allocation to female function by hermaphroditic flowers (Table 2-3). The number of stamens and the number of ovules per flower were counted under a stereomicroscope. Then, the number

of stamens per flower was divided by the number of ovules to obtain the stamen/ovule ratio, which was used as an index of the number of stamens per flower.

To measure the pollination success of a raceme on each flowering date, fruit set on hermaphrodite ramets of each morph was determined for each flowering date in 2018. Blooming flowers on each raceme were marked with different colored tape on each survey day. Fruit set of all hermaphroditic ramets whose flowering date had been identified was determined.

To compare the seed output between hermaphroditic and female ramets, I counted the number of racemes, flowers, pistils, and ovules and determined fruit set only in morph I, which has both hermaphroditic and unisexual female ramets.

In addition, a generalized linear model (GLM) analysis was performed with the fruit set at each flowering start date as a response variable. Analysis was performed for the fruit set of hermaphrodites in morph I, II, and III, and the female ramet of morph I. Flowering start date, population male sex ratio, and insect visitation rate were used as explanatory variables. Although the flowering start date usually refers to male stage flowers, in the hermaphrodite of morph I and morph II, the receptive female stage flowers opened approximately two survey days (ca. 1 week) after the flowering start date. Therefore, in the case of hermaphrodite of morph I and morph II, I referred male sex ratio and the visitation rate to the values on two survey days after the flowering start date.

2-3-5 Male flower excision test in morph II

To investigate the effect of male flowers on the reproductive success of lateral racemes of hermaphroditic ramets in morph II, I performed a male flower excision experiment at Susuki (138°5’36” E, 36°12’15” N) in 2017. Fruit set of the lateral racemes of an

unmanipulated hermaphroditic ramet at Sakura (138°5’10” E, 36°12’57” N) in 2017 was used as a control (Wilcoxon signed-rank test). In general, the function of male flowers is to attract flower visitors and to overcome any pollen limitation (Wilson and Price, 1977; Wilson, 1983; Solomon, 1985; Podolsky, 1992; Podplsky, 1993; Elle and Meagher, 2000; Barrett, 2002; Vallejo-Marín and Rausher, 2007). Therefore, I expected that the excision of unisexual male flowers would result in a pollen limitation and reduce the seed set of hermaphroditic lateral racemes that bloomed at the same time as flowers on unisexual male racemes. This experiment was carried out on morph II, which has large numbers of unisexual male flowers. The Susuki (male flower excision site) and Sakura (control site) populations each occupy an area of about 100 m × 200 m, and the straight-line distance between the two sites is approximately 1.5 km. At both sites, the number of ramets was approximately 100, and the ratio of the number of hermaphroditic ramets to the number of andromonoecious ramets was approximately 1:1 in 2016. At Susuki, after the lateral racemes had bloomed, flowers on the male racemes were excised at intervals of 3–4 days by scissors. The natural fruit set recorded at both sites in 2016 was also used for comparison (Wilcoxon signed-rank test).

2-4 Results

2-4-1 Pollinator quality and quantity

In the single-visitation experiment carried out to evaluate pollinator quality, *Bombus beaticola beaticola* (Hymenoptera) and *Vespula flaviceps* (Hymenoptera) pollinated morph I flowers, and pollinator quality was high; fruit set per single visit was about 0.8–0.9. *Parantica sita* (Lepidoptera), *Argynnis paphia* (Lepidoptera) and Diptera visited morph II flowers, but pollinator quality was low; fruit set per single visit was about 0.3.

Comparison of fruit set per single pollinator visit showed that the pollinator quality of Hymenoptera (*B. beaticola beaticola* and *V. flaviceps*) visiting morph I was relatively high (Figure 2-3a). Fruit set following a single visitation by Lepidoptera (*A. paphia* and *P. sita*) and Diptera (Syrphidae spp.) to morph II did not differ significantly from the control. The number of touched flowers per single visit showed that the pollinator quality of Hymenoptera was high, whereas that of Lepidoptera and Diptera was not (Figure 2-3b).

In 2017 and 2018, I investigated seasonal changes in the flower visitation rate and found that each morph grew in a different pollinator environment. Pollinators of morph I tended to visit flowers at high frequency throughout the flowering season, with an average visitation rate of 1.91–2.62 visitors per minute per raceme (Figures 2-4j, 2-5j). The visitation rate to morph II was low in the early and late flowering season, but the average visitation rate throughout the flower season was 0.72–1.26 visitors per minute per raceme (Figures 2-4k, 2-5k). The visitation rate to morph III was low throughout the flowering season, averaging 0.09 visitors per minute per raceme. These trends were similar in both 2017 and 2018 (Figures 2-4l, 2-5l). A breakdown of the insects visited on each survey day is given in Figure 2-6.

2-4-2 Flowering phenology and sex ratio changes

Morph I showed a seasonal change in the male sex ratio from a high value (i.e., male dominant) to a lower value (female dominant) (Figures 2-4a, 2-5a). In addition, in the first half of the flowering season, when the majority of the hermaphroditic ramets were in the male phase, many unisexual female ramets were also in flower, whereas in the second half of the flowering season, when the majority of the hermaphroditic ramets were

in the female phase, only a few male flowers on andromonoecious ramets were in flower (Figures 2-4d, 2-5d). When I considered hermaphroditic ramets only, the male sex ratio of the morph I population changed clearly from a high value to a low value, but when I also considered unisexual ramets, the male sex ratio was relatively stable over the course of the flowering season (Figures 2-4g, 2-5g), with a mean ratio during the flowering season of 0.46–0.50.

In morph II, the male sex ratio showed a seasonal change from a high value to a lower value (Figures 2-4b, 2-5b). In addition, in the first half of the flowering season, when the majority of hermaphroditic ramets were in the male phase, and in the second half of the flowering season, when the majority of hermaphroditic ramets were in the female phase, many unisexual male flowers on andromonoecious ramets were also in bloom (Figures 2-4e, 2-5e). Although the male sex ratio changed clearly from a high value to a low value when I considered only the hermaphroditic ramets, when I considered unisexual flowers as well, the male sex ratio was relatively stable (Figures 2-4h, 2-5h) with a mean ratio during the flowering season of 0.70–0.74.

The sex phase change occurred a shorter transition period in morph III than in morphs I and II (Figures 2-4c, 2-5c). The male and female phases were not completely separate in time; the female phase began 1–2 days after flowering began, and a few unisexual male flowers on lateral racemes bloomed in the second half of the flowering season (Figures 2-4f, 2-5f). The male sex ratio of the morph III population did not change as much as it did in morphs I and II (Figures 2-4i, 2-5i); the mean ratio during the flowering season was 0.41–0.55.

2-4-3 Seasonal changes in the stamen/ovule ratio and fruit set

In each morph, the stamen/ovule production ratio of hermaphroditic ramets increased toward the end of the 2017 flowering season. The slope of a regression line fitted to the data was 7.94×10^{-3} for morph I, 8.36×10^{-2} for morph II, and 1.43×10^{-1} for morph III (Figures 2-4m–o, regression coefficient significance test, $P < 0.01$). However, in morph III, the stamen/ovule production ratio varied greatly among individual flowers, so a regression analysis may not be appropriate. The number of pollen grains per anther and the seed size did not differ among the morphs (Table 2-3), so in each morph the stamen/ovule production ratio can be regarded as an indicator of the allocation to the male sexual function.

In 2018, fruit set in morph I was high (0.95–0.99) for each first flowering date (Figure 2-5m). In morph II, fruit set was 0.88–0.98 on average, and it decreased slightly during the second half of the flowering season (Figure 2-5n). In morph III, fruit set was 0.79–0.98 on average, and it also decreased in the second half of the flowering season (Figure 2-5o). GLM analysis with fruit set as a response variable showed that only the morph II result rate was significantly affected by the flowering start date (Table 2-4).

2-4-4 Male flower excision test in morph II

The average fruit set of the lateral racemes of hermaphroditic ramets at Sakura, the control site, was 0.92. The average fruit set of the lateral racemes of hermaphroditic ramets at Susuki, the male flower excision site, was 0.77. This difference between the control and excision treatments was statistically significant (Figure 2-7, Wilcoxon signed-rank test, $P < 0.01$). In morph II, fruit set by the lateral racemes of hermaphroditic ramets decreased when male flowers were not available. Fruit set on lateral racemes of hermaphroditic ramets at Susuki in 2017, when male flowers were ablated, was

significantly reduced compared with fruit set on lateral racemes of hermaphroditic ramets at the same site in 2016, when no male flowers were ablated (Wilcoxon signed-rank test, $P < 0.01$).

2-5 Discussion

As shown by Pellmyr (1987), unisexual female ramets in morph I flower in the first half of the flowering season. In these results, unisexual male flowers on andromonoecious ramets in morphs I and II flowered in the second half of the flowering season (Figures 2-4d–e, 2-5d–e). This flowering phenology of unisexual flowers is consistent with Pellmyr's (1987) 'minority sex' hypothesis, but it does not fully explain the reproductive system differences among the morphs. Here, I examine the relationship between the pollinator environment and the reproductive system of each morph of *C. simplex* because it has been shown experimentally that different reproductive systems evolve in different pollinator environments (Gervasi and Schiestl, 2017).

2-5-1 Why does morph I have many unisexual female ramets but few unisexual male racemes?

Are female ramets maintained by morph I because they have a relative fitness advantage? First, classical studies have suggested that unisexual female ramets are not maintained in a gynodioecious population unless they produce twice as many seeds as the hermaphroditic ramets (Lewis, 1941). The sexual system of morph I, which has both hermaphroditic and unisexual female ramets, can be described as gynodioecy. Estimated seed production by hermaphroditic ramets in morph I, however, was higher than that by unisexual female ramets (Table 2-5), so female ramets were inferior to hermaphroditic

ramets in seed productivity. Second, if hermaphroditic ramets have a high selfing rate and suffer inbreeding depression, unisexual female ramets, which produce seeds through outcrossing, are considered to be more advantageous than hermaphroditic ramets (Lloyd, 1975; Charlesworth and Charlesworth, 1978). This scenario is not applicable to morph I, however, because the outcrossing rate of its hermaphroditic ramets exceeds 90% (Toji and Itino, 2020). These findings suggest that female ramets are maintained in the morph I population because the timing of their flowering is frequency-dependent and favorable. The same seasonal advantage should exist for morph II, but unisexual female ramets are unique to morph I. Why are there no female ramets in morph II, and why are there many unisexual male flowers in morph II but few in morph I?

Bumblebees, which frequently visited the morph I population, are effective pollinators of many plants (Schulke and Waser, 2001; Mayfield et al., 2001), and the pollinator quality of *V. flaviceps*, one of the visitors to morph I flowers of *C. simplex*, was higher than that of the other pollinators (Figure 2-3). In addition, the visitation rate of all pollinators to morph I flowers was high throughout the season (1.907–2.617 individuals per minute per raceme, Figures 2-4j, 2-5j). Thus, both the quality and quantity of pollinators of morph I flowers were high. These results suggest that in the morph I population, unisexual female ramets, which likely receive ample pollen from hermaphroditic flowers via their excellent pollinators (*Bombus* and *Vespula*), can be easily maintained in the population.

In the gynodioecious species *Daphne laureola*, the number of pollen grains on the stigmas of both hermaphroditic and female flowers decreases with increasing altitude (Alonso, 2005). The higher the altitude at which *D. laureola* grows, the lower the proportion of unisexual female ramets is in the population. This finding suggests that the

proportion of unisexual female ramets is influenced by the amount of pollen received. In fact, Asikainen and Mutikainen (2005) have proposed that a pollen limitation influences the evolution of the sex ratio of gynodioecious plants. In general, enough pollinators of unisexual female ramets be available in order to maintain gynodioecy in a population (Stone and Olson, 2018). In morph I, because pollinator quality and quantity were both excellent, unisexual female ramets receive a sufficient amount of pollen for them to be maintained in the population. I thus conclude that the presence of unisexual female ramets in morph I can be explained by both the seasonal advantage that they confer and the excellent pollinator environment.

Contrary to Pellmyr's (1987) 'minority sex' hypothesis, morph I had only a few andromonoecious ramets with male flower that bloomed in the second half of the flowering season, when male phase flowers on hermaphroditic ramets were rare and male racemes should be advantageous (Figures 2-4d, 2-5d). Surprisingly, the fruit set of hermaphroditic ramets was high regardless of the flowering date (Figure 2-5m). This result suggests that the presence or absence of male flowers in morph I has no effect on the reproductive success of the hermaphroditic ramet, perhaps because the population is visited by many excellent pollinators. As a result, morph I plants do not need to produce unisexual male flowers and their proportion in the population is low.

However, genetic and demographic factors, such as the association between the sex determination nuclear gene and the multiple cytoplasmic male sterility (CMS) genes, are known to influence the population sex ratio in the gynodioecy plant (Bailey and Delph, 2007). The mechanism of sex determination in *C. simplex* is still unclear, and this needs to be elucidated and discussed. In addition, future approaches to compare the quality and quantity of visitors within each morph may be more useful for discussion. For example,

when the pollination rate and visitor quality are low in several morph I populations, the sex ratio is expected to be skewed toward males in the populations. Based on the present results, the sex ratio did not affect the reproductive success of morph I individuals (Table 2-4). Since these results examined reproductive success only in terms of fruit set (female fitness), it is a future challenge to include the dynamics of success as pollen parents (male fitness) in the analysis.

2-5-2 Why does morph II have many unisexual male racemes but no unisexual female ramets?

In general, female ramets should be advantageous in the first half of the flowering season when female phase flowers on hermaphroditic ramets are scarce. However, the visitation rate by pollinators to flowers in the morph II population tended to be low in both the early and late parts of the flowering season (Figures 2-4k, 2-5k). If plants with unisexual female ramets invaded the morph II population, they would bloom early in the flowering season, when flower visitors are infrequent. This situation might give rise to a pollen limitation such that the unisexual female ramets might not be able to set fruit (In the case of dioecy plant see Yu and Lu, 2019). Moreover, the pollinator quality of flower visitor insects to the morph II population is relatively low (Figure 2-3). Together, these results suggest that unisexual female ramets are less likely to be maintained in the morph II population, even in the early season when female phase flowers on hermaphroditic ramets are scarce.

The male sex ratio was high in the morph II population throughout the flowering season (Figures 2-4h, 2-5h). This high ratio may be an adaptation of morph II to a pollinator-scarce environment, because maintenance of a high male sex ratio may help to

overcome pollen limitation. Male flowers have to main functions: to overcome any pollen limitation and to attract flower visitors (Willson, 1983). Male flowers in morph II may be more likely to reproduce successfully because a pollen limitation is caused by a decreased number of pollinators during the second half of the flowering season. In addition, by having abundant male flowers in bloom, the morph may attract more pollinators. In fact, morph II has more racemes per ramet than morphs I and III (number of racemes per ramet, mean ± SE: morph I, 1.36 ± 0.09, morph II, 5.32 ± 0.34, morph III, 1.18 ± 0.18, Tukey's HSD, $P < 0.01$). An abundance of blooming flowers increasing the floral display size is known to attract pollinators (Willson and Price, 1977; Grindeland et al., 2005; Lobo et al., 2016).

The stamen/ovule ratio of hermaphroditic ramets of morph II increased greatly toward the end of the flowering season in 2017 (Figure 2-4n), indicating that allocation to male function (pollen) on hermaphroditic ramets became greater. This result is consistent with other characteristics of morph II, which produces many unisexual male flowers in the second half of the flowering season. Many studies have shown that in protandrous hermaphroditic plants, the pollen/ovule ratio, male phase duration, or floral display size increase toward the end of the flowering season (Kudo et al., 2001; Ishii and Sakai, 2002; Garcia, 2003; Hiraga and Sakai, 2007; Zhao et al., 2008; Ishii and Harder, 2012).

In addition, in male flower excision experiment, fruit set of the lateral racemes of hermaphroditic ramets in the male flower excision area was significantly decreased compared with the control group (Figure 2-7). This result suggests that the male flowers provide pollen to hermaphroditic flowers that bloom later in the flowering season. Because excision was performed at 3–4 day intervals, a considerable amount of pollen may still have been transported from male flowers to hermaphroditic flowers. In addition,

only flowers on ramets within a 100 m × 200 m plot were ablated, but *C. simplex* plants were also present outside the plot; thus, pollen was probably also transferred from plants external to the plot. Considering these two points, I can conclude that effect of male flower excision was underestimated in our experiment. Nevertheless, the significant reduction of fruit set on the lateral racemes of hermaphroditic ramets that was observed emphasizes the importance of male flowers in the morph II population. Whether these male flowers primarily help morph II plants to overcome a pollen limitation or to attract more insects, or both, requires further investigation.

Several studies have suggested that the optimal allocation of investment to male and female functions varies in the context of a pollen limitation (Ashman et al., 2004; Burd, 2008; Ezoe and Washizu, 2009; Harder and Aizen, 2010). When the number of available pollinators across a population is low, the allocation to attractive floral organs is increased. Conversely, as the visitation increases, the optimal strategy is to reduce the allocation to attractive floral organs (Ezoe and Washizu, 2009; Harder and Aizen, 2010). In general, the blooming of male flowers increases the floral display size and functions as a pollinator attractor (Wilson and Price, 1977; Wilson, 1983; Solomon, 1985; Podolsky, 1992; Podolsky 1993; Elle and Meagher, 2000; Barrett, 2002; Vallejo-Marín and Rausher, 2007). Thus, an allocation to male flowers can be understood as allocation to attractive floral organs. Many high-quality pollinators visit the morph I population (Figures 2-3, 2-4j, 2-5j), and it produces few male racemes to contribute to the floral display (Figures 2-4d, 2-5d). In contrast, the quality of pollinators visiting morph II is low, and the pollinator quantity is unstable (Figures 2-3, 2-4k, 2-5k), but many male flowers and racemes increase the size of the floral display (Figures 2-4e, 2-5e). The relationships between reproductive system and the pollinator environment of morphs I and II of *C. simplex* are

thus consistent with the theoretical model. This needs to be clarified because the sex determination mechanism in morph II is unknown. In addition, it would be particularly important to assess male adaptation by pollen parental analysis in morph II, where male flowers are abundant.

2-5-3 High selfing rate and loss of protandry in morph III

Flower visitors to morph III were rare throughout the flowering season during the 2 years of observations (Figures 2-4l, 2-5l). This result suggests that the high selfing rate of morph III may be a consequence of the extremely low abundance of flower visitors, as previously suggested by Toji and Itino (2020). Morph III grows in a dark forest floor environment and blooms in late autumn, when the temperature is low and relatively few pollinators are active. When the pollinator visitation rate is low, floral traits that enhance selfing are likely to evolve (Darwin, 1876; Baker, 1955; Fausto et al., 2001; Kalisz and Vogler, 2003; Kameyama and Kudo, 2009).

In the examination of the flowering phenology of morph III, I found that the transition from the male phase to female phase occurred within a rather short time span (Figures 2-4c, 2-5c). This weakened protandry in morph III may have evolved to promote selfing.

According to the model proposed by Ezoe and Washizu (2009), when self-pollination of flowers can occur and the flower visitation rate is extremely low, the allocation to attractive floral organs is abandoned and the flowers specialize in producing self-pollinated seeds. As the flower visitation rate increases, the allocation to attractive floral organs also increases, but if the visitation rate continues to increase, investment in attractive floral organs eventually plateaus.

For a more detailed evaluation of this hypothesis, it is necessary to show that sex ratio

and reproductive system changes occur within each morph of *C. simplex* when the pollinator environment changes. For example, if the examination of other morph I populations revealed reduced visitation rates, in those populations, female flowers should be less common and male flowers more common. Pellmyr (1986) observed flower visits by bumblebees to morph III populations, but he did not examine differences between survey sites. Pellmyr (1986) conducted surveys at two sites located 180 km apart (straight-line distance). If high-quality pollinators such as bumblebees frequently visit a morph III population, that population might be expected to reproduce mainly by outcrossing, rather than by selfing. In a future study, our hypothesis should be tested further by conducting surveys in other mountain areas and regions and by comparing different populations of the same morphs.

2-6 Tables

Table 2-1. Differences in the ecological characteristics of the three ecotypes of *C. simplex*. Information from previous studies (Pellmyr, 1986; Kuzume and Itino, 2013; Toji and Itino, 2020) and the information obtained in this study are included.

Ecotype	Main pollinator	Altitudinal distribution	Habitat	Flowering season	Mating systems	Sex expressions
Morph I	bumblebees	1350–2370 m	Sunny open environments	Late July – Early September	Outcrossing	Hermaphrodite, Female, Andromonoecy (rare)
Morph II	butterflies, flies and syrphids	920–1500 m	Sunny forest edges	Early September – Early October	Outcrossing	Hermaphrodite, Andromonoecy
Morph III	flies and syrphids	650–1350 m	Dark forest floors	Early October – Early November	Selfing	Hermaphrodite, Andromonoecy (rare)

Table 2-2. Number of studied ramets for each sex expression in the three ecotypes of *C. simplex*.

	Morph I			Morph II		Morph III	
Year	Hermaphrodite	Female	Andromonoecy	Hermaphrodite	Andromonoecy	Hermaphrodite	Andromonoecy
2017	118	75	4	83	49	39	5
2018	127	72	4	74	47	40	3

Table 2-3. One-way ANOVA test results for seed size and the number of pollen grains per anther among ecotypes in *C. simplex*. Seed size was compared among morph I hermaphroditic ramets, morph I female ramets, morph II hermaphroditic ramets, and morph III hermaphroditic ramets. The major axis of 10 seeds per individual ramet was measured with a digital calliper, and the average was used as the seed size per individual ramet. Ten individuals were examined in each group of ramets. The number of pollen grains per anther was compared among morph I hermaphroditic ramets, morph II hermaphroditic ramets, morph II male flowers on andromonoecious ramets, and morph III hermaphroditic ramets. One unopened anther per individual ramet was selected, placed in 100 μL of methylene blue solution, and broken open with tweezers. After the pollen grains became suspended and dispersed in the solution, 1.6 μL of solution was removed and applied to a cell counter plate (WATSON). Then the pollen grains were counted under an optical microscope (with 40×). Ten ramets in each population were examined. For statistical analysis, the number of pollen grains in the diluted solution was used directly.

Source	df	ss	*F*	*p*
Seed size	3	1.37×10^{-3}	0.03	0.99
Residuals	36	5.42×10^{-1}		
Number of pollen per anther	3	359.28	0.77	0.52
Residuals	36	5624.50		

Table 2-4. Results of generalized linear model analysis using the fruit set as a response variable. Flowering start date (Time), population male sex ratio, and insect visitation rate were used as explanatory variables. Although the flowering start date usually refers to male stage flowers, in the hermaphrodite of morph I and morph II, the receptive female stage flowers opened approximately two survey days after the flowering start date. Therefore, In the case of hermaphrodite of morph I and morph II, I referred male sex ratio and the visitation rate to the values on two survey days after the flowering start date. Asterisks indicate significant level*; $P < 0.05$

Factor	Coefficient	SE	*t*	*P*
Morph I				
Hermaphrodite				
Flowering start date	-6.097×10^{-4}	1.372×10^{-4}	-0.444	0.680
Male sex ratio	6.065×10^{-2}	6.121×10^{-2}	0.991	0.378
Visitation rate	6.535×10^{-3}	6.347×10^{-3}	1.030	0.361
Female				
Flowering start date	-7.988×10^{-4}	2.414×10^{-3}	-0.331	0.763
Male sex ratio	-2.176×10^{-2}	9.863×10^{-2}	-0.221	0.840
Visitation rate	-1.787×10^{-2}	1.302×10^{-2}	-1.372	0.264
Morph II				
Hermaphrodite				
Flowering start date	-5.454×10^{-3}	1.576×10^{-3}	-3.461	0.026*
Male sex ratio	-5.465×10^{-2}	4.247×10^{-2}	-1.287	0.268
Visitation rate	8.182×10^{-4}	9.373×10^{-3}	0.087	0.935
Morph III				
Hermaphrodite				
Flowering start date	-1.077×10^{-2}	3.121×10^{-3}	-3.452	0.075
Male sex ratio	-8.426×10^{-2}	1.902×10^{-1}	-0.443	0.701
Visitation rate	8.271×10^{-2}	2.192×10^{-1}	0.377	0.742

Table 2-5. Evaluation of seed output between hermaphroditic and unisexual female ramets of morph I. Values are means (± SE). Differences were evaluated by using the Wilcoxon signed-rank tests.

		Hermaphroditic ramet		Unisexual female ramet		*P*
a	Number of racemes	1.355 (± 0.086)	(*n* = 93)	1.368 (± 0.143)	(*n* = 38)	0.975
b	Flowers per raceme	86.356 (± 4.032)	(*n* = 87)	71.923 (± 4.077)	(*n* = 52)	0.022
c	Pistils per small flower	5.542 (± 0.155)	(*n* = 48)	5.469 (± 0.229)	(*n* = 32)	0.760
d	Ovules per pistil	7.147 (± 0.171)	(*n* = 48)	6.397 (± 0.185)	(*n* = 32)	< 0.01
e	Fruit set per ramet	0.961 (± 0.013)	(*n* = 17)	0.998 (± 0.001)	(*n* = 35)	0.761
	Seed output per ramet (a ×b × c × d × e)	4453.95		3435.33		< 0.01

2-7 Figures

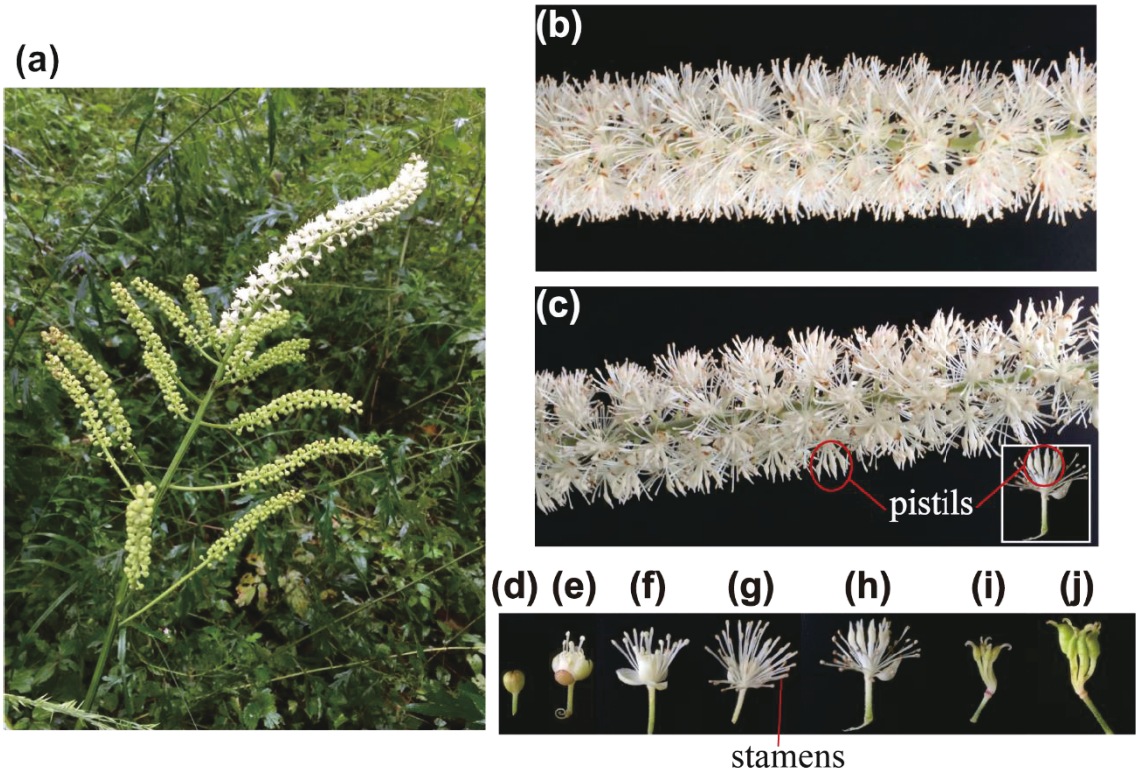

Figure 2-1. Photographs of a hermaphroditic ramet of *C. simplex* and seasonal sexual changes of the hermaphroditic raceme. (a) Morph II hermaphroditic ramet. Only the primary raceme is blooming. The lateral racemes will flower after the primary raceme has set fruit. (b) Enlarged view of the hermaphroditic raceme in the male phase. All of the many small flowers on the raceme are male. (c) Enlarged view of the hermaphroditic raceme in the female phase. Pistils protrude from all of the flowers. Some of the pistils are emphasized by red circles. (d)–(f) Flower development from bud to flowering. (g) Flower in the male phase that is beginning to release pollen. (h) Flower in the female phase that is ready to receive pollen. The pistils are markedly elongated. (i) Flower after blooming. Pistils that are not pollinated remain in this state. (j) Successfully pollinated pistils swell and form fruit.

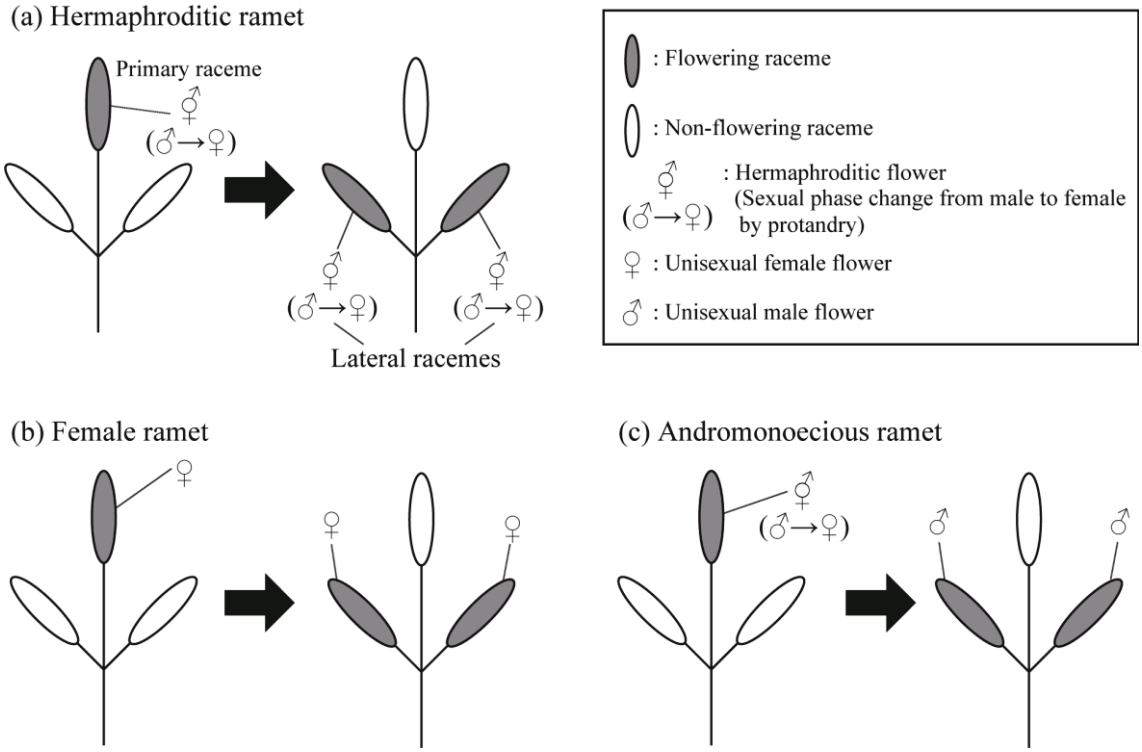

Figure 2-2. The order of flowering on a ramet. The black arrows indicate the passage of time. Regardless of the sex expression, the primary raceme of a ramet blooms first, followed by the lateral racemes. (a) A sexual phase change from male to female occurs in protandrous hermaphroditic flowers on both primary and lateral racemes. (b) Unisexual female ramet. All racemes function only as female. (c) Primary racemes of the andromonoecious ramets have hermaphroditic flowers, and the lateral racemes have unisexual male flowers. The hermaphroditic flowers of the primary raceme are protandrous.

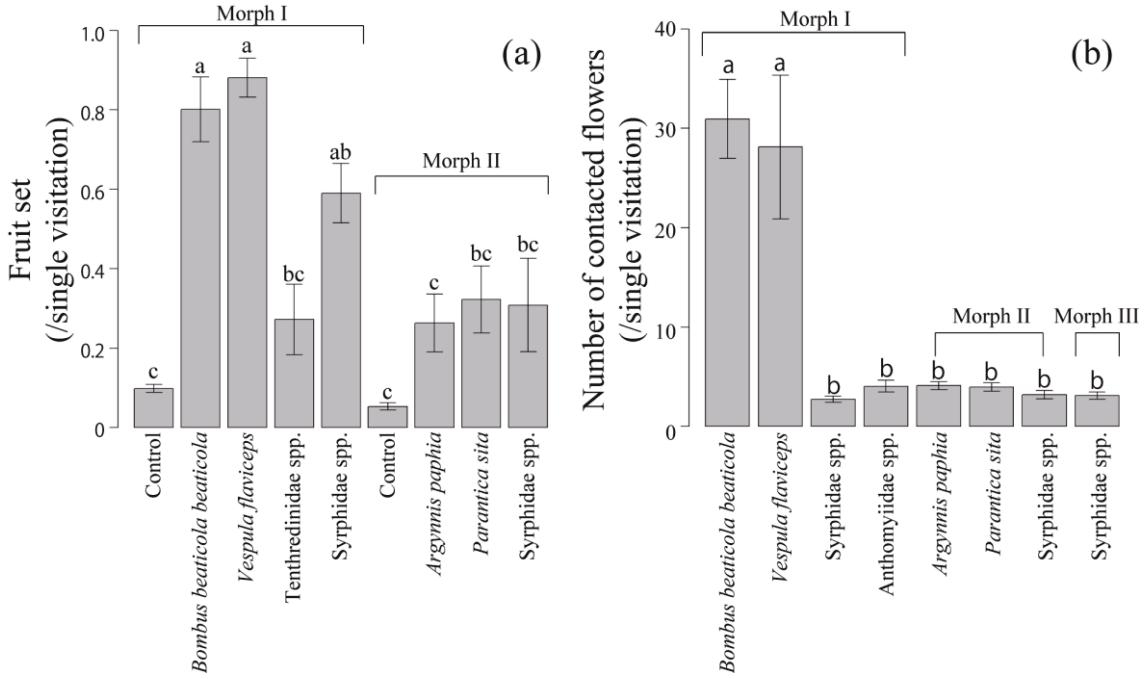

Figure 2-3. Pollinator quality in *C. simplex*. (a) Flower fruit set per single visitation of each visitor type. Only flowers that the visitor contacted were counted. Control flowers were bagged throughout the flowering season. (b) Numbers of visited flowers during a single visitation to a raceme. Different lowercase letters indicate significant differences between the populations (mean ± SE, Tukey's HSD, $P < 0.05$). Hymenoptera: *Bombus beaticola beaticola*, *Vespula flaviceps*, Tenthredinidae ssp. Lepidoptera: *Argynnis paphia*, *Parantica sita* Diptera: Syrphidae spp. Anthomyiidae spp.

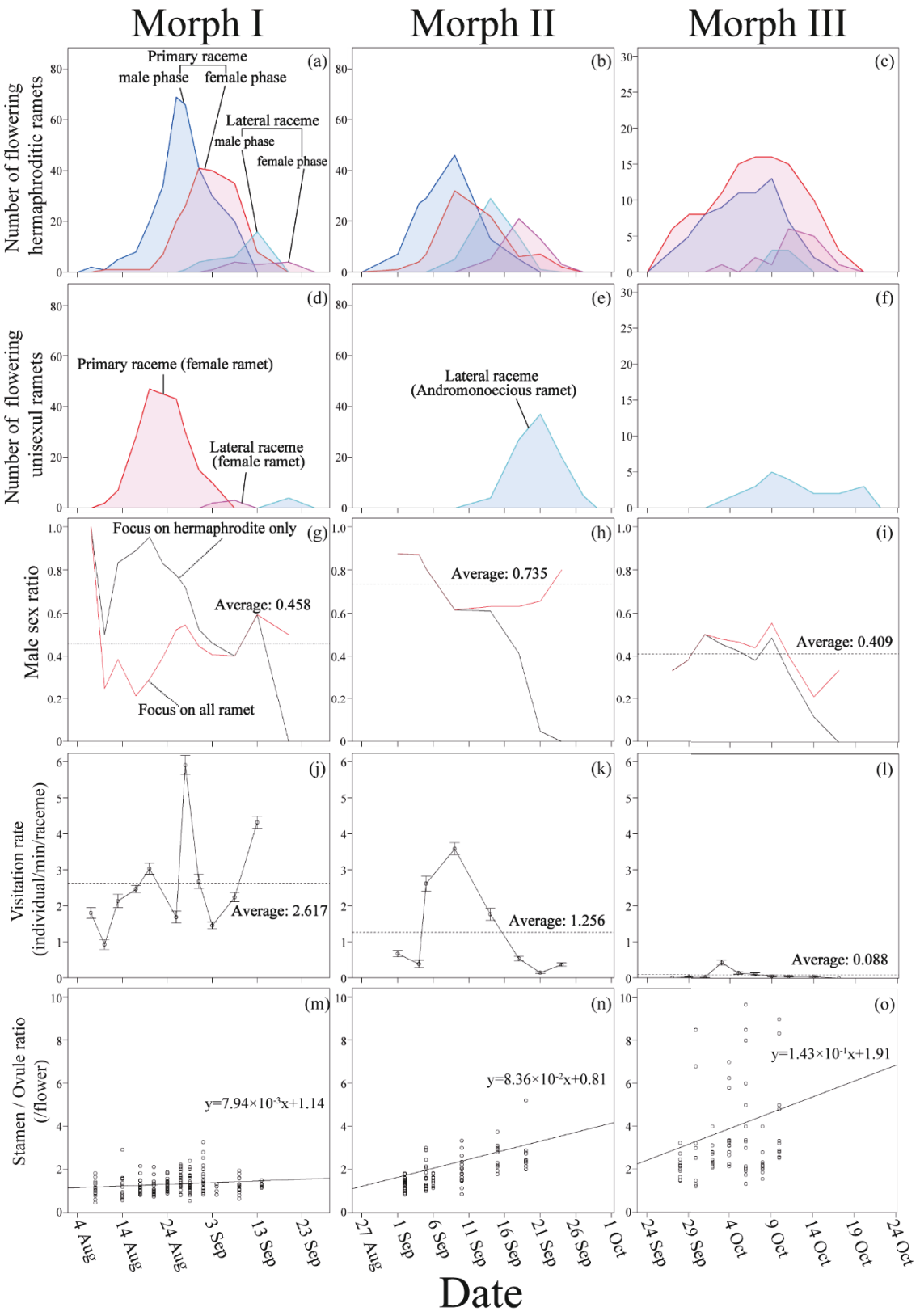

Figure 2-4. Seasonal changes in the sex ratio, the abundance of visiting insects, and the

stamen/ovule ratio of each morph in 2017. (a)–(c) Flowering phenology of hermaphroditic ramets. The numbers of flowering plants and male or female phase and racemes were counted; primary and lateral racemes were counted separately. (d)–(f) Flowering phenology of ramets with unisexual flowers. Here, the numbers of ramets with unisexual male flowers and unisexual female ramets were counted. (g)–(i) Male sex ratio considering only hermaphroditic ramets (black lines) and considering all ramets (red lines) in the population. The horizontal dashed lines represent the average male sex ratio of the population during the flowering season. (j)–(l) Seasonal variation in the abundance of visiting insects (number of insect visitors per minute). The dashed lines represent the seasonal average visitation rate of the population. (m)–(o) Seasonal changes in the stamen/ovule production ratio of hermaphroditic ramets. This ratio is an indicator of the male function (pollen) allocation of hermaphroditic ramets. The flowering start date is shown on the horizontal axis. The slope of each regression line is significant (regression coefficient significance test, $P < 0.01$).

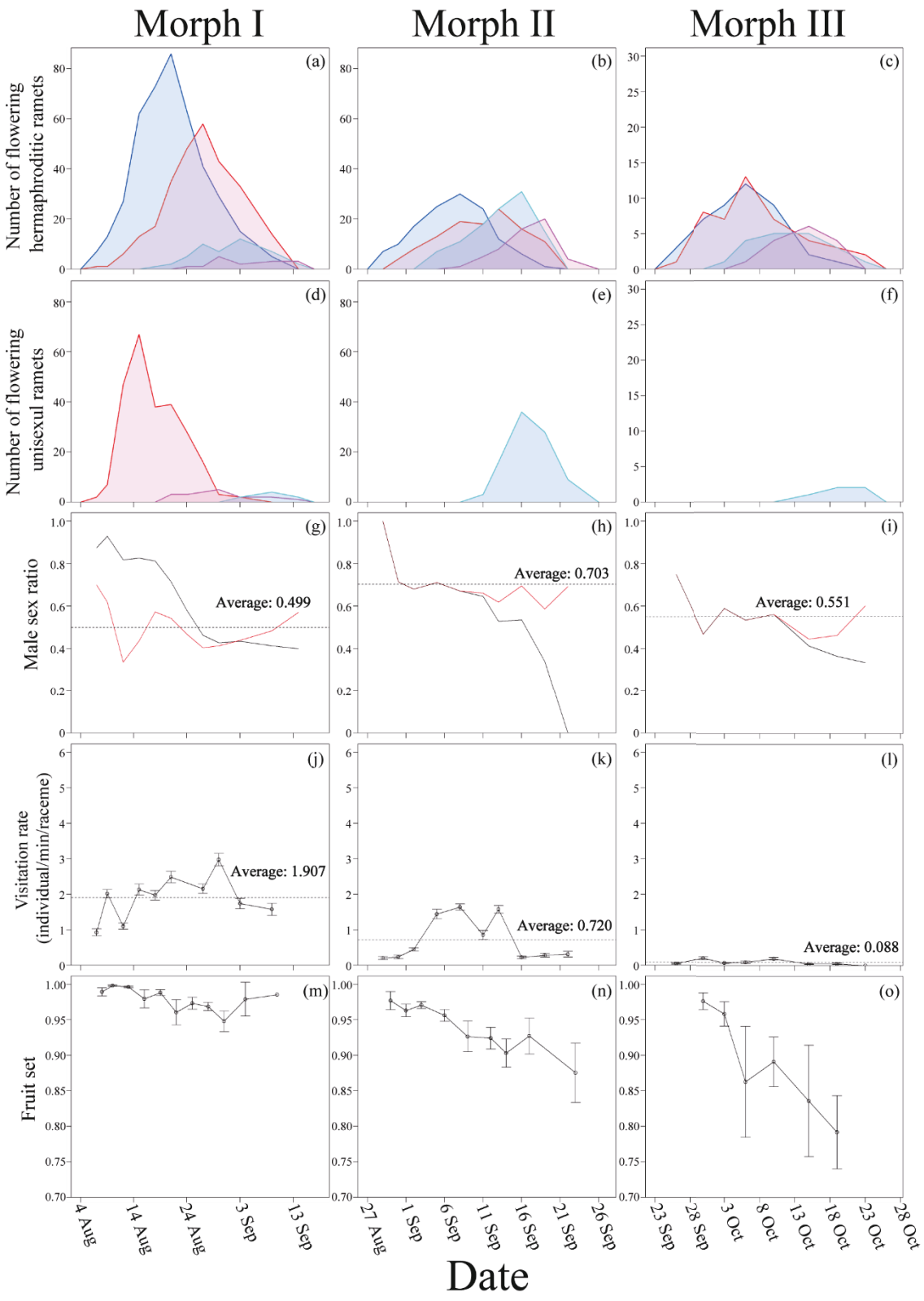

Figure 2-5. Seasonal changes in the sex ratio, the abundance of visiting insects, and fruit set of hermaphroditic ramets of each morph in 2018. (a)–(c) Flowering phenology of

hermaphroditic ramets. The numbers of flowering plants and male or female phase and racemes were counted; primary and lateral racemes were counted separately. (d)–(f) Flowering phenology of ramets with unisexual flowers. Here, the numbers of ramets with unisexual male flowers and unisexual female ramets were counted. (g)–(i) Male sex ratio considering only hermaphroditic ramets (black lines) and considering all ramets (red lines) in the population. The dashed lines represent the seasonal average male sex ratio of the population. (j)–(l) Seasonal variation in the abundance of visiting insects (number of visiting insects per minute). The dashed lines represent the seasonal average visitation rate to the population. (m)–(o) Fruit set on hermaphroditic ramets for each flowering start date.

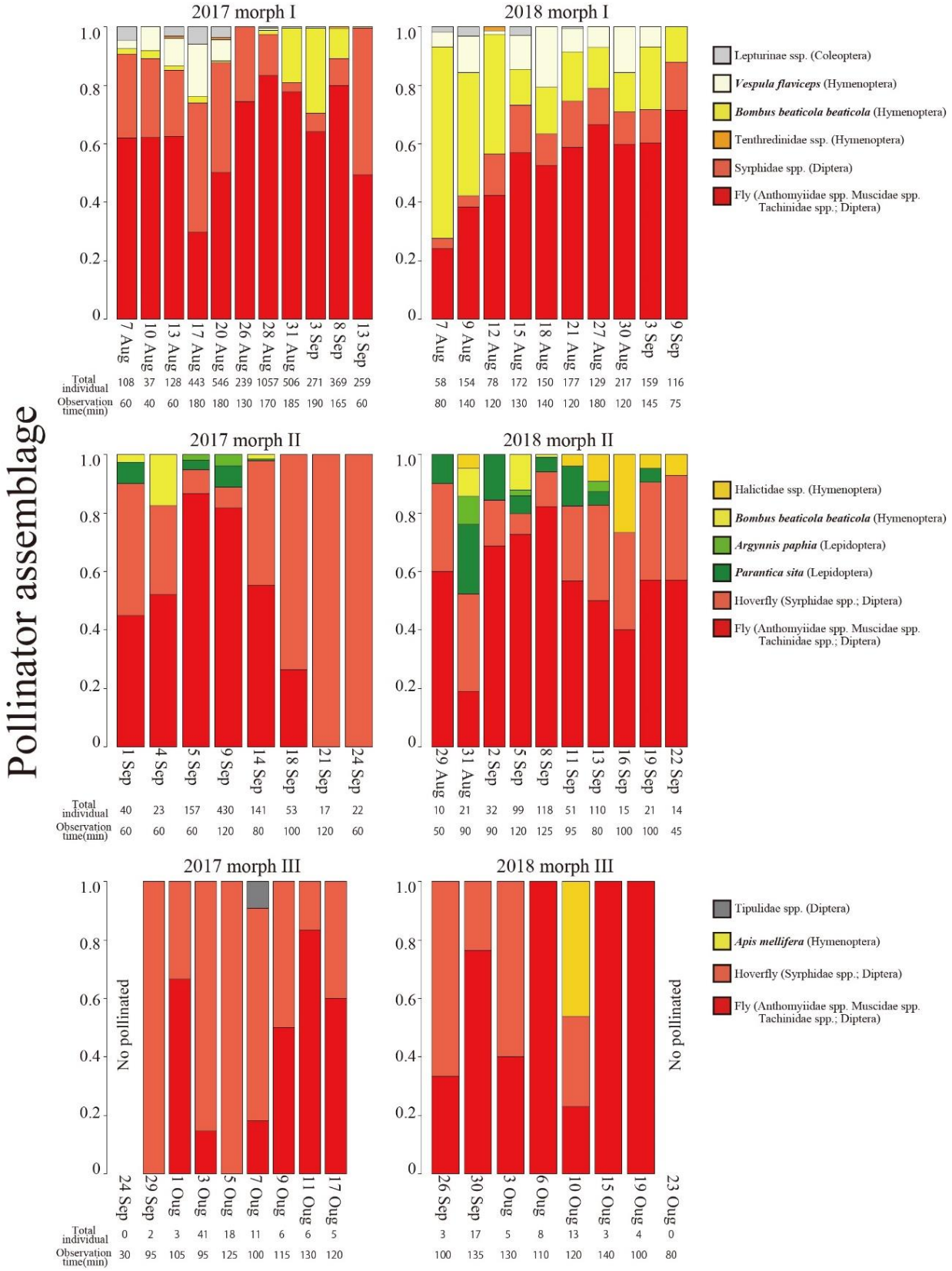

Figure 2-6. Flower visitor insects on all survey days to all populations. The insect taxa

are color-coded. The number of insects that visited and the observation time on each survey day are shown below each bar. These data were obtained at the same time as the visitation rates were measured (described in the text). The visually determined pollinator taxon was recorded.

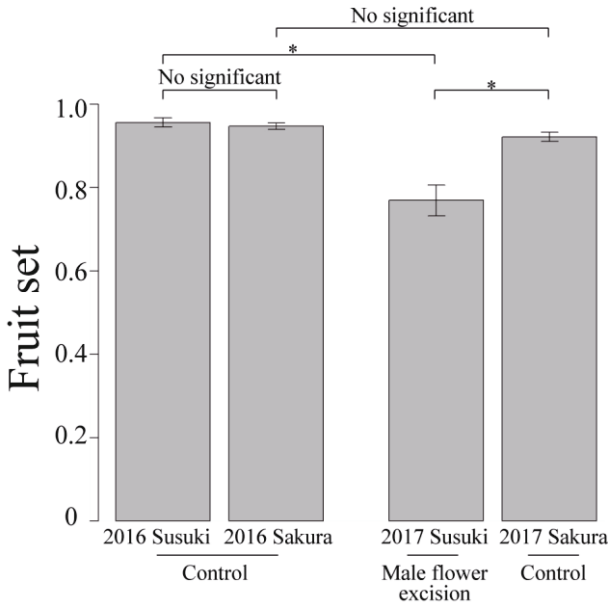

Figure 2-7. Effect of excision of unisexual male flowers on andromonoecious ramets on fruit set of hermaphroditic ramets. Fruit set of the lateral racemes of hermaphroditic ramets were measured in two populations of morph II (Susuki and Sakura). The natural fruit set at each of the two sites was measured in 2016 as a control. Then in 2017, unisexual male flowers of andromonoecious ramets were excised at Susuki, and fruit set of lateral racemes of hermaphroditic ramets was measured. As an additional control, natural fruit set of the lateral racemes of hermaphroditic ramets was again measured in 2017 at Sakura. Asterisks indicate significant differences (Wilcoxon signed-rank test, $P < 0.01$).

Chapter 3

Intraspecific convergence of floral size correlates with pollinator size on different mountains: a case study of a bumblebee-pollinated *Lamium* (Lamiaceae) flowers in Japan

3-1 Abstract

Geographic differences in floral size sometimes reflect geographic differences in pollinator size. However, I know little about whether this floral size specialization to the regional pollinator size occurred independently at many places or occurred once and then spread across the distribution range of the plant species. I investigated the relationship between the local floral size of flowers and local pollinator size in 12 populations of *Lamium album* var. *barbatum* on two different mountains in the Japan Alps. Then, using 10 microsatellite markers, I analyzed genetic differentiation among the 12 populations. The results showed that local floral size was correlated with the average size of relevant morphological traits of the local pollinators: floral size was greater in populations visited frequently by the largest flower visitors, *Bombus consobrinus* queens, than it was in other populations. I also found that the degree of genetic similarity between populations more closely reflected interpopulation geographic proximity than interpopulation similarity in floral size. Although genetic similarity of populations was highly associated with geographic proximity, floral size varied independently of geographic proximity and was associated with local pollinator size. These results suggest that in *L. album* var. *barbatum*, large floral size evolved independently in populations on different mountains as a convergent adaptation to locally abundant large bumblebee species.

3-2 Introduction

Plant–pollinator interaction, one of the main mutualistic relationships between angiosperms and animals, greatly influences the reproductive success of plants (Galen. 1996; Scobell and Scott, 2002; Dohzono et al., 2004; Herrera et al., 2006; Inoue et al., 2007; Gómez et al., 2009; Nattero et al., 2011). Floral adaptation to pollinators is thought to be a key mechanism leading to the diversification of flower traits and speciation in angiosperms (Grant and Grant, 1965; Stebbins, 1970; Galen and Newport, 1987; Johnson, 2010). Accordingly, variations in floral characteristics, including in flower shape (Gómez et al., 2006; Nagano et al., 2014), size (Hodges, 1997; Fenster et al., 2004), color (Campbell et al., 1997; Newman et al., 2012), and odor (Pellmyr, 1986; Majetic et al., 2009), have been recognized to have resulted from adaptation to pollinators. In fact, many studies have shown that geographic variation of flower traits is associated with geographic variation of pollinator assemblages (Steiner and Whitehead, 1991; Alexandersson and Johnson, 2002; Herrera et al., 2006; Anderson and Johnson, 2008, 2009; Gómez et al., 2009; Pauw et al., 2009; Anderson et al., 2010a; I. Dohzono and Suzuki, 2010; Johnson and Anderson, 2010; Thompson et al., 2013; Boberg et al., 2014; Nagano et al., 2014; Kuriya et al., 2015; Egawa et al., 2020). These have been interpreted as the consequences of adaptation of floral traits to pollinators.

Local adaptation of plants to pollinators can lead to plant speciation through the establishment of prezygotic reproductive isolation, because specialization to specific pollinators may preclude pollinator sharing between related plant lineages (Herrera et al., 2006; Anderson and Johnson, 2008; Newman et al., 2015). In fact, according to the Grant–Stebbins model of floral divergence (Grant and Grant, 1965; Stebbins, 1970; Johnson, 2010; Anderson et al., 2014), prezygotic reproductive isolation through pollinator-based selection is the main pathway of floral trait diversification. The Grant–

Stebbins model proposes that local adaptation of plants to local pollinator assemblages results in trait diversification and reinforcement of reproductive isolation. Thus, a geographic mosaic of flower visitors may promote allopatric divergence of plants leading to the emergence of different ecotypes. Accordingly, if divergence in allopatry is followed by secondary contact, I can hypothesize that local adaptation to pollinators may prevent gene flow between the two ecotypes even after the secondary contact (Pellmyr, 1986; Majetic et al., 2009). One useful approach to understanding trait diversification and speciation in angiosperms, therefore, is to combine an ecological evolutionary analysis of local plant adaptations with an analysis of population genetics to assess the degree of genetic isolation between populations. Given that about 25% of angiosperm diversification events may be associated with a shift in pollinators (Van der Niet and Johnson, 2012), this combination of analytical approaches can shed considerable light on the origin of plant diversity (Thompson, 2005, 2013). Nevertheless, researchers focusing on plant diversification have only recently begun to use these two approaches in combination (Anderson et al., 2014; Briscoe Runquist and Moeller, 2014; Van der Niet et al., 2014). In particular, knowledge of the patterns of morphological changes associated with intraspecific genetic structures can contribute to our understanding of the early stages of divergence (Anderson et al., 2014).

In this study, I posit two hypotheses to explain geographic differences in floral characteristics. The first hypothesis is ‘secondary contact’ hypothesis. It assumes that allopatric floral size differentiation occurred between populations with large-sized flowers where plants were pollinated by large pollinators, and populations with small-sized flowers where plants were pollinated by small pollinators. In this scenario, the different-sized flowers have already been reproductively isolated because of the different

pollinators, their distribution range secondarily overlapped, and currently gene flow occurs only between similar-sized flowers. The second hypothesis is 'independent local adaptation of floral size'. In contrast to 'secondary contact' hypothesis, it assumes that the local floral size is the results of current adaptation selected by local pollinator size and the gene flow occurs mainly between nearby populations because no reproductive isolation between different-sized flowers evolved yet. In this scenario, the degree of genetic similarity among populations should reflect geographic proximity rather than floral size similarity. Based on this hypothesis, I assume that the floral size has evolved independently among mountain regions.

Lamium album (Lamiaceae) is native to Europe and Asia. In Europe, it is reported to be visited mainly by bumblebees, small wild bees and honeybees (Sulborska et al., 2014). The Asian subspecies, *L. album* var. *barbatum*, is visited mainly by bumblebees (Hattori et al., 2015). In Japan, floral size varies geographically in *L. album* var. *barbatum* (Hattori et al., 2015). Flower–pollinator trait matching has been demonstrated in a Japanese population of *L. album* var. *barbatum* by Hattori et al. (2016), who observed that as the difference between bumblebee tongue length and the floral size of *L. album* var. *barbatum* becomes larger in a population, fruit set per single pollinator visit becomes smaller. Thus, I expect floral size to be greater in Japanese populations of *L. album* var. *barbatum* visited by larger pollinators, and I can expect to find a relationship between floral size and the size of relevant pollinator traits in those populations.

In this study, I investigated the relationship between floral size and pollinator size in 12 populations of *L. album* var. *barbatum* in two different mountain areas and confirmed plant–pollinator trait matching in these populations: plants in populations visited by long-tongued pollinators characteristically had long corolla tubes, whereas

plants in populations visited by short-tongued pollinators had short corolla tubes. In addition, using 10 microsatellite markers, I estimated the population genetic structures of the 12 *L. album* var. *barbatum* populations and found that floral size correlated with local pollinator size but not with the genetic similarity of populations. This finding supports convergent intraspecific floral trait evolution: the second of the two hypotheses formulated above.

3-3 Materials and Methods

3-3-1 Plant species

Lamium album L. var. *barbatum* (Lamiaceae) is a perennial herb that grows along forest edges throughout East Asia (Hayashi, 2009). It produces creamy white, two-lipped, entomophilous, and self-incompatible flowers (Sulborska et al., 2014; Hattori et al., 2015). The flowers are frequently visited by various bumblebee species, and in Japan, bumblebees are their main pollinators (Hattori et al., 2015). Flower–pollinator morphological matching has been reported to improve seed set in a population of *L. album* var. *barbatum* located near the populations of this study (Hattori et al., 2016). A bumblebee visiting a flower of *L. album* var. *barbatum* inserts its tongue into the inner part of the corolla tube to forage for nectar and in the process rubs its head and thorax against the anthers and the stigma. In addition to bumblebees, honeybees and wild bees have been observed to visit European (Poland) *L. album* flowers (Sulborska et al., 2014).

3-3-2 Study site

Populations of *L. album* var. *barbatum* were surveyed at 12 sites in two mountain areas in Matusmoto, Nagano Prefecture, the central Japan Alps. All surveys were conducted

between April and July, during the flowering season of each population, in 2018 or 2019. The two mountain areas were around Mt. Norikura, west of the Matsumoto basin (the "west area"), and around the Utsukushigahara highland, which is east of the basin (the "east area") (Figure 3-1). Each population of *L. album* var. *barbatum* was a geographically cohesive group of densely distributed plants located along a forest road in deciduous broad-leaved forest. The distance between the populations ranged from 0.4 to 52.4 km. I conducted the following measurements during the flowering peak of each population.

3-3-3 Floral size measurement

First, 18-170 individuals from each population were haphazardly selected and marked with color tape. Then, following the method of Hattori et al., (2015), I measured the floral size of 1–6 flowers per individual plant with a digital caliper (precision, 0.01 mm). The floral size was defined as the distance from the flower's base at the stem to its tip (Figure 3-2). Preliminary measurements showed that the variation of floral size among flowers on an individual plant was less than the variation among plants. Therefore, I used the average value of the measured floral sizes of 1–6 flowers on an individual plant as the floral size of that plant. I also measured plant height, as a proxy for plant resource status, of 20 haphazardly selected individuals in each population. Average floral sizes were compared between populations by using Tukey's honestly significant difference (HSD) test. In addition, I used the Moran's I test for spatial autocorrelation to determine to what degree correlations could be explained by the sampling of populations in close proximity to one another. For this test, I used the moran.test function in the "spdep" package in the R software environment ver. 4.0.2 (R Core Team, 2013).

3-3-4 Pollinator assemblages and size variation

To observe the pollinator assemblages of *L. album* var. *barbatum*, I selected the largest patch of plants (ranging in area from about 10 to 200 m^2) in each of the 12 populations and haphazardly established a 1 m × 1 m quadrat (about 100 individuals) within the patch on each census day (Table 3-1). I then recorded the insects that visited the flowers in this quadrat. Observations were made on several days between 8:00–14:00 local time, when flower visitors were active in each population. At each location, I observed all flower visitors for a total of 90–660 minutes spread over 1–4 days during the peak flowering period. Since bumblebee species (*Bombus* spp.) can be easily distinguished while they are visiting a flower, the species of each bumblebee was recorded as they visited a flower, and the observed species were recorded. In contrast, it is difficult to distinguish among *Eucera* spp. and species of small bees during their flower visits, so I estimated the species-level pollinator assemblage of these taxa from capture survey results (see below).

To define the size of each pollinator species, I measured morphological traits of each species relevant to the pollinating behavior of that species. For this survey, flower-visiting insects were haphazardly captured following their flower visitation, and the size of each of the selected traits was measured with a digital caliper (precision, 0.01 mm). *Bombus* spp., *Eucera* spp., and *Apis cerana japonica* (hereafter, "large bees") are "thrust pollinators"; they forage for nectar by thrusting their heads into flowers and extending their tongues. Thus, I defined the pollinator size of large bees as the sum of the tongue length and the head length. (Figure 3-2). In contrast, *Ceratina* spp., *Lasioglossum* spp., and *Andrena* spp. (hereafter, "small bees") are "whole-body pollinators"; they forage for nectar by crawling into the corolla tube. The small bees first land at the entrance to the flowers (upper or lower lip), and then crawl into the flowers to forage, moving through

the anthers and stigma to the nectary. As a result, pollen grains become attached to both the head and the ventral side of the abdomen of small bees; thus, I defined the pollinator size of small bees as the body length from the tip of its tongue to the caudal end of the abdomen (Figure 3-2). Nectar robbers (*Apis mellifera*, *Bombus hypocrita*, *Xylocopa appendiculata circumvolans*) and small bees on which I did not observed attached pollen grains (*Euodynerus nipanicus*, *Lasioglossum nipponense*, *L. occidens, Nomada comparata* at Onosawa, *Nomada* spp. at Onosawa) were excluded from this calculation of average pollinator size. I checked for attached pollen grains soon after a bee's visit to a flower and identified *L. album* var. *barbatum* pollen grains under a microscope (× 2–10). The bees were observed in a motionless state after anesthesia. Pollen grains were observed by visual inspection, and the pollen grains of *L. album* var. *barbatum* had a very distinct color against the body color of the bees. Pollinator size was measured separately for each plant population, even for insects of the same species. Although *B. diversus* workers were observed in the quadrat surveys at Onosawa and Norikura, and *B. honshuensis* workers at Ohmizusawa, they were not captured and their sizes in those populations were not measured. Therefore, the mean size of all *B. diversus* (*B. honshuensis*) individuals captured from the other populations was used as the size of *B. diversus* at Onosawa and Norikura (*B. honshuensis* at Ohmizusawa).

As the average pollinator size for each plant population, the weighted arithmetic mean was calculated from the relative abundance of each pollinator species in the pollinator assemblage and the size of that species:

$$\text{Average pollinator size} = \sum_{i=1}^{n} Pi(Ni/Nt)$$

where n = the total number of insect species visiting a *L. album* var. *barbatum* population

(patch), *Pi* = mean size of the *i*th insect species, *Ni* = the number of flowers in the patch that the *i*th insect species visited, and *Nt* = the number of flowers in the patch that any of the insect species visited. Thus, *Ni*/*Nt* is the relative abundance of the *i*th insect species visiting the population. For each population, average pollinator size was calculated for three groups of flower visitors: all flower visitors, only large bees, and only small bees.

3-3-5 Factors influencing local floral size

To examine factors influencing floral size, I used a linear mixed model (LMM) with a Gaussian error distribution and identity as the link function. Before this analysis, I tested the effect of the variables by likelihood ratio tests. First, I prepared a model with all variables as follows: floral size of each individual was the response variable, and the average pollinator size (all pollinators), average pollinator size (only large bees), average pollinator size (only small bees), average plant height of each population, and the altitude of each population were predictive variables. I treated the altitude as a proxy for clinal abiotic environmental changes (e.g. meteorological changes). In addition, I treated plant individual and sampling data (year and month) as random effects. The variance inflation factor (VIF) statistic was used to confirm the correlation among predictive variables with VIF = 0.5 as a threshold value (Neter et al., 1996). No VIFs above the threshold were detected. A likelihood ratio test using the parametric bootstrap method (Hoel et al., 1971) was performed for models that included all variables and models that lacked one of each predictive variable and random effect. Variables were selected from the difference in deviance between the models obtained by 1000 bootstrap calculations. As a likelihood ratio test results, the average pollinator size (all pollinators), average pollinator size (only large bees) and altitude remained as predictive variables.

The LMM analysis was performed with the lmer function in the "lme4" package in the R software environment ver. 4.0.2 (R Core Team, 2013). I further conducted a model selection approach based on AIC. First, I performed model selection on the entire dataset using brute force approach (trying every possible model), starting from a global model including all remained predictive variable by likelihood ratio test, and plant individual and sampling data (year and month) as random effects. These are the explanatory variables that were judged to be valid in the likelihood ratio test results. I then compared the global model with all simpler models based on AIC (i.e. comparing all the combinations of explanatory variables) using the dredge function in the "MuMIn" package in the R software environment ver. 4.0.2 (R Core Team, 2013). This function returned the model with the lowest Akaike information criterion (AIC), and I adopted this model (Table 3-2). The results of this model selection procedure informed which average pollinator size variable (all pollinators or only large bees) was used in a least-squares regression analysis. Using these results, therefore, I explored covariation between corolla tube length and the average pollinator size of only large bees across populations by a least-squares regression analysis.

3-3-6 Genetic similarities of *Lamium album* var. *barbatum* populations

To examine the genetic structure of *L. album* var. *barbatum*, I used 10 polymorphic microsatellite primers originally developed for *L. album* (Horsley, 2013) (Table 3-3). For this analysis, fresh leaf material was collected randomly from 8–16 individual plants in each of the 12 *L. album* var. *barbatum* populations during 2018–2019. DNA was extracted by the CTAB method (Doyle and Doyle, 1990), and the extracted DNA was diluted or concentrated to a final concentration of 10 μg/ml.

Each of the forward microsatellite primers was synthesized after adding one of four different universal fluorescent sequences: 5'-GCCTCCCTCGCGCCA-3', 5'-GCCTTGCCAGCCCGC-3', 5'-CAGGACCAGGCTACCGTG-3', or 5'-CGGAGAGCCGAGAGGTG-3' (Blacket et al., 2012). Polymerase chain reaction (PCR) analyses were performed in a thermal cycler using a reaction mixture consisting of 1 μl template DNA, 3 μl of 2 × Type-it Microsatellite PCR Kit (QIAGEN, Valencia, California, USA), 0.7 μl of 0.1 μM forward primer, 0.7 μl of 0.2 μM reverse primer, and 0.7 μl of 0.1 μM fluorescent-labeled universal primer. The DNA amplification program consisted of an initial denaturation step of 5 min at 95 °C, followed by 35 cycles at 95 °C for 30 s, 60 °C for 90 s, and 72 °C for 30 s, and final elongation at 60 °C for 30 min. The PCR products were detected by using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA) and GeneScan™ 500 LIZ™ dye Size Standard (Applied Biosystems). Fragment lengths were calculated with GeneMapper version 4.0 software (Applied Biosystems).

I tested two analysis of molecular variance (AMOVA) models estimating the percentage of molecular variance accounted for by each level of the nested sampling hierarchy. First model, 12 populations were divided according to the two mountain areas (east or west areas). Second model, 12 populations were divided the six floral size groups. Floral size groups were constructed based on the results of Tuley's HSD comparison of the average floral size among the populations. Floral size groups were divided into six groups with significantly different flower sizes (see Table 3-1, alphabet a, b, c, d, e, fg). AMOVA was run using Arlequin ver 3.5.2.2 (Excoffier and Lischer, 2010). The significance of variance components in the AMOVA models was tested by 1000 random permutations.

In addition, a Bayesian clustering analysis of the fragment length datasets was performed with STRUCTURE software version 2.3.4 (Pritchard et al., 2000; Falush et al., 2003). I used this analysis to determine the genetic cluster to which each individual is assigned. Simulations were conducted with 100 k burn-in iterations and 100 k Markov chain Monte Carlo repetitions. The number of genetic clusters (K) was calculated 10 times for each of 1–12, and the ΔK value (Evanno et al., 2005) was used as the criterion for selecting the appropriate number of clusters, that is, the number of genetic clusters from which the 12 populations of *L. album* var. *barbatum* were derived.

3-4 Results

3-4-1 Geographic variation of floral size

I found that floral size of *L. album* var. *barbatum* and the pollinator assemblage greatly differed among populations (Tukey's HSD, $P < 0.05$; Table 3-1). There was no spatial autocorrelation of average floral size between populations (Moran's I = –0.028; $P = 0.332$).

3-4-2 Pollinator size variation

In the survey of insect visitors, large bees, small bees (whole-body pollinators), small bees (without attached pollen grains), and nectar robbers were observed (Table 3-4). In particular, only small bees visited flowers of the Shimashima I population. In contrast, only large bees visited flowers of the Ougisawa and Hirokoba populations. In our analysis, I treated only the first two groups as valid pollinators. The average pollinator size varied among populations: for all pollinators (first two groups only), it was 10.05–24.91 mm; for large bees, it was 12.08–26.72 mm, and for small bees, it was 8.85–12.26 mm (Table 3-1). The largest bees were queens of *Bombus consobrinus*, which were observed in

particularly high proportions in the Mitsumata, Ougisawa, and Hirokoba populations (Table 3-4). Bees that were not considered to contribute to pollination were excluded from the size measurements. These included small bees without attached pollen grains (*E. nipanicus*, *L. nipponense*, *L. occidens*, *N. comparata*, and *Nomada* spp.), which were observed only at Onosawa and Fujiidani, and nectar robbers (*A. mellifera*, *B. hypocrita*, *X. appendiculata circumvolans*), which forage for nectar by drilling a hole in the lower part of the corolla tube (Table 3-4).

3-4-3 Factors influencing local floral size

As a variable selection result, average pollinator size (only small bees) and plant height were selected as ineffective variables, so these variables were excluded from LMM analysis (Likelihood ratio test, $P < 0.01$). The model with the lowest Akaike information criterion (AIC) value and occupied high weight was that in which the average pollinator size (only large bees) was included as only predictive variable (Table 3-2). In this model, the average pollinator size (only large bees) was a statistically significant variable (Table 3-5). By a regression analysis between floral size and the average pollinator size (only large bees), I detected a strong relationship (least squares regression, $R^2 = 0.807$, LMM, $P < 0.001$; Figure 3-3)

3-4-4 Genetic structure of *Lamium album* var. *barbatum* populations

The analysis of molecular variance (AMOVA) result based on 10 microsatellite loci also indicated a significant difference in genetic structure between the two mountain areas (Table 3-6; $\Phi_{CT} = 0.031$; $P < 0.022$). However, in the AMOVA result, most of the genetic variation was detected within populations (79.56%) and among populations within areas

(17.31%). In the STRUCTURE analysis result, the most appropriate number of genetic clusters was $K = 2$ (Figure 3-4a), and, for the most part, the populations in the east area were found to differ genetically from those in the west area (Figure 3-4b). However, the Shimashima I population, although located in the west area, was genetically closer to populations in the east area, whereas the Fujiidani population, which was in the east area, was genetically closer to populations in the west area.

3-5 Discussion

3-5-1 Relationship between floral size and pollinator size

Both the floral size and pollinator assemblages of *L. album* var. *barbatum* showed geographic variations (Table 3-1; Figure 3-1), but the lack of any spatial autocorrelation of floral size suggests that populations that are spatially close are not necessarily similar in floral size. In fact, the model that best explained floral size of a population was that in which the average size of large bees was the only explanatory variable (Table 3-5). Moreover, in the regression analysis of the 12 populations, floral size was strongly correlated with the average size of large bee pollinators (Figure 3-3).

Unlike large bees, small bees can forage successfully in flowers with both short and long corolla tubes because they crawl into the flower tube to forage. Therefore, a match between the body size of small bees and floral size is not necessary for successful pollination. Interspecific variation in body size and tongue length is a prominent feature of large bees, *Bombus* spp., and many studies have demonstrated correlations between floral size in a plant species and the *Bombus* species composition of its pollinator assemblage (Dohzono et al., 2004; Suzuki et al., 2007; Nagano et al., 2014; Kuriya et al., 2015). Our results indicate that in *L. album* var. *barbatum*, floral size at a particular

location is correlated with the local average body size of large bees. However, it is possible that the correlation between floral size and local pollinator size reflects selection on a co-varying characteristic or selection mediated by other agents (Wade and Kalisz, 1990). In this context, the observation that the correlation between floral size and local pollinator size was associated with seed set per single visit by a bumblebee in a *L. album* var. *barbatum* population at Norikura (Hattori et al., 2016) is good evidence that variation in this floral trait represents an adaptation to pollinator size.

At the Mitsumata and Hirokoba locations, the herb *Meehania urticifolia*, which has a long corolla tube (over 40 mm), was abundant, and *B. consobrinus* queens visited the flowers of this herb during its flowering season, just prior to that of *L. album* var. *barbatum*. Similarly, at Ougisawa, the shrub *Weigela hortensis*, which also has a long corolla tube, blooms a little earlier than *L. album* var. *barbatum*, and *B. consobrinus* queens were observed to visit flowers of both species (T. Toji personal observation). Thus, at sites with populations of *L. album* var. *barbatum* flowers having long corolla tubes, other flower species also tended to have long corolla tubes. These observations suggest that the local evolution of long floral size in *L. album* var. *barbatum* may reflect interactions with large bumblebees in these local areas.

Our results add to this classic flower-pollinator trait matching result (Herrera et al., 2006; Nagano et al., 2014), and I show that the selection by flower visitors is the evolutionary background of change in floral size (Figure 3-3). The mechanism through which pollinators exert the selective pressures have been shown to be selection in pollen export. A recent meta-analysis has shown that the amount of investment in petals evolves via strong competition for pollen export (Paterno et al., 2020). It is conceivable that this complex of factors may have resulted in selection for floral size. However, the very strong

linear relationship between floral size and pollinator size still suggests that selection by flower visitors is the evolutionary background of floral size (Figure 3-3).

3-5-2 Genetic structure and independent floral size adaptation

The STRUCTURE analysis and AMOVA results suggest that, in general, populations within each mountain area were more closely related to each other than they were to populations in the other mountain area (Table 3-6; Figures 3-4, 3-5). The largest flower visitors, *B. consobrinus* queens, visited four populations, Ohmizusawa, Mitsumata and Ougisawa in the west area and Hirokoba in the east area, and floral size in these four populations was significantly longer than it was in other populations (Table 3-1). However, in the genetic clustering analysis results, Ohmizusawa, Mitsumata and Ougisawa belonged to one of the two genetic clusters detected whereas Hirokoba belonged to the other (Figure 3-4). This result suggests that floral size in *L. album* var. *barbatum* evolved independently in each genetic cluster.

The large genetic gap between the Shimashima I and Shimashima II populations is interesting because these two populations are only 0.4 km apart in straight line distance (Figure 3-1). This genetic difference may reflect a history of colonization. In these two populations, *L. album* var. *barbatum* plants bloom at different times of the year (Table 3-1), and the pollinator assemblages and floral size distributions also differ between them. Given these differences in the timing of flowering and in the flower visitor assemblages, I infer that these populations are able to maintain genetic independence despite their proximity. Similarly, in Matsumoto, Japan, the shrub *Cimicifuga simplex* comprises multiple parapatric ecotypes that appear to be maintained by differences in the flowering season and flower visitor assemblage among the ecotypes (Pellmyr, 1986; Toji and Itino,

2020). Further study is needed to determine what factors maintain the genetic differentiation between the Shimashima I and II populations in *L. album* var. *barbatum*. Although Shimashima I is located in the west area, it is genetically more closely related to populations in the east area. Similarly, Fujiidani is in the east area but is genetically more closely related to populations in the west area (Figure 3-4b). Clear evidence to explain these discrepancies in the genetic structure of these populations is currently lacking.

The most striking aspect of our results is that the evolutionary geographic mosaic displayed by flower tube length variation reflects the regional distribution of the large bumblebee *B. consobrinus*, whereas the genetic similarity among populations reflects geographic proximity rather than flower trait similarity. Our results thus support the second hypothesis (floral size ecotypic 'speciation' did not occur, and trait divergence is independent of population genetic structure: convergent intraspecific floral trait evolution) proposed in the introduction. Sympatric ecotypic divergence in different mountain areas in Japan has also been reported in the alpine herb *Potentilla matsumurae* (Hirao et al., 2019). In this species, two ecotypes have been found, one favoring growth in fellfields and the other favoring growth in snowbeds. This ecotype divergence has occurred independently in at least two geographically separated mountain areas in Japan (Hokkaido and Tohoku), and the different ecotypes in the same region are genetically close. This pattern is similar to the results of this study. Thus, the independent divergence of floral traits can be detected by comparing floral traits and genetic structures across mountain ranges.

3-6 Conclusions

I presented evidence for convergent intraspecific floral trait evolution by showing that changes in floral morphology in populations of *L. album* var. *barbatum* were associated with a shift to a morphologically different pollinator assemblage, but did not reflect the degree of genetic relatedness among the *L. album* var. *barbatum* populations. This study showed that a comparative approach to plant traits and genetic structure between mountain areas can be useful for demonstrating intraspecific genetic divergence and convergence of plant traits. To verify the Grant-Stebbins model, described in the introduction, it will be necessary in the future to examine a larger clade with more transitions in pollinating systems together with information on pollinator ranges, plant migration patterns (biogeography), and the direction of pollination system transitions (Van der Niet et al., 2014).

3-6 Tables

Table 3-1. Survey results from the 12 *L. album* var. *barbatum* populations. Pollinator visitation frequencies in each 1 m × 1 m quadrat during the indicated observation time. The census days of each population are within the approximate peak flowering period of that population. Different lowercase letter superscripts to average floral size indicate significant differences between the populations (Tukey's honestly significant difference (HSD) test, $P < 0.05$)

	Location											
	West area							East area				
	Shimashima I	Shimashima II	Ohmizusawa	Onosawa	Mitsumata	Norikura	Ougisawa	Fujiidani	Santanda	Ushifuse	Nakayamasawa	Hirokoba
Visitation frequency												
Small bees (whole-body pollinators) total	7	87	189	20	7	13	-	29	26	28	16	-
Large bees (thrust pollinators) total	0	7	63	3	28	61	4	33	2	16	33	2
Eucera ssp. & *Apis* ssp.	-	7	-	-	-	-	-	33	2	16	-	-
Bombus ardens worker	-	-	-	-	-	12	-	-	-	-	-	-
B. honshuensis worker	-	-	14	-	-	40	-	-	-	-	4	-
B. honshuensis queen	-	-	29	-	1	-	-	-	-	-	4	-
B. diversus worker	-	-	-	3	-	1	-	-	-	-	12	-
B. diversus queen	-	-	-	-	-	-	-	-	-	-	1	-
B. consobrinus worker	-	-	3	-	-	8	2	-	-	-	12	1
B. consobrinus queen	-	-	17	-	27	-	2	-	-	-	-	1
Observation time (min)	540	230	600	270	215	357	90	450	450	410	660	130

Visitation rate (individual/h)	0.78	26.35	31.50	5.78	17.58	22.69	5.33	12.67	4.00	8.78	7.45	1.85
Average pollinator size (mm, all visitors)	11.38	11.78	13.41	10.05	23.61	14.05	24.91	12.44	11.91	12.57	15.72	23.46
Average pollinator size (mm, only large bees)	-	13.45	19.11	17.48	26.72	15.43	24.91	12.66	12.08	13.12	17.73	23.46
Average pollinator size (mm, only small bees)	11.38	11.65	11.60	11.11	11.18	8.85	-	12.20	11.89	12.26	11.59	-
Average floral size (mm ± SD)	25.91 (± 0.57)[a]	28.57 (± 0.90)[d]	29.21 (± 1.44)[e]	27.71 (± 0.76)[c]	30.53 (± 1.26)[fg]	28.55 (± 1.03)[d]	31.12 (± 0.92)[g]	27.06 (± 0.86)[b]	25.93 (± 0.79)[a]	26.78 (± 0.84)[b]	28.32 (± 1.35)[d]	30.01 (± 1.18)[f]
Census days	4 Apr–16 May 2018	20 May–6 Jun 2018	11 May –18 Jun 2018	1-6 Jun 2019	4–14 Jun 2019	17 Jun–9 Jul 2018	26 Jun–3 Jul 2019	17–23 May 2019	2–30 May 2019	10–31 May 2018	1–12 Jun 2019	21 Jun–1 Jul 2019

Table 3-2. Results of the LMM model selection using the dredge function in the "MuMIn" package.

Predictive variables and coefficients								
Intercept	Altitude	Average pollinator size (all pollinators)	Average pollinator size (only large bees)	Degrees of freedom	Log likelihood	AIC	Delta	Weight
23.07			0.3076	5	-5215.281	10440.6	0	0.904
23.02		0.03595	0.2792	6	-5216.528	10445.1	4.49	0.096
23	0.0001866		0.2998	6	-5222.382	10456.8	16.2	0
22.99	0.00009301	0.03134	0.279	7	-5224.185	10462.4	21.81	0
23.68		0.3109		5	-5334.679	10679.4	238.8	0
23.63	0.0001612	0.3025		6	-5341.986	10696	255.41	0
25.15	0.002868			5	-5542.36	11094.7	654.16	0
28.16				4	-5684.964	11377.9	937.37	0

Table 3-3. Information on the 10 microsatellite markers used in this study. These markers were developed by Horsley (2013).

Marker name		Primer sequences (5'-3')	Repeat motif	Size range	GenBank accession no.
LA5	F:	tgccaaacggcccatattc	aat	218-315	KC621919
	R:	actgaatttgcacagtgatcttg			
LA7	F:	gaagcctagtgaggcggtg	aag	190-221	KC621925
	R:	ctccctaagtcgtttctcgtg			
LA16	F:	agtcacatggaactgatggaag	aat	324-372	KC621927
	R:	ctgtacggcgcagatttcg			
LA25	F:	ggaagggatgtcagtcaggg	aatt	276-337	KC621921
	R:	gttggctcctgtaagatgcac			
LA34	F:	cgtacgctacaggcagaac	att	246-258	KC621926
	R:	agacacaatgctagccatcc			
LA35	F:	tctccactcgttaatcgcac	aatc	220-274	KC621923
	R:	attacatgatgggattaggacaac			
LA54	F:	caactggtgaagaccatcgc	acat	262-342	KC621922
	R:	gacaattctcgctccaaccg			
LA55	F:	tccagagcttcccgatacc	acat	241-273	KC621924
	R:	actatggcgctcagcaaatg			
LA58	F:	tcatcacaagaaatggtcgacag	agc	110-182	KC621929
	R:	cctgcgagtcgttgtttcc			
LA63	F:	agcctcgaacactgactcc	att	213-257	KC621928
	R:	cactcactctgccaatagcc			

Table 3-4. Sizes of the captured flower visitors (pollinators) in each population (mean ± SE). Insects using the "whole-body" visitation mode are small bees, and those using the "thrust" visitation mode are large bees (See Table 3-1). Castes of *Bombus* spp. are indicated by W, worker, or Q, queen. An asterisk following the species name indicates that no pollen grains were found on the bodies of insects of that species.

West area				
Location	Species	Visitation mode	Insect size (mm, mean ± SE)	*N* =
Shimashima I	*Ceratina megastigmata*	Whole-body	7.27 ± 0.07	2
	Ceratina japonica	Whole-body	11.75 ± 0.36	13
	Lasioglossum occidens	Whole-body	14.8	1
Shimashima II	*Lasioglossum speculinum*	Whole-body	6.98	1
	C. japonica	Whole-body	12.58 ± 0.56	5
	Eucera nipponensis	Thrust	13.45	1
Ohmizusawa	*Andrena liridiloma*	Whole-body	12.04	1
	Bombus honshuensis Q	Thrust	16.16 ± 0.16	2
	Bombus consobrinus W	Thrust	19.74 ± 0.40	3
	B. consobrinus Q	Thrust	27.26 ± 0.26	3
	Bombus hypocrita Q*	Nectar robber	16.62	1
	C. japonica	Whole-body	12.24 ± 0.24	16
	Ceratina megastigmata	Whole-body	11.19 ± 0.18	26
Onosawa	*Bombus diversus* Q	Thrust	21.76	1
	C. japonica	Whole-body	12.05 ± 0.31	14
	C. megastigmata	Whole-body	11.32 ± 0.24	8
	L. speculinum	Whole-body	8.13 ± 0.15	5
	*Lasioglossum nipponense**	Whole-body	9.52	1
	*L. occidens**	Whole-body	12.87	1
	Nomada spp.*	Whole-body	5.77	1
Mitsumata	*B. consobrinus* Q	Thrust	27.09 ± 0.62	10
	B. honshuensis Q	Thrust	16.62	1
	C. megastigmata	Whole-body	12.97 ± 0.19	2

	L. speculinum	Whole-body	7.6	1
Norikura	*Andrena omogensis*	Whole-body	8.85	1
	Bombus ardens W	Thrust	11.01 ± 0.50	4
	B. consobrinus W	Thrust	21.18 ± 0.61	6
	B. honshuensis W	Thrust	15.56 ± 1.10	9
Ougisawa	*B. consobrinus* W	Thrust	21.95	1
	B. consobrinus Q	Thrust	27.86	1

East area				
Location	Species	Visitation mode	Insect size (mm, mean ± SE)	*N* =
Fujiidani	*Apis cerana japonica*	Thrust	12.55 ± 0.19	8
	B. ardens W	Thrust	11.47	1
	B. hypocrita Q*	Nectar robber	13.73	1
	C. japonica	Whole-body	11.93 ± 0.21	7
	*Euodynerus nipanicus**	Whole-body	no mesured	1
	Euc. nipponensis	Thrust	11.94 ± 0.25	8
	Eucera spurcatipes	Thrust	14.05 ± 0.21	5
	Lasioglossum mutilum	Whole-body	14.09	1
	*Nomada comparata**	Whole-body	no mesured	1
	*Xylocopa appendiculata circumvolans**	Nectar robber	13.85 ± 0.34	8
Santanda	*Apis mellifera**	Nectar robber	9.45 ± 0.15	26
	C. japonica	Whole-body	12.21 ± 0.15	19
	Euc. nipponensis	Thrust	12.08 ± 0.91	4
	L. speculinum	Whole-body	5.88	1
	*X. appendiculata circumvolans**	Nectar robber	9.85 ± 0.18	3
Ushifuse	*A. mellifera**	Nectar robber	9.08 ± 0.15	15
	B. hypocrita Q*	Nectar robber	14.5	1
	C. japonica	Whole-body	12.83 ± 0.18	21

	C. megastigmata	Whole-body	9.24 ± 1.10	4
	Euc. nipponensis	Thrust	11.85 ± 0.11	2
	Euc. spurcatipes	Thrust	13.44 ± 0.15	8
Nakayamasawa	*Ap. mellifera**	Nectar robber	9.60 ± 0.05	2
	B. consobrinus W	Thrust	17.71 ± 0.87	3
	B. consobrinus Q	Thrust	27.69 ± 0.90	2
	B. diversus W	Thrust	17.48 ± 0.48	8
	B. diversus Q	Thrust	23.46 ± 0.90	3
	B. honshuensis W	Thrust	13.53 ± 0.09	2
	B. honshuensis Q	Thrust	16.31	1
	B. hypocrita W*	Nectar robber	11.39 ± 0.30	8
	B. hypocrita Q*	Nectar robber	15.58 ± 0.36	9
	C. japonica	Whole-body	11.91 ± 0.24	2
	C. megastigmata	Whole-body	11.27 ± 0.26	2
	*X. oppendiculata circumvolans**	Nectar robber	13.56	1
Hirokoba	*B. consobrinus* W	Thrust	19.62	1
	B. consobrinus Q	Thrust	27.3	1

Q: queen

W: worker

*: no pollen grains were found on the bodies of these insects

Table 3-5. Outcome of the linear mixed model with the lowest AIC value (Table 3-2). Testing the effect of the average pollinator size (only large bees) to floral size of *L. album* var. *barbatum*.

Factor	Coefficient	SE	*t*	*P*-value
Intercept	23.07	0.532	43.39	0.009
average pollinator size (only large bees) (mm)	3.076×10^{-1}	8.031×10^{-3}	38.31	2.00×10^{-16}

Table 3-6. Analysis of molecular variance (AMOVA) results for the 12 *L. album var. barbatum* populations.

Source of variance	df	SS	Variation (%)	Φ statistic	*p*-value
Among mountain areas: west and east area	1	34.52	3.13	$\Phi_{CT} = 0.031$	0.022
Among populations within areas	10	170.24	17.31	$\Phi_{SC} = 0.179$	<0.001
Within populations	494	832.75	79.56	$\Phi_{ST} = 0.204$	<0.001
Among floral size groups: based on Tukey's HSD	5	92.231	-1.10	$\Phi_{CT} = -0.001$	0.621
Among populations within floral size groups	6	112.528	20.28	$\Phi_{SC} = 0.201$	<0.001
Within populations	494	832.749	80.81	$\Phi_{ST} = 0.192$	<0.001

3-8 Figures

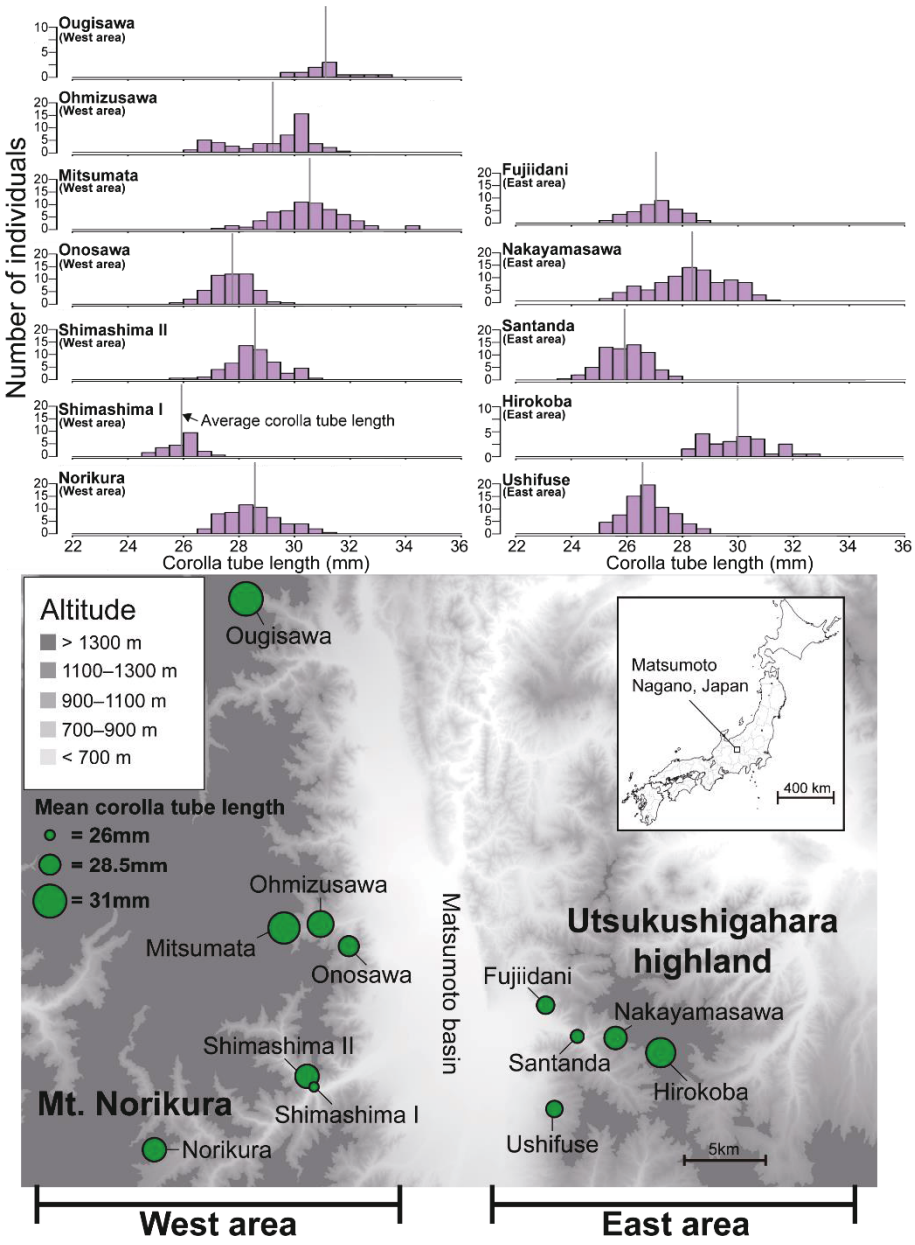

Figure 3-1. Study sites and mean floral size in each population. Distribution of floral size in the 12 populations (top) and the locations of the studied *L. album* var. *barbatum* populations (bottom). The vertical gray line in each histogram indicates the average floral size in that population. The size of the circles on the map indicates the average floral size of the indicated population. The west area comprises populations in the Mt. Norikura region, and the east area comprises populations in the Utsukushigahara highland region.

This map is based on the Digital Topographic Map published by Geospatial Information Authority of Japan (https://www.gsi.go.jp/).

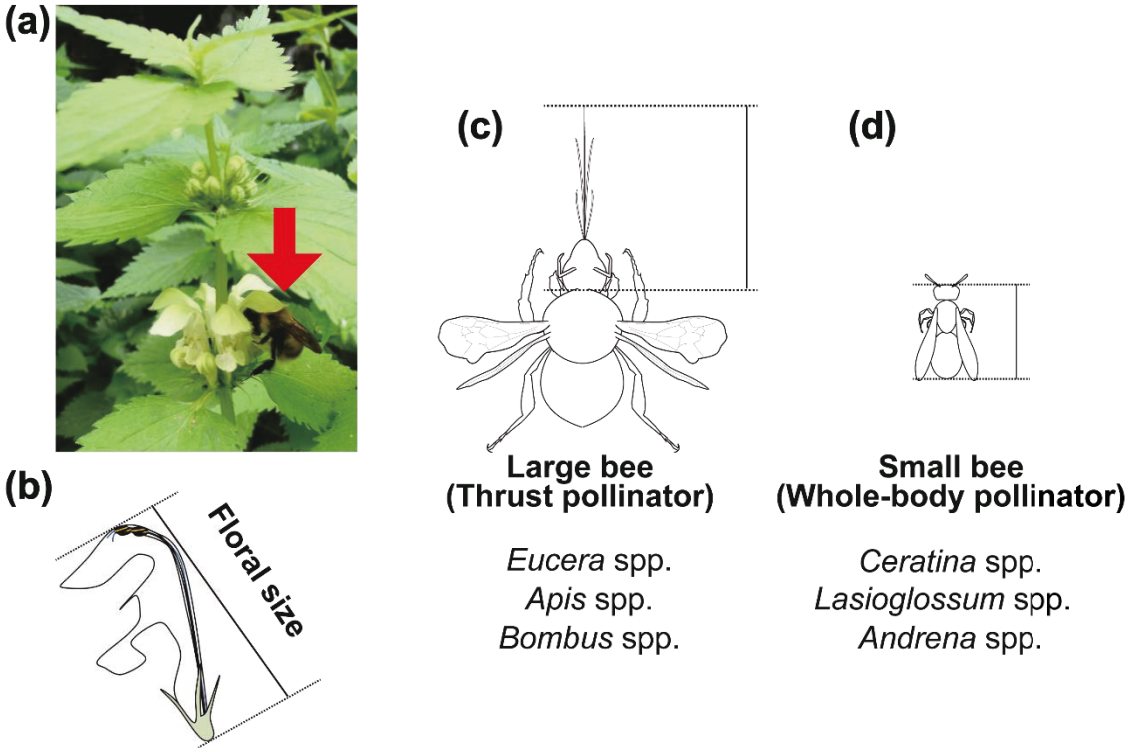

Figure 3-2. *L. album* var. *barbatum* flowers and pollinators. (a) A *Bombus consobrinus* queen (red arrow) visiting a *L. album* var. *barbatum* flower in the Mitsumata population. (b) Measurement of floral size. (c) Mouthpart measurement in large bees that forage for nectar by thrusting their head into the flowers. (d) Measurement of body size of small bees that forage for nectar by crawling into the flowers.

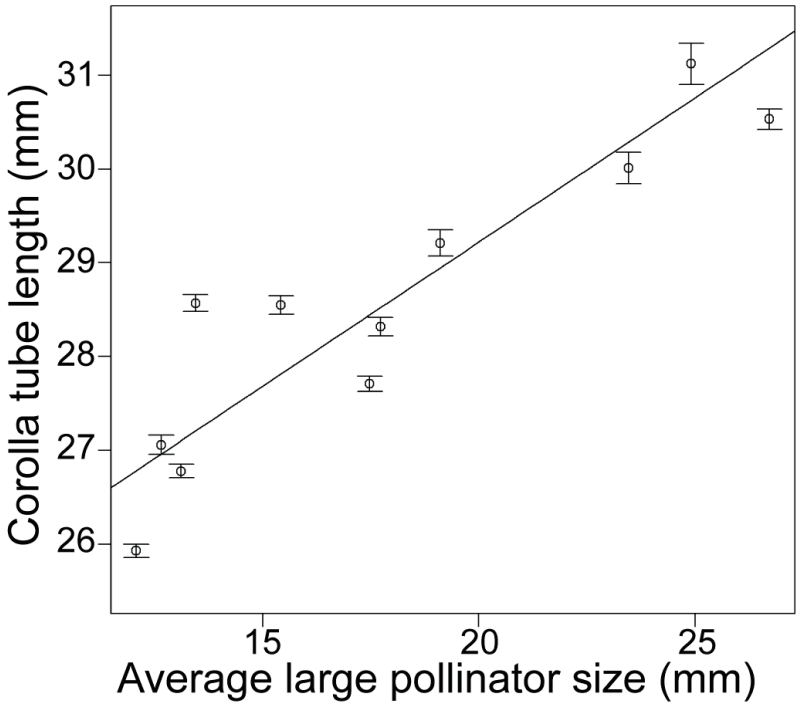

Figure 3-3. Relationship between floral size and average size of large bee pollinators. The line was fitted to the data by LMM result ($P < 0.001$). Data for Shimashima I, where no large bees visited the flowers, were not included in the regression analysis and are not shown in the figure. Error bars for average population floral size indicate the standard error.

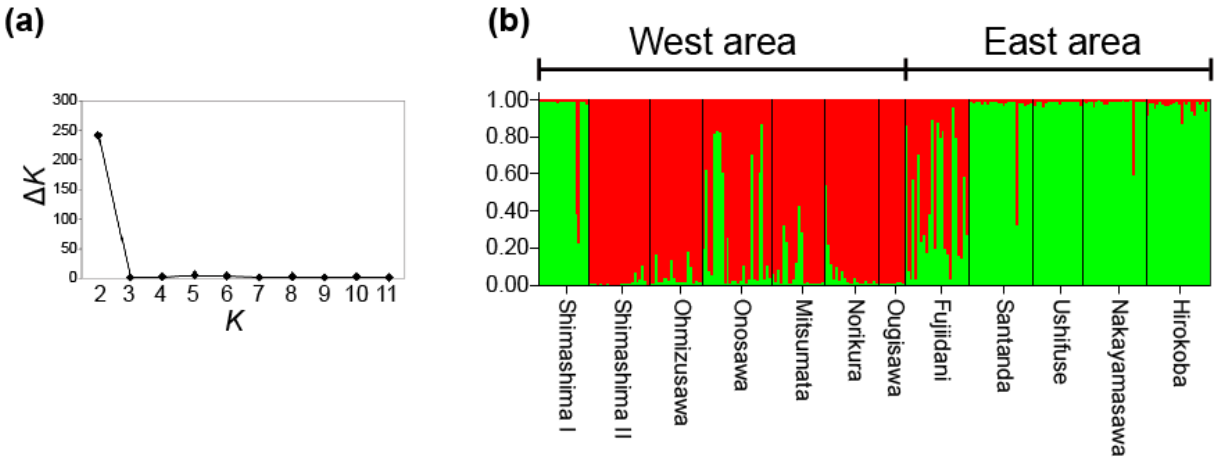

Figure 3-4. Population genetic structure of *L. album* var. *barbatum* populations. (a) Δ*K*, an index used to determine the appropriate number of genetic clusters (*K*), peaked at *K* = 2. (b) Genetic structure of *L. album* var. *barbatum* inferred by using Bayesian clustering implemented in STRUCTURE with *K* = 2. Different genetic clusters are represented by different colors.

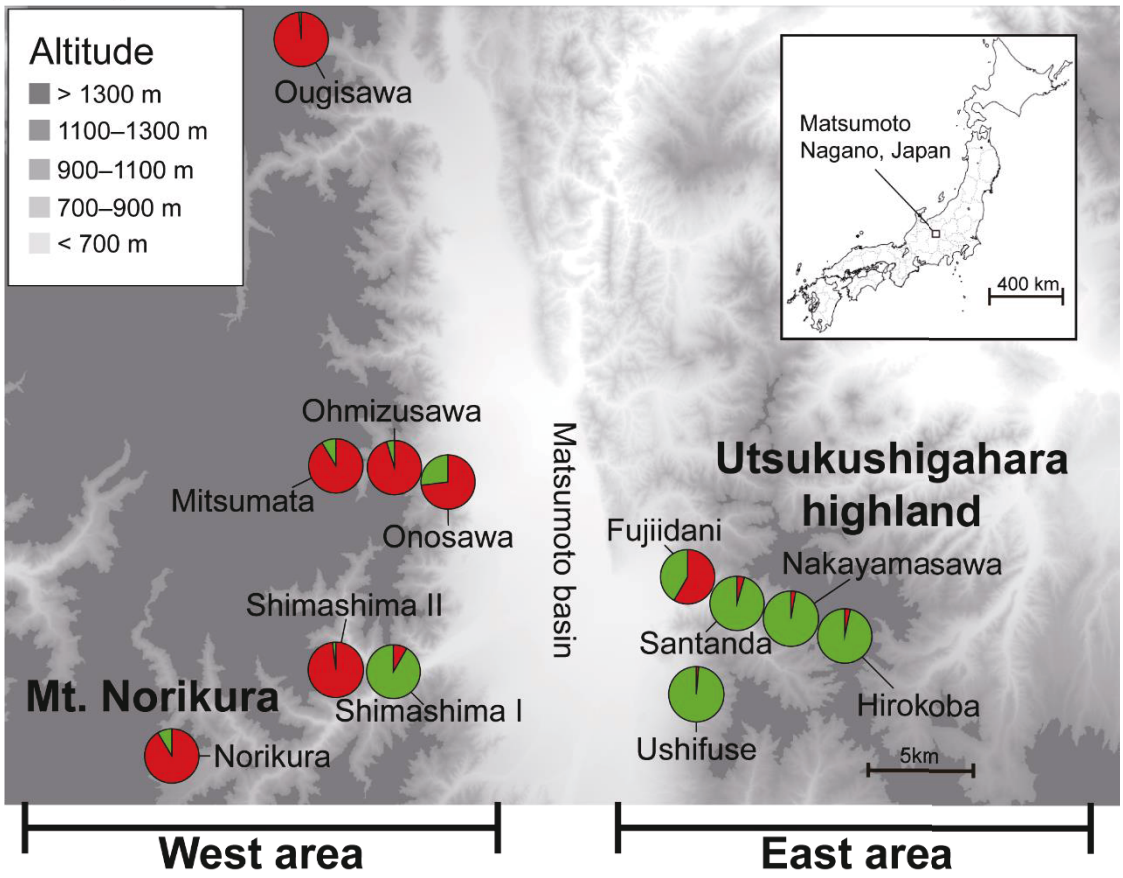

Figure 3-5. Geographic genetic structure on study sites. Pie charts indicate that the proportion of the two clusters identified by STRUCTURE as averaged for each population. The west area comprises populations in the Mt. Norikura region, and the east area comprises populations in the Utsukushigahara highland region.

Chapter 4

Intraspecific independent evolution of floral spur length in response to local flower visitor size in Japanese *Aquilegia* in different mountain regions

4-1 Abstract

Geographic differences in floral traits may reflect geographic differences in effective pollinator assemblages. Independent local adaptation to pollinator assemblages in multiple regions would be expected to cause parallel floral trait evolution, although sufficient evidence for this is still lacking. In this study, I investigated the relationship between flower spur length and pollinator size in 16 populations of *Aquilegia buergeriana* var. *buergeriana* distributed in four mountain regions in the Japanese Alps. I also examined the genetic relationship between yellow- and red-flowered individuals, to see if color differences caused genetic differentiation by pollinator isolation. Genetic relationships among 16 populations were analyzed based on genome-wide single-nucleotide polymorphisms. Even among populations within the same mountain region, pollinator size varied widely, and the average spur length of *A. buergeriana* var. *buergeriana* in each population was strongly related to the average visitor size of that population. Genetic relatedness between populations was not related to the similarity of spur length between populations; rather, it was related to the geographic proximity of populations in each mountain region. Our results indicate that spur length in each population evolved independently of the population genetic structure but in parallel in different mountain regions. Further, yellow- and red-flowered individuals of *A. buergeriana* var. *buergeriana* were not genetically differentiated. Unlike other *Aquilegia* species in Europe and America visited by hummingbirds and hawkmoths, this species is

consistently visited by bumblebees in Japan. As a result, genetic isolation by flower color has not occurred.

4-2 Introduction

Pollination mutualism is one of the major interaction systems between plants and animals, and through this interaction, flower visitors contribute to the reproduction of plants in different ways (Galen, 1996; Scobell and Scott, 2002; Herrera et al., 2006; Inoue et al., 2007; Gómez et al., 2009; Dohzono and Suzuki, 2010; Nattero et al., 2011). Adaptation to locally different pollinator assemblages within the distribution range of a plant species leads to local morphological specialization, which may cause trait diversification and speciation in the plants (Grant and Grant, 1965; Stebbins, 1970; Galen and Newport, 1987). Geographic variation in floral traits such as flower size and shape (Gómez et al., 2006; Nagano et al., 2014), corolla tube size (Hodges, 1997; Fenster et al., 2004), odor (Pellmyr 1986; Majetic et al., 2009), and color (Campbell et al., 1997; Newman et al., 2012) are considered to have evolved as a local adaptation to regional pollinators. In particular, morphological matching between floral spur length and pollinator proboscis length is well known, with the textbook example being Darwin's hawkmoth and orchid (Darwin, 1877; Nilsson, 1988). In fact, geographic correlations between floral size and pollinator size have been reported in a variety of plant taxa (Alexandersson and Johnson, 2002; Herrera et al., 2006; Anderson and Johnson, 2008; Johnson and Anderson, 2010; Boberg et al., 2014; Nagano et al., 2014; Kuriya et al., 2015).

Local adaptation of floral traits to pollinators may have occurred across multiple regions, but there is little evidence as to whether variation in floral traits has occurred

independently among regional populations (but see Anderson et al., 2014; Toji et al., 2021). As in textbook examples of ecological speciation (Nosil, 2012), one useful approach to understand the interaction between trait diversification and speciation in angiosperms is to combine a field analysis of local plant evolutionary adaptations with a population genetic analysis that examines genetic relationships among populations. In particular, local adaptation of floral traits to pollinators may lead to speciation via the establishment of prezygotic reproductive isolation (Grant-Stebbins model; Grant and Grant, 1965; Stebbins, 1970; Johnson and Anderson, 2010; Anderson et al., 2014), because one possible result of specialization of a trait to a particular pollinator is a lack of pollinator sharing among plant populations (Herrera et al., 2006; Anderson and Johnson, 2008; Newman et al., 2015). About 25% of plant diversification events may be associated with pollinator shifts (Van der Niet and Johnson, 2012); thus, combined analyses of local adaptation of floral traits and population genetics can shed light on the mechanisms of plant diversity (Thompson, 2005; Thompson et al., 2013). In this study, I conducted both trait and genetic analyses to determine whether the differentiation of floral traits (flower size) among plant populations in different mountain regions was the result of secondary contact between two differentiated lineages with long and short flowers, or whether flower size evolved recently in each population as an adaptation to the local pollinator size.

In genus *Aquilegia* (Ranunculaceae), adaptive radiation to different pollinators (bumblebees, hummingbirds, and hawkmoths) has occurred. Mainly, flower color, spur length, flower orientation, and pistil length have evolved to adapt to each pollinator (Fulton and Hodges, 1999; Hodges et al., 2004). Moreover, molecular phylogenetic evidence also indicates that pollinator shifts have led to morphological diversification and

speciation within this genus (Whittall and Hodges, 2007). According to Whittall and Hodges (2007), a more ancestral floral state of *Aquilegia* is purple, downward facing, short-spurred flowers, which are pollinated by bumblebees. From plants with this floral state, taxa with red, downward facing flowers with protruding stamens and intermediate length spurs, which are pollinated by hummingbirds, were derived. Then, taxa with white and yellow long-spurred, lateral and upward facing flowers, which are pollinated by hawkmoths, were derived from those taxa. Their results reveal an interesting patten of species-level diversification as a consequence of pollinator shifts, although evidence for flower trait diversification at the earlier stages of speciation is lacking. To observe early stages of speciation, it is useful to investigate the pattern of evolutionary morphological diversification within a single species (Sobel and Streisfeld, 2015).

In this study, I focused on evolutionary processes leading to spur length and flower color differentiation in *A. buergeriana* var. *buergeriana*. In this species, geographic variation in spur length has previously been observed in six populations in two mountain regions, but the relationship between spur length and flower visitors in these populations is not known (Hattori et al., 2014). Yellow-flowered individuals are dominant in this species, and bumblebees seem to be the main flower visitors. In some populations, red-flowered individuals occur orthotopically with yellow-flowered individuals, but the genetic relationship between red- and yellow-flowered individuals is unknown. Differences in flower color in *Aquilegia* can lead to genetic isolation even between neighboring or sympatric populations and is likely to be important in speciation (Schemske and Bradshaw, 1999; Hopkins and Rausher, 2012). Here, I first investigated the correspondence between variation in floral spur length and flower-visiting insect size in 16 *Aquilegia* populations in four mountain regions. The results showed a

morphological correlation between spur length and average visitor size in each population, even within the same mountain region; spur lengths were shorter in populations visited by smaller flower visitors, and spur lengths were longer in populations visited by larger flower visitors. Next, I identified genome-wide single-nucleotide polymorphisms (SNPs) by the MIG-seq (multiplexed inter-simple sequence repeats genotyping by sequencing) method (Suyama and Matsuki, 2015) to clarify the genetic relationships among the populations. These results showed that genetic relationships tended to be clustered by mountain region and, therefore, that spur length evolved in parallel in each mountain region. Individuals with different flower colors were not differentiated in the genome-wide SNPs analysis, however. This result suggests that pollinator isolation by flower color has not occurred in these populations. Instead, the red flower color is maintained in various populations in which most individuals have yellow flowers.

4-3 Materials and Methods

4-3-1 Plant species and study site

Aquilegia buergeriana var. *buergeriana* f. flavescens is a perennial, protandrous herb endemic to Japan. The spur and sepals of its flowers are pale yellow (yellow-flowered individual) or reddish brown (red-flowered individuals) (Figure 4-1a, b). Flowers of both colors face downward. In the study area, in the central Japanese Alps, yellow-flowered individuals are more common. Japanese *Aquilegia* species are mainly visited by bumblebees (Tamura and Shimizu, 1999; Itagaki and Sakai, 2006; Hattori et al., 2014), even though, in general, yellow-flowered *Aquilegia* are pollinated by hawkmoths (Hodges et al., 2004). Unlike most *Aquilegia* with yellow flowers, however, the yellow flowers of Japanese *A. buergeriana* do not have protruding anthers and pistils and are not

visited by hawkmoths (T. Toji personal observation by camera trap).

I studied *Aquilegia* populations in four mountain regions (Utsukushigahara, Norikura, Ontake, and Iizuna) of the central Japanese Alps (Figure 4-1d; Table 4-1). Field surveys were conducted during the flowering season, from July to September, in 2018 and 2019: Populations in the Utsukushigahara, Norikura, and Ontake mountain regions were surveyed in 2018, and populations in the Iizuna mountain region were surveyed in 2019.

4-3-2 Measurement of traits

Spur length was measured of all flowering individuals in each population, including both red- and yellow-flowered individuals. The spur lengths of 1–3 randomly selected flowers per plant were measured with a digital caliper (precision 0.01 mm), and the average length of the measured spurs was used as the spur length of that individual (Figure 4-1c). Corolla diameter and petal width of each individual were measured at the same time. The variation in floral traits was visualized by principal component analysis (PCA) and compared among populations. Preliminary observations showed that the three floral traits did not differ among flowers within an individual, and petal width did not differ among the five petals of each flower. I considered spur length to be the most important trait because of its relation to visitor size. Therefore, in subsequent analyses I focused on spur length. The multiple comparison Steel-Dwass test was used to compare average spur length between populations.

I also examined spatial autocorrelation (i.e., whether the variation in spur length could be explained by physical distance) by using the "moran.test" function in the "spdep" package in the R Software Environment ver. 4.0.2 (R Core Team, 2013) to run Moran's I test. This analysis used the average spur length and the latitude and longitude of each

population.

4-3-3 Flower visitor assemblages and size variation

To investigate the flower visitors of *A. buergeriana* var. *buergeriana*, I walked through each population and captured insects that were visiting flowers. This survey was conducted during 7:00–14:00 local time, when flower visitors are active. Each population was observed a total of 60–180 min over 1–3 days at the peak of the flowering season. Captured insects were measured from the tip of the proboscis to the end of the abdomen with a digital caliper (precision, 0.01 mm) to determine visitor size (Figure 4-1c). Observations were made of visitors to both yellow and red flowers to confirm that there were no differences in visitor assemblage or visiting frequency between differently colored flowers.

As the average visitor size for each plant population, the weighted arithmetic average was calculated from the relative abundance of each visitor species and the size of that species:

$$\text{Average visitor size} = \sum_{i=1}^{n} Pi(Ni/Nt)$$

where n = the total number of insect species visiting a *A. buergeriana* var. *buergeriana* population, Pi = the average size of the ith insect species, Ni = the number of flowers in the population that the ith insect species visited, and Nt = the number of flowers in the population that any of the insect species visited. Thus, Ni/Nt is the relative abundance of the ith insect species visiting the population. Observations of flower visitor frequency showed that large bumblebees (*Bombus* spp.) were the main visitors, although small bees (*Ceratina* spp.) also visited occasionally. Bumblebees, which extend their proboscis to

the nectar source at the tip of the spur to suck nectar, visited both male and female phases and both yellow and red flowers (T. Toji personal observation). Smaller bees could not reach the spur tip to forage for nectar. Furthermore, although they sometimes collected pollen from the flowers, they did not contribute to pollination because they rarely moved between plant individuals and did not visit female-phase flowers. Because the pollen visitation patterns of the bumblebees and small bee species were very different, I calculated average visitor size for all visitors (i.e., bumblebees plus small bees) and for bumblebees only.

4-3-4 Factors influencing local spur length

The factors affecting spur length in *A. buergeriana* var. *buergeriana* were estimated by a linear mixed model (LMM) analysis. In this analysis, average spur length of each population was used as the objective variable, and average visitor size (all visitors), average visitor size (only bumblebees), plant height, number of flowers per individual, and altitude of each population were used as explanatory variables. Here, plant height and the number of flowers per individual were used as indicators of the nutritional status of the plant, and altitude was used as a representative indicator of non-biotic environmental factors (e.g. meteorological changes). Population was added to the model as a random effect.

Before conducting the LMM analysis, the variance inflation factor (VIF) statistic was calculated to check for correlation (multicollinearity) between variables, using VIF = 0.5 as the threshold (Neter et al., 1996). For all variables, VIF was less than 0.25, confirming that no multicollinearity existed. Next, I conducted a likelihood ratio test using the parametric bootstrap method (Hoel et al., 1971) to select the effective variables. In this

test, for each variable, the difference in deviance, obtained by 1000 bootstrap calculations, between the global model with all variables and a model lacking that variable was determined. No variables were removed as a result of this analysis.

For appropriate model selection, I first prepared a global model that included all of the following variables: average spur length of the population, average visitor size (all visitors) of the population, average visitor size (only bumblebees) of the population, plant height, number of flowers per individual, and altitude. Then, using the "dredge" function of the R package "MuMIn", I compared the global model to simple models with fewer explanatory variables. Then, I adopted the model with the lowest Akaike Information Criterion (AIC) was adopted. I excluded the 9 ON-1000 population, which was not visited by bumblebees, from this analysis, treating it as a missing value.

4-3-5 Genetic structure of *A. buergeriana* var. *buergeriana* populations

Leaf samples were obtained from 6–21 individuals in each study population, and DNA was extracted by the CTAB method (Doyle and Doyle, 1990). I performed MIG-seq (Suyama and Matsuki, 2015) to detect genome-wide SNPs. A MIG-seq library was prepared following to the protocol of Suyama et al. (2021). A first PCR was performed to amplify inter-simple sequence repeat regions using MIG-seq primer set 1 (Suyama and Matsuki, 2015), and then a second PCR was performed on the purified/equalized first PCR product to add the sequences necessary for sequencing on the MiSeq system and for sample identification. The second PCR products were pooled and fragments of 350 bp or more were isolated. I used MiSeq Reagent Kit v3 (150 cycle, Illumina) and performed sequencing with an Illumina MiSeq Sequencer (Illumina, San Diego, CA, USA) following the manufacturer’s protocol. I used the "DarkCycle" option to skip sequencing

of the first 17 bases of reads 1 and 2 (simple sequence repeat primer regions and anchors).

Low-quality reads and extremely short reads containing adaptor sequences were removed by using trimmomatic 0.39 (Bolger et al., 2014). *De novo* SNP discovery was performed by using the Stacks 2.41 software pipeline (Catchen et al., 2013; Rochette et al., 2019). For *de novo* SNP discovery, I used the following parameters: minimum depth of coverage required to create a stack (m) = 3, maximum distance between stacks (M) = 2, and maximum mismatches between loci when building the catalog (n) = 2. Three different filtering criteria were applied for quality control of the SNP data. First, SNPs that were retained by 80% or more samples were included in the SNP dataset. Second, SNPs with a minor allele frequency of less than 0.05 were removed. Third, loci containing SNPs with extremely high observed heterozygosity ($H\text{o} \geq 0.6$) were removed. Fourth, after performing a Hardy-Weinberg equilibrium test on each population, I excluded loci where allele frequencies deviated from the Hardy-Weinberg equilibrium at $P < 0.01$ in three or more populations.

The following population genetic statistics of SNP sites for each population were calculated with the Stacks populations module: expected heterozygosity *H*e, observed heterozygosity *H*o, nucleotide diversity π, and inbreeding coefficient F_{IS} (Hartl and Clark, 1997). The population genetic structure was examined by PCA using PLINK 1.9 (Purcell et al., 2007). In addition, a Bayesian clustering analysis was performed with STRUCTURE software version 2.3.4 (Pritchard et al., 2000; Falush et al., 2003). For the STRUCTURE analysis, simulations were performed with 100k burn-in iterations and 100k Markov chain Monte Carlo iterations. The number of genetic clusters (*K*) was calculated 10 times for each possible *K* value from 1 to 10, and the appropriate number of clusters was estimated based on the ΔK value (Evanno et al., 2005). Then, to examine

the genetic structure within each mountain region in more detail, I performed additional STRUCTURE analyses. First, SNP re-detection was performed in each of three mountain regions, the Utsukushigahara, Norikura+Ontake, and Iizuna regions based on results of the initial analysis. The population structure obtained based on all samples, with the above filtering criteria used in SNP detection. Second, 10 independent STRUCTURE analysis runs were performed for each mountain region with 100,000 burn-in steps and an additional 100,000 steps with the admixture model; log-likelihood values were estimated for each possible K value (K = 1–10), and the appropriate number of clusters was estimated based on the ΔK.

4-3-6 Isolation by distance and isolation by phenotype

I investigated whether the genetic structure of *A. buergeriana* var. *buergeriana* reflects geographic distance or trait differences. In general, populations separated by greater distances are more genetically differentiated than populations close together (Wright, 1943). On the other hand, if populations with similar traits are also genetically similar, then I can expect to find a correlation between differences in traits between populations and the degree of genetic differentiation. I used GenoDive software version 3.0 (Meirmans, 2020) to calculate the genetic isolation (F_{ST}) between populations. The geographic distance between populations was calculated from the latitude and longitude of the populations, and the difference in the average spur length of each population was used as the trait difference. I calculated the relationship between pairwise F_{ST} or $F_{ST}/(1 - F_{ST})$ and geographic distance between populations, as well as the relationship between pairwise F_{ST} or $F_{ST}/(1 - F_{ST})$ and trait difference between populations, following methods in Rousset (1997) and Noutsos et al. (2014). The relationship between genetic isolation

and geographic or trait distance was tested by Mantel tests using the R package "ade4" with 10,000 Monte-Carlo permutations.

4-4 Results

4-4-1 Spur length and flower visitor size

The average spur length of each population of *A. buergeriana* var. *buergeriana* varied in the range of 32.85–40.31 mm, confirming the presence of diversity in spur length within this species (Figure 4-1d; supplementary material, Figure 4-2, Table 4-2). No spatial autocorrelation of average spur length among populations was detected (Moran's I statistic = –0.160, $P = 0.757$). PCA results for spur length, corolla diameter, and petal width roughly indicated a morphological separation among populations (supplementary material, Figure 4-3), but yellow- and red-flowered individuals could not be clearly separated on the basis of variations in flower morphology (supplementary material, Figure 4-3).

The average visitor size of each population varied in the range of 8.69–40.80 mm (bumblebees plus small bees) and 31.84–40.80 mm (only bumblebees) (supplementary material, Table 4-1). Five types of bumblebees were observed, in descending order of size: *B. consobrinus* queen, *B. diversus* queen, *B. consobrinus* worker, *B. diversus* worker, and *B. honshuensis* worker. Flower visits by small bees of the genus *Ceratina* were observed in several populations (supplementary material, Table 4-1). Average plant height of each population varied in the range of 54.84–98.01 cm.

4-4-2 Factors influencing local spur length

The LMM model with the lowest AIC value was the model that included only average

visitor size (only bumblebees) as an explanatory variable (Table 4-3, 4-4). A very strong linear relationship was found between average spur length and average visitor size (only bumblebees) ($P < 0.0001$, Figure 4-4).

4-4-3 Genetic structure of *A. buergeriana* var *buergeriana* populations

A total of 16,033,406 raw reads (69,109 ± 731 reads per sample) were obtained by MIG-seq, and after quality control, 15,510,825 reads (66,587 ± 713 reads per sample) were used for further analyses. After *de novo* SNP detection and filtering, the MIG-seq dataset of 232 samples from 16 populations contained 190 SNPs, distributed among the mountain regions as follows: Utsukushigahara region (63 individuals, 175 SNPs), Norikura+Ontake region (126 individuals, 167 SNPs), Iizuna region (43 individuals, 175 SNPs). Norikura and Ontake regions were combined based on the initial STRUCTURE results. The values of the population genetics parameters varied among populations (*H*e, 0.0904–0.1450; *H*o, 0.0593–0.2154; π, 0.0616–0.2248; F_{IS}, –0.0608 to 0.2041; supplementary material, Table 4-5).

In the PCA results for 190 SNPs of 232 individuals from 16 populations of *A. buergeriana* var. *buergeriana*, principal components 1 and 2 (PC1 and PC2, respectively) explained 28.78% of the variance. The geographical structure of the populations is clearly reflected in a plot of PC2 against PC1 (Figure 4-5), but within populations of *A. buergeriana var. buergeriana*, red and yellow- and red-flowered individuals did not clearly show genetic isolation. On the basis of the PCA results, the populations could be separated into three regional groups: Utusuhigahara, Norikura+Ontake, and Iizuna populations. The STRUCTURE analysis of all populations showed that, based on Δ*K*, the appropriate number of genetic clusters was $K = 2$ (most likely), or $K = 3$ (next most likely)

(supplementary material, Figure 4-6). The STRUCTURE analysis results also clearly reflected the geographical structure in each region (Figure 4-6, 4-7, 4-8). The appropriate number of genetic clusters in the Utsukushigahara (63 individuals, 175 SNPs), Ontake+Norikura (126 individuals, 167 SNPs), and Iizuna (43 individuals, 175 SNPs) mountain regions were $K = 3$, 3, and 2, respectively, based on ΔK (supplementary material, Figure 4-6). Structure among populations within the same mountain region was also detected (Figure 4-7b, d). In particular, the populations in Norikura+Ontake region could be separated into Norikura and Ontake groups. These two groups were not separated in the initial STRUCTURE analysis. Yellow- and red-flowered individuals in a population were not genetically distinguished in the STRUCTURE analysis results.

4-4-4 Isolation by distance and isolation by phenotype

A significant relationship between geographic distance and genetic isolation (F_{ST}, $F_{ST}/(1 - F_{ST})$) was detected for all combinations of variables (Table 4-6). On the other hand, trait differences between populations were not related to genetic isolation.

4-5 Discussion

4-5-1 Intraspecific independent evolution of spur length among mountain regions

The spur length of *A. buergeriana* var. *buergeriana* was correlated with the average flower visitor size (only bumblebees) of the population; spur length varied greatly with the average visitor size even among spatially close populations in the same mountain region (Figures 4-4, 4-7). The PCA results for the three floral traits (spur length, corolla width, and petal width), showed that the floral traits tended to be differentiated even among populations within the same mountain region (supplementary material, Figure 4-

3). The genetic results obtained by PCA and the STRUCTURE analysis of genome-wide SNPs suggest that populations within each mountain region are more closely related to each other than to populations in other mountain regions (Figures 4-5, 4-7, 4-8). Genetic isolation was proportional to geographical distance and did not reflect trait differences (Table 4-6). These results suggest that spur length of *A. buergeriana* var. *buergeriana* evolved independently in each mountain region. The Ontake and Norikura regions are part of a series of volcanic massifs called the Norikura volcanic chain (Kimura and Yoshida, 1999; Sekiguchi and Yamagishi, 2013), and the colonization history of the two regions seems to be very closely related. The close genetic relationship detected between the populations of these two mountain regions may be related to the related origin of the massifs. In the Iizuna region, populations at different altitudes seem to belong to different genetic clusters (Figure 4-7d); further, flowers in lower altitude populations were visited by *B. diversus* and those in higher altitude populations were visited by *B. consobrinus* (Table 4-1). These results suggest that genetic differentiation may occur between higher and lower altitude populations because of a lack of pollinator sharing. Further studies are needed to determine whether gene flow by pollination is hindered between populations at higher and lower altitudes in the Iizuna region.

Hodges et al. (2002) have reported a genetic basis for spur length in two *Aquilegia* species (*A. formosa* and *A. pubescens*), and they have performed quantitative trait locus mapping for spur length variation. In addition, the functions of some of the quantitative genes that cause spur length variation in *A. coerulea* have been elucidated (Zhang et al., 2020). Therefore, I think it highly likely that spur length has a genetic basis in *A. buergeriana* var. *buergeriana*, and that the evolution of spur length is facilitated by flower visitors.

In anole lizards, leg length has evolved independently on different islands to suit local habitats (Losos, 2010), and in stickleback fishes, the evolution of marine to freshwater forms (sticklebacks that move between rivers and the sea) occurred independently in different marine and freshwater locations in various regions of the world (Jones et al., 2012). I propose that plant species distributed across a wide geographic range with site-specific, different-sized pollinators constitute another model suitable for testing independent adaptive radiation. I have demonstrated that spur length in an *Aquilegia* species may have evolved independently among mountain regions by using a population genetics approach to compare traits among mountain regions. Independent evolution in different mountain regions has recently been examined in various model systems: for example, the independent evolution of upland and short-winged forms of scorpionfly *Panorpodes* (Panorpodidae) (Suzuki et al., 2019), the independent evolution of *Potentilla matsumurae* (Rosaceae) in fellfield and snowbed environments on different mountains in Japan (Hirao et al., 2019), and the independent evolution of alpine morphology in *Antirrhinum* species (Antirrhineae) (Durán-Castillo et al., 2021). Further, I recently presented a case in which I used microsatellite markers to show the independent adaptation of floral tube size in *Lamium album* var. *barbatum* (Lamiaceae), associated with flower visitor size, in the Utsukushigahara and Norikura regions of the Japanese Alps (Toji et al., 2021). These examples show that comparisons between mountain regions can be used to study independent trait evolution in various organisms, and similar patterns might be found in many places around the world.

4-5-2 Flower color does not contribute to genetic isolation

Although red-flowered individuals were observed in some populations, genetic

analyses (STRUCTURE and PCA) based on neutral genes did not differentiate red- and yellow-flowered individuals in those populations. These results suggest that red flower color is maintained in each population merely as a flower color polymorphism. Throughout the diversification history of *Aquilegia*, flower color changes have been shown to be associated with pollinator shifts (Whittall and Hodges, 2007). Another well-known example is the *Mimulus aurantiacus* species complex, in which flower color influences pollinator preference, which in turn leads to genetic isolation. Within the *M. aurantiacus* species complex, there are two ecotypes, one with red flowers, which are preferred by hummingbirds, and the other with yellow flowers, which are preferred by hawkmoths. Although these two ecotypes are very closely related, cluster analysis by RAD-seq (restriction site-associated DNA sequencing) based on genome-wide SNP data has shown that they are genetically distinct (Sobel and Streisfeld, 2015). In the hybrid zone between the two ecotypes, the *MaMyb2* gene, which is involved in the synthesis of flower pigments, is geographically maintained despite neutral gene flow occurred (Streisfeld and Kohn, 2005; Sobel and Streisfeld, 2015; Stankowski and Streisfeld, 2015). Gene flow between yellow and red flower *M. aurantiacus* ecotypes in the early stages of speciation seems to be limited mainly by differences in pollinator preference (Sobel and Streisfeld, 2015). Similarly, gene flow between two closely related *Aquilegia* species: hummingbird-pollinated, red-flowered *A. formosa* and hawkmoth-pollinated, yellow-flowered *A. pubescens* is also limited by pollinator isolation when the two species are distributed parapatrically (Fulton and Hodges, 1999; Noutsos et al., 2014).

Why have yellow- and red-flowered individuals in *A. buergeriana* var. *buergeriana* not become genetically isolated? In the central Nagano region, where this study was conducted, bumblebees appear to be abundant and many flowers depend on bumblebees

for pollination (e.g. Egawa and Itino, 2020), whereas potential pollinators such as birds and butterflies that prefer red flowers are scarce. In another Japanese mountain region (the Taisetsu mountains), flowers are dominantly visited by bees and flies at the community level (Mizunaga and Kudo, 2017). A recent review has reported that Lepidoptera account for less than 10% of insect visitors to flowers in many parts of Asia, whereas bees and flowers account for more than half (Funamoto, 2019). It is possible that in the central Japanese Alps, because only the locally abundant bumblebees contribute to pollination of *A. buergeriana* var. *buergeriana* irrespective of the flower color, pollinator shifts to other taxa such as birds have not triggered the evolution of extreme traits. Whether bumblebees cause selection or act neutrally with respect to flower color requires further investigation, but the maintenance of small numbers of red-flowered individuals in some populations suggests that the frequency of red flowers may be determined by genetic drift. The maintenance of this small number of different flower-color polymorphisms in some populations might become a driving force for a pollinator shift should the plants be faced with a new pollinator environment.

4-6 Conclusions

Two main conclusions follow from our results that 1) the evolution of spur length in *A. buergeriana* var. *buergeriana* has occurred independently in different mountain regions, and 2) the few red-flowered phenotypes that occur within the species has not led to genetic differentiation. First, given that the independent evolution of floral size in different mountain regions has also recently been reported in *L. album* var. *barbatum* (Toji et al., 2021), the independent evolution of floral size among mountain regions may be a generalized event that occurs commonly in different taxa. The approach used here to test

for independent evolution among mountain regions is applicable to any taxon and a variety of traits. In particular, morphological analyses combined with MIG-seq (Suyama and Matsuki, 2015), which can be used to obtain genome-wide SNPs from non-model organisms, constitute a powerful method for elucidating patterns of morphological and genetic diversification within species. Second, I found no relationship between flower color and the degree of genetic differentiation, despite the fact that pollinator isolation caused by differences in flower color has been reported in two closely related species of *Aquilegia* (Fulton and Hodges, 1999; Noutsos et al., 2014). I infer that in the mountainous region of Japan, where bumblebees are locally abundant large pollinators, shifts to different pollinator taxa are unlikely to occur, and the polymorphism in *A. buergeriana* var. *buergeriana* flower color is likely maintained by random genetic drift. Thus, our results are an important exception to diversification in genus *Aquilegia*, which is well known to have occurred by both flower-color and pollinator shifts (Whittall and Hodges, 2007).

4-7 Tables

Table 4-1. Overview of the study site. Location information and numbers of flower visiting insects observed (W, worker; Q, Queen).

Pop No.	Population name	Mountain region	Latitude	Longitude	Observed flower visitors						Observation time (min)
					Small bees	*Bombus honshuensis* W	*B. diversus* W	*B. diversus* Q	*B. consobrinus* W	*B. consobrinus* Q	
1	UT-1080	Utsukushigahara	36.222378	138.070700	-	-	-	-	5	-	180
2	UT-1300	Utsukushigahara	36.250953	138.034308	-	-	3	1	-	-	150
3	UT-1370	Utsukushigahara	36.215731	138.088757	-	-	-	-	4	1	100
4	UT-1640	Utsukushigahara	36.246782	138.055615	3	2	-	-	4	-	120
5	NR-930	Norikura	36.539493	137.785174	-	-	2	1	-	-	140
6	NR-1120	Norikura	36.129036	137.719438	2	-	2	-	-	-	60
7	NR-1600	Norikura	36.144650	137.628887	-	4	-	-	4	-	180
8	NR-1700	Norikura	36.109987	137.607450	9	-	4	2	-	-	100
9	ON-1000	Ontake	35.800620	137.564363	1	-	-	-	-	-	100
10	ON-1340	Ontake	35.842808	137.541307	-	-	-	-	-	-	-
11	ON-1760	Ontake	35.866656	137.525053	2	-	2	-	2	-	155
12	ON-2160	Ontake	35.869647	137.500463	-	-	-	-	3	-	150
13	IZ-1520	Iizuna	36.731352	138.125567	-	-	4	-	-	-	120
14	IZ-1630	Iizuna	36.733123	138.126941	-	-	3	-	-	-	120
15	IZ-1750	Iizuna	36.734757	138.127005	-	-	-	-	3	-	120
16	IZ-1870	Iizuna	36.736391	138.129494	-	-	-	-	3	-	120

Table 4-2. Continued. Average spur length and average plant height in each population. Statistically significant differences between populations are indicated by different lowercase letters (Steel-Dwass test, $P < 0.05$).

Pop No.	Population name	Average pollinator size (mm)		Average spur length (mm)	Average plant height (cm)
		All pollinators	Only bumblebees		
1	UT-1080	38.09	38.09	39.67 ^{a}	86.05 ^{abcd}
2	UT-1300	33.86	33.86	36.20 ^{c}	81.82 ^{abcd}
3	UT-1370	40.80	40.80	38.15 ^{abc}	98.01 ^{a}
4	UT-1640	27.47	33.76	35.99 ^{c}	76.14 ^{bcd}
5	NR-930	34.90	34.90	36.49 ^{c}	92.41 ^{ab}
6	NR-1120	20.30	31.84	35.77 ^{c}	80.26 ^{abcd}
7	NR-1600	34.00	34.00	37.04 ^{bc}	69.59 ^{de}
8	NR-1700	27.81	34.54	38.28 ^{abc}	72.62 ^{cde}
9	ON-1000	8.69	-	32.85 ^{d}	66.32 ^{de}
10	ON-1340	-	-	-	-
11	ON-1760	25.68	35.15	36.87 ^{bc}	57.51 ^{e}
12	ON-2160	32.18	40.72	40.31 ^{a}	56.56 ^{e}
13	IZ-1520	36.61	32.08	36.61 ^{c}	54.84 ^{e}
14	IZ-1630	31.90	31.90	36.82 ^{c}	58.95 ^{e}
15	IZ-1750	38.35	38.35	38.36 ^{ab}	67.71 ^{de}
16	IZ-1870	38.08	38.08	39.08 ^{ab}	63.67 ^{de}

Table 4-3. The GLM model that best explained variation in average spur length among populations of *A. buergeriana* var. *buergeriana*. This model had the lowest AIC value among the tested models; see Table 4-4 for the model comparison results.

	Coefficient	SE	*t*	*P*-value
Intercept	23.662	3.159	7.489	<0.0001
Average visitor size (only bumblebees)	0.388	0.088	4.420	<0.0001

Table 4-4. GLM model selection results obtained by using the dredge function in the "MuMIn" package.

Predictive variables and coefficients										
Altitude	Plant height	Number of flowers per ramet	Average visitor size (all visitors)	Average visitor size (only bumblebees)	Intercept	Degrees of freedom	Log likelihood	AIC	Delta AIC	Weight
				0.38760	23.66	4	-514.391	1036.8	0	0.895
			-0.02337	0.41750	23.35	5	-516.194	1042.4	5.61	0.054
					37.57	3	-519.135	1044.3	7.49	0.021
	0.00861			0.38480	23.14	5	-517.756	1045.5	8.73	0.011
		0.00003		0.38760	23.66	5	-518.076	1046.2	9.37	0.008
			0.11770		33.7	4	-519.376	1046.8	9.97	0.006
0.00087				0.35590	23.47	5	-520.011	1050	13.24	0.001
	0.00930		-0.03049	0.42370	22.69	6	-519.466	1050.9	14.15	0.001
		0.00009	-0.02338	0.41750	23.35	6	-519.875	1051.8	14.97	0.001
	0.01184				36.71	4	-522.019	1052	15.26	0
		0.00127			37.55	4	-522.776	1053.6	16.77	0
	0.01131	-0.00674		0.38570	23	6	-521.131	1054.3	17.48	0
	0.01065		0.11230		33.1	5	-522.454	1054.9	18.13	0
0.00188					34.7	4	-523.65	1055.3	18.52	0
0.00087			0.00004	0.35560	23.47	6	-521.819	1055.6	18.86	0
		0.00113	0.11760		33.69	5	-523.023	1056	19.27	0
0.00195			0.12220		30.57	5	-523.562	1057.1	20.34	0

0.00132	0.01277			0.33520	22.59	6	-522.743	1057.5	20.7	0
0.00090		0.00197		0.35440	23.45	6	-523.662	1059.3	22.54	0
	0.01217	-0.00717	-0.03302	0.42790	22.51	7	-522.815	1059.6	22.85	0
	0.01451	-0.00700			36.61	5	-525.369	1060.7	23.96	0
0.00236	0.01571				32.82	5	-525.84	1061.7	24.9	0
0.00134	0.01289		0.00281	0.33070	22.62	7	-524.533	1063.1	26.28	0
	0.01314	-0.00640	0.11140		33.04	6	-525.834	1063.7	26.89	0
0.00241	0.01495		0.11570		29	6	-525.893	1063.8	27	0
0.00191		0.00324			34.59	5	-527.248	1064.5	27.71	0
0.00090		0.00193	0.00097	0.35280	23.47	7	-525.465	1064.9	28.15	0
0.00132	0.01541	-0.00666		0.33600	22.46	7	-526.123	1066.2	29.46	0
0.00199		0.00370	0.12220		30.46	6	-527.151	1066.3	29.52	0
0.00236	0.01831	-0.00680			32.72	6	-529.202	1070.4	33.62	0
0.00133	0.01555	-0.00675	0.00010	0.33550	22.45	8	-527.905	1071.8	35.03	0
0.00241	0.01722	-0.00588	0.11480		28.95	7	-529.301	1072.6	35.82	0

Table 4-5. Population genetics parameters of each population.

Pop No.	Population	Number of analyzed individuals	Observed heterozygosity (*Ho*)	SE	Expected heterozygosity (*He*)	SE	Nucleotide diversity (π)	SE	Fixation index (F_{IS})	SE
1	UT-1080	16	0.1347	0.0174	0.1155	0.0139	0.1199	0.0144	-0.0339	0.1249
2	UT-1300	10	0.1235	0.0154	0.1437	0.0129	0.1526	0.0138	0.0954	0.0795
3	UT-1370	16	0.1437	0.0137	0.1578	0.0132	0.1637	0.0137	0.0558	0.1236
4	UT-1640	21	0.1450	0.0120	0.2112	0.0136	0.2169	0.0140	0.2041	0.1382
5	NR-930	16	0.0786	0.0132	0.0897	0.0117	0.0929	0.0121	0.0360	0.0928
6	NR-1120	15	0.1198	0.0131	0.1531	0.0132	0.1587	0.0137	0.1186	0.0984
7	NR-1600	18	0.0919	0.0114	0.1378	0.0125	0.1422	0.0129	0.1636	0.1225
8	NR-1700	16	0.1254	0.0147	0.1443	0.0137	0.1497	0.0142	0.0799	0.1350
9	ON-1000	16	0.0951	0.0108	0.1581	0.0132	0.1641	0.0137	0.1967	0.1515
10	ON-1340	11	0.1024	0.0106	0.1470	0.0129	0.1549	0.0136	0.1416	0.0895
11	ON-1760	18	0.1447	0.0137	0.1632	0.0137	0.1688	0.0142	0.0549	0.1469
12	ON-2160	16	0.0904	0.0179	0.0593	0.0110	0.0616	0.0114	-0.0608	0.1432
13	IZ-1520	6	0.1352	0.0131	0.2154	0.0152	0.2248	0.0158	0.2018	0.1109
14	IZ-1630	17	0.1311	0.0144	0.2089	0.0154	0.2213	0.0163	0.2026	0.1100
15	IZ-1750	6	0.1258	0.0165	0.1843	0.0149	0.2026	0.0163	0.1861	0.0576
16	IZ-1870	14	0.1244	0.0148	0.1616	0.0147	0.1684	0.0153	0.1092	0.1306

Table 4-6. Mantel test results for the relationships between F_{ST} or $F_{ST}/(1 - F_{ST})$ and geographic distance or trait differences. For each test, statistically significant *P*-values are shown in bold.

	F_{ST}	$F_{ST}/(1 - F_{ST})$
Geographic distance	**< 0.0001**	**< 0.0001**
Log (Geographic distance)	**< 0.0001**	**0.0032**
Trait differences	0.9748	0.9545

4-8 Figures

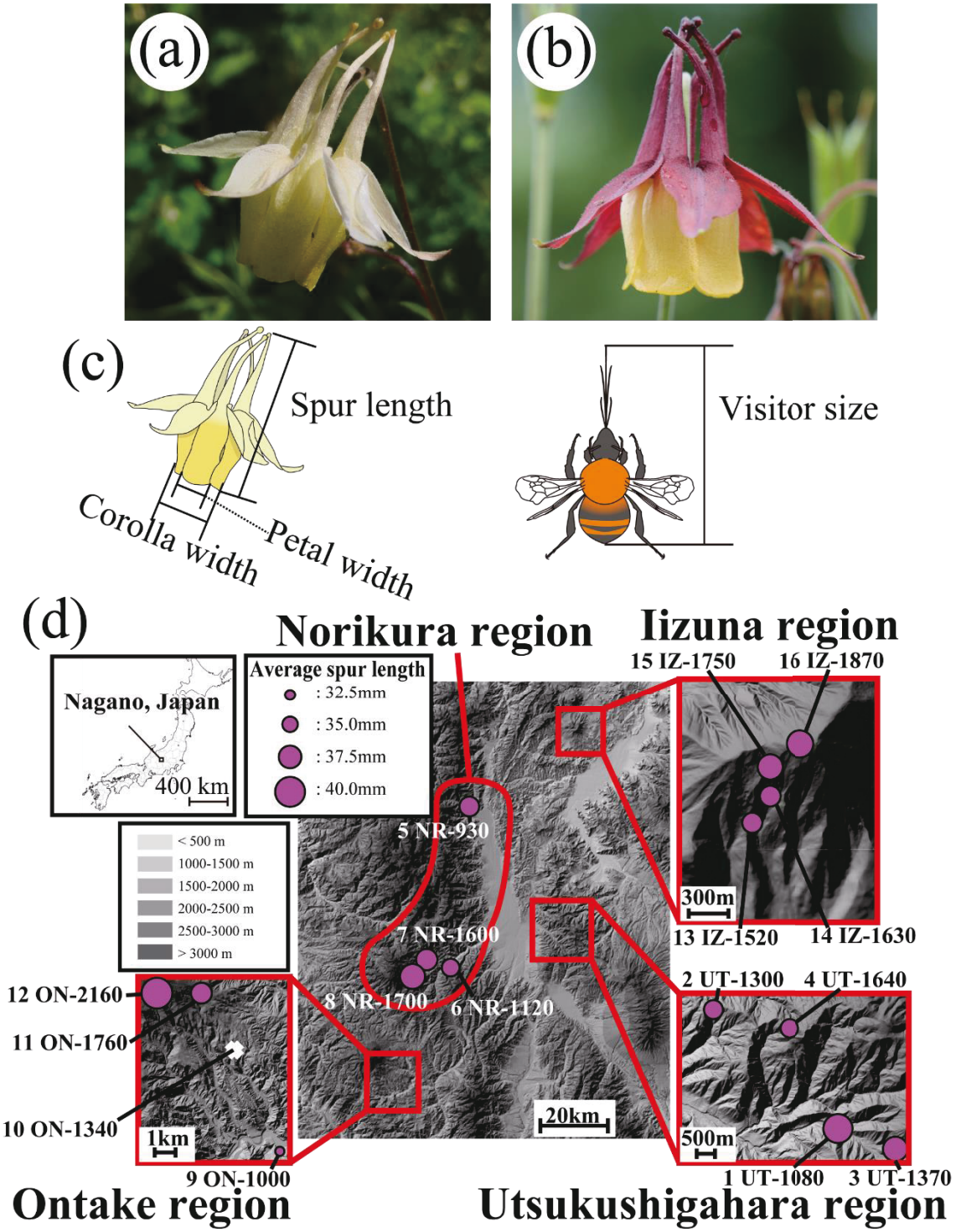

Figure 4-1. Study species and the study sites. (a) Yellow flower and (b) red flower of *A. buergeriana* var. *buergeriana*. (c) Measurement of floral spur length and bumblebee

size. (d) Locations of the 16 surveyed populations in the four mountain regions (populations are indicated by "Population no. Region abbreviation-altitude [in meters]". The size of the purple circle at each site indicates the average spur length of the flowers at that site (spur length was not observed in the 10 ON-1340 population).

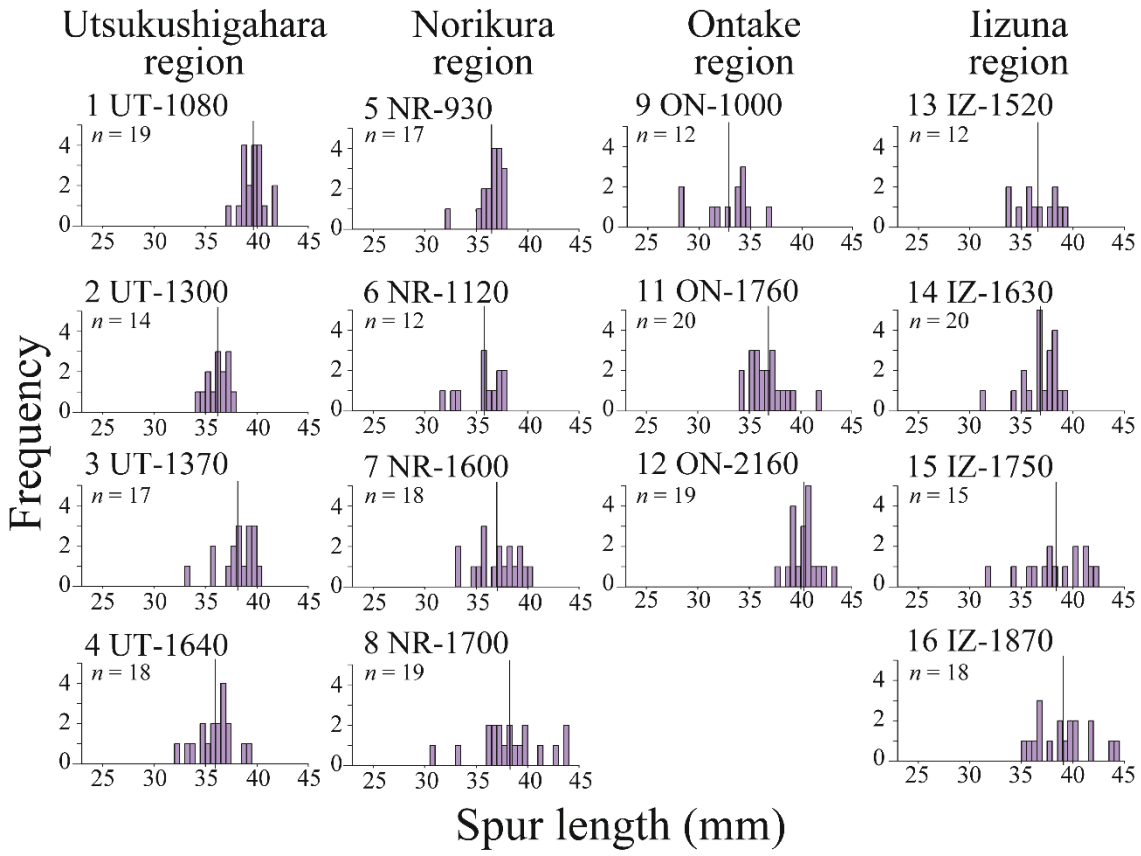

Figure 4-2. Frequency distributions of spur length in populations of *A. buergeriana var. buergeriana*. The vertical line in each histogram indicates the mean spur length in that population. *n* indicates the sample size. Spur length was not measured in the 10 ON-1340 population.

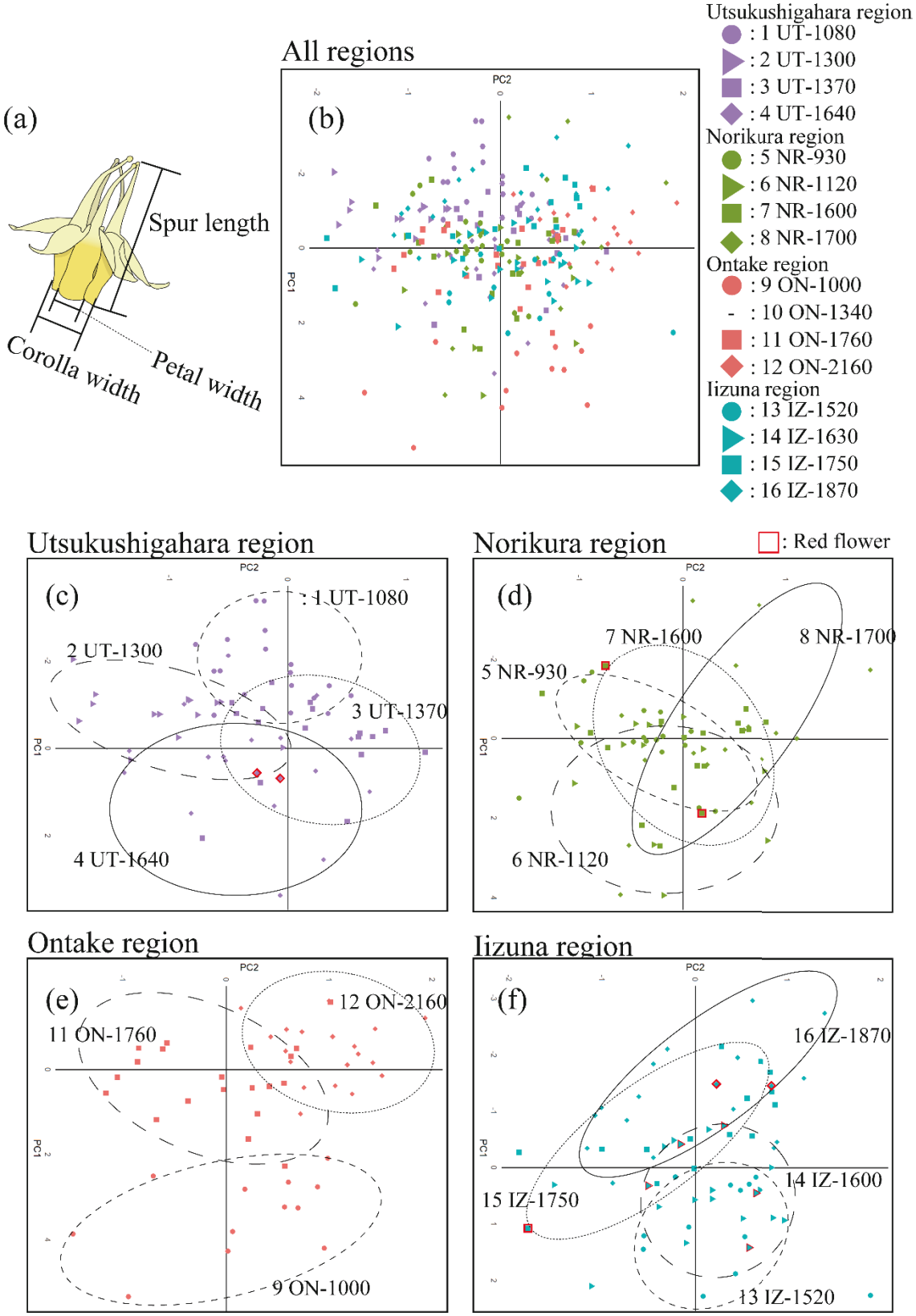
(a)
Spur length
Corolla width
Petal width
All regions
(b)
PC2
PC1
Utsukushigahara region
: 1 UT-1080
: 2 UT-1300
: 3 UT-1370
: 4 UT-1640
Norikura region
: 5 NR-930
: 6 NR-1120
: 7 NR-1600
: 8 NR-1700
Ontake region
: 9 ON-1000
: 10 ON-1340
: 11 ON-1760
: 12 ON-2160
Iizuna region
: 13 IZ-1520
: 14 IZ-1630
: 15 IZ-1750
: 16 IZ-1870
Utsukushigahara region
(c)
1 UT-1080
2 UT-1300
3 UT-1370
4 UT-1640
Norikura region
: Red flower
(d)
7 NR-1600
8 NR-1700
5 NR-930
6 NR-1120
Ontake region
(e)
12 ON-2160
11 ON-1760
9 ON-1000
Iizuna region
(f)
16 IZ-1870
14 IZ-1600
15 IZ-1750
13 IZ-1520

Figure 4-3. Principal component analysis (PCA) results for three floral traits in *A. buergeriana var. buergeriana*. (a) Measurement of each trait. (b) PCA results for all floral traits of individuals in all populations. (c–f) PCA results for all floral traits of individuals in the populations of each mountain region. Ellipses indicate the different population groupings. Symbols for red-flowered individuals are outlined in red.

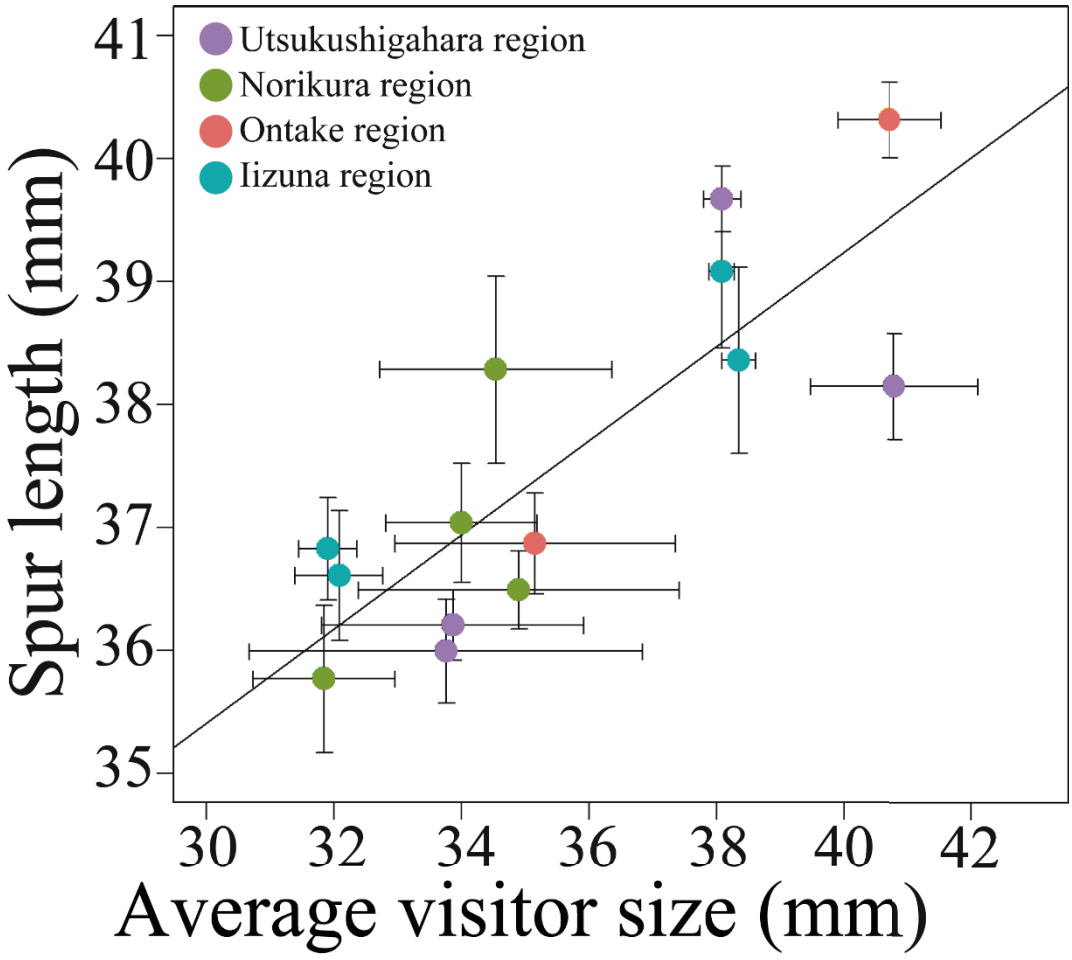

Figure 4-4. Relationship between average spur length and average visitor size (only bumblebees) in populations of *A. buergeriana* var. *buergeriana*. A regression line was fitted to the data with reference to the LMM results ($P < 0.0001$). The 12 ON-1000 population was not visited by bumblebees, so it was excluded from the analysis. Error bars indicate standard errors.

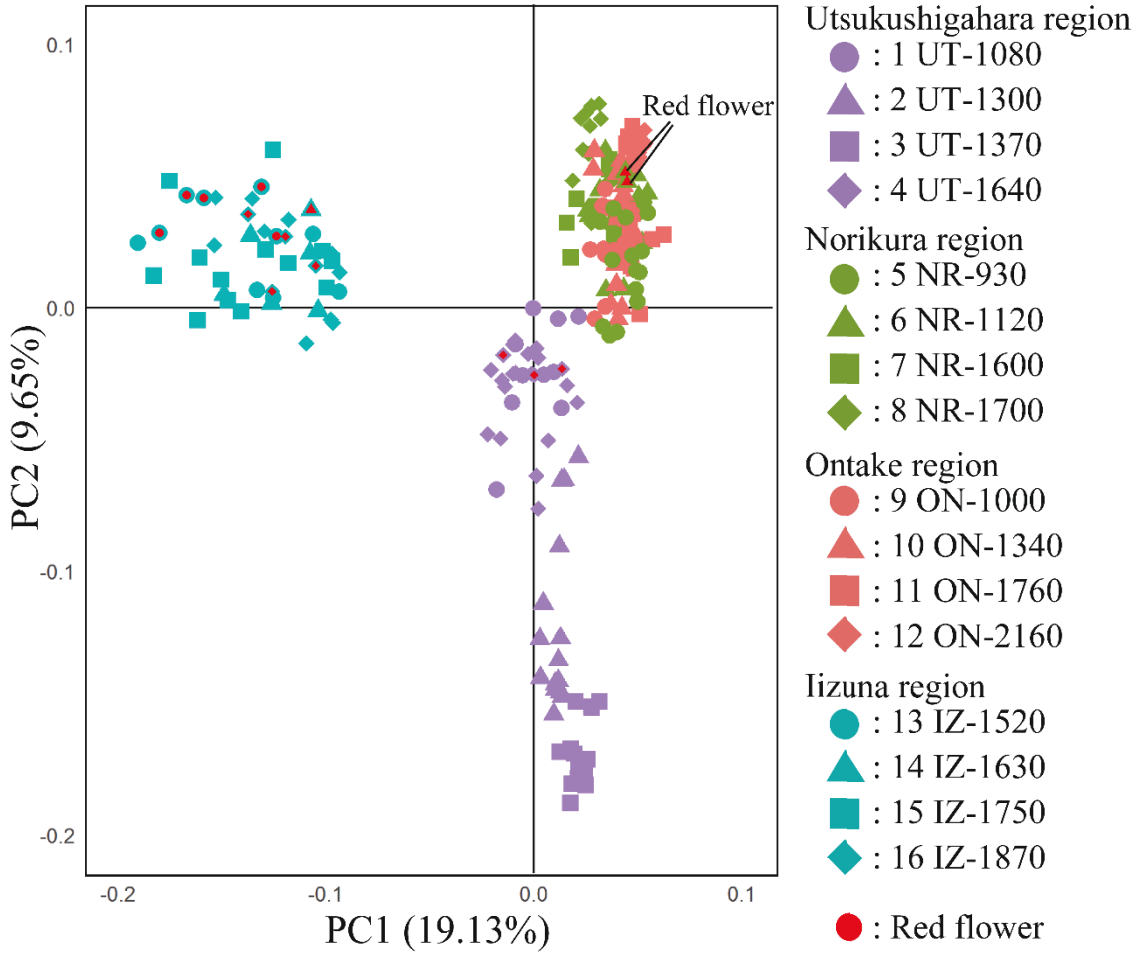

Figure 4-5. Principal component analysis results for all populations. Principal component 1 (PC1; contribution rate 19.13%) is plotted on the horizontal axis and PC2 (contribution rate 9.65%) on the vertical axis. The red-flowered individuals sampled in some populations are shown by symbols with a red center.

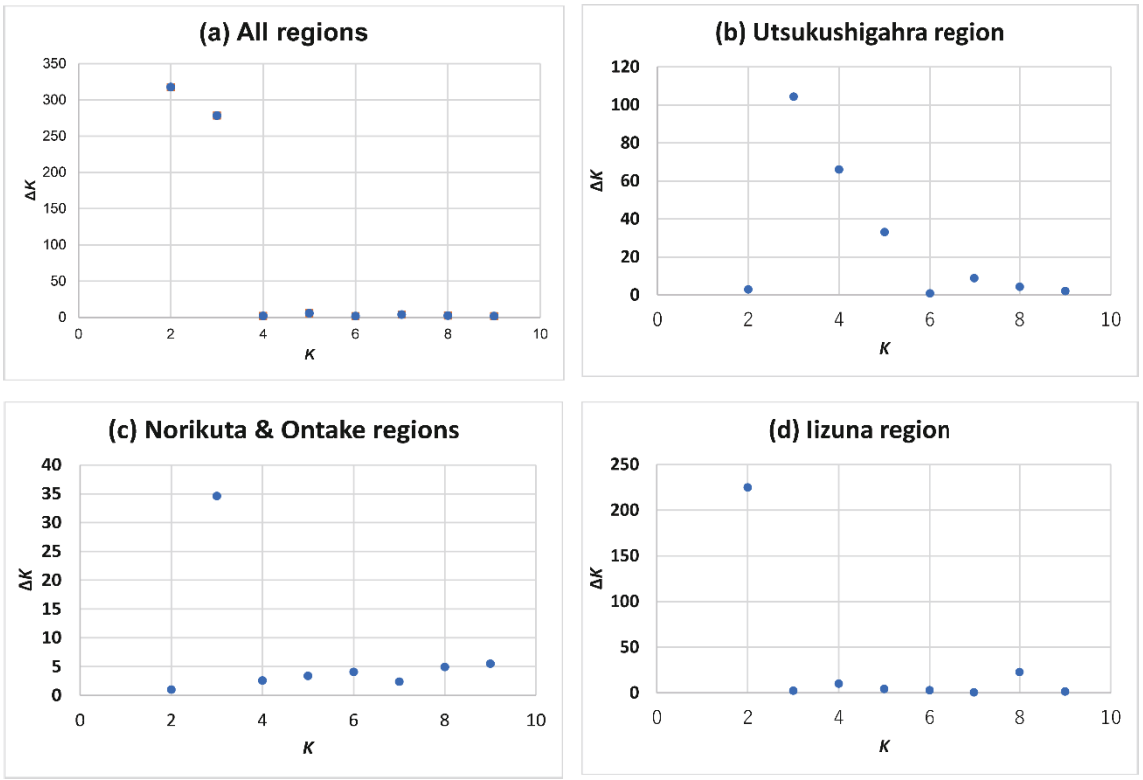

Figure 4-6. Number of appropriate clusters (K) determined by the STRUCTURE analysis based on ΔK. (a) When all populations were included in the analysis, $K = 2$ or $K = 3$ was inferred to be the appropriate number of clusters. (b) For populations in the Utsukushigahara region only, $K = 3$ was appropriate. (c) For Norikura+Ontake populations only, $K = 3$ was appropriate. (d) For Iizuna populations only, $K = 2$ was appropriate.

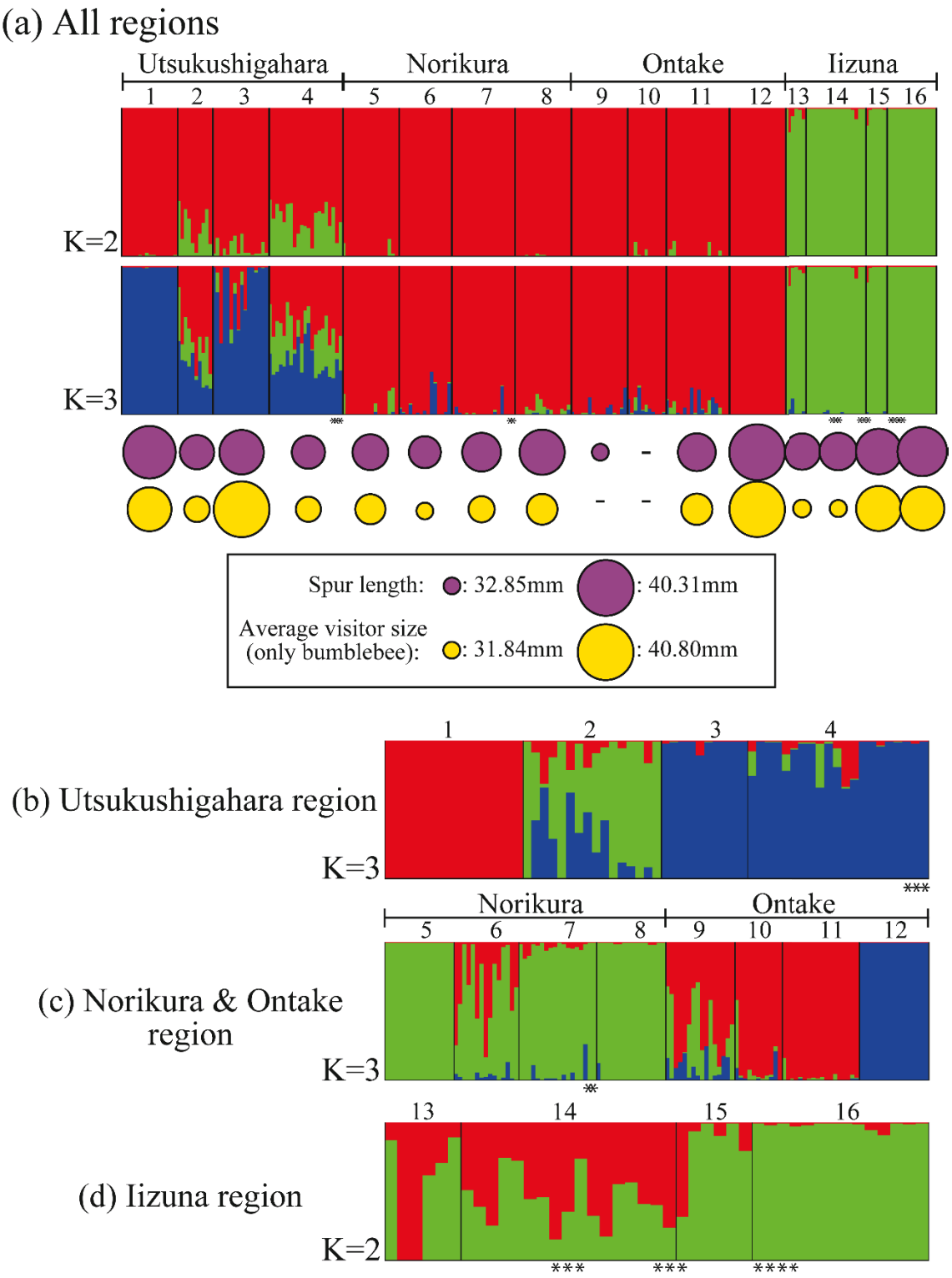

Figure 4-7. STRUCTURE analysis results. In each case, the appropriate value of *K* was determined from Δ*K*. Population numbers, corresponding to those in Figure 1, are shown

along the top of each panel. Populations in which red-flowered individuals were sampled are indicated by asterisks along the bottom of each panel. (a) Cluster analysis results for SNPs in all populations (K = 2, 3). The relative relationship between average spur length relative and average visitor size (only bumblebees) in each population is shown by the relative sizes of the purple and yellow circles. Cluster analysis results for SNPs of only the (b) Utsukushigahara (K = 3), (c) Norikura+Ontake (K = 3), and (d) Iizuna (K = 2) regions.

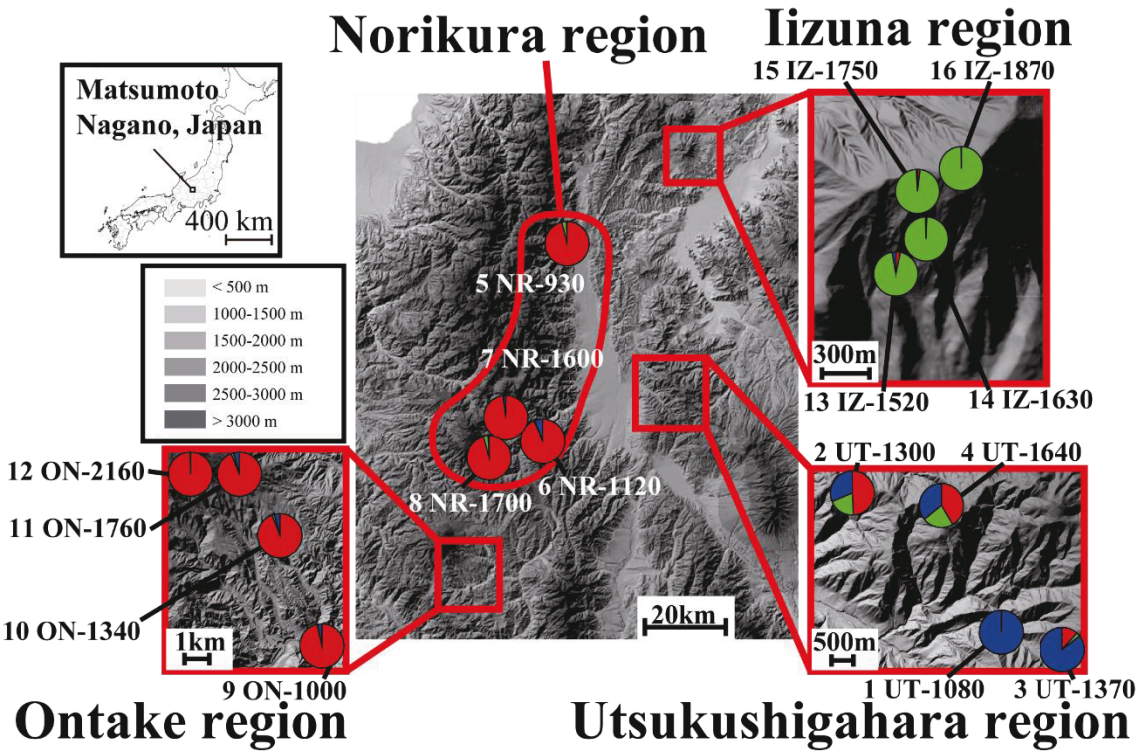

Figure 4-8. The results of the STRUCTURE analysis for all populations with $K = 3$. For each population, the circle graph shows that the relative probability of the population belonging to each of the four clusters (indicated by different colors). To obtain the probability of the population belonging to a cluster, the probabilities that the individuals in the population belonged to the cluster were averaged.

Chapter 5

Bimodal floral size within a population caused by disruptive selection through pollinator floral size preferences

5-1 Abstract

Understanding the mechanisms that generate and maintain trait variation within natural populations has long been a major goal in evolutionary biology. Intra-specific and -population trait dimorphism has attracted considerable attention in perspective of speciation but has rarely been reported. I found that a bimodal floral size distribution was observed in a population of *Lamium album* var. *barbatum* over 2 years. In this population, pollinators, small and large bee species, tended to visit and pollinate flowers with a floral size matching their size. As a result of this pollinator preference, the fitness of ramets with floral size of intermediate length was lower than that of ramets with long or short floral size. Microsatellite analysis revealed a slight genetic differentiation between ramets with long or short floral size. Additional genetic analysis showed no evidence of secondary contact with allopatric populations with long or short floral size. These results strongly suggest that, in the population, the bimodal distribution of floral size has sympatric origin and is maintained by disruptive selection resulting from the pollinator preferences to floral size. This study, for the first time, demonstrates that sympatric differences in floral size can alter the behavior of flower visitors.

5-2 Introduction

Floral trait divergence is generally understood to result from geographic variation of pollinators. When the pollinator species composition differs within the distribution range

of a plant species, a geographic mosaic of selection pressures is created, resulting in the formation of pollinator ecotypes with different morphological traits (Anderson et al., 2014). Local adaptation to geographically different pollinators reduces the sharing of pollinators between populations and results in the establishment of prezygotic isolation (Herrera et al., 2006; Anderson and Johnson, 2008; Newman et al., 2015). The Grant-Stebbins model of floral divergence (Grant and Grant, 1965; Stebbins, 1970; Johnson, 2010), which is based on this idea, is the most convincing hypothesis to explain the early stages of allopatric speciation in angiosperms. Geographical variation in floral tube length is the most common examples of floral divergence, and many studies have shown their correspondence to geographically different pollinator sizes (Anderson and Johnson, 2008; Pauw et al., 2009; Anderson et al., 2014; Boberg et al., 2014; Nagano et al., 2014; Kuriya et al., 2015). It is conceivable that disruptive selection for floral traits might occur within a single population by a similar mechanism if multiple pollinators are present with different morphological traits. However, this possibility has received little attention (but see (Campbell et al., 1997; Rymer et al., 2010). Disruptive selection is selection for extreme traits in a population (Conner and Hartl, 2004). For example, if I postulate sympatric populations of plants with large or small flowers, each of which has a reproductive advantage over ones with intermediate-sized flowers, then disruptive selection might cause these phenotypes to become reproductively isolated, thus leading to sympatric speciation (Maynard Smith, 1966; Rosenzweig, 1978; Dieckmann and Doebeli, 1999).

Many evolutionary ecologists have long believed that pollinator behavior (floral preferences) causes morphological isolation of floral traits, thereby leading to allopatric or sympatric speciation (Gegear and Burns, 2007). However, there is little evidence of

disruptive selection caused by differences in pollinator preference. In one of the few examples in the literature, Anderson et al. (2010a) showed that small and large flower ecomorphs with different floral scents within one population of the iris *Gladiolus longicollis* were visited by species of hawkmoth with varying proboscis length, and that these differences in flowering phenology were linked to the flower preferences of the pollinators. In a subsequent study, Rymer et al. (2010) showed, by a paternal parent analysis in a population of *G. longicollis* with low plant density, assortative mating occurs between flowers of similar size, and they suggested that differences in the behavioral patterns of pollinators might result in non-random mating within a population. Campbell et al. (1997) reported that in a hybrid zone between *Ipomopsis aggregata* (a species with a wide red corolla favoured by hummingbirds) and *I. tenuituba* (with a narrow white corolla favoured by hawkmoths), disruptive selection for corolla width occurred in hybrid individuals. Although hybrid individuals displayed great trait variation, the different corolla widths preferred by hummingbirds and hawkmoths caused disruptive selection for floral traits. As a result, trait differences between the two species were maintained by pollinator preference. These examples, however, focus on secondary contact zones between two separately occurring ecomorphs.

There are two possible explanations for this trait bimodality: a pattern of disruptive selection occurring in sympatry (e.g., Cichlid (Martin, 2012)), or a pattern of secondary contact between populations with different traits, as in the examples above. It is generally believed that trait bimodality is more likely to occur as a result of secondary contact (Coyne and Orr, 2004). As an example validated in floral size, one study used allozyme markers to show that the bimodal floral-tube length distribution in a population of *Lapeirousia anceps* (Iridaceae) originated through secondary contact (Anderson et al.,

2016). Interestingly, the only pollinator of this population was the bee fly *Moegistorhynchus longirostris*, which has a long proboscis, and premating isolation of the two phenotypes appears to be caused by the pollen's adhering to different parts of the bee fly when it visits long and short corollas (Minnaar et al., 2019). However, clear examples of bimodality of flower traits occurring in sympatry have been lacking. In any case, for bimodality of traits to be maintained in a population, a strong reproductive barrier causing disruptive selection must exist between widely different phenotypes within a population (Maynard Smith, 1966; Kingsolver et al., 2001; Gavrilets, 2004).

Meta-analysis suggested that disruptive selection is as common as stabilizing selection which is the most common mechanism in natural population (Kingsolver et al., 2001). However, although there is increasing evidence for disruptive selection in natural populations, most early studies were theoretical (Maynard Smith, 1962; Ajar, 2003; Zhang et al., 2013) (but see (Bolnick, 2004; Calsbeek and Smith, 2008; Hendry et al., 2009; Martin and Pfennig, 2009; Martin, 2012; Anderson et al., 2016)), and bimodally distributed traits in natural populations have often been overlooked. To rectify this situation, populations with bimodally distributed traits need to be identified and empirical studies to identify the origin of the bimodality need to be conducted.

Geographical variation in both floral size and pollinator size as well as trait matching between plants and pollinators are known to occur in *Lamium album* var. *barbatum* (Hattori et al., 2015; Hattori. et al., 2021; Toji et al., 2021). In 2018, Toji et al. (2021) measured floral size in 12 populations of *L. album* var. *barbatum*, but found a bimodal floral size distribution in only one population. I was motivated by this finding to conduct another study to examine how the bimodal floral size distribution was maintained in this population. First, I confirmed that the bimodal floral size distribution observed in 2018

(Toji et al., 2021) was maintained in the population in 2019. Then, by combining the data collected over the two years, I sought answers to the following questions. (1) Do pollinators of this population show a species-specific floral size preference; for example, do large pollinators visit only large flowers? (2) Does the fitness (seed set) of plants with different floral sizes show evidence of disruptive selection by selection gradient analysis? (3) Does genetic isolation occur in sympatry between plants with large and small flowers? (4) Did the population with a bimodal floral size distribution arise in sympatry through disruptive selection, or through secondary contact between two populations with different floral sizes?

5-3 Materials and Methods

5-3-1 Plant species and study site

Lamium album L. var. *barbatum* (Lamiaceae) is a perennial herb pollinated by bumblebees and a small bee species. Its creamy-white, two-lipped, self-incompatible flowers (Sulborska et al., 2014; Hattori et al., 2015) are frequently visited by bumblebee species, and flower–pollinator trait size matching has been observed: that is, the larger the difference between the tongue length of the bumblebees and the floral size of *L. album* var. *barbatum*, the smaller the seed set per single bumblebee visit (Hattori et al., 2021). When a bumblebee visits a flower of this species and inserts its tongue into the inner corolla tube to forage for nectar, its head and thorax rub against the anthers and stigma.

Among the 12 populations studied in 2018, Toji et al. (2021) found a bimodal floral size distribution in only the Ohmizusawa population (137°78’68” E, 36°30’53” N, 1000 m a.s.l.). In this study, I collected survey data of a second year and then conducted a selection gradient analysis of the relationship between floral size and seed set. I also

observed pollinator behavior in relation to floral size. Floral size was measured during the May–June flowering period in both 2018 ((Toji et al., 2021)) and 2019 (this study). Pollinator behavior was observed and seed set was measured during May–July 2019.

5-3-2 Floral size and pollinator size

I measured floral size with digital callipers (precision, 0.01 mm) by the method of Hattori et al. (2015) (Figure 5-1a). The size of 1–5 flowers on 99 randomly selected ramets was measured in 2018 (Toji et al., 2021) and on 202 ramets in 2019 (this study) in the Ohmizusawa population. Statistically, floral size within ramets did not differ significantly (Toji et al., 2021), so the average length of the 1–5 measured corolla tubes was used as the floral size of the ramet. Ramets with a floral size larger than 28.00 mm were defined as "large flowers" and those with a floral size smaller than 28.00 mm as "small flowers" (Figure 5-1b). Also, if necessary, "intermediate" is used for ramets with flowers within 28.00 ± 0.50 mm. Within the population, large and small flower ramets were mixed and randomly distributed. The Silverman test for multimodality (Silverman 1981), where the null hypothesis was that "the number of modes in the frequency distribution is *n* or less", was used to test the floral size distribution in both 2018 and 2019 for *n* = 1–5 modes. The first *n* for which the null hypothesis cannot be rejected in this test can be adopted as the number of modes (Silverman 1981; Efron and Tibshirani 1994).

To investigate the pollinator assemblage of the population, I established a quadrat of approximately 1 m × 1 m (containing about 100 ramets) and recorded flower-visiting insects within the quadrat. In addition, I walked around the entire study area (10 m × 20 m) and sequentially captured insects to determine the size of the flower-visiting insects both 2018 and 2019. When a large bee (bumblebee) inserts its head into the nectary of a

flower, its head touches both the anthers and pistil of the flower. Therefore, for large bee pollinators, I used the combined proboscis length and head length as the pollinator size. A small bee crawls into the flower, and its whole body may touch the anthers and pistil. Therefore, for small bees, I used the whole-body length, from the tip of the proboscis to the end of the abdomen, as the pollinator size.

In 2018, floral size measurements were made on four days between 11 May and 7 June. Therefore, it is possible to test whether temporal isolation between large and small flowers occurred through differences in flowering time. In the 2019 survey, however, floral size was measured only on 13 and 14 June, at the flowering peak season, so I could not compare flowering time between large and small flowers.

5-3-3 Pollinator behavior

To investigate the pollinator preference in relation to the floral size of *L. album* var. *barbatum*, I conducted behavioral observations of pollinators by examining the floral size of flowers visited by pollinators in the study area (about 10 m × 20 m). I observed the behaviors of four major pollinators, *Bombus honshuensis* worker (large bee), *B. honshuensis* queen (large bee), *B. consobrinus* queen (large bee), and *Ceratina japonica* (small bee), during a total of 299 observations of bee–flower interactions in 2019. During these observations, I classified the observed behaviors into types. The three large bees (bumblebees) exhibited two behaviors: "Visit" and "Avoid". The "Visit" behavior was defined as when a bumblebee puts its head inside a flower to forage for nectar (Figure 5-1c), and the "Avoid" behavior was defined as when a bumblebee flies to within 10 cm of a flower but does not alight, or when it briefly touches the flower but then leaves it without foraging. The small bee, *C. japonica*, exhibited three behaviors: "Legitimate visit",

"Nectar robber", and "Pollen foraging". A "Legitimate visit" was defined as when the small bee landed on the upper part of the flower and then crawled across the anthers and pistil on its way to the nectary (Figure 5-1d). "Nectar robber" was defined as when a small bee landed on the lower lip of the flower and then crawled directly into the nectary, bypassing the anthers and pistil, so not contributing to pollination (Figure 5-1e). "Pollen foraging" was defined as when a small bee licked the anthers or collecting pollen grains without going to the nectary (Figure 5-1f). I examined whether a pollinator exhibited different behaviors towards large (>28 mm) and small (<28 mm) flowers by a chi-square test.

5-3-4 Reproductive success and selection gradient analysis

To measure the fitness of *L. album* var. *barbatum* in relation to floral size, I marked the 100 ramets whose floral size was measured in 2019 with tape. Then, after the flowers were finished, I collected the fruits and measured seed set. Some of the marked ramets died without developing fruit. Thus, I eventually collected 82 ramets whose seed set could be measured. At the same time, I collected leaf samples from 83 of the marked ramets for genetic analysis (see the next section). The number of mature seeds (MS) and immature seeds (IM) were counted in up to 10 fruits per plant, and the ratio MS/ (MS + IM) was used as the ramet seed set. Each *L. album* var. *barbatum* flower has four ovules; thus, the seed set of each flower was easily checked visually.

I considered the seed set per ramet as female fitness and conducted a selection gradient analysis to examine selection for floral size. I standardized seed set and floral size ($\bar{x}$=0, σ=1) and then conducted a quadratic regression analysis on the resulting data set. To estimate the selection coefficients, I doubled the quadratic regression coefficients

(Stinchcombe et al., 2008). In the regression analysis, β is the first-order regression coefficient, and non-zero values suggest the possibility of directional selection; and γ is the second-order regression coefficient, and a value greater than zero suggests stabilizing selection, whereas a value less than zero suggests disruptive selection (Conner and Hartl, 2004).

The coefficients of the regression curve obtained by the selection gradient analysis were not significant, so I applied a smoothing spline (Eubank, 1988) to the relationship between seed set and floral size. To calculate the appropriate smoothing parameter (SP), I applied generalized cross-validation (GCV) to the regression model (Golub et al., 1979). The lower the SP, the smoothed spline curve becomes (i.e., the fit to the data plot is improved). However, if the SP is too low, then the prediction accuracy of the model will be poor, so the determination of the optimal SP value by application of GCV is appropriate.

5-3-5 Genetic isolation between sympatric ramets with large and small flowers and the possibility of secondary contact

To estimate the degree of genetic differentiation between ramets with large and those with small flowers, I used 10 microsatellite markers, which were developed for *L. album* (Horsley, 2013). I used the fresh leaf samples collected from 83 ramets of known floral size in 2019 for the analysis. DNA extraction and genotyping were conducted as described by Toji et al. (2021).

Given that ramets of large and small flowers have different genetic pools, I tested for significant differences in allele frequencies using the 'genic differentiation' option in Genepop v. 4.7 (Rousset, 2008). I also used the F-statistic (F_{ST}) and the R-statistic (R_{ST}) to estimate genetic differentiation (Weir and Cockerham, 1984; Slatkin, 1995). In addition,

I calculated genetic distances D_A (Nei et al., 1983) and D_{SW} (Shriver et al., 1995) using POPTREE2 (Takezaki et al., 2010). D_{SW} takes into account the number of repeats in the microsatellite region.

I compared D_{SW} between populations to evaluate whether secondary contacts with populations surrounding the Ohmizusawa population could account for the bimodal floral size distribution in the Ohmizusawa population. I calculated the genetic distances between plants with large and small flowers in the Ohmizusawa population and plants in the Ougisawa, Mitsumata, Onosawa, and Norikuta populations, which are geographically and also genetically (using microsatellites) close to Ohmizusawa (Toji et al., 2021). If secondary contact gave rise to bimodality of the Ohmizusawa population, I expected that plants with small and large flowers in the Ohmizusawa population would be genetically close to different surrounding populations. I used genotype data from Toji et al. (2021) for populations other than Ohmizusawa. The microsatellite genotype data for the Ohmizusawa population were collected as part of the present study, which corresponds to the ramets whose floral size were measured. I used POPTREE2 to calculate the genetic relatedness between populations from the allele frequency data. The unweighted pair-group with arithmetic mean method (Sneath and Sokal, 1973) and the D_{SW} values between populations, taking account of the number of microsatellite repeats, were used to draw a dendrogram. Support values for the dendrogram were calculated by performing 1000 bootstrap calculations.

To examine the individual based genetic structure, I performed a Bayesian clustering analysis was performed with STRUCTURE software version 2.3.4. (Pritchard et al., 2000 ; Falush et al., 2003). The fragment length dataset of the six populations used in the above analysis was used directly for this analysis. I used this analysis to determine the

genetic cluster to which each individual is assigned. Analysis was conducted with 100 k burn-in iterations and 100 k Markov chain Monte Carlo repetitions. The number of genetic clusters (K) was calculated 10 times for each of 1–10, and the ΔK value (Evanno et al., 2005) was used as the criterion for selecting the appropriate number of clusters.

5-4 Results

5-4-1 Floral size and pollinator size

Floral size distributions with two peaks were obtained in both the 2018 and 2019 study years (Figure 5-2a). I created Silverman test P-value plots for the floral size distribution, where the significance level was $\alpha = 0.05$. As a result, the null hypothesis that the number of modes was one or less was rejected for both years (Figure 5-2b), but the null hypothesis that the number of modes was 2–5 or less could not be rejected. Thus, it is appropriate to consider the floral size distribution to have two modes in each of the two years. The results of the 2018 floral size survey showed that large flower ramets bloomed from the beginning to the end of the flowering season, whereas small flower ramets appeared to start blooming slightly later (Figure 5-3).

Small bees accounted for 97% of the pollinator assemblage in 2018, and for 75% of the assemblage in 2019 (Figure 5-2c). The small bee *Ceratina megastigmata* (average pollinator length 11.19 mm) was collected in large numbers in 2018, whereas in 2019, the small bee *C. japonica* (average pollinator length 12.24 mm) was mainly collected. Among large bees, the average head plus proboscis length was 16.16 mm in *B. honshuensis* workers, 19.74 mm in *B. honshuensis* queens, and 27.26 mm in *B. consobrinus* queens in 2018 (Toji et al., 2021).

5-4-2 Pollinator behavior

The composition of behaviors exhibited by all the pollinator species differed between large (> 28 mm) and small (< 28 mm) flowers (Figure 5-4; chi-square test; *B. honshuensis* worker, $\chi^2 = 15.9$, $P < 0.01$; *B. honshuensis* queen, $\chi^2 = 25.2$, $P < 0.01$; *B. consobrinus* queen, $\chi^2 = 41.6$, $P < 0.01$; *C. japonica*, $\chi^2 = 47.7$, $P < 0.01$). Large bees (bumblebees) visited large flowers at a high rate (82.7–98.1%) and avoided small flowers at a high rate (63.3–72.0%). The small bee *C. japonica* exhibited the Nectar robber behavior at a high rate (91.4%) in large flowers (visiting the nectary without touching the anthers and pistil), whereas 100% (23/23) touched the anthers and pistil of small flowers (Legitimate visit). Rarely, small bees exhibited pollen foraging behavior in flowers of intermediate size (around 28 mm; 13.4%, 9 out of 67 observations of small bee behaviors).

5-4-3 Reproductive success and selection gradient analysis

The measured floral size and seed sets of 82 ramets were used for the selection gradient analysis. A convex regression curve was fitted to the relationship between seed set and floral size, but the regression coefficients did not indicate statistically significant disruptive selection or directional selection (Figure 5-5; $\gamma \pm \mathrm{SE} = 0.068 \pm 0.111$, $P = 0.543$; $\beta \pm \mathrm{SE} = 0.214 \pm 0.113$, $P = 0.063$).

I therefore applied a smoothing spline to the floral size and seed set data set. The GCV value was lowest when the smoothing parameter (SP) was SP = 0.007, so this SP value was used in the smoothing spline model (Figure 5-6). The smoothing spline model significantly explained the change in seed set with respect to floral size (Figure 5-7; $P < 0.05$). In addition, the smoothing spline model significantly improved the accuracy of the fit of a line to the data plot (ANOVA, df = 6.98, F = 2.35, $P < 0.05$). According to the

spline curve, the greatest reduction in fitness was for a floral size of 27.60 mm.

5-4-4 Genetic isolation between sympatric ramets with large and small flowers and the possibility of secondary contact

The genetic differentiation analysis using 10 microsatellite markers between ramets with small and large flowers in the Ohmizusawa population detected a genetic difference in the two loci (LA5 and LA63) and in the loci overall ($P < 0.05$; Table 1). At three loci (LA35, LA54, and LA58), all individuals in Ohmizusawa had one fixed allele. After the application of the Bonferroni correction to the results, only the LA63 locus met the statistical significance level ($\alpha = 0.05$). The calculation of genetic distance D_{SW} between the Ohmizusawa population and surrounding six populations showed that the genetic distance between small (< 28 mm) and large (> 28 mm) flowers of the Ohmizusawa population was smaller ($D_{SW} = 0.012$) than that between the Ohmizusawa population and any of the other populations ($D_{SW} = 0.872$–2.878). The monophyly of the Ohmizusawa population was supported on the D_{SW} dendrogram by a high bootstrap value (Figure 5-8). Based on ΔK, the Bayesian clustering analysis STRUCTURE results supported that K=2 was appropriate (Figure 5-9). Cluster analysis suggested that Ohmizusawa and its surrounding populations assigned different genetic clusters (Figure 5-8). Although gene flow from the Ohmizusawa population to the Onosawa population was suggested, little gene flow between clusters seemed to have occurred.

5-5 Discussion

A statistically significant multimodality of the frequency distribution of floral size in the Ohmizusawa population was found in both years (Figure 5-2b). Two distinct peaks

were observed in both 2018 and 2019 (Figure 5-2a), and significant differences in pollinator behavior between large and small flowers were observed during 2019 (Figure 5-4). Application of a smoothing spline to the relationship between seed set and floral size showed that the female fitness of flowers with an intermediate floral size was decreased (Figure 5-7). These results suggest that large and small pollinators may change their behavior depending on the floral size of the flower being visited, leading to the disruptive selection of floral size. In 2018, both large and small flowers were abundant on 1 June, the peak of the 2018 flowering season. Large flowers tended to start blooming slightly earlier than small flowers, but a large temporal isolation of flowering time between large and small flowers was not observed (Figure 5-3).

5-5-1 Differences in pollinator behavior in relation to floral size

Our observations of pollinator behavior showed that large bees (bumblebees) tended to visit large flowers and avoid small flowers (Figure 5-4). Bumblebees are generally known to follow an optimal foraging strategy (Heinrich, 1979; Ohashi and Yahara, 1998), and they have been shown to be more efficient at foraging when they visit flowers that are a match for their body size (Heinrich, 1979; Inouye, 1980; Dohzono et al., 2011). The largest pollinator, *B. consobrinus* queens, did not visit flowers smaller than 27.7 mm (Figure 5-4), but the relatively small bumblebee species, *B. honshuensis* workers and queens, occasionally visited small flowers. Overall, the relatively large bumblebees may avoid small *L. album* var. *barbatum* flowers because they are less efficient when foraging in small flowers. Bumblebees may recognize the floral size of *L. album* var. *barbatum*, either visually or once they touch the flower. I observed bumblebees to avoid a flower after hovering, facing the flower squarely, and then flying away, or after first touching a

flower and then flying away without foraging.

The small bee *C. japonica* was observed to forage on flowers of any size, but its behavior differed depending on the flower size (Figure 5-4). The behaviors of small bees visiting small flowers can result in pollination and thus are profitable for the flowers, but the nectar robbing behavior of small bees visiting larger flowers is detrimental to the flowers. Why did the behavior of small bees change with floral size? Flowers with longer corolla tubes are likely to have a larger flower entrance. As a result of this ease of access, bees may be more likely to first alight on the lower lip when they visit a large flower (as shown in Figure 5-1e). Conversely, the flower entrance of smaller flowers is narrower, which may make access to the nectary from the lower lip more difficult. As a result, small bees may alight instead on the top of the flower (as shown in Figure 5-1d). The pollen foraging behavior in small bees was rare compared to the other behaviors, and it is unclear whether this behavior contributes to pollination.

Optimal foraging strategies have been reported for larger bees (Heinrich, 1979), but little information is available for smaller bees. In our observations, they often stayed on leaves and showed little interest in foraging, which suggests that they were not following an optimal foraging strategy (T. Toji personal observation). *C. japonica* has been documented to have a 'polylectic' habit, in which they visit various flowers in a disorderly manner (Miyamoto, 1961). Although at our study site, *C. japonica* was more numerous than the bumblebees, its pollination efficiency was probably lower than that of bumblebees, because it appeared to visit flowers sporadically. In general, bumblebees are considerably more efficient than other insects in terms of the number of flowers visited per hour and pollination efficiency per visit (Heinrich, 1979; Mayfield et al., 2001; Toji et al., 2020). Small flowers constituted a smaller part of the Ohmizusawa population than

large flowers, perhaps because they are pollinated by small bees such as *C. japonica*, which are less efficient pollinators. However, it remains unclear why large and small flowers can exist sympatrically. The flower-visiting insect fauna is highly variable from year to year (Dupont et al., 2009; Kudo and Ida, 2013), and this uncertainty may affect fitness with regard to floral size. For example, in a year with extremely few bumblebees, large flowers would be at a relative disadvantage. In fact, during our two-year survey, variation in the pollinator assemblage was large (Figure 5-2c). In the future, it would be useful to examine whether the bimodal floral size is maintained over a longer term and to examine the annual variation of the fitness of ramets with large/small flowers.

5-5-2 Reproductive success and floral size

After the application of a smoothing spline, floral size could significantly explain the change in seed set (Figure 5-7). The spline curve showed that fitness was decreased for an intermediate floral size (around 28 mm). Flowers with an intermediate floral size were not effectively pollinated by either large or small bees, which may have caused a reduction in their fitness. However, our results are only for female plant fitness, whereas the overall fitness of a plant is the sum of paternal fitness (male reproductive success through pollen export and receipt), and maternal fitness (female reproductive success through seed production) (Christopher et al., 2020). Therefore, to measure overall fitness, the success of the paternal parent must be analysed using genetic markers to determine paternity (Briscoe Runquist et al., 2017; Christopher et al., 2020). However, in *L. album* var. *barbatum* the anthers and pistil are very close together in the same flower (Figure 5-1a); thus, pollen receipt and pollen export may be strongly correlated.

5-5-3 Genetic isolation and sympatric origin of large and small flowers

Genetic differentiation analysis showed a difference in allele frequencies between large and small flowers (Table 1). As a similar case of this study, beak size in Darwin's finch (*Geospiza fortis*) on Santa Cruz Island, Galápagos is known to show a bimodal distribution, with many individuals having large or small beaks but few with intermediate sized beaks (Huber et al., 2007). This bimodal distribution of beak size may have resulted from disruptive selection for two morphologies specialized to different preference of feeding seeds, and it may represent an early stage of speciation (Herrel et al., 2005). Genetic differentiation analysis showed that genetic isolation between large and small beaked finches, with F_{ST} and R_{ST} values between the two morphologies larger than those detected in this study between plants with large and small flowers (Darwin's finch: F_{ST} = 0.017, R_{ST} = 0.040 (Huber et al., 2007); *L. album* var. *barbatum*: F_{ST} = 0.006, R_{ST} = 0.002; Table 1). In the case of our results, the genetic differentiation analysis results after Bonferroni correction were mainly not statistically significant, and F_{ST} and R_{ST} values were small. Thus, the genetic differentiation between large and small flowers was slight and may have originated relatively recently. The D_{SW} dendrogram and Bayesian clustering analysis STRUCTURE does not support the interpretation that the large and small flower traits were derived from other populations, and it seems unlikely that they could have resulted from secondary contact (Figure 5-8). he subtlety of the genetic differentiation and the rejection of the secondary contact hypothesis together suggest that the bimodality of the floral size distribution in the Ohmizusawa population is likely to be of relatively recent sympatric origin.

5-6 Conclusions

Our results suggest that pollinator preference can initiate sympatric trait bimodality. Until now, there has been little evidence of disruptive selection of floral traits resulting from differences in pollinator preference within a population. Despite the many studies on regional trait matching between floral size and flower visitor size (Anderson and Johnson, 2008; Pauw et al., 2009; Anderson et al., 2014; Boberg et al., 2014; Nagano et al., 2014; Kuriya et al., 2015), this is the first study to show that sympatric differences in floral size alter flower visitor behavior. Theoretical studies (Zhang et al., 2013) have shown that one of the requirements for the evolution of floral trait bimodality is the presence of pollinators with a short proboscis that prefer to forage from short-tubed flowers, and others with a long proboscis that prefer long-tubed flowers. Our results, which are close to this theoretical situation, suggest that the stochasticity of flower visitor behavior in relation to floral size is also a very important parameter.

5-7 Tables

Table 5-1. Genetic distances D_A and D_{SW}, F_{ST} and R_{ST} statistics, and genetic differentiation (differences in allele frequencies) results between ramets with small and large flowers in the Ohmizusawa population. The symbol "-" indicates a locus with a fixed allele.

Locus	D_A	D_{SW}	F_{ST}	R_{ST}	Genetic differentiation (p)
LA5	0.070	0.013	-0.002	0.044	0.035
LA7	0.004	-0.033	-0.004	-0.010	0.601
LA16	0.039	0.004	0.006	-0.006	0.290
LA25	0.003	0.000	-0.007	-0.007	0.331
LA34	0.001	-0.005	-0.018	-0.018	0.727
LA35	0.000	0.000	-	-	-
LA54	0.000	0.000	-	-	-
LA55	0.014	0.002	0.018	0.032	0.115
LA58	0.000	0.000	-	-	-
LA63	0.084	0.139	0.037	0.017	0.003
overall	0.021	0.012	0.006	0.002	0.009

5-8 Figures

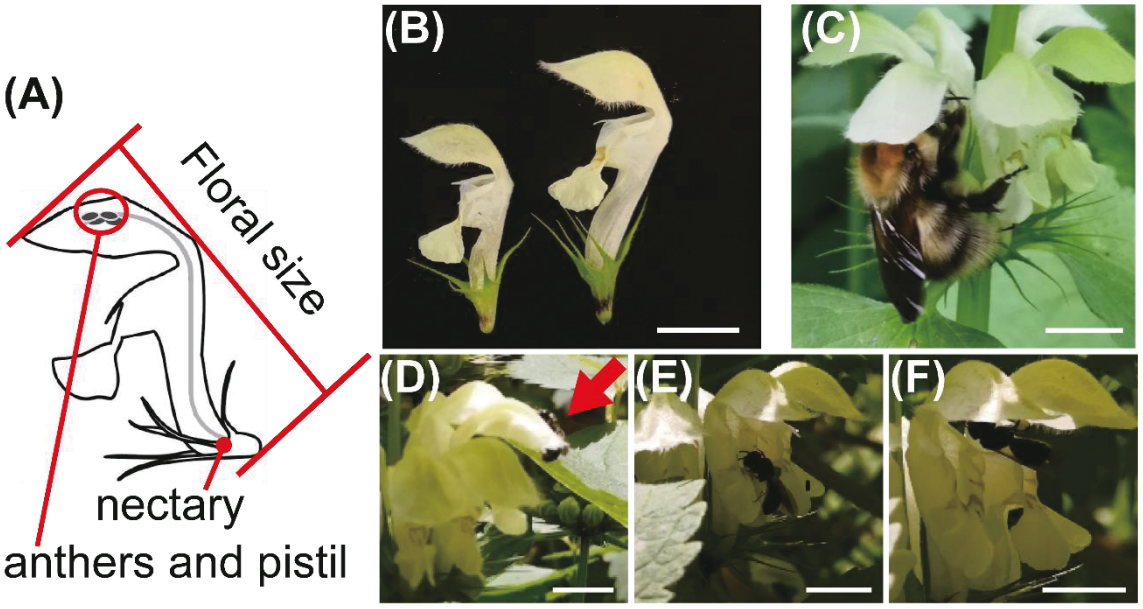

Figure 5-1. Flowers and pollinators of *L. album* var. *barbatum*. (A) Floral size determination, and the positions of the anthers, pistil, and nectary. (B) Small (26.02 mm) and large (30.95 mm) flowers from the Ohmizusawa population. (C) A *Bombus consobrinus* queen (large bee) visiting a flower. (D) A small bee, *Ceratina japonica*, alighting on the upper part of a flower and touching the anthers and pistil (legitimate visit). (E) A small bee alighting on the lower lip and not touching the anthers or pistil (nectar robber). (F) A small bee feeding on pollen (pollen foraging). Scale bars, 1 cm.

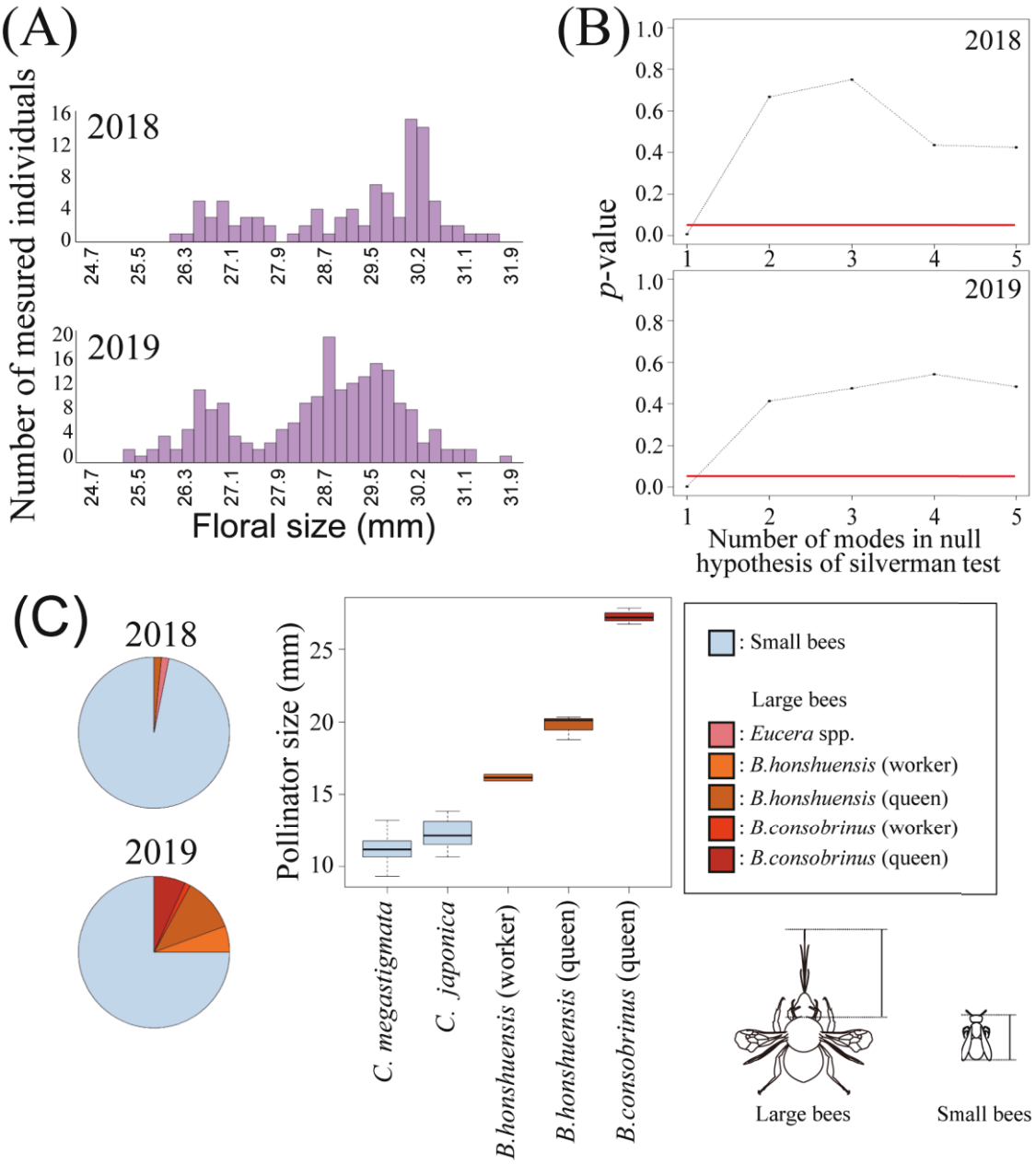

Figure 5-2. (A) Bimodal frequency distributions of flower size in the Ohmizusawa population in 2018 (n = 99 ramets) and 2019 (n = 202 ramets). (B) Silverman test p-value plots for the frequency distribution of flower size. In both years, the null hypothesis that the number of modes of the flower size frequency distribution was ≤1 was rejected, whereas the null hypothesis that the number of modes was ≤2–5 was not rejected. The horizontal red line indicates the significance level (α = 0.05). (C) Pie charts, showing the

pollinator assemblage in each year, and a box plot of pollinator size: median (bar), lower and upper quartiles (box ends), and the lower and upper quartile ± 1.5 × interquartile range (whiskers). The bee diagrams show that the total body length of small bees was measured, whereas for large bees, the length of the head plus proboscis was measured.

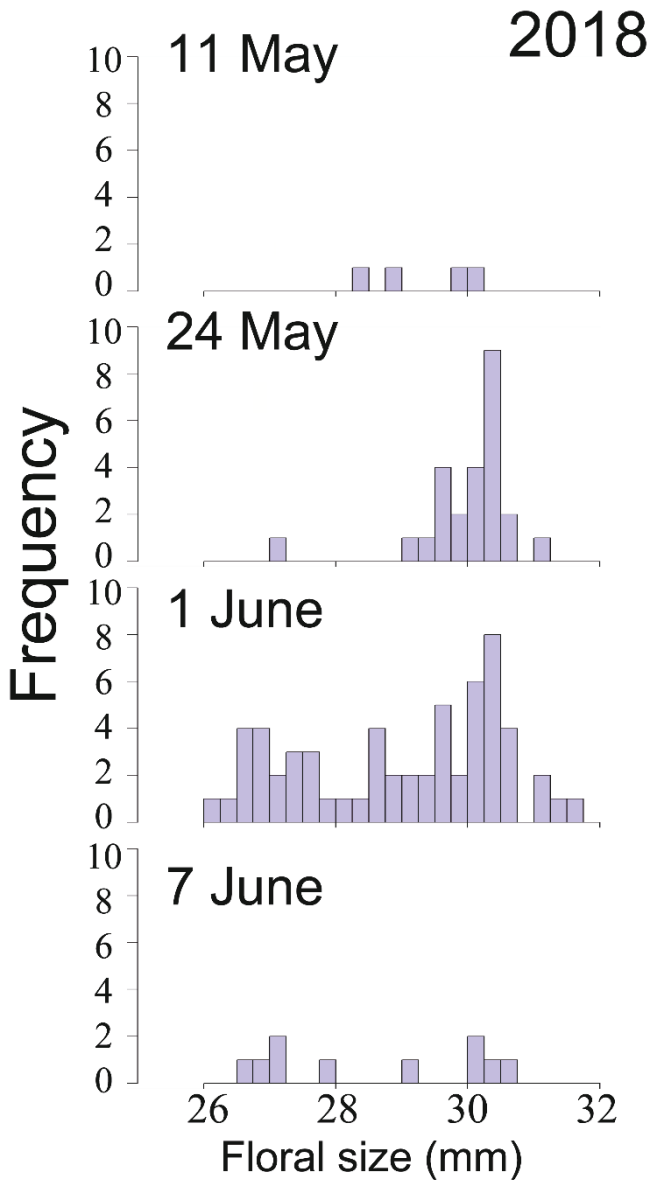

Figure 5-3. Frequency histograms of floral size measured on four days during the 2018 flowering season. The size of 2–5 flowers per ramet was measured, and the mean value was used as the flower size of the ramet (n = 1). The date on which floral size was measured is shown in the upper left corner of each graph.

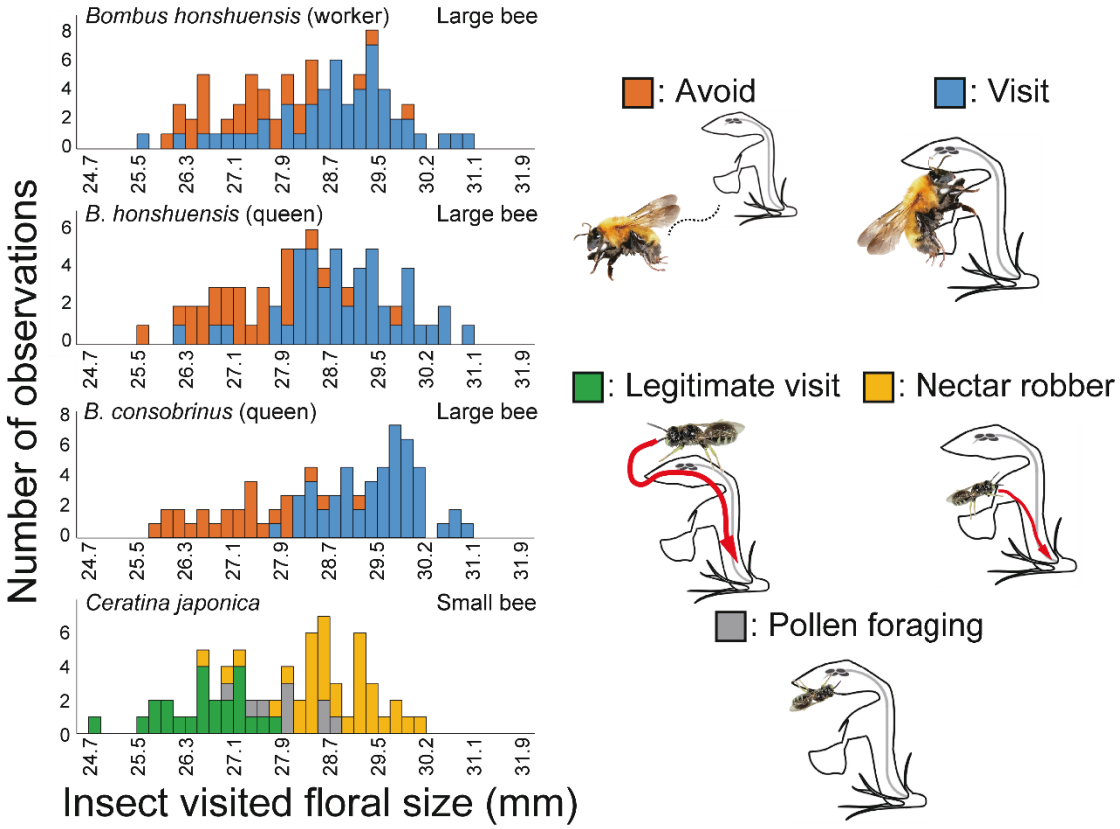

Figure 5-4. In 2019, pollinator behavior varied depending on the flower size of *L. album* var. *barbatum*. Bumblebees (large bees) exhibited two behaviors: "Avoid" and "Visit". "Avoid" was defined as approaching within 10 cm or touching a flower but not foraging. "Visit" was defined by the bumblebee's inserting the head into the flower and foraging, so that the bee's head touched the anthers and pistil. The small bee *C. japonica* exhibited three behaviors: "Legitimate visit", "Nectar robber" and "Pollen foraging". During a "Legitimate visit", the bees initially landed on top of the flower and approached the nectary via the anthers and pistil. A "Nectar robber" alighted first on the lower lip of the flower and foraged without touching the anthers and pistil. "Pollen foraging" bees collected pollen directly from the anthers.

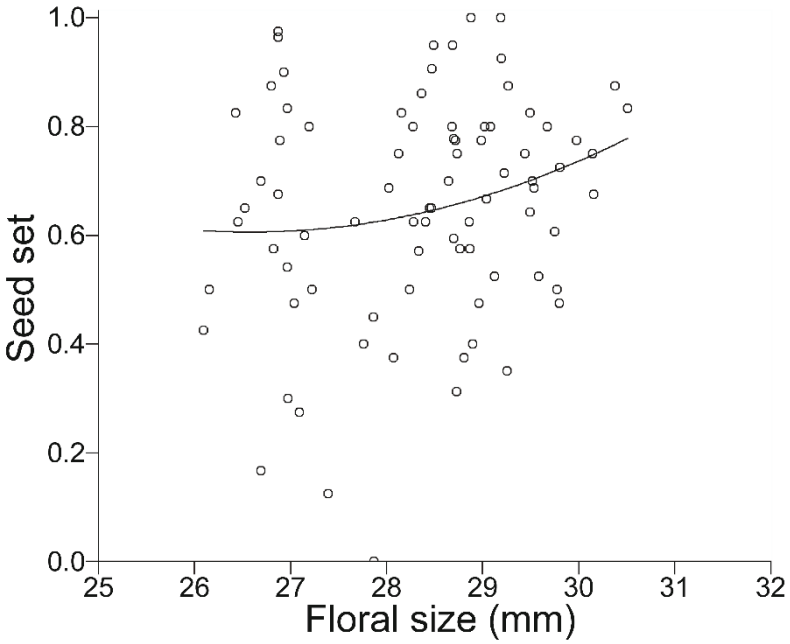

Figure 5-5. Relationship between flower size and seed set of *L. album* var. *barbatum* in the Ohmizusawa population in 2019 (n = 82 ramets) showing the quadratic regression curve fitted to the data (standardized quadratic regression coefficient $\gamma \pm$ SE = 0.068 ± 0.111, P = 0.5431; standardized linear regression coefficient $\beta \pm$ SE = 0.214 ± 0.113, P = 0.063).

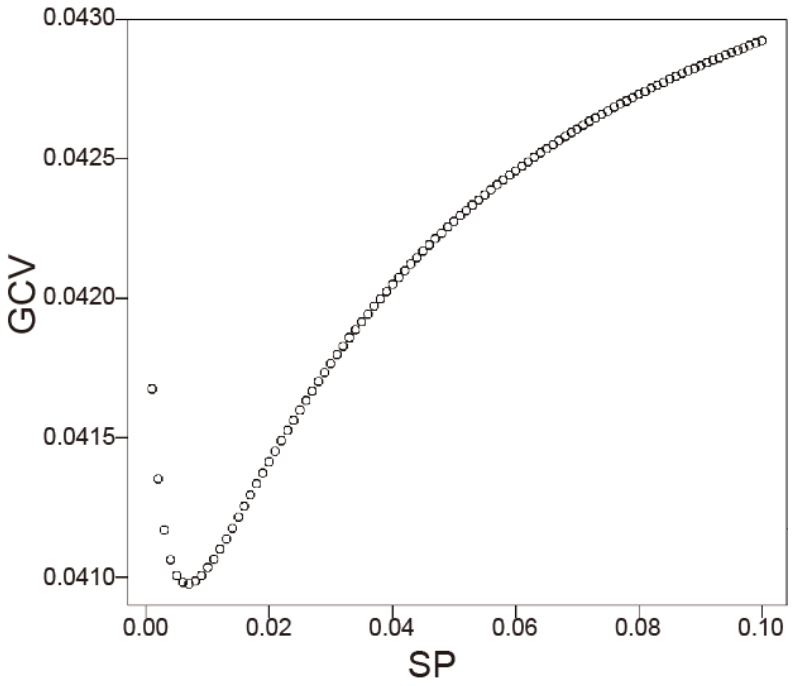

Figure 5-6. The appropriate smoothing parameter (SP) for application of a smoothing spline to the relationship between seed set and flower size was determined from the relationship between generalized cross-validation (GCV) values and calculated smoothing parameter (SP) values. The SP value (SP = 0.007) associated with the lowest GCV value was selected and used to draw the spline curve shown in Figure 4.

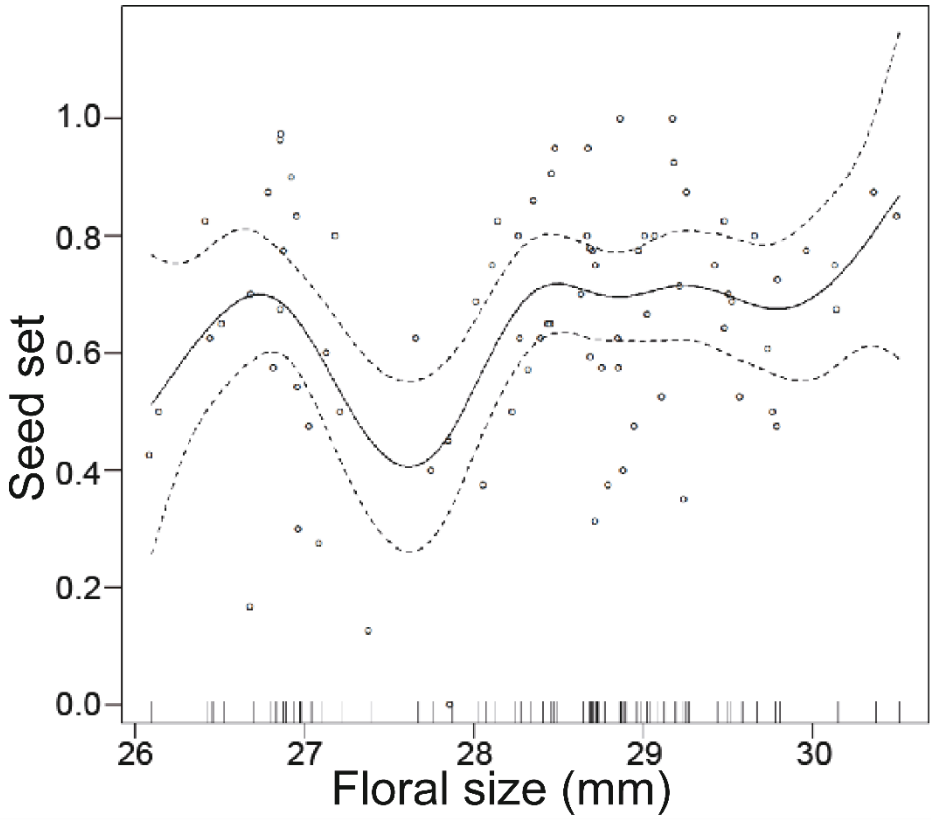

Figure 5-7. Relationship between fitness (seed set) and flower size (n = 82 ramets; approximate significance of the smoothing terms, $p < 0.05$) of *L. album* var. *barbatum* in the Ohmizusawa population in 2019. Solid lines represent the estimated mean values and dashed lines indicate the 95% confidence interval for the mean. The density of the floral size data is indicated along the lower horizontal axis. The appropriate smoothing parameter was determined by generalized cross-validation (GCV; see Figure 5-6).

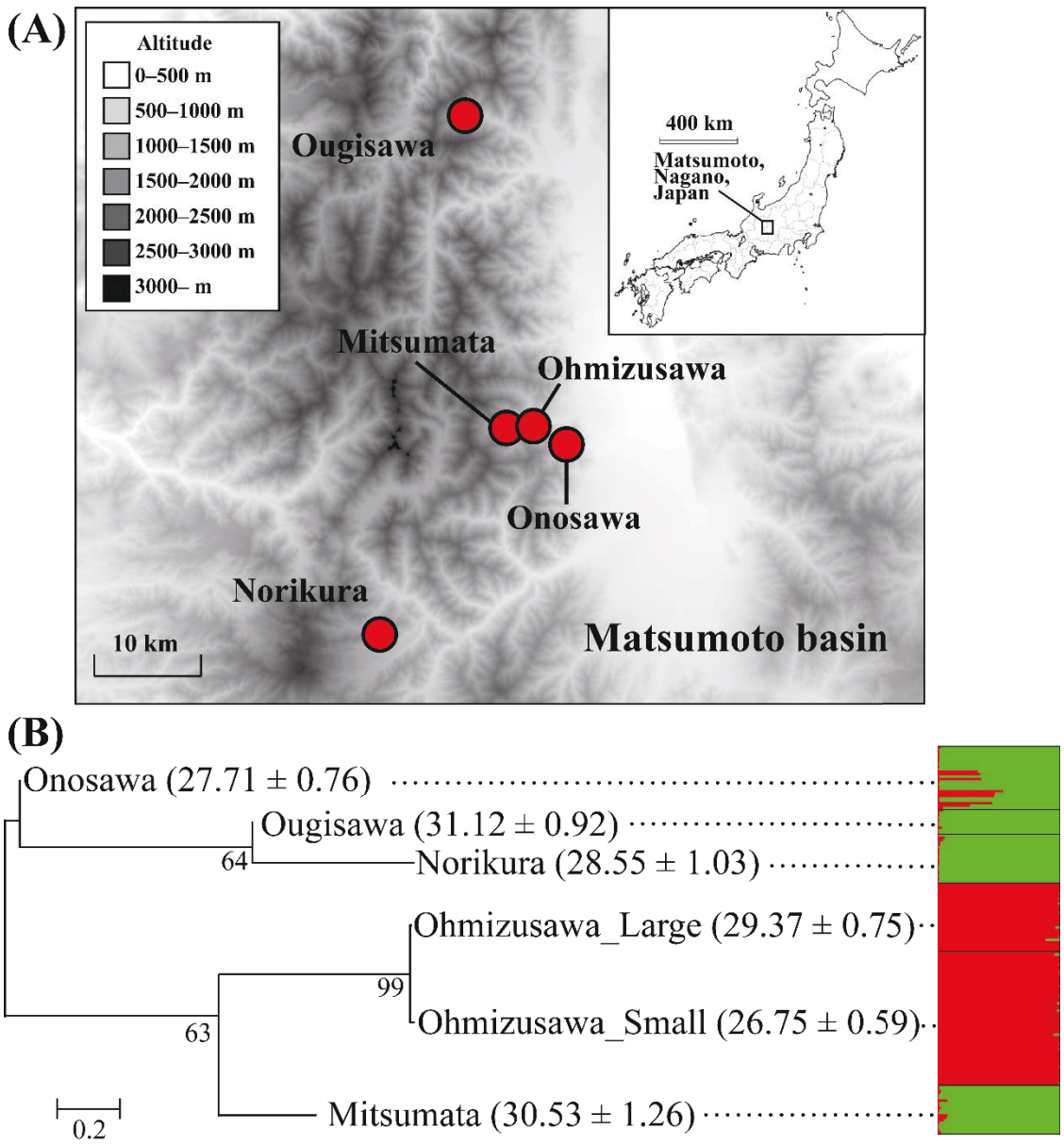

Figure 5-8. (A) Map showing *L. album* var. *barbatum* populations around the Ohmizusawa population. (B) Dendrogram drawn by the neighbour-joining method and based on the genetic distance (D_{SW}) among six populations. The right color figure shows the results of Bayesian clustering analysis STRUCTURE (K=2). It has been shown that there is little gene flow between genetic clusters. The Ohmizusawa population has been divided into small- (< 28 mm) and large-flower (> 28 mm) populations. The number by each node is the bootstrap value for that node. The numbers in parentheses after each

population indicate floral size (mean ± SD, mm). The floral size data for the Ohmizusawa population floral were obtained in 2019. Data for the other populations were collected by Toji et al. (2021) in 2018.

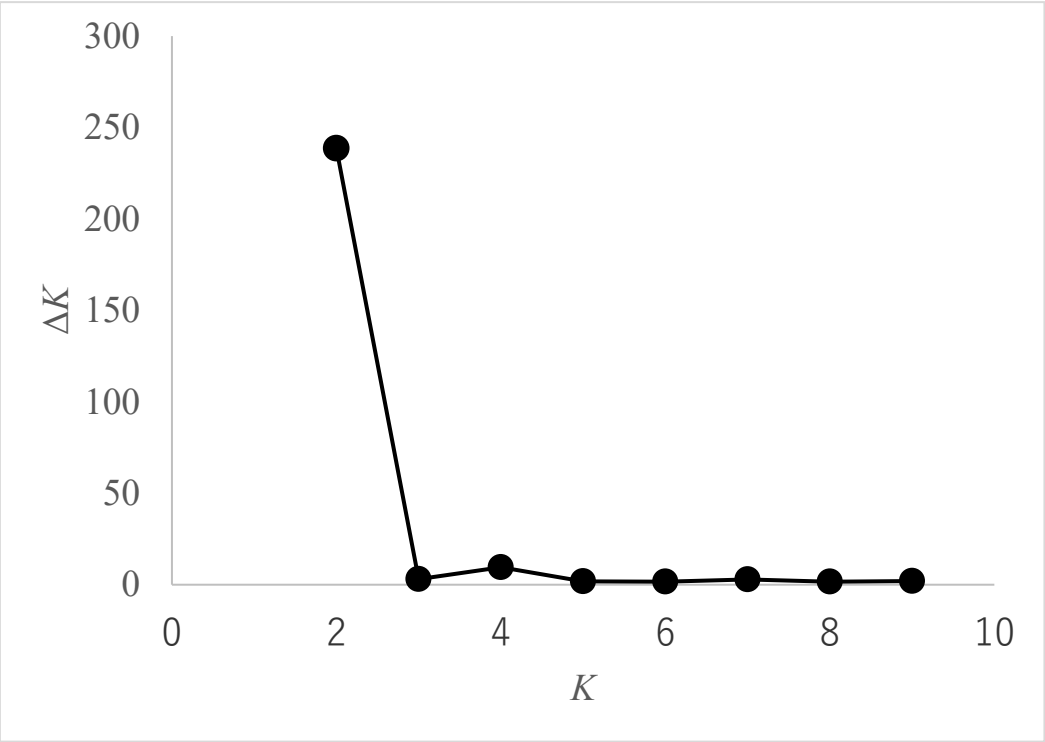

Figure 5-9. Population genetic structure of *L. album var. barbatum* 6 populations. Δ*K* value used to determine the appropriate number of genetic clusters (*K*), peaked at *K* = 2.

General discussion

I confirmed that intraspecific floral trait variation occurs at various scales, including between ecotypes (Chapters 1 and 2), within species (Chapters 3 and 4), and within a population (Chapter 5). The results of these chapters commonly pointed out that flower visitor fauna affects floral trait variation.

In Chapter 1, I used the genetically and ecologically distinct three *Cimicifuga simplex* pollination morphs to investigate differences in reproductive systems among the morphs. Field studies showed that floral sex expression tended to be different among the three morphs. In addition, estimation of selfing rate using microsatellite markers revealed that only one type has high selfing rate. In Chapter 2, I discussed the factors that cause the differences in reproductive systems among the three morphs of *C. simplex* in terms of the quality and quantity of pollinators. I found that insects visiting each of the three *C. simplex* morphs differed greatly in their seasonal visitation rate and in their qualitative ability to transport pollens. These pollinator differences are likely to result in the differences in reproductive systems. I explored this possibility based on the similarities of our dataset with the theoretical models (Ezoe and Washizu, 2009, Harder and Aizen, 2010). Chapters 1 and 2 are good examples of how plant reproductive systems are influenced by flower visitor communities that vary greatly in quality and quantity over seasons and locations.

In Chapter 3, I investigated the geographic variation of floral size in *L. album* var. *barbatum* and found a clear correlation between floral size and local flower visitor size. I found a clear correlation between the floral size of *L. album* var. *barbatum* and local flower visitor size, suggesting that the floral size of *L. album* var. *barbatum* has evolved as a result of natural selection, since flower visitor size is a selective pressure on floral size (Hattori et al., 2021). Furthermore, population genetic analysis using microsatellite

markers revealed that *L. album* var. *barbatum* in the western and eastern mountain region belong to different genetic clusters. These results suggest that floral size of *L. album* var. *barbatum* evolved independently according to visitor size in both of the western and eastern mountain regions.

In Chapter 4, I applied the same study design to *Aquilegia buergeriana* var. *buergeriana* as I did to *L. album* var. *barbatum* in Chapter 3 with MIG-seq method (Suyama and Matsuki, 2015) to analyze genome-wide SNPs. As a result, floral size in *A. buergeriana* var. *buergeriana* was strongly correlated with local visitor size, like the results obtained for *L. album* var. *barbatum*. Genetic differentiation among the four mountain regions was also detected. These results suggest that floral size evolved independently among mountain regions according to pollinator size in *A. buergeriana* var. *buergeriana*. The results in Chapters 3 and 4 are good examples of how floral traits evolved with spatially different flower visitor communities. Examples of parallel evolution, in which species with similar traits appear independently in different mountain regions, have recently become evident, such as in *Antirrhinum* (Durán-Castillo et al., 2021) and in scorpionfly (Suzuki et al., 2020). A series of studies in Chapters 3 and 4 have shown that independent evolution can occur between different mountain regions even at the level of intraspecific variation in plants. This suggests that floral traits evolved rapidly in response to the flower visitor fauna, even for a very short time without speciation.

In Chapter 5, I focused on the bimodality of sympatric floral size in *L. album* var. *barbatum*. In this chapter, I studied small and large flower individuals without temporal or geographical isolation. I explained how the two types of plant individuals are maintained in the population by the behavioral patterns of flower visitors. Small bees effectively pollinated (touched the stamen and pistils) on small flowers but acted as a

nectar robber on large flowers. On the other hand, large bees effectively pollinated on large flowers but ignored small flowers. Weak genetic differentiation between small and large flowers was also detected. These results suggest that the evolution and maintenance of sympatric floral size bimodality as well as the genetic differentiation are caused by the large and small flower visitors.

In summary, Chapters 1 and 2 focus on plant reproductive strategies in response to temporal changes in flower visitor quantity and quality: optimizing floral sexual expression and mating systems (selfing or outcrossing). Chapters 3 and 4 focus on the adaptive evolution of floral size in response to the geographic variation in flower visitor size. Chapter 5 focuses on the evolution and maintenance of floral size bimodality in a plant population. Based on the results of all chapters, I will discuss the effects of temporal and spatial changes in pollinator communities on trait evolution of reproductive systems and floral size of the plants.

Detailed discussions have already been made in each chapter. Here, I will relate my results to other research from a broader perspective and discuss what kind of further research should be conducted in the future.

Prospect to further research on the relationship between flower visitor communities and reproductive systems of flowering plants

In Chapter 1, I used *C. simplex* as a material to detect the differentiation of reproductive systems among the three morphs. In Chapter 2, I compared the quantity and quality of flower visitors among the three morphs, and I found significant differences in the flower visitor environments surrounding the three morphs. In the series of studies, I found a relationship between the quality and quantity of flower visitors and the reproductive

systems of plants, although there is no direct evidence.

Brassica rapa has been found to evolve different plant traits when grown in several generations under three conditions: fly-mediated, bee-mediated, and self-pollinated by hand-pollination (Gervasi and Schiestl, 2017; Ramos and Schiestl, 2019). Specifically, autonomous selfing rates increased and the number of individuals with the fragrant *p*-anisaldehyde decreased under fly-borne environments. Under the bee-pollinated environment, the plant height increased and the fragrant *p*-anisaldehyde and indole in the flowers increased. In the hand-pollinated environment, self-incompatibility was alleviated, and the number of seeds produced by selfing increased. These evolutions occurred in a very short period (11 generations) and it is good examples of how plants evolve adaptive strategies to different flower visitor environments. Future research could include cultivation experiments of many species using this approach. If we can follow the evolution of plant traits in real time under such cultivation conditions, it will provide more direct evidence that pollination environments promote the evolution of reproductive systems in plants.

Prospect to further researches on the size matching between flowers and flower visitors

In Chapters 3 and 4, I added new insights into this research field by added genetic analysis to classical flower-visitor size matching. The possibility of a mismatch between floral size and flower visitor size has been discussed as a future development in this field (Anderson et al., 2010b, Nattero et al., 2010, Moré et al., 2012). However, there is no new research that follows these studies. Anderson et al. (2010b) showed that flower-visitor size mismatch occurs commonly, with the floral size tending to be larger than the visitor

size. One possible reason for the paucity of studies on such morphological mismatches is that such facts are treated as negative research data and do not receive much attention.

In *Campanula punctata* var. *hondoensis*, a population was found to have a mismatch between flower and flower visitor size (Nagano et al., 2014). This population with a relatively large floral size is frequently visited by many small-sized bumblebees. Clarifying what kind of selection pressure to floral size is present in this population will help to clarify the mechanism of size-mismatch between flowers and flower visitors.

Future research on sympatric floral size bimodality

The content of Chapter 5 was basically based on the classical flower-pollinator size-matching, but it studied a very narrow range compared to previous studies in that it tracked the detailed flower visitor's behavior within a population. It is very important phenomenon because of studies on the sympatric trait bimodality are very limited.

In this section, I will review some of the related published papers and discuss why sympatric floral size bimodality has been overlooked until now. The reviewed papers studied corolla length and spur length, and they discussed the relationship between flower visitor size and floral morphology (Table 6-1). For these studies, I summarized the plant and visitor species, a number of studied populations, how they express the floral size data (e.g., means, histograms, box plots), flower-pollinator size matching, and bimodality in the floral traits or not. I found 21 papers and 86 plant species, and 425 populations surveyed. I found 8 papers (3 of which were by T. Toji) that represented the frequency distribution of floral size. This research studied a total of 8 species and 44 populations. The 3 of them papers focused on sympatric floral size bimodality. About 90% of the plant species and study populations had floral size data summarized as mean ± SE or SD. It is

suggested that present floral size bimodality may have been overlooked in these papers. There were few papers (only 2 papers) which we can access the raw data.

These results suggest that the problem with the research community studying flower-pollinator size-matching is that they do not pay much attention to the frequency distribution of floral size, but only discuss the mean value. The one-to-one relationship is very beautiful, as in Darwin's orchid and hawkmoth example (Darwin, 1877; Nilsson, 1988), but because of this too-beautiful example, we may regard the floral size and flower visitor size as a one-to-one relationship in any situation and discuss the mean size of flower vs mean size of flower visitor. In fact, if the flower traits are multimodal or visitors of various sizes visit the flowers, a many-to-many relationship should be considered. A further problem is that it is difficult to visualize the data depending on the research design. In the study of Hiraiwa and Ushimaru (2017) on a wide range of plant species, only a few individuals from a single site were sampled for floral morphology. To draw a histogram, many samples from a single location were required.

Perhaps because of the above background, it is likely that the bimodal trait is quite often overlooked. It may be that the frequency distributions are simply not published in the papers, but this may be due to the fact that traits with bimodal frequency distributions are very difficult to interpret, and in a sense, have a negative data element. This is also true for other traits besides floral size. Examples of studies of trait bimodality in other organisms, including plants, are quite rare, as mentioned in the introduction and discussion in Chapter 5. In the future, we should focus on the frequency distribution of traits in various species and ask how common bimodality of traits is. In addition, we need to clarify what mechanisms are at work when bimodal traits occur. For this purpose, data visualization and accessibility to raw data through online repositories are recommended.

Table 6-1. Papers used in the review. Summarized the plant and visitor species, number of studied populations, how they express the floral size data (e.g., means, histograms, box plots), flower-pollinator size matching, and bimodality in the floral traits or not.

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Amaryllidaceae	*Nerine humilis*	11	fly, bee	mean±SE	○		raw data in Dryad repository	Newman et al., 2015
Balsaminaceae	*Impatiens noli-tangere*	10	bumblebee	mean±SE				Hattori et al., 2016
Balsaminaceae	*Impatiens textori*	11	bumblebee	mean±SE				Hattori et al., 2016
Iridaceae	*Gladiolus longicollis*	1		histogram	○	○		Rymer et al., 2010
Iridaceae	*Lapeirousia anceps*	1	fly	histogram	○	○		Alexandersson and Johnson, 2002 Anderson et al., 2016
Iridaceae	*Lapeirousia anceps*	10	fly	mean±SD	○			Pauw et al., 2009
Iridaceae	*Tritoniopsis revoluta*	11	fly, bee	histogram	○			Anderson et al., 2014
Lamiaceae	*Isodon effusus*	3	bumblebee	Box plot	○			Suzuki, 1992
Lamiaceae	*Isodon logitubus*	2	bumblebee	Box plot	○			Suzuki, 1992
Lamiaceae	*Isodon shikokianus*	5	bumblebee	Box plot	○			Suzuki, 1992
Lamiaceae	*Isodon umbrosus*	15	bumblebee	mean±SD				Dohzono and Suzuki, 2010
Lamiaceae	*Isodon umbrosus*	13	bumblebee	Box plot	○			Suzuki, 1992

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Lamiaceae	*Lamium album* var. *barbatum*	7	bumblebee	mean±SE	○			Hattori et al., 2015
Lamiaceae	*Lamium album* var. *barbatum*	12	bumblebee, bee	histogram	○			Toji et al., 2021
Lamiaceae	*Lamium album* var. *barbatum*	1	bumblebee, bee	histogram		○	One of the 12 populations in Toji et al. 2021	Chapter 5
Lamiaceae	*Meehania urticifolia*	6	bumblebee	mean±SE				Hattori et al., 2015
Lamiaceae	*Prunella vulgaris*	7	bumblebee	mean±SE	○			Kuriya et al., 2015
Lamiaceae	*Prunella vulgaris*	12	bumblebee	mean±SD	○			Egawa et al., 2020
Liliaceae	*Hosta sieboldiana*	1	bumblebee	Box plot	○			Suzuki et al., 2002
Orchidaceae	*Habenaria gourlieana*	1	moth	histogram				Moré et al., 2012
Orchidaceae	*Habenaria johannensis*	1	moth	histogram				Moré et al., 2012
Orchidaceae	*Habenaria sagittifera*	1	moth	mean±SE	○			Sakagami and Sugiura, 2019
Orchidaceae	*Habenaria paulistana*	1	moth	histogram				Moré et al., 2012
Orchidaceae	*Platanthera bifolia*	12	moth	mean±SD	○			Boberg et al., 2014
Ranunculaceae	*Aquilegia buergeriana* var. *buergeriana*	6	bumblebee	mean±SE				Hattori et al., 2014
Ranunculaceae	*Aquilegia buergeriana* var. *buergeriana*	15	bumblebee	histogram	○			Toji et al., 2021

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Scrophulariaceae	*Zaluzianskya microsiphon*	16	fly	mean±SE	○			Anderson and Johnson, 2008
Aizoaceae	*Tetragonia tetragonoides*	2	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Amaryllidaceae	*Crinum asiaticum* var. *japonicum*	6	long- or short-tongued pollinators*1	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Aster microcephalus* var. *littoricola*	5	vaious pollinators*2	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Aster microcephalus* var. *ovatus*	3	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Bidens pilosa* var. *pilosa*	2	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Chrysanthemum* × *marginatum*	2	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Asteraceae	*Chrysanthemum pacificum*	7	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Cirsium hachijoense*	2	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Cirsium japonicum*	1	long- or short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Cirsium maritimum*	1	long-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Crepidiastrum platyphyllum*	4	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Eclipta thermalis*	1	medium-tongued pollinators*3	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Erigeron philadelphicus*	4	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Asteraceae	*Farfugium japonicum*	8	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Ixeris japonica*	1	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Ixeris repens*	4	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Ixeris stolonifera*	3	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Lactuca raddeana* var. *elata*	1	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Melanthera prostrata*	7	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Nipponanthemum nipponicum*	2	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Asteraceae	*Picris hieracioides* subsp. *japonica*	6	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Solidago altissima*	4	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Sonchus oleraceus*	8	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Taraxacum officinale*	5	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Taraxacum platycarpum*	2	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Asteraceae	*Youngia japonica*	4	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Brassicaceae	*Brassica rapa* var. *nippo-oleifera*	1	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Brassicaceae	*Nasturtium officinale*	1	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Brassicaceae	*Raphanus sativus* var. *hortensis* f. *raphanistroides*	3	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Campanulaceae	*Adenophora triphylla var. japonica* f. *glabra*	2	medium- or short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Campanulaceae	*Campanula microdonta*	5	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Caprifoliaceae	*Lonicera japonica*	8	long- or short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Caryophyllaceae	*Dianthus japonicus*	3	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Caryophyllaceae	*Silene gallica* var. *gallica*	3	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Caryophyllaceae	*Stellaria media*	1	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Convolvulaceae	*Calystegia soldanella*	8	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Convolvulaceae	*Cuscuta campestris*	2	medium- or short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Ericaceae	*Rhododendron indicum*	1	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Fabaceae	*Canavalia lineata*	2	long-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Fabaceae	*Lathyrus japonicus*	7	mainly long-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Fabaceae	*Lotus corniculatus* var. *japonicus*	3	medium- or short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Fabaceae	*Trifolium repens*	2	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Geraniaceae	*Geranium thunbergii*	1	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Iridaceae	*Crocosmia* × *crocosmiiflora*	3	long-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Lamiaceae	*Vitex rotundifolia*	8	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Liliaceae	*Lilium maculatum*	3	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Lythraceae	*Lythrum anceps*	1	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Onagraceae	*Oenothera laciniata*	8	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Oxalidaceae	*Oxalis corniculata* var. *trichocaulon*	8	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Oxalidaceae	*Oxalis debilis* subsp. *corymbosa*	4	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Pittosporaceae	*Pittosporum tobira*	1	medium-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Plantaginaceae	*Linaria japonica*	2	long-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Polygonaceae	*Fallopia japonica* var. *hachidyoensis*	2	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Polygonaceae	*Persicaria chinensis*	11	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Primulaceae	*Lysimachia mauritiana*	8	mainly short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Rosaceae	*Rhaphiolepis indica* var. *umbellata*	3	mainly medium-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Rubiaceae	*Diodia teres*	1	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Rubiaceae	*Paederia scandens* var. *maritima*	5	long- or short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Solanaceae	*Lycium chinense*	3	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Verbenaceae	*Lantana camara* var. *aculeata*	2	mainly long-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Verbenaceae	*Phyla nodiflora*	1	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Vitaceae	*Ampelopsis glandulosa* var. *hancei*	7	vaious pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017

Family	Plant species	Number of surveyed populations	Flower visitors	Floral size summarization methods	Flower-pollinator size matching	Bimodality	Notes	Reference
Vitaceae	*Cayratia japonica*	1	medium- or short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017
Xanthorrhoeaceae	*Hemerocallis fulva* var. *littorea*	4	short-tongued pollinators	mean			raw data in Dryad repository	Hiraiwa and Ushimaru, 2017

*1 short-tongued pollinators: small bees (*Lasioglossum*, *Ceratina*), small dipterans, and beetles

*1 long-tongued pollinators: *Bombus*, *Amegilla*, and lepidopterans

*2 various pollinators: All types of pollinators listed above were visited

*3 medium-tongued pollinators: *Apis*, *Megachile*, and large dipterans

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References

Aizen, M. A. (2001) Flower sex ratio, pollinator abundance, and the seasonal pollination dynamics of a protandrous plant. Ecology 82: 127–144.

Ajar, É. (2003) Analysis of disruptive selection in subdivided populations. BMC Evolutionary Biology 3: 22.

Alexandersson, R., Johnson, S. D. (2002) Pollinator-mediated selection on flower-tube length in a hawkmoth-pollinated *Gladiolus* (Iridaceae). Proceedings of the Royal Society B: Biological Sciences 269: 631–636.

Alonso, C. (2005) Pollination success across an elevation and sex ratio gradient in gynodioecious *Daphne laureola*. American Journal of Botany 92: 1264–1269.

Anderson, B., Johnson, S. D. (2008) The geographical mosaic of coevolution in a plant-pollinator mutualism. Evolution 62: 220–225.

Anderson, B., Johnson, S. D. (2009) Geographical covariation and local convergence of flower depth in a guild of fly-pollinated plants. New Phytologist 182: 533–540.

Anderson, B., Pauw, A., Cole, W. W., Barrett, S. C. (2016) Pollination, mating and reproductive fitness in a plant population with bimodal floral-tube length. Journal of Evolutionary Biology 29: 1631–1642.

Anderson, B., Alexandersson, R., Johnson, S. D. (2010a) Evolution and coexistence of pollination ecotypes in an African *Gladiolus* (Iridaceae). Evolution 64: 960–972.

Anderson, B., Terblanche, J. S., Ellis, A. G. (2010b) Predictable patterns of trait mismatches between interacting plants and insects. BMC Evolutionary Biology, 10: 1–14.

Anderson, B., Ros, P., Wiese, T. J., Ellis, A. G. (2014) Intraspecific divergence and

convergence of floral tube length in specialized pollination interactions. Proceedings of the Royal Society B: Biological Sciences 281: 20141420.

Ashman, T. L., Knight, T. M., Steets, J. A., Amarasekare, P., Burd, M., Campbell, D. R., Dudash, M. R., Johnston, M. O. et al. (2004) Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. Ecology 85: 2408–2421.

Asikainen, E., Mutikainen, P. (2005) Pollen and resource limitation in a gynodioecious species. American Journal of Botany 92:487–494.

Bailey, M. F., Delph, L. F. (2007) A field guide to models of sex-ratio evolution in gynodioecious species. Oikos 116: 1609–1617.

Baker, H. G. (1955) Self-compatibility and establishment after 'long-distance' dispersal. Evolution 9: 347–348.

Barrett, S. C. (2002) Evolution of sex: the evolution of plant sexual diversity. Nature Reviews Genetics 3: 274–284.

Barrett, S. C. H. (2002) Sexual interference of the floral kind. Heredity 88:154–159.

Bell, G. 1985. On the function of flowers. Proceedings of the Royal Society B: Biological Sciences 224: 223–265.

Blacket, M. J., Robin, C., Good, R. T., Lee, S. F., Miller, A. D. (2012) Universal primers for fluorescent labelling of PCR fragments–an efficient and cost-effective approach to genotyping by fluorescence. Molecular ecology resources 12: 456–463.

Boberg, E., Alexandersson, R., Jonsson, M., Maad, J., Agren, J., Nilsson, L. A. (2014) Pollinator shifts and the evolution of spur length in the moth-pollinated orchid *Platanthera bifolia*. Annals of Botany 113: 267–275.

Bolger, A. M., Lohse, M., Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30: 2114–2120.

Bolnick, D. I. (2004) Can intraspecific competition drive disruptive selection? An experimental test in natural populations of sticklebacks. Evolution 58: 608–618.

Briscoe Runquist, R. D., Moeller, D. A. (2014) Floral and mating system divergence in secondary sympatry: testing an alternative hypothesis to reinforcement in *Clarkia*. Annals of Botany 113: 223–235.

Briscoe Runquist, R. D., Geber, M. A., Pickett-Leonard, M., Moeller, D. A. (2017) Mating system evolution under strong pollen limitation: evidence of disruptive selection through male and female fitness in *Clarkia xantiana*. The American Naturalist 189: 549–563.

Brunet, J. (1996) Male reproductive success and variation in fruit and seed set in *Aquilegia caerulea* (Ranunculaceae). Ecology 77: 2458–2471.

Brunet, J., Charlesworth, D. (1995) Floral sex allocation in sequentially blooming plants. Evolution 49: 70–79.

Burd, M. (2008) The Haig-Westoby model revisited. The American Naturalist 171: 400–404.

Busch, J. W., Schoen D. J. (2008) The evolution of self-incompatibility when mates are limiting. Trends in plant science 13: 128–136.

Calsbeek, R., T. B. Smith. (2008) Experimentally replicated disruptive selection on performance traits in a Caribbean lizard. Evolution 62: 478–484.

Campbell, D. R., Waser, N. M., Melendez-Ackerman, E. J. (1997) Analyzing pollinator-mediated selection in a plant hybrid zone: hummingbird visitation patterns on three spatial scales. The American Naturalist 149: 295–315.

Casimiro-Soriguer I., Buide M. L., Narbona E. (2015) Diversity of sexual systems within different lineages of the genus *Silene*. AoB Plants 7: plv037

Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., Cresko, W. A. (2013) Stacks: an analysis tool set for population genomics. Molecular Ecology 22: 3124–3140.

Charlesworth, B., Charlesworth, D. (1978) A model for the evolution of dioecy and gynodioecy. The American Naturalist 112: 975–997.

Christopher, D. A., Mitchell, R. J., Karron, J. D. (2020) Pollination intensity and paternity in flowering plants. Annnals of Botany 125: 1–9.

Conner, J. K., Hartl, D. L. (2004) *A primer of ecological genetics*, Sinauer Associates, Sunderland, MA, USA.

Coyne, J. A., Orr, H. A. (2004) *Speciation*. Sinauer Associates, Sunderland, MA, USA.

Culley, T. M., Klooster, M. R. (2007) The cleistogamous breeding system: a review of its frequency, evolution, and ecology in angiosperms. The Botanical Review 73: 1–30.

Darwin, C. (1876) *The effect of cross and self fertilization in the vegetable kingdom*. John Murray, London, England, UK.

Darwin, C. (1877) *On the various contrivances by which British and foreign orchids are fertilised by insects*: John Murray, London, England, UK.

Desfeux, C., Maurice, S., Henry, J. P., Lejeune, B., Gouyon P. H. (1996) Evolution of reproductive systems in the genus *Silene*. Proceedings of Royal Society B: Biological Sciences 263: 409–414.

Devlin, B., Stephenson, A. G. (1987) Sexual variations among plants of a perfect-flowered species. The American Naturalist 130: 199–218.

Dieckmann, U., Doebeli, M. (1999) On the origin of species by sympatric speciation.

Nature 400: 354–357.

Dobzhansky T. (1937) *Genetics and the Origin of Species*. Columbia University Press, NY, USA.

Dohzono, I., Suzuki, K. (2010) Morphological and genetic differentiation in Isodon umbrosus by altitudinal variation in bumblebee pollinator assemblages. Plant Species Biology 25: 20–29.

Dohzono, I., Takami, Y., Suzuki, K. (2011) Is bumblebee foraging efficiency mediated by morphological correspondence to flowers? International Journal of Insect Science 3: 1–10.

Dohzono, K., Suzuki, K., Murata, J. (2004) Temporal changes in calyx tube length of *Clematis stans* (Ranunculaceae): A strategy for pollination by two bumble bee species with different proboscis lengths. American Journal of Botany 91: 2051–2059.

Doyle, J. J., Doyle, J. L. (1990) Isolation ofplant DNA from fresh tissue. Focus 12: 39–40.

Dupont, Y. L., Padrón, B., Olesen, J. M., Petanidou, T. (2009) Spatio-temporal variation in the structure of pollination networks. Oikos 118: 1261–1269.

Durán-Castillo, M., Hudson, A., Wilson, Y., Field, D. L., Twyford, A. D. (2021) A phylogeny of *Antirrhinum* reveals parallel evolution of alpine morphology. New Phytologist.

Efron, B., Tibshirani, R. J. (1994) *An introduction to the bootstrap*, CRC press, Boca Raton, Florida, USA.

Egawa, S., Hirose, K., Itino, T. (2020) Geographic changes in pollinator species composition affect the corolla tube length of self-heal (*Prunella vulgaris* L.):

Evidence from three elevational gradients. Ecological Research 35: 819–825.

Elle, E., Meagher, T, R. (2000) Sex allocation and reproductive success in the andromonoecious perennial *Solanum carolinense* (Solanaceae). II. Paternity and functional gender. The American Naturalist 156: 622–636.

Emura, K. M. (1970) Cytotaxonomical study on the Eurasiatic Species of the genus *Cimicifuga*. 2. The Journal of Japanese Botany 45: 362–377.

Eubank, R. L. (1988) *Spline smoothing and nonparametric regression*. M. Dekker, New York, USA.

Evanno, G., Regnaut, S., Goudet, J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14: 2611–2620.

Excoffier, L., Lischer, H. E. (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular ecology resources 10: 564–567.

Ezoe, H. Washizu, N. (2009) Effect of stochasticity in the availability of pollinators on the resource allocation within a flower. Journal of theoretical biology 258: 630–636.

Falush, D., Stephens, M., Pritchard, J. K. (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164: 1567–1587.

Fausto, J. A., Eckhart, V. M., Geber, M. A. (2001) Reproductive assurance and the evolutionary ecology of self-pollination in *Clarkia xantiana* (Onagraceae). American Journal of Botany 88: 1794–1800.

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., Thomson, J. D. (2004)

Pollination syndromes and floral specialization. Annual Review of Ecology, Evolution, and Systematics 35: 375–403.

Fulton, M., Hodges, S. A. (1999) Floral isolation between *Aquilegia formosa* and *Aquilegia pubescens*. Proceedings of the Royal Society B: Biological Sciences 266: 2247–2252.

Funamoto, D. (2019) Plant-pollinator interactions in East Asia: a review. Journal of Pollination Ecology 25: 46–68.

Galen, C. (1996) Rates of floral evolution: adaptation to bumblebee pollination in an alpine wildflower, Polemonium viscosum. Evolution 50: 120–125.

Galen, C., Newport, M. (1987) Bumble bee behavior and selection on flower size in the sky pilot, Polemonium viscosum. Oecologia 74: 20–23.

García, M. B. (2003) Sex allocation in a long-lived monocarpic plant. Plant Biology 5: 203–209.

Gavrilets, S. (2004) *Fitness landscapes and the origin of species*. Princeton University Press. Princeton, NJ, USA.

Gegear, R. J., Burns, J. G. (2007) The birds, the bees, and the virtual flowers: can pollinator behavior drive ecological speciation in flowering plants? The American Naturalist 170: 551–566.

Gervasi, D. D. Schiestl, F. P. (2017) Real-time divergent evolution in plants driven by pollinators. Nature Communications 8: 14691.

Golub, G. H., Heath, M., Wahba, G. (1979) Generalized cross-validation as a method for choosing a good ridge parameter. Technometrics 21: 215–223.

Grant, V., Grant, K. A. (1965) *Flower Pollination in the Phlox Family*. Columbia University Press, New York, USA.

Grindeland, J. M., Sletvold, N., Ims, R. A. (2005) Effects of floral display size and plant density on pollinator visitation rate in a natural population of *Digitalis purpurea*. Functional Ecology 19: 383–390.

Gómez, J. M., Abdelaziz, M., Camacho, J. P. M., Muñoz-Pajares, A. J., Perfectti, F. (2009) Local adaptation and maladaptation to pollinators in a generalist geographic mosaic. Ecology Letters 12: 672–682.

Gómez, J. M., Perfectti, F., Camacho, J. P. M. (2006) Natural selection on *Erysimum mediohispanicum* flower shape: insights into the evolution of zygomorphy. The American Naturalist 168: 531–545.

Gómez, J. M., Perfectti, F., Bosch, J., Camacho, J. (2009) A geographic selection mosaic in a generalized plant–pollinator–herbivore system. Ecological Monographs 79: 245–263.

Good, J. M., Handel, M. A., Nachman, M. W. (2008) Asymmetry and polymorphism of hybrid male sterility during the early stages of speciation in house mice. Evolution 62: 50–65.

Harder, L. D., Aizen, M. A. (2010) Floral adaptation and diversification under pollen limitation. Philosophical Transactions of the Royal Society of London B: Biological Sciences 365: 529–543.

Hartl, D. L., Clark, A. G. (1997) *Principles of population genetics* (Vol. 116). Sinauer associates, Sunderland, MA, USA.

Hattori, M., Nagano, Y., Itino, T. (2014) Geographic flower trait variation of *Aquilegia buergeriana* Sieb. et Zucc. var. *buergeriana* on Mt. Norikura and the Utsukushigahara Highland, Japan. American Journal of Plant Sciences 5: 2686–2692.

Hattori, M., Nagano, Y., Itino, T. (2015) 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). American Journal of Plant Sciences 6: 737–745.

Hattori, M., Nagano, Y., Shinohara, Y., Itino, T. (2016) Pattern of flower size variation along an altitudinal gradient differs between *Impatiens textori* and *Impatiens noli-tangere*. Journal of Plant Interactions 11: 152–157.

Hattori., M., Tamada, Y., Itino, T. (2021) Effect of pollinator size on seed set in *Lamium album* var. *barbatum*. Plant Ecology and Evolution 154: 201–206.

Hayashi, Y. (2009) *Nihon-no Yasou*. Yama to Keikoku Sha. Tokyo. Japam.

Heinrich, B. (1979) *Bumblebee economics*. Harvard University Press, Cambridge, Massachusetts, USA.

Hendry, A. P., Huber, S. K., De Leon, L. F., Herrel, A., Podos, J. (2009) Disruptive selection in a bimodal population of Darwin's finches. Proceedings of the Royal Society B: Biological Sciences 276: 753–759.

Herrel, A., Podos, J., Huber, S., Hendry, A. (2005) Bite performance and morphology in a population of Darwin's finches: implications for the evolution of beak shape. Functional Ecology 19: 43–48.

Herrera, C. M. (1987) Components of pollinator" quality": comparative analysis of a diverse insect assemblage. Oikos 50: 79–90.

Herrera, C. M., Castellanos, M. C., Medrano, M. (2006) *Geographical context of floral evolution: towards an improved research programme in floral diversification* (Barret, S. C. H., Harder, L. D. Ed.). Oxford University Press, Oxford, England, UK.

Hiraga, T., Sakai, S. (2007) The effects of inflorescence size and flower position on biomass and temporal sex allocation in *Lobelia sessiliflora*. Plant Ecology 188: 205–214.

Hiraiwa, M. K., Ushimaru, A. (2017) Low functional diversity promotes niche changes in natural island pollinator communities. Proceedings of the Royal Society B: Biological Sciences 284: 20162218.

Hirao, A. S., Shimono, Y., Narita, K., Wada, N., Kudo, G. (2019) Ecotypic divergences of the alpine herb *Potentilla matsumurae* adapted to fellfield–snowbed habitats across a series of mountain sky islands. American Journal of Botany 106: 772–787.

Hodges, S. A. (1997) A rapid adaptive radiation via a key innovation in *Aquilegia*. In *Molecular evolution and adaptive radiation* (pp. 391–405): Cambridge University Press, Cambridge, UK.

Hodges, S. A., Fulton, M., Yang, J. Y., Whittall, J. B. (2004) Verne Grant and evolutionary studies of *Aquilegia*. New Phytologist 161: 113–120.

Hodges, S. A., Whittall, J. B., Fulton, M., Yang, J. Y. (2002) Genetics of floral traits influencing reproductive isolation between *Aquilegia formosa* and *Aquilegia pubescens*. The American Naturalist 159: S51–S60.

Hoel, P., Sidney, P., Stone, C. (1971) *Introduction to Statistical Theory*. Brooks/Cole Publishing, Pacific Grove, California, USA.

Hopkins, R., Rausher, M. D. (2012) Pollinator-mediated selection on flower color allele drives reinforcement. Science 335: 1090–1092.

Horsley, C. A. (2013) Pollinator-mediated interactions between native plants and the invasive alien Himalayan balsam. University of Leeds, Ph.D thesis.

Huber, S. K., León, L. F. D., Hendry, A. P., Bermingham, E., Podos, J. (2007) Reproductive isolation of sympatric morphs in a population of Darwin's finches. Proceedings of the Royal Society B: Biological Sciences 274: 1709–1714.

Inoue, M. N., Ushijima, J., Yokoyama, J. (2007) Effect of *Weigela hortensis* (Caprifoliaceae) floral morphology on pollinator behavior. Plant Species Biology 22: 77–86.

Inouye, D. W. (1980) The effect of proboscis and corolla tube lengths on patterns and rates of flower visitation by bumblebees. Oecologia 45: 197–201.

Ishii, H. S., Harder, L. D. (2012) Phenological associations of within-and among-plant variation in gender with floral morphology and integration in protandrous *Delphinium glaucum*. Journal of Ecology 100: 1029–1038.

Ishii, H. S., Sakai, S. (2002) Temporal variation in floral display size and individual floral sex allocation in racemes of *Narthecium asiaticum* (Liliaceae). American Journal of Botany 89: 441–446.

Itagaki, T., Sakai, S. (2006) Relationship between floral longevity and sex allocation among flowers within inflorescences in Aquilegia buergeriana var. oxysepala (Ranunculaceae). American Journal of Botany 93: 1320–1327.

Johnson, S. D. (2006) *Pollinator-driven speciation in plants*. Pages 295–310 *in* L. D. Harder, S. C. H. Barrett, editors. *The ecology and evolution of flowers*. Oxford University Press, Oxford, England, UK.

Johnson, S. D. (2010) The pollination niche and its role in the diversification and maintenance of the southern African flora. Philosophical Transactions of the Royal Society B: Biological Sciences 365: 499–516.

Johnson, S. D., Anderson, B. (2010) Coevolution between food-rewarding flowers and

their pollinators. Evolution: Education and Outreach 3: 32–39.

Johnson, S. G., Delph, L. F., Elderkin, C. L. (1995) The effect of petal-size manipulation on pollen removal, seed set, and insect-visitor behavior in *Campanula americana*. Oecologia 102: 174–179.

Jones, F. C., Grabherr, M. G., Chan, Y. F., Russell, P., Mauceli, E., Johnson, J., Swofford, R., Pirun, M., et al. (2012) The genomic basis of adaptive evolution in threespine sticklebacks. Nature 484: 55–61.

Kalisz, S., Vogler, D. W. (2003) Benefits of autonomous selfing under unpredictable pollinator environments. Ecology 84: 2928–2942.

Kameyama, Y., Kudo, G. (2009) Flowering phenology influences seed production and outcrossing rate in populations of an alpine snowbed shrub, *Phyllodoce aleutica*: effects of pollinators and self-incompatibility. Annals of Botany 103: 1385–1394.

Kimura, J. I., Yoshida, T. (1999) Magma plumbing system beneath Ontake Volcano, central Japan. Island Arc 8: 1–29.

Kingsolver, J. G., Hoekstra, H. E., Hoekstra, J. M., Berrigan, D., Vignieri, S. N., Hill, C., Hoang A., Gibert, P., et al. (2001) The strength of phenotypic selection in natural populations. The American Naturalist 157: 245–261.

Kudo, G., Maeda, T., Narita, K. (2001) Variation in floral sex allocation and reproductive success within inflorescences of *Corydalis ambigua* (Fumariaceae): pollination efficiency or resource limitation? Journal of Ecology 89: 48–56.

Kudo, G., Ida, T. Y. (2013) Early onset of spring increases the phenological mismatch between plants and pollinators. Ecology 94: 2311–2320.

Kuriya, S., Hattori, M., Nagano, Y., Itino, T. (2015) Altitudinal flower size variation correlates with local pollinator size in a bumblebee-pollinated herb, *Prunella*

vulgaris L. (Lamiaceae). Journal of Evolutionary Biology 28: 1761–1769.

Kuzume, H., Itino, T. (2013) Agreement of subdivisions inferred from different data types in *Cimicifuga simplex* (Ranunculaceae): pollination morphs and genotypes based on internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA. Journal of Japanese Botany 88: 176–181.

Lewis, D. (1941) Male sterility in natural populations of hermaphrodite plants the equilibrium between females and hermaphrodites to be expected with different types of inheritance. New Phytologist 40: 56–63.

Lloyd, D. G. (1975) The maintenance of gynodioecy and androdioecy in angiosperms. Genetica 45: 325–339.

Lobo, J. A., Lacerda-Ramos, D., Braga, A. C. (2016) Visitation rate of pollinators and nectar robbers to the flowers and inflorescences of *Tabebuia aurea* (Bignoniaceae): effects of floral display size and habitat fragmentation. Botanical Journal of the Linnean Society 181: 667–681.

Losos, J. B. (2010) Adaptive radiation, ecological opportunity, and evolutionary determinism: American Society of Naturalists EO Wilson Award address. The American Naturalist 175: 623–639.

Majetic, C. J., Raguso, R. A., Ashman, T. L. (2009) The sweet smell of success: floral scent affects pollinator attraction and seed fitness in *Hesperis matronalis*. Functional Ecology 23: 480–487.

Martin, C. H. (2012) Weak disruptive selection and incomplete phenotypic divergence in two classic examples of sympatric speciation: Cameroon crater lake cichlids. The American Naturalist 180: E90–E109.

Martin, R. A., Pfennig, D. W. (2009) Disruptive selection in natural populations: the

roles of ecological specialization and resource competition. The American Naturalist 174: 268–281.

Mayfield, M. M., Waser, N. M., Price, M. V. (2001) Exploring the ‘most effective pollinator principle' with complex flowers: bumblebees and *Ipomopsis aggregata*. Annals of Botany 88: 591–596.

Maynard Smith, J. (1962) Disruptive selection, polymorphism and sympatric speciation. Nature 195: 60–62.

Maynard Smith, J. (1966) Sympatric speciation. The American Naturalist 100: 637–650.

Medan, D., Bartoloni, N. (1998) Fecundity effects of dichogamy in an asynchronically flowering population: a genetic model. Annals of Botany 81: 373–383.

Meirmans, P. G. (2020) genodive version 3.0: Easy-to-use software for the analysis of genetic data of diploids and polyploids. Molecular ecology resources 20: 1126–1131.

Minnaar, C., de Jager, M. L., Anderson, B. (2019) Intraspecific divergence in floral-tube length promotes asymmetric pollen movement and reproductive isolation. New Phytologis 224: 1160–1170.

Miyamoto, S. (1961) Flower-visiting habit of *Ceratina japonica* Cockerell : Biological studies on Japanese bees XXIV. Japanese Journal of Entomology 29: 28–35.

Mizunaga, Y., Kudo, G. (2017) A linkage between flowering phenology and fruit-set success of alpine plant communities with reference to the seasonality and pollination effectiveness of bees and flies. Oecologia 185: 453–464.

More, M., Amorim, F. W., Benitez-Vieyra, S., Medina, A. M., Sazima, M., Cocucci, A. A. (2012) Armament imbalances: match and mismatch in plant-pollinator traits of

highly specialized long-spurred orchids. PLoS One 7: e41878.

Morgan, M. T., Schoen, D. J. (1997) The role of theory in an emerging new plant reproductive biology. Trends in Ecology & Evolution 12: 231–234.

Nagano, Y., Abe, K., Kitazawa, T., Hattori, M., Hirao, A. S., Itino, T. (2014) Changes in pollinator fauna affect altitudinal variation of floral size in a bumblebee-pollinated herb. Ecology and Evolution 4: 3395–3407.

Naghiloo, S., Nikzat-Siahkolaee, S., Esmaillou, Z. (2021) Size-matching as an important driver of plant–pollinator interactions. Plant Biology 23: 583–591.

Nakai, T. (1916) Notulæ ad plantas Japoniæ et Koreæ XI. The Botanical Magazine 30: 145–147.

Nattero, J., Cocucci, A. A., Medel, R. (2010) Pollinator-mediated selection in a specialized pollination system: matches and mismatches across populations. Journal of Evolutionary Biology 23: 1957–1968.

Nattero, J., Malerba, R., Medel, R., Cocucci, A. (2011) Factors affecting pollinator movement and plant fitness in a specialized pollination system. Plant Systematics and Evolution 296: 77–85.

Ne'eman, G., Jürgens, A., Newstrom-Lloyd, L., Potts, S. G., Dafni, A. (2010) A framework for comparing pollinator performance: effectiveness and efficiency. Biological Reviews 85: 435–451.

Nei, M., Tajima, F., Tateno, Y. (1983) Accuracy of estimated phylogenetic trees from molecular data. Journal of molecular evolution 19: 153–170.

Neter, J., Kutner, M. H., Nachtsheim, C. J., Wasserman, W. (1996) *Applied linear statistical models, 4th edn*. McGraw-Hill, New York, USA.

Newman, E., Anderson, B., Johnson, S. D. (2012) Flower colour adaptation in a

mimetic orchid. Proceedings of the Royal Society B: Biological Sciences 279: 2309–2313.

Newman, E., Manning, J., Anderson, B. (2015) Local adaptation: mechanical fit between floral ecotypes of *Nerine humilis* (Amaryllidaceae) and pollinator communities. Evolution 69: 2262–2275.

Nilsson, L. A. (1988) The evolution of flowers with deep corolla tubes. Nature 334: 147–149.

Nosil, P. (2012) *Ecological speciation*. Oxford University Press, Oxford, England, UK.

Noutsos, C., Borevitz, J., Hodges, S. A. (2014) Gene flow between nascent species: geographic, genotypic and phenotypic differentiation within and between *Aquilegia formosa* and *A. pubescens*. Molecular Ecology 23: 5589–5598.

Ohashi, K., Yahara, T. (1998) Effects of variation in flower number on pollinator visits in *Cirsium purpuratum* (Asteraceae). American Journal of Botany 85: 219–224.

Ornduff, R. (1969) Reproductive biology in relation to systematics. Taxon 18: 121–133.

Pauw, A., Stofberg, J., Waterman, R. J. (2009) Flies and flowers in Darwin's race. Evolution 63: 268–279.

Pellmyr, O. (1986) Three pollination morphs in *Cimicifuga simplex*; incipient speciation due to inferiority in competition. Oecologia 68: 304–307.

Pellmyr, O. (1987) Multiple sex expressions in *Cimicifuga simplex*: dichogamy destabilizes hermaphroditism. Biological Journal of the Linnean Society 31: 161–174.

Pettengill, J. B., Briscoe Runquist, R. D., Moeller, D. A. (2016) Mating system divergence affects the distribution of sequence diversity within and among

populations of recently diverged subspecies of *Clarkia xantiana* (Onagraceae). American Journal of Botany 103: 99–109.

Pettengill, J. B., Moeller, D. A. (2012) Tempo and mode of mating system evolution between incipient *Clarkia* species. Evolution 66: 1210–1225.

Podolsky, R. D. (1992) Strange floral attractors: pollinator attraction and the evolution of plant sexual systems. Science 258: 791–793.

Podolsky, R. D. (1993) Evolution of a flower dimorphism: How effective is pollen dispersal by" male" flowers? Ecology 74: 2255–2260.

Pritchard, J. K., Stephens, M., Donnelly, P. (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945–959.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A., Bender, D., Maller, J., Sklar, P., et al. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. The American journal of human genetics 81: 559–575.

R Core Team. (2013) R: A language and environment for statistical computing. Vienna: R Foundation for statistical Computing.

Rader, R., Bartomeus, I., Garibaldi, L. A., Garratt, M. P., Howlett, B. G., Winfree, R., Cunningham, S. A., Mayfield, M. M., et al. (2016) Non-bee insects are important contributors to global crop pollination. Proceedings of the National Academy of Sciences 113: 146–151.

Ramos, S. E., Schiestl, F. P. (2019) Rapid plant evolution driven by the interaction of pollination and herbivory. Science 364: 193–196.

Renner, S. S. (2014) The relative and absolute frequencies of angiosperm sexual systems: dioecy, monoecy, gynodioecy, and an updated online database. American

Journal of Botany 101: 1588–1596.

Ritland, K. (2002) Extensions of models for the estimation of mating systems using *n* independent loci. Heredity 88: 221.

Rochette, N. C., Rivera-Colón, A. G., Catchen, J. M. (2019) Stacks 2: Analytical methods for paired-end sequencing improve RADseq-based population genomics. Molecular Ecology 28: 4737–4754.

Rosenzweig, M. L. (1978) Competitive speciation. Biological Journal of the Linnean Society 10: 275–289.

Rousset, F. (1997) Genetic differentiation and estimation of gene flow from *F*-statistics under isolation by distance. Genetics 145: 1219–1228.

Rousset, F. (2008) genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Molecular ecology resources 8: 103–106.

Rymer, P. D., Johnson, S. D., Savolainen, V. (2010) Pollinator behaviour and plant speciation: can assortative mating and disruptive selection maintain distinct floral morphs in sympatry? New Phytologist 188: 426–436.

Sakagami, K., Sugiura, S. (2019) Noctuid moths as pollinators of *Habenaria sagittifera* (Orchidaceae): Floral adaptations for the transfer of pollinaria on the thoraxes of moths. The Science of Nature 106: 1–7.

Schemske, D. W., Bradshaw, H. (1999) Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). Proceedings of the National Academy of Sciences 96: 11910–11915.

Schulke, B., Waser, N. M. (2001) Long-distance pollinator flights and pollen dispersal between populations of *Delphinium nuttallianum*. Oecologia 127: 239–245.

Scobell, S. A., Scott, P. E. (2002) Visitors and floral traits of a hummingbird-adapted

cactus (Echinocereus coccineus) show only minor variation along an elevational gradient. The American midland naturalist 147: 1–15.

Sekiguchi, T., Yamagishi, N. (2013) Land condition map of Ontakesan Volcano. Geospatial Information Authority of Japan Report Jiho 123: 61–80.

Servedio, M. R., Noor, M. A. F. (2003) The role of reinforcement in speciation: theory and data. Annual Review of Ecology, Evolution, and Systematics 34: 339–364.

Setoguchi, H., Kajimura, G. (2004) Leaf morphology of the rheophyte, *Rhododendron indicum* f. otakumi (Ericaceae). Acta Phytotaxomica et Geobotanica 55: 45–54.

Shimizu, K. K, Shimizu-Inatsugi, R., Tsuchimatsu, T., Purugganan, M. D. (2008) Independent origins of self-compatibility in *Arabidopsis thaliana*. Molecular Ecology 17: 704–714.

Shriver, M. D., Jin, L., Boerwinkle, E., Deka, R., Ferrell, R. E., Chakraborty, R. (1995) A novel measure of genetic distance for highly polymorphic tandem repeat loci. Molecular biology and evolution 12: 914–920.

Silverman, B. W. (1981) Using kernel density estimates to investigate multimodality. Journal of the Royal Statistical Society: Series B (Methodological) 43: 97–99.

Slatkin, M. (1995) A measure of population subdivision based on microsatellite allele frequencies. Genetics 139: 457–462.

Sneath, P. H., Sokal, R. R. (1973) *Numerical taxonomy: The principles and practice of numerical classification.* W. H. Freeman, San Francisco, California, USA.

Sobel, J. M., Streisfeld, M. A. (2015) Strong premating reproductive isolation drives incipient speciation in Mimulus aurantiacus. Evolution 69: 447–461.

Solomon, B. P. (1985) Environmentally influenced changes in sex expression in an andromonoecious plant. Ecology 66: 1321–1332.

Spencer, S. C., Rieseberg, L. H. (1995) The importance of flowering time and flower number in the relative fitness of males and hermaphrodites in *Datisca glomerata* (Datiscaceae). Plant Species Biology 10: 65–69.

Stankowski, S., Streisfeld, M. A. (2015) Introgressive hybridization facilitates adaptive divergence in a recent radiation of monkeyflowers. Proceedings of the Royal Society B: Biological Sciences 282: 20151666.

Stebbins, G. L. (1970) Adaptive radiation of reproductive characteristics in angiosperms, I: pollination mechanisms. Annual Review of Ecology and Systematics 1: 307–326.

Steiner, K. E., Whitehead, V. (1991) Oil flowers and oil bees: further evidence for pollinator adaptation. Evolution 45: 1493–1501.

Stinchcombe, J. R., Agrawal, A. F., Hohenlohe, P. A., Arnold, S. J., Blows, M. W. (2008) Estimating nonlinear selection gradients using quadratic regression coefficients: double or nothing? Evolution 62: 2435–2440.

Stone, J. L. (1996) Components of pollination effectiveness in *Psychotria suerrensis*, a tropical distylous shrub. Oecologia 107: 504–512.

Stone, J. D., Olson, M. S. (2018) Pollination context alters female advantage in gynodioecious *Silene vulgaris*. Journal of Evolutionary Biology 31: 111–122.

Streisfeld, M. A., Kohn, J. R. (2005) Contrasting patterns of floral and molecular variation across a cline in *Mimulus aurantiacus*. Evolution 59: 2548–2559.

Sulborska, A., Dmitruk, M., Konarska, A., Weryszko-Chmielewska, E. (2014) Adaptation of *Lamium album* L. flowers to pollination by Apoidea. Acta Scientiarum Polonorum-Hortorum Cultus 13: 31–43.

Suyama, Y., Matsuki, Y. (2015) MIG-seq: an effective PCR-based method for genome-

wide single-nucleotide polymorphism genotyping using the next-generation sequencing platform. Scientific reports 5: 1–12.

Suyama, Y., Hirota, S. K., Matsuo, A., Tsunamoto, Y., Mitsuyuki, C., Shimura, A., Okano, K. (2021) Complementary combination of multiplex high-throughput DNA sequencing for molecular phylogeny. Ecological Research 37: 171–181.

Suzuki, K. (1992) Bumblebee pollinators and pollination ecotypes of *Isodon umbrosus* and *I. shikokianus* (Lamiaceae). Plant species biology 7: 37–48.

Suzuki, K., Dohzono, I., Hiei, K. (2007) Evolution of pollinator generalization in bumblebee-pollinated plants. Plant Species Biology 22: 141–159.

Suzuki, K., Dohzono, I., Hiei, K., Fukuda, Y. (2002) Pollination effectiveness of three bumblebee species on flowers of *Hosta sieboldiana* (Liliaceae) and its relation to floral structure and pollinator sizes. Plant Species Biology 17: 139–146.

Suzuki, T., Suzuki, N., Tojo, K. (2019) Parallel evolution of an alpine type ecomorph in a scorpionfly: Independent adaptation to high-altitude environments in multiple mountain locations. Molecular Ecology 28: 3225–3240.

Takezaki, N., Nei, M., Tamura, K. (2010) POPTREE2: Software for constructing population trees from allele frequency data and computing other population statistics with Windows interface. Molecular biology and evolution 27: 747–752.

Tamura, M., Shimizu, T. (1999) *Wild Flowers of Japan: Herbaceous Plants (Including Dwarf Subshrubs*. Heibonsha, Tokyo, Japan.

Thompson, J. N. (2005) *The geographic mosaic of coevolution*: University of Chicago Press, Chicago, USA.

Thompson, J. N. (2013) *Relentless evolution*. University of Chicago Press, Chicago, USA.

Thompson, J. N., Schwind, C., Guimarães, P. R., Friberg, M. (2013) Diversification through multitrait evolution in a coevolving interaction. Proceedings of the National Academy of Sciences 110: 11487–11492.

Thomson, J. D., Barrett, S. C. (1981) Temporal variation of gender in *Aralia hispida* Vent. (Araliaceae). Evolution 35: 1094–1107.

Timerman, D., Barrett, S. C. (2019) The spatial ecology of sex ratios in a dioecious plant: Relations between ramet and genet sex ratios. Journal of Ecology 107: 1804–1816.

Toji, T., Kameyama, Y., Hirao, A. S. Itino, T. (2018) Development and characterization of microsatellite markers for three pollination morphs of *Cimicifuga simplex* (Ranunculaceae). American Journal of Plant Sciences 9: 599–605.

Toji, T., Itino, T. (2020) Differences in sex expression and mating systems in three pollination morphs of *Cimicifuga simplex*. Plant Species Biology 35: 112–119.

Toji, T., Ishimoto, N., Egawa, S., Nakase, Y., Hattori, M., Itino, T. (2021) Intraspecific convergence of floral size correlates with pollinator size on different mountains: a case study of a bumblebee-pollinated *Lamium* (Lamiaceae) flowers in Japan. BMC ecology and evolution 21: 1–13.

Toji, T., Ishimoto, N., Itino, T. (2020) Seasonal change of flower sex ratio and pollinator dynamics in three reproductive ecotypes of protandrous plant. Ecosphere 11: e03251.

Torices, R., Méndez, M., Gómez, J. M. (2011) Where do monomorphic sexual systems fit in the evolution of dioecy? Insights from the largest family of angiosperms. New Phytologist 190: 234–248.

Ueno, N., Suyama, Y., Seiwa, K. (2007) What makes the sex ratio female-biased in the

dioecious tree *Salix sachalinensis*? Journal of Ecology 95: 951–959.

Usukura, M., Imaichi, R., Kato, M. (1994) Leaf morphology of a facultative rheophyte, *Farfugium japonicum* var. *luchuense* (Compositae). Journal of Plant Research 107: 263–267.

Vallejo-Marín, M., Rausher, M. D. (2007) The role of male flowers in andromonoecious species: Energetic costs and siring success in *Solanum carolinense* L. Evolution 61: 404–412.

Van der Niet, T., Johnson, S. D. (2012) Phylogenetic evidence for pollinator-driven diversification of angiosperms. Trends in Ecology & Evolution 27: 353–361.

Van der Niet, T., Pirie, M. D., Shuttleworth, A., Johnson, S. D., Midgley, J. J. (2014) Do pollinator distributions underlie the evolution of pollination ecotypes in the Cape shrub *Erica plukenetii*? Annals of Botany 113: 301–315.

Wade, M. J., Kalisz, S. (1990) The causes of natural selection. Evolution 44: 1947–1955.

Weir, B. S., Cockerham, C. C. (1984) Estimating *F*-statistics for the analysis of population structure. Evolution 38: 1358–1370.

Wells, M. S., Lloyd, D. G. (1991) Dichogamy, gender variation and bet-hedging in *Pseudowintera colorata*. Evolutionary Ecology 5: 310–326.

Whittall, J. B., Hodges, S. A. (2007) Pollinator shifts drive increasingly long nectar spurs in columbine flowers. Nature 447: 706–709.

Willson, M. F. (1983) *Plant reproductive ecology*. John Wiley & Sons, New York, USA.

Willson, M. F., Price, P. W. (1977) The evolution of inflorescence size in *Asclepias* (Asclepiadaceae). Evolution 31: 495–511.

Wirth, L. R., Graf, R., Gugerli, F., Landergott, U., Holderegger, R. (2010) Lower selfing rate at higher altitudes in the alpine plant *Eritrichium nanum* (Boraginaceae). American Journal of Botany 97: 899–901.

Wright, S. I., Kalisz, S., Slotte, T. (2013) Evolutionary consequences of self-fertilization in plants. Proceedings of Royal Society B: Biological Sciences 280: 20130133.

Wright, S. (1943) Isolation by distance. Genetics 28: 114.

Yu, L., Lu, J. (2019) Effects of population size and structure on reproductive success of a *Pistacia chinensis* population. Plant Biosystems - An International Journal Dealing with All Aspects of Plant Biology 1–8.

Zhang, F., Hui, C., Pauw, A. (2013) Adaptive divergence in Darwin's race: how coevolution can generate trait diversity in a pollination system. Evolution 67: 548–560.

Zhang, R., Min, Y., Holappa, L. D., Walcher-Chevillet, C. L., Duan, X., Donaldson, E., Kong, H., Kramer, E. M. (2020) A role for the Auxin Response Factors *ARF6* and *ARF8* homologs in petal spur elongation and nectary maturation in *Aquilegia*. New Phytologist 227: 1392–1405.

Zhao, Z. G., Meng, J. L., Fan, B. L., Du, G. Z. (2008) Reproductive patterns within racemes in protandrous *Aconitum gymnandrum* (Ranunculaceae): potential mechanism and among-family variation. Plant Systematics and Evolution 273: 247–256.