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

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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*. We investigated seasonal sex ratio changes and pollinator environments of the three morphs to verify and expand the minority sex hypothesis. We investigated flowering phenology, pollinator quantity and quality, seasonal population sex ratio dynamics, and stamen/ovule ratios in hermaphroditic flowers. We 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.

Key words: andromonoecy; dichogamy; gynodioecy; pollinator dynamics; pollinator quality; pollinator quantity; reproductive ecology; selfing; sex ratio.

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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, Fausto 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 a driving force 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 we 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, we focused on *Cimicifuga simplex* Wormsk. (Ranunculaceae), a protandrous plant with three morphs, each with a different reproduction system and a different pollinator assemblage. First, we 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, we 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, and 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 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, a morph II plants with low-quality flower visitors is expected to have a high investment ratio in males. We 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, we propose expanding the minority sex hypothesis.

In this study, we 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. We 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. We 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, we 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, we measured the frequency of flower visits by insect pollinators per minute. We monitored these parameters in populations of the three morphs during two years.

Methods

Plant species and the survey area

Cimicifuga simplex is a perennial herb distributed in eastern and northeastern Asia (Nakai 1916, Emura 1970). In this species, a hermaphroditic ramet has many flowers arranged in a simple raceme (Fig. 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 (Fig. 1). The male and female phases each last for 3–4 d. The lateral raceme 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 (Fig. 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

Table 1. Differences in the ecological characteristics of the three morphs of *Cimicifuga simplex*.

Morph	Main pollinator	Altitudinal distribution (m)	Habitat	Flowering season	Mating systems	Sex expressions	Survey population
Morph I	Bumblebees	1350–2370	Sunny open environment	Late July–Early September	Outcrossing	Hermaphrodite, Female, Andromonoecy (rare)	2340 m a.s.l., 137°34′19″ E, 36°7′18″ N
Morph II	Butterflies, flies, and syrphids	920–1500	Sunny forest edge	Early September–Early October	Outcrossing	Hermaphrodite, Andromonoecy	1350 m a.s.l., 138°2′28″ E, 36°15′7″ N
Morph III	Flies and syrphids	650–1350	Dark forest floor	Early October–Early November	Selfing	Hermaphrodite, Andromonoecy (rare)	1000 m a.s.l, 138°0′39″ E, 36°9′22″ N

Note: Information from previous studies (Pellmyr 1986, Kuzume and Itino 2013, Toji and Itino 2020) and the information obtained in this study are included.

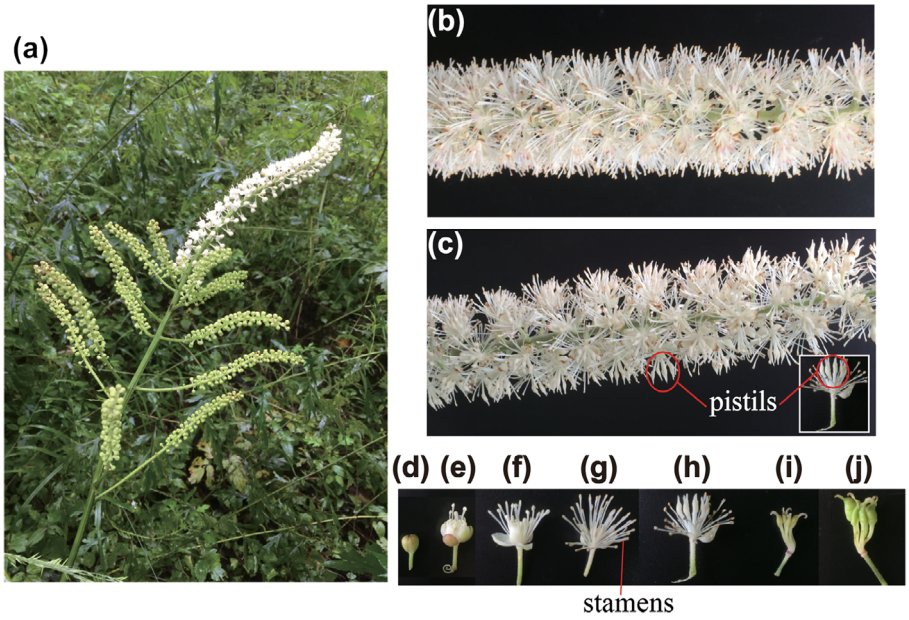


Fig. 1. Photographs of a hermaphroditic ramet of *Cimicifuga simplex* and seasonal sexual changes of the hermaphroditic raceme. (a) Morph II hermaphroditic ramet. Only the primary raceme is blooming. The lateral racemes will flower after the primary raceme has set fruit. (b) Enlarged view of the hermaphroditic 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.

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 1; Pellmyr 1986, Kuzume and Itino 2013, Toji and Itino 2020).

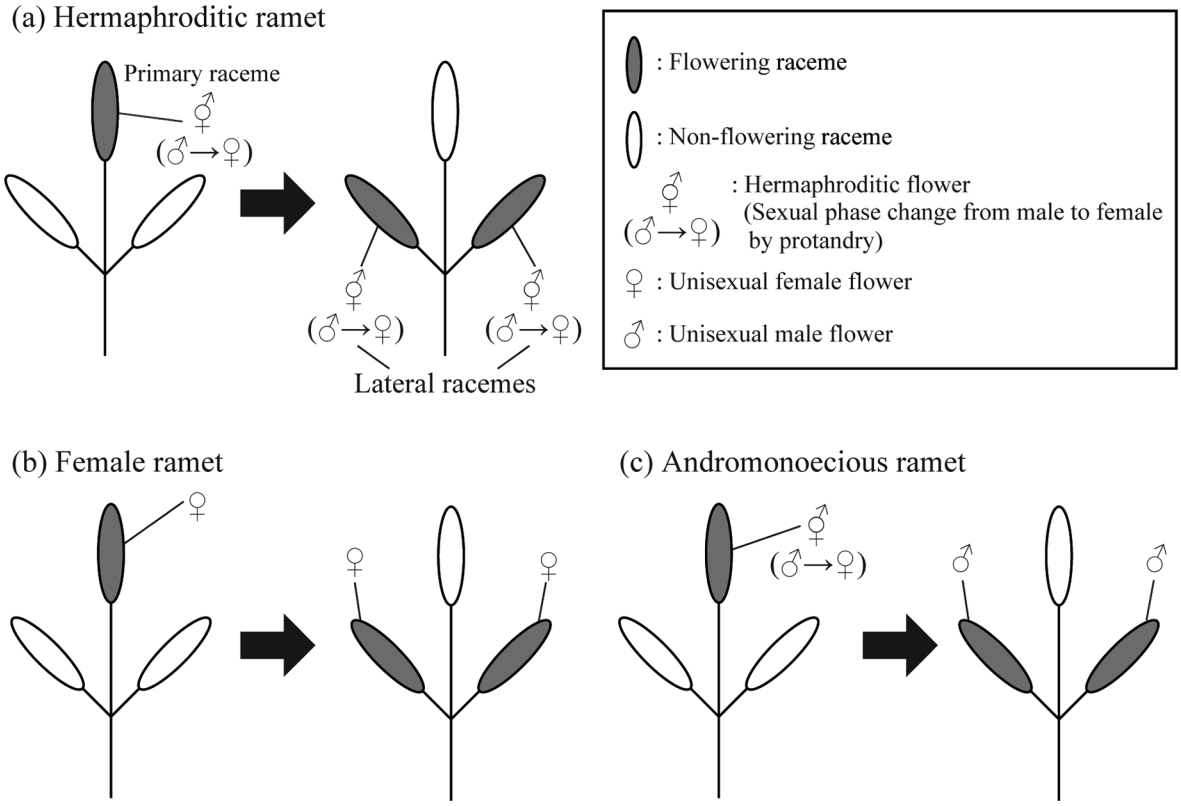


Fig. 2. The order of flowering on a ramet. The black arrows indicate the passage of time. Regardless of the sex expression, the primary raceme of a ramet blooms first, followed by the lateral racemes. (a) A sexual phase change from male to female occurs in protandrous hermaphroditic flowers on both primary 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.

In each population, we examined ramets on plants growing within a 100 m × 200 m quadrat at intervals of 2–7 d (Table 2). We 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, during 2 yr (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 studies, we 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

Table 2. Number of ramets for each sex expression in the three morphs of *Cimicifuga 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

morph on multiple sites consistently had a similar reproductive system, we selected one site for each morph as our study site.

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. We 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, we 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, we 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. We 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, we counted the flower visitation rate at each periodic survey site. We 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. We 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, we 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. We observed flower visitors from 9:00 a.m. to 14:00 p.m. local time on a sunny day.

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 (Fig. 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 was calculated. The male sex ratio was determined for each population as follows:

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

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, we calculated the male sex ratio without considering unisexual ramets as follows:

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

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.

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, we 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, we 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 (Appendix S1: Table S1), we 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 (Appendix S1: Table S1), we considered the number of ovules to be an effective measure of allocation to female function by hermaphroditic flowers (Appendix S1: Table S1). 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, we 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, 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 morphs 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 (~1 week) after the flowering start date. Therefore, in the case of hermaphrodite of morph I and morph II, we referred male sex ratio and the visitation rate to the values on two survey days after the flowering start date.

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, we 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, 1993, Elle and Meagher 2000, Barrett 2002, Vallejo-Marín and Rausher 2007). Therefore, we 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 × 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 d by scissor.

The natural fruit set recorded at both sites in 2016 was also used for comparison (Wilcoxon signed-rank test).

Results

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 (Fig. 3 a). 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 (Fig. 3b).

In 2017 and 2018, we 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 (Figs. 4j, 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 (Figs. 4k, 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 (Figs. 4l, 5l). A breakdown of the insects visited on each survey day is given in Appendix S1: Fig. S1.

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; Figs. 4a, 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 (Figs. 4 d, 5d). When we 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 we also considered unisexual ramets, the male sex ratio was relatively stable over the course of the flowering season (Figs. 4g, 5g), with a mean ratio during the flowering season of 0.46–0.50.

In morph II, the male sex ratio a seasonal changed from a high value to a lower value (Figs. 4b, 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 (Figs. 4e, 5e). Although the male sex ratio changed clearly from a high value to a low value when we considered only the hermaphroditic ramets, when we considered unisexual flowers as well, the male sex ratio was relatively stable (Figs. 4h, 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 (Figs. 4c, 5c). The male and female phases were not completely separate in time; the female phase began 1–2 d after flowering began, and a few unisexual male flowers on lateral racemes bloomed in the second half of the flowering season (Figs. 4f, 5f). The male sex ratio of the morph III population did not change as much as it did in morphs I and II (Figs. 4i, 5i); the mean ratio during the flowering season was 0.41–0.55.

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,

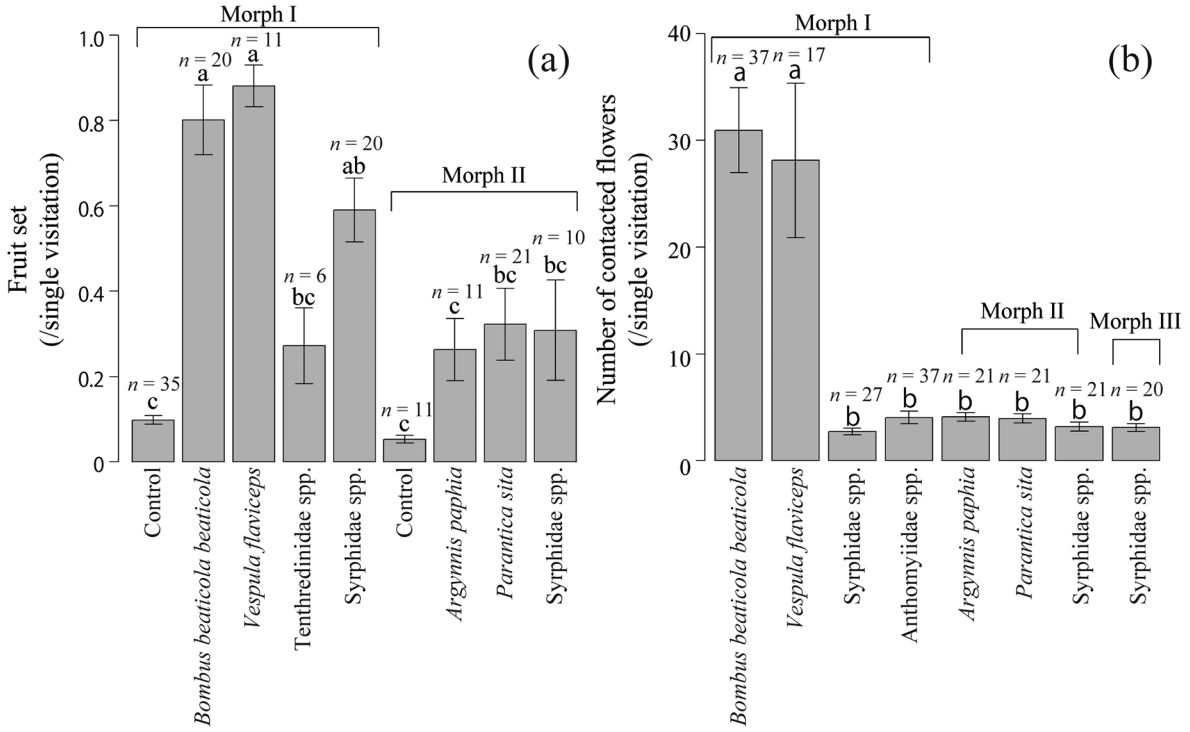


Fig. 3. Pollinator quality in *Cimicifuga simplex*. (a) Flower fruit set per single visitation of each visitor type. Only flowers that the visitor contacted were counted. Control flowers were bagged throughout the flowering season. (b) Numbers of visited flowers during a single visitation to a raceme. 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.

and 1.43×10^{-1} for morph III (Fig. 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 (Appendix S1: Table S1), 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 (Fig. 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 (Fig. 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 (Fig. 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 3).

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 (Fig. 6, 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

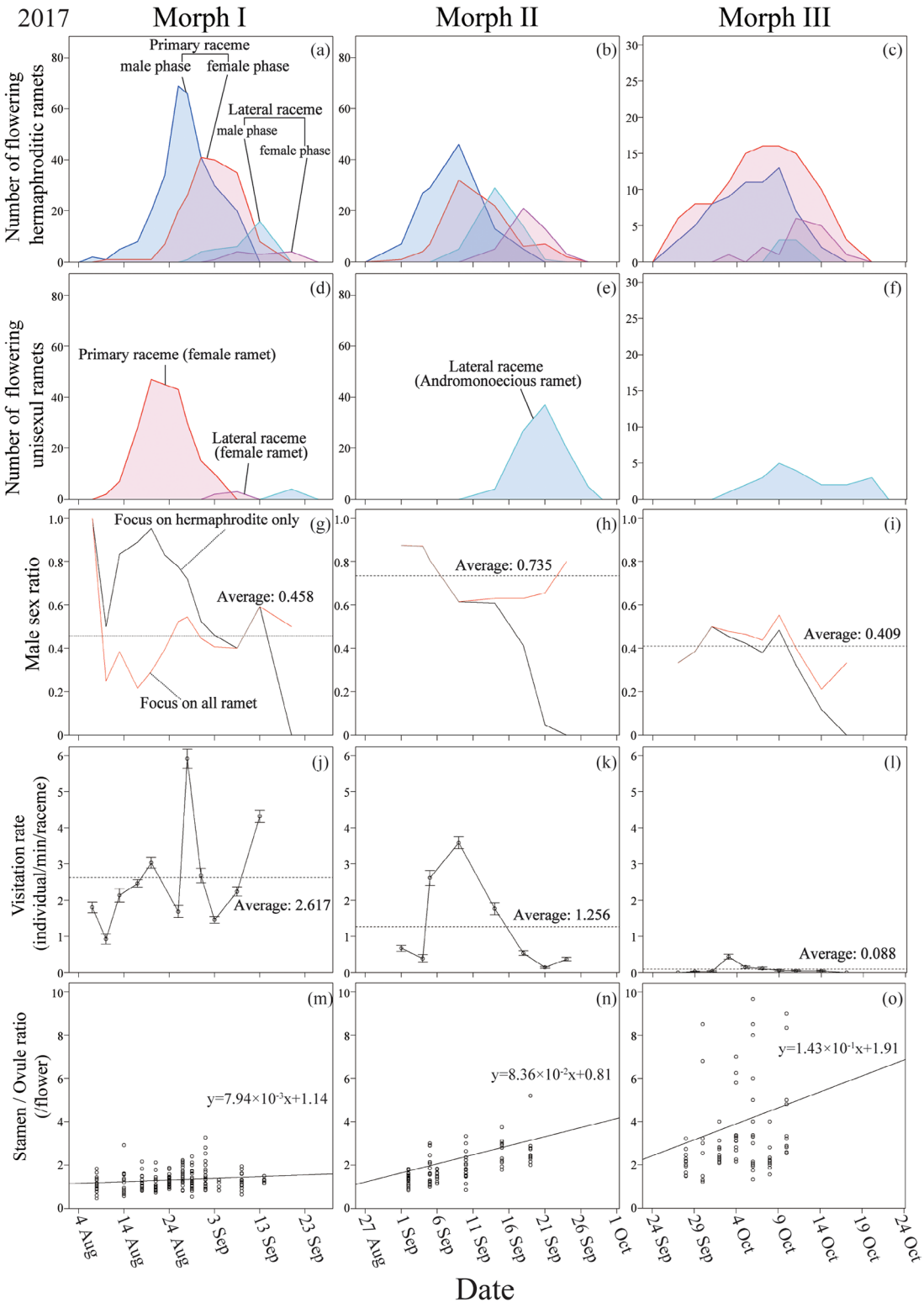


Fig. 4. Seasonal changes in the sex ratio, the abundance of visiting insects, and the stamen/ovule ratio of each

(Fig. 4. *Continued*)

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, $P < 0.01$).

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, $P < 0.01$).

Discussion

As shown by Pellmyr (1987), unisexual female ramets in morph I flowered in the first half of the flowering season. In our results, unisexual male flowers on andromonoecious ramets in morphs I and II flowered in the second half of the flowering season (Figs. 4d–e, 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, we 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).

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 4), 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). Our 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 (Fig. 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, Figs. 4j, 5j). Thus, both the quality and quantity of pollinators of morph I flowers were high. These results suggest that in

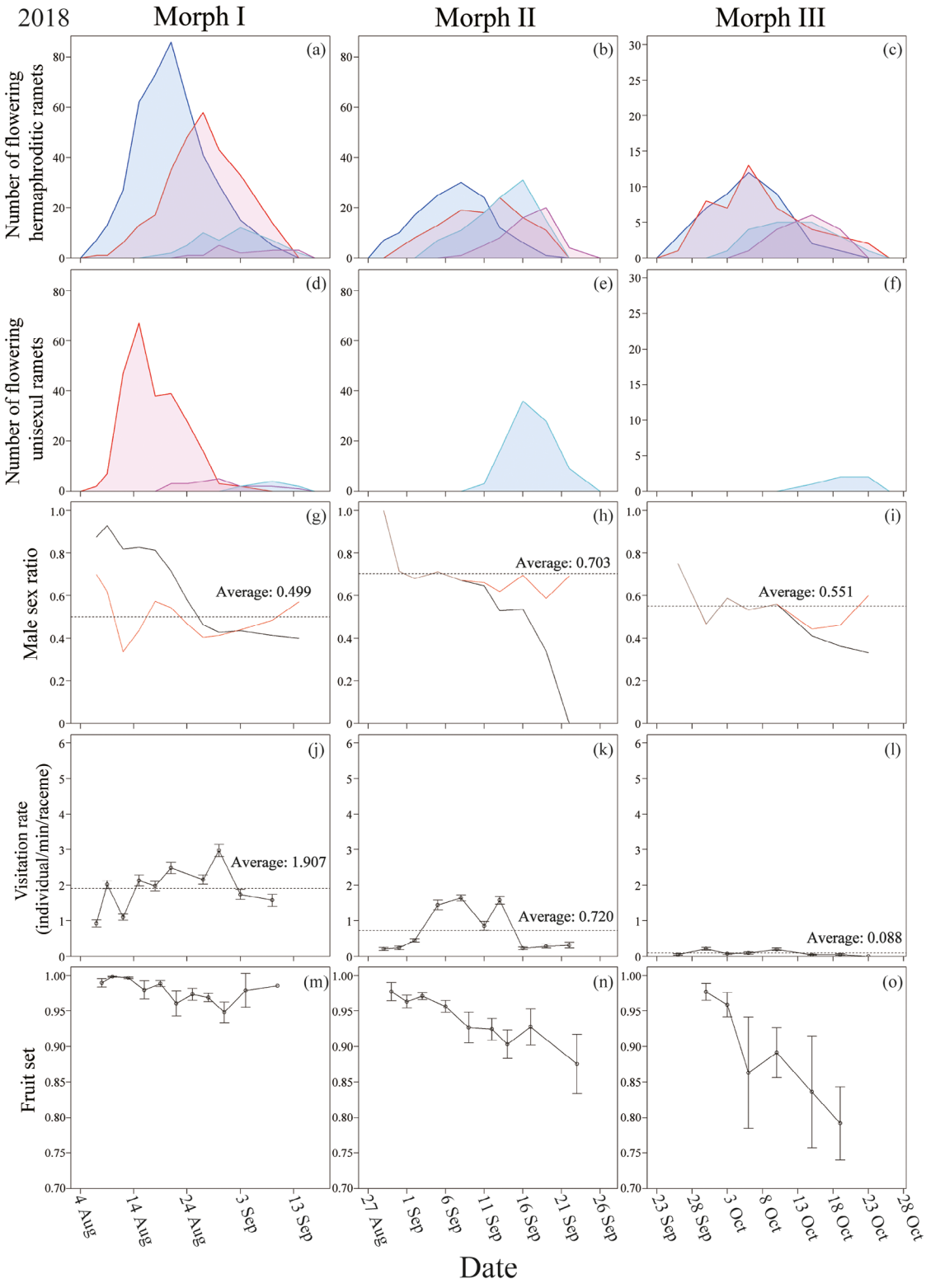


Fig. 5. Seasonal changes in the sex ratio, the abundance of visiting insects, and fruit set of hermaphroditic

(Fig. 5. *Continued*)

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.

Table 3. Results of generalized linear model analysis using the fruit set as a response variable.

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

Notes: 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, we referred male sex ratio and the visitation rate to the values on two survey days after the flowering start date. Asterisks indicate significant label *; $P < 0.05$.

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. We 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.

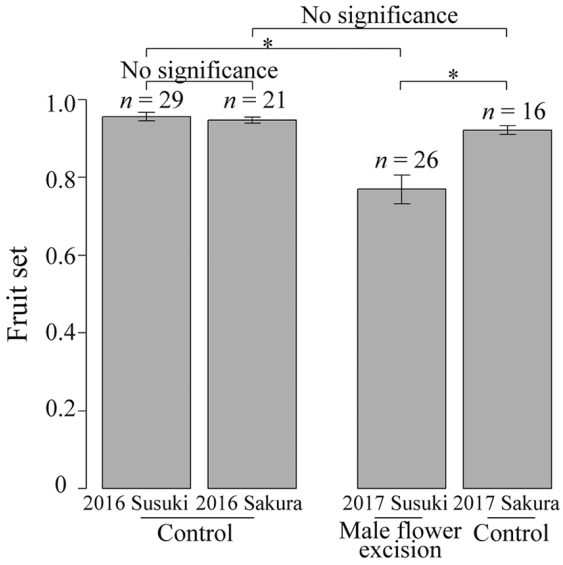


Fig. 6. Effect of excision of unisexual male flowers on andromonoecious ramets on fruit set of hermaphroditic ramets. Fruit set of the lateral racemes of hermaphroditic ramets was measured in two populations of morph II (Susuki and Sakura). The natural fruit set at each of the two sites was measured 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, $P < 0.01$).

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 (Figs. 4d, 5d). Surprisingly, the fruit set of hermaphroditic ramets was high regardless of the flowering date (Fig. 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 of 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 3). Since our 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.

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

Table 4. Evaluation of seed output between hermaphroditic and unisexual female ramets of morph I.

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

Note: Differences were evaluated by using the Wilcoxon signed-rank test.

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 (Figs. 4k, 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 (Fig. 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 (Figs. 4h, 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 $\pm$ 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 (Fig. 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, García 2003, Hiraga and Sakai 2007, Zhao et al. 2008, Ishii and Harder 2012).

In addition, in our 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 (Fig. 6). 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- to 4-d 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 100×200 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, we can conclude that effect of male flower excision was underestimated in our experiment. Nevertheless, the significant reduction in fruit set on the lateral racemes of hermaphroditic ramets that were 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 (Figs. 3, 4j, 5j), and it produces few male racemes to contribute to the floral display (Figs. 4d, 5d). In contrast, the quality of pollinators visiting morph II is low, and the pollinator quantity is unstable (Figs. 3, 4k, 5k), but many male flowers and racemes increase the size of the floral display (Figs. 4e, 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.

High selfing rate and loss of protandry in morph III

Flower visitors to morph III were rare throughout the flowering season during the 2 yr of observations (Figs. 4l, 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 our examination of the flowering phenology of morph III, we found that the transition from the male phase to female phase occurred within a rather short time span (Figs. 4c, 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 our 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 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.

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Supporting Information

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