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

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Abstract

Cimicifuga simplex (Ranunculaceae) has three genetically distinct pollination morphs. Here, we 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. A 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.

KEYWORDS

high elevation, plant reproductive system, Ranunculaceae, selfing rate, sex expression

1 INTRODUCTION

Angiosperms have various sexual systems from hermaphroditism to dioecy, and mating systems from outcrossing to predominant self-fertilization (Culley & Klooster, 2007; Renner, 2014; Wright, Kalisz, & Slotte, 2013). 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 (Charlesworth & Charlesworth, 1978; Lloyd, 1975), 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 (Casimiro-Soriguer, Buide, & Narbona, 2015; Desfeux, Maurice, Henry, Lejeune, & Gouyon, 1996). In the genera Collinsia and Arabidopsis, directional evolution of the reproductive system (such as outcrossing to selfing) occurred several times (Shimizu, Shimizu-Inatsugi, Tsuchimatsu, & Purugganan, 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, flowers' sex expression is often consistent within species (Desfeux et al., 1996), although the selfing rate is often variable within species (e.g., Wirth, Graf, Gugerli, Landergott, & Holderegger, 2010).

In C. simplex Wormsk. (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. ² WILEY PLANT SPECIES BIOLOGY

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.

Cimicifuga simplex also comprises three genetically differentiated pollination morphs (Kuzume & Itino, 2013; Pellmyr, 1986), 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 (Kuzume & Itino, 2013; Pellmyr, 1986), although the visitation rates are low.

In this study, we examined the relationship between sex expression and the pollination morphs of C. simplex. We found that sex expression and outcrossing rate differed among the three pollination morphs. Morph I, which has high outcrossing rates, comprised hermaphroditic ramets and only female ramets. Morph II, which has high outcrossing rates, comprised hermaphroditic ramets and andromonoecious ramets. Morph III, which has low outcrossing rates, comprised hermaphroditic ramets. Such intraspecific variation of sex expressions has scarcely been TOJI AND ITINO

investigated, so further research on this system would shed light on the evolution of plant sex expression. In addition, as shown in many studies, it was suggested that mating limitation due to the lack of a pollinator is related to the acquisition of selfing in morph III.

2 MATERIALS AND METHODS

2.1 *Cimicifuga simplex* and the study sites

Cimicifuga simplex is a perennial herb distributed in eastern and northeastern Asia (Emura, 1970; Nakai, 1916). 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 & 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).



FIGURE 1 Three pollination morphs of *Cimicifuga simplex*. (a) Morph I is distributed in a high alpine zone (in the Norikura_5 population, 2,340 m, a.s.l.). (b) Morph II is distributed in midland forest edge (visited by the butterfly Parantica sita in the Fukashi population, 1,350 m, a.s.l.). (c) Morph III is distributed in shaded lowland (in the Gake population, 920 m, a.s.l.)

FIGURE 2 Distribution sites of three pollination morphs of Cimicifuga simplex in Matsumoto, Nagano, Japan. The study sites are accompanied by the names of the populations (see Table 1). The not studied but only distribution checked sites are shown without names



In 2016, we studied 10 C. simplex populations in Nagano, central Japan (Figure 2): we 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.

Sex expression and 2.2 inflorescence size

To determine population composition, during the 2016 flowering season, we 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, we measured the length of the primary inflorescence (the inflorescence at the top of the ramet) of each marked ramet.

Flower visitation rate of insects 2.3

To assess pollinator composition of each morph, during the peak flowering period of each population, we observed flower visitors from 9:00 a.m. to 12:00 noon local time on a fine day. We 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).

Evaluation of outcrossing rate 2.4

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To estimate the multilocus outcrossing rate (t_m) , eight microsatellite loci (Cisi 1 to Cisi 8; Toji, Kameyama, Hirao, & Itino, 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; morph III, Misuzu and Hora) and five to six seeds per plant were haphazardly collected for analysis. Genomic DNA was extracted from ovules with a DNeasy Plant Mini Kit (Oiagen, Germantown, Maryland) and a polymerase chain reaction analysis for genotyping was conducted following the method of Toji et al. (2018).

Statistical analyses 2.5

A chi-squared test was used to compare differences in sex expression between morphs. Tukey's honestly significant difference (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) we 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 (The R Project for Statistical Computing; http://www.r-project.org/).

3 RESULTS

3.1 | Sex expression and inflorescence size

Each of the three pollination morphs of C. simplex differed with respect to sex expression and mating system

						Frequency of plant rame	ts	
Pollination morph	Population name	Altitude (m)	Latitude (N)	Longitude (E)	Number of ramets in the population	with hermaphroditic flowers	with only female flowers	with hermaphroditic and male flowers
Ι	Norikura_1	2,050	36°12′29′′	137°58′48′′	24	0.92	I	0.08
I	Norikura_2	2,120	36°12′19′′	137°57′98′′	24	0.54	0.46	I
Ι	Norikura_3	2,200	36°12′05′′	137°57′40′′	19	0.74	0.26	Ι
I	Norikura_4	2,300	36°11′97′′	137°57'25''	81	0.95	0.05	Ι
I	Norikura_5	2,340	36°12′18′′	137°57′19′′	118	0.64	0.32	0.04
Π	Fukashi	1,350	36°25′13′′	$138^{\circ}04'04''$	108	0.77		0.23
Π	Sakura	1,300	36°21′60′′	$138^{\circ}08'38''$	92	0.49	I	0.51
III	Misuzu	1,000	36°26'32''	138°01'26''	41	0.98		0.02
III	Hora	700	36°28'03''	137°98′47′′	11	1.00	I	I
III	Gake	920	36°15′64′′	$138^{\circ}01'08''$	111	0.93		0.07
Note: Location, po	pulation size and sex rati	.o.						



FIGURE 3 Observed forms of sex expression in the three pollination morphs of *Cimicifuga simplex* (the sum of all populations). An asterisk indicates that the combination of forms of flower sex expression differs between the morph pairs (chi-squared test, p < .01)

(Figure 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-squared test: morph I vs. morph II, $\chi^2 = 120.8$, p < .01; morph I vs. morph II, $\chi^2 = 42.2$, p < .01; morph II, $\chi^2 = 46.4$, p < .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 4a). The inflorescence size of morph III was significantly smaller than that of the other morphs (Tukey's HSD, p < .05).

3.2 | 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 4b). Morph III was visited significantly less frequently than the other morphs (Tukey's HSD, p < .05). Many dipteran insects were recorded as visiting morphs I–III, most of which were Syrphinae (Table 2). Hymenopteran insects,

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	Percentage of	flower visitors								
	Morph I popu	lations				Morph II pc	opulations	Morph III	populatio	su
	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%					

TABLE 2 Flower visitors to the three pollination morphs of *Cimicifuga simplex* in 10 populations

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FIGURE 4 (a) Inflorescence size, (b) visitation rates of each insect order (number of insect visits per inflorescence per minute, mean \pm standard error [*SE*]) and (c) multilocus outcrossing rate (t_m) in the three pollination morphs of *Cimicifuga simplex*. The box plots in (a) and (c) show the median (bar), the lower and upper quartiles (box ends), lower and upper quartile $\pm 1.5 \times$ interquartile range (whiskers) and outliers (circles). Different lowercase letters indicate significant differences between the populations (Tukey's honestly significant difference (HSD), p < .05)

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*.

3.3 | 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 4c). The outcrossing rate was significantly lower in morph III than in the other morphs (Tukey's HSD, p < .05).

4 | DISCUSSION

We 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, we did not find this sex expression.

Bumblebees, which are excellent pollinators of herbaceous plants in general (Mayfield, Waser, & Price, 2001; Schulke & Waser, 2001), visited morph I flowers frequently (Figure 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 3). Dipteran insects (mostly syrphid flies) were frequent visitors to morph II flowers (Figure 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 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? Why were no ramets with only female flowers recorded?

In general, male flowers are decorative; their role is to attract pollinators (Willson, 1983). We 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. We 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 4b) and the small size of its inflorescences (Figure 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 the male phase to female phase occurred quickly (T. 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, we confirmed that the overlap of the male phase and female phase of morph III lasts for more than 4 days. We suggest that the rapid sexual phase change of morph III is responsible for the high selfing rate. If C. simplex has self-incompatibility as mentioned in Pellmyr (1987), self-incompatibility may have been 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 disabled by natural selection (Busch & 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 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 and populations. It is suggested that the optimal resource allocation strategy changes due to exposure to different pollinator environments (Ezoe & Washizu, 2009; Harder & Aizen, 2010). The difference in the sex ratio between morphs and 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 for the evolution of plant mating systems. Similar to Gervasi and Schiestl (2017), we suggest that different pollinator environments affect the selfing rate of C. simplex, but more importantly, we suggest that different pollinator environments also affect flower sex expressions of C. simplex.

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