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Site-specific clonal structure influences the seed production of an alpine shrub *Rhododendron aureum*: implications for geitonogamous pollination

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Abstract

Pollination success and seed production of clonal plants will depend on the clonal structure of local populations if it affects the degree of geitonogamous pollination. We compared the clonal structure and reproductive performance of an alpine dwarf shrub, *Rhododendron aureum*, between local populations with different snowmelt conditions in the Taisetsu Mountains of northern Japan. This species is an outcrosser and many self-fertilised ovules are aborted before maturation. Flowering at early snowmelt sites (fellfield and shrubby habitats) occurs in mid-June, when overwintered bumble-bee queens are the major pollinators, whereas flowering at late snowmelt sites (snowbed habitat) occurs after mid-July, when bumble-bee workers are the main pollinators. Fruit-set rates were larger in the early-flowering populations than in the late-flowering populations due to larger queen abundance than usual years. However, seed production in ripe fruits differed between the habitat types in the early-flowering populations, where fellfield population with continuous patch distribution showed higher seed production than shrubby population with fragmented patch distribution. It was supposed that frequent geitonogamous pollination in the fragmented population resulted in higher abortion of self-fertilised seeds. Flower number per inflorescence was similar between flowering times, but ovule number per flower was significantly higher in the early-flowering populations than in the late-flowering populations. The production of many ovules was expected to be advantageous for the early-flowering population to ensure the seed production in environments with fewer flower visitors in usual years. In conclusion, heterogeneous ecological situations in the alpine ecosystem lead to habitat-specific seed production pattern among conspecific populations.

Keywords: alpine plant, clonal structure, geitonogamous pollination, ovule, Rhododendron, seed production

Introduction

In alpine zones dominated by dwarf shrubs, clonal growth is an important trait for the survival and persistence of many alpine plants, since harsh climatic conditions often limit seed production and the establishment of seedlings (Bliss 1971; Körner 2021). Although the question of whether clonal plants are more prevalent in alpine ecosystems than in lower altitude ecosystems remains controversial (Klimešová and Doležal 2011), clonal growth could help ensure the survival of local populations. The seed production of clonal plants is influenced by patch size, ramet density and patch distribution within a population (Vallejo-Marín et al. 2010). Achieving a balance between the positive effects of larger clonal patches on pollinator attraction and the negative effects of geitonogamous pollination is crucial (Harder et al. 2004). Geitonogamous pollination is defined as pollen transfer between flowers on the same plant (Hessing 1988). Although a large clonal patch with many flowers often attracts pollinators, seed production in outcrossing plants may be limited by frequent geitonogamous pollination within the clonal patch (Handel 1985; Charpentier 2001). In clonal patches of alpine dwarf shrubs, many flowers are produced densely, which attracts pollinators, but geitonogamous pollination is also likely to be intense. Many alpine plants growing in Japan are obligate outcrossers and have a self-compatibility index (SCI: seeding or fruiting rate by selfing / seeding or fruiting rate by outcrossing) of less than 0.2 (Kudo 2022). Therefore, the negative effects of geitonogamous pollination are likely to be strongly expressed in clonal dwarf shrubs.

Rhododendron aureum grows clonally through frequent branching of creeping stems, with large variations in clone size, shoot length, and leaf number observed among populations (Tikhonova and Polezhaeva 2023). This suggests significant plasticity in clonal structure and morphological performance in response to the growing environment (Wang et al. 2018). Alpine vegetation in northern Japan can be broadly classified into three major habitat types: exposed

fellfield, alpine dwarf pine (*Pinus pumila*) shrub, and late-snowmelt snowbed. Interestingly, *R. aureum* grows in all habitat types. In the fellfield habitat with little snow cover throughout the year, the soil surface is exposed to strong winds and freezing temperatures in winter (Kudo 2021). This results in dry, nutrient-poor soil with low organic content (Vertelina et al. 1996). Clonal patches of *R. aureum* are continuously distributed on open sites in the fellfield habitat. The shrubby habitat consists of rocky, bare ground with little snow cover. *Pinus pumila* mitigates strong winds and acts as a nurse plant for other species (Sugimoto et al. 2024). In the shrubby habitat, small, fragmented patches of *R. aureum* are distributed around the edge of dwarf pine clumps. In the snowbed habitat, where there is thick snow cover, the soil temperature remains close to 0°C in winter, resulting in moist, nutrient-rich soil conditions (Rosbakh et al. 2022). Late-flowering populations occupy the snowbed habitat, where individual plants are distributed across open sites. This study focused on the difference in the clonal structure of *R. aureum* (fragmented vs. continuous) and investigated the effects of geitonogamous pollination in each population.

The degree of geitonogamous pollination also varies depending on the type of pollinators (Utelli and Roy 2000; Brunet and Sweet 2006; Barman et al. 2018). Previous studies have shown that the foraging behaviour of bumble-bee queens and workers (*Bombus* spp.) affects the seed production of *R. aureum* differently, as demonstrated by comparisons of the flowering phenology, pollination situation, and reproductive success between fellfield populations and snowbed populations (Kudo 1993, 2021; Hirao et al. 2006; Kudo et al. 2011). Although *R. aureum* can produce some fruits through self-pollination, pollinator visits are still crucial (Kudo 1993). This species is mainly pollinated by bumble bees, but the visitation frequency and the caste of bees (queen or worker) vary throughout the flowering season (Kudo 1993). In the fellfield habitat, flowering occurs from mid-June to late June, when only overwintered queens

visit the flowers at low frequency. In contrast, flowering in the snowbed habitat occurs from mid-July to August, when workers visit the flowers frequently. Most hand-pollinated flowers produce fruits regardless of the pollination treatment (i.e., self-, outcross or mixed pollen), but self-pollinated seeds often fail to mature due to early-acting inbreeding depression (Hirao et al. 2006; Kudo et al. 2011). Thus, seed production is strongly depressed by self-pollination. In fellfield populations, the fruit-set rate per inflorescence is lower than in snowbed populations, but the seed-set rate per fruit is higher (Hirao et al. 2006; Kudo et al. 2011). This is because queens tend to fly longer flight distances between inflorescences during foraging than workers do, resulting in a lower frequency of geitonogamous pollination (Kudo et al. 2011). Previous studies have not considered the effects of clonal structure on geitonogamous pollination because they were conducted in populations with a continuous distribution of plant patches in both habitats. The theory of optimal foraging strategy predicts that bumble bees will stay on a single patch for longer when flower patches are sparsely distributed over a large area (Pyke 1980). In the fragmented patches of the shrubby habitat, queens may even cause geitonogamous pollination.

Inflorescence density, flower number per inflorescence and ovule number per flower are important factors affecting seed-set success via geitonogamous pollination. However, they have been rarely investigated or compared between habitats under natural conditions. It is expected that the reproductive traits of *R. aureum* will differ between populations if their pollination situations differ significantly. One possibility is the regulation of flower number per inflorescence or clonal patch. The optimal size of floral displays may be determined by a trade-off between the advantage of pollinator attraction and the risk of geitonogamous selfing (Harder et al. 2004). While the production of many inflorescences per plant or many flowers per inflorescence may attract more pollinators, it can also lead to increased pollen movement within

a plant due to pollinators staying longer per visit. This raises the risk of self-pollination (Karron et al. 2004; Williams 2007). As applied to *R. aureum* in northern Japan, early-flowering populations suffer from intense pollen limitation, while late-flowering populations have low seed-set rates due to high abortion rates of selfed seeds caused by geitonogamous pollination (Hirao et al. 2006). These patterns predict greater optimal flower production in early-flowering populations than in late-flowering ones. However, as inflorescence density in clonal plants depends on genet size (Schmid et al. 1995), this expectation may not be realised. On the other hand, the number of flowers per inflorescence can vary between populations independently of genet size.

A second possibility for a habitat-specific reproductive strategy is the regulation of ovule number per flower. In situations of low and stochastic pollination, increasing number of ovules per flower is advantageous in order to maximise seed production when pollinator visits are infrequent and unpredictable (the ovule packaging strategy hypothesis; Burd 1995). Therefore, ovule number per flower is expected to be higher in early-flowering populations than in late-flowering populations. To understand the effects of flowering time on reproductive traits, we took fruiting data from a previous study site (Mt. Kaun), as well as from a study site for clonal structure (Mt. Akadake).

The present study aims to elucidate the effects of clonal structure and flowering period on the success of seed production in *R. aureum*. More specifically, we investigate the following questions: (1) Is genet size, i.e., area of genetically same clone patches, smaller in the fragmented population in the shrubby habitat than in the continuous population in the snowbed habitat, reflecting the size distribution of plant patches in each population? (2) How does seed-set rate vary depending on flower number per inflorescence, inflorescence number per genet, floral density around the inflorescence and/or clonal structure (fragmented vs. continuous

distribution)? (3) Do the early-flowering populations produce more flowers per inflorescence in order to be more attractive to pollinators? (4) Is ovule number in each flower greater in the early-flowering populations, where visitation frequency of overwintered queens is low and unpredictable?

Materials and methods

Study site

A field survey was conducted in 2023 in the Taisetsu Mountains, Hokkaido, northern Japan. We observed flower visitors, measured fruit-set rates, sampled mature fruits, and investigated the clonal structure of populations. One site was located on Mt. Akadake (43º40'N, 142º56'E), where one early-flowering population (1,840 m a.s.l.) and one late-flowering population (1,780 m a.s.l.) were selected (Appendix Fig. S1). The distribution of snow during the summer depends more on the direction and shape of slopes than on elevation alone. The early-flowering population was situated on a plateau dominated by alpine dwarf pine (*P. pumila*), with small patches of *R. aureum* distributed around the edges of dwarf pine clumps (Fig. 1a). The late-flowering population was situated in a snowbed environment where snow remained until mid-July, with *R. aureum* distributed continuously on a gentle slope, uninterrupted by shrubs (Fig. 1b).

To enable comparison with our previous studies (Kudo et al. 2011; Kudo 2021), an additional survey of floral traits and pollination success was conducted in 2023 at a different location in the Taisetsu Mountains, on Mt. Kaun (43º33'N, 142º52'E), where our previous studies were conducted. The early-flowering population at this site was situated in an exposed fellfield habitat (1,900 m a.s.l.; plot O in Kudo 2021; Fig. 1c), while the late-flowering population was situated in a snowbed habitat (1,810 m a.s.l.; plot D in Kudo 2021; Fig. 1d). In

both populations, *R. aureum* was distributed continuously without interruption by shrubs.

Study species

Rhododendron aureum (Ericaceae) is an evergreen shrub widely distributed in the alpine regions of Russia, Mongolia, China, the Korean Peninsula and Japan (Polezhaeva et al. 2021). In the alpine regions of Japan, the flowering season varies from early June to early August depending on the timing of snowmelt (Kudo 2021). The flowers are pale yellow, with a corolla measuring 2-3 cm in length and 3-4 cm in width. Flower buds form the year before flowering and there are usually three to eight flowers per inflorescence. Various insects visit *R. aureum* flowers, including bumble bees, butterflies, syrphid flies and other flies; however, bumble bees are the most effective pollinators (Kudo et al. 2011). The fruits mature approximately two months after pollination, and the seeds are dispersed by wind like other *Rhododendron* species (Stephenson et al. 2007).

Observation of pollinators

In order to assess the seasonal pattern of pollinator activity, we observed the visitation frequency of bumble bees at the Akadake site (1,776–1,842 m a.s.l.). Pollen delivery was not observed rigorously in this study. The observation of bumble bees was done on calm days (during 8:00–14:30) on 21 and 22 June and 9 and 20 July. We moved the observation point every 30 minutes. Each observation was conducted within an area of approximately 5-6 m^2 for the early-flowering population (8 observation points) and 6-60 m^2 for the late-flowering population (10 observation points). The size of the observation area was determined according to the patch size in each population. The number of inflorescences per observation area ranged from 26 to 130. The observation date, *Bombus* species, caste, and the number of inflorescences

visited during a stay in each visit were recorded. *Bombus* species could be visually identified in the field. Visitation frequency was calculated per inflorescence every 30 minutes. Observations of pollinators at the Kaun site were limited due to unstable weather conditions and they were not included in the analysis.

Fruit and seed formation

In both the Akadake and Kaun sites, 40-49 inflorescences were randomly selected in each population and the fruit-set rate (fruit to flower ratio) was calculated for each inflorescence during the fruiting season. Mature fruits were also collected from 20 randomly selected infructescences in each population. In the laboratory, the individual fruits were separated into paper bags and dried at room temperature. If fruits did not dehisce within two weeks, they were treated as aborted fruits with no seed production. Following fruit dehiscence, mature seeds, aborted (undeveloped) seeds and unfertilised ovules were counted to determine the seed-set rate (seed to ovule ratio). The unfertilised ovules were brownish in colour and so small that they appeared powdery. The aborted seeds were smaller and more rounded than mature seeds. As aborted seeds and unfertilised ovules were sometimes difficult to distinguish, they were treated as one group. The mature seeds in each fruit were weighed using an electronic balance with an accuracy of 0.1 mg (MS204S, Mettler-Toledo International Inc., Switzerland) and the mean individual seed mass (total seed mass/seed number) was calculated for each fruit.

Clonal structure measurement

The clonal structure, or distribution of clonal patch, was compared between the early-flowering population, which was fragmented into small patches within the shrubby habitat, and the late-flowering population, which was continuous within the snowbed habitat, at the Akadake site.

Within the early-flowering population, 14 patches were selected along a 500 m footpath. In the late-flowering population, sampling was conducted within a 13 m × 6 m of continuous plant cover (25 m × 6 m). To estimate the number of plants (genets) and the size of individual genets, leaf samples were taken at the intersections of the 1 m grid in each patch or area for plant genotyping. The number of inflorescences and flowers were then recorded in each grid. To estimate the number of inflorescences per genet, the inflorescences in each grid were divided into four equal 50 cm × 50 cm parts and assigned to the genet with the closest intersection, as determined by leaf DNA data. In a genetic analysis using seven microsatellite markers, the individuality (genet) of the plants was identified based on the following two criteria: (1) all waveform peaks at each locus should be matched, and (2) any peak appearing at other than the target locus should be in the same position. Details of the DNA extraction process and the microsatellite markers are provided in the next section. Clonal structure was not investigated in the Kaun populations due to practical limitations, but both of the fellfield and snowbed populations were continuously distributed without physical isolation by other vegetation.

Estimation of outcrossing rate

The outcrossing rate was examined to confirm the mating system of *R. aureum* populations. The outcrossing rates of fruits under natural pollination were compared between the early-flowering and late-flowering populations at both sites, based on a genetic analysis using microsatellite markers. Leaf DNA data were used for mother plant genotyping. The outcrossing rate was measured using 11-22 seeds from 20 fruits selected in each population, with fruits containing fewer than 24 seeds being excluded from the experiment. DNA extraction was attempted from 24 seeds per fruit. Of these, 62.2% (1,195 out of 1,920 seeds in total) produced accurate waveform peaks.

DNA was extracted using the cetyltrimethylammonium bromide extraction method (CTAB method; Stewart and Via 1993). The following seven microsatellite markers were used: RM9D1, RM9D6 (Naito et al. 1998), RM9D9 (Hirao et al. 2006), RA31, RA65, RA85, and RA99 (Kwak et al. 2015). The extracted DNA products were amplified by two-step multiplex PCR using a TaKaRa PCR Thermal Cycler Dice Gradient (Takara Bio Inc., Otsu, Shiga, Japan). In the first step, 0.4 µl each of forward tailed and reverse pigtail primers were added to 194.4 µl of distilled water to form a 200 µl primer set. The first PCR step was performed with 1 µl of primer set, 1 µl of DNA extract liquid, 2.5 µl of 2 × Type-it Multiplex PCR Master Mix (QIAGEN, Hilden, Germany) and 0.5 µl of distilled water (total 5.0 µl). The thermocycling steps were as follows: initial denaturation at 95.0℃ for 5 min; 22 cycles of denaturation at 95.0℃ for 30 s, annealing at 52.0℃ for 90 s and extension at 72.0℃ for 30 s. In the second step, 4.8 µl each of four fluorescence dye-labelled universal primers (Blacket et al. 2012) and seven reverse pigtail primers were added to 47.2 µl of distilled water to make a 100 µl R + D set. 0.5 µl of R + D set and 2 × Type-it Multiplex PCR Master Mix were added to each PCR product and subjected to a second PCR step (total 6.0 µl): 18 cycles of denaturation at 95℃ for 30 s, 52.0℃ for 90 s and extension at 72.0℃ for 30 s; a final extension at 60.0℃ for 30 min. Final PCR products were analysed using an Applied Biosystems 3730 DNA Analyser with a GeneScan 500 LIZ Size Standard (Thermo Fisher Scientifc Inc., Waltham, MA, USA). Samples were genotyped using Peak Scanner software version 2.0 (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Genetic diversity of seeds was assessed by calculating the number of alleles per locus (A), observed heterozygosity (H_o), expected heterozygosity (H_e) and Wright's inbreeding coefficient (F_{is}) using FSTAT version 2.9.4 (Goudet, 1995, 2003). F_{is} for each locus was tested whether it differed from the expected value under the Hardy–Weinberg equilibrium using 1000 randomizations. Outcrossing rate (multilocus genotypes, t_m) was determined using MLTR

version 3.2 (Ritland 2002; Ritland and Jain 1981). The analyses of outcrossing rate in each fruit and entire population were conducted separately. Standard errors (SE) for the parameters were estimated from 1,000 bootstraps.

Statistical analyses

All statistical analyses were performed using R software version 4.4.2 (R core team 2024). Values of reproductive success, i.e., inflorescence fruit-set rate, fruit seed-set rate, seed outcrossing rate and seed number per fruit, were compared between the early-flowering and late-flowering populations. Analysis of the fruit-set rate was performed using a generalised linear model (GLM) with a binomial error distribution and a logit-link function in which the explanatory variables were flowering time and site (Akadake and Kaun), including an interaction. Analyses of the seed-set and outcrossing rates were performed using generalised linear mixed models (GLMMs) assuming a binomial error distribution (with a logit-link function) using the *lme4* package (Bates et al. 2015). The analysis of seed number was performed by a GLMM with a Poisson error distribution and a log-link function. In the GLMMs, the explanatory variables were the same as in the GLM for the fruit-set rate analysis, with inflorescence ID set as a random effect.

The reproductive characteristics of the early-flowering and late-flowering populations were compared. These characteristics included flower number per inflorescence, ovule number per flower, and mean individual seed mass per fruit. The analysis of flower number was performed using a GLM with a Poisson distribution and a log-link function. Ovule number was analysed by using a GLMM with a Poisson distribution and a log-link function. The explanatory variables in the GLM and GLMM were the same as in the GLM of fruit-set rate. Seed mass was analysed using a GLMM with a gamma distribution and a log-link function, including seed-set

rate as an additional explanatory variable alongside flowering time, site, and their interaction, in order to test the relationship between seed size and seed number. The best-fit model was selected based on AIC values. In the GLMMs, inflorescence ID was included as a random effect.

The Wilcoxon rank sum test was used to compare genet size between the early-flowering and late-flowering populations, since the data deviated significantly from a normal distribution (Shapiro-Wilk test, $W = 0.769$, $p < 0.0001$). Inflorescence density was compared between populations using a GLM with a Poisson distribution and a log-link function. The factors influencing the seed-set rate were analysed using a GLMM, with the explanatory variables being flower number per inflorescence, inflorescence number per genet, flower density, and flowering time and interactions with flower traits and flowering time. Inflorescence ID was included as a random effect. The best-fit model was selected based on AIC values.

Results

Visitation frequency of bumble bees

Visitation frequency and the composition of queen and worker bees varied considerably between habitat types (Table 1). The peak flowering period in the early-flowering population was mid-June, when only queens were present, whereas the peak flowering period in the late-flowering populations was mid-July, by which time workers had emerged. In the early-flowering population, 26 queens of *Bombus hypocrita* ssp. *sapporoensis* and two queens of *B. hypnorum* ssp. *koropokkrus* were recorded during eight censuses (4 hours) on 21 and 22 June. In contrast, only one *B. hypocrita* worker was recorded during two censuses (1 hour) on 9 July, when the late-flowering population began to flower. On 20 July, three queens and 24 workers of *B. hypocrita*, one worker of *B. hypnorum*, three workers of *B. beaticola* ssp. *moshkarareppus* and 13 workers of *B. yezoensis* were recorded during eight censuses (4 hours) in the late-

flowering population.

Fruit-set and seed-set success

Reproductive outputs in each population of the Akadake and Kaun sites are summarised in Figure 2. Contrary to our expectation, fruit-set rates (averaging between 0.59 and 0.83) were significantly higher in the early-flowering populations than in the late-flowering populations at both sites (slope: -0.915, $z = -3.32$, $p < 0.001$; Table 2a), and were higher at the Akadake site than at the Kaun site (slope: -0.841, $z = -3.19$, $p < 0.01$; Table 2a).

The seed-set rates of ripe fruits were similar between the early-flowering and late-flowering populations at the Akadake site (averaging 0.22-0.23), whereas the rates were higher in the early-flowering population (averaging 0.46) than in the late-flowering population (averaging 0.17) at the Kaun site (Fig. 2). This suggests that the seed-set success in early-flowering populations differed between sites, i.e., shrubby habitat at the Akadake site vs. fellfield habitat at the Kaun site (flowering time × site interaction; slope: -1.283, $z = -5.16$, $p < 0.0001$; Table 2b). Outcrossing rates of ripe fruits were 0.62 ± 0.07 SE in the early-flowering population and 0.79 ± 0.06 in the late-flowering population at the Akadake site, while they were 0.67 ± 0.06 in the early-flowering population and 0.61 ± 0.07 in the late-flowering population at the Kaun site. The late-flowering population had a higher outcrossing rate than the early-flowering populations at the Akadake site, whereas outcrossing rates were similar between the early-flowering and the late-flowering populations at the Kaun site (flowering time × site interaction; slope: -4.136, $z = -2.59$, $p < 0.01$; Table 2c). The F_{is} displayed negative values in four populations, indicating the dominance of outcrossing in this species (Table 3). The early-flowering population had a higher number of seeds in ripe fruits than the late-flowering population at both sites, but this trend was more pronounced at the Kaun site (flowering time ×

site interaction; slope: −0.793, z = −3.67, p < 0.001; Table 2d). At the Kaun site, fruits in the fellfield habitat contained three times more seeds than those in the snowbed habitat (Fig. 2). These results suggest that the seed-set pattern of the early-flowering populations differed greatly between the shrubby and fellfield habitats, even when flowering times were similar.

Reproductive performance

The reproductive characteristics of each population at the Akadake and Kaun sites are shown in Table 4. There was no significant difference in the number of flowers per inflorescence between the early-flowering and late-flowering populations (slope: −0.033, z = −0.93, p = 0.35; Appendix Table S2a). However, the number of flowers at the Kaun site was marginally greater than that at the Akadake site (slope: 0.139, z = 1.89, p = 0.06). The number of ovules within flowers was significantly higher in the early-flowering populations than in the late-flowering populations at both sites (slope: −0.206, z = −2.97, p < 0.01; Appendix Table S2b). There was no significant difference in individual seed mass between the early-flowering and late-flowering populations (slope: 0.088, t = 1.43, p = 0.15; Appendix Table S2c). Individual seed mass was found to be dependent of seed-set rate, suggesting that there is trade-off relationship between seed size and seed number.

Clonal structure

In the early-flowering population of the shrubby habitat, which consisted of small patches, 18 genets were detected in 14 groups (E1-14; Fig. 3a). The individual patches contained 1-3 genets and the same genets were observed in different patches separated by approximately 1 m (E5 and E6, E9 and E10). Individual genets measured 1.70 ± 0.24 m^2 in size (mean ± SE, n = 20), and the inflorescence density was 11.0 ± 1.7 m^{-2} (n = 34). In the late-flowering population of

the snowbed habitat, where the plants were distributed continuously, 60 genets were detected within a 62 m^2 area (L1; Fig. 3b). The size of individual genets was 1.04 ± 0.11 m^2 (n = 60), and the inflorescence density was 8.3 ± 0.9 m^{-2} (n = 62). Genet size was significantly larger (W = 721.5, p = 0.005) and inflorescence density was significantly higher in the early-flowering population (slope: -0.287, $z = -4.23$, $p < 0.0001$; Fig. 4, Appendix Table S3a).

The number of inflorescences per genet ranged from zero to 104 across all populations. A GLMM performed for seed-set rates indicated that inflorescence number negatively affected seed-set rate only in the late-flowering population (a significant negative interaction between inflorescence number and late-flowering population; slope: -0.026, $z = -3.13$, $p < 0.01$; Fig. 5, Appendix Table S3b). However, the number of flowers per inflorescence and flower density around the inflorescence were removed from the explanatory variables in the best-fit model selected by AIC (AIC of the adopted model: 2911.9, the model added flower number per inflorescence: 2912.3, the model added flower density: 2913.5). The number of flowers per inflorescence (from 1 to 6) varied less than the number of inflorescences per genet (from 1 to 104), suggesting that the effect of flower number on geitonogamous pollination was smaller than that of inflorescence number.

Discussion

The present study revealed differences in the clonal structure and reproductive performance of *R. aureum* across local populations. In answer to the questions posed in the introduction section, (1) contrary to the size distribution of plant patches, genet size was larger in the fragmented population in the shrubby habitat than in the continuous population in the snowbed habitat. (2) Seed-set rate was only negatively affected by inflorescence number per genet in the continuous population. (3) Early-flowering populations did not produce more flowers per inflorescence.

(4) The number of ovules per flower was greater in the early-flowering populations than in the late-flowering populations. We discuss the relationship between seed production and population structure in clonal plants in terms of geitonogamous pollination below.

Pollination situation and fruit-set success

In contrast to our previous studies (Hirao et al. 2006; Kudo et al. 2011), the visitation frequency of bumble bees was similar between the early-flowering and late-flowering populations in 2023. Generally, the abundance of overwintered queens is lower than that of workers due to the annual life-cycle of eusocial insects (Heinrich 1976; Pyke et al. 2011). However, the abundance of overwintered queens in 2023 was the highest in the last 13 years, while the abundance of workers was the second lowest at the Akadake site (Appendix Table S4). A high frequency of queens and a low frequency of workers were also observed at the Kaun site (data is not shown). Thus, the pollinator situation in this year was very different to that in standard years.

The fruit-set success of bee-pollinated alpine plants is strongly influenced by the seasonal dynamics of bumble bees. Early-flowering plants, which are visited by overwintered queens, generally have lower fruit-set rates than late-flowering plants, which are visited by workers (Kudo 2022). Long-term monitoring of *R. aureum* fruit-set rates at the Kaun site (Kudo 2021) revealed that the mean fruit-set rate of the fellfield population (35%) was much lower than that of the snowbed population (71%). However, in 2023, the fruit-set rates were higher in the early-flowering populations (68-83%) than in the late-flowering populations (59-67%) at both sites. This contrasting pattern may be due to the unusual population dynamics of bumble bees in 2023. Bumble bee abundance often varies from year to year depending on weather and floral resource conditions during colony development (Ogilvie et al. 2017). In 2022, the abundance of workers was twice the usual amount (Appendix Table S4). Successful colony development

could lead to a higher abundance of overwintered queens next year.

Effect of clonal structure on seed production

Analysis of the clonal structure at the Akadake site revealed that the early-flowering population in the shrubby habitat consisted of small, fragmented patches of a few genets. In contrast, the late-flowering population in the snowbed habitat was continuously distributed, with many genets mixed together. A previous study has reported that individual *R. aureum* genets can sometimes be up to 10 m^2 in size (Wang et al. 2018), but the present study found that the average genet size was less than 2 m^2 in both populations. In the shrubby habitat, separate patches were sometimes composed of the same genets. These patches were often found alongside the same dwarf pine clumps, suggesting that the expansion of the dwarf pine cut through the creeping branches of *R. aureum*.

The seed-set patterns of ripe fruits are different from the fruit-set patterns between sites. Despite the higher fruit-set rate in the early-flowering population, the seed-set rates of ripe fruits were similarly low between the early-flowering (averaging 0.23) and the late-flowering (averaging 0.22) populations at the Akadake site. Conversely, seed-set rates at the Kaun site were higher in the early-flowering population (averaging 0.46) than in the late-flowering population (averaging 0.17). The abortion of self-pollinated seeds due to geitonogamous pollination is a major limiting factor in seed production in *R. aureum*, particularly in late-flowering populations where workers are frequently present (Kudo et al. 2011). However, frequent geitonogamous pollination occurs even when the population was visited by queens, in the populations with fragmented clonal structure. Actually, queens repeatedly visited small patches in the early-flowering population at the Akadake site (K. Takahashi, personal observation). This behaviour differs greatly from that observed in the early-flowering fellfield

population at the Kaun site in our previous study (Kudo et al. 2011), suggesting flexible foraging behaviour of bumble bees. Also in workers collecting pollen, foraging behaviour often varies depending on the population structure of targeting plant species (Mayer et al. 2012). The site-specific difference in seed-set patterns observed in this study highlight the importance of the clonal structure of individual populations in affecting foraging behaviour and the frequency of geitonogamous pollination (Charpentier 2001).

Although there were some variations in the outcrossing rates between populations at the Akadake site, the overall outcrossing rate across all populations was found to be between 0.61 and 0.79. In a previous study, the outcrossing rates at the Kaun site were found to be between 0.69 and 0.85 (Kudo et al 2011). These results suggest that *R. aureum* is an outcrosser, which is consistent with reports on other *Rhododendron* species (Hirao 2010; Delmas et al. 2014; Takahashi and Kudo 2025). As self-pollination strongly reduce seed production through outcrossing, the seed-set success of *R. aureum* should be assessed in terms of both qualitative (pollinator abundance) and quantitative (legitimate pollen receipt) pollination success. The present study indicates that fruit-set rate is an index of quantitative pollen limitation and that seed-set rate of ripe fruits is an index of qualitative pollen limitation in *R. aureum*.

Comparison of reproductive performance

We expected the early-flowering population to exhibit a greater floral display, as this would be beneficial under pollinator limitation. While inflorescence density was significantly higher in the early-flowering population, the number of flowers per inflorescence did not differ. As the number of flowers per inflorescence had no effect on the seed-set rate, the degree of geitonogamous pollination would not drive evolution in the number of flowers per inflorescence in *R. aureum*. The high number of inflorescences per genet in the early-flowering

population is probably due to the large genet size and may contribute to pollinator attraction during periods of low insect activity. The increased risk of geitonogamous pollination associated with larger genet size may be offset by the increased opportunity for outcrossing due to greater attractiveness (Stephens et al. 2020). Conversely, the seed-set rate in the late-flowering population was negatively correlated with the number of inflorescences per genet. The occurrence of geitonogamous pollination often depends on the floral display size of the genets (Karron et al. 2004; Liao et al. 2009). In the late-flowering population, even small individuals can attract pollinators due to the continuous arrangement of many genets, where the formation of large clonal patches is disadvantageous.

At both the Akadake and Kaun sites, the number of ovules within a flower was higher in the early-flowering populations than in the late-flowering populations. This supports the ovule packaging strategy hypothesis (Burd 1995): the production of many ovules in each flower is advantageous when pollinator activity is low and visits are unpredictable. This increases the likelihood of producing more seeds when pollinators visit the flowers. As mentioned above, the high frequency of visits to the early-flowering population in 2023 was exceptional compared to the long-term record. Variation in ovule number between populations has also been reported for *Rhododendron ferrugineum* and *Rhododendron semibarbatum* (Escaravage et al. 1997; Ono et al. 2008). In *R. ferrugineum* growing in the French Alps, for example, the lowland population has more ovules per flower than the upland population. This variation is explained by differences in environmental conditions, such as soil temperature and fertility (Escaravage et al. 1997). In the alpine zone of the Taisetsu Mountains, weather and edaphic conditions are harsher in the fellfield (early flowering) than in the snowbed (late flowering). Therefore, environmental conditions may not limit ovule production in *R. aureum*.

Conclusions

Plant species that are distributed across a wide range of snow conditions exhibit significant variation in flowering phenology and in their interactions with their pollinators (Spence 1989; Moriwaki et al. 2020). Furthermore, the present study showed that population structure significantly affects seed production, even when the flowering period is the same. Populations with fragmented clone patches tend to have lower seed production than continuous populations due to frequent geitonogamous pollination. However, some issues remain unresolved and should be addressed in future research. The most important of these is the difficulty of clearly separating temporal variation in pollinator type (queens vs. workers) from spatial variation caused by population structure (continuous vs. fragmented clonal patches) in the present study. First, information on the visitation frequency and foraging behaviour of bumble bees is limited at the Akadake site, where pollinator observations were only conducted during an unusual year. Second, generalisation of habitat-specific population structures is difficult because clonal structure was only measured at one site. Finally, it needs to be verified whether the number of ovules per flower is a genetically determined trait, independent of environmental conditions, between populations. Further evaluation is required to determine whether ovule number has evolved in response to the pollination situation.

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Author contributions

KT and GK designed this study and wrote the manuscript. KT conducted the field survey at the Akadake site and genetic analysis, and GK conducted the field survey at the Kaun site. No other person is entitled to authorship.

Conflict of interest

The authors declare that they have no conflict of interest.

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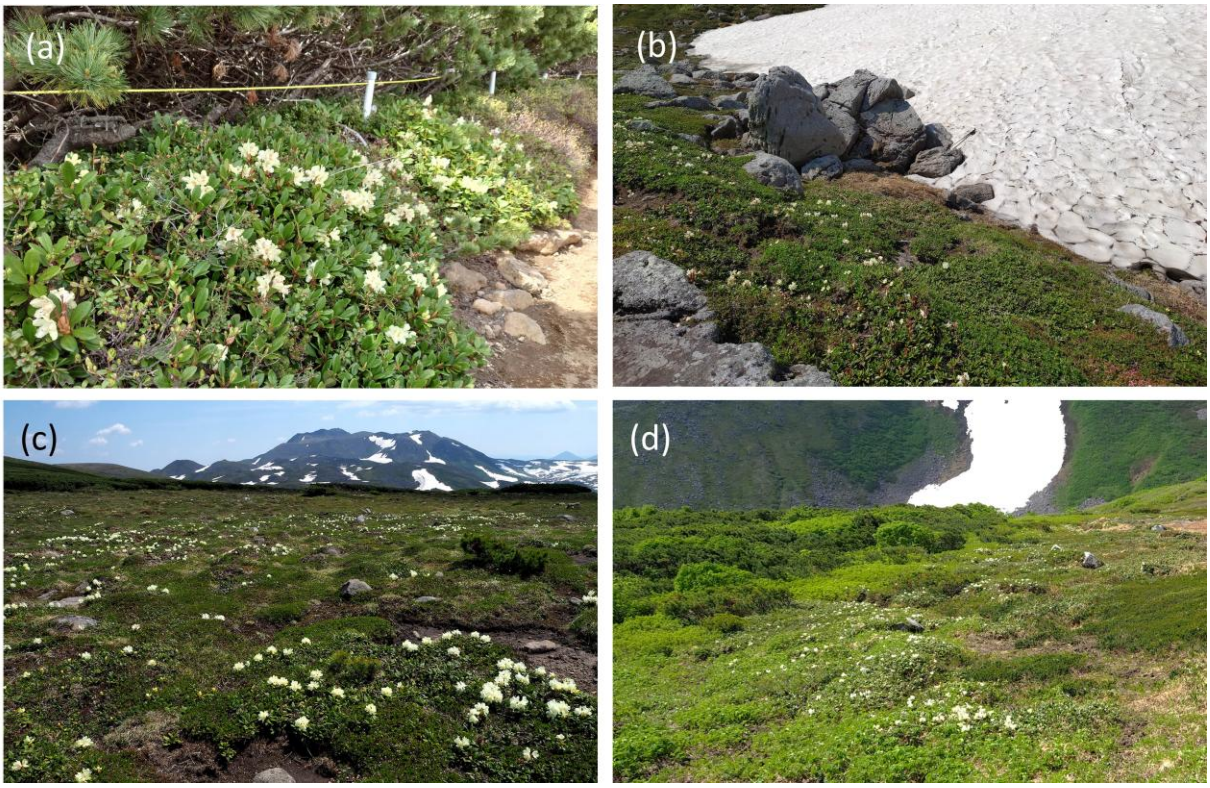


Fig. 1 Appearance of *Rhododendron aureum* populations in the study site. (a) Shrubby habitat and (b) snowbed habitat at the Akadake site. (c) Fellfield habitat and (d) snowbed habitat at the Kaun site.

Table 1 Visitation frequency (mean ± SE, per inflorescence per 30 minutes) of bumble bees on *Rhododendron aureum* flowers in the shrubby habitat (early-flowering population) and the snowbed habitat (late-flowering population) at the Akadake site.

Habitat	Observation date	Observation period	Visitation frequency	
			Queen	Worker
Shrubby	21 & 22 June	4.0 h	0.41 ± 0.10	0
Snowbed	9 July	1.0 h	0	0.07 ± 0.07
Snowbed	20 July	4.0 h	0.02 ± 0.01	0.60 ± 0.14

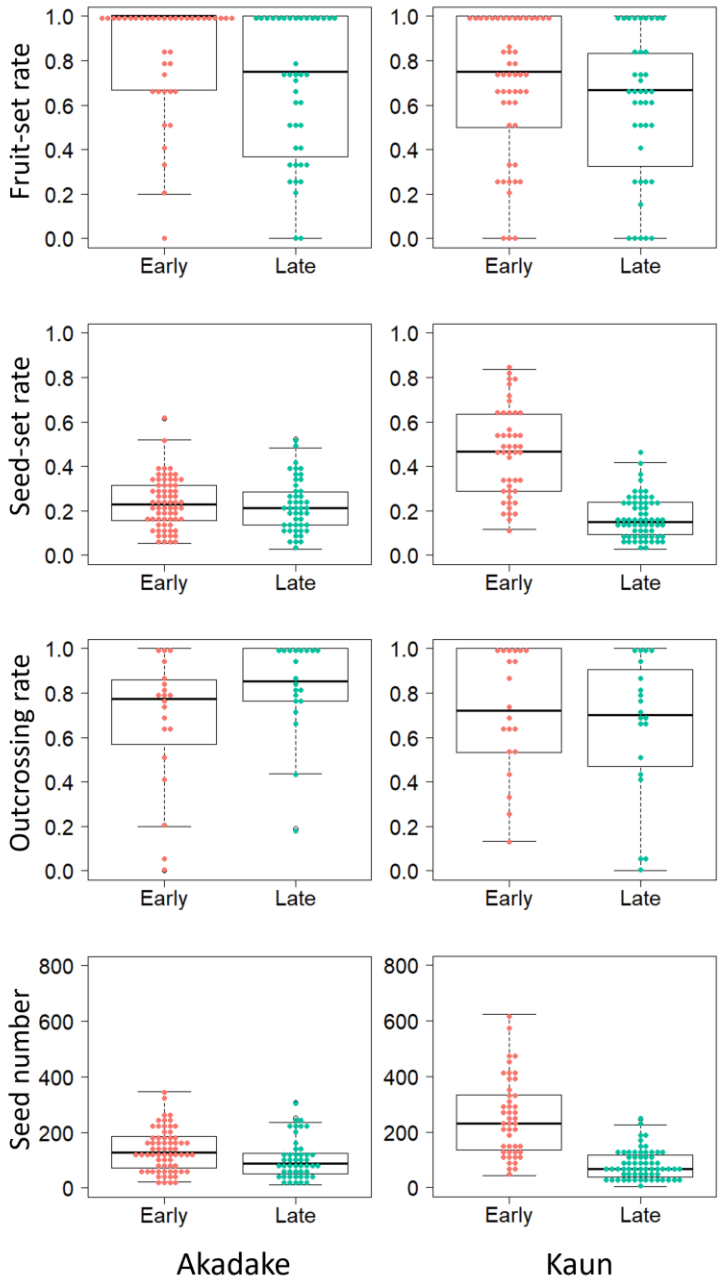


Fig. 2 Fruit-set rate, seed-set rate, outcrossing rate and seed number in the early-flowering and late-flowering populations at the Akadake and Kaun sites. Refer to Table 2 for the statistical results.

Table 2 Results of GLM performed for the comparison of fruit-set rates (a) and GLMMs performed for the comparison of seed-set rates (b), outcrossing rates (c) and seed number per ripe fruit (d) between populations and between sites.

(a) Fruit-set rate

Variable	Coefficient	SE	*z* value	*p* level
Intercept (Early-flowering population, Akadake site)	1.664	0.218	7.63	<0.0001
Late-flowering population	−0.915	0.276	−3.32	<0.001
Kaun site	−0.841	0.264	−3.19	<0.01
Late-flowering population × Kaun site	0.519	0.347	1.50	0.13

(b) Seed-set rate per fruit

Variable	Coefficient	SE	*z* value	*p* level
Intercept (Early-flowering population, Akadake site)	−1.226	0.124	−9.91	<0.0001
Late-flowering population	−0.081	0.176	−0.46	0.64
Kaun site	0.961	0.178	5.41	<0.0001
Late-flowering population × Kaun site	−1.283	0.249	−5.16	<0.0001

(c) Outcrossing rate of seeds within a fruit

Variable	Coefficient	SE	*z* value	*p* level
Intercept (Early-flowering population, Akadake site)	0.946	0.775	1.22	0.22
Late-flowering population	2.724	1.134	2.40	<0.05
Kaun site	1.731	1.121	1.54	0.12
Late-flowering population × Kaun site	−4.136	1.596	−2.59	<0.01

(d) Seed number per fruit

Variable	Coefficient	SE	*z* value	*p* level
Intercept (Early-flowering population, Akadake site)	4.813	0.108	44.77	<0.0001
Late-flowering population	−0.282	0.153	−1.85	0.06
Kaun site	0.557	0.154	3.62	<0.001
Late-flowering population × Kaun site	−0.793	0.216	−3.67	<0.001

Table 3 Characteristics of the seven microsatellite markers used for the genetic analysis of *Rhododendron aureum* populations at Akadake site (a) and Kaun site (b).

(a) Akadake site	Early-flowering population (20 fruits, 302 seeds)				Late-flowering population (20 fruits, 288 seeds)			
Locus	*A*	H_o	H_e	F_{is}	*A*	H_o	H_e	F_{is}
RM9D1	5	0.318	0.277	−0.150	6	0.465	0.396	−0.174
RM9D6	14	0.615	0.553	−0.112	13	0.739	0.682	−0.084
RM9D9	7	0.578	0.584	0.010	7	0.608	0.574	−0.059
RA31	15	0.521	0.546	0.047	12	0.691	0.634	−0.089
RA65	4	0.433	0.365	−0.186	3	0.476	0.404	−0.176
RA85	17	0.551	0.500	−0.100	13	0.766	0.667	−0.149
RA99	10	0.745	0.626	−0.189	10	0.500	0.441	−0.133
Overall	10.3	0.537	0.493	−0.089	9.1	0.606	0.543	−0.117

(b) Kaun site	Early-flowering population (20 fruits, 302 seeds)				Late-flowering population (20 fruits, 303 seeds)			
Locus	*A*	H_o	H_e	F_{is}	*A*	H_o	H_e	F_{is}
RM9D1	10	0.324	0.294	−0.102	3	0.019	0.019	−0.036
RM9D6	14	0.745	0.666	−0.119	11	0.733	0.672	−0.091
RM9D9	10	0.614	0.565	−0.085	5	0.564	0.540	−0.046
RA31	18	0.731	0.697	−0.048	12	0.676	0.615	−0.100
RA65	3	0.505	0.452	−0.116	3	0.316	0.258	−0.225
RA85	18	0.724	0.664	−0.091	14	0.566	0.556	−0.017
RA99	14	0.752	0.631	−0.193	13	0.747	0.630	−0.187
Overall	12.4	0.628	0.567	−0.107	8.7	0.517	0.470	−0.101

Note: F_{is} per locus did not differ significantly from the expected values under Hardy–Weinberg equilibrium ($p > 0.05$, 1000 randomizations). Abbreviations: *A*, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{is}, Wright's inbreeding coefficient.

Table 4 Flower number per inflorescence, ovule number per flower, seed number per fruit and mean individual seed mass (mean ± SE) at the shrubby habitat (early-flowering population) and the snowbed habitat (late-flowering population). Numbers in parentheses refer to sample size.

Site	Flowering time	Habitat	Flower number	Ovule number	Individual seed mass (mg)
Akadake	mid-June	Shrubby	3.78 ± 0.07 (375)	561.0 ± 11.7 (67)	0.089 ± 0.002 (67)
Akadake	mid to late July	Snowbed	3.66 ± 0.04 (513)	459.6 ± 22.3 (47)	0.096 ± 0.003 (47)
Kaun	mid-June	Fellfield	4.35 ± 0.16 (49)	527.1 ± 16.0 (46)	0.079 ± 0.002 (46)
Kaun	mid to late July	Snowbed	4.75 ± 0.15 (40)	465.6 ± 10.6 (64)	0.104 ± 0.002 (64)

Refer to Appendix Table S2 for the statistical results.

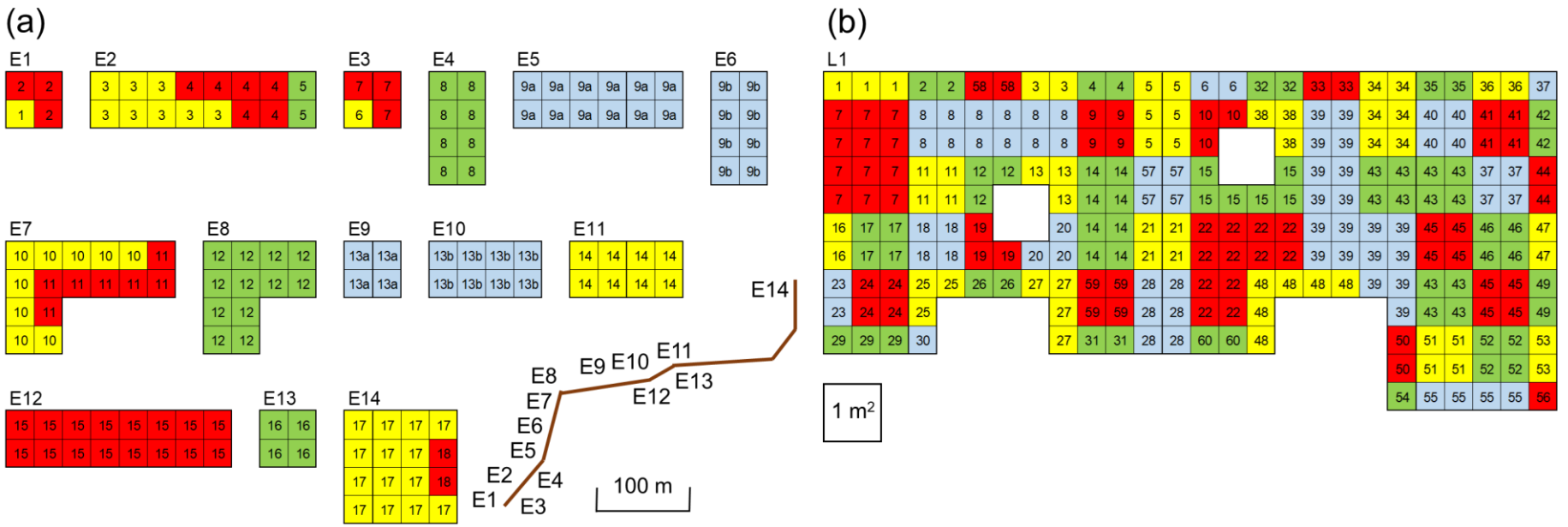


Fig. 3 Clonal structure of *Rhododendron aureum* groups. (a) E1-14 and (b) L1 represent the group IDs of the early-flowering population in the shrubby habitat and the late-flowering population in the snowbed habitat, respectively. In the early-flowering population (a), location of each group (E1-E14) is illustrated, where a brown line indicates footpath. E5 and E6 groups, and E9 and E10 groups are recognised as the same genet, respectively. The size of a single cell is 50 cm × 50 cm since genets were estimated from the leaves at the four corners of one square meter. The numbers in the cells are the individual (genet) IDs. The four colours are painted on each individual to increase visibility.

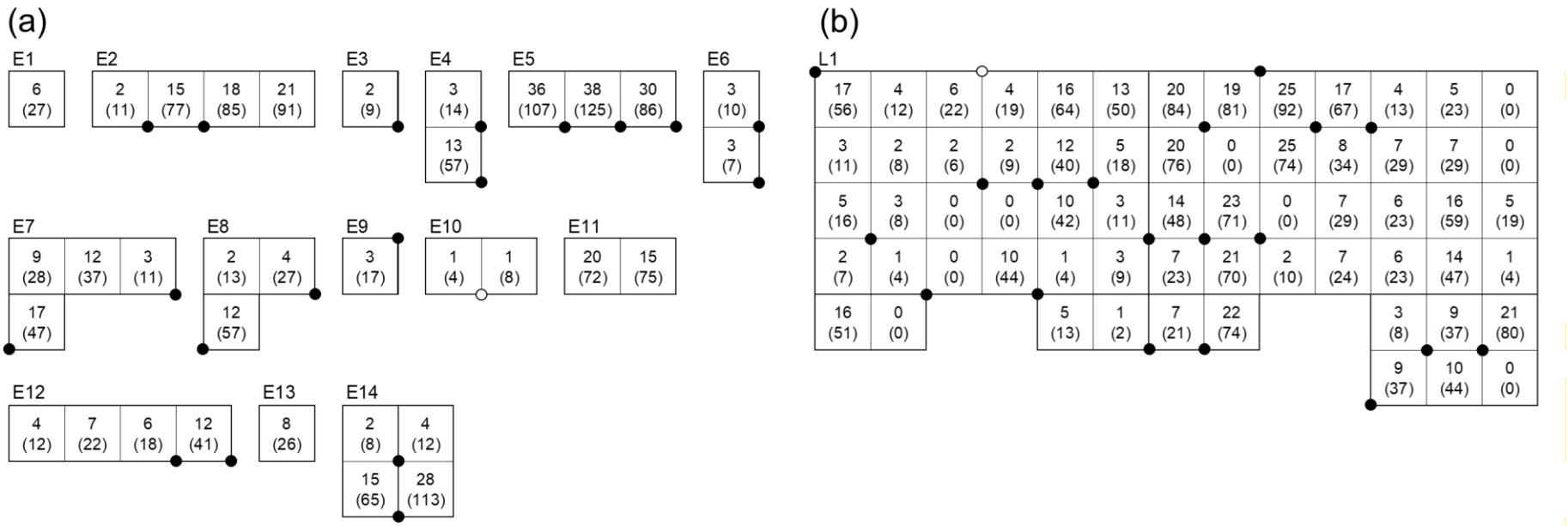


Fig. 4 Inflorescence number (upper row) and flower number (lower row, in brackets) per square meter in *Rhododendron aureum* populations. (a) E1-14 and (b) L1 represent the group IDs of the early-flowering and late-flowering populations, respectively. Filled (black) circles indicate inflorescences taken fruits. Open (white) circles indicate inflorescences that failed to produce fruit due to abortion or seed feeding damage.

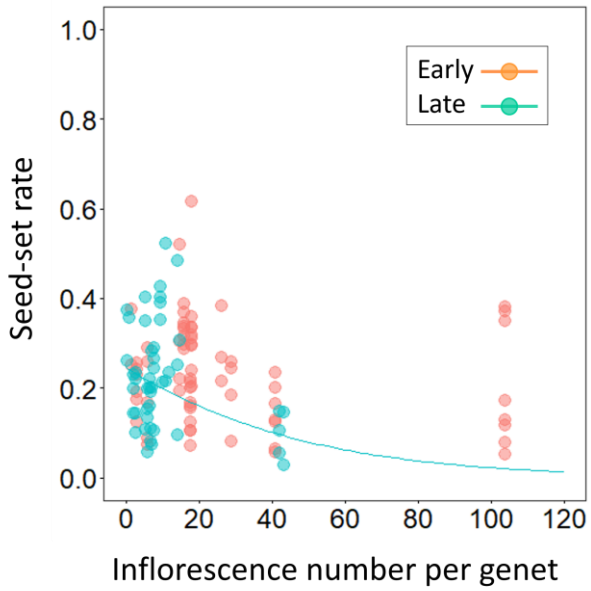
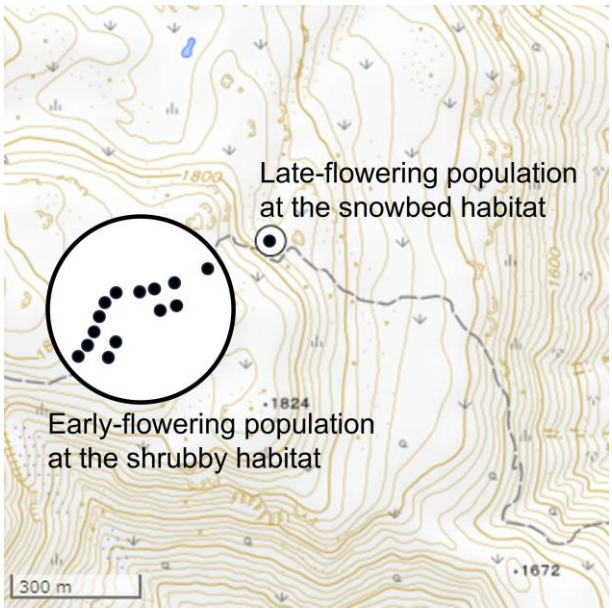


Fig. 5 Effects of inflorescence density on the seed production of *Rhododendron aureum*. Refer to Appendix Table S3b for the statistical result.



Appendix Fig. S1 Map of the Akadake site in the Taisetsu Mountains (created by editing the GSI Maps; Geospatial Information Authority of Japan 2024). Black circles indicate *Rhododendron aureum* groups.

Appendix Table S2 Results of GLM performed for the comparison of flower number per inflorescence (a) and GLMMs performed for the comparisons of ovule number per fruit (b) and individual seed mass (c) between populations and between sites.

(a) Flower number per inflorescence

Variable	Coefficient	SE	*z* value	*p* level
Intercept (Early-flowering population, Akadake site)	1.331	0.027	50.13	<0.0001
Late-flowering population	−0.033	0.035	−0.93	0.35
Kaun site	0.139	0.073	1.89	0.06
Late-flowering population × Kaun site	0.121	0.106	1.15	0.25

(b) Ovule number per flower

Variable	Coefficient	SE	*z* value	*p* level
Intercept (Early-flowering population, Akadake site)	6.313	0.049	128.68	<0.0001
Late-flowering population	−0.206	0.070	−2.97	<0.01
Kaun site	−0.056	0.070	−0.80	0.42
Late-flowering population × Kaun site	0.067	0.099	0.10	0.49

(c) Individual seed mass

Variable	Coefficient	SE	*t* value	*p* level
Intercept (Early-flowering population, Akadake site)	−2.357	0.047	−49.67	<0.0001
Seed-set rate	−0.249	0.076	−3.27	<0.01
Late-flowering population	0.088	0.061	1.43	0.15
Kaun site	−0.072	0.064	−1.13	0.26
Late-flowering population × Kaun site	0.094	0.089	1.06	0.29

Appendix Table S3 Results of GLM performed for the comparison of inflorescence densities (a) and GLMM performed for the comparison of seed-set rates (b) between populations at the Akadake site. Effects of flower number per inflorescence and flower density around that inflorescence were removed by the AIC model selection in the GLMM.

(a) GLM of inflorescence density

Variable	Coefficient	SE	*z* value	*p* level
Intercept (Early-flowering population)	2.401	0.052	46.49	<0.0001
Late-flowering population	−0.287	0.068	−4.23	<0.0001

(b) GLMM of seed-set rate

Variable	Coefficient	SE	*z* value	*p* level
Intercept (Early-flowering population)	−1.124	0.142	−7.89	<0.0001
Inflorescence number per genet	−0.003	0.003	−1.02	0.31
Late-flowering population	0.191	0.201	0.95	0.34
Inflorescence number × Late-flowering population	−0.026	0.008	−3.13	<0.01

Appendix Table S4 Long-term monitoring data of bumble-bee frequency along the fixed trail (3.4 km length, 1,490–1,845 m elevation) at the Akadake site (G. Kudo, unpublished data). Bee monitoring was conducted at approximately 7-10 day intervals during the summer season (from early June to early September). The maximum number of bumble bees observed during a single census is shown for each year. In 2023, when this study was conducted, queen abundance was the highest and worker abundance was the second lowest during the 13 years, suggesting higher pollination services in early season and lower pollination services in mid-summer than usual.

Year	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	Mean
Mid- to late June (queen)	19	5	5	6	3	6	5	1	6	6	10	3	29	8
Mid- to late July (worker)	80	40	120	128	81	14	117	97	58	372	106	259	34	116