RESEARCH ARTICLE

Short title: Effectiveness of diurnal and nocturnal pollinators in early spring

Night and day: Contributions of diurnal and nocturnal visitors to pollen dispersal, paternity diversity, and fruit set in an early-blooming shrub, *Daphne jezoensis*

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ABSTRACT

Premise: Under uncertain pollinator visit conditions, plants often exhibit long flowering periods and generalized pollination systems. Flowering of the gynodioecious shrub *Daphne jezoensis* occurs in early spring in cool temperate forests. Pollination by nocturnal moths is expected, given the species' tubular-shaped flowers with sweet fragrance and nectar. However, the effectiveness of nocturnal moths under cool conditions is unknown. We evaluated the relative importance of diurnal and nocturnal visitors as pollinators in early spring.

Methods: We investigated flowering duration, flower visitors, and floral scents in a natural population. We experimentally exposed flowers to visitors only during daytime or nighttime using bagging treatments and evaluated the contributions of diurnal and nocturnal insects to fruit set, pollen dispersal distance, and paternity diversity using 16 microsatellite markers.

Results: Female flowers lasted ~3 wk, which was ~8 d longer than the flowering period of hermaphrodites. Various insects, including Coleoptera, Diptera, Hymenoptera, and Lepidoptera, visited the flowers during both daytime and nighttime. Flowers emitted volatiles, such as lilac aldehyde isomers and β-ocimene, which are known to attract moths. Fruit-set rate in the night-open treatment was similar to or higher than that in the day-open treatment. However, pollen dispersal distance in the night-open treatment was shorter than that in the day-open treatment. Paternity diversity was similar in day-open and night-open treatments.
Conclusions: Early-blooming plants ensure pollen receipt and dispersal by having a long flowering period and using both diurnal and nocturnal flower visitors, suggesting the importance of a generalized pollination system under uncertain pollinator visit conditions.

KEYWORDS
early-blooming, floral scent, flower longevity, generalized pollination, gynodioecy, nocturnal moth, paternity diversity, pollen dispersal, Thymelaeaceae

INTRODUCTION

Animal-pollinated plants with long flowering periods and generalized pollination systems generally have an advantage under uncertain or limited pollinator visit conditions (Herrera, 2002; Darling and Barrett, 2011). This is because a generalized pollination system increases the opportunity for pollen transfer via a wide range of insect groups (Rathcke, 2003; Darling and Barrett, 2011). These reproductive characteristics have been reported frequently in early-blooming and alpine plants, whose flowering season is characterized by low temperatures and unstable weather conditions (Schemske et al., 1978; Alonso, 2004; Rosati et al., 2019; Funamoto and Sugiura, 2020).

Plants with a generalized pollination system must attract various insects by using different cues to promote their flowers. For instance, diurnal bees primarily use visual cues, such as flower color and shape, whereas nocturnal moths mostly use scent as a cue (Milet-Pinheiro et al., 2012; Jürgens et al., 2014). However, highly attractive flowers, such as those producing large amounts of floral scent and nectar, may increase not only pollinator visits but also the risk of herbivory and nectar-robber visits (Theis, 2006; Kessler et al., 2015) and have high energy costs (Gershenzon, 1994). Therefore, it is expected that plants regulate the attractiveness to a certain level to balance the tradeoff between the benefit and loss gained by higher attractiveness. Some plant species alter the amount of floral scent produced between daytime and nighttime, depending on the daily pattern of pollinator activity (Dötterl et al., 2012; Lemaitre et al., 2014; Chapurlat et al., 2018; Okamoto et al., 2022).

The contribution of flower visitations to pollination success and the efficiency of pollen transfer, which is affected by pollen carryover, flight distance, and floral constancy,

may differ between diurnal and nocturnal insects because of different taxonomic groups and foraging behavior (Barthelmess et al., 2006; Giménez-Benavides et al., 2007; Jürgens et al., 2014). For instance, dipteran and hymenopteran insects are active during daytime, whereas lepidopteran insects are active during both daytime (butterflies) and nighttime (moths). Previous studies have reported that bumble bees and hawkmoths are particularly important pollinators of day-flowering and night-flowering plants, respectively, because their long-distance flight ability and high floral constancy promote outcrossing and wider pollen dispersal, resulting in the maintenance of genetic diversity and gene flow (Austin et al., 2019; Skogen et al., 2019; Lewis et al., 2023). Nevertheless, hawkmoths are not common in forests, especially in early spring. Alternatively, small settling moths may be important pollinators in early spring, as they are active even under cool conditions (Sayama et al., 2012; Funamoto and Sugiura, 2020) and visit flowers of various species (Singh et al., 2022). However, their actual contribution as pollinators, especially their pollen dispersal ability, remains mostly unknown.

For early-blooming plants growing in cool temperate and boreal forests, pollination success is often unstable due to low availability of pollinators under cool and unstable weather conditions, especially for obligate outcrossing plants (Schemske et al., 1978; Barrett and Helenurm, 1987; Kudo et al., 2008). *Daphne jezoensis* (Thymelaeaceae) is a gynodioecious shrub inhabiting the understory of cool temperate forests, and flowering occurs early in spring soon after snowmelt. The tubular-shaped flowers are pale-yellow in color, emit sweet fragrance, produce small amounts of nectar, and remain open during daytime and nighttime. The stigma is located inside the tubular flower below the anthers. Therefore, insects with a long proboscis, such as bees, burterflies, and moths, are anticipated to be effective pollinators.

In this study, we aimed to clarify the reproductive strategy and contributions of diurnal and nocturnal insects to pollination success in *D. jezoensis*. We predicted that a long flowering period and a generalized pollination system are advantageous for the pollination success of early-blooming plants. We also predicted that diurnal insects contribute substantially to the pollination of early-blooming plants, whereas the contribution of nocturnal insects as pollinators may be limited because of their lower activity and diversity in early spring. To examine these predictions, we recorded the flowering period and flower visitors; determined the volatile composition of floral scent during daytime and nighttime; and compared fruit set, pollen dispersal distance, and paternity diversity in plants exposed to either diurnal or nocturnal flower visitors in a natural population of *D. jezoensis*.

MATERIALS AND METHODS

Study species and study site

Daphne jezoensis Maxim. is a gynodioecious shrub distributed in the snowy regions from central to northern Japan and Sakhalin (Iwatsuki et al., 1999). Flowering occurs early in spring soon after snowmelt but before leaf emergence of the canopy trees, and fruits mature 2 mo later. The average number of inflorescences per plant was ~5 (range: 1–70) and the average number of flowers per inflorescence was ~6 (range: 1–13). Hermaphroditic plants produce perfect flowers in which developed anthers and a pistil exist, but the fruiting ability is very small (Shibata et al., 2021). Female plants produce similarly shaped flowers, but the flower size is smaller, and anthers are shrunken without pollen. Hence, hermaphroditic plants mostly act as pollen donors (male), and female plants (the seed producers) are pollen recipients. Individual fruits containing a single seed are produced.

A field survey was conducted in a natural deciduous broad-leaved forest within the Nopporo forest park, Hokkaido, northern Japan (43°02′N, 141°31′E), in 2018 and 2020. Measurements were performed using a permanent plot (50 m × 60 m) established in 2009, where all individuals were tagged, mapped, and genotyped using microsatellite markers (Appendix S1). A total of 225 plants (93 female and 132 hermaphroditic plants) and 183 plants (81 female and 102 hermaphroditic plants) produced flowers within the plot in 2018 and 2020, respectively. The study population was isolated from other populations by ~3 km, and the plot was located at a large gap space in a deciduous forest. All 67 hermaphroditic individuals that were flowering outside of the plot in 2018 and 2020 were also mapped and genotyped as candidate pollen donors of the population (Appendix S1). The farthest candidate pollen donors from the recipient plants within the plot were located ~50 m away.

Flower longevity

Potential flower longevity (i.e., the maximum flowering period without pollen receipt) of female and hermaphroditic plants was investigated in 2018. One inflorescence in each of the five female and nine hermaphroditic plants was tagged and covered with a fine-meshed bag before flowering to prevent natural pollination. Soon after flower opening, hermaphroditic flowers were emasculated to prevent self-pollination and covered again. The number of remaining flowers was recorded every 3 or 5 d until the shedding of all flowers. In 2015, we recorded the total number of open flowers on 76 female and 90 hermaphroditic plants every ~10 d through the flowering period to determine the general trend of flower longevity at the population level.

Flower visitors

Direct observations of diurnal flower visitors were conducted between 0900 and 1700 hours in 2018. One observer walked slowly around 50 flowering *D. jezoensis* plants within a 20 m × 20 m area and recorded the insects visiting the flowers during a 15 min period at a time (census). In total, eight censuses were conducted on 3 d with calm weather. Family, genus, or

species of the observed insects was identified in the field if possible. Dipteran insects were classified into beeflies (*Bombylius major*), syrphid flies (Syrphidae), and other flies. Small beetles were collected and transferred to the laboratory for further identification.

In 2020, we recorded flower visitors during daytime and nighttime using interval photography. Two cameras (WG-70, Ricoh, Tokyo, Japan) were placed in front of female and hermaphroditic inflorescences and set to automatically take pictures at 45 s intervals. Diurnal observation was conducted from 0630 hours (1.5 h after sunrise time) to 1829 hours (sunset time), and nocturnal observation was conducted from 1830 to 0629 hours. In total, 15,145 and 13,189 pictures were captured during daytime and nighttime, respectively. Insects observed to visit the same plant consecutively were not counted.

Floral scent

To analyze the floral scent of female and perfect flowers during daytime and nighttime, we sampled floral volatiles from plants grown at the experimental garden in Higashikawa, central Hokkaido (43°42′N, 142°33′E). Volatile sampling was conducted continuously during daytime (between 1200 and 1600 hours) and nighttime (between 2000 and 2330 hours) on 25 April 2022. Measurements were conducted for 5- or 6-yr-old plants that were grown from the seeds harvested from the Nopporo population. Five female and five hermaphroditic plants were randomly selected, and the same plants were used for daytime and nighttime volatile sampling. Daytime sampling was always carried out before nighttime sampling. The number of flowers varied from 11 to 44, depending on the plants.

All inflorescences per plant (including some leaves) were covered and enclosed with polyethylene terephthalate (PET) bags (180 mm × 250 mm; Mitsubishi Gas Chemical Company, Tokyo, Japan). A glass cartridge (3.0 mm internal diameter, 160 mm long) filled with 100 mg of Tenax-TA (GL Sciences, Tokyo) was inserted into the outlet of each PET bag, on the other side, connected to an air pump (MP-Σ30NII, SIBATA Scientific Technology, Tokyo), and the floral headspace was aspirated (100 mL/min airflow) for 1 h. The PET bags were renewed between day and night samplings to prevent contamination of diurnal floral volatiles with nocturnal floral volatiles. In the present study, volatile samplings from the ambient air (as a blank sample) and vegetative plant parts (such as leaves) were not conducted. This might prevent the detection of potential contaminants in the floral samples (see below).

The volatile samples were analyzed via gas chromatography/mass spectrometry (GC/MS; GC: Agilent 6890 with an HP-5MS capillary column: 30 m long, 0.25 mm inner diameter, and 0.25 μm film thickness; MS: Agilent 5973 mass selective detector, 70 eV with helium as the carrier gas; Agilent, Santa Clara, California, USA) and a thermal desorption cold-trap injector (TCT; CP4010; Chrompack, Middelburg, The Netherlands) following the previous study (Shiojiri et al., 2021). The oven temperature of the gas chromatograph was

programmed to increase from 40°C (5 min hold) to 280°C at 15°C /min. The compounds were identified by comparing their mass spectra with those of compounds from the Wiley7N database (John Wiley & Sons, Hoboken, New Jersey, USA), and their retention times and mass spectra were compared with those of synthetic compounds. The peak area of the gas chromatograph output (total ion intensities per flower per minute) was used as an estimate of the total emission intensity of floral scents.

Pollination exposure experiment

The contribution of diurnal and nocturnal insects to fruit set (female success) was assessed by conducting a pollination exposure treatment in 2018 and 2020. Before flowering, 12 and 11 female plants with sufficient numbers of flower buds were selected for the day and night exposure experiment in 2018 and 2020, respectively, and their inflorescences were covered with fine-meshed bags to prevent pollinator visits. Additionally, we selected 12 and 9 female plants for outcross pollination experiment, while the remaining 71 and 74 female plants were exposed to natural pollination, in 2018 and 2020, respectively. The following four treatments were conducted: (1) natural pollination, in which flowers were exposed to flower visitors during the entire day (71 and 74 plants in 2018 and 2020, respectively); (2) outcross pollination, in which all flowers were outcrossed by hand (12 and 9 plants in 2018 and 2020, respectively); (3) day-open treatment, in which bags on the inflorescences were removed between 0630 and 1829 hours to expose flowers to diurnal visitors (40 and 64 inflorescences on 12 and 11 plants in 2018 and 2020, respectively); and (4) night-open treatment, in which bags of the inflorescences were removed between 1830 and 0629 hours to expose flowers to nocturnal visitors (40 and 64 inflorescences on 12 and 11 plants in 2018 and 2020, respectively). The day-open and night-open treatments were performed on the same plants in which half of the inflorescences were used for each treatment (Appendix S1). The natural pollination and outcross pollination treatments were conducted on an individual basis. In the outcross pollination treatment, pollen was collected from ~10 hermaphroditic donors ≥10 m away from the recipient plants and artificially deposited onto the stigmas of all female flowers soon after opening. The exposure and outcross pollination treatments were conducted on calm days without rainfall. The experimental periods of the day-open and night-open treatments were 3 d and 4 d (exposure period: 37 h and 32.5 h) and 8 d and 8 d (95.5 h and 91 h) in 2018 and 2020, respectively. Mature fruits were counted and harvested for genotyping in mid-July. Fruit set was calculated as the ratio of fruit to flower numbers at the plant level (for the natural and outcross pollination treatments) or inflorescence level (for the day-open and night-open treatments).

Genotyping

To genotype parental plants, we collected one leaf from all flowering plants within the permanent plot. One leaf was also collected from all hermaphroditic plants around the plot. The leaf samples were stored in a desiccator with silica gel at room temperature (20–25°C). For the fruits harvested from females, plumule and radicle samples were collected from seeds and stored at −60°C, because cotyledons and other seed tissues alter the results of PCR analyses. DNA extraction and PCR analyses were conducted following Shibata et al. (2021). DNA extraction was performed using the cetyltrimethylammonium bromide protocol (Stewart and Via, 1993). The extracted DNA was amplified using a TaKaRa PCR Thermal Cycler Dice Gradient (Takara Bio, Otsu, Shiga, Japan) using 16 microsatellite markers. We used eight primer pairs, (DpJe010, DpJe020, DpJe044, DpJe059, DpJe061, DpJe074, DpJe076, and DpJe082) according to our previous study (Shibata et al., 2021), and another eight primer pairs (DpJe003, DpJe012, DpJe018, DpJe024, DpJe028, DpJe029, DpJe054, and DpJe077) (Appendix S2). A two-step multiplex PCR was performed using universal primers for fluorescent labeling of PCR fragments (Blacket et al., 2012). In the first step, a final 5 μL volume containing 1 μL of extracted DNA, 2.5 μL of 2 × Type-it Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 0.5 μL of forward tailed primers (0.6 μM for each locus) tagged with a specific sequence (universal tail) attached at the 5′ end, 0.5 μL of reverse primers (2.4 μM for each locus), and 0.5 μL of water was analyzed at 95°C for 5 min, and then 23–24 cycles were run at 95°C for 30 s, 60°C for 90 s, and 72°C for 40 s, followed by holding at 8°C. The number of cycles changed depending on the quality of DNA (i.e., 23 cycles for leaves and 24 cycles for seeds). Subsequently, 0.5 μL of 2 × Type-it Multiplex PCR Master Mix (QIAGEN) and 0.5 μL of fluorescence-labeled universal primers (1.2 μM for each color) that included the same universal tails were added for the second step. The second step was performed using three cycles of 95°C for 30 s, 60°C for 90 s, and 72°C for 40 s, followed by 60°C for 30 min. The DNA fragments were analyzed using an Applied Biosystems 3730 Genetic Analyzer with a GeneScan 500 LIZ Size Standard (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and scored using the R package "Fragman" (Covarrubias-Pazaran et al., 2016). In 2018 and 2020, 199 and 169 unique genotypes were detected from hermaphroditic flowering ramets, respectively.

Paternity assignment

The paternity of seeds (n = 257 in 2018, n = 327 in 2020) was estimated using the genotype and phenotype information (i.e., sex type and spatial location of flowering plants) and the R package "MasterBayes" (Hadfield et al., 2006). In total, 199 and 169 plants of the hermaphroditic genotype were used as candidate pollen donors in 2018 and 2020, respectively. In "MasterBayes," the probability of siring was assumed using an exponential function $e^{\beta d}$, where d is the pairwise distance between mother and candidate pollen donors and β is the vector of the parameter. The posterior distributions of β, paternity, unsampled

sire number, and genotyping error rate were jointly estimated using Markov chain Monte Carlo methods. We ran three Markov chains, each of which was converged after 150,000 iterations, with a burn-in interval of 30,000 iterations and a thinning interval of 100. Convergences of the chains were evaluated using the package "coda" in R. Mean values, standard errors, and 95% confidence intervals for β and unsampled sire number were obtained from the posterior distributions. Paternity assignments with a probability exceeding 0.95 were used to obtain complementary estimates of pollen dispersal. To evaluate the diversity of the paternity of seeds within the maternal plants, we calculated the Shannon's diversity index *H*. For this calculation, we used the maternal plants that produced more than two seeds (seven and six plants for the natural pollination, four and five plants for the day-open treatment, and eight and three plants for the night-open treatment in 2018 and 2020, respectively).

Statistical analyses

All statistical analyses were conducted using R version 4.1.2 (R Core Team, 2021). To compare the total scent emission per flower between daytime and nighttime in five female and five hermaphroditic plants, a linear model was employed, in which the total volatile was the response variable and the sex type (female or hermaphrodite), time of day (daytime or nighttime), and their interaction were set as explanatory variables. The significance of the explanatory variables was evaluated by performing an *F*-test using the package "car" (Fox and Weisberg, 2019). To compare the floral scent profiles between the sex types and between daytime and nighttime, dissimilarities in the composition of floral volatiles were determined based on the Bray-Curtis dissimilarity indices according to the square-root transformed relative peak areas and visualized in two dimensions with nonmetric multidimensional scaling (NMDS) using the package "vegan" (Oksanen et al., 2022). We further performed a permutational multivariate analysis of variance (PERMANOVA) of the Bray-Curtis indices based on 9999 permutations to test the differences in scent composition between the sex types and between daytime and nighttime using "vegan." The interaction of sex type and time of day was also included as the explanatory variable.

We examined the effect of pollination treatment on fruit set using a generalized linear model (GLM) and a generalized linear mixed-effects model (GLMM) with a negative binomial error distribution with a log-link function using the package "glmmTMB" (Brooks et al., 2017). First, fruit set at individual level was compared between the natural and outcross pollination treatments using a GLM. Among the naturally pollinated plants, 15 individuals were randomly selected in each year to obtain a sample size similar to the outcross pollination treatment. In the GLM, fruit set was the response variable and pollination treatment (natural or outcross), year (2018 or 2020), and their interaction were set as explanatory variables. The significance levels of the explanatory variables were evaluated

using the likelihood-ratio test and "car". Next, fruit set at the inflorescence level was compared between the day-open and night-open treatments using a GLMM. The maternal plant ID was set as a random factor in the GLMM. The significance of the explanatory variables was evaluated using the Wald chi-square test. GLMM was performed separately for each year when the interaction term was significant.

The distribution of the observed pollen dispersal distances between females (pollen recipient) and hermaphrodites (pollen donor) was compared to the distribution of random mating pairs using the Kolmogorov-Smirnov test. The distribution of observed pollen dispersal was also compared between the day-open and night-open treatments using the Kolmogorov-Smirnov test.

The relationship between the harvested seed number and distinct father number was analyzed using a GLM with a Poisson error distribution with a log-link function. In the GLM, the distinct father number was set as the response variable, and the harvested seed number, pollination treatment (natural, day-open or night-open), and their interactions were set as the explanatory variables. The significance levels of the explanatory variables were evaluated using the likelihood-ratio test.

RESULTS

Flower longevity and flower visitors

Females tended to retain flowers longer than hermaphrodites when flowers were emasculated and bagged (Figure 1). The same trend was observed under natural conditions in 2015 (Appendix S3). Female flowers lasted ~3 wk or longer. Flower longevity of hermaphrodites exhibited a large variation among individuals, from 10 d to 3 wk.

In total, 86 flower visitors belonging to the following four orders were observed: Coleoptera, Diptera, Hymenoptera, and Lepidoptera (Table 1). Small sap-feeding beetles, *Epuraea* spp. (Nitidulidae), were observed during both daytime and nighttime, and they entered flowers to feed on pollen or nectar. Diverse insect species visited flowers during daytime. Beeflies (*Bombylius major*), bumble bees (*Bombus hypocrita sapporoensis*), and lepidopteran insects (butterflies and moths) were observed to insert their proboscis into the tubular flowers. However, syrphid and other flies commonly fed on pollen directly from the upper-positioned anthers of perfect flowers, suggesting a low contribution to pollination. During nighttime, nocturnal moths (*Cleora leucophaea*) were frequently recorded, and they tended to visit several flowers on a plant.

Floral scent during daytime and nighttime

We successfully obtained the volatile composition data during daytime and nighttime from four and four females and three and five hermaphrodites, respectively. The exact data for

some volatile samples could not be obtained because the peak of volatiles exceeded the measurement limit due to extremely strong floral scent. The mean (± SE) value of the total volatiles of floral scent (peak area) during daytime and nighttime were $19.1 \times 10^5 \pm 6.6 \times 10^5$ and $1.2 \times 10^5 \pm 1.1 \times 10^5$ in female flowers and $18.3 \times 10^5 \pm 17.9 \times 10^5$ and $6.6 \times 10^5 \pm 3.1 \times 10^5$ in perfect flowers, respectively. There was no difference in total floral scent emission between sex type and time of day (sex type: $F_{1,12} = 0.01$, $P = 0.94$; time of day: $F_{1,12} = 2.92$, $P = 0.11$; sex type × time of day: $F_{1,12} = 0.17$, $P = 0.69$).

A total of 25 compounds were identified from the floral scent of *D. jezoensis* (Appendix S4), among which β-ocimene, lilac aldehyde isomers, and trans-allo-ocimene were predominant. Decanal was detected only in female flowers, and methyl salicylate was detected only in perfect flowers. Cineole was detected only during daytime in both sex types. Significant sexual differences were detected in the composition of the floral scent, whereas no difference was found between daytime and nighttime (sex type: $F_{1,12} = 2.71$, $R^2 = 0.16$, $P < 0.05$; time of day: $F_{1,12} = 1.35$, $R^2 = 0.08$, $P = 0.24$; sex type × time of day: $F_{1,12} = 0.99$, $R^2 = 0.06$, $P = 0.40$ in PERMANOVA; Appendix S5).

Pollination success by diurnal and nocturnal pollinators

Fruit set at individual level showed no increases by outcross pollination, indicating that female plants were not pollen-limited during the 2 yr (pollination treatment: $\chi^2 = 0.38$, $P = 0.54$; year: $\chi^2 = 0.01$, $P = 0.91$; pollination treatment × year: $\chi^2 = 0.13$, $P = 0.72$; Figure 2). When fruit set at the inflorescence level was compared between the day-open and night-open treatments, a significant interaction between the treatment and year was detected ($\chi^2 = 5.23$, $P < 0.05$; Figure 3). Fruit set was significantly higher in the night-open inflorescences than in the day-open inflorescences, but only in 2018 ($\chi^2 = 9.53$, $P < 0.01$ in 2018; $\chi^2 = 0.78$, $P = 0.38$ in 2020).

Pollen dispersal by diurnal and nocturnal pollinators

A total of 584 seeds sampled from 86 plants were genotyped, and the paternity of 470 seeds from 82 plants (80.5%) was identified at the 95% confidence level (Table 2). The vector of the distance parameter β was estimated as −0.13 (95% CI: −0.15 to −0.11) in 2018 and −0.14 (95% CI: −0.16 to −0.12) in 2020, indicating that the probability of paternity declined with distance. The number of unsampled sires was estimated as 33.5 (95% CI: 21.7–48.2) in 2018 and 34.8 (95% CI: 25.7–45.8) in 2020.

Pollen dispersal distance was calculated for each pollination treatment using the most likely paternity (Table 2). The distribution of the observed pollen dispersal distance was different from that of random mating pairs among the target females and flowering hermaphrodites in all treatments for both years (Kolmogorov-Smirnov test: $P < 0.001$; Figure 4). The mean and maximum distances of the observed pollen dispersal were shorter than

those of random mating pairs. A large portion of mating pairs existed within a 10 m distance (66.7% and 71.2% for natural, 81.3% and 80.8% for day-open, and 91.1% and 95.7% for night-open pollination treatments in 2018 and 2020, respectively).

Mean and maximum pollen dispersal distances tended to be shorter during nighttime than during daytime (Table 2). The distribution of the observed pollen dispersal was significantly different between the day-open and night-open treatments in 2020, but no difference was observed in 2018 (Kolmogorov-Smirnov test: $P = 0.85$ in 2018, $P < 0.05$ in 2020; Figure 4). The number of distinct fathers (pollen donors) was 2.4 ± 0.24 (mean ± SE) for natural pollination, 2.4 ± 0.42 for day-open treatment, and 2.3 ± 0.23 for night-open treatment. The paternity diversity (Shannon's diversity index) was 0.94 ± 0.09 (mean ± SE) for natural pollination, 0.88 ± 0.21 for day-open treatment, and 0.81 ± 0.06 for night-open treatment. The number of distinct fathers increased with the number of seeds produced by maternal plants, irrespective of the pollination treatment (number of seeds: $\chi^2 = 55.02$, $P < 0.001$; treatment: $\chi^2 = 1.08$, $P = 0.58$; number of seeds × treatment interaction: $\chi^2 = 2.70$, $P = 0.26$; Figure 5).

DISCUSSION

Long flowering periods in early-blooming plants

Anthesis of female flowers lasted >3 wk when pollinator visits were restricted. Similar trends were reported in other plant species flowering in winter or early spring, when pollinator activity was uncertain due to cool and unstable weather conditions (Herrera et al., 2001; Alonso, 2004; Darling and Barrett, 2011). Because pollen limitation was not detected in the present study, a long flowering period might successfully compensate for the uncertain pollination situations in the study years. Fruit set was relatively low even when outcross hand-pollination was performed. Moreover, fruit set was independent of plant size and light availability, suggesting little resource limitation. This might be due to the potentially low fruiting ability of *D. jezoensis* (Shibata and Kudo, 2022). The longest pollen dispersal distance was recorded in the natural pollination treatment (i.e., open for the entire flowering period) in both years. Therefore, longer flowering duration, resulting in the visitations of various insect groups, might increase the probability of long pollen dispersal.

Flower longevity was consistent with a balance between the benefit of increased pollination success and the cost of flower maintenance (Ashman and Schoen, 1994; Charnov, 1996). The longer flowering trend in females than in hermaphrodites in this species suggests that females can receive more pollen that results in higher seed-set success by longer floral duration, whereas hermaphrodites, which function as males, do not maintain flowers after pollen release. Interestingly, the congener *D. laureola* has longer anthesis in hermaphrodites than in females, and the hermaphrodites have higher fecundity than females (Alonso, 2004).

Hence, the extended longevity of individual flowers may act as a reproductive assurance for seed production when pollinator availability is uncertain.

Various flower visitors and diverse floral scent compounds

Our results indicate that *D. jezoensis* has a generalized pollination system. Beeflies, bumble bees, and butterflies were diurnal flower visitors, whereas moths were common nocturnal flower visitors, and all these insects have a long proboscis. Furthermore, sap-feeding beetles were commonly observed during both daytime and nighttime. Because their body size was sufficiently small to enter the flowers and touch the anthers and stigma, they could be pollinators. Because short-stay pollinators, such as beeflies and bumble bees, might be missed and underestimated when using interval photography, an interval of <45 s may be required for more detailed recording of flower visitors.

Flowers emitted various compounds that attract several types of pollinators. Lilac aldehyde isomers have been reported in moth-pollinated flowers, and the antennal responses of moths to these compounds have been demonstrated (Dötterl et al., 2006; Jürgens et al., 2014). Limonene, β-ocimene, β-myrcene, linalool, α-pinene, and benzaldehyde are commonly found in floral scents of plants (Knudsen et al., 2006). In particular, β-ocimene was reported in bee-, moth-, and butterfly-pollinated plants (Kaiser and Tollsten, 1995; Jürgens et al., 2014), and β-myrcene was reported in moth-pollinated plants (Meagher and Landolt, 2008). Therefore, various floral scent compounds emitted during daytime and nighttime can potentially act as attractants for a wide range of insects in *D. jezoensis*. In particular, lilac aldehyde isomers, which form moths' favorite scent (Dötterl et al., 2006; Jürgens et al., 2014), were emitted during both daytime and nighttime, they may attract not only nocturnal but also diurnal moths, such as *C. leucophaea* and *Hedina psychina* in this study. Although we detected a difference in the composition of floral scent between female and perfect flowers, we cannot exclude the possibility of contamination during the measurements, such as volatile compounds from tissues other than flowers. Furthermore, a small sample size limited our ability to clarify the ecological functions of the diverse floral scents detected in this species.

Contribution and efficiency of diurnal and nocturnal pollinators

Fruit set in the night-open treatment was similar to or higher than that of the day-open treatment, indicating that both diurnal and nocturnal insects contributed to the pollination success of *D. jezoensis*. Yearly variation in fruit set was larger in night-open flowers than in day-open flowers, suggesting that the pollinator activity was more unstable during nighttime in early spring, due to cool temperature. Several studies have reported that settling moths were inefficient or less important as pollinators in early-blooming plants (Alonso, 2004; Jürgens et al., 2014; Funamoto and Sugiura, 2020). However, in the present study, both

diurnal and nocturnal pollinators were effective in *D. jezoensis*. This plant species may improve its pollination success by attracting both diurnal and nocturnal pollinators using visual and olfactory cues in early spring.

Pollen dispersal distance tended to be shorter during nighttime, and short-distance pollen transfers (<10 m) were more frequent during nighttime than during daytime. Although the relative importance of settling moths and sap-feeding beetles as nocturnal pollinators was unclear in this study, both settling moths and beetles tended to stay for a longer period on the same plants and moved shorter distances than diurnal insects. We observed that the same nocturnal moth (*C. leucophaea*) individuals stayed on the same plants for ~30 min. Only a few studies have investigated pollen dispersal distance of nocturnal settling moths, revealing that distances were short and resulted in high geitonogamy (Marr et al., 2000; Alamo-Herrera et al., 2022). In previous studies, nocturnal pollen dispersal distance was commonly estimated by using a fluorescent dye or by conducting the study in an experimental garden (Young, 2002; Barthelmess et al., 2006; but see Rhodes et al., 2017; Alamo-Herrera et al., 2022). To our knowledge, the present study is the first to examine nocturnal pollen dispersal of early-blooming plants using genetic markers in a natural population.

The number of distinct paternities per maternal plant (i.e., paternity diversity) was similar between the day-open and night-open flowers. Paternity diversity was approximately half of the produced seed number in this species. This value was relatively high compared with that of other plant species, such as *Bombax ceiba* (3.7 paternity for 31.3 seeds/plant) and *Anigozanthos humilis* (3.5 paternity for 9.2 seeds/plant), but it was less than that of *Arabidopsis halleri* (22.4 paternity for 26.7 seeds/plant; Llaurens et al., 2008; Kestel et al., 2021; Xiang et al., 2022). Depositions of pollen grains by multiple insects or by insects that have a large pollen carryover may result in higher paternity diversity (Karron et al., 2006; Mitchell et al., 2013). Multiple paternity may be beneficial for plants because genetically compatible pollen can fertilize ovules via pollen tube competition after pollination, and females can select only high-quality embryos to develop into seeds through selective abortion (i.e., avoidance of biparental inbreeding; Delph and Havens, 1998; Pannell and Labouche, 2013). The relatively high paternity in *D. jezoensis* can be attributed to the following factors. First, the paternity analysis was conducted only for seeds produced by female plants in the absence of self-pollination. Second, because each flower contains only one ovule, a single visit by a pollinator results in the fertilization of one seed, irrespective of whether various pollen are deposited onto the stigma. This indicates that multiple visits by pollinators are required for the fertilization of all flowers during the long floral duration. Finally, high plant density within a population might cause frequent pollen delivery from multiple neighboring plants (Llaurens et al., 2008) even when pollen dispersal is short.

CONCLUSIONS

Both diurnal and nocturnal insects contribute to pollination success in *D. jezoensis*. Early-blooming species can ensure pollination success, multiple paternities, and wide pollen dispersal by extending the flowering period and attracting both diurnal and nocturnal insects under uncertain pollination situations.

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AUTHOR CONTRIBUTIONS

A.S. designed the study and collected and analyzed the data. A.S. and G.K. discussed the results. A.S. wrote a first draft of the manuscript, and G.K. revised the manuscript.

DATA AVAILABILITY STATEMENT

All data and R script used for this study are available in Zenodo: https://doi.org/10.5281/zenodo.8253921.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Locations of individuals within the permanent plot and genotyped candidate pollen donors.

Appendix S2. Sequences of additional primer pairs.

Appendix S3. Flowering periods of female and hermaphroditic plants in *Daphne jezoensis* within a natural population. Open flower numbers per plants in 2015 are indicated. Mean values are indicated by larger symbols and lines.

Appendix S4. Compounds of floral scent in females and hermaphrodites of *Daphne jezoensis*. Mean values of relative areas (SE) are indicated as percentage. The number of samples used and the number of samples containing each compound are indicated in the brackets.

Appendix S5. Nonmetric multidimensional scaling (NMDS) based on Bray–Curtis indices of floral scent during daytime and nighttime in females (F) and hermaphrodites (H) of *Daphne jezoensis*. $n = 4$ for F and $n = 3$ for H during daytime, and $n = 4$ for F and $n = 5$ for H during nighttime.

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Table 1. Numbers of insect visitors on *Daphne jezoensis* flowers during daytime and nighttime recorded by direct observation and interval photography.

Species	Direct observation	Interval photography	
	Day	Day	Night
Coleoptera			
Epuraea spp.	12	16	17
Diptera			
Bombylius major	2	0	0
Diptera spp.	2	10	0
Syrphidae spp.	0	3	0
Hymenoptera			
Bombus hypocrita	1	0	0
Lepidoptera			
Cleora leucophaea	0	0	18
Hedina psychina	0	1	0
Inachis io	1	0	0
Nymphalidae sp.	2	0	0
Pieridae sp.	0	1	0

Table 2. Sample size for paternity analysis and pollen dispersal distance in each treatment in each year.

Year	Treatment	Number of seeds (number of genets)		Random mating distance (m)		Pollen dispersal distance (m)	
		Genotyped	Identified	Mean ± SE	Maximum	Mean ± SE	Maximum
2018	Natural	158 (32)	132 (30)	36.2 ± 0.34	118.1	10.8 ± 1.23	52.3
	Day-open	37 (11)	32 (11)	34.3 ± 0.62	114.7	5.5 ± 0.96	24.8
	Night-open	62 (11)	45 (10)	34.3 ± 0.65	114.7	5.6 ± 0.67	17.3
2020	Natural	199 (32)	163 (32)	35.7 ± 0.32	119.0	9.19 ± 1.21	80.9
	Day-open	67 (7)	52 (7)	35.7 ± 0.66	118.0	7.49 ± 1.57	52.5
	Night-open	61 (8)	46 (5)	35.3 ± 0.73	104.3	3.58 ± 1.15	41.5

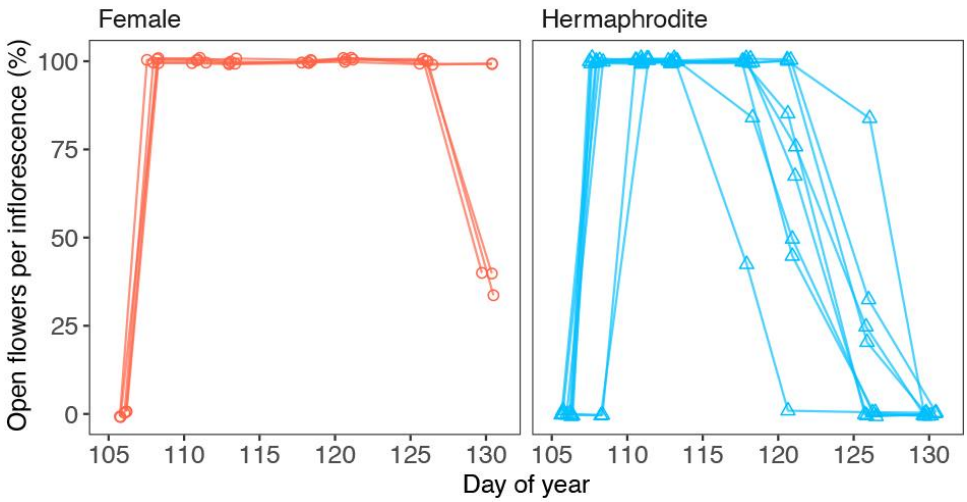

Figure 1. Proportion of flowers per inflorescence open during the flowering period (Julian date) of female (*n* = 5) and hermaphroditic (*n* = 9) *Daphne jezoensis*.

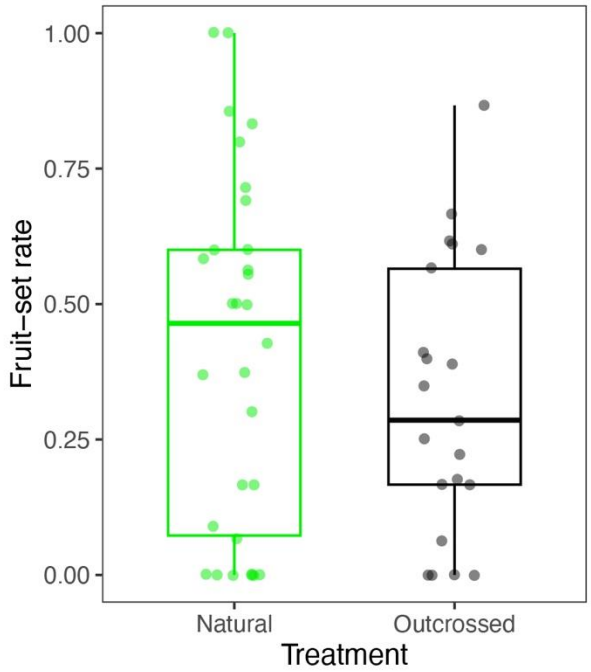

Figure 2. Fruit-set rates of female *Daphne jezoensis* when natural (*n* = 15 in 2018, *n* = 15 in 2020) and outcrossed (*n* = 12 in 2018, *n* = 9 in 2020) pollination treatments were conducted at the individual level.

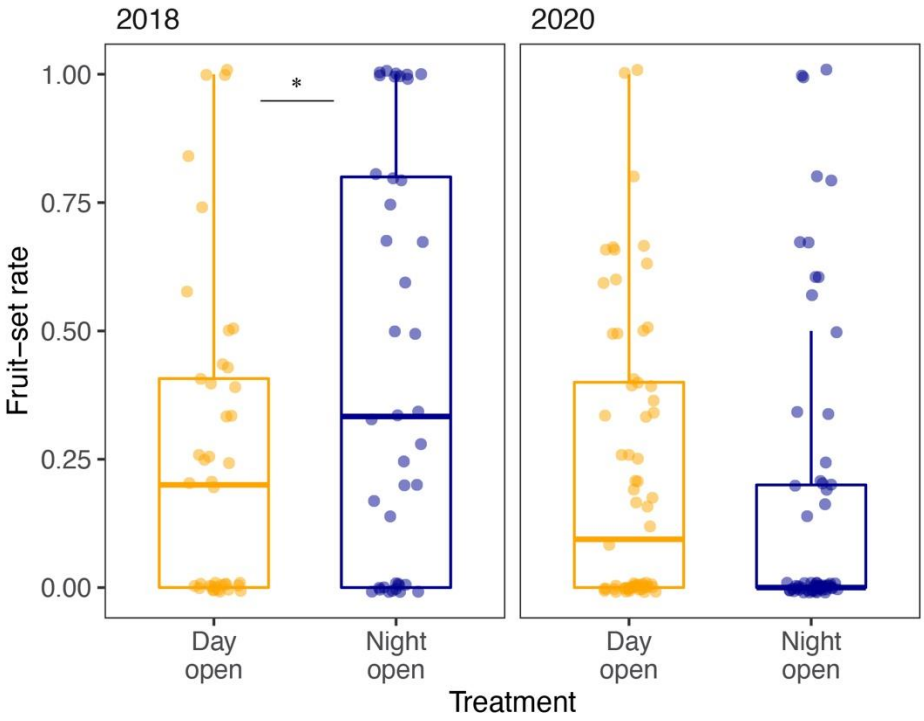

Figure 3. Fruit-set rates on female inflorescences of *Daphne jezoensis* when day-open and night-open treatments were conducted (n = 40 and n = 64 for each treatment in 2018 and 2020, respectively). Asterisk indicates significant difference based on GLMM ($P < 0.05$).

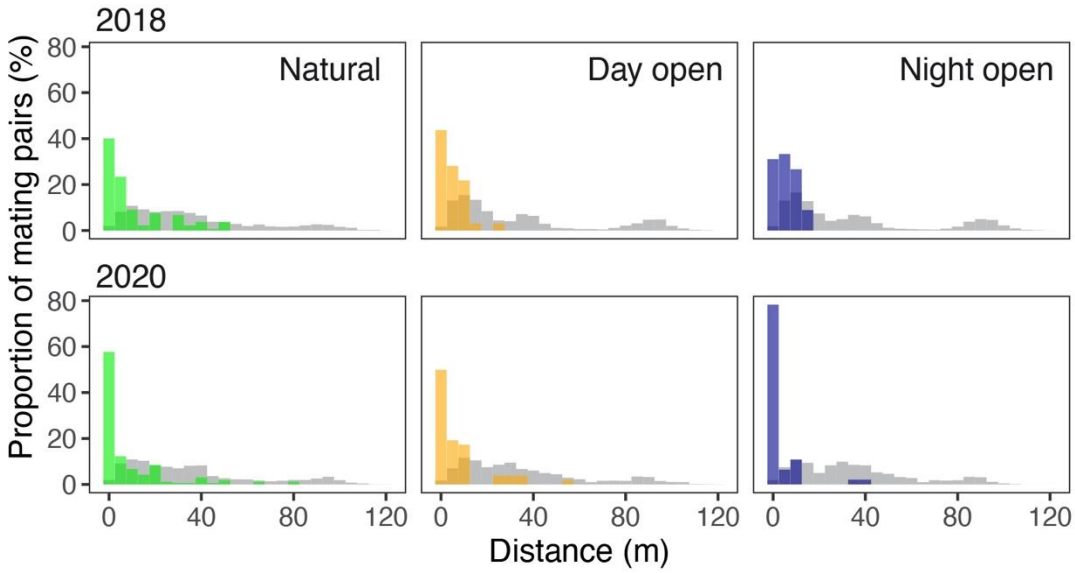

Figure 4. Proportion of mating pairs by pairwise distance among the target females and flowering hermaphrodites for each pollination treatment and each year in *Daphne jezoensis*. Observed pollen dispersals (green, orange, and blue bars) and random mating pairs (gray bars) are indicated.

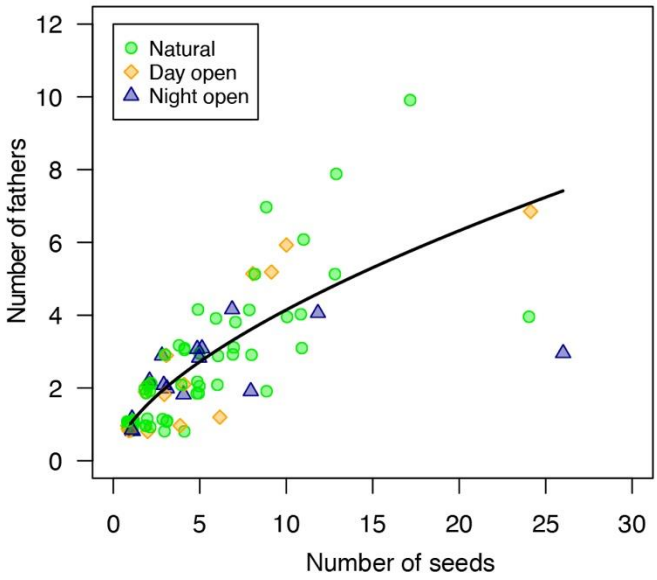

Figure 5. Relationship between numbers of harvested seeds and distinct fathers per maternal plant for each pollination treatment in *Daphne jezoensis*. Line indicates the relationship predicted by GLM (n = 13 for natural pollination, n = 9 for day-open, and n = 11 for night-open treatments).