



HOKKAIDO UNIVERSITY

Title	Nocturnal moth pollination in an alpine orchid, <i>Platanthera tipuloides</i>
Author(s)	Shibata, Akari; Kudo, Gaku
Citation	Plant Species Biology, 40(1), 16-25 https://doi.org/10.1111/1442-1984.12487
Issue Date	2024-09-18
Doc URL	https://hdl.handle.net/2115/96000
Rights	This is the peer reviewed version of the following article: Akari Shibata, Gaku Kudo (2024). Nocturnal moth pollination in an alpine orchid, <i>Platanthera tipuloides</i> . Plant Species Biology, Volume40, Issue1, Pages 16-25, which has been published in final form at https://doi.org/10.1111/1442-1984.12487 . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions. This article may not be enhanced, enriched or otherwise transformed into a derivative work, without express permission from Wiley or by statutory rights under applicable legislation. Copyright notices must not be removed, obscured or modified. The article must be linked to Wiley's version of record on Wiley Online Library and any embedding, framing or otherwise making available the article or pages thereof by third parties from platforms, services and websites other than Wiley Online Library must be prohibited.
Type	journal article
File Information	2024_Shibata_Kudo_Nocturnal moth pollination in an alpine orchid <i>Platanthera tipuloides</i> _HUSCAP.pdf



Original Article

Title: Nocturnal moth pollination in an alpine orchid, *Platanthera tipuloides*

Authors:

Akari Shibata^{1, 2, *} and Gaku Kudo³

¹ Graduate School of Environmental Science, Hokkaido University, Sapporo,
Hokkaido, 060-0810, Japan

² Fukui City Museum of Natural History, Fukui, Fukui, 910-8006, Japan (Present
address)

³ Faculty of Environmental Earth Science, Hokkaido University, Sapporo, Hokkaido
060-0810, Japan

* Corresponding author: Email aka.11mbbc28@gmail.com

Abstract

Pollination success of alpine plants is often restricted by low and unpredictable pollinator activity because of harsh and unstable weather conditions, where nocturnal pollination is rare. The alpine orchid, *Platanthera tipuloides* (Orchidaceae), has inconspicuous greenish-yellow flowers with a sweet scent and a long spur that contains nectar. These floral traits are expected to be related to nocturnal moth pollination. To elucidate the pollination mode and reproductive characteristics of this species, we measured floral traits (spur length, nectar content in the spur, floral scent), documented flower visitors using camera traps, and quantified self-compatibility and the degree of pollen limitation through controlled pollinations at two study plots in the Taisetsu Mountains, northern Japan. It was revealed that *P. tipuloides* is self-incompatible and

pollen limitation was absent at one of the study plots. The flowers emitted more volatile substances during the night, including lilac aldehyde isomers, which are known to attract moths. A nocturnal moth, *Entephria amplicosta*, was observed foraging nectar from the flowers, while no diurnal visitors were observed. The proboscis of *E. amplicosta* was shorter than the spur length, but it was long enough to access the accumulated nectar in the spurs. These results suggest that nocturnal pollination by moths is possible and can be efficient even in an alpine ecosystem with harsh environmental conditions.

Keywords: alpine; floral scent; nocturnal moth; orchid; self-incompatibility

Introduction

Flowering plants have evolved diverse floral traits through plant–pollinator interactions (Fenster et al., 2004; Harder & Johnson, 2009). In particular, Orchidaceae is a species-rich family with diverse floral traits, and many orchid species have specialized pollination systems that depend on single taxonomical groups of pollinators, such as moths and bees (Ackerman et al., 2023; Nilsson, 1992). Pollinators of a single orchid species are often limited to a few insect groups that are specialized to specific floral traits, such as shape, color, and scent (Schiestl & Schluter, 2009). Therefore, it is often possible to speculate on the main pollinator groups of individual orchid species based on their floral properties.

Flowers pollinated by nocturnal moths are often characterized by the emission of sweet scents during the evening and night, nectar presentation in long spurs or floral tubes, and white- or greenish-colored flowers (Balducci et al., 2020; Hapeman & Inoue, 1997; Little et al., 2005). In orchids that attract nocturnal moths through nectar presentation, spur length is a key trait that affects pollination efficiency (Trunschke et al. 2019, 2020). It is predicted that the relationship between spur length and pollination success follows a saturating curve, whereby pollination success increases with spur length as long as the spur is shorter than the proboscis of pollinators, while longer spurs beyond the saturation point have no further benefit (Boberg et al. 2009; Nilsson 1988; Trunschke et al., 2020). On the other hand, accessibility to nectar for insects varies depending on the nectar level in the spurs. Pollinators with a shorter proboscis than spur length are expected to contribute to pollination in their attempt to probe nectar. Therefore, the variation in the distance to nectar in the spurs can affect the diversity of pollinators and pollination success of plants.

Most entomophilous alpine plants are predominantly visited by flies and bees, where generalist pollinators are common and plant species with specialized pollination systems are rare (Encinas-Viso et al., 2022; Kudo, 2016; Totland, 1993). A recent study on the mating systems of Japanese alpine plants demonstrated that a large number of entomophilous plant species are obligate outcrossers that depend on insect pollination (Kudo, 2022). This indicates that pollinator interactions are a key issue that affects the seed production of alpine plants. However, pollination studies of alpine plants that depend on insects other than bees and flies are scarce, especially regarding nocturnal pollination. The mating system and pollination ecology of orchids growing at higher elevations have been reported in some species (Claessens & Seifert, 2017; Li et al., 2010; Schiestl & Glaser, 2012), but orchid species specialized for the pollination by nocturnal moths are unknown in typical alpine regions above the treeline. Our understanding of the adaptation of alpine orchid pollination systems, especially evolutionary trend in generalization or specialization under harsh environmental conditions, remains limited.

Platanthera tipuloides var. *sororia* (*P. tipuloides*) is a perennial orchid that grows in alpine and subalpine meadows, with inconspicuous greenish-yellow flowers that have a sweet scent and nectar secretion in a long spur. Based on these floral characteristics, we hypothesized that it may be pollinated by nocturnal moths. However, alpine environments are characterized by cool temperature, strong wind, high humidity, and unstable weather conditions, even during the summer, resulting in low and unpredictable pollinator activity and pollination success (Arroyo et al., 1985; McCall & Primack, 1992; Wang et al., 2023). Therefore, it is questionable whether the pollen transfer only by nocturnal moths is sufficient for *P. tipuloides*. In this study, we

investigated the pollination mode and reproductive characteristics of *P. tipuloides* in alpine environments. We addressed the following three research questions: (1) Is this species self-incompatible and pollen-limited? (2) Is this species predominantly visited by nocturnal moths, and does it emit volatile substances that are preferred by moths, especially at night? (3) To what extent does the spur length or the distance to nectar in the spurs overlap with the proboscis length of the nocturnal moths?

Methods

Study species and sites

Platanthera tipuloides var. *sororia* (Schltr.) Soó is distributed in the subalpine and alpine meadows from central to northern Japan, Sakhalin, and Kamchatka. The plant height is 20–40 cm, the flower is a greenish-yellow color with a sweet scent, and nectar is secreted in the long spurs (Figure 1a). Flowering occurs during mid-summer, and the seeds mature one month later. This study was performed at an alpine snow-meadow above the treeline in the central part of the Taisetsu Mountains, Hokkaido, northern Japan (43° 33'N, 142° 53'E) in 2020. We conducted field surveys at two subpopulations (Hisago plot, a 20 m × 100 m area at 1790 m above sea level; Kaun plot, a 30 m × 50 m area at 1840 m above sea level) located approximately 1 km apart. The plots were chosen to have similar environmental conditions and vegetation types. Air temperature and wind speed during the flowering periods were measured at a nearby weather station (at 1700 m above sea level).

Floral shape measurement

For the measurements of spur length, width and nectar content in the spurs, 30 flowering plants were selected per plot, and one inflorescence of each plant was photographed using a digital camera (Nikon D5200). To adjust the measurement scale, graph paper was placed in the background of the inflorescence (Figure 1b). The length and width of the spurs and nectar content in the spurs were measured from the photographs using ImageJ software (<http://rsb.info.nih.gov/ij/>). The nectar content in the spurs is visible from the outside through the semi-transparent spurs (Figure 1b). The nectar volume was calculated as a cylinder using the spur width and nectar depth. The distance to the nectar in the spur —the distance from the entrance to the surface of the accumulated nectar —, was calculated by subtracting the nectar depth from the spur length.

Floral scent measurement

We sampled the floral volatile substances from plants at the Kaun plot during the middle of the flowering season using the solid phase absorbent. Volatile sampling was performed during the night (between 18:00 and 5:45) and day (between 6:00 and 17:45) on August 8 and 9, 2020. Night sampling was always carried out before day sampling. A Mono Trap RCC18 block (tubular solid phase absorbance, 5 mm long, 2.9 mm diameter, GL Science Inc., Japan) was hung from a narrow wire on a flower stalk with an inflorescence. The inflorescence and Mono Trap without leaves were covered and enclosed with a clean polyethylene terephthalate (PET) bag for approximately 12 h, and the Mono Trap was then collected in a glass vial. The Mono Trap and PET bag were replaced between the day and night sampling periods to avoid contamination between the diurnal and nocturnal floral volatiles. Four plants were randomly selected, and the

same plants were used for both day and night volatile sampling. The number of flowers per plant varied from 7 to 13. An ambient air sample was collected by covering and enclosing only the Mono Trap in a clean PET bag to identify any ambient contaminants. We handled plants as gently as possible to avoid release of volatile compounds induced by damage, that could contribute to differences between day and night samples. The Mono Trap samples were stored at -20°C until analysis.

The volatile samples were analyzed using gas chromatography/mass spectrometry (GC/MS) (GC: 7890N with a DB-5ms column (30 m long $\times$ 0.25 mm inner diameter $\times$ 0.25 μm film thickness); Agilent Technologies, California, USA; MS: JMS-Q1000GC; JEOL Ltd., Tokyo, Japan). The oven temperature of the gas chromatograph was programmed to increase from 40°C to 180°C at $6^{\circ}\text{C}/\text{min}$ (4-min hold) and from 180°C to 270°C at $15^{\circ}\text{C}/\text{min}$ (5-min hold). The compounds were identified according to the retention time and mass spectra using the NIST 11 database (mass search program ver. 2.0). The peak area of the gas chromatograph output (total ion intensities per flower per minute) was used to determine the strength of the floral volatile.

Pollination experiment

To assess the degree of pollen limitation and self-compatibility, the following three treatments were performed: (1) open pollination, where all flowers were exposed to flower visitors during the flowering period (30 and 27 plants at the Hisago and Kaun plots, respectively); (2) artificial cross-pollination, where the flowers were outcrossed by hand (17 plants at each of the Hisago and Kaun plots); and (3) artificial self-pollination, where the flowers were self-pollinated by hand (17 plants at the Kaun plot). The plants that were used for the self-pollination treatment were tagged and covered

with fine-mesh bags before flowering to prevent visits by pollinators, and the plants used for open pollination and cross-pollination were tagged during the flowering period. For the hand-pollination treatment, pollinia were taken out from the flowers of the donor plants and transferred to the stigmas of the recipient flowers using fine forceps. Pollinia of ten donors, located >10 m apart from the recipient plants, were used for the cross-pollination treatment. Pollinia from the same recipient plants were used for the self-pollination treatment. The hand-pollination treatment was performed on all open flowers per plant during a peak flowering day. After the pollination treatment, all remaining floral buds on the inflorescences (0–4 per plant) were removed to avoid confusion between treated and untreated flowers, and the self-pollinated plants were covered again with fine-mesh bags. The number of flowers was recorded for all plants at the time of tagging or pollination treatments. The fruits were counted and collected when they matured and before seed dispersal. For the mature fruits, developed seeds and depauperate seeds (including unfertilized ovules) were counted under a stereomicroscope in the laboratory. We classified well-rounded brown seeds with wings as developed seeds, and small withered seeds (or undeveloped ovules) as depauperate seeds (or unfertilized ovules). Fruit-set rate was calculated as the ratio of fruit to flower numbers per inflorescence, and seed-set rate was calculated as the ratio of developed seeds to original ovule numbers per fruit.

Flower visitors

Flower visitors were recorded using interval photography at the Kaun plot. Two cameras (WG-70, Ricoh, Tokyo, Japan) were placed in front of flowering plants (1–5 plants for each camera) and programmed to automatically capture images at 45-s

intervals. Diurnal observations were performed from 6:00 (1.5 h after sunrise time) to 17:59 (1 h before sunset time) on eight days, and nocturnal observations were performed from 18:00 to 5:59 on six nights (Table S1). A total of 3,926 and 11,280 images were recorded during the day and night, respectively. Conspecific insects that were continuously recorded on the same plants were considered to be the same individual.

To identify the nocturnal flower visitors, two LED light traps (mosquito killers) were set up at the center of the Kaun plot during five nights of the flowering period. The trapped nocturnal moths were collected in the following morning and identified in the laboratory. The proboscis of the moths was cut with scissors, mounted on a glass slide with a small piece of fuchsin-tinted glycerin jelly, and inspected for the presence of pollen grains of *P. tipuloides* using a microscope (BX43, Olympus, Tokyo). The mounted proboscis was photographed, and its length was measured using ImageJ software.

Statistical analyses

All statistical analyses were performed using R version 4.2.1 (R Core Team, 2022). We examined the correlation between spur length and width using Pearson's correlation coefficient. To compare the floral scent profiles between day and night, the Bray–Curtis dissimilarity indices were calculated based on the relative peak areas of the floral volatiles. The dissimilarity was visualized in two dimensions using nonmetric multidimensional scaling (NMDS) with the Vegan package (Oksanen et al., 2022). We also performed permutational multivariate analysis of variance (PERMANOVA) of the Bray–Curtis dissimilarity indices based on 9,999 permutations to evaluate the

differences in the volatile compositions between day and night using the Vegan package.

To assess the degree of pollen limitation and self-compatibility, the fruit-set and seed-set rates were compared between the pollination treatments using generalized linear models (GLMs) and generalized linear mixed-effect models (GLMMs) with a negative binomial error distribution and a log-link function using the glmmTMB package (Brooks et al., 2017). Because the timing of flowering was one week earlier at the Hisago plot than at the Kaun plot, we expected that the degree of pollen limitation might differ between populations. First, the fruit-set rates were compared at the individual level between the pollination treatments using GLMs. In the GLM to test pollen limitation, fruit number was the response variable, flower number was set as the offset term, and the pollination treatment (open and cross), plot (Hisago and Kaun), and their interaction were set as the explanatory variables. GLM was performed separately for each plot when the interaction term was significant. In the GLM to evaluate self-compatibility, fruit number was the response variable, flower number was set as the offset term, and the pollination treatment (self and cross) was set as the explanatory variable. Because the self-pollination treatment was conducted only for the Kaun plot, the GLM for self-compatibility was performed only for the Kaun plot. Next, the seed-set rates were compared between the pollination treatments using GLMMs. In the GLMM to test pollen limitation, seed number was the response variable, ovule number was set as the offset term, and the pollination treatment (open and cross), plot (Hisago and Kaun), and their interaction were set as the explanatory variables. In the GLMM to evaluate self-compatibility, seed number was the response variable, ovule number was set as the offset term, and the pollination treatment (self and cross) was set as the

explanatory variable. The plant ID was set as a random factor in the GLMMs. The significance level of the explanatory variables was evaluated using the likelihood-ratio test and Wald chi-square test with the car package in the GLMs and GLMMs, respectively.

Results

Floral traits

Flowers of *P. tipuloides* had long, narrow spurs (Table 1; Figure 1b) and viscidium — the sticky part of the pollinium — located on the inside of the spur entrance (Figure 1c). The spur lengths were 19.3 ± 0.19 mm and 19.8 ± 0.18 mm (mean $\pm$ SE) at the Hisago and Kaun plots, respectively (Table 1). Spurs contained nectar up to about a quarter or a third of their height, and the distance to nectar was 13.4 ± 0.25 mm and 13.5 ± 0.21 mm at the Hisago and Kaun plots, respectively. The length and width of the spurs were not significantly correlated in either plot ($r = -0.14$, $P = 0.46$ at Hisago; $r = 0.10$, $P = 0.13$ at Kaun).

The total volatile emission (mean $\pm$ SE of peak area) during the day and night were 0.18 ± 0.07 (range: 0.05–0.38) and 4.69 ± 1.83 (2.28–10.12), respectively. Differences in the total volatile emission between the day and night within a plant were 2.18 (day: 0.10, night: 2.28) in minimum and 9.74 (day: 0.38, night: 10.12) in maximum. A total of 11 compounds were identified in the floral volatile substances (Table 2). The major components were lilac aldehyde isomers, benzaldehyde, indole, and phenylethyl alcohol. Lilac aldehyde isomers could not be distinguished based on the similarity search using the database. The composition of the floral volatiles tended to differ between day and night ($F = 5.02$, $R^2 = 0.46$, $P = 0.06$ in PERMANOVA; Figure 2).

Some floral volatile substances tended to be emitted more during the night than during the day, especially lilac aldehyde isomers, which accounted for 40% of the total nocturnal volatiles (Table 2). However, the percentage of benzaldehyde, benzyl alcohol, and indole was higher during the day than during the night.

Mating system

The fruit-set success of open- and cross-pollinated flowers was $6.6 \pm 2.7\%$ (mean $\pm$ SE) and $24.9 \pm 6.1\%$ at the Kaun plot, and $24.4 \pm 4.3\%$ and $33.0 \pm 7.6\%$ at the Hisago plot, respectively. In the comparison of fruit-set rates between the open- and cross-pollinated plants, a significant interaction between pollination treatment and plot was detected ($\chi^2 = 3.85$, $P < 0.05$), indicating different levels of pollen limitation between plots. The fruit-set success of cross-pollinated plants was significantly higher than that of open-pollinated plants at the Kaun plot ($\chi^2 = 5.62$, $P < 0.05$; Figure 3a), but not at the Hisago plot ($\chi^2 = 0.30$, $P = 0.59$). Therefore, fruit production was pollen-limited only at the Kaun plot. The fruit-set success of self-pollinated plants ($6.6 \pm 3.3\%$) was lower than that of the cross-pollinated plants at the Kaun plot ($\chi^2 = 5.14$, $P < 0.05$; Figure 3a).

The seed-set success of open- and cross-pollinated flowers was $40.2 \pm 9.2\%$ and $53.0 \pm 4.7\%$ at the Kaun plot, and $40.2 \pm 3.7\%$ and $42.5 \pm 3.7\%$ at the Hisago plot, respectively. The seed-set success of open- and cross-pollinated flowers did not differ in any of the two plots, as indicated by non-significant terms for both the interaction (pollination treatment $\times$ plot, $\chi^2 = 0.34$, $P = 0.56$) and main effects (pollination treatment, $\chi^2 = 0.52$, $P = 0.47$; Figure 3b). In addition, the seed-set success of self-pollinated fruits ($7.3 \pm 1.8\%$) was remarkably lower than that of cross-pollinated fruits

($\chi^2 = 63.76$, $P < 0.001$; Figure 3b). These results suggest that this species is nearly self-incompatible.

Flower visitors

There were no insect visitors to the *P. tipuloides* flowers during the day throughout the measurement period. However, visitation by the nocturnal moth, *Entephria amplicosta* (Geometridae), was recorded during the night through interval photography (Figure 1d, e). In total, three individuals were recorded on two different days. Although we recognized in several pictures that the nocturnal moths inserted their proboscis into the flowers, it was unclear whether the pollinia were attached to their proboscises.

Among the 55 moth individuals caught by the light traps, 54 could be identified (Table S2), and 22 of these were *E. amplicosta*, with a proboscis length of 7.88 ± 0.10 mm (mean $\pm$ SE; range 6.6–8.7 mm; Table S2). The deposition of pollinia was not observed on the proboscises of these moths.

The distance from the entrance to the surface of the accumulated nectar in the spur ranged from 6.0 to 23.4 mm among flowers. Ten flowers from nine plants had a distance shorter than 8.7 mm to the nectar, making it accessible to *E. amplicosta* (Figure 4). Because proboscis lengths of 49 moth individuals from nine species, including *E. amplicosta*, were longer than 6.0 mm, they could access the nectar when spurs were filled (Figure 4; Table S2). The nocturnal moths were more likely to be caught and photographed when the night temperature was warmer than 10°C, especially under less windy conditions (Figure S1).

Discussion

Based on the following three findings from this study, nocturnal moths are most likely the main pollinators of *P. tipuloides*: (1) self-incompatible flowers set seeds when naturally pollinated, (2) nocturnal moths fed on *P. tipuloides* flowers, but no other insects did, and (3) flowers emitted similar volatiles to those of other plants pollinated by nocturnal moths. Lilac aldehyde isomers, known to attract moths (Dötterl et al., 2006; Jürgens et al., 2014; Plepys et al., 2002), are found in other nocturnal moth-pollinated *Platanthera* species (Esposito et al., 2018; Plepys et al., 2002; Tollsten & Bergström, 1993). Several *Platanthera* species are also known to be pollinated by nocturnal moths; however, they commonly grow in forest and meadow habitats in lowland and subalpine areas (Esposito et al., 2018; Little et al., 2005; Nilsson, 1983; Suetsugu & Hayamizu, 2014).

This study is the first report of specialized nocturnal moth pollination in an alpine orchid species growing above the treeline. So far, ants and bumblebees have been reported as available pollinators of alpine orchids (Claessens & Seifert, 2017; Li et al., 2010; Schiestl & Glaser, 2012), suggesting an adaptation to pollinators in harsh alpine environments. Although several studies have reported the moth pollination at higher elevations for some orchid species, such as *Habenaria limprichtii*, *Gymnadenia odoratissima*, and *Platanthera bifolia*, these studies were conducted in subalpine meadows below the treeline (Boberg et al. 2014; Huber et al. 2005; Sun et al. 2013; Tao et al. 2018). The present study indicated that nocturnal moths were active only when nighttime temperature was above 10°C under less windy conditions (Figure S1). On the other hand, pollen limitation was absent at one plot, suggesting that *P. tipuloides* could receive sufficient pollination services even under the unstable alpine environmental conditions of northern Japan.

Although several *Platanthera* species have been reported to be highly self-compatible (Boberg & Ågren, 2009; Brzosko, 2003; Little et al., 2005), *P. tipuloides* exhibited self-incompatibility and required between-plant pollen transfer by insects. In alpine regions, self-compatibility has been tested for two orchid species, and both were found to be self-compatible (Li et al. 2010; Sletvold et al. 2010). Autonomous self-pollination and self-compatibility can act as reproductive assurance when pollinators and/or reproductive partners are absent (Lloyd 1992; Morgan et al. 2005). In our study, significant pollen limitation was detected at one of the two plots (Kaun). Although these plots located about 1 km apart from each other in the snow-meadows with similar vegetation, flowering at the Hisago plot occurred one week earlier than at the Kaun plot in the study year. Because weather conditions are often variable and unpredictable in alpine regions, site-specific pollen limitation observed in our study might reflect the differences in weather conditions during the flowering periods. To clarify the pollination situation, further studies over several years and across multiple populations are required.

In this study, the nocturnal moth, *E. amplicosta*, was the most common flower visitor, but we did not find any pollinia on the proboscis of this moth in either the interval photographs or the specimens caught in the light traps. The placement of the viscidia inside the spur entrance ensures that the viscidia will attach to the proboscis of moths during nectar collection (Johnson & Edwards, 2000; Nilsson, 1983). Although the proboscis length of *E. amplicosta* (7.9 mm in average, range 6.6–8.7 mm; Table S2) was shorter than the spur length (19.5 mm, range 11.0–27.2 mm), the distance to the nectar (13.5 mm, 6.0–23.4 mm) varied considerably between the flowers depending on the nectar volume, making some accessible to *E. amplicosta*. Because moths with short

proboscis can collect only a small amount of nectar from flowers with long spurs, the moths may move quickly between flowers, leading to higher outcrossing success. A short proboscis relative to the spur length may also improve the functional fit between stigma and pollinia attached to proboscis, resulting in increased pollination efficiency (Trunschke et al. 2020). Furthermore, the remaining nectar in the spur can be used by other moths with longer proboscis. Therefore, flowers can be visited by multiple moth individuals by accumulating a large amount of nectar within a long and narrow spur. Although *E. amplicosta* was the most frequently captured species, 12 other nocturnal moth species were also recorded during the flowering period. Five of these species, *Aplocera perelegans*, *Syngrapha ottolenguii*, *Catocala dissimilis*, *Anaplectoides virens*, and *Palpita nigropunctalis*, had longer proboscis than *E. amplicosta*. To clarify the importance of the other nocturnal moth species as pollinators of *P. tipuloides*, further studies are required.

The present study suggests that *P. tipuloides* depends on a specific pollinator group, nocturnal moths, in the alpine habitat. The flowering of this species occurs from late July to early August, which corresponds with the warmest period of the short summer. Thus, the flowering period of *P. tipuloides* may be limited to the most active season of the nocturnal moths in alpine environments. To elucidate the general importance of nocturnal pollinators in alpine ecosystems, further studies on the activity of nocturnal flower visitors and their pollination efficiency are required.

Acknowledgments

We would like to thank M. Sakurai for his help with the identification of the moths, H. Kudoh for his helpful comments on the manuscript, and the associate editor and two anonymous reviewers for their valuable comments and suggestions. We thank Instrumental Analysis Division, Global Facility Center, Creative Research Institution, Hokkaido University for the floral volatile analysis using the JMS-Q1000GC system and providing insight and expertise that greatly assisted this research. This study was supported by the Japan Society for the Promotion of Science (JSPS KAKENHI 19J10583; 22H02695).

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Ackerman, J. D., Phillips, R. D., Tremblay, R. L., Karremans, A., Reiter, N., Peter, C. I., Bogarín, D., Pérez-Escobar, O. A., & Liu, H. (2023). Beyond the various contrivances by which orchids are pollinated: global patterns in orchid pollination biology. *Botanical Journal of the Linnean Society*, 202, 295–324. doi: 10.1093/botlinnean/boac082.
- Arroyo, M. T. K., Armesto, J. J., & Primack, R. B. (1985). Community studies in pollination ecology in the high temperate Andes of central Chile II. Effect of temperature on visitation rates and pollination possibilities. *Plant Systematics and Evolution*, 149, 187–203. doi: 10.1007/BF00983305.
- Balducci, M. G., Van Der Niet, T., & Johnson, S. D. (2020). Diel scent and nectar rhythms of an African orchid in relation to bimodal activity patterns of hawkmoth pollinators. *Annals of Botany*, 126, 1155–1164. doi: 10.1093/aob/mcaa132.
- Boberg, E., & Ågren, J. (2009). Despite their apparent integration, spur length but not perianth size affects reproductive success in the moth-pollinated orchid *Platanthera bifolia*. *Functional Ecology*, 23, 1022–1028. doi: 10.1111/j.1365-2435.2009.01595.x.
- Boberg, E., Alexandersson, R., Jonsson, M., Maad, J., Ågren, J., & Nilsson, L. A. (2014). Pollinator shifts and the evolution of spur length in the moth-pollinated orchid *Platanthera bifolia*. *Annals of Botany*, 113, 267–275. doi: 10.1093/aob/mct217.
- Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H., Mächler, M., & Bolker, B. M. (2017). glmmTMB balances

403 speed and flexibility among packages for zero-inflated generalized linear mixed
 404 modeling. *R Journal*, 9, 378–400. doi: 10.32614/rj-2017-066.

405 Brzosko, E. (2003). The dynamics of island populations of *Platanthera bifolia* in the
 406 biebrza National Park (NE Poland). *Annales Botanici Fennici*, 40, 243–253.

407 Claessens, J., & Seifert, B. (2017). Significant ant pollination in two orchid species in
 408 the Alps as adaptation to the climate of the alpine zone? *Tuexenia*, 37, 363–374.
 409 doi: 10.14471/2017.37.005.

410 Dötterl, S., Burkhardt, D., Weißbecker, B., Jürgens, A., Schütz, S., & Mosandl, A.
 411 (2006). Linalool and lilac aldehyde/alcohol in flower scents. Electrophysiological
 412 detection of lilac aldehyde stereoisomers by a moth. *Journal of Chromatography*
 413 *A*, 1113, 231–238. doi: 10.1016/j.chroma.2006.02.011.

414 Encinas-Viso, F., Bovill, J., Albrecht, D. E., Florez-Fernandez, J., Lessard, B.,
 415 Lumbers, J., Rodriguez, J., Schmidt-Lebuhn, A., Zwick, A., & Milla, L. (2022).
 416 Pollen DNA metabarcoding reveals cryptic diversity and high spatial turnover in
 417 alpine plant–pollinator networks. *Molecular Ecology*, 1–17. doi:
 418 10.1111/mec.16682.

419 Esposito, F., Vereecken, N. J., Gammella, M., Rinaldi, R., Laurent, P., Tyteca, D.
 420 (2018). Characterization of sympatric *Platanthera bifolia* and *Platanthera*
 421 *chlorantha* (Orchidaceae) populations with intermediate plants. *PeerJ*, 2018, 1–34.
 422 doi: 10.7717/peerj.4256.

423 Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., & Thomson, J. D. (2004).
 424 Pollination syndromes and floral specialization. *Annual Review of Ecology,*
 425 *Evolution, and Systematics*, 35, 375–403. doi:
 426 10.1146/annurev.ecolsys.34.011802.132347.

427 Hapeman, J. R., & Inoue, K. (1997). Plant-pollinator interactions and floral radiation in
 428 *Platanthera* (Orchidaceae). In T. Givnish and K. Systma (Eds.), *Molecular*
 429 *Evolution and Adaptive Radiation*, (pp. 433–454). Cambridge, UK: Cambridge
 430 University Press.

431 Harder, L. D., & Johnson, S. D. (2009). Tansley review Darwin’s beautiful
 432 contrivances: evolutionary and functional evidence. (pp. 530–545).

433 Huber, F. K., Kaiser, R., Sauter, W., Schiestl, F. P. (2005). Floral scent emission and
 434 pollinator attraction in two species of *Gymnadenia* (Orchidaceae). *Oecologia*, 142,
 435 564–575. doi: 10.1007/s00442-004-1750-9.

436 Johnson, S. D., & Edwards, T. J. (2000). The structure and function of orchid pollinaria.
 437 *Plant Systematics and Evolution*, 222, 243–269. doi: 10.1007/BF00984105.

438 Jürgens, A., Glück, U., Aas, G., & Dötterl, S. (2014). Diel fragrance pattern correlates
 439 with olfactory preferences of diurnal and nocturnal flower visitors in *Salix caprea*
 440 (Salicaceae). *Botanical Journal of the Linnean Society*, 175, 624–640. doi:
 441 10.1111/boj.12183.

442 Kudo, G. (2016). Landscape structure of flowering phenology in alpine ecosystems:
 443 significance of plant–pollinator interactions and evolutionary aspects. In G. Kudo,
 444 (ed.) *Structure and Function of Mountain Ecosystems in Japan*, (pp. 41–62).
 445 Berlin: Springer. doi: 10.1007/978-4-431-55954-2_3.

446 Kudo, G. (2022). Outcrossing syndrome in alpine plants: implications for flowering
 447 phenology and pollination success. *Ecological Research*, 37, 288–300. doi:
 448 10.1111/1440-1703.12314.

449 Li, P., Zheng, G. L., Dafni, A., & Luo, Y. B. (2010). Reproductive biology of an alpine
 450 orchid *Phaius delavayi*. *Plant Systematics and Evolution*, 286, 167–173. doi:
 451 10.1007/s00606-010-0296-z.

452 Little, K. J., Dieringer, G., & Romano, M. (2005). Pollination ecology, genetic diversity
 453 and selection on nectar spur length in *Platanthera lacera* (Orchidaceae). *Plant*
 454 *Species Biology*, 20, 183–190. doi: 10.1111/j.1442-1984.2005.00137.x.

455 Lloyd, D. G. (1992). Self- and cross-fertilization in plants. II. The selection of self-
 456 fertilization. *International Journal of Plant Sciences*, 153, 370–380.

457 McCall, C., & Primack, R. B. (1992). Influence of flower characteristics, weather, time
 458 of day, and season on insect visitation rates in three plant communities. *American*
 459 *Journal of Botany*, 79, 434–442. doi: 10.1002/j.1537-2197.1992.tb14571.x.

460 Morgan, M. T., Wilson, W. G. (2005). Self-fertilization and the escape from pollen
 461 limitation in variable pollination environments. *Evolution*, 5, 1143–1148. doi:
 462 10.1080/00313029400169831.

463 Nilsson, L. A. (1983). Processes of isolation and introgressive interplay between
 464 *Platanthera bifolia* (L.) Rich and *P. chlorantha* (Custer) Reichb. (Orchidaceae).
 465 *Botanical Journal of the Linnean Society*, 87, 325–350.

466 Nilsson, L. A. (1988). The evolution of flowers with deep corolla tubes. *Nature*, 334,
 467 147–149. doi: 10.1038/334147a0.

468 Nilsson, L. A. (1992). Orchid pollination biology. *Trends in Ecology and Evolution*, 7,
 469 255–259. doi: 10.1016/0169-5347(92)90170-G.

470 Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., Ribeiro
 471 Cunha E, Smith T, Stier A, & Ter Braak C. W. J. (2022). vegan: Community
 472 Ecology Package. R Package Version 2.6-2.

473 Plepys, D., Ibarra, F., & Löfstedt, C. (2002). Volatiles from flowers of *Platanthera*
 474 *bifolia* (Orchidaceae) attractive to the silver Y moth, *Autographa gamma*
 475 (Lepidoptera: Noctuidae). *Oikos*, 99, 69–74. doi: 10.1034/j.1600-
 476 0706.2002.990107.x.

477 R Core Team. (2022). R: A language and environment for statistical computing. R
 478 foundation for statistical computing.

479 Schiestl, F. P., & Glaser, F. (2012). Specific ant-pollination in an alpine orchid and the
 480 role of floral scent in attracting pollinating ants. *Alpine Botany*, 122, 1–9. doi:
 481 10.1007/s00035-011-0098-0.

482 Schiestl, F. P., & Schluter, P. M. (2009). Floral isolation, specialized pollination, and
 483 pollinator behavior in orchids. *Annual Review of Entomology*, 54, 425–446. doi:
 484 10.1146/annurev.ento.54.110807.090603.

485 Sletvold, N., Ågren, J. (2010). Pollinator-mediated selection on floral display, spur
 486 length and flowering phenology in the deceptive orchid *Dactylorhiza lapponica*.
 487 *International Journal of Plant Sciences*, 171, 999–1009. doi: 10.1086/656597.

488 Suetsugu, K., & Hayamizu, M. (2014). Moth floral visitors of the three rewarding
 489 *Platanthera* orchids revealed by interval photography with a digital camera.
 490 *Journal of Natural History*, 48, 1103–1109. doi: 10.1080/00222933.2013.861940.

491 Sun, M., Gross, K., Schiestl, F. P. (2013). Floral adaptation to local pollinator guilds in
 492 a terrestrial orchid. *Annals of Botany*, 113, 289–300. doi: 10.1093/aob/mct219.

493 Tao, Z. Bin, Ren, Z. X., Bernhardt, P., Wang, W. J., Liang, H., Li, H. D., & Wang, H.
 494 (2018). Nocturnal hawkmoth and noctuid moth pollination of *Habenaria*
 495 *limprichtii* (Orchidaceae) in sub-alpine meadows of the Yulong Snow Mountain

496 (Yunnan, China). *Botanical Journal of the Linnean Society*, 187, 483–498. doi:
 497 10.1093/botlinnean/boy023.
 498 Tollsten, L., & Bergström, L. G. (1993). Fragrance chemotypes of *Platanthera*
 499 (Orchidaceae) - the result of adaptation to pollinating moths? *Nordic Journal of*
 500 *Botany*, 13, 607–613. doi: 10.1111/j.1756-1051.1993.tb00105.x.
 501 Totland, O. (1993). Pollination in alpine Norway: Flowering phenology, insect visitors,
 502 and visitation rates in two plant communities. *Canadian Journal of Botany*, 71,
 503 1072–1079. doi: 10.1139/b93-124.
 504 Trunschke, J., Sletvold, N., Ågren, J. (2019). The independent and combined effects of
 505 floral traits distinguishing two pollination ecotypes of a moth-pollinated orchid.
 506 *Ecology and Evolution*, 9, 1191–1201. doi: 10.1002/ece3.4808.
 507 Trunschke, J., Sletvold, N., Ågren, J. (2020). Manipulation of trait expression and
 508 pollination regime reveals the adaptive significance of spur length. *Evolution*, 74,
 509 597–609. doi: 10.1111/evo.13927.
 510 Wang, Y., Gao, E., Hou, M., Ghazoul, J., & Zhao, Z. (2023). Pollen limitation and
 511 context-dependent alleviating mechanisms in a co-flowering alpine grassland
 512 community. *Oikos*, 2023, 1–12. doi: 10.1111/oik.09758.
 513

Table 1. Floral traits of *Platanthera tipuloides* measured at two plots. Sample sizes of plants (N_{plants}) and flowers (N_{flowers}), and mean $\pm$ SE values are shown.

Components	Site	N_{plants}	N_{flowers}	Mean $\pm$ SE
Flower number	Hisago	47	-	7.21 $\pm$ 0.28
	Kaun	78	-	7.76 $\pm$ 0.18
Spur length (mm)	Hisago	30	208	19.3 $\pm$ 0.19
	Kaun	30	214	19.8 $\pm$ 0.18
Spur width (mm)	Hisago	30	208	0.84 $\pm$ 0.009
	Kaun	30	214	1.01 $\pm$ 0.011
Nectar depth (mm)	Hisago	29	136	5.94 $\pm$ 0.17
	Kaun	30	177	6.17 $\pm$ 0.16
Nectar volume (mm ³)	Hisago	29	136	3.40 $\pm$ 0.13
	Kaun	30	177	5.31 $\pm$ 0.21
Distance to nectar (mm)	Hisago	29	136	13.4 $\pm$ 0.25
	Kaun	30	177	13.5 $\pm$ 0.21

Table 2. Compounds of floral scent in *Platanthera tipuloides*. Means $\pm$ SE values of relative areas (percentage) for daytime and nighttime samples are shown.

Compound	Day (<i>n</i> = 4)	Night (<i>n</i> = 4)
Benzaldehyde	50.09 $\pm$ 3.99	31.15 $\pm$ 6.53
Benzyl alcohol	2.35 $\pm$ 0.59	0.80 $\pm$ 0.19
2,4,6-Cycloheptatrien-1-one, 4-methyl-	0.75 $\pm$ 0.10	1.26 $\pm$ 0.33
Linalyl acetate	1.75 $\pm$ 0.76	1.46 $\pm$ 0.87
Phenylethyl Alcohol	10.60 $\pm$ 1.53	11.06 $\pm$ 1.30
1,4-Cyclohexadiene, 3-ethenyl-1,2-dimethyl-	0.88 $\pm$ 0.36	1.41 $\pm$ 0.24
Lilac aldehyde isomer 1 ^a	12.37 $\pm$ 4.63	35.60 $\pm$ 7.02
Lilac aldehyde isomer 2 ^a	0.88 $\pm$ 0.34	2.04 $\pm$ 0.34
Lilac aldehyde isomer 3 ^a	0.66 $\pm$ 0.18	1.26 $\pm$ 0.67
Carveol	0.95 $\pm$ 0.48	1.39 $\pm$ 0.27
Indole	18.71 $\pm$ 4.10	12.57 $\pm$ 2.26
Total	100	100

^a Three lilac aldehyde isomers could not be distinguished.

Relative areas are indicated in bold, when they differ by two or more times between daytime and nighttime.

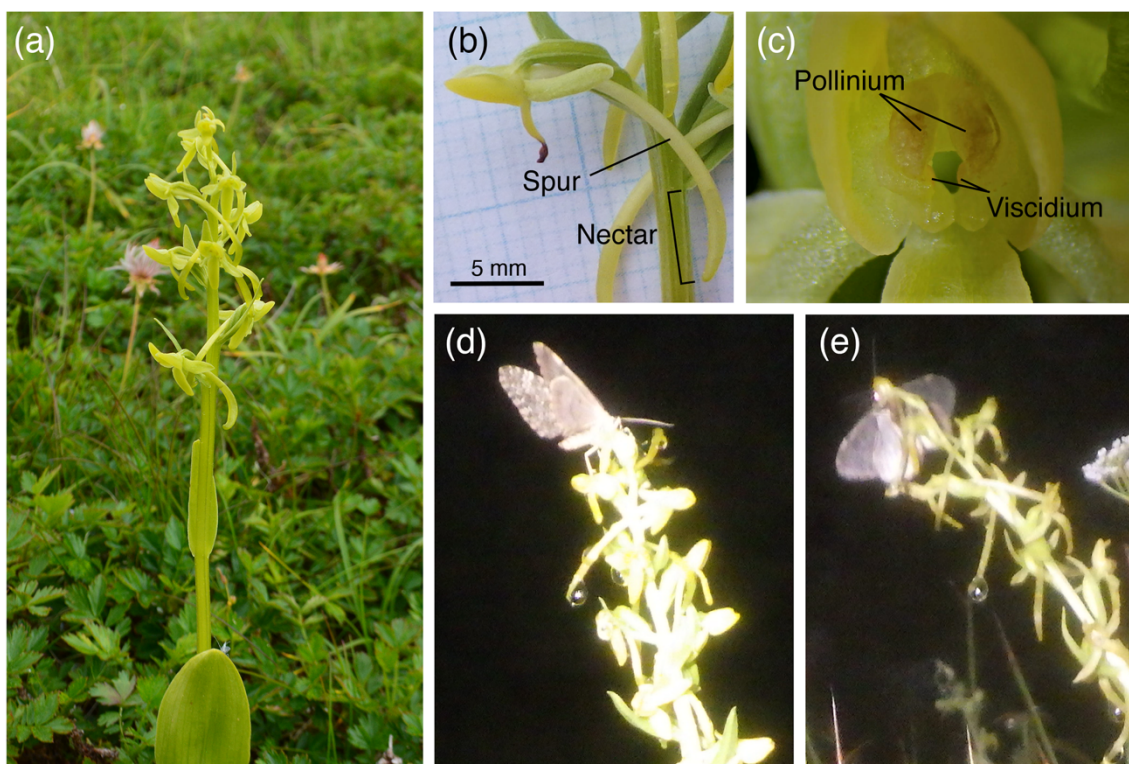


Figure 1. Flowers and flower visitors of *Platanthera tipuloides*. (a) Inflorescence; (b) spur and nectar; (c) pollinium and viscidium; (d, e) nocturnal moths, *Entephria amplicosta*, visiting the flowers.

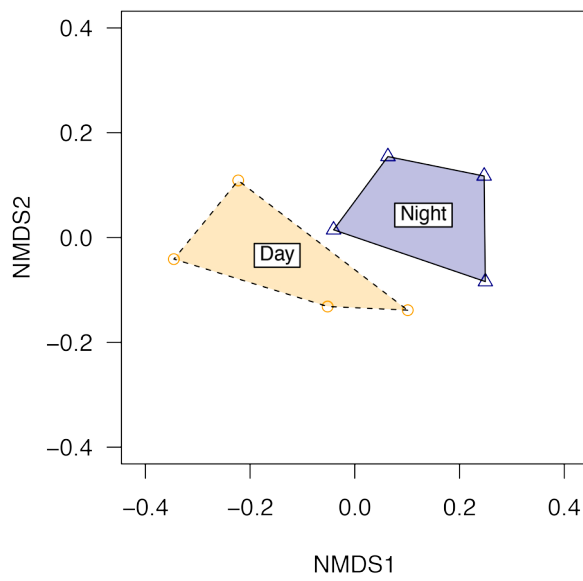


Figure 2. Nonmetric multidimensional scaling (NMDS) based on the Bray–Curtis indices of floral scent during the day and night of *Platanthera tipuloides*. $n = 4$ for each day and night sampling.

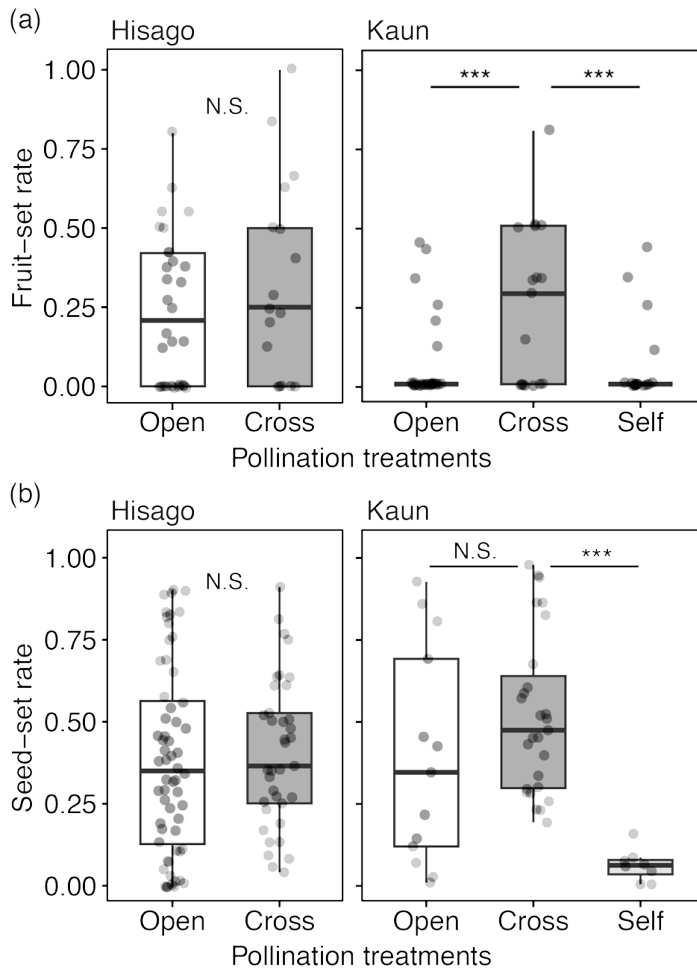


Figure 3. Fruit-set rate (a) and seed-set rate of mature fruits (b) under the open-, cross-, and self-pollination treatments at the Hisago and Kaun plots of *Platanthera tipuloides*.

In the box plots, 25%, 50%, and 75% of the data are indicated by hinges and centerlines. The whiskers extending from the hinges to the highest and lowest values are within 1.5× of the interquartile range. Significant differences based on the generalized linear models (GLMs) and generalized linear mixed-effect models (GLMMs) are indicated by: ***, $P < 0.001$; N.S., not significant.

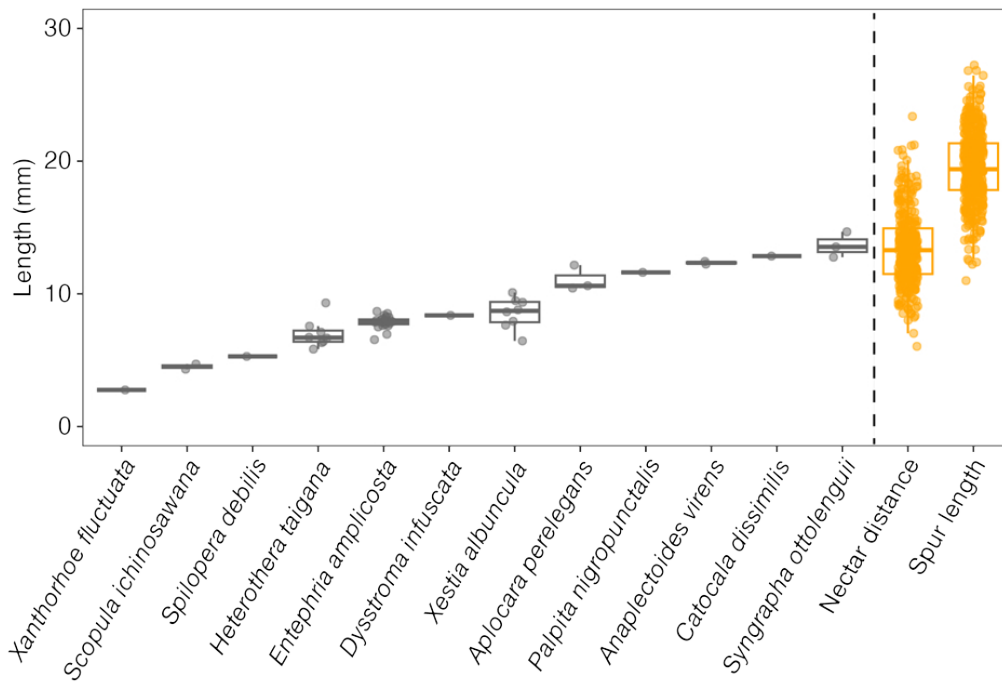


Figure 4. Proboscis length of nocturnal moths caught by the light traps, the distance to nectar, and spur length of *Platanthera tipuloides*. Each grey and orange point indicates an individual moth and flower, respectively.