



Seasonal Changes in Flower Characteristics of *Oenothera laciniata* Naturalized in Japan

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Abstract

The present study aimed to investigate the possibility of partial outcrossing in *Oenothera laciniata*, which is a naturalized plant in Japan. We investigated the changes in the morphology of the petals, stamens, and pistils of *O. laciniata* flowers under different environmental conditions and seasons. Contrary to the existing knowledge that flowers of *O. laciniata* bloom at evening and nighttime, our study found that they also bloomed at daytime in April, when several insects visit these flowers. We found that the petal length was longer, that is, flower size is larger in April, than that in June and August. Flowers of *O. laciniata* likely attract pollinators by flowering at daytime with larger size in the spring season. Further, we found the occurrence of three atypical types of flowers in *O. laciniata* including 1) the pin type, which has longer stamens compared to pistils, 2) the thrum type, which has longer pistils compared to stamens, and 3) the close type, whose column head does not open. These atypical flowers likely help increase the opportunity of outcrossing, and cultivation test results suggest that they may be genetically associated. However, normal flowers also co-existed on plants with atypical flowers. The frequency of the occurrence of these atypical flowers were random and did not exhibit any trend with season or natural habitats. Based on these observations, we conclude that these atypical flowers are not distinctly distylous, but rather present a variation of form of the flowers of *O. laciniata*.

Keywords: *Oenothera laciniata*, naturalized plants, distyly, petal, stamen, pistil.

Introduction

Oenothera laciniata, which is native to North America, likely invaded Japan in the late 19th century and is presently, widely distributed across the country (Sakamoto and Hirota, 2015). It is identified as an invasive alien species under the Invasive Alien Species Act of the Ministry of the Environment of Japan; however, its reproductive physiology in Japan is not clear (Ministry of the Environment, 2022).

Oenothera laciniata is popularly known as "Matsuyoigusa" in Japan, which translates to "flower waiting night," as it blooms from dusk until dawn (Sakamoto and Hirota, 2015). It is also known as "Tsukimisou," which means

"moon-viewing flower" (Shimizu, *et al.*, 2001; Shimizu, 2003; Kadota, 2013). *Oenothera* species that bloom at night are pollinated by moths such as Sphingidae (hawkmoth) and Noctuidae. Their flowers have characteristics that attract moths, such as fluorescent colors that stand out at night and strong fragrance, and their pollen threads have an appropriate structure to allow hovering insects to easily transfer the pollen.

In contrast, *Oenothera* species produce cloned seeds before flowering by automatic self-pollination in breeding experiments in their natural habitats (Cleland, 1972). This mode of reproduction is known as 'permanent

translocation heterozygotes' (Raven, *et al.*, 1979). Several individuals of *Oenothera* species—which have 'automatic self-pollination' and 'permanent translocation heterozygotes'—do not produce seeds from outcross pollination in natural habitats (Brown and Levin, 2011). Naturalized plants often have low population densities in new habitats, and hence, few plants are available for reproduction, highlighting the advantage bisexual individuals that can reproduce by self-pollination (Baker, 1967). In fact, naturalized *Oenothera* species in Japan such as *O. laciniata* grow wild in areas with less natural habitat, which may make it difficult for moths to visit. Naturalized *Oenothera* species in Japan also exhibit 'automatic self-pollination' and 'permanent translocation heterozygotes' (Sakamoto and Hirota, 2015). These characteristics likely contributed to expanding the distribution of *Oenothera* species in Japan. Particularly, *O. laciniata* grows in urbanized environments compared to other *Oenothera* species, suggesting that it likely exhibits 'automatic self-pollination' and 'permanent translocation heterozygotes'.

In contrast, Sakamoto and Hirota, (2015) reported the presence of 'thrum type' flowers in *O. laciniata* in Japan; it has shorter pistils than stamens. They highlighted the possibility that *O. laciniata* may exhibit distyly, which is a trait evolution that avoids self-fertilization and promotes outcrossing (Ganders, 1979; Thompson, *et al.*, 1998; Sakamoto and Hirota, 2015). Hence, *O. laciniata* in Japan might be not only show self-pollination but also partial outcrossing. The natural distribution environment of *O. laciniata* is wider than other *Oenothera* species and it grows in urban, rural, and seaside areas. In addition, the flowering period of *O. laciniata* is long and lasts from spring to autumn, whereas other *Oenothera* species flower only in summer (Inagaki, unpublished). Hence, the success of outcrossing in *O. laciniata* likely depends on the distribution and the timing of occurrence of pollinators.

The present study aimed to investigate the changes in the characteristics of *O. Laciniata* flowers, such as flower size and length of stamen and pistil under different environmental conditions and seasons in Japan.

Material and Methods

Study Area

Two areas where *O. laciniata* growing naturally were identified in the Suruga ward of Shizuoka city, Japan. These included: 1) Area A near Ohyagawa River on the east side of the Shizuoka Plain (Ohya) (34.9644N, 138.4266E), and 2) area B near the Abe River on the west side of the Shizuoka Plain (Mukoshikiji) (34.9623N, 138.3590E). In each area, three different natural habitat environments were selected, including "roadside," "park" managed by mowing, and "sand" containing sand and gravel soils. For "sand," areas A and B included beach and riverbank, respectively.

Seasonal Changes in Flowering Time

The surveys to study seasonal changes were conducted April 19-21, June 21-23, and August 21-23, 2021. The flowering times of *O. laciniata* in all three habitats of area B were observed hourly from 5:00 AM to 7:00 PM.

Seasonal Changes of Flower Characteristics

A total of 30 plants were randomly selected from six natural populations in areas A and B, and flowers were collected from one by one from one plant on April 27, July 22, and August 25, 2021. Thereafter, the lengths of petals, stamens, and pistils of the collected flowers were measured. For convenience, we classified flowers into three types based on the ratio of stamen length to pistil length: "normal type," "thrum type," and "pin type" with ratios between 0.8 and 1.2, < 0.8, and > 1.2, respectively. Furthermore, we also identified some flowers as "close types" if the column head of the flower does not open. Note that the flowers of *O. laciniata* are divided into four parts.

Cultivation Test

Plants which have three different type of flowers, namely the pin type, thrum type, and

close type, were collected from sand in area B on June 21, 2021. The collected plants were grown in a greenhouse at the Center for Education and Research in Field Sciences, Shizuoka University (Kariyado, Fujieda city, Shizuoka Prefecture, Japan; 34.9059N, 138.2722E), and the type of all flowers that flowered between June 21 and July 21, 2021 were recorded.

Data Analysis

Data from this study were analyzed using BellCurve for Excel 5.0 software (Social Survey Research Information Co., Ltd.). To analyze seasonal changes in petal length, an analysis of variance was conducted, and then the Tukey’s multiple range test was used to detect significant differences among the treatments with a probability of 95% ($\alpha = 0.05$). The multi-way analysis of variance was used to analyze the factors affecting petal length and percentage of normal type flowers.

Results

Seasonal Change of Flowering Time

Fig. 1 shows the seasonal changes in the flowering time of *O. laciniata* for all three habitats in area B. Air temperatures in the figure represent data from April 19, June 21, and August 21, 2021, which were the first days of each investigation period. The three habitats of area B showed the same flowering time for all investigation periods. In April, all flowers bloomed until 9:00 AM. Thereafter, some flowers wilted between 10:00 AM to 4:00 PM, but others continued blooming. During daytime, *Eristalomyia tenax* and *Stomorhina obsoleta* visited the flowers (data not shown). New flowers of *O. laciniata* bloomed around 5:00 PM. In July, wilted flowers were already observed at 5:00 AM, no blooming flowers were observed at 7:00 AM. In August, wilted flowers were observed at 5:00 AM, and no blooming flowers were already observed at 6:00 AM.

Times of Day		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Apr.19	air temp.(°C)	7.3	8.1	10.8	13.1	14.8	15.7	17.8	18.7	19.0	18.8	18.5	18.5	17.4	17.0	16.4	14.5
	Park	○	○	○	○	○	△	△	△	△	△	△	△	○	○	○	○
	Roadside	○	○	○	○	○	△	△	△	△	△	△	△	○	○	○	○
	Sand	○	○	○	○	○	△	△	△	△	△	△	△	○	○	○	○
Jun.19	air temp.(°C)	21.0	21.3	22.8	25.1	25.8	25.0	25.9	25.9	26.3	25.8	24.8	24.8	23.7	23.8	23.1	22.8
	Park	△	△	×	×	×	×	×	×	×	×	×	×	×	×	○	○
	Roadside	△	△	×	×	×	×	×	×	×	×	×	×	×	×	○	○
	Sand	△	△	×	×	×	×	×	×	×	×	×	×	×	×	○	○
Aug.21	air temp.(°C)	22.8	23.1	24.2	24.7	24.4	25.2	27.2	27.6	27.8	28.5	27.5	27.8	27.5	27.1	27.1	26.4
	Park	△	×	×	×	×	×	×	×	×	×	×	×	×	×	○	○
	Roadside	△	×	×	×	×	×	×	×	×	×	×	×	×	×	○	○
	Sand	△	×	×	×	×	×	×	×	×	×	×	×	×	×	○	○

○: Blooming
 △: Wilted and blooming
 ×: No blooming

Fig 1: Seasonal flowering times for three natural populations, including park, roadside, and sand

Seasonal Changes in the Petal Length of Flowers in Park, Roadside, and Sand

Fig. 2 shows the seasonal changes in the petal length of flower in three natural populations, including park, roadside, and sand, for both areas A and B. No difference was observed in the petal length between the three habitats in April in area A (Fig.2 a). Here, petal length tended to decrease for all three populations,

as the season progressed through June and August. The petal length reduction was more pronounced in flowers collected from sand, with significantly shorter petal length of the flowers collected from sand habitat in June and August compared to those park and roadside.

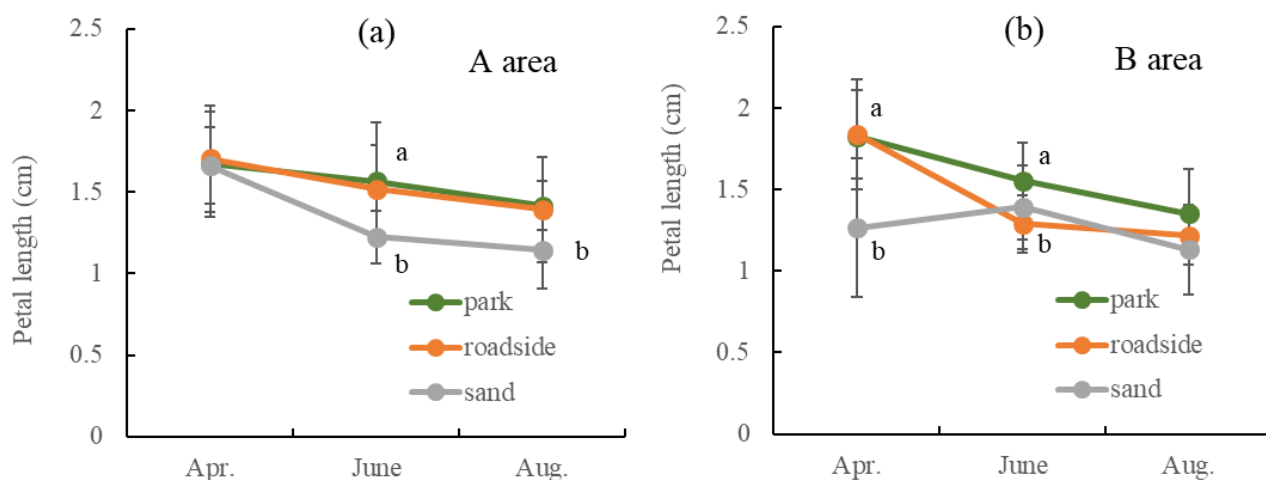


Fig 2: Seasonal changes in the petal length of *O. laciniata* flowers in three natural populations, including park, roadside, and sand, among areas A and B. Error bar represents standard deviation. Different letters indicate significantly different at $p < 0.05$ (Tukey's multiple range test)

In area B, the petal length was significantly shorter in sand than in roadside and park in April (Fig.1 b). The flowers from roadside and park showed reduction in petal lengths as the season progressed through June and August. The petal length reduction was more pronounced in the flowers collected from roadsides than those from parks. The petal length of flowers were shorter on roadsides and sand in June than in parks. No differences in petal lengths were observed among the three populations in August.

Seasonal Changes of Proportion of Flower Types in Three Natural Populations, Including Park, Roadside, and Sand

Variations in the lengths of stamens and pistils were observed. In addition to "normal type," "thrum type," "pin type," and "close type," we study differentiated pin and thrum flower types with close column heads as Pin-close type and Thrum-close type, respectively.

Fig. 3 shows the proportion of each flower types, including normal type, pin type, thrum type, close type, pin-close type, and thrum-close type, in park, roadside, and sand. In park of area A, the percentage of normal type and thrum type in April was 68% and 32 %, respectively. In June, the percentage of normal type was approximately the same as in April, and in addition to the thrum type, there was also the occurrence of pin type. The roadside habitat of area A showed contrary trend to the park as the percentage of normal type decreased as the season progressed. Here, the percentages of pin type and thrum type flowers were approximately similar. In the sand habitat in area A, the percentage of normal type was approximately 50 % throughout the investigation period and did not vary significantly with season. In addition, while both pin type and thrum type were observed in April, only pin type was observed from June onwards in the sand in area A.

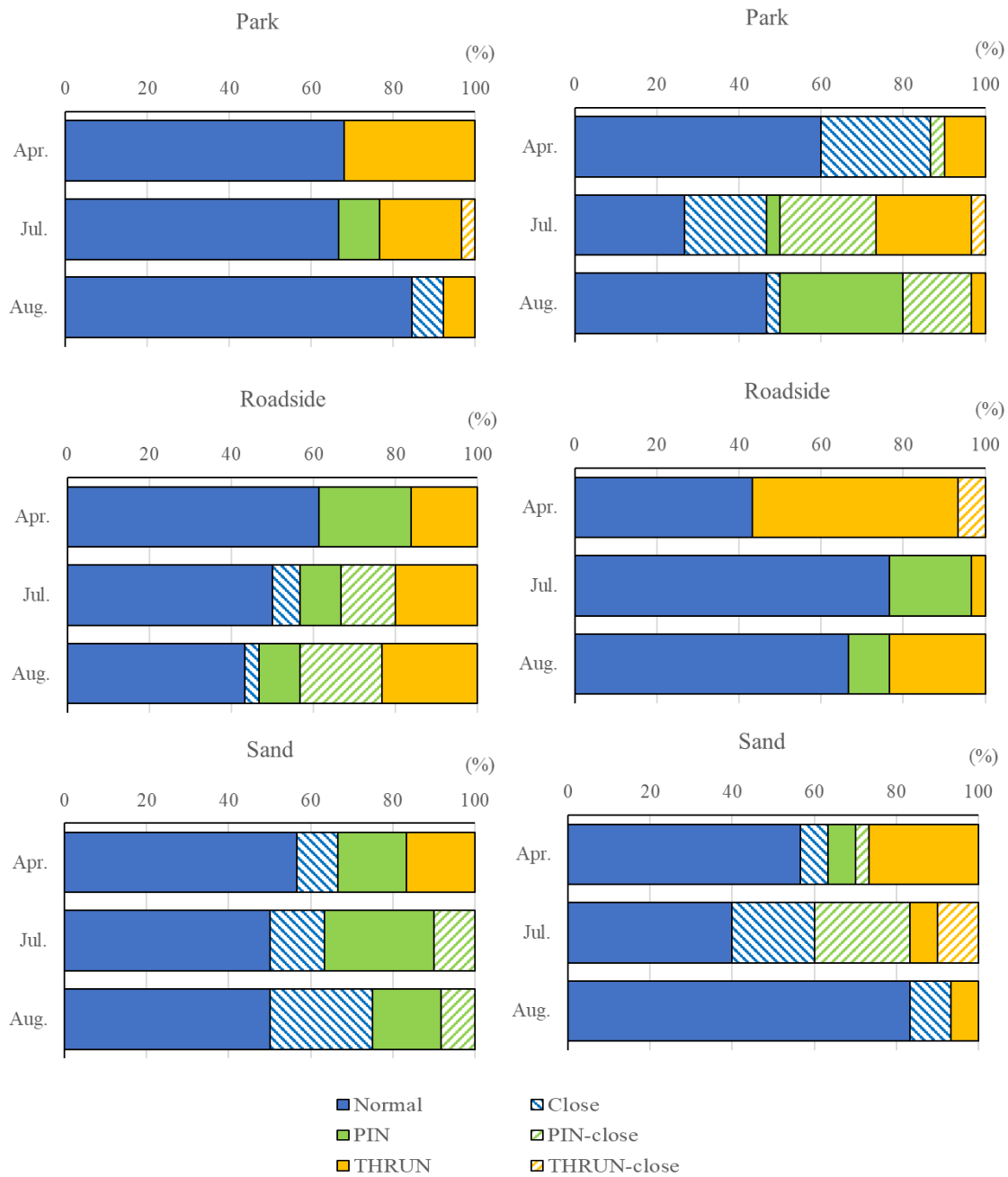


Fig 3: The proportion of each flower types, including normal type, pin type, thrum type, close type, pin- close type, and thrum- close type, of *O. laciniata* in three natural populations, including park, roadside, and sand in (a) area A, and (b) area B

In the park of area B, the percentage of normal type decreased in June and August compared with that in April. In addition, the park was characterized by the frequent appearance of close type. In contrast, there was little occurrence of closed type in the roadsides in area B. In the roadside in B area, the percentage of thrum type was high, and the percentage of normal types was low in April, whereas the percentage of normal type

increased in June and August. In addition, pin type also occurred in June and August. In the sand in B area, there are both pin type and thrum type in April. In June, the percentage of close type increased, whereas percentage of normal type decreased. In contrast, the percentage of normal type increased in August.

Discussion

Most *Oenothera* species are generally pollinated by moth (Sakamoto and Hirota, 2015), but *O. laciniata* has likely developed self-pollination (Cleland, 1972). It is well known that *O. laciniata* blooms from evening to nighttime; however, the results of the present study indicated that though the flowers of *O. laciniata* bloomed from evening to night in June and August, they continued to bloom in the morning and throughout the daytime in April. In addition, we observed that the pollinators such as hoverflies and fly visit the flowers of *O. laciniata* during the daytime in April. Hoverflies prefer yellow flowers, which may be the reason behind many yellow flowers blooming in early spring, when the hoverflies are primarily active as pollinators rather than bees. The yellow flower color of *O. laciniata* is intended to make it stand out at night, which attracts hoverfly. Moreover, *O. laciniata* likely blooms during the daytime in spring to actively attract a mate for the pollinators. In fact, this study indicated that the flower size of *O. laciniata* is larger in spring compared with that in summer, and largeflowers have the advantage for attracting more pollinators (Kudoh and Whigham, 1998). This suggests the possibility that *O. laciniata* can reproduce by partial outcrossing in the spring by pollinator, such as hoverfly.

A previous study has reported the presence of "thrum type" flowers of *O. laciniata* in Japan (Sakamoto and Hirota, 2015). The presence study also found the presence of 'pin type' flowers in *O. laciniata*. To the best of our knowledge, this is a first report highlighting the occurrence of such variation in the flowers of *O. laciniata*. However, the occurrences ratio of pin type and thrum type varied with season and natural habitats. Therefore, it was considered to be variation in the length of the pistil and stamens and not distyly. We also reported the presence of close type of flowers whose column head did not open. In addition, it was indicated close type are compatible with pin type and thrum type and pin type. These characteristics can likely be associated with genetics, i.e., plants having pin type did

not produce thrum type flowers, and plants having thrum type did not produce pin type flowers. However, these plants having pin type or thrum type also exhibited normal type flowers. This variation of length of the pistil and stamens is a disadvantageous trait for automatic self-pollination (Sakamoto and Hirota, 2015). This suggests that the variation of length of the pistil and stamens may be partially assisting outcrossing, while based on self-pollination. Although the self-pollination is beneficial for plants in the short term, loss of genetic diversity is disadvantageous in the long term (Stebbins, 1957). It was reported that the game theoretic model indicated that partial outcrossing is most advantageous, and in fact, there are many examples of autogamous plants that reproduce altogether with outcrossing and show intermediate rates of self-fertilization (Sakai and Ishii, 1999). While *O. laciniata* is also acquiring self-pollination, it may be developing a new mechanism that allows it to outcross.

In the present study, we assumed that parks are relatively easy for pollinators to visit, whereas roadsides and sandy areas are environments where pollinators are scarce, and hence, self-pollination is required. In addition, it is also inferred that it has a tendency to outcross in the spring and an increased tendency to self-pollinate in the summer, if *O. laciniata* is outcrossing in the spring. However, the occurrence of such these atypical flower columns flower such as pin type, thrum type, and close type, in *O. laciniata* is independent of season and natural habitats, and it is completely random. Therefore, it is unclear to what extent the appearance of these atypical flower columns affects the fitness. Previous studies have shown that eggplant exhibits variation in the length of stamens and pistil that are similar to the pin type and thrum type (Saito, 1963), and flower having shorter the pistil increase under poor nutrient soil condition (Koga, *et al.*, 2010). This suggests that the variation of length of stamens and pistil of *O. laciniata* may depend on environmental conditions.

Conclusion

It is suggested that *O. laciniata* may have developed self-pollination, which is an advantageous trait for a naturalized plant (Cleland, 1972). The findings of the present study suggest that the flowers of *O. laciniata* are in bloom during daytime and pollinators visit the flowers in spring. In addition, flower size in spring is larger than that in summer, and flowers likely have a tendency to undergo partially outcross pollination during spring. Moreover, in addition to thrum type flowers of *O. laciniata*, which have been reported previously, we found the presence of pin type and close type flowers of *O. laciniata*. However, we considered that these atypical flowers do not show distyly, but are structural variations in flowers. We suggest that these variations of flowers increase opportunity of outcrossing. It would be interesting to explore whether these variations of flower are characteristics of naturalized *O. laciniata* populations in Japan or if they also occur in the native populations. In future, it will be necessary to elucidate such variations in characteristics of flowers in other natural habitats, including the native habitats.

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