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Asai, Takeshi
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Specialized petal with conspicuously fringed margin influences reproductive success in *Habenaria radiata* (Orchidaceae)

Kenji Suetsugu¹  | Yusuke Abe¹ | Takeshi Asai² | Shuji Matsumoto² | Masahiro Hasegawa³

¹Graduate School of Science, Kobe University, Kobe, Japan

²Himeji Tegarayama Botanical Garden, Himeji City, Japan

³Osaka Museum of Natural History, Osaka, Japan

Correspondence

Kenji Suetsugu

Email: kenji.suetsugu@gmail.com

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Most angiosperms depend on animals for pollination and have evolved floral traits tailored to pollinators with diverse morphologies and behaviors. Although petal diversity is often assumed to have co-evolved with the visual preferences of pollinators, this does not necessarily account for all floral diversity. Petals can serve many functions, including storing nectar, ensuring pollen attachment to pollinators, releasing scent attractants, or serving as landing platforms or footholds for pollinators (reviewed by Endress & Matthews, 2006; Katsuhara et al., 2017). In the Orchidaceae, the morphologically specialized petal known as a lip or labellum is likely to serve as a visual attractant and landing platform (Rudall & Bateman, 2002).

Orchids exhibit a marked diversity of lip structures, with variations in size, form, and markings that substantially differ from the two lateral petals (Rudall & Bateman, 2002). For instance, the common name for *Habenaria radiata*—the white egret orchid—is derived from its exquisite lip that resembles the graceful flight of a white egret. The lip has three lobes, with two side lobes extending laterally that are highly fringed and have a characteristic plume-like or wing-like shape, whereas the central lobe lacks ornamented margins (Figure 1a,b; Tachibana et al., 2021). According to legend, the spectacular floral morphology was born of the

soul of an exhausted egret (Kimura, 1982). However, the actual processes that drove the evolution of the white egret-like floral morphology remain elusive.

The tremendous variation in floral presentation among different orchid species is often ascribed to specialized interactions between each orchid and its narrow spectrum of pollinators (Cozzolino & Widmer, 2005). We predicted that the complex lip morphology of *H. radiata* had evolved to increase its pollinator-mediated reproductive success. *Habenaria radiata* is pollinated primarily by nocturnal hawkmoths, *Theretra japonica* and *Theretra nessus* (Sphingidae) (Shigeta & Suetsugu, 2020) and diurnal skipper butterflies, *Parnara guttata*, *Polytremis pellucida*, and *Pelopidas mathias* (Hesperiidae) (Ikeuchi et al., 2015; Suetsugu & Tanaka, 2014). The hawkmoths typically hover on the flowers without landing on them, whereas the skippers perch on the flowers when seeking nectar (Ikeuchi et al., 2015; Suetsugu & Tanaka, 2014). Consequently, we hypothesized that the lip is vital as a visible marker for hawkmoths and a visual marker and landing place for skippers.

Although the adaptive significance of floral traits can be assessed by examining naturally occurring variation, this often provides no evidence of natural selection,





FIGURE 1 *Habeneria radiata* and its pollinators. (a, b) Intact flower. (c) Flower after fringe removal. (d) The skipper *Pelopidas mathias* visiting an intact flower, with pollinaria attached to its compound eye. (e) The hawkmoth *Theretra oldenlandiae* visiting an intact flower. (f) The hawkmoth *T. oldenlandiae* visiting a flower after fringe removal.

probably because phenotypic variance is relatively constrained under natural conditions. By contrast, experimental manipulation of floral characteristics can circumvent this limitation, providing conclusive evidence for selection (Cuartas-Domínguez & Medel, 2010; Lamborn & Ollerton, 2000). Therefore, to investigate whether the highly fringed lip morphology of *H. radiata* affects its reproductive success, we experimentally manipulated the floral characteristics by removing the lobe fringes of flowers in their natural populations (Figure 1c). The fringe removal did not affect fruit set (the number of fruits/number of flowers [%]), but it did reduce seed production (particularly the proportion of well developed seeds or seed set [%] and marginally the weight of total number of seeds in a capsule [from this point forward seed weight; mg]). Therefore, to investigate the reasons for the reduction in seed production,

we observed the pollination behavior of the hawkmoth and skipper in the laboratory in detail (Appendix S1).

Our field studies showed that no thrips-mediated fruit set occurred in the populations investigated in this study, although thrips occasionally deliver pollen grains to *H. radiata* stigmas (Shigeta & Suetsugu, 2020). By contrast, the *H. radiata* pollinarium, or multiple pollinaria, were observed to be attached to the compound eyes of the hawkmoths and skippers, confirming that they are effective pollinators (Figure 1d,e). Nonetheless, the skipper proboscis (16.9 ± 1.2 [mean \pm SD] mm in *Parnara guttata*, $n = 6$; 16.2 ± 0.4 mm in *Pelopidas mathias*, $n = 6$; and 16.0 ± 0.02 mm in *Polytremis pellucida*, $n = 2$) is much shorter than the depth from the *H. radiata* spur entrance to the nectar (26.3 ± 3.9 mm, $n = 171$). Therefore, despite its nectariferous habit, skippers gain no rewards from *H. radiata*. The skippers typically visited a



few flowers before leaving, potentially after realizing the lack of a nectar reward. By contrast, the hawkmoth proboscis length (34.9 ± 2.5 mm in *Theretra japonica*, $n = 15$; 36.1 ± 0.7 mm in *Theretra oldenlandiae*, $n = 4$) was similar to that of the *H. radiata* spur (35.6 ± 3.3 mm, $n = 171$), indicating that hawkmoths can easily access the nectar. Their proboscis is also not long enough to enable them to steal nectar without providing pollination services. These hawkmoths often visit dozens of flowers to obtain nectar, collecting multiple pollinaria on their compound eyes. This suggests that *H. radiata* is morphologically more adapted to pollination by hawkmoths than that by skippers, and that hawkmoths are likely to be more reliable *H. radiata* pollinators.

Contrary to our expectations, our experimental manipulation revealed that the fringed lip structures played a limited role in pollinator attraction. Fruit set in flowers without fringe removal was even higher, although not significantly (Figure 2a; 44.0%, $n = 75$, $p = 0.104$) than that in flowers with intact fringes (31.2%, $n = 77$; Appendix S1: Table S1). It is known that nocturnal hawkmoths require visual cues for approaching flowers and probing them (Goyret et al., 2007), indicating that a visual display may be crucial even for nocturnal moth-pollinated plants. Deeply divided or fringed petal lobes are particularly common among nocturnal hawkmoth-pollinated species (Mitchell et al., 2015). The repeated evolution of a fringed lip in *Platanthera* (Orchidaceae) is associated with hawkmoth pollination (Hapeman & Inouye, 1997). In addition, in *Trichosanthes* (Cucurbitaceae), the loss of the long-fringed flower coincides with a pollinator shift from predominantly nocturnal sphingids to diurnal bees or butterflies (de Boer et al., 2012). Given that flowers with fringes should be able to conserve resources better than those of the same diameter without fringes, fringed petals have been considered an energy-efficient adaptation to exploit the hawkmoth preference for large flowers with deeply divided margins (Mitchell et al., 2015). However, we also found that the lip area did not affect the fruit set ($p = 0.34$). Therefore, in *H. radiata*, the visual display of individual flowers may not be crucial for attracting pollinators.

By contrast, seed weight (Figure 2b; 8.1 ± 5.7 mg, $n = 31$, $p = 0.06$) and the proportion of well developed seeds (Figure 2c; $40.5 \pm 30.9\%$, $n = 31$, $p < 0.0001$) were lower in flowers without fringes than those in flowers with intact fringes ($58.9 \pm 30.0\%$ and 11.7 ± 5.7 mg, respectively, $n = 23$; Appendix S1: Table S1). Our artificial pollination experiments revealed that fringe removal does not affect seed production ($p > 0.5$; Appendix S1: Table S1). Therefore, differential seed production is caused by differences in pollen quality or quantity received

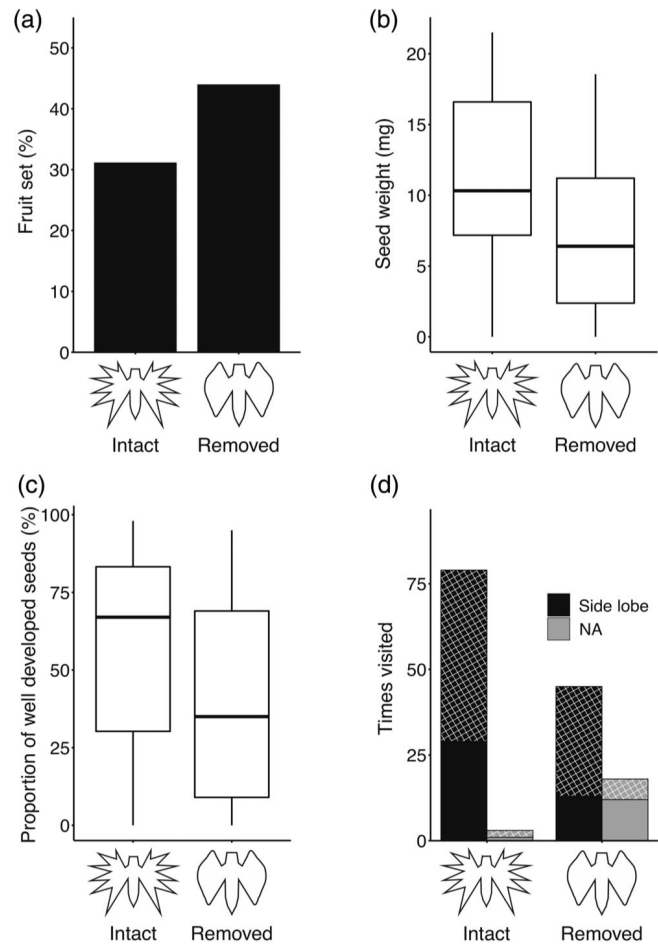


FIGURE 2 Effects of fringe removal on reproductive success [(a) Fruit set, (b) seed weight, and (c) proportion of well developed seeds], and (d) hawkmoth behavior. Black bars: frequency of hawkmoths grasping the side lobe with the midlegs. Gray bars: frequency of hawkmoths touching no floral parts while visiting flowers. Filled bars: experiments with real flowers. Cross-hatched bars: experiments with artificial flowers.

and not the damage caused by fringe removal. If fringe removal reduces the pollen quality, this may be because flowers without fringes are a lower priority for pollinators and are visited after their genetically related intact neighbors. This hypothesis was based on the observation that well developed seeds occurred less frequently in self-pollinated flowers ($18.3 \pm 15.5\%$, $n = 17$, $p < 0.0001$) compared with cross-pollinated flowers ($47.1 \pm 23.8\%$, $n = 16$; Appendix S1: Table S1). However, our presentation experiment revealed that fringe removal affected neither the general floral choice (152 vs. 160 visits to flowers without fringes vs. intact flowers, $p = 0.81$ in hawkmoths; and 5 vs. 4, $p = 1$, in skippers) nor the first floral choice (6 vs. none in hawkmoths and 2 vs. 1 in skippers). Therefore, reduced seed production following fringe removal may be caused by a reduction in the number of pollen grains received per visit.



Our experiment may also explain why flowers without fringes received fewer pollen grains per visit. Most studies have indicated that the hawkmoth forages while hovering like a hummingbird. However, we have shown that the hawkmoth usually lands on the *H. radiata* flower, and the claws on its legs cling to the lateral lobe fringes or lateral petals. When sucking nectar from intact flowers, the hawkmoths always grasped the lateral petals or lip fringes with their forelegs (91/91) and the lip fringe with their midlegs (Figures 1e and 2d; Video S1; 79/82). The similarity in length between the hawkmoth proboscis and *H. radiata* spur brings the hawkmoth very close to the flowers, probably making continuous hovering impossible, and causing the moth to grasp during foraging. However, when sucking nectar from flowers with their fringes removed, the hawkmoth midlegs occasionally failed to grip any floral parts (Figures 1f and 2d; Video S2; Appendix S1: Table S1; 18/63, $p < 0.0001$). This observation strongly suggested that the hawkmoth adopts an unstable posture when the fringe is absent. By contrast, all the skippers landed and perched on the side lobe of the lip, regardless of the presence of fringes. Therefore, lower seed production is likely to be due to the hawkmoths' difficulty in grasping the lip without the presence of fringes. This possibility needs to be verified by counting the number of pollen grains transferred to the stigma after hawkmoth visits to flowers with and without fringes.

To the best of our knowledge, this study is the first to investigate experimentally the adaptive significance of long fringes in orchids. Given that the hawkmoths have been considered to hover while foraging, we first hypothesized that the conspicuously fringed lip served as a visual guide. However, our experimental manipulation of floral characteristics demonstrated that the fruit set was not lower following fringe removal, whereas seed production was reduced significantly. In addition, our experiment indicated that the hawkmoths often grasped fringes while foraging, although they still continued to beat their wings. Taken together, our results strongly suggested that the fringe primarily stabilized the hawkmoth's posture, thereby increasing pollen deposition on the stigma. It is well known that angiosperm petals act as visual attractions, but their other functions have not received much attention. Further experiments are needed to gain a deeper understanding of petal function and diversity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data (Suetsugu et al., 2022) are available in Figshare: <https://doi.org/10.6084/m9.figshare.19386548.v1>.

ORCID

Kenji Suetsugu  <https://orcid.org/0000-0002-7943-4164>

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SUPPORTING INFORMATION

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

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