

Floral Morphology and Pollinator Fauna of Sister Species *Primula takedana* and *P. hidakana* in Hokkaido Island, Japan

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Abstract We compared the floral morphologies and pollinator fauna of two sister taxa, *Primula hidakana* and *P. takedana*, to obtain a better understanding of the pollination systems of these species. Observations of their floral characteristics revealed that both species are strictly distylous. The flower shape of *P. hidakana* has a much larger corolla than that of its sister species, and the diameter of its corolla tube mouth was approximately half that of *P. takedana*. Observations of flower visitors revealed that pollinators of *P. takedana*, which were small, short-tongued insects, varied more than those of *P. hidakana* did. The diversity of pollinator fauna in *P. takedana* implies that *P. takedana* is unable to restrict the type of insect visitor due to flower structure constraints. We conclude that differences in floral structure between these sister primrose species are associated with pollinator fauna differentiation, which may have contributed to their speciation.

Key words: distyly, floral morphology, pollinator, *Primula*, time lapse photography.

Introduction

Primula hidakana (Miyabe & Kudo) Nakai and *P. takedana* Tatew. (Primulaceae) are perennial clonal herbs endemic to Hokkaido Island, Japan. *Primula hidakana*, which is found in southern Hokkaido Island, grows on wet, shaded rocky cliffs in the mountains, whereas *P. takedana* appears on denuded slopes of gray clay mixed with sharp grit from serpentine rocks in northern Hokkaido Island (Fig. 1a). Recent molecular phylogenies and karyotyping have indicated that these two species represent sister taxa (Richards, 2003; Yamamoto *et al.*, 2017),

and our ongoing population demographic study based on multiple microsatellite loci suggests that *P. takedana* evolved from *P. hidakana* during the late Pleistocene (Yamamoto *et al.*, unpublished).

As in most *Primula* species, *P. takedana* and *P. hidakana* flowers are distylous (Richards, 2003), displaying long- and short-styled morphs (Fig. 1b) within a population. Many distylous plants share a common floral morphology involving radial symmetry, including a simple open corolla with a restrictive floral tube (Lloyd and Webb, 1992), which may play an important role

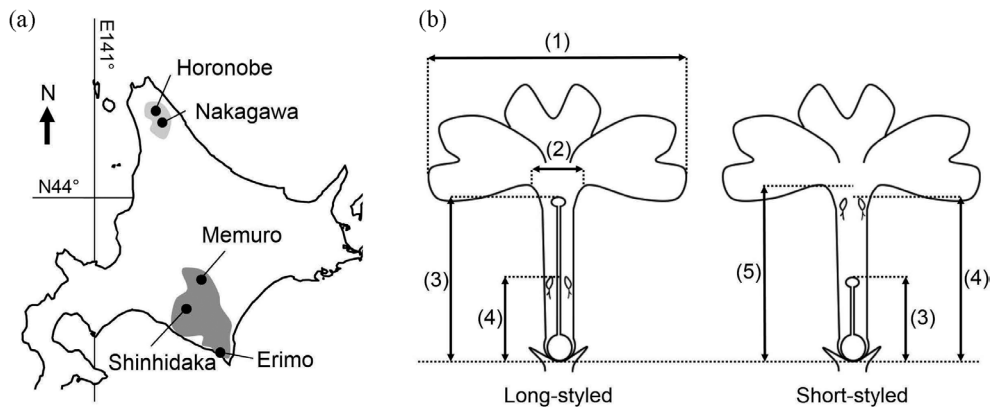


Fig. 1. (a) Presumed distribution ranges of *Primula hidakana* (dark gray) and *P. takedana* (light gray), and locations of study sites. (b) Diagram of distylous flowers and morphometric parts measured in flowers: (1) corolla width; (2) diameter of corolla tube mouth; (3) stigma height; (4) anther height; (5) tube length.

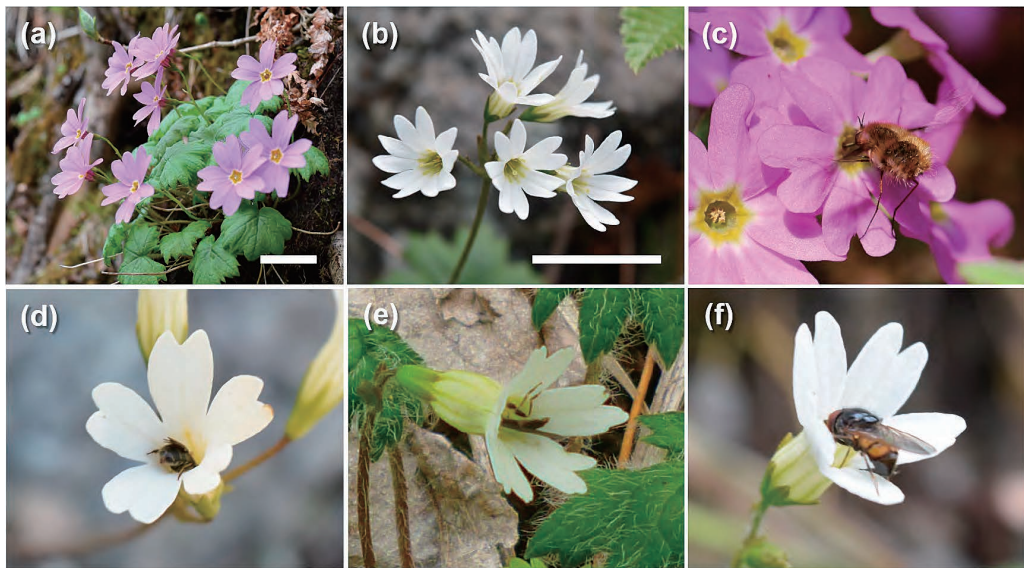


Fig. 2. Photographs of *P. hidakana* and *P. takedana* pollination systems. (a) *P. hidakana*. (b) *P. takedana*. (c) Bee fly (Bombyliidae sp.) visiting *P. hidakana*. (d) Sweet bee (*Lasioglossum* sp.) visiting *P. takedana*. (e) Syrphid fly (*Melanostoma* sp.) visiting *P. takedana*. (f) Syrphid fly (*Rhinga laevigata*) visiting *P. takedana*. Scale bars = 20 mm.

in ensuring efficient pollination between morphs by the segregation of pollen grains from different morphs on the pollinator's body (Lloyd and Webb, 1992). Accordingly, legitimate pollination requires long-tongued insect visitors such as bumblebees, butterflies, and bee flies (Washitani *et al.*, 1995; Huang *et al.*, 2015). Although molecular phylogeny supports the clustering of

P. hidakana and *P. takedana* and their recent evolution, their floral structures exhibit distinct differentiation. The *P. hidakana* flower is a typical primrose with simple, pink petals and a narrow corolla tube (Fig. 2a), whereas the *P. takedana* flower is white, sweetly fragrant, and bell-shaped, with a funnel-shaped corolla tube (Fig. 2b) (Yamazaki, 1993; Richards, 2003). Although

these marked differences in floral structure between sister species have been recognized, no quantitative analysis of their flower morphology or visiting insect fauna has been conducted.

The objective of this study was to survey the floral morphologies and pollinator fauna of *P. hidakana* and *P. tookadana* to clarify the relationship between differences in their floral morphology and pollination systems.

Materials and Methods

Floral morphology

Measurements of floral traits and pollinator observations were conducted in 2016 among three populations each of *P. hidakana* and *P. tookadana* on Hokkaido Island, Japan (Fig. 1a and Table 1). To examine the floral traits of the two morphs (pin: long styled and thrum: short styled) of each species, 25 flowers each of pin and thrum were randomly collected from each population. The collected samples were fixed in 70% FAA solution (5% formaldehyde, 5% acetic acid, and 90% alcohol). In total, 300 flowers were sampled and classified by flower morph. We measured five characteristics of the sampled flowers using digital calipers: corolla width, diameter of corolla tube mouth, tube length, anther

height, and stigma height (Fig. 1b). To evaluate the flower color of each species, flower petal reflectance was measured between 300–700 nm using a spectrometer (Ocean Optics, Dunedin, FL, USA). Reflectance measurements were conducted for three individuals of each species.

Pollinator observations

Observations of floral visitors to *P. hidakana* and *P. tookadana* flowers were conducted using time-lapse images taken with a waterproof digital camera (Pentax Optio WG-1, Tokyo, Japan). Each camera was mounted on a tripod in front of the plant; the time interval between images was 30 seconds. Each insect visiting a flower was counted, and the photographs were used to identify the insect. Because no night visiting insects to each primrose were found in a preliminary phase, all pollinator observations were performed during daytime. At the same time as the camera recording, we captured insects visiting *P. tookadana* flowers using butterfly nets for species identification. The direct observation was conducted with the exception of individuals for the camera recording. Each collected insect was placed in a microtube containing a piece of cotton wool soaked with chloroform and preserved at -18°C until observation.

Table 1. Study sites and details of the time-lapse photography conducted in this study

Site	Date	Time	Number of		
			Flowers	Images	Insect visits
<i>Primula hidakana</i>					
Memuro	2016/5/9	07:00~19:00	11	5,951	1
Erimo	2016/5/12	14:00~21:00	22	8,397	0
	2016/5/13	10:00~17:00	30	9,142	2
	2016/5/14	09:00~16:00	29	9,586	1
Shinhidaka	2016/5/17	11:00~17:00	29	6,125	0
	2016/5/18	07:00~15:00	32	8,947	9
Total			153	48,148	13
<i>P. takedana</i>					
Nakagawa	2016/5/24	10:00~16:00	12	3,868	8
	2016/5/26	07:00~15:00	22	7,606	10
	2016/5/27	09:00~16:00	27	8,002	2
Horonobe A	2016/5/26	07:00~20:00	21	14,263	22
	2016/5/27	09:00~16:00	29	8,650	10
Horonobe B	2016/5/29	07:00~21:00	36	21,692	21
	2016/5/30	07:00~15:00	29	10,636	15
Total			176	74,717	88

Table 2. Mean $\pm$ standard deviations (SD) of floral characters in the floral morphs of *P. takedana* and *P. hidakana*

Character (mm)	<i>P. takedana</i>					<i>P. hidakana</i>				
	Long-styled (75)		Short-styled (75)		Significance between morphs	Long-styled (75)		Short-styled (75)		Significance between morphs
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Corolla width	15.13	1.85	13.99	1.63	*	27.35	3.53	26.25	3.08	*
Diameter of corolla tube mouth	3.51	0.49	4.06	0.43	*	2.23	0.51	2.25	0.36	n.s
Tube length	8.57	1.02	10.56	1.14	*	10.14	0.98	11.88	1.05	*
Anther height	5.22	0.79	8.47	0.99	*	6.37	0.61	11.07	0.89	*
Stigma height	9.20	1.04	4.26	0.58	*	10.00	1.00	5.31	0.61	*

Values in parentheses represent sample sizes.

Asterisks (*) denote significant differences (Mann–Whitney U-test) between morphs ($P < 0.05$).

Results

Floral morphology

Measurements of the floral morphology characters of the two *Primula* morphs are summarized in Table 2. All floral morphology characters differed significantly between the two morphs within each taxon, except mouth diameter in *P. hidakana*. In particular, we observed clear reciprocal herkogamy between flower morphs in each species (Table 2); *i.e.*, the anther height of the short-styled morph was significantly greater than those of the long-styled morph, and the stigma heights of the long-styled morph was significantly greater than those of the short-styled morph.

We compared the three measurements of corolla shape (corolla width, mouth diameter and tube length) at the species level (box-and-whisker plots, Fig. 3a–c). Overall, significant morphological differentiation was found between the sister species in all characters. *P. hidakana* had a significant larger corolla than did *P. takedana* (Fig. 3a, c), whereas the mouth diameter of *P. hidakana* was approximately half that of *P. takedana* (Fig. 3b).

Corolla reflectance was significantly different in *P. hidakana* than in *P. takedana*, suggesting significant floral color differentiation between the sister species (Fig. 3d).

Pollinator faunas

We collected a total of 122,865 images, of

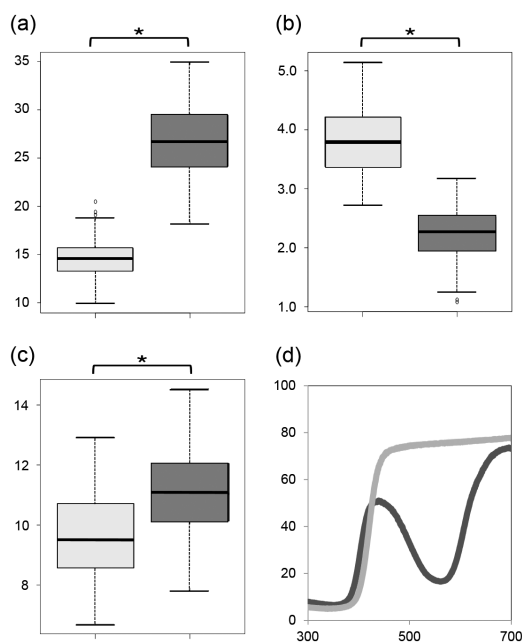


Fig. 3. (a)–(c) Box-and-whisker plots comparing three floral traits (mm) between *Primula* species: (a) corolla width; (b) mouth diameter; (c) tube length. Asterisks denote significant differences (Mann–Whitney U-test) found between species ($P < 0.001$). (d) Reflectance spectra of flowers: Y-axis represents reflectance percentages; X-axis represents wavelength (nm). In all figures, *P. hidakana* and *P. takedana* are shown in dark gray and light gray, respectively.

which 101 included insects visiting *P. hidakana* and *P. takedana* (Table 1). We identified 18 taxa in 12 families and four orders based on the photographs and specimens (Table 3). The photo-

Table 3. Number and identity of each floral visitor to *P. hidakana* and *P. takedana* flowers, identified by photographs and collection

Visitor*	<i>P. hidakana</i>			<i>P. takedana</i>		
	Memuro	Erimo	Shinhidaka	Nakagawa	Horonobe A	Horonobe B
Hymenoptera						
<i>Ceratina</i> sp.				1	1	2
<i>Ceratina megastigmata</i>						
Formicidae <i>Myrmica</i> sp.				5	2	8
Halictidae sp.		2	2		1	1
<i>Lasioglossum</i> sp.				8	3	8
<i>Lasioglossum duplex</i>						
<i>Lasioglossum problematicum</i>						
Ichneumonidae sp.						
Diptera						
Anthomyzidae sp.						
Asilidae sp.				1		4
Bombyliidae sp.			6			
Empididae sp.						
Muscidae sp.						
<i>Cheilosia abdominalis</i>						
<i>Episyrphus balteatus</i>						
<i>Melanostoma</i> sp.					11	2
<i>Melanostoma mellinum</i>						
<i>Melanostoma interruptum</i>						
<i>Rhingia laevigata</i>					5	4
<i>Sphegina violovitshi</i>						
Plecoptera						
Nemouridae sp.						
Coleoptera						
Nitidulidae sp.				4	2	2
Unidentified very small visitors	1	1	1	1	6	6

*Insect species shown in bold were collected directly on *P. takedana* flowers.

graphic evidence indicated that floral visitor fauna differed between the two species; most insect visitors to *P. takedana* were dipterans and hymenopterans, with a variety of species within these groups, whereas fewer small bees and a bee fly (Bombyliidae sp., Fig. 2c) visited *P. hidakana*.

From the photographic evidences, the most frequent hymenopteran visitors were the sweet bee *Lasioglossum* spp. (Halictidae, Fig. 2d) (19 individuals) and ants (*Myrmica* spp.; Formicidae). Among dipterans, two syrphid flies *Melanostoma* spp. (Syrphidae, Fig. 2e) (13 individuals) and *Rhingia laevigata* (Syrphidae, Fig. 2f) (9 individuals) were recognized as floral visitors, as was sweet bees. Most floral visitors to *P. takedana* were found in the corolla tube, unlike visitors to *P. hidakana* (Fig. 2d–f). The bee fly was not recorded visiting *P. takedana* despite its

being the most abundant insect found on *P. hidakana* flowers.

Discussion

The floral structures of *P. hidakana* and *P. takedana* indicated that they are strict distylous plants, which is likely a consequence of pollination between the two morphs, intermediated by efficient insect visitors. Bee flies have been frequently reported in previous pollinator observations studies of primroses in England (*P. elatior*, *P. veris*, and *P. vulgaris*: Christy, 1922; Woodell, 1960) and Japan (*P. sieboldii*: Takahashi *et al.*, 1992), and many previous works have noted the importance of long-tongued pollinator insects to mediate pollen transfer among morphs in *Primula* (e.g., Washitani *et al.*, 1995; Huang *et al.*, 2015).

The *P. hidakana* flower was characterized by a large, open, pink corolla with a restrictive floral tube. These floral traits are typical of distylous plants (Lloyd and Webb, 1992), and require long-tongued insect visitors to perform efficient legitimate pollination among morphs. Considering that floral tube diameter and length restrict the type of pollinator able to reach and obtain nectar and pollen inside the corolla (Dos Santos and Wittmann, 2000), the long-tongued flies (Bombyliidae spp., Fig. 2c) that we frequently observed may be an important pollinator for *P. hidakana*.

The size of the white, funnel-shaped *P. takedana* flower was significantly different from that of the *P. hidakana* flower. The pollinators observed on *P. takedana* flowers were small, short-tongued insects such as sweet bees and syrphid flies (Table 3). Although the relative contributions of these small, short-tongued insects to *P. takedana* pollination are still uncertain, previous investigations of *P. takedana* reproduction reported high fertility in each morph in natural habitats (Tsukui *et al.*, 1994). Thus, our results may differ from those of previous studies that found an insignificant contribution from short-tongued bees and syrphid flies to pollination. Campbell *et al.* (1986) indicated the importance of the syrphid fly *Helophilus groenlandicus* (Syrphidae) as an effective pollinator for *P. laurentiana* in Northeastern North America; however, this primrose is self-compatible, with long homostylous flowers. Zhu *et al.* (2015) suggested asymmetrical disassortative pollination in the distylous primrose, *P. secundiflora*, in alpine regions of southwestern China, and indicated that syrphid flies, *R. binotata* (Syrphidae), contributed to their pollination, with only syrphid flies moving pollen grains from the short-styled to the long-styled morph.

Thus, the diversity of *P. takedana* pollinators suggests that the primrose may not be able to restrict the type of insect visitors due to flower structure constraints, whereas *P. hidakana*, with its large, open, pink corolla and restrictive floral tube can accept only long-tongued insect visitors. Floral structure differentiation between these sis-

ter primrose taxa is associated with pollinator fauna differentiation that will likely cause further divergence between these species. Since Darwin (1877), *Primula* has been studied as a model plant for the evolution of heterostyly. However, pollination biology has been investigated in only a few *Primula* species (*e.g.*, Campbell *et al.*, 1986; Washitani *et al.*, 1995; Van Rossum *et al.*, 2006; Huang *et al.*, 2015; Zhu *et al.*, 2015). In this study, we identified distinct differentiation in pollinator fauna between the sister species of *Primula*, *P. hidakana* and *P. takedana*, as well as differences in floral traits. Further biological study is needed to elucidate the pollination biology of these species, such as direct observations of pollen grain adherence on the surface of insect bodies and the relationship between female fertility and guilds of floral visitors. Differentiation in floral color and scent are known to be triggers that can lead to pollinator shifts (*e.g.*, Vereecken *et al.*, 2010). Although we examined differences in the reflectance spectra *Primula* corolla colors (white/pink), floral scent should be also examined: *P. takedana* flowers emit a unique sweet scent that may contribute to attracting insect visitors. Thus, the combination of multiple floral traits and their potential effect on pollinator shifts should be considered in these sister species to determine the causes of the drastic evolution in floral traits that occurred in *P. takedana*.

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