Floral ecology of *Oreocharis acaulis* (Gesneriaceae): An exceptional case of "preanthetic" protogyny combined with approach herkogamy

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ABSTRACT

Protogyny is supposed to represent the ancestral form of dichogamy in the angiosperms, but is rare in advanced groups such as the Asteridae, in which protandry prevails by far. Here we report on an unusual form of protogyny combined with herkogamy in a Chinese species of Gesneriaceae (Asteridae–Lamiales): *Oreocharis acaulis* (formerly *Opithandra acaulis*). This is characterized by a conspicuous protrusion of the style from the flower bud and the stigma becoming receptive before corolla opening (female-only stage; preanthetic protogyny) and both sexes staying functional during anthesis (hermaphroditic stage), with the stigma presented above the anther level (approach herkogamy). The plants studied were found to be self-compatible, but autonomous self-pollination and apomixis were not observed. Successful pollination was found to depend fully on the presence of insect pollinators (*Bombus* sp.). The visiting frequency was higher in the hermaphroditic stage (in which also more nectar was produced) than in the female-only stage. The out-crossed flower buds opened earlier and had a shorter flowering period than selfed flowers. Similarly, the outcross-pollen germinated earlier and the pollen tube growth was faster than in self-pollen. Anthers, pistil and corolla of *O. acaulis* obviously form an integrative functional unit in which the elongated style plays a key role both in pollen-dispensing and pollen-deposition. The combination of preanthetic protogyny with herkogamy has probably arisen through selection for promoting out-crossing and prolonging the exposition time of the receptive stigma in order to capture a higher amount of pollen grains. This may be understood as a strategy to cope with scarcity of pollinators in the plants' habitat.

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Introduction

Dichogamy (maturation of the sexes in flowers at different times) and herkogamy (spatial separation of the sexes) are generally considered as floral strategies for reducing self-fertilization and promoting outcross-pollination. However, recent studies suggest that dichogamy and herkogamy may entail additional functions (Lloyd and Webb, 1986; Sargent and Otto, 2004; Webb and Lloyd, 1986). Lloyd and Webb (1986) pointed out that the temporal or spatial separation of pollen and stigmas reduces the interference during their presentation. Pollen-stigma interference may occur when stamens restrict access to stigmas or remove incoming pollen, or when self-pollen causes stigma clogging; similarly, gynoecia may restrict access to pollen or cause outgoing pollen to be redeposited (Webb and Lloyd, 1986). Mechanisms avoiding self-interference primarily promote paternal fitness, whereas mechanisms preventing self-fertilization primarily increase maternal fitness. Bertin and Newman (1993) argued that three factors

might be involved in promoting dichogamy: (1) selection for avoidance of pollen-pistil interference, (2) selection for avoidance of self-fertilization, and (3) selection for synchrony of pollen discharge and stigma receptivity in the different flower types of diclinous species. However, the causes and consequences of dichogamy are still not sufficiently understood (Sargent and Otto, 2004).

As is well known, there are two forms of dichogamy: protogyny (the stigmas becoming receptive before pollen release), and protandry (vice versa). Protogyny is considered as a more effective anti-selfing mechanism than protandry because in self-compatible taxa outcross pollen is received before self-pollen is shed (Bertin, 1993; Bertin and Newman, 1993; Griffin et al., 2000; Lloyd and Webb, 1986; McKone et al., 1995; Pickering, 1997; Stace, 1995). Phylogenetically, protogyny is apparently the ancestral condition in angiosperms, because almost all basal angiosperms are protogynous (Endress, 2010; Gottsberger et al., 1980; Lloyd and Webb, 1986). However, in the angiosperms as a whole, protogyny is relatively rare, while protandry prevails by far, especially in animal-pollinated plants (Bertin and Newman, 1993; Endress, 2010; Lloyd and Webb, 1986; Sargent and Otto, 2004). In particular, protandry is strongly associated with advanced subclasses such as the Asteridae (Bertin and Newman, 1993). Notable are combinations of

dichogamy and herkogamy. Protogyny combined with 'approach herkogamy' (Lloyd and Webb, 1986) [style(s) + stigma(s) distinctly sticking out from the anther level] was so far only found in a few members of the asterids: Gentianaceae, Scrophulariaceae, Boraginaceae and Solanaceae (Barker, 1982; Robertson and Lloyd, 1991). However, many more protogynous species in animal-pollinated asterids probably may be discovered, because as compared to their high species number and enormous floral diversity, the pollination biology of only a small proportion of taxa has been studied.

During our fieldwork in southern (subtropical) China, we found a new form of protogyny combined with approach herkogamy, involving the elongation of the style and the presentation of the receptive stigma prior to anthesis (as conventionally done, the start of anthesis is equated here with corolla opening, though one could argue that in the present case anthesis starts with the style protrusion from the flower bud). This was found in a species of Gesneriaceae, *Oreocharis acaulis*, a small perennial herb endemic to the Guangdong Province of South China (Wang et al., 1998). Gesneriaceae is one of the more basal families of the order Lamiales. Protandry is prevalent in this and other families of the order (Martén-Rodríguez and Fenster, 2008; Podolsky, 1993; Sanmartin-Gajardo and Sazima, 2005; Weber, 2004). Although protogyny is known from a few species of *Gesneria* (Martén-Rodríguez and Fenster, 2008; Skog, 1976), *O. acaulis* is, as far as we know, the only protogynous species of Gesneriaceae in which a combination of protogyny and herkogamy occurs and in which the stigma becomes exposed and receptive before flower opening. Our study addresses the floral biology and breeding system of *O. acaulis* aiming to contribute to a better understanding of the particular dichogamy phenomenon in this species. We sought to answer the following specific questions: (1) What are the details of floral morphology, development and reproductive biology of *O. acaulis*? (2) What is the pollination mechanism of *O. acaulis*? (3) Does protogyny, especially in the present incomplete form in which the receptive stigma protrudes from the "flower bud", promote outcrossing?

Materials and methods

Plant material, study period and research sites

Oreocharis acaulis (Merr.) Mich. Möller & A. Weber was formerly known as *Opithandra acaulis* (Merr.) B. L. Burtt, but recently included in *Oreocharis* by Möller et al. (2011). The plants are small deciduous, perennial acaulescent herbs growing on rocky outcrops in subtropical humid monsoon forests (Fig. 1A). Flowering time is from March to April. The flowers are pink without a noticeable scent and broadly tubular with slight widening (but no inflation) toward the zygomorphic limb. The two stamens are adnate to the adaxial side of corolla tube (in contrast to most other diandrous Gesneriaceae it is the adaxial stamen pair that is fertile), inserted roughly in the middle, and the anthers cohere at the apex. The ovary is narrow-cylindrical and puberulent outside, and the style is long exserted.

Fieldwork was carried out during the flowering seasons 2007, 2008, 2009 and 2010 in two populations in the Province of Guangdong, South China: one located in the forest park Gui-feng Shan (GFS – 114°03′29.3″E, 23°48′82.6″N, alt. 377 m) and the other in the nature reserve Nan-kun Shan (NKS – 113°51′48.6″E, 23°36′54.8″N, alt. 715 m).

Flowering phenology and floral ecology

In each population (studied 2007–2009), ten to twenty flowering individuals were marked and monitored from 0630 to 1800 h every day. For each flower (bud), the position (height) of stigma and anthers, the duration of anthesis (time from corolla opening to withering), the time of anther dehiscence, and the flower withering were recorded at 0700, 1200 and 1700 h every day. The number of flowers per inflorescence and the number of inflorescences per individual were also examined.

In the GFS population, we collected 20 anthetic flowers from different plants to study their pollen viability, stigma receptivity, nectar volume and sugar concentration at 1200 h every day. Pollen viability was assessed by means of the MTT (dimethylthiazol-diphenyl-tetrazolium bromide) method by which exclusively viable pollen is stained (Dafni, 1992). Pollen from different flowers in the same flowering periods was mixed, and five random samples of at least 500 pollen grains per sample were counted. Simultaneously, their stigmatic receptivity was measured via the MTT test (Rodriguez-Riaño and Dafni, 2000). Nectar production was measured in flowers bagged before stigma protrusion. Nectar volume was extracted with a 1-μL microcapillary, and sugar concentration (percentage sucrose, w/v) was tested with a handheld temperature-compensated refractometer (Brix 0 – 32%, ATAGO, Honcho Itabashiku, Tokyo, Japan). Pollen grains, ovules and P/O ratios per flower were obtained by dissecting fresh flowers as described by Wang et al. (2004). One freshly opened flowers from each of the 20 randomly sampled plants were collected and fixed in FAA (70% ethanol:formaldehyde solution:acetic acid = 90:5:5). Then the anthers were soaked in 1 M HCl for 3 h, squashed to release the pollen grains in a calibrated tube, and this was finally filled with 1 mL of detergent solution. The suspension was vortexed for 60 s, and 10 separate 1 μL subsamples were transferred onto slides and the pollen grains were counted. The ovules were counted by dissecting the ovaries in each flower, and the P/O ratio (number of pollen grains divided by the number of ovules) was calculated for each flower.

Influence of different pollination treatments on flower development

To assess the influence of pollination on flower development, we performed different pollination treatments (i.e. open pollinated, bagged unpollinated, out-crossed, selfed) on flower buds from twenty randomly selected flowering individuals as soon as their stigmas protruded from the flower bud. We monitored the changes of the flowering periods and the height of pistil and stamens during anthesis in both populations.

Flower visitors and their behavior

Flowers were monitored for insect visitors between 0730 and 1800 h on 1, 6–8 April 2007, 11–13 April 2008, and 27–28 March and 4 April 2009 in the GFS population. The observation periods ranged from 30 min to 1 h every 2 h per flower. In addition to these observation periods, occasional checks for flower visitors were made in the two populations in 2007, 2008 and 2009. The behavior of the flower visitors and their visiting frequencies (visits inflorescence^{-1} h^{-1}) were recorded. At least three individuals of each kind of visitor were captured for identification. In order to assess whether visitors prefer the flowers in different sex phases, we recorded visiting times to flowers in the female-only and the hermaphroditic stage and compared the visiting frequency of all visitors between flowers in the female-only and the hermaphroditic stage applying independent-samples t test.

Deposition and removal of pollen

To find out whether protogyny promotes out-crossing, we identified and counted the conspecific pollen grain number on the stigmas of 34 flowers in the female-only stage from
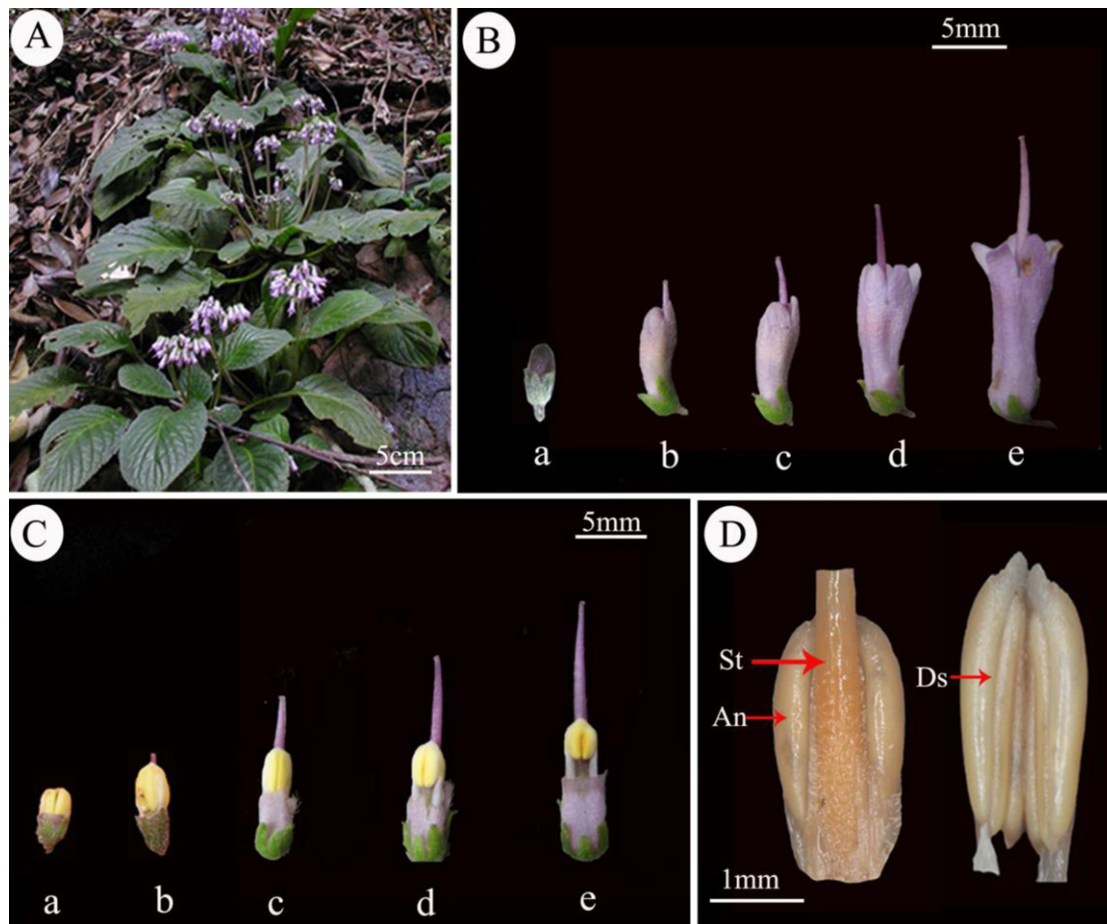
Fig. 1. *Oreocharis acaulis*, flowers and flower development. (A) Plant growing on a rock outcrop. (B and C) Flowers at different developmental stages. (B) The intact flower (bud) showing stigma protrusion from corolla. (C) The flower (bud) with corolla cut showing the close proximity of the stigma and anthers; (a) flower bud; (b and c) stigma protrusion in the unopened flower bud (female-only stage 1); (d) opened flower before anther dehiscence (female-only stage 2); (e) opened flower after anther dehiscence (hermaphrodite flower stage). (D) Anthers and style. St, style; An, anther; Ds, dehiscence slit of pollen sac.

randomly chosen 34 plants in the two populations with the aid of a dissecting microscope. In order to test whether the style elongation helps to increase pollen deposition, the pollen deposition on stigmas was examined by collecting stigmas of flowers in different developmental stages, i.e. female-only stage 1 (flower bud showing style/stigma protrusion), female-only stage 2 (anthetic flower before anther dehiscence) = day 1, and hermaphroditic stage (anthetic flower after anther dehiscence) = day 2, day 3, day 4. In order to test whether the style elongation helps to promote dispensing of pollen, the pollen removal from anthers was examined by collecting anthers of flowers in different developmental stages. These flowers were open-pollinated throughout the flowering period, or they were bagged before the stigmas protruded from the unopened flower buds and then exposed to open-pollination for one day. Also compared was the pollen deposition on virgin stigmas (day 3) in the intact and the emasculated flowers after a single pollinator visit. Similarly, we compared the pollen removal from anthers of day 3 flowers in intact flowers and flowers with styles cut off. The stigmas were embedded in a drop of aniline blue on microscope slides, and the pollen grains were counted under a microscope at $20\times$ magnification. The anthers were collected and the remaining pollen was determined. The pollen-counting procedure was the same as used for counting the pollen grains per flower. Pollen removal was determined by comparing the remaining pollen with the pollen produced in different flowers from the same plant individual (Dudash, 1991).

Breeding system experiments

To determine the breeding system, artificial pollination experiments were carried out and compared with open pollination. In the two populations under study we performed the following pollination experiments and analyzed fruit and seed set during March to April 2007, 2008 and 2009: (1) open pollination: flowers were marked before anthesis and then left untouched; (2) autonomous autogamy: flower buds were bagged before style/stigma protrusion; (3) autogamy: freshly opened flowers (day 1), which were bagged before stigma protrusion, were hand-pollinated with self-pollen and then enclosed in bags; (4) xenogamy: pollen from plants at least 50 m away was placed directly onto the stigmas of freshly opened flowers (day 1), which were bagged before stigma protrusion; (5) agamospermy: stamens were removed from flower buds before stigma protrusion, and the flowers were then enclosed in bags.

Pollen germination and pollen-tube growth rates

To find out whether different pollen donors (autogamy and xenogamy) affect the pollen tube growth rate in the pistil, we tested the pollen germination and pollen-tube growth rates of self- and cross-pollinated flowers. 5–10 hand-pollinated flowers, in which pollen collected from self or other plants (at least 50 m away) was placed directly onto the stigmas of flower buds bagged before

Table 1
Oreocharis acaulis. Duration of flowering stages in different pollination experiments (hours, mean ± standard error).

Treatments	Unopened flower bud showing stigma protrusion (female-only stage 1)	Opened flower		
		Female-only stage 2 (day 1)	Hermaphroditic stage (day 2–day 4)	Total (day 1–day 4)
Open pollinated flowers	83.11 ± 17.79[a] (*n* = 19)	6.82 ± 8.07[a] (*n* = 17)	87.92 ± 37.28[a] (*n* = 12)	95.92 ± 37.24[ab] (*n* = 12)
Bagged unpollinated flowers	72.46 ± 16.51[ab] (*n* = 13)	8.23 ± 9.45[a] (*n* = 13)	112.46 ± 36.46[a] (*n* = 13)	120.69 ± 31.35[a] (*n* = 13)
Out-crossed flowers	56.13 ± 12.09[c] (*n* = 15)	6.27 ± 7.20[a] (*n* = 12)	81.64 ± 35.95[a] (*n* = 11)	87.99 ± 33.87[b] (*n* = 11)
Selfed flowers	71.23 ± 16.86[b] (*n* = 13)	6.69 ± 6.29[a] (*n* = 13)	92.54 ± 21.69[a] (*n* = 13)	101.15 ± 18.89[ab] (*n* = 13)

Note: Identical letters indicate that the mean values among different treatments are not significantly different.

pollination. These were then enclosed in bags, collected and fixed in FAA at different time intervals (1, 2, 4, 8, 16, 24 h). Pistils were cleared and softened with 15 M NaOH for about 3 h, rinsed in tap water for 1 h to remove the NaOH, and then stained with aniline blue previously decoloured with K_2HPO_4 for approximately 12 h. The samples were observed with an epifluorescence photomicroscope (AXIO Imager A1, Carl Zeiss, Jena, Germany), and the length of the pistil and pollen tubes were measured. The pollen tube growth rate was calculated by dividing the length of the pollen tubes by the length of the pistil (%).

Data analysis

We used ANOVA to detect variation among treatments in SPSS 11.5. Differences in visiting frequencies (between the female-only stage and hermaphroditic stage), the pollen depositions (between the intact flowers and emasculated flowers), the pollen removals (between the intact flowers and style-cut flowers), and the fruit sets and seed sets (between out-crossed and selfed) were tested using independent-samples *t* test. Differences in the nectar volumes, sugar concentrations, the pistil growth rates (among open-pollinated, out-crossed and selfed), the durations of each flowering stage (among open-pollinated, bagged, out-crossed and selfed), the pollen depositions and pollen removals (among different stages), and the pollen germination and pollen-tube growth rates (among different pollination times) were compared with one-way ANOVA.

Results

Phenology and flower production

Oreocharis acaulis flowers between early March and early May, differing to some degree in the years of observation and the populations examined. The plants studied produced 1–16 (mean 3.78 ± 2.50, *n* = 60) axillary inflorescences (pair-flowered cymes) with 1–20 (mean 5.97 ± 3.83, *n* = 60) flowers (Fig. 1A) in each inflorescence. There were 1–38 (mean 10.22 ± 10.34, *n* = 32) flowers open at one time in a plant. The number of flowers produced per flowering period and plant varied considerably, namely from 1 to 188 (mean 17.53 ± 28.71, *n* = 60). In conclusion, there is great variation in the number of inflorescences produced by a plant, in the number of flowers per inflorescence and the total number of flowers produced in a flowering season.

Flower development

The flowers of *O. acaulis* are protogynous in the preanthetic stage in that the style protrudes from the still closed corolla and the stigma becomes receptive (Fig. 1A–C). Throughout anthesis they are hermaphroditic. More precisely, the flower development can be divided into four stages (Fig. 1B and C; Table 1): bud stage (corolla closed, style with terminal stigma enclosed; 5–7 d); female-only stage 1 (protrusion of the style from the corolla bud and the stigma becoming receptive; 83.1 ± 17.8 h); female-only stage 2 (stage from corolla opening to anther dehiscence; 6.8 ± 8.1 h); hermaphroditic stage (stage from anther dehiscence
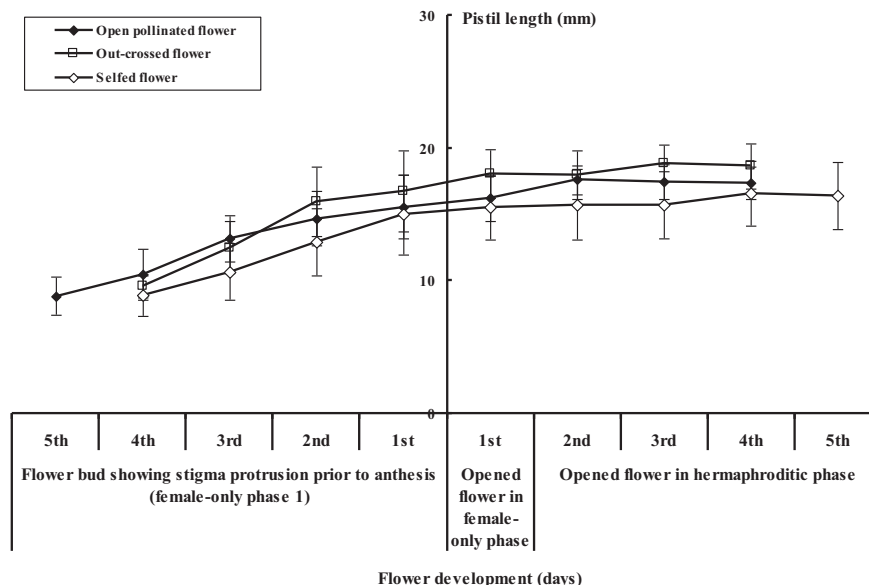

Fig. 2. *Oreocharis acaulis*. Height of pistil at flowers of different development stages and during different pollination experiments (mean ± standard error, *n* = 20).
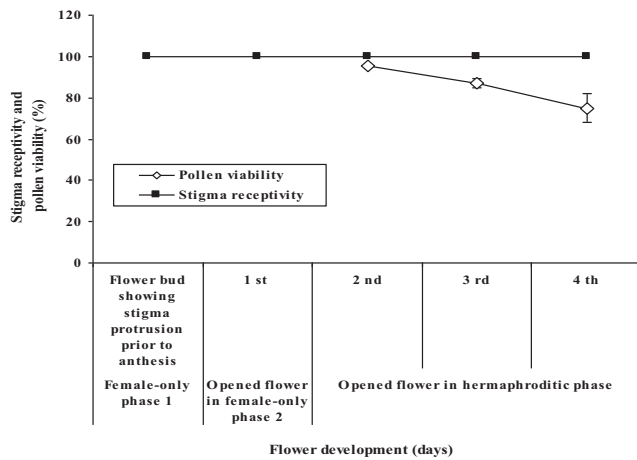
Fig. 3. *Oreocharis acaulis.* Pollen viability and stigma receptivity of flowers during anthesis (mean ± standard error, $n = 20$).

until flower withering; 87.9 ± 37.3 h). Corolla opening begins between 0800–1000 h ca. 3–4 (occasionally 6) days after the stigma has started protruding from the closed corolla. A flower thereafter stays open for further 4 (–6) days (Fig. 2). During that time the style continues to elongate (Figs. 1B and C and 2), but elongation is slight. The anthers start to dehisce at 1700 h, about 6–7 (occasionally 8) h after corolla opening. At this time the style reaches roughly its full length (Fig. 2). During the lifetime of the flower, the stigma stays positioned above the anthers, with the stamen position remaining relatively stable (Figs. 1B and C and 2).

P/O ratio, pollen viability and stigma receptivity

We counted/calculated $327{,}215 \pm 129{,}167$ pollen grains and 526 ± 161 ovules per flower. The resulting P/O ratio was 677 ± 341 ($n = 40$). Based on the MTT test, the stigma receptivity was 100% from the flower bud (female-only stage) up to corolla abscision, and pollen viability also remained at a high level (>75.00%) during anthesis (Fig. 3).

Nectar

No nectar was produced in flower buds prior to anthesis. With progressing anthesis nectar volume increased (Fig. 4). Before anther dehiscence the nectar volume produced in a flower was only 0.92 ± 0.68 μL ($n = 20$), with a sugar concentration of $10.6 \pm 2.3\%$. However, after anther dehiscence the nectar volume increased slightly with progressing anthesis and finally reached
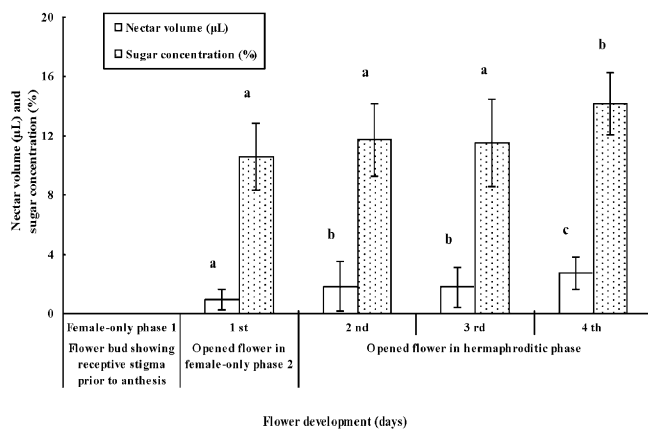

Fig. 4. *Oreocharis acaulis.* Nectar production and sugar concentration of flowers bagged before stigma protrusion (mean ± standard error, $n = 20$). Identical letters indicate that the means among the flowering stages are not significantly different.

Table 2
Pollen deposition on stigmas and pollen removal from anthers of flowers in different development stages (number of pollen grains, mean ± standard error).

Treatments		Unopened flower bud showing stigma protrusion (female-only stage 1)	Opened flower in female-only stage 2	Opened flower in hermaphroditic stage		
			Day 1	Day 2	Day 3	Day 4
Pollen deposition	Open pollination	10.09 ± 25.44^c ($n=34$)	11.86 ± 9.21^c ($n=29$)	23.14 ± 31.48^c ($n=42$)	107.61 ± 115.09^b ($n=38$)	154.16 ± 112.72^a ($n=38$)
	Bagged before stigma protrusion and open pollinated for one day	16.70 ± 38.58^c ($n=50$)	13.12 ± 25.97^c ($n=25$)	21.92 ± 21.14^{bc} ($n=39$)	68.57 ± 105.44^a ($n=46$)	53.41 ± 88.04^{ab} ($n=22$)
Pollen removal	Open pollination	–	–	$215{,}830 \pm 33{,}072.62^b$ ($n=40$)	$261{,}090 \pm 10{,}609.75^c$ ($n=40$)	$276{,}140 \pm 5119.62^a$ ($n=40$)
	Bagged before stigma protrusion and open pollinated for one day	–	–	$161{,}120 \pm 36{,}648.70^b$ ($n=40$)	$227{,}468 \pm 21{,}210.95^c$ ($n=50$)	$261{,}228 \pm 17{,}177.01^a$ ($n=25$)

Note: Identical letters indicate that the mean values among the flowering stages are not significantly different.

Table 3
Pollen deposition on stigmas and pollen removal from anthers of flowers in different experiments (number of pollen grains, mean ± standard error).

Treatments		Flowers of day 3 after a single pollinator visit	Flowers of day 3 open pollinated for one day	Flowers open pollinated through anthesis
Pollen deposition	Intact flowers	84.71 ± 63.03 (n = 41)	133,768.40 ± 69,450.32 (n = 33)	276.03 ± 230.39 (n = 32)
	Emasculated flowers	71.00 ± 69.19 (n = 37)		89.12 ± 113.58 (n = 25)
	t	0.916		3.714
	P	0.363		0.000
Pollen removal	Intact flowers		294,342.20 ± 33,973.62 (n = 42)	72.90 ± 73.98 (n = 43)
	Style-cut flowers		155,015.91 ± 100,950.84 (n = 35)	63.81 ± 54.34 (n = 32)
	t		8.401	0.578
	P		0.000	0.565
	Intact flowers		50,597.56 ± 88,924.27 (n = 36)	351,304.50 ± 6960.46 (n = 40)
	Style-cut flowers	4.302		338,666.90 ± 10,076.21 (n = 36)
		0.000		6.414
				0.000

$2.10 \pm 1.44\,\mu L$ ($F_{3,76} = 6.834$, $P = 0.000$) with a sugar concentration of $12.5 \pm 2.8\%$ ($F_{3,76} = 7.587$, $P = 0.000$) (Fig. 4).

Results of pollination experiments

As shown in Table 1, the out-crossed flower buds opened significantly earlier ($F_{3,56} = 7.915$, $P = 0.000$) and had a slightly shorter flowering period ($F_{3,45} = 2.516$, $P = 0.070$) than the flowers of other treatments. However, there was no difference in the female-only stage 2 and the hermaphroditic stage between different pollination treatments. This indicates that pollination had no influence on the female-only stage 2 ($F_{3,51} = 0.214$, $P = 0.886$) and the hermaphroditic stage ($F_{3,45} = 1.982$, $P = 0.130$). The bagged flower buds opened earlier than the open-pollinated flowers. But their flowering period was significantly longer than that of other treatments. The results (Fig. 2) also showed that the pistil growth rate in out-crossed flowers was significantly higher than in open-pollinated flowers ($F_{2,200} = 4.601$, $P = 0.006$) and selfed flowers ($F_{2,200} = 4.601$, $P = 0.012$) before anther dehiscence (hermaphroditic stage), but there was no significant difference in the open-pollinated and selfed flowers ($F_{2,200} = 4.601$, $P = 0.924$). In all experiments the style length reached its maximum while anthers started to dehisce after corolla opening.

Flower visitors and their visiting frequency in the particular floral stages

According to our observations (in 2007, 2008 and 2009), the most frequent flower visitors were bumblebees (*Bombus* sp.) (Fig. 5A–D), who visited the flowers for foraging pollen (by buzzing) and/or taking up nectar. The visiting frequency reached its peak at 1.13 ± 0.71 visits per inflorescence per hour. During the 10 days of observation, only one hawkmoth (*Macroglossum* sp.; Fig. 5E) and one bee (an unidentified species of *Apidae*) were recorded to visit the flowers. They can be considered as visitors not effecting pollination. Syrphid flies (*Baccha maculata*) were abundant in the habitat, but landed infrequently on the corolla and only sucked remaining rain drops (Fig. 5F). They also can be considered as visitors not effecting pollination. The visiting frequency on the flowers in the hermaphroditic stage was significantly higher than in the female-only stage (0.45 ± 0.29 vs 0.05 ± 0.10 visits inflorescence^{-1} 30 min^{-1}, $n = 15$; $t = 2.987, P = 0.017$).

Floral construction and pollination mechanism

The two fertile stamens of *O. acaulis* are the posterior ones, thus adnate to the adaxial side of corolla tube, inserting near the middle of the tube. The anthers cohere at the apex and thus form a functional unit, in which the elongated, longitudinally dehiscing thecae tightly enclose the style between them (Fig. 1D). The long and strong style locks the dehiscence slits of the anthers (Fig. 1D), and serves as a kind of lever to occlude or open the pollen chambers. This floral construction forms a key-lock mechanism of pollen-dispensing and pollen-deposition, and the operator (=opener of the anther slits) is the pollinator. When a bumblebee visits the flower, it hangs upside down, clasping the corolla with its feet, and then puts its head into the flower in order to forage pollen or nectar (Fig. 5A and B). During this process, the stigma situated on top of the long style touches the underside of the bumblebee (Fig. 5C and D). If pollen carried along from another flower is present, pollination is successful. Meanwhile, and due to the pressure on the stigma during the bumblebee's buzzing, the powdery pollen grains are released and fall along the style onto the

Fig. 5. *Oreocharis acaulis*, flower visitors. (A–D) A bumble bee (*Bombus* sp.) visiting the flower. (E) A hawkmoth (*Macroglossum* sp.) sucking nectar. (F) A syrphid fly (Baccha maculata) visiting the flower. Bar = 1 cm.

underside of the bumblebee. These pollen grains are then delivered to the stigma of another flower by the bumblebee.

Pollen deposition in the particular stages of anthesis

Our results (Table 2) show that pollen grains are deposited on the stigma both in the female-only stage 1 and female-only stage 2 (day 1). A significant difference was not found in the pollen deposition between the two female stages, neither in open-pollinated flowers, nor in flowers bagged before stigma protrusion and open-pollinated for only one day. Table 2 also shows that the amount of pollen grains deposited on stigma increases significantly with the progress of anthesis. In all the above cases the peak of pollen deposition occurred in the flowers of day 3 and day 4 when the style had reached its full length. Moreover, there was no difference in pollen deposition on virgin stigmas of intact and emasculated flowers after a single pollinator visit (Table 3). Likewise, no significant difference was found in the amount of pollen grains on stigmas of day 3 flowers, in intact flowers and emasculated flowers open-pollinated and exposed only one day (Table 3). However, throughout anthesis the pollen deposition on intact flowers was significantly higher than in emasculated flowers (Table 3).

Pollen removal in the particular stages of anthesis

As shown in Table 2, pollen removal from the anthers increased significantly with the progress of anthesis and style elongation after anther dehiscence both in the open pollinated flowers and in the flowers bagged before stigma protrusion and open-pollinated for

only one day. This might be partly due to an increase of pollinator visitations with the flowering process. However, the amount of pollen grains removed in the flowers of day 3 and day 4, in which the style length had reached its maximum, was significantly higher than that in the flowers of day 2. The results (Table 3) also show that the amount of pollen grains removed from anthers of style-cut flowers (day 3) was significantly less than that of intact flowers, both in flowers open-pollinated throughout the flowering period and in flowers bagged before stigma protrusion and open-pollinated for only one day. Similarly, pollen removal from virgin anthers of the style-cut flowers (day 3) was also significantly less than that of intact flowers after a single visit of pollinators.

Breeding system

The results of our pollination experiments (Table 4) reveal that *O. acaulis* is self-compatible, and that between experimental self- and outcross-pollinated flowers there is no significant difference both in fruit set ($t = -0.573$, $P = 0.598$) and seed set ($t = -1.433$, $P = 0.156$). As proven by the absence of fruit and seed set in the flowers bagged before style/stigma protrusion, with stamens removed or not, apomixis and autonomous selfing can be excluded.

Pollen germination and pollen tube growth

Our results show that pollen grains do germinate on stigmas of flowers in the female stage 1. Outcross-pollen on the stigmas of flower buds germinated earlier, and their pollen tube growth

Table 4
Fruit and seed set in the pollination experiments (percentage, mean ± standard error).

Treatments	No. of flowers (plants)	Fruit set (%)	Seed set (%)
Open pollination	533 (34)	26.00 ± 9.07	54.63 ± 28.28
Autonomous autogamy (bagging before stigma protrusion)	435 (47)	0.81 ± 1.41	0
Test for agamospermy (stamens removed before stigma protrusion, flowers bagged)	125 (27)	0.00	0
Experimental test for autogamy	318 (68)	23.92 ± 9.33	21.42 ± 16.64
Experimental test for xenogamy	260 (81)	29.91	28.07 ± 22.01
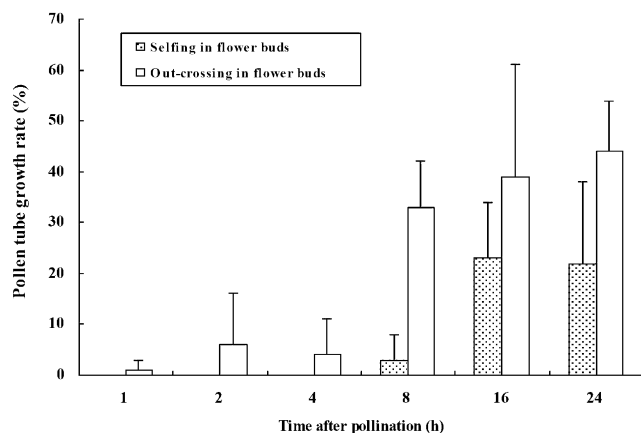
Fig. 6. *Oreocharis acaulis.* Pollen tube growth rate following self- and cross-pollination (mean ± standard error, $n = 10$).

rate was also significantly higher than selfing-pollen ($F_{3,95} = 8.347$, $P < 0.005$) within 16 h after pollination (Fig. 6).

Discussion

Floral construction and the key-lock mechanism of pollen-dispensing and pollen-deposition

Flowers are functional units for sexual reproduction balancing several conflicting demands (i.e. offering pollen as a floral attractant vs. saving it for reproduction; availability of resources vs. the need of an elaborate display in order to attract pollinators; securing production of offspring by self-pollination vs. promoting genetic diversity by outcrossing – Claßen-Bockhoff, 2007). In consequence, each flower can be regarded as a species-specific compromise for managing sexual reproduction (Claßen-Bockhoff, 2007; Reith et al., 2007). The precision of pollen deposition can be increased by various mechanisms. In many members of Gesneriaceae and other Lamiales the anthers are in close contact, fuse post-genitally and form a functional unit (Weber, 2004; Westerkamp and Claßen-Bockhoff, 2007). Further elaboration leads to sophisticated devices to shed the pollen in an economic way (e.g., the salt-shaker mechanism of *Drymonia* species, see Wiehler, 1983). The flower of *Oreocharis acaulis* can be functionally interpreted as an ingenious device of pollen-dispensing and pollen-deposition, in other words: a key-lock mechanism of pollen-dispensing and pollen-deposition. Here, the precision of pollen deposition and pollen-dispensing is greatly enforced by the anthers, the pistil, and the corolla forming an integrative functional unit. The high precision of pollen deposition may be associated with the reduction of the fertile stamens to two. Such a reduction is found in many Old World Gesneriaceae (Weber, 2004) and in other Lamiales (Westerkamp and Claßen-Bockhoff, 2007), but *O. acaulis* (along with some other species formerly placed in the genus *Opithandra*) is special by having the anterior (rather than the posterior) stamens reduced. Our results of the breeding system experiments indicate that apomixis and autonomous selfing does not occur in *O. acaulis*. Its successful

pollination unambiguously depends on the presence of pollinators. Pollination is sternotribic (inverted), differing from that of most "bilabiate blossoms" which are prone to nototribic (upright) pollen deposition. During the process of sternotribic pollination, the strongly elongated style of *O. acaulis* plays a key role both in pollen deposition and removal. As to our pollen deposition experiments, pollen deposition increased with the progression of anthesis and reached its maximum when the style had elongated to its full length. This suggests that the elongating style promotes pollen deposition, because the elongating style exposes the stigma to touch closely the pollen-dusted underside of a pollinator when it hangs upside down on the flower, clasping the corolla with its feet when foraging for pollen or nectar. On the other hand, the pollen removal experiments also showed that the amount of pollen removed from the anthers increased with progressing anthesis after anther dehiscence, and reached its maximum when the style elongated at its full length. Moreover, pollen removal in flowers with styles cut off was significantly lower than in intact flowers both after a single visit and throughout anthesis. All these facts allow the conclusion that the elongating style may facilitate pollen removal. When a *Bombus* bee pollinator visits a flower, its buzzing activity generates enough pressure on the stigma to remove the style from the dehiscing slits of the anthers and the pollen grains are released.

Functional significance of protogyny combined with approach herkogamy

In general, protogyny is viewed as a more effective mechanism in preventing self-fertilization within a flower than protandry, and it is more common in self-compatible taxa than in self-incompatible taxa (Aizen and Basilio, 1995; Bertin and Newman, 1993; Griffin et al., 2000; Lloyd and Webb, 1986). Our results show that *O. acaulis* is self-compatible and that there were some pollen grains deposited on the stigmas of flower both in the female-only stages 1 and 2. This suggests that protogyny does promote out-crossing, because the prior cross-pollen may increase the probability of cross-fertilization (Griffin et al., 2000). However, protogyny will be relatively ineffective as to the reduction of selfing if pollinators tend to avoid flowers in the female phase. This is particularly likely to occur if pollinators collect only pollen or if flowers produce less nectar in the female than in the male phase (Aizen and Basilio, 1998; Bell et al., 1984; Gonzalez et al., 1995; Griffin et al., 2000; Inoue et al., 1995; Klinkhamer and De Jong, 1990). Our study revealed similar results for *O. acaulis*, namely that the flowers produced less nectar production during the female-only than in the hermaphroditic stage, and most pollinators preferred to visit the hermaphroditic stage flowers rather than the flowers in the female-only stages. Our experiment of pollen deposition also showed that stigmas receive significantly less pollen during the female-only stage than during the hermaphroditic stage, and that the stigmas of intact flowers receive significantly higher pollen loads than emasculated flowers during the whole course of anthesis. Furthermore, our experiments also showed that there was no significant difference between the emasculated and intact flowers in the pollen deposition of both the virgin stigmas after a single pollinator visit

and the stigmas of day 3 flowers open pollinated for one day. This implies that there is no interference in pollen import between the anther and pistil within a flower and the protogyny combined with approach herkogamy avoids self-pollination within a flower. However, as there are often many open flowers (in different anthetical stages) present within a plant in *O. acaulis*, geitonogamy is not effectively prevented. Selfing may occur as the stigmas remain receptive and as unfertilized ovules are still available when anthers shed the self-pollen (Griffin et al., 2000). This study also shows that ovule number of *O. acaulis* can reach up to 526 per flower, and the stigma stays receptive throughout the flower's life-span; but the amount of pollen grains deposited on the stigma before anther dehiscence was only 11.9. All the above shows that protogyny combined with approach herkogamy may represent a floral mechanism to limit self-pollination within a flower (though geitonogamy cannot be avoided).

The special form of protogyny of *O. acaulis*, with the receptive stigma emerging before flower opening and the style elongating until anther dehiscence, differs from that of most protogynous species among angiosperms where receptive stigmas are presented only after flower opening. There are some conflicting reports on exserted stigmas becoming receptive before anthesis (Bashaw and Funk, 1987; Shafer et al., 2000; Snyder et al., 1955). However, our experiments relating to stigma receptivity, pollen viability and germination indicate that the stigma of *O. acaulis* is apparently receptive as soon as it protrudes from the unopened flower bud, and remains receptive throughout anthesis. Style/stigma protrusion prior to corolla opening may be understood as a floral device to prolong the presentation of receptive stigmas in order to capture a larger number pollen grains and to increase the possibility of cross-fertilization. Some studies suggest that outcrossing can shorten the life span of flowers as compared with selfing (Aizen, 1993; Devlin and Stephenson, 1984; Gregg, 1991; Richardson and Stephenson, 1989) and that flower longevity is prolonged to increase the amount of cross pollen and thus promotes genetic diversity (Aizen, 1993; Lankinen et al., 2006). Similarly, our results showed that the out-crossed flower buds of *O. acaulis* significantly opened earlier after stigma protrusion and had a significantly shorter flowering period than selfed flowers. Outcross-pollen on the stigmas of flower buds also germinated earlier and the pollen tube growth was also significantly faster than in self-pollen. Our experiments, in which the flowering period in bagged (and thus unpollinated) flower buds was significantly longer than that in outcrossed and self-pollinated flowers, also indicate that the absence of pollination may prolong the flower longevity and thus increases the chance for pollination. All the above implies that the special kind of protogyny found in *O. acaulis* possibly arose through selection for promoting out-crossing and prolonging the presentation of receptive stigmas in order to capture a greater amount of pollen. This may be understood as a strategy to cope with scarcity of pollinators in the habitat of the species. In fact, our observations made during three years in two populations of *O. acaulis* revealed that pollinators were rare and the visiting frequency was very low.

Acknowledgements

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