Patterns and consequences of stigma height variation in a natural population of a distylous plant, *Primula sieboldii*

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Summary

1. In order to reveal the direction and magnitude of phenotypic selection on the stigma height of heterostylous morphs in a natural population of *Primula sieboldii* E. Morren (Primulaceae), morphological traits including stigma and anther heights, flowering phenology, local density of potential mating partners, size of ramet, number of pollen grains deposited from the opposite morph and seed set were measured in two flowering seasons.

2. Both components of female reproductive success, pollen receipt and seed set, were significantly larger in the long-styled than in the short-styled morph in both years.

3. Selection analyses based on bivariate correlation and path-analysis models revealed positive relationships between stigma height and pollination or seed set in the short-styled morph only, although the effect was statistically significant only in one year. In contrast, seed set in the long-styled morph was correlated with ramet size.

4. Morph difference in reproductive success and the natural selection revealed for higher stigmas in the short-styled morph should be ascribed to the narrow corolla tube of the species that strictly restrict the path of the proboscis of the pollinators.

Key-words: Female reproductive success, floral morphology, heterostyly, natural selection, stigmatic pollen load

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Introduction

Most floral traits, including corolla colour and shape, flowering phenology and the positions of reproductive organs within the flower, may evolve in response to natural selection through biological interactions with animal pollinators. Among the floral traits, stigma exsertion is thought to affect female reproductive success through influence on quantity and quality of pollen receipt (Campbell, Waser & Price 1996; Thomson & Stratton 1985), and thus may be a particular target of natural selection (Campbell 1989; Johnston 1991). Highly exserted stigmas which promote contact with pollinators' bodies may be advantageous for effective pollination, especially in plants with tubular corollas (Campbell *et al*. 1996; Stone & Thomson 1994).

Heterostyly is a genetically controlled floral polymorphism (Barrett 1992). In the case of distyly, two morphs, long- and short-styled, produce flowers that differ reciprocally in stigma and anther height. The floral polymorphism is usually genetically linked with a diallelic self-incompatibility system. Avoidance of self-pollination and promotion of intermorph pollination may be major selective pressures responsible for the evolution of spatial separation of stigma and anthers within the flower (herkogamy) and between-morph reciprocity of positions of these organs, respectively (Darwin 1877; Lloyd & Webb 1992a).

In the tubular flowers typical of heterostylous species (Lloyd & Webb 1992b), the low stigmas of the short-styled morph are likely to contact the pollinator's body less effectively and thus receive less pollen. In most studies in which pollen loads on stigmas have been measured for distylous plants, the total amount of pollen load in the long-styled morph exceeded that in the short-styled morph, although the ratio of opposite-morph ('legitimate') pollen grains to total pollen load was significantly smaller in the long-styled morph (Nicholls 1986; Piper & Charlesworth 1986). Stone & Thomson (1994) investigated pollination mechanism and pollen flow in artificial flowers and found that higher stigmas contacted pollinators' bodies more

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readily, and received more pollen grains than lower stigmas. Thus the long-styled phenotype is better for pollen receipt. In the short-styled morph, selection for herkogamy and selection for exsertion would conflict.

Although considerable within-morph variation in the length of the style and stamens has been studied in some heterostylous plants (Dulberger 1992; Fenster & Barrett 1994), natural selection (in the sense of Lande & Arnold 1983) for stigma and anther heights, i.e. the relationships between the heights of these organs and pollination or seed set, have not yet been studied. To assess the causal mechanism of natural selection in a natural population, traits other than the target trait, as well as environmental factors, should be considered as they also influence reproductive success (Mitchell-Olds & Shaw 1987). Therefore multivariate statistical techniques such as path analysis (Kingsolver & Schemske 1991; Mitchell 1993) have been used to study natural selection (Herrera 1993; Mitchell 1994; Schemske & Horvitz 1988). The spatial structure of the plant population (that is, the size and/or density of the population), which may profoundly affect mating success through the availability and range of mating partners (House 1992; Nishihiro & Washitani 1998; Wyatt & Hellwig 1979), should be also included in the analysis.

Natural selection on stigma height and several other floral traits was measured in a natural population of *Primula sieboldii* E. Morren (Primulaceae), a clonal distylous herb with a deep corolla tube. Heterostyly provides certain advantages for study, including the ease of determining potential mating partners for individual plants, and the ability to distinguish the parent morphs of pollen grains deposited on stigmas. *Primula sieboldii* has another advantage: pollinator visitations to individual flowers can be easily assessed by claw marks left by the predominant pollinators, queen bumblebees (Washitani *et al.* 1994a).

Our hypothesis on natural selection for stigma height in *P. sieboldii* was that plants with higher stigma are advantageous for pollen receipt and seed set. Based on the results of the measurements of natural selection, we discuss selective pressures responsible for the evolutionary determination of the positions of stigmas and anthers in heterostylous tubular flowers.

Materials and methods

SPECIES AND STUDY SITE

Primula sieboldii is a clonally growing herb that occurs in a range of moist habitats throughout Japan. Each genet is composed of various numbers of physiologically independent ramets. In the Hidaka region of the southern Hokkaido, *P. sieboldii* is associated with maritime deciduous forests dominated by *Quercus dentata*, which is typical of the region. This study was carried out in a 50×50 m plot set in a fragment (50×600 m) of the forest dominated by *Q. dentata* in

1995–97. Leaf emergence of *P. sieboldii* is in early May, immediately before the leaf expansion of *Q. dentata* when the forest floor is relatively well illuminated. Flowering starts in mid- to late May and ends in late June. Each flowering ramet bears 5–10 flowers on a 15–30 cm flowering stalk.

Primula sieboldii produces two distinct mating types: long- and short-styled morphs. Typical for heterostylous species, it requires insect pollinators for pollination between different morphs. A previous study has shown that queen bumblebees (especially *Bombus diversus tersatus*) are the predominant visitors to the flowers of this species and facilitate intermorph pollination (Washitani *et al.* 1994a).

POPULATION SPATIAL STRUCTURE

All the flowering ramets of individual genets in the study plot were mapped in spring 1996 and 1997. Locations of the individual ramets were recorded to ±10 cm with reference to a square grid mapped onto the study plot. Individual genets were distinguished according to flower appearance (size, colour and shape; Washitani *et al.* 1991, 1994b) and leaf morphology, which varied considerably from genet to genet. The floral morph of each genet was determined by the relative position of stigma and anthers; ramets with flowers with the stigma seen around the mouth of the corolla tube were classified as long-styled; those with anthers around the mouth as short-styled. In three census periods (early, middle and late) during each flowering season, the number of flowers was recorded for all flowering ramets in the genets with < 20 flowering ramets. For larger genets, 15–20 flowering ramets were arbitrarily chosen for flower counts. In addition, the position, number and floral morph of flowering ramets in the additional 50×25 m area adjacent to the study plot in the longitudinal direction of the forest were similarly recorded.

For each genet in the study plot, the number of flowering ramets of the opposite morph that occurred within a circular neighbourhood area of various sizes (from 2·5 to 25 m in radius) was determined by checking the coordinates of individual flowering ramets occurring in the plot.

FLOWERING PHENOLOGY

In 1995, 1996 and 1997, all inflorescences within the plot were inspected every 2–4 days during the whole flowering season. At each inspection, newly opened and newly faded flowers were marked with small, dated strips of vinyl chloride tapes at the pedicels. Flowering duration was defined as the length between the flowering and closing dates.

Flowering synchrony for individual flowers was evaluated as the relative degree of overlapping of flowering with all the opposite-morph flowers in the study plot. In 1996, however, the flowering dates of almost all 282

genets studied were so similar that we did not estimate synchrony. The intermorph flowering synchrony (S) of flower i was calculated as:

$$S_i = \sum^{n_0} e_0 / (f_i \cdot n_0)$$

where e_0 is the number of days during which both flower i and any flower of the opposite morph are simultaneously in bloom, f_i is the flowering duration of flower i, and n_0 is the total number of opposite morph flowers in the population. If a flower is completely synchronized with all the opposite flowers of the population, the synchrony equals 1, while the value is 0 in case of completely asynchronized flowering. The mean flowering synchrony for a genet was calculated as the mean S_i for all the flowers within the genet.

FLORAL MORPHOLOGY

In 1996, floral morphological traits [stigma and anther heights (maximum height from the base of ovary), corolla breadth (corolla diameter) and corolla tube length], were measured to ±0·1 mm for 1068 flowers from 113 genets using a pair of digital-slide callipers. Stigma heights were measured with flowers still attached to the pedicels, but the other traits were measured with the corolla collected immediately after the flower closed. In 1997, 20 genets each from the long- and short-styled morphs were arbitrarily chosen, and corolla diameter, corolla tube length and anther and stigma heights of three flowers each from three ramets for individual genets were measured. Relative anther and stigma heights were evaluated as the ratios of anther and stigma heights to length of corolla tube (Washitani *et al.* 1994b).

CLAW MARKS AND STIGMATIC POLLEN LOAD

At the time of closure of individual flowers in 1996 and 1997 we recorded the presence or absence of claw marks on the petals. These distinctive marks are made by tarsal claws of bumblebees visiting the flower.

Stigmas were collected for pollen-load measurements using fine forceps after the stigma height measurement, when the corolla of the flowers would fall if lightly touched. At this stage fertilization of ovules is complete, but ungerminated pollen and exines of germinated pollen still remain on the stigma. Immediately after collecting, the stigmas were mounted on glass slides and sealed with transparent nail enamel. In 1996 stigmas were collected from three or more flowers without any symptoms of damage by predators or pathogens, from three to 10 arbitrarily chosen ramets of 42 genets each of the long- and short-styled morphs. In total the pollen load was measured for 407 stigmas. In 1997, pollen loads on a total of 360 stigmas from 20 genets of each morph were similarly collected after floral morphological traits had been measured.

One anther from each of three to 10 arbitrarily chosen flowers was also collected from the individual genets; the stigmas were collected for the pollen-load measurement and similarly mounted on a glass slide.

Under the microscope, *P. sieboldii* pollen grains appear roughly triangular. In the laboratory the longest side (hereafter referred to as pollen size) of 200 arbitrarily chosen grains from each anther sample was measured for all the genets examined, using a fluorescence microscope (BX50, Olympus, Tokyo) equipped with a video-micrometer system (VM30, Olympus). The genet mean and the standard deviation of the size of anther pollen were obtained for each genet. There was a significant difference in the genet mean of pollen size between morphs (mean ± SD: 13·3 ± 1·2 μm, $n = 42$ in the long-styled morph; 19·2 ± 1·8 μm, $n = 42$ in the short-styled morph, in 1996). For stigma samples, sizes were measured for all the pollen grains on stigmas with < 200 pollen grains loaded, or 200–400 chosen grains from stigmas with more than 200 grains. A deposited grain was judged as being from the opposite morph (legitimate pollen grain) if it differed from the mean size of pollen grains of the genet by 2 SD. We assumed that most of the pollen grains from the same morph were deposited by intraflower and/or intragenet pollination. For stigmas which received > 200 pollen grains, the total number of grains received by intermorph pollination was estimated from the proportion of legitimate pollen grains among all those for which size was measured, and the total number of grains loaded on the stigma.

FRUIT AND SEED SET

Fifteen to 20 infructescences from individual genets, or all the infructescences from genets with < 20 ramets, were harvested in mid-July in 1995, 1996 and 1997, and fruit and seed set were measured. The numbers of matured capsules, vestigial flowers and seeds in individual matured capsules were counted. The presence or absence of damage to capsules by herbivores or pathogens was also recorded.

SIZE OF RAMET

At the end of flowering seasons of 1996 and 1997, the maximum length and width of the individual leaf blades were recorded for all the ramets measured for seed set. Preliminary examination revealed that the product of maximum length and width of leaf blade was well correlated with leaf area ($r = 0.91$, $P < 0.0001$, $n = 281$) and its summation over all the leaves of the individual ramet, hereafter referred to as ramet size, was significantly correlated with the dry mass of the whole ramet ($r = 0.81$, $P < 0.0001$, $n = 63$).

OVULE NUMBER

In 1997 three flowers were collected from each of five ramets, chosen from four genets of each morph

in the study plot. The number of ovules contained in each flower was counted under a dissecting microscope. Corolla diameter, corolla tube length and stigma and anther heights were also measured on the same flowers.

LIGHT AVAILABILITY

In 1995 photosynthetically active photon-flux density (PPFD) was measured under diffuse light on three overcast days during the flowering season of *P. sieboldii* (5, 16 and 30 June) using four quantum sensors (IKS-25, Koito, Tokyo) arranged in a $3 \times$ cm square, at the height of the leaves of each flowering ramet. The PPFD under open sky was measured simultaneously at a height of 3 m near the forest. All sensors were connected to a data logger (Thermodac-E, Eto-Denki, Tokyo). Relative PPFD, an appropriate index for microsite light availability at the forest floor (Washitani & Tang 1991), was calculated as the ratio of PPFD at the individual measuring point in the forest to that under open sky.

DATA ANALYSIS

We used nested ANOVA to test the effects of morph and genet within morph on the number of legitimate pollen grains loaded, the number of pollen grains loaded, and the seed set per flower in each of the years studied.

We analysed correlations between the genet mean of the local density of potential mating partners and that for each component of female reproductive success, in order to test the effect of availability of potential mating partners. To examine the relationships between floral traits and reproductive success, simple bivariate correlations between trait values and the components of female reproductive success were calculated. Because spatial patchiness in trait and fitness values can affect the estimation of significance levels for correlation coefficients (Thomson *et al.* 1996), we corrected these correlations for spatial autocorrelation by the Clifford–Richardson–Hémon procedure (Clifford, Richardson & Hémon 1989).

Path analyses were used to explore the relative importance of morphological and environmental factors as determinants of female fitness and the relationship between pollination and seed set using the CALIS procedure of SAS 6·10.

The number of pollen grains loaded on stigmas and seed set per flower were normalized by $\log_{10}(x + 1)$ transformation before parametric analyses.

Results

FLOWER NUMBER

There was no statistically significant difference in the number of flowering genets between the long- and short-styled morphs in 1995–97 (Table 1). However, the short-styled morph had more flowering ramets per genet than the long-styled morph. As there was no significant difference in the mean number of flowers per flowering ramet ($5 \cdot 5 \pm 1 \cdot 5$, $n = 1601$ in the long-styled morph; $5 \cdot 6 \pm 1 \cdot 6$, $n = 1967$ in the short-styled morph), short-styled flowers were more abundant than long-styled in the study plot (Table 1).

FLORAL MORPHOLOGY AND MORPH CLASSIFICATION

There were no bimodal distributions in genet means for corolla diameter, corolla tube length, stigma height or anther height (Fig. 1a–d), except for that of herkogamy, i.e. the height difference between stigma and anther (Fig. 1e) in the 1996 measurement. An exactly similar pattern was obtained in 1997 for 20 genets of each morph (data not shown).

Most of the floral morphological traits measured were positively correlated with each other (Table 2). Correlations between anther height and corolla tube length were the highest in all the cases examined (Table 2). Although genet means for relative stigma height were distributed continuously, those for relative anther height showed marked dimorphism (Fig. 1f,g). Morphological scoring of a plant's style–length morph based on the dimorphism in relative anther height or in herkogamy was consistent with scoring based on the criteria adopted in the field inspection (see Materials and methods).

Although stigma height varied within morphs, the mean and range of the genet means for stigma height in the long- and short-styled morph (long-styled, mean 12·1 mm, range 8·9–14·2 mm; short-styled, 9·6,

Table 1. Number of genets with flowering ramets, number of flowering ramets, and number of flowers of the long- and short-styled morphs in the study plot in the 3 years. Results of χ^2 goodness-of-fit tests for equality of the two morphs are also shown: ** $P < 0.01$, *** $P < 0.001$, df = 1

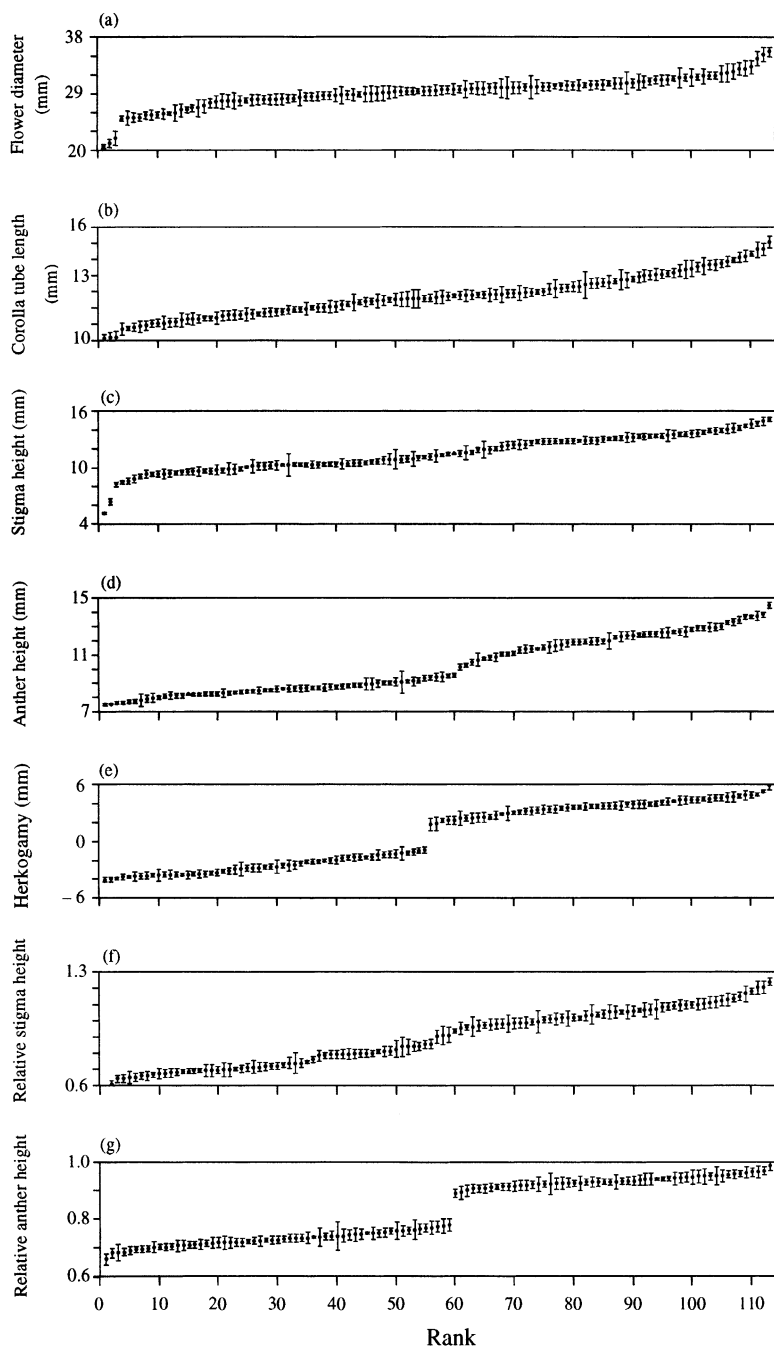|	1995			1996			1997		
Parameter	Long	Short	χ^2	Long	Short	χ^2	Long	Short	χ^2
Flowering genets	111	110	0·00	152	147	0·04	185	211	0·73
Flowering ramets	705	855	7·23**	941	1230	18·8***	723	1030	26·6***
Flowers	4060	5100	59·3***	5730	7680	142***	4010	5400	104***

Fig. 1. Genet means (dots) and 1 SD (bars) of flower diameter (a), corolla tube length (b), stigma height (c), anther height (d), herkogamy (e), relative stigma height (f), and relative anther height (g) for 113 genets in the *Primula sieboldii* population are plotted against their rank. Relative stigma and anther heights were calculated as the ratios of stigma and anther heights to corolla tube length.

5·1–12·5 mm) roughly coincided with that for anther height in the opposite morph (long-styled, 8·6, 6·8–12·5 mm; short-styled, 12·1, 8·6–14·5 mm).

OVULE NUMBER

The number of ovules in individual flowers varied from 77 to 172, with a mean of 120 ($n = 120$). Nested ANOVA revealed that although variation among genets was

Table 2. Correlation coefficients between genet means of floral morphological traits, corolla diameter, corolla-tube length, stigma height, and anther height. Asterisks denote statistical significance for the correlations; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Traits	Tube length	Stigma height	Anther height
1996			
Long-styled morph ($n = 42$)			
Corolla diameter	0·33*	0·41**	0·25
Tube length		0·11	0·54***
Stigma height			0·08
Short-styled morph ($n = 42$)			
Corolla diameter	0·42**	0·44**	0·44**
Tube length		0·46**	0·88***
Stigma height			0·42**
1997			
Long-styled morph ($n = 20$)			
Corolla diameter	0·41	0·58**	0·02
Tube length		0·24	0.78***
Stigma height			−0·02
Short-styled morph ($n = 20$)			
Corolla diameter	0·68***	0·29	0·46*
Tube length		0·67***	0·85***
Stigma height			0·73***

significant (df = 7, $F = 18.23$, $P < 0.001$), between-morph variation was not (df = 1, $F = 2.59$, $P = 0.11$). None of the floral morphological traits was correlated significantly with ovule number in a flower in each morph (test of significance of correlation coefficients by Fisher's z-transformation at $P = 0.05$).

COMPONENTS OF FEMALE REPRODUCTIVE SUCCESS

Claw marks

During the flowering seasons, queens of three bumble-bee species, *B. diversus tersatus*, *B. schrencki albidipleuralis* and *B. deuteronymus deuteronymus*, were observed to visit *P. sieboldii* flowers. *Bombus diversus tersatus* was observed most frequently and is likely to be the main pollinator in the study plot (Washitani *et al*. 1994a). Claw marks were found on the petals of 83, 93 and 91% of flowers inspected in 1995, 1996 and 1997, respectively.

Pollination

More pollen grains were loaded on stigmas and more legitimate pollen grains were loaded in the long-styled than in the short-styled morph (Table 3). Although effects of morph and genet nested within morph were significant, most variances were explained by the effects of the morph in each year (Table 4). The between-morph difference in the number of legitimate

Table 3. Pollen load on the stigmas of the flowers of *Primula sieboldii* in the population. Means and standard errors of total number of pollen grains loaded on the stigmas and number of legitimate pollen grains loaded on the stigmas shown for long- and short-styled morphs

Morph	n	Total pollen load		Legitimate pollen load	
		Mean	SE	Mean	SE
1996					
Long	182	9860	867	811	59·2
Short	224	2240	230	289	17·6
1997					
Long	180	9930	604	700	34·1
Short	180	2280	193	249	15·6

Table 4. Results of nested ANOVA for total number of pollen grains and number of legitimate pollen grains loaded on stigmas, between morphs and among genets in each year. The effect of genet was nested within morph. Both parameters of pollination were log-transformed before the analyses

Source	df	SS	F	P
Total number of pollen grains loaded				
1996				
Morph	1	190	374	< 0·0001
Genet (Morph)	105	163	3·02	< 0·0001
Residual	418	212		
1997				
Morph	1	76·2	299	< 0·0001
Genet (Morph)	38	119	12·3	< 0·0001
Residual	320	81·5		
Number of legitimate pollen grains loaded				
1996				
Morph	1	214	678	< 0·0001
Genet (Morph)	105	114	3·44	< 0·0001
Residual	418	132		
1997				
Morph	1	42·1	555	< 0·0001
Genet (Morph)	38	85·2	29·6	< 0·0001
Residual	320	24·3		

pollen grains deposited was significant, even allowing for the difference in the number of flowers examined ($\chi^2 = 113·4$, df = 1, $P < 0·001$ in 1996; $\chi^2 = 95·0$, df = 1, $P < 0·001$ in 1997).

Seed set

The harvested capsules or the vestigial flowers were classified into one of five categories: all or a part of the fruit consumed ('consumed'); infected by smut fungi ('infected'); withered with immature seeds ('withered'); fruit lost ('fallen'); or ripened with matured seeds or intact but with the failed flower vestige ('undamaged'). No or few seeds were recorded for the categories other than undamaged (Table 5). The categories other than undamaged constituted 24, 30 and 55% of all the samples in 1995, 1996 and 1997, respectively. In 1997, relatively many fruits were withered, and seed set in undamaged fruits was the least in that year (Table 5). To avoid confusing the factors affecting seed set, the damaged fruits were neglected in the following analyses. The fraction of damaged fruits for each genet had no significant relationship with flower morphology or phenology.

Seed set per flower in undamaged flowers varied significantly between morphs and among genets (Table 6). Seed set was significantly greater in the long-styled morph ($31·6 \pm 31·3$, $n = 1675$ in 1995; $49·5 \pm 30·5$, $n = 1933$ in 1996; $22·5 \pm 29·5$, $n = 1446$ in 1997) than in the short-styled morph ($22·0 \pm 28·7$, $n = 2470$ in 1995; $29·2 \pm 30·9$, $n = 2212$ in 1996; $9·5 \pm 21·8$, $n = 1957$ in 1997).

In the long-styled morph there was no significant relationship between genet means for the number of legitimate pollen grains loaded and seed set per flower (Fig. 2), but in the short-styled morph there was significant positive correlation ($r = 0·54$, $P < 0·001$; Fig. 2).

FACTORS AFFECTING FEMALE REPRODUCTIVE SUCCESS OF INDIVIDUAL GENET

Spatial availability of mating partners

In the short-styled morph, correlations between the genet means of each parameter for female reproductive success and the number of flowering ramets of the opposite morph depended on the spatial scale; with neighbourhood areas specified by circles of different radius drawn around focal genets, correlations were

Table 5. Seed set in flowers belonging to the categories undamaged, consumed, infected, withered and fallen (see text). Mean and SD with number of flowers in each category and its fractions (%)

Category	1995			1996			1997		
	Mean ± SD	n	(%)	Mean ± SD	n	(%)	Mean ± SD	n	(%)
Undamaged	25·9 ± 30·2	4145	(76)	38·6 ± 32·4	4088	(70)	15·0 ± 26·1	3404	(45)
Consumed	1·96 ± 8·95	524	(10)	5·79 ± 15·9	789	(13)	0·98 ± 5·98	1062	(14)
Infected	0·00	22	(0)	0·00	24	(0)	2·11 ± 12·5	71	(1)
Withered	0·83 ± 5·28	394	(7)	0·13 ± 3·02	569	(10)	0·00	2232	(30)
Fallen	0·00	375	(7)	0·00	377	(6)	0·00	721	(10)

Table 6. Results of nested ANOVA for seed set in undamaged flowers of *Primula sieboldii* between morphs and among genets in each year. The effect of genet was nested within morph. Data for seed set were log-transformed before analysis

Source	df	SS	F	P
1995				
Morph	1	57·6	99·2	< 0·0001
Genet (Morph)	139	334	4·10	< 0·0001
Residual	4004	2320		
1996				
Morph	1	201	500	< 0·0001
Genet (Morph)	257	617	6·02	< 0·0001
Residual	3829	1540		
1997				
Morph	1	127	288	< 0·0001
Genet (Morph)	284	436	3·47	< 0·0001
Residual	3118	1380		

significant for radii less than 10 m (Fig. 3). The local availability of mating partners was better correlated with the number of legitimate pollen grains loaded than with the seed set in most of the significant cases examined in the short-styled morph (Fig. 3). In the long-styled morph, however, no significant correlations were detected (Fig. 3).

Light availability

With the progress of seasonal leaf regeneration in both the tree layer of *Q. dentata* and the herbaceous
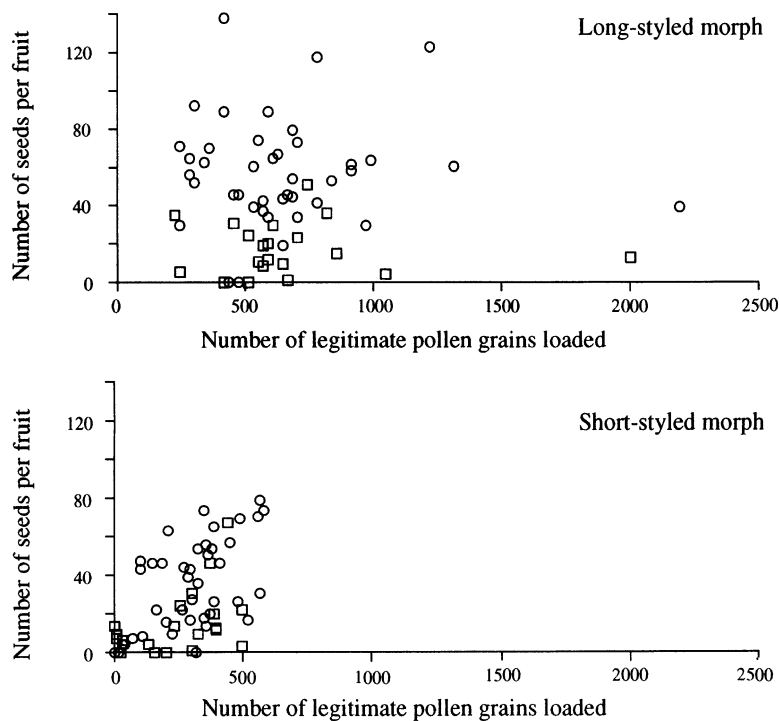

Fig. 2. Relationships between the mean number of legitimate pollen grains loaded on stigma and the mean seed set per undamaged flower for the same genet of long- and short-styled morphs. Circles and squares represent data for 1996 and 1997, respectively.

and shrub layers, the average microsite light availability for *P. sieboldii* (at 15 cm above ground) declined from 31 to 6% during its flowering period in 1995. Autocorrelation of the light availability of the genet between dates was significantly positive ($n = 153$, $r > 0·32$, $P < 0·0001$), with the light availability at the forest edge being consistently greater throughout the measuring period.

There was no significant correlation between the microsite light availability and the genet mean of seed set of the undamaged flower in both morphs in 1995 ($r = 0·06$, $P = 0·68$ in the long-styled morph; $r = -0·08$, $P = 0·58$ in the short-styled morph).

Flowering phenology

None of the parameters of flowering phenology of genets – duration of flowering, median date of flowering and flowering synchrony – were significantly correlated with the components of female reproductive success, i.e. the genet mean of the number of legitimate pollen grains loaded and seed set per undamaged flower in all the years examined (Fisher's *z*-test, $P > 0·05$).

Flower morphology

Correlation coefficients between the components of female reproductive success and the floral morphological parameters are shown in Table 7. There was a significant positive correlation between the height of the stigma and the number of legitimate pollen grains deposited only in the short-styled morph in 1996, but not in the long-styled morph (Table 7). There were significant positive correlations between the height of the stigma and the seed set per flower in both morphs in 1996 (Table 7). Other floral characters – corolla diameter, corolla tube length and anther height – were not significantly correlated with components of female reproductive success in either morph or years (Table 7).

Ramet size

The index of ramet size (leaf size summed over all the leaves of individual ramets) was positively correlated with the number of flowers per ramet ($r = 0·18$, $P = 0·01$), while none of the other floral traits was correlated with ramet size in both morphs. The correlation between the genet mean of ramet size and seed set per flower was significant only in the long-styled morph ($n = 42$, $r = 0·45$, $P < 0·01$ in 1996; $n = 20$, $r = 0·50$, $P < 0·05$ in 1997).

RELATIVE IMPORTANCE OF FLORAL MORPHOLOGY AS A FITNESS DETERMINANT

Hypothetical path models to explain among-genet variation in seed set per undamaged flower are shown in
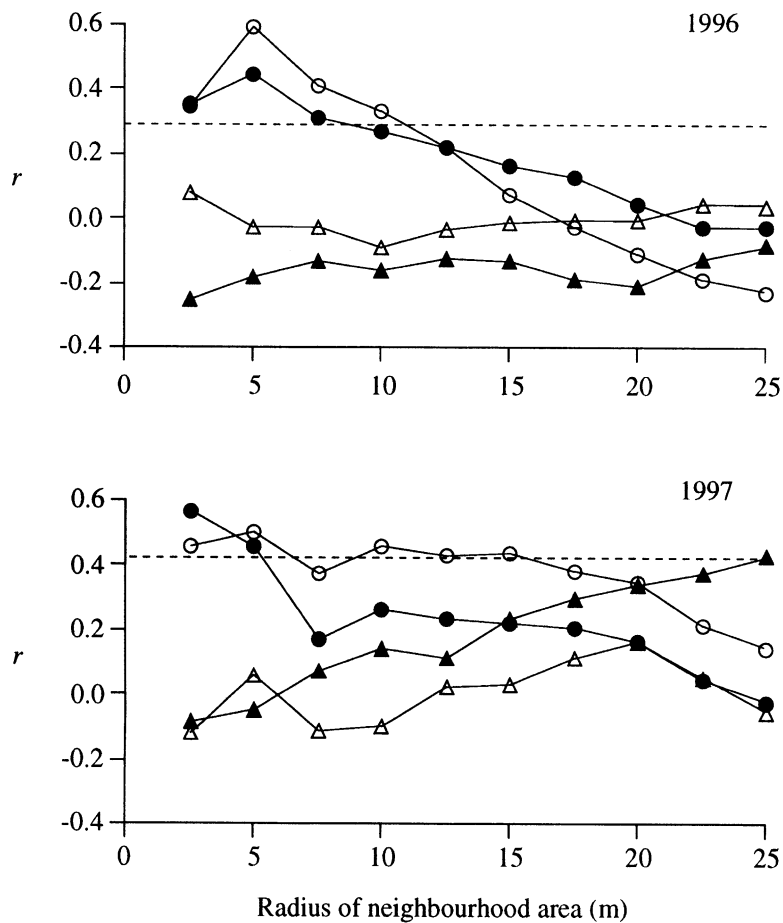
Fig. 3. Correlation coefficients between the number of the flowering ramets of the opposite morph located within various distances (2·5–25 m) from the centre of the focal genet and its mean of the seed set per undamaged flower (closed triangles and circles for long- and short-styled morphs, respectively) and mean number of legitimate pollen grains loaded (open triangles and circles for long- and short-styled morph, respectively) in 1996 and 1997. Values above the dashed lines are statistically significant (Fisher's *z*-transformation, $P < 0.05$). Sample size: $n = 42$ and $n = 20$ in each calculation of correlation coefficient in 1996 and 1997, respectively. The data for number of flowering ramets of the opposite morph located within 2·5 and 10 m were log-transformed before the correlation calculation as they skewed to the right.

Table 7. Correlation between genet mean of floral morphological traits (corolla diameter, corolla-tube length, stigma height, anther height, herkogamy) and genet mean of components of female reproductive success [number of legitimate pollen grains loaded (pollination) and seed set per flower]. Correlation coefficients represented with significance level corrected by the Clifford–Richardson–Hémon procedure for spatial structure; *$P < 0.05$, **$P < 0.01$. Data for pollination and seed set were log-transformed before the analyses

Floral traits	1996 ($n = 42$)		1997 ($n = 20$)	
	Pollination	Seed set	Pollination	Seed set
Long-styled morph				
Corolla diameter	−0·18	0·12	−0·06	0·07
Tube length	−0·12	0·20	−0·07	0·09
Stigma height	−0·05	0·39*	0·01	0·24
Anther height	−0·05	0·20	−0·08	0·03
Short-styled morph				
Corolla diameter	0·17	0·25	−0·16	0·06
Tube length	0·06	−0·02	−0·19	0·32
Stigma height	0·48*	0·53**	0·01	0·12
Anther height	0·10	0·02	−0·05	0·33

Fig. 4. We hypothesized that floral morphological traits and the local density of potential mating partners, i.e. the number of the ramets of the opposite morph within 5 m, influenced seed set through the effect on the number of legitimate pollen grains loaded. Ramet size was suggested to influence seed set directly, as the bivariate correlation analyses revealed that the genet mean ramet size was correlated with seed set but not with the number of legitimate pollen grains loaded in the long-styled morph, and the ramet size could therefore affect the resource availability for seed maturation.

In both 1996 and 1997, in the short-styled morph the effects of local density of the opposite-morph ramets were significant, even in the multivariate model in which the effects of the floral morphology were included (Fig. 4, Table 8). Among the effects of floral morphological traits, only the stigma height of the short-styled morph affected the variation in number of legitimate pollen grains loaded in 1996, but the effect was not significant in 1997 (Fig. 4; Table 8). The effects of ramet size on seed set were significant only in the long-styled morph in both years (Fig. 4; Table 8).

Discussion

NATURAL SELECTION THROUGH FEMALE FUNCTION

In hermaphroditic plants, fitness components of both functions should be measured in order to evaluate the evolutionary consequences of selection, as evolutionary modification of reproductive traits can be selected through both male and female functions (Bell 1985). In contrast to female reproductive success, which can easily be measured by seed set, male reproductive success is difficult to measure directly. In most studies that have attempted to measure natural selection through male function in natural plant populations, reproductive success was evaluated indirectly through the delivery of pollen analogues to stigmas (Campbell 1989) or pollen removal from anthers (O'Connell & Johnston 1998; Stanton, Snow & Handel 1986).

Since we dealt only with estimates of female reproductive success, the evolutionary consequences of the selection measured cannot be fully evaluated. However, when seed set is pollen-limited, natural selection may act on reproductive traits affecting pollen receipt to enhance female reproductive success, provided there is no trade-off between male and female fitness. Seed set in the short-styled morph of *P. sieboldii* was inferred to be pollen-limited because the number of legitimate pollen grains deposited was correlated with seed set per flower. Therefore the relationship between floral traits and pollen receipt allows us to examine the potential of evolutionary modification to maximize female fitness in the short-styled morph.

In the long-styled morph, however, there was no correlation between the number of legitimate pollen
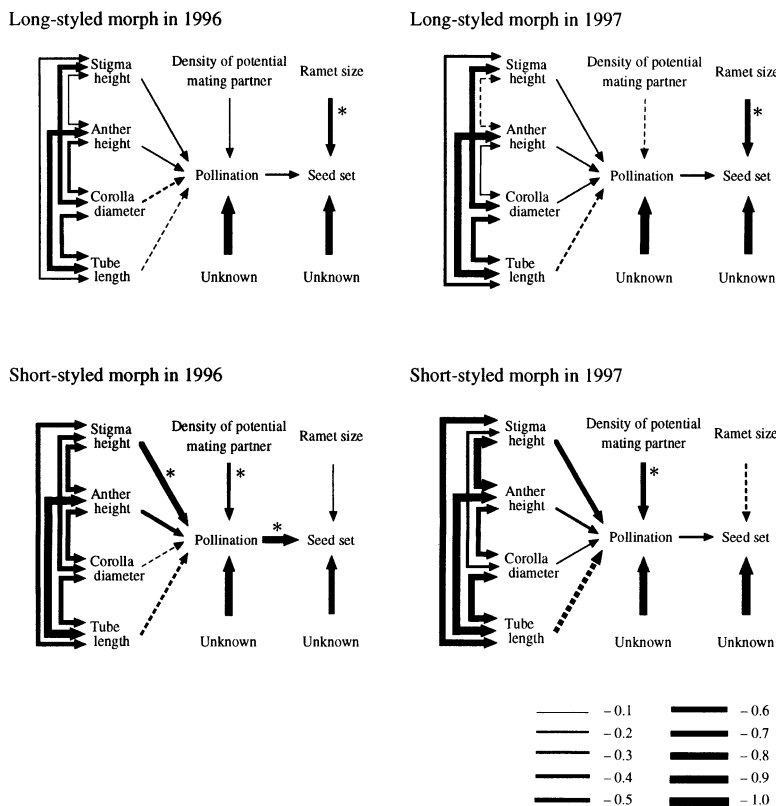
Long-styled morph in 1996

Long-styled morph in 1997

Short-styled morph in 1996

Short-styled morph in 1997

(Diagram labels: Stigma height, Anther height, Corolla diameter, Tube length, Density of potential mating partner, Ramet size, Pollination, Seed set, Unknown)

Legend:
—— – 0.1   ▬ – 0.6
—— – 0.2   ▬ – 0.7
—— – 0.3   ▬ – 0.8
—— – 0.4   ▬ – 0.9
—— – 0.5   ▬ – 1.0

Fig. 4. Path diagram for the effects of floral morphological traits (genet means), number of ramets of the opposite morph located within the 5 m distance from the centre of the focal genets (density of potential mating partner), and genet mean for ramet size, on genet mean for number of legitimate pollen grains loaded (pollination) and seed set per undamaged flower. Residual variables representing the unmeasured factors that affect a given dependent variable are represented by 'unknown'. Single-headed arrows depict path coefficients; double-headed arrows, correlations. Positive effects, solid line; negative effects, dotted line. The magnitude of path coefficients is coded using different line widths, and paths differing significantly from zero (t-test, $P < 0.05$) are indicated with asterisks. Actual values for the coefficients are shown in Table 8.

grains deposited and seed set per flower. Rather, seed set depended on plant size. Partial or cryptic self-compatibility of the long-styled morph (Washitani *et al.* 1991, 1994b) could partly explain these results. The absence of pollen limitation for seed set in the long-styled morph is also supported by the fact that the stigmas of the long-styled morph received abundant pollen grains, far in excess of the ovule number.

RELATIONSHIP BETWEEN STIGMA HEIGHT AND POLLEN RECEIPT

Highly positioned or well exposed stigmas can receive pollen more easily than concealed stigmas because they make better contact with pollinators' bodies (Campbell *et al.* 1996; Stone & Thomson 1994). More pollen grains were deposited on the stigma and more legitimate pollen grains were deposited in the long-styled morph of *P. sieboldii*. Furthermore, the stigma height of the short-styled morph correlated with the number of legitimate pollen grains deposited, although the relationship was statistically significant only in 1996. These results suggest that relatively tall stigmas are more beneficial in pollen receipt, at least within the present range of variation of stigma height in *P. sieboldii*.

The relationship between stigma height and the number of legitimate pollen grains deposited probably arises from the way the insect's body contacts the stigma. The relatively narrow corolla tube of the *P. sieboldii* flower (< 2 mm in diameter at its opening; Washitani *et al.* 1994b) may restrict the entry/exit path of the proboscis of effective pollinators (queens of long-tongued bumblebees) foraging for nectar secreted at the bottom of the corolla. When inserting and withdrawing the

Table 8. Direct effect (DE), indirect effect (IE) and proportion of variance explained (*r*) from linear regression of standardized variables for the path models illustrated in Fig. 4. Data for pollination, seed set and local density were log-transformed before the path analyses

Dependent variables	Independent variables	1996 Long-styled			Short-styled			1997 Long-styled			Short-styled		
		DE	IE	r^2	DE	IE	r^2	DE	IE	r^2	DE	IE	r^2
Pollination	Stigma height	0·03	0	0·04	0·47	0	0·36	0·01	0	0·02	0·36	0	0·33
	Anther height	0·04	0		0·24	0		0·04	0		0·16	0	
	Corolla diameter	−0·19	0		−0·07	0		0·04	0		0·10	0	
	Tube length	−0·07	0		−0·28	0		−0·19	0		−0·55	0	
	Local density	0·03	0		0·33	0		−0·06	0		0·51	0	
Seed set	Stigma height	0	0·00	0·21	0	0·36	0·58	0	0·00	0·26	0	0·09	0·07
	Anther height	0	0·01		0	0·18		0	0·00		0	0·04	
	Corolla diameter	0	−0·03		0	−0·05		0	0·00		0	0·02	
	Tube length	0	−0·01		0	−0·21		0	−0·02		0	−0·13	
	Local density	0	0·00		0	0·25		0	−0·01		0	0·12	
	Ramet size	0·45	0		0·03	0		0·50	0		−0·13	0	
	Pollination	0·13	0		0·76	0		0·11	0		0·25	0	

proboscis, the bee may contact the stigma with a more apical region of the proboscis than part contacting when it is inserted more deeply. Therefore the higher the stigma, the larger the area of that part of the proboscis which can contact with it.

The pattern of pollen distribution along the proboscis of a queen of *B. diversus tersatus*, after visiting flowers of both *P. sieboldii* morphs (Washitani *et al.* 1994a), clarifies the advantage of higher stigmas in pollen receipt: more pollen grains were located on the proximal parts than on the distal parts of the proboscis.

SELECTION OF HETEROSTYLOUS FLORAL MORPHOLOGY

The most beneficial placement of stigmas may also depend on the anther height of the mating partner. In heterostylous plants with intramorph incompatibility, natural selection can result in the position of stigmas at the same height as anthers of the opposite morph (Darwin 1877; Lloyd & Webb 1992a). The mean and range of the stigma and anther heights of a *P. sieboldii* morph coincided with those of the anther and stigma heights, respectively, of the opposite morph. This suggests strong selection for between-morph reciprocity in the heights of these organs. The actual heights of the stigmas and anthers should be evolutionarily determined under the influence of selective pressures other than that for reciprocity. The advantage for high stigmas in the short-styled morph is opposed by the advantage of herkogamy to reduce self-pollination and/or self-interference. Stigma height in the short-styled morph, thus evolutionarily shaped under the balance of these opposing selection pressures, might then determine the optimal anther height of the long-styled morph through selection for reciprocity.

It can be imagined that high reproductive organs, the stigmas of the long-styled morph and the anthers of the short-styled morph, are also influenced by two opposite selective pressures: promotion of herkogamy leading to higher position, and avoidance of excess exposure due to the risks of mechanical or physiological damage. Evolution of the heterostylous floral morphology of *P. sieboldii* with a tubular corolla can be explained as the result of these selective pressures.

Acknowledgements

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