Chapter 3.

Heterostylous morph differences in pollen transfer and deposition patterns in *Primula sieboldii* on a visitation by a queen bumblebee, measured with a semi-natural experimental system

Introduction

Heterostyly is a genetically controlled floral polymorphism in which populations are composed of two or three morphs that differ in a suite of floral characters (Ganders 1979). In the case of distyly, the population consists of two morphs, long- and short-styled morphs having stigmas and anthers differing in the height, reciprocally. The floral polymorphism is usually genetically linked with a diallelic self-incompatibility system. Avoidance of self-pollination and promotion of inter-morph pollination are believed to be major selective forces responsible for the evolution of spatial separation of stigma and anthers within the flower and the between-morph reciprocity of positions of these organs, respectively (Darwin 1877; Lloyd & Webb 1992).

Marked difference in pollen grain size between the heterostylous morphs provides a suitable system to study pollen transfer patterns and their consequences (e.g. Ganders 1974, 1976; Wolfe & Barrett 1989). Studies of pollen deposition on naturally pollinated stigmas of heterostylous species have often revealed asymmetries in pollen transfer between the floral morphs (reviewed by Lloyd & Webb 1992). In 13 of 17 distylous species of which pollen flow has been measured, significantly greater pollen transfer from the short-styled to

the long-styled morph flowers than in the reverse direction was demonstrated (Stone & Thomson 1994).

In the tubular flowers typical of distylous species (Ganders 1979), one of the most important factors causing this asymmetrical pollen flow is thought to be the way the pollinator's body contacts the stigmas or anthers, i.e., more protruded reproductive organs can make better contact with the pollinators' bodies (e.g. Ganders 1974, 1976; Stone & Thomson 1994). The asymmetrical pollen flow is thought to result in a gender difference between the morphs, so that the short-styled morph performs better as a male parent, while the long-styled morph attains more reproductive success through the female function at least in the pollination stage (Kohn & Barrett 1992a, b). Understanding the mechanisms of gender variation is important because genetically based variation in gender roles can lead to the evolution of separate sexes from hermaphroditism (Beach & Bawa 1980; Casper & Charnov 1982; Charlesworth 1989).

On the other hand, the unbalanced pollen flow has been hypothesized to be one of the important factors leading to morph-specific natural selection on floral morphology. Nishihiro et al. (2000) investigated the relationships between the stigma height and female reproductive success of genets at both stages of pollen receipt and seed-set in a natural population of distylous *Primula sieboldii* E. Morren (Primulaceae), and found that female success was significantly larger in the long-styled than in the short-styled morph at both stages. In addition, only in the short-styled morph, significant relationships between intra-morph variations in the stigma height and pollination or seed set success were revealed. They concluded that in the short-styled morph, selection for herkogamy and selection for stigma exposure would be somewhat conflicting. Since they only estimated female reproductive success, the evolutionary consequences of the selection revealed cannot be fully evaluated. The factors causing variation in both female and male reproductive successes

between the morphs should be analyzed before we can fully understand how the selection acts on floral morphology. Possibly, at every stage of pollination, i.e., pollen production in the anthers, pollinator visitation on the flowers, pollen removal from the flowers by the pollinators, and pollen deposition on the stigmas of the flowers, some factors could act either alone or in concert with others to cause the asymmetrical pollen transfer and the different gender roles.

Between-morph pollen transfer patterns are inevitably affected by the relative abundance of flowers of the different morph(s) within a population. Therefore, in order to evaluate between-morph pollen transfer patterns, analysis of stigmatic pollen loads following known sequences of visits, or measurement with artificial populations with equal morph ratios are required (Stone & Thomson 1994). The former approach enabled me to measure these patterns during individual pollination processes. Although a great deal of effort has been made on the analysis of pollination process in heterostylous species (reviewed by Lloyd & Webb 1992), no studies have been attempted to measure whole elements of the pollination process under conditions independent of influences from morph ratio.

The rate of pollinator visits to flowers has been used as a common index of a pollinator contribution to pollination success (Kearns & Inouye 1993). However, this index cannot always provide direct information on the contribution to pollination success due to the following reasons; variation in pollination effectiveness among different pollinators (e.g., Motten 1986) and non-additive effects of pollinators on pollen delivery (Young & Young 1992). The contribution of visitation rates to pollination success can be estimated on the basis of the measurements of the pollen load and exchange after a single pollinator visit.

The contribution of a pollinator to female reproductive success of plant species at pollination should be evaluated by both pollen load size and donor diversity (mixed

pollination) because mixed pollination is an important necessary condition of multiple paternity (Campbell 1998). Despite the important consequences of multiple paternity in plants, i.e., increase of fertility (Marshall 1991), reduction of genetic relatedness among offspring (Ritland 1989), and augmentation of maternal fitness (Marshall & Ellstland 1986), the mechanisms by which it occurs remain to be clarified. Two different mechanisms are suggested to result in mixed pollination; deposition of a mixed pollen load due to pollen carryover from flower to flower (Marshall & Ellstrand 1985; Campbell 1998) and multiple pollinator visits in close succession of time (Dudash & Ritland 1991). The extent of pollen carryover should be evaluated as well as pollinator visitation rates before we can answer which mechanism of mixed pollination is more important (Campbell 1998).

I evaluated between-morph patterns of pollen transfer based on the measurements at the individual pollination stages with an experimental system of *Primula sieboldii* and its effective pollinator queen bumblebee in order to reveal: (1) Which stage of pollination produces the asymmetry in pollen transfer between the morphs of *P. sieboldii*? (2) How effective is a single visit of pollinator for the reproductive success at pollination quantitatively and qualitatively? I studied the between-morph patterns of pollen transfer in detail by measuring the number of pollen grains produced in the anthers of the flower and both the amounts of pollen removed from flowers and loaded on the stigmas on a single visit by the bee. To evaluate the third stage, stigmatic pollen loads deposited on stigmas of the opposite-morph flowers along the sequence of visit by the bee should be measured since all the pollen grains from a donor flower are not transferred to the first visited opposite-morph flower (pollen carryover).

The system of *P. sieboldii* and the queen bumblebees provides several advantages for direct measurements of pollen transfer; a clear dimorphism in pollen size in *P. sieboldii* which allows the investigator to distinguish the parent morphs of pollen grains deposited on

the stigma (Washitani et al. 1994b; Nishihiro et al. 2000), and suitable plant and flower size and morphology for observation, i.e., an erect, single-stalked rosette plant (15-30 cm tall) with 3 cm corolla diameter.

Materials and Methods

SPECIES AND STUDY SITE

Primula sieboldii E. Morren (Primulaceae) is a clonally growing geophyte that occurs in a range of moist habitats throughout Japan. Each genet is composed of various numbers of physiologically independent ramets. In the southern Hokkaido, *P. sieboldii* is associated with maritime deciduous forests dominated by *Quercus dentata*.

The present study was carried out in a natural habitat of *P. sieboldii* (5 ha) in the Hidaka region of the southern Hokkaido. Daily mean, maximum and minimum air temperatures recorded at the standard meteorological station at Monbetsu (42° 30' N, 142° 0' E) for early June, the peak flowering season of *P. sieboldii* (averages for 1995-97), are 11.3, 14.5, and 8.4℃, respectively.

Nectar-feeding queen bumblebees (especially *Bombus diversus tersatus*) are the predominant visitors of the species and facilitate inter-morph pollination between the long- and short-styled morphs (Washitani et al. 1994a). The flower has a deep corolla tube topped by spreading petal lobes. The length of proboscis of the bumblebee is known to be well correlated with the corolla-tube length of the flower (approximately 13 mm, Washitani et al. 1994a). The mean number of ovules per flower is approximately 120 (Nishihiro et al. 2000).

It is safe to say that the bumblebees did not discriminate between the morphs when they visited the flowers because pollinator availability, which was assessed by the proportion of flowering inflorescences with claw marks, did not differ between the morphs in the relatively large populations (genets $\geqq$ 7) of *P. sieboldii* (Chapter 1, Matsumura & Washitani 2000).

MEASUREMENTS OF POLLEN DISPERSAL PATTERNS

FLOWERS AND BUMBLEBEES

The measurement of pollen carryover was performed in a flight cage placed above naturally growing patches of *P. sieboldii* genets using the queens of *B. diversus tersatus* collected locally and trained to feed *P. sieboldii* flowers. Individual genets were distinguished by flower appearance (size, color, and shape; Washitani et al. 1994b) which varied considerably from genet to genet.

After collecting, the experimental bumblebee queens (experimental bees) were kept individually in a small polypropylene box with blotting paper placed at the bottom of the box (bee box) and stored in a cooled container at the 4-8℃ in order to suppress bee activity to avoid excess energy consumption during the storage. The experimental bees were fed with a few drops of sugared water (30% sucrose) twice a day at 8:00 and at 22:00.

Floral morphology, pollen size for the experimental *P. sieboldii* genets, and the proboscis length and head breadth of the experimental bees are presented in Tables 1, 2, and 3, respectively.

BUMBLEBEE TRAINING

An outdoor flight cage ($1.8 \times 1.8 \times 1.8$ m^3) which was made with a steel frame and cotton meshes with a 1.04 mm square opening was placed above a natural patch of a *P. sieboldii* genet consisted of approximately 50 ramets with fresh flowers.

In the cage, we opened the lid of the bee box and put a bouquet of *P. sieboldii* flowers (containing 10 flowers) close to the bee in order to stimulate her to feed the flowers. When the bee began to feed on the flowers of the bouquet, the bouquet was

brought near to the flowers on the ground, and then the bee was allowed to visit the flowers freely. Such training was repeated until the bees voluntarily foraged the flowers at their release from the boxes.

The flowers used in this training were enriched in nectar with honey diluted with water (40% sucrose, original sugar concentration of nectar 25-36%; Washitani et al. 1994a).

MEASUREMENT OF POLLEN CARRYOVER

In order to estimate pollen carryover along the visitation sequence of an experimental bee, the number of pollen grains from donor flowers was counted for all the stigmas of recipient flowers of the opposite morph which were visited by the experimental bee along her visitation sequence.

In each experimental run, the flight cage was placed to accommodate one recipient genet of *P. sieboldii* which consisted of 20-50 ramets with fresh unvisited flowers. In order to exclude previous insect visitation, the budding flowers had been wrapped with cotton meshes with 0.16 mm square opening until just before the experiment.

All the ramets of each recipient genet (Table 2) were marked at the stalk with numbered strips of vinyl chloride tape and the flowers were also individually marked at their pedicels with smaller strips of the tape (2 mm in width).

In each run, the bee was first invited to visit to a bouquet of donor flowers of one morph. After the bee had visited 1-6 donor flowers and a pollen load on the bee proboscis was confirmed, the bee was guided to the opposite-morph recipient flowers. The order of the ramets and flowers she visited was recorded, together with the timing of grooming behavior. Each run was continued until the bee stopped foraging. After each run, the bee was cleaned with pieces of glycerine jelly.

A total of 29 experimental runs were carried out using seven queen bumblebees in

1997-98. However, I only analyzed data from the 20 runs in which the visitation sequence was not intercepted with grooming behavior. When the bee visited the same recipient flower more than once (13.7% of the visited flowers), only the previous to this event was included in the analysis.

STIGMATIC POLLEN LOAD

Immediately after each run, stigmas of all flowers of the recipient genet were collected by using a fine forceps. The stigmas were sealed with transparent nail enamel on glass slides for pollen counting in the laboratory. Five anthers each from 3 arbitrarily chosen flowers of 10 ramets were also collected and sealed with transparent nail enamel for measurement of the pollen size of each genet.

POLLEN COUNTING

In the laboratory, the widest diameter of each pollen grain (hereafter called pollen size) of 300 arbitrarily chosen grains from each anther specimen was measured under a fluorescence microscope (BX50, Olympus, Tokyo) equipped with a video-micrometer system (VM30, Olympus). The genet mean and standard deviation of the size of anther pollen grains were calculated for each genet (Table 2).

For each stigma collected from the recipient flowers, all the pollen grains deposited on it were counted by morph being referred to their sizes; judgement criteria are given in Table 2.

POLLEN BALANCE SHEET

MEASUREMENT OF POLLEN REMOVAL AND WASTAGE

The number of pollen grains deposited on a self stigma, those were shed on a corolla or inside a corolla-tube (pollen wastage), and those transferred to the bee proboscis (pollen removal) on a single visit by the bee were measured, as described below.

The flight cages were placed to contain the long- (P7) and short- (T7) styled morph genets consisting of approximately 50 ramets with fresh, unvisited flowers.

In each run, the experimental bee was incited to feed a single flower. Immediately after the bee withdrew her proboscis from the flower, she was captured and all the deposited pollen grains on the proboscis were collected with pieces of glycerine jelly containing basic fuchsin (Beattie 1971) to be fixed and stained. The stigma was collected and sealed with transparent nail enamel on a glass slide. The pollen grains shed on the corolla or inside the corolla-tube of the flower were also collected separately with pieces of basic fuchsin glycerine jelly. The anthers were carefully pinched off by a fine forceps and dipped into a vial with 70% ethanol individually.

In the laboratory, the stained pollen grains transferred to the bee proboscis, and those shed on the corolla or inside the corolla-tube were counted under a light microscope ($\times$100).

The numbers of pollen grains on the stigmas were counted under a fluorescence microscope ($\times$100, BX50, Olympus).

After the ethanol was evaporated, individual anthers which had been preserved in the vials were cut up under a dissecting microscope and pollen grains were collected from the anthers with pieces of basic fuchsin glycerine jelly to be fixed and stained on glass

slides. The pollen grains which remained inside the vials were also collected. The numbers of the grains were counted under a light microscope (×100).

MEASUREMENT OF POLLEN PRODUCED PER FLOWER

In order to quantify the pollen produced per flower, one of 5 anthers each from 1-6 arbitrarily chosen flowers of 1-6 ramets were collected before anthesis in each genet used in the carryover measurement in 1998, and stored individually in 70% ethanol. After the anthers were naturally dried, these were cut up under a dissecting microscope. All the pollen grains in the individual anthers were fixed and stained on glass slides with basic fuchsin glycerine jelly and the numbers of the grains were counted by Monte Carlo method with counting unit of 100 grains under a light microscope (×100). Pollen produced per flower was estimated from the number of pollen grains per anther.

For estimating large number of pollen grains, counting acetolyzed pollen grains using hemacytometers has been the standard means (Kearns & Inouye 1993). In a preliminary experiment, approximately 60% of pollen grains per flower of *P. sieboldii* were destroyed by acetolysis (Matsumura C. unpublished data, 1999). Thus, I counted total pollen grains under a light microscope to estimate the number of the grains more exactly.

DATA ANALYSIS

Significant differences in the sums of donor pollen grains loaded on the recipient stigmas among 3 groups of flowers, i.e., early (from the 1st to the 10th), middle (from the 11th to the 20th) and late (from the 21st to the 30th) visited flowers were tested by Kruskall-Wallis test.

Significant differences between the morphs in the total, legitimate (opposite morph) and illegitimate (self and/or geitonogamous) pollen grains on stigmas of the flowers visited

once in the pollen carryover measurement were tested by Mann-Whitney *U*-test.

The significance of the relationship between the sum of the pollen grains loaded on the stigmas of recipient flowers and the total number of flowers visited once by the bee during each run for the pollen carryover measurement was examined by product moment correlation coefficient.

Between-morph differences in the pollen grains produced per flower, the pollen remaining in anthers, the self stigmatic pollen load, the pollen deposition on bee proboscis, and the self pollen grains on the corolla or inside the corolla-tube for individual flowers after a single visit by bee were tested by Mann-Whitney *U*-test. Significant differences between visited and unvisited flowers in these components were tested by Mann-Whitney *U*-test.

Results

POLLEN LOAD PATTERNS ALONG VISITING SEQUENCES

No pollen grains were observed on the stigmas of 75% and 70% of the unvisited recipient flowers of the long- and short-styled morphs, respectively (Fig. 1), while the size and composition of pollen loads deposited on the stigmas of the visited recipient flowers varied greatly from flower to flower within each run and among the runs (Fig 2).

In the 18 runs in which the bee visited more than 9 recipient flowers without grooming, the number of pollen grains on recipient stigmas derived from donor flowers was significantly different between flowers visited early (from the 1st to the 10th), middle (from the 11th to the 20th), and late (from the 21st to the 30th) in the visiting sequence (Kruskall-Wallis test, $p < 0.01$) irrespective of the recipient morphs. The summed number of stigmatic legitimate pollen grains of the early group flowers was 3.8 or 4.0 times larger than those of the middle or late group, respectively. However, some stigmas at the late stage in the flower sequence contained pollen grains exceeding the ovule number (Fig. 3).

In the 20 runs in which the visitation sequence was not interrupted by grooming behavior, the stigmas of the long-styled morph flowers received significantly more total (mean ± s.d., 2572.26 ± 1841.24, n = 184, Mann-Whitney *U*-test, $U = 4713.5$, $p < 0.0001$) and illegitimate (mean ± s.d., 2425.15 ± 1783.79, n = 184, Mann-Whitney *U*-test, $U = 4735.0$, $p < 0.0001$) pollen grains than those of the short-styled morph (mean ± s.d., 623.36 ± 512.44 and 537.63 ± 448.47, respectively, n = 216). There was a significant between-morph difference (Mann-Whitney *U*-test, $U = 12853.0$, $p < 0.0001$) in the number of stigmatic legitimate pollen grains (mean ± s.d., 147.11 ± 248.75, n = 184 in the long-styled morph; 85.74 ± 209.14, n = 216 in the short-styled morph).

The number of legitimate pollen grains found on recipient stigmas increased with increasing total number of flowers visited by the bee (Fig. 4), but the correlation was significant only for the long-styled recipient flowers ($r = 0.845$, $p = 0.001$, $n = 10$, Fig. 4).

Stigmatic pollen grains exceeding the ovule number of pollen grains were found on 27% and 17% of the once visited recipient flowers of the long- and short-styled morphs, respectively, while negligible numbers of legitimate pollen grains were observed on 13% and 42% of the visited flowers of the long- and short-styled morphs, respectively (Fig. 5).

POLLEN BALANCE SHEET

The pollen balance sheet for a flower after a single visit by bee, i.e., the number of pollen grains produced per flower, those remaining in anthers, those transferred to the bee proboscis, those loaded on the self stigma, and those shed on the corolla or inside the corolla-tube are summarized in Table 4.

The number of pollen grains produced per flower was twice larger in the long-styled morph than in the short-styled morph (Table 4). The means of pollen load on the bee proboscis were not significantly different between the morphs, and these were only a small fraction of pollen produced (approximately 6%, 13500 in the long-styled and 10000 in the short-styled morph, Table 4). Mean stigmatic self pollen deposition was several times larger in the long-styled morph than in the short-styled morph (Table 4). For each morph, apparent pollen wastage, i.e., the sum of the pollen grains deposited on the self stigma and shed on other parts of the flower on a single visit by bee was approximately 10% of pollen grains produced per flower, and if 'the lost' was added to the fraction, the wastage was estimated to be 70% of pollen grains produced per flower.

Discussion

THE POLLINATION PROCESSES RESPONSIBLE FOR POLLEN TRANSFER ASYMMETRY BETWEEN THE MORPHS

It has been hypothesized that the asymmetrical pollen transfer between the morphs is attributed to the greater accessibility to contact by insects of long-styled morph than short-styled morph stigmas (e.g. Ganders 1974, 1976; Stone & Thomson 1994).

Most of the previous studies, in which the roles of architectural variation in floral traits in reproductive success of heterostylous plants were analyzed, have used correlative approaches to explore relationships between morphological variation and reproductive success (reviewed by Ganders 1979; Lloyd & Webb 1992). These approaches are useful for generating hypotheses about the causes of reproductive variation among morphs, but cannot demonstrate that the traits under study are the causes. Between-morph comparison of reproductive success at the whole stages of pollination can supplement these studies and enables us to evaluate importance of floral morphological traits. The results of my investigation of pollen transfer patterns in the '*P. sieboldii*-bumblebee experimental system' strongly support the hypothesis that the asymmetrical pollen transfer is largely ascribed to the floral architecture.

In the pollination process, reproductive success of a flower through female function is dependent on the receipt of sufficient pollen grains to fertilize the ovules of the flower. Reproductive success as a male parent is dependent on dispersal of pollen grains to as many ovules of recipient flowers as possible (Harder & Barrett 1996). In *P. sieboldii*, despite twice the larger number of pollen grains produced in the long-styled morph compared to that of the short-styled morph, no significant difference between the morphs was found

in pollen removal from a flower on a single visit by the bee. The long-styled morph with anthers placed deep within the corolla tube produced larger number of pollen grains than the short-styled morph, thereby compensating for their disadvantage in pollen removal by pollinators. No significant difference in pollen removal between the morphs means that potential number of pollen grains delivered from a flower to other flowers is not biased between the morphs in *P. sieboldii*.

However, the stigmas of the long-styled morph received significantly more legitimate pollen grains than those of the short-styled morph on a single visit by the pollinator. The short-styled morph donated pollen to compatible morph more efficiently than the long-styled morph. Therefore, the short-styled morph may have higher male fitness and the long-styled morph may be relatively female. The between-morph difference in stigma height is likely to account for the pollen transfer asymmetry in *P. sieboldii*, suggested by Nishihiro et al (2000).

The relatively narrow corolla tube of the *P. sieboldii* flower may tightly restrict the entry/exit path of the proboscis of the effective pollinator bee queens foraging for nectar secreted at the bottom of the corolla. When proboscis is inserted into and withdrawn from the flower, the bee must extensively contact the stigma surface with a more apical region of the proboscis than 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 (Nishihiro et al. 2000).

DIFFERENT VULNERABILITY TO POLLEN LIMITATION

In both morphs, stigmatic pollen load after a single visit by the bee may be insufficient for maximum seed set. Several visits by the pollinators in both morphs would be required for receiving pollen grains far more than the ovule number, which is a necessary condition for full fertilization of the ovules. The short-styled morph is thought to be more vulnerable to

legitimate pollen shortage than the long-styled morph in *P. sieboldii* because of much smaller legitimate pollen load on their stigmas. Previous studies for natural populations of *P. sieboldii* (Washitani et al. 1994b; Chapter 1, Matsumura & Washitani 2000) demonstrated the vulnerability to pollen limitation of the short-styled morph compared to the long-styled morph.

POTENTIAL OF MIXED POLLINATION AND FEMALE SUCCESS

Although most of the pollen grains from donor flowers were deposited on the first several recipient flowers were subsequently visited by the bee, considerable amounts of grains traveled much further. The number of the donor pollen grains loaded on the recipient stigmas increased with increase of the total number of flowers visited by the bee during an experimental run. The extensive pollen carryover thus demonstrated suggested that a mixed pollen load would be brought about by a single visit by the bee which carries mixture of pollen from many flowers of different genets on its proboscis in the population which consists of a number of genets.

In *P. sieboldii*, an artificial pollination study revealed that not only pollen load size but also mixed pollination are favorable for female reproductive success (Kawakami M., Nishihiro J. & Washitani I., in preparation). In natural populations of *P. sieboldii*, pollinator visitation rate is relatively low, since average visit rates by queen bumblebees were observed to be 0.1 or at best 0.6 visit per patch per hour (Matsumura C. unpublished data, 1996). Thus, mixed pollen deposition attained by a single visit would be advantageous for female reproductive success.

In the species of which the mixed pollination is brought about by the simultaneous deposition of a mixed load, the factors which influence the composition of a pollen load: the plant density, spatial pattern, and frequency of mating groups in a population (Antonovics

& Levin 1980; Kunin 1992, 1993, 1997; Aizen & Feinsinger 1994; Ågren 1996; Groom 1998; Aizen 2001) would be important for female success. Female success of *P. sieboldii* is thought to be sensitive to habitat fragmentation and isolation (Chapter 1, Matsumura & Washitani 2000).

MERIT OF SEMI-NATURAL EXPERIMENTAL SYSTEM IN POLLEN CARRYOVER MEASUREMENT

Pollen carryover has long been recognized as an important factor that affects the extent of gene flow in plant populations (Schaal 1980; Karron et al. 1995). Despite the necessity of obtaining real patterns of pollen carryover to estimate its effect on gene flow, most of data in the previous studies were obtained from the measurements under rather artificial settings, i.e., with fluorescent dyes as pollen analogue of which the dynamics was not correspondent with pollen (Thomson et al. 1986), or with emasculated recipient flowers which might itself influence pollen dispersal (Price & Waser 1982). Experimental systems permitting measurements under more natural conditions, as I used in the present study, may contribute to the future studies of the pollination mechanisms and their consequences in flowering plants.

Table 1 Mean ± S.D. of floral morphological traits of *Primula sieboldii* genets used in the experiments in 1997 and 1998. 'n' = number of flowers measured.

Genet	Morph	Year	n	Corolla diameter (mm)	Corolla-tube length (mm)	Corolla-tube breadth (mm)	Stigma height (mm)	Anther height (mm)
P0	Long	1997	10	25.45±1.84	12.48±0.40	1.74±0.13	12.24±0.23	9.46±0.28
P1	Long	1997	10	23.08±0.72	11.65±0.43	1.58±0.16	10.79±0.29	8.64±0.42
P2	Long	1997	10	29.51±1.44	13.17±0.54	1.79±0.11	11.53±0.38	9.98±0.58
P3	Long	1997	10	28.17±2.16	12.64±0.71	1.56±0.19	12.31±0.51	9.56±0.35
P4	Long	1997	10	28.15±2.13	12.59±0.58	1.52±0.11	11.35±0.49	8.92±0.46
P5	Long	1998	8	27.36±0.98	12.71±0.24	1.67±0.14	12.01±0.30	9.56±0.32
P6	Long	1998	9	26.27±3.58	11.20±0.73	1.44±0.17	11.08±0.42	7.87±0.84
P7	Long	1998	15	27.10±1.38	13.65±0.41	1.34±0.13	11.88±0.23	10.17±0.47
P10	Long	1998	17	23.46±3.24	11.23±0.71	1.54±0.12	10.47±0.80	8.12±0.38
T0	Short	1997	10	28.02±1.48	13.19±0.34	1.6±0.17	8.81±0.31	12.73±0.38
T1	Short	1997	10	27.63±1.61	13.05±0.47	2.1±0.18	7.9±0.44	12.12±0.36
T2	Short	1997	11	24.37±0.59	13.06±0.40	1.72±0.08	8.58±0.23	12.11±0.31
T3	Short	1997	10	26.5±1.30	12.89±0.41	2.45±0.14	8.04±0.25	12.33±0.28
T4	Short	1997	10	27.64±0.93	14.43±0.29	1.68±0.11	8.61±0.28	13.64±0.42
T7	Short	1998	18	27.22±1.89	15.54±0.55	1.55±0.17	8.31±0.33	14.29±0.58
T8	Short	1998	10	26.64±2.62	12.85±0.53	2.29±0.16	8.75±0.29	11.81±0.42

Table 2 Pollen size parameters for combinations of donor and recipient genets of individual runs in the experiment for measurement of pollen carryover in 1997 and 1998. For each combination, the critical value and judgement error assumed for the descrimination of donor and recipient pollen grains by their sizes are shown. 'n' = number of pollen grains measured.

	Donor			Recipient				Judgement error (%)		
Run	Genet	Pollen size (μm)	n	Genet	Pollen size (μm)	n	Critical value (μm)	Long-styled	Short-styled	Year
1	P0	11.82 ±0.90	1800	T2	17.52 ±1.37	3000	14.27	0.50	0.53	1997
2	P0	11.82 ±0.90	1800	T3	16.86 ±1.37	3000	13.82	1.39	1.40	1997
3	P0	11.82 ±0.90	1800	T1	15.97 ±1.30	3000	13.48	2.94	3.03	1997
4	P4	11.44 ±0.82	3000	T4	15.83 ±1.27	2800	13.32	1.50	1.54	1997
5	P7	12.16 ±1.15	600	T7	18.03 ±1.58	600	14.90	1.67	1.50	1998
6	P4	11.44 ±0.82	3000	T4	15.83 ±1.27	2800	13.32	1.50	1.54	1997
7	P7	12.16 ±1.15	600	T7	18.03 ±1.58	600	14.90	1.67	1.50	1998
8	P7	12.16 ±1.15	600	T8	18.12 ±1.64	600	14.75	2.33	2.00	1998
9	P7	12.16 ±1.15	600	T8	18.12 ±1.64	600	14.75	2.33	2.00	1998
10	P7	12.16 ±1.15	600	T8	18.12 ±1.64	600	14.75	2.33	2.00	1998
11	T0	16.47 ±1.89	1300	P3	11.34 ±0.86	3000	12.90	3.06	2.92	1997
12	T0	16.47 ±1.89	1300	P1	10.37 ±0.90	2500	12.44	1.36	1.31	1997
13	T8	18.12 ±1.64	600	P6	11.22 ±1.00	600	14.00	0.33	0.33	1998
14	T4	15.83 ±1.27	2800	P4	11.44 ±0.82	3000	13.32	1.50	1.54	1997
15	T7	18.03 ±1.58	600	P10	13.01 ±1.54	600	15.70	5.00	7.33	1998
16	T7	18.03 ±1.58	600	P5	10.50 ±1.45	600	14.88	1.83	1.50	1998
17	T4	15.83 ±1.27	2800	P4	11.44 ±0.82	3000	13.32	1.50	1.54	1997
18	T7	18.03 ±1.58	600	P5	10.50 ±1.45	600	14.88	1.83	1.50	1998
19	T0	16.47 ±1.89	1300	P2	11.38 ±1.03	3000	13.22	4.67	4.62	1997
20	T0	16.47 ±1.89	1300	P2	11.38 ±1.03	3000	13.22	4.67	4.62	1997

The morphs are coded as follows: P = long-styled morph, T = short-styled morph.

Table 3 Morphological traits of the queens of *Bombus diversus tersatus* used in the experiments in 1997 and 1998.

Bee individual	Proboscis length (mm)	Head breadth (mm)	Year
1	13.45	3.33	1997
2	13.32	3.46	1997
3	12.82	3.57	1997
4	12.81	3.05	1997
5	12.66	3.32	1998
6	12.94	3.37	1998
7	12.42	3.36	1998

Table 4 Pollen balance sheet of a flower of *Primula sieboldii* on a single visit by a queen bumblebee with the reference of the unvisited flowers.

Components	Long-styled morph					Short-styled morph						
	Visited	n	Unvisited	n	*P*	Visited	n	Unvisited	n	*P*	Test	*P*
Pollen produced per flower (estimate)			281198±60512	20				149055±28124	18		MW	<.0001
Pollen deposition on bee proboscis	13475±4804	12				10031± 5171	13				MW	=.07
Pollen remaining in anthers per flower	55485±13225	16	84705±26198	6	<.05	37646±15683	14	67554±13224	6	<.01	MW	<.01
Self pollen load on stigma	3138±1269	16	545±621	6	<.001	659±559	14	10±17	6	<.001	MW	<.0001
Self pollen grains on the corolla	2956±2136	16	205±195	6	<.05	662±1024	14	286±361	6	=.31	MW	<.01
Self pollen grains inside the corolla	24850±5876	16	23200±4343	6	=.53	10900±4648	14	7783±4270	6	=.19	MW	<.0001

Notes on statistical tests: significance was assumed from the magnitude of the difference in means between the visited and unvisited flowers in each morph by Mann-Whitney *U*-test, significance was assumed from the magnitude of the difference in means of the visited flowers between the morphs; MW = Mann-Whitney *U*-test. Blank indicates absence of data.

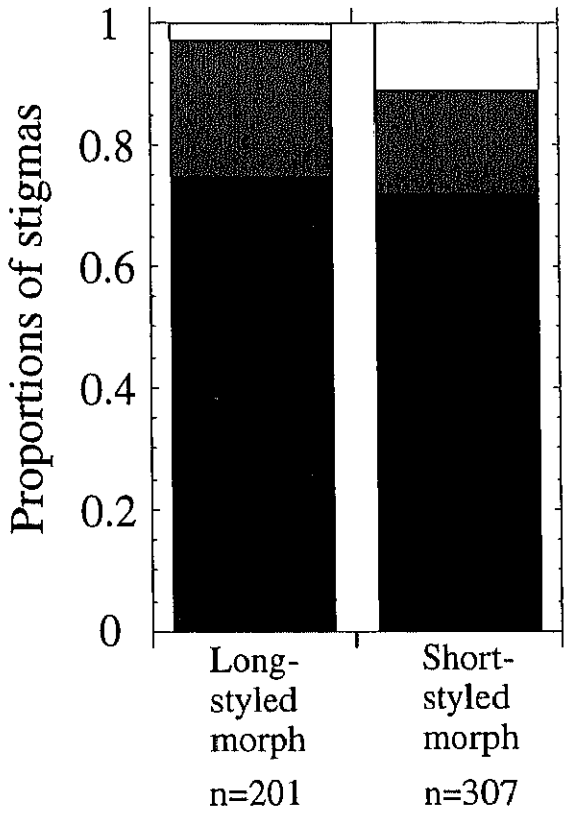
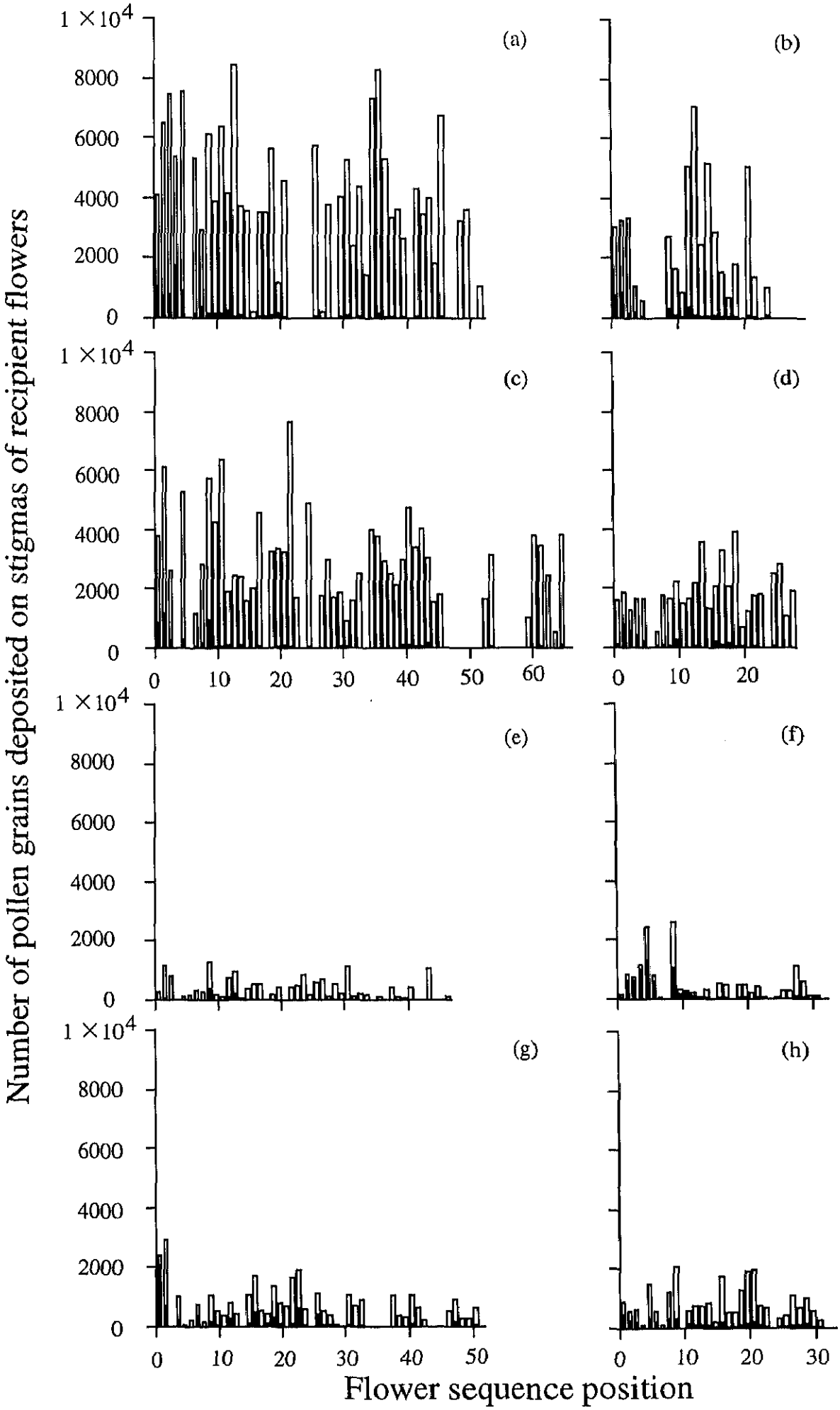


Fig. 1 The proportions of three types of stigma status for the unvisited *Primula sieboldii* flowers during the runs for the measurement of pollen carryover. The groups were set on the basis of the average number of ovules per flower of *Primula sieboldii* (120, Nishihiro et al. 2000). ■; with no pollen, ■; with 1 to 120 pollen grains, □; with > 120 pollen grains.

Fig. 2 Deposition of donor (filled bars) and illegitimate (open bars) pollen grains on the stigmas of the long-styled morph recipients of *Primula sieboldii* (run numbers; (a) 11; (b) 13; (c) 12; (d) 15 in Table 2) or short-styled morph flowers (run numbers; (e) 5; (f) 2; (g) 1; (h) 3 in Table 2) plotted against flower sequence position. Among the 20 runs in which the visitation sequence is consecutive with no grooming behavior, the 8 runs with relatively long visitation sequences are shown here. When the same recipient flower is visited two or three times by a bee in an experimental run, only a flower sequence position of the first visitation is indicated in the figure and void sequence positions are due to the second and/or the third visitation.


Number of pollen grains deposited on stigmas of recipient flowers
1 ×10^4
8000
6000
4000
2000
0
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
0 10 20 30 40 50 60
0 10 20
0 10 20 30 40 50
0 10 20 30
Flower sequence position

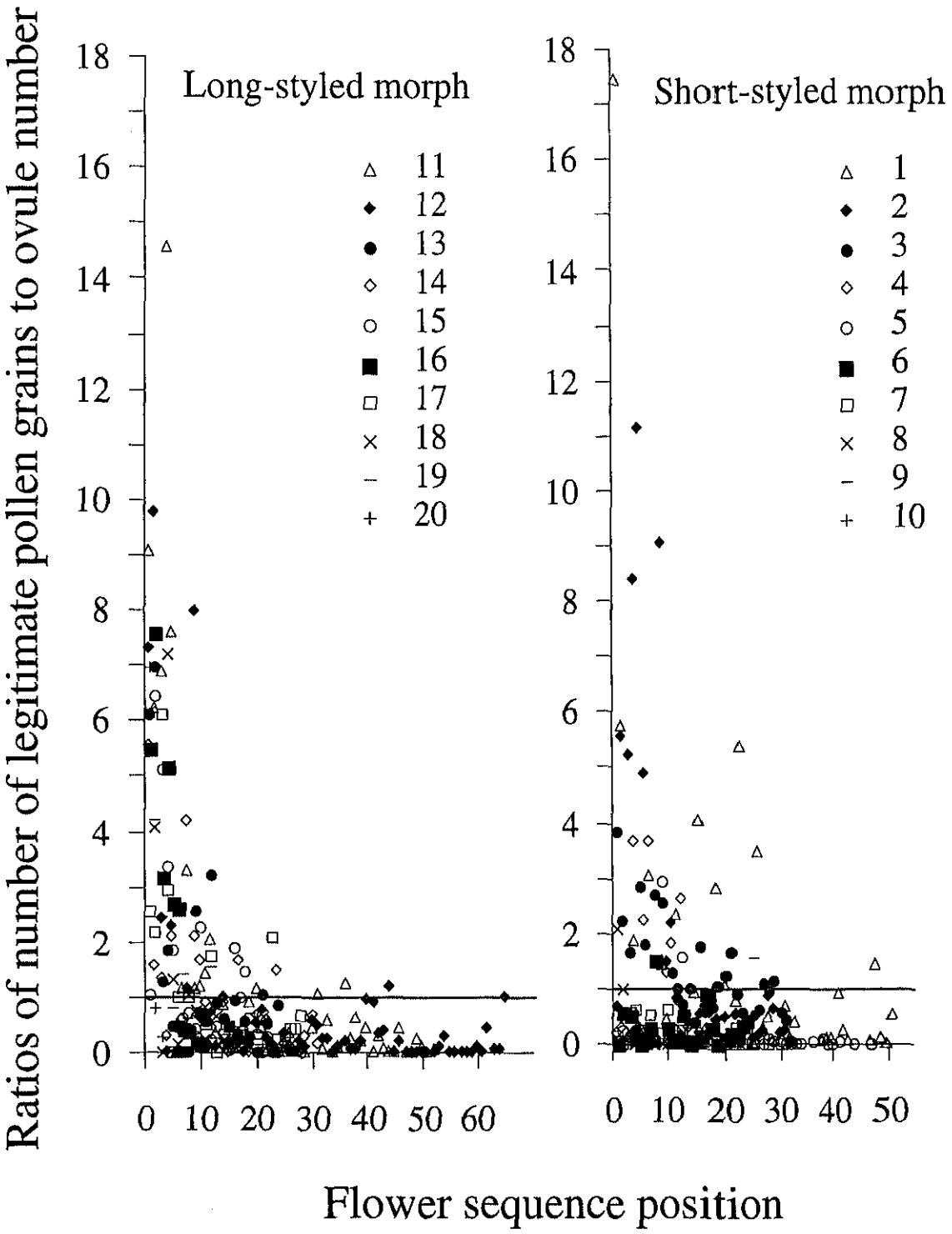


Fig. 3 The ratio of the number of donor pollen grains deposited on the stigmas of the recipient flowers to the average number of ovules per flower of *Primula sieboldii* (120; Nishihiro et al. 2000) plotted against flower sequence position. Different symbols indicate different runs. The numbers assigned to the runs correspond to those listed in Table 2.

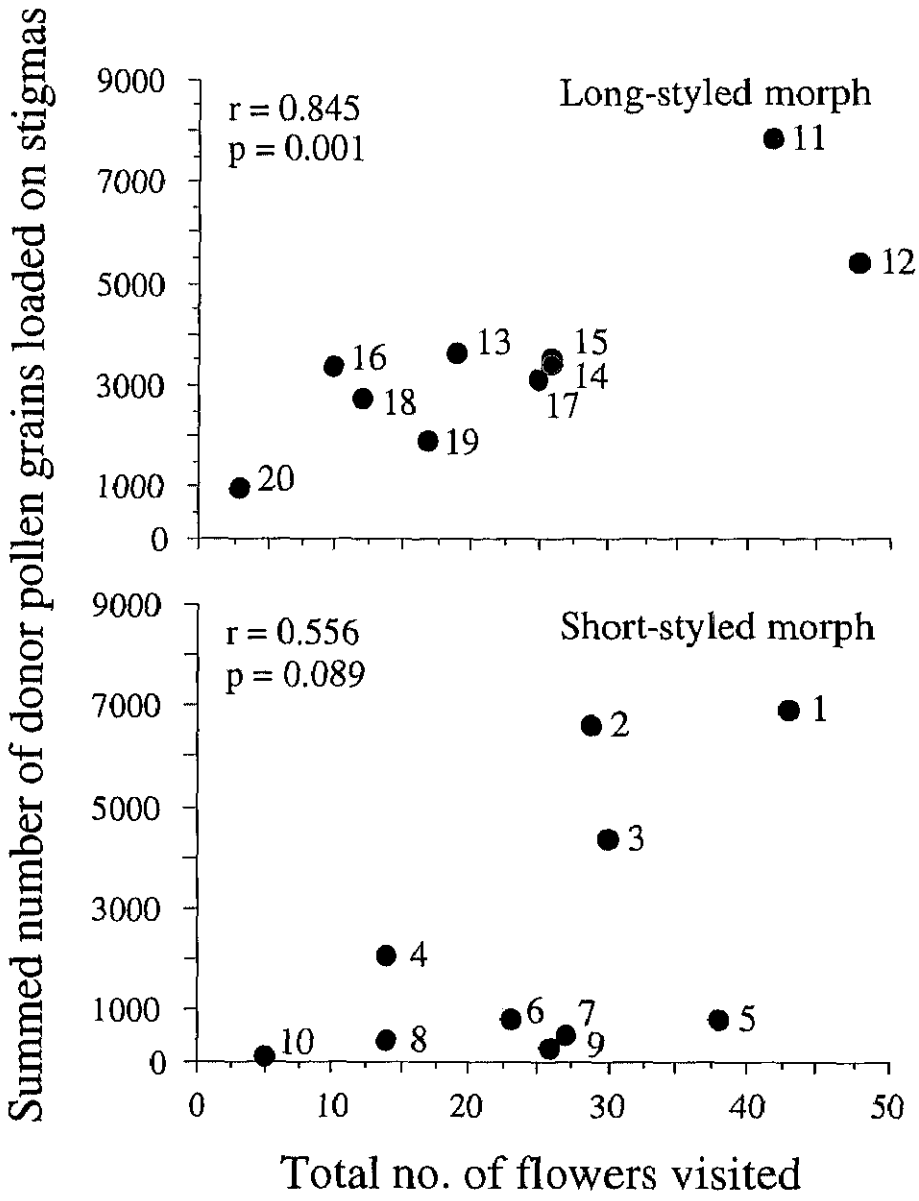


Fig. 4 Relationship between the summed number of the donor pollen grains loaded on the stigmas during a visitation sequence and the total no. of flowers visited. Numbers in the right of individual symbols assigned to the runs correspond to those listed in Table 2. Correlation coefficient and its significance level are also shown.

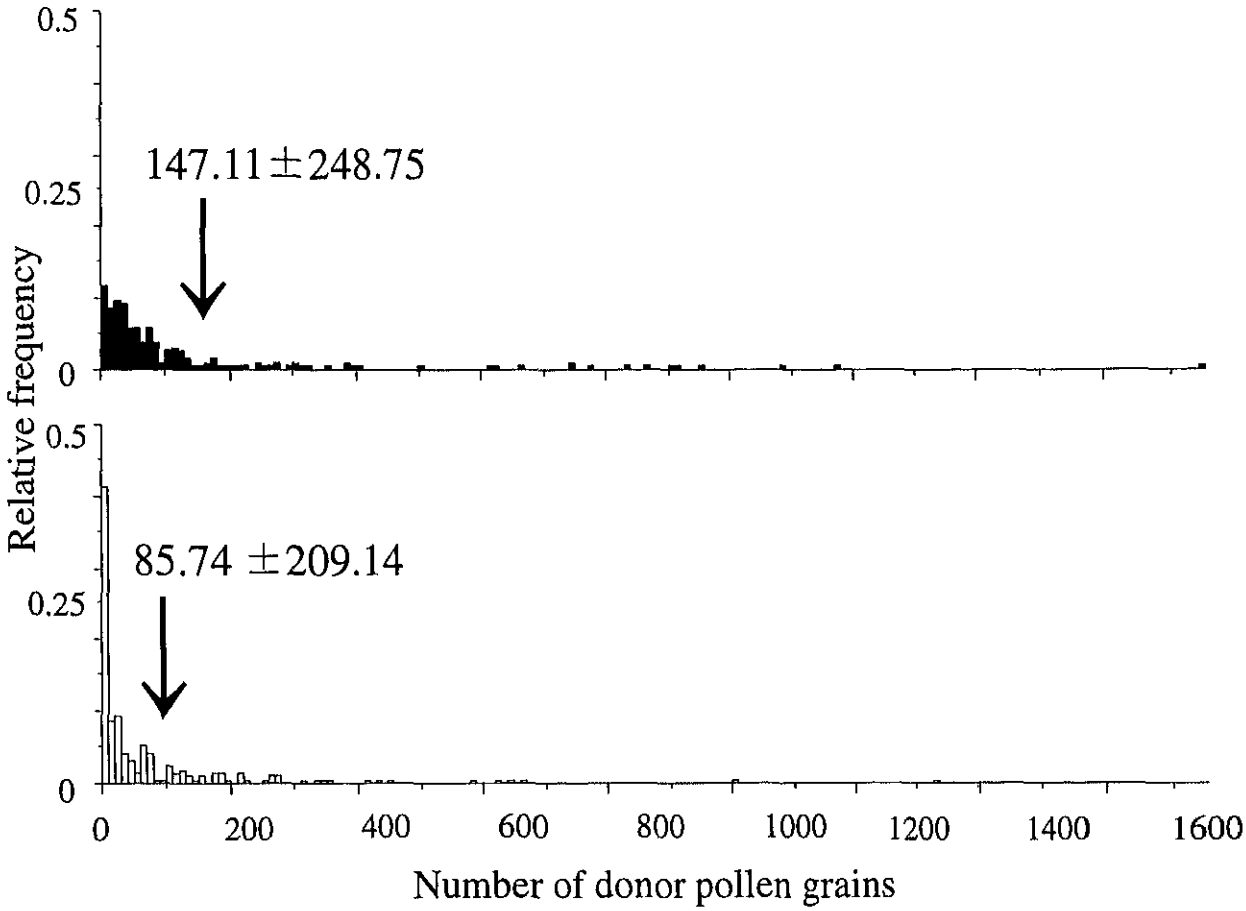


Fig. 5 Relative frequency distribution of number of legitimate pollen grains loaded on the stigmas of the long-styled (above, number of stigmas; 184) and the short-styled (below, number of stigmas; 216) morph flowers of *Primula sieboldii* which were visited once during the 20 runs in the experiment for measurements of pollen carryover. An arrow in each histgram indicates the average number of legitimate pollen deposition on the stigmas of the recipient flowers.