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Aspects of sexual failure in the reproductive processes of a rare bulbiferous plant, *Titanotrichum oldhamii* (Gesneriaceae), in subtropical Asia

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Abstract *Titanotrichum oldhamii* produces both flowers and asexual bulbils on its inflorescences. However, field observations and herbarium collections indicate that seed set is infrequent and that most reproduction is from vegetative bulbils. We have investigated the failure of sexual reproduction and identified four major causes: (1) in the wild, the seed:ovule ratio for open pollination was only 1.9%, in contrast to 10.1% for artificial cross-pollination, implying poor pollinator services. (2) The overall reproductive success was 5–10 times greater in glasshouse pollination treatments compared to field treatments. This suggests suboptimal environmental conditions for seed set in natural habitats, which may eliminate natural seed set completely. (3) Pollination experiments showed that outcrossed populations set significantly more seed and had higher germination rates than intra-population and self crosses, in both field and glasshouse experiments. *T. oldhamii* thus appears to benefit from wide outcrossing. Pollen transfer between populations, however, seems to be infrequent because of the rarity and scattered distribution of *Titanotrichum* populations. (4) Flowers near the apex of the inflorescence are less likely to set seed, es-

pecially late in the season when inflorescences convert to bulbil production. In these late flowers, pollen tubes showed poor guidance as they approach the micropyle of the ovules. Even under optimal glasshouse conditions, the average outcrossing success was only 0.229. Almost one-half of the ovules remained undeveloped and 13.5% of ovules aborted after pollination, indicating a strong shift of resource allocation toward vegetative bulbils and rhizome development. Efficient reproduction from asexual bulbils may thus have released *Titanotrichum* from strong selection for efficient sexual reproduction. However, occasional seed set observed in the wild may be very important for maintaining some genetic diversity in populations, and promoting overall fitness.

Keywords Asexual reproduction · Vivipary · Bulbil · Pollen tube guidance

Introduction

Titanotrichum oldhamii (Hemsl.) Soler. (Gesneriaceae) is a tropical bulbiferous plant. The inflorescence can convert to producing seed-like vegetative dispersal structures termed “bulbils”, by the conversion of a floral meristem into bulbil primordia (Wang and Cronk 2003). In a study of flower and bulbil development of *T. oldhamii*, Wang and Cronk (2003) found that a single floral meristem converts into a cluster of ca. 60–80 bulbils, unlike other bulbiferous plants such as *Polygonum viviparum* and *Mimulus gemmiparus*, in which a single flower is replaced by a single bulbil (Diggle 1997; Moody et al. 1999). As many as 20–30 clusters are produced per side branch and 20 or more branches can be present per plant (Fig. 1). Thus, *T. oldhamii* can very effectively produce thousands of bulbils on a single plant. Although extensive asexual reproduction by bulbils is evident in *T. oldhamii*, the plant is a rare endemic of Taiwan, southern Ryukyu and the adjacent area of China (Walker 1976; Li and Kao 1998; Wang et al. 1998). It occurs in fragmented populations, often along small creeks. Even though some

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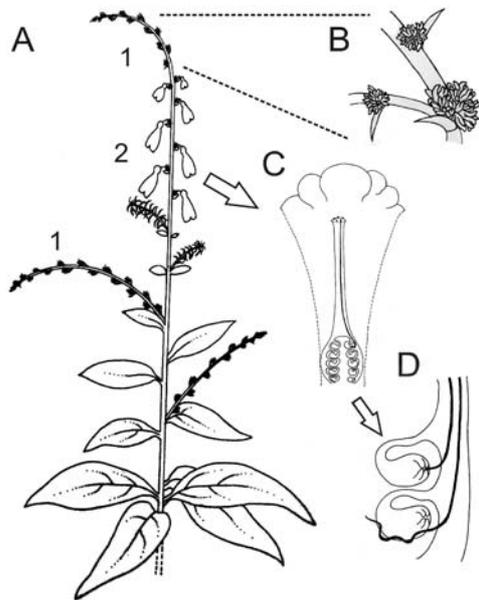


Fig. 1A–D Flower and bulbil arrangement in *Titanotrichum oldhamii*. **A** Drawing of flowering shoot showing bulbil cluster (1) and flowers (2), after Wang and Cronk (2003). **B** Schematic drawing of bulbil clustering (not to scale). **C** Schematic drawing of flower cut open to reveal ovules. **D** Schematic drawing of ovules showing normal fertilisation (top) and pollen tube guidance failure (bottom)

15–30 showy flowers (each possessing up to a few thousand ovules) can be produced per raceme, seed set in the wild is rare. At the end of the flowering season (late August) or under short daylength conditions, bulbil production in the inflorescences becomes the dominant reproductive mode in almost all natural populations (Wang and Cronk 2003; Wang et al. 2004a). The plants also perennate by means of vigorous rhizomes.

In many arctic and temperate bulbiliferous plants there is a significant reduction in the number of fertile flowers produced compared to their close relatives growing in more southern, temperate regions (Kerner 1904; Diggle et al. 1998). The reduction in flower number alone may cause pollination failure simply because the few flowers fail to attract sufficient pollinators, which are anyway limited in arctic regions (Kerner 1904). However, *T. oldhamii* grows in the tropics, where flowers are produced abundantly. There are only a few studies on the sexual reproduction and pollination of plants that are primarily bulbiliferous, and the conclusions drawn are equivocal. Studies by Brochmann and Håpnes (2001) on the pollination of two bulbiliferous plants, *Saxifraga cernua* and its bulbiliferous hybrid *Saxifraga svalbardensis* (a cross with *Saxifraga rivularis*), suggested that these two taxa are fertile although natural seed set has rarely been observed in either the hybrid or the parent. Artificial pollination increased seed set greatly, suggesting that pollinator activity was the limiting factor in seed set. A study of *Saxifraga granulata* in Danish populations revealed that these hermaphrodite flowers showed over 70% seed set upon both selfing and crossing, although bulbil production was still the most important mode of reproduction

(Hansen and Molau 1994). However, another study on English populations of the same species growing in a harsh habitat showed a high degree of sexual sterility and inbreeding depression upon selfing of hermaphrodites of these gynodioecious populations (Stevens 1988). Outcrossing species usually suffer a large genetic load because mutational recessive lethals can be maintained by heterozygosity, thus decreasing fitness compared to their selfing relatives. For example, *Epilobium angustifolium* aborted 60–80% embryos after pollination, while its inbreeding relatives have virtually no abortion after selfing (Wiens et al. 1987). The flower of *T. oldhamii* is showy, implying potential for outcrossing, but most *T. oldhamii* populations are small and, to a high degree, clonal (Wang et al. 2004b). The low seed set in the wild could also be the result of inbreeding. It could also result from resource re-allocation due to the numerous developing vegetative bulbil meristems, if the latter are a stronger sink for photosynthates or perturb the hormonal balance within the inflorescence in a way unfavourable to developing seeds.

To investigate the causes of sexual failure in *T. oldhamii*, observations and experiments on pollination and fertilisation processes and seed performance were conducted both in the field and in the glasshouse.

Materials and methods

Floral phenology and pollination studies

Field experiments

Field observations on more than 50 individuals were used to establish floral phenology and the timing of sexual stages (anther dehiscence and stigma receptivity) were recorded.

One population of *T. oldhamii* in the Yangmingshan National Park, Kuanfu lo, Taipei, northern Taiwan (25° 09' N, 121° 32' E) was selected for pollination study during the summer of 1999 (June–August). The population genetic structure of this population was surveyed in our previous study and found to consist of several different clones (Wang et al. 2004b). The within-population pollination treatments avoid crossing within the same clone. Day length in summer was about 14 h. The average summer temperature was about 28°C during the day and 20°C at night (reports from the nearest weather station). This population was well protected inside the natural reserve with ca. 200 mature individuals, and frequent insect flower visitors were observed. Pollinators observed visiting *Titanotrichum* flowers were caught and identified.

Pollination experiments set up in the field in 1999 were designed to investigate the possible breeding strategy of *Titanotrichum*. Six treatments were investigated: (1) pollen exclusion to test for agamospermy, (2) pollination with pollen from the same flower (within-flower selfing), (3) pollination with pollen from different flowers of the same individual (geitonogamous selfing), (4) selected flowers labelled and emasculated (open pollination), (5) cross pollination between different clones within populations, and (6) cross pollination between populations.

Up to eight flowers in one to two inflorescences were selected on randomly chosen individuals. As it was observed that fruit set (proportion of enlarged capsules) of *Titanotrichum* on herbarium sheets was limited mainly to the lower part of the inflorescence (when bulbils were present at the upper part of the inflorescence), flowers near the top of the raceme were excluded from these experiments to avoid any possible effect of bulbil development on seed set. To investigate possible agamospermy (treatment 1, apomixis), flowers emasculated at the bud stage were bagged in hand-

Table 1 Localities of nine populations of *Titanotrichum oldhamii* used for glasshouse and field pollination experiments

Population code	Country	Locality (region)	Latitude	Longitude
A ^a	Taiwan (north)	Kuanfu lo (Taipei county)	25.09	121.32
B	Taiwan (north)	Juansi waterfall (Taipei county)	25.10	121.33
C	Taiwan (north)	Wulai (Taipei county)	24.52	121.33
D	Taiwan (north-central)	Ronhua (Taoyuan county)	24.48	121.20
E	Taiwan (north-central)	Tawan (Taoyuan county)	24.36	121.19
F	Taiwan (central)	Pahsien shan (Taichung county)	24.10	121.01
G	Taiwan (south)	Patunkuan trail (Taitung county)	23.21	121.11
H	Taiwan (east)	Tairoko (Hualien county)	24.09	121.37
I	China	Yungchun (Fujian province)	25.19	118.17

^a The single population on which field pollination studies were carried out

made nets. As dichogamy was observed (3 days after anther dehiscence, the stigma becomes receptive and curves downward to be temporarily in close proximity to the anthers), within flower selfing (treatment 2; selfing within the same flower) was investigated by applying pollen from the same flower directly onto the stigma, and the flower was then bagged. Artificial selfing with pollen from another flower (treatment 3) within the same individual (geitonogamy) was also tested and flowers bagged as above. The extent of natural pollination (to determine pollinator efficiency) was investigated by collecting capsules from open emasculated marked flowers (treatment 4). Flowers for both within and between population crossings (xenogamy) were first emasculated at the bud stage and bagged, and a few days later pollen from individuals of the same population (treatment 5), or the nearest adjacent population (treatment 6; population Juan-si waterfall, ca. 1 mile west of the study site, population code B in Wang et al. 2004b) was applied. Pollen for treatments 3, 4, 5, and 6 came from newly opened flowers, on the 1st day of anthesis. After pollination, the flowers were bagged again, harvested when mature (1.5 months after pollination), and dried at room temperature (ca. 20°C). The number of enlarged capsules [fruit/flower (Fr/FI) ratio], seed set per capsule (*T. oldhamii* has only one ovary per capsule), and seeds/ovule (S/O) ratios were also recorded. Aborted seeds appear hollow and transparent yellow in colour, and are distinguishable from brown mature seeds and from undeveloped ovules. The abortion rate (AR) was calculated as the ratio between aborted seeds and total ovule numbers. Harvested mature seeds were germinated in Petri dishes on filter paper at 22°C and 16 h daylength. We have observed that seeds of *T. oldhamii* usually germinate within 2–4 weeks. The proportion of germinated seeds (radicle emergence) was thus counted after 14 days (early germination rate, germination vigour) and 30 days (total germinability). No further seeds germinated after 30 days.

The product of Fr/FI, S/O and proportion of total germination is a measure of total reproductive success (RE) (Wiens et al. 1987). We use this to compare the reproductive success of each treatment.

Glasshouse experiments

A total of 55 individuals from 9 natural populations, all samples from our field study, were collected and cultivated in the living research collections at the Royal Botanical Garden, Edinburgh (RBGE) (Table 1). These populations had all previously been investigated for their population genetic structure (Wang et al. 2004b). As there is some clonal structure within each population, the individuals chosen from each population avoid the same genotype. The minimum temperature was set to 20°C (day) and 18°C (night). We focused on differences between (1) selfing-geitonogamy, (2) within population crosses, and (3) between population crosses.

All pollen came from flowers on the 1st day of anther dehiscence. Pollen viability checks were performed with 0.1% cotton blue or 0.1% acetocarmine (Dafni 1992). In all cases a high per-

centage (80–90%) of normally formed stained grains was found (data not shown). The receptivity of the stigma was verified visually (the stigma becomes sticky and the style starts to curve down) prior to pollen application in all pollination treatments.

During the summer of 2000, 320 flowers were pollinated in different treatments (for methods, see the corresponding field experiments) and most treatments were repeated in the summer of 2001. Data for the 2 years were combined for analysis. Capsules were harvested when mature. Seed set and germination analysis followed that of our field study.

Seed set in relation to flower position

On herbarium sheets, fruit set of *Titanotrichum* was observed mainly in the lower part of the inflorescence when bulbils were present in the upper part of the inflorescence. To examine the effect of bulbil development and flower position on seed set, we cross-pollinated, over a period of 6 weeks, all flowers within three inflorescences of one individual from population Wulai (population C) cultivated at RBGE with pollen from one individual of population Ronhua (population D). Seed set per capsule was then determined and correlated to the exact flower position in the raceme. This combination of genotypes results in regular high seed set, thus allowing us to check any seed set difference before and after bulbil initiation.

Pollination and fertilisation processes in the style and ovary

To investigate potential fertilisation failure, we traced pollen tube development among different treatments after reciprocal pollination of individuals of Juansi (population B), Patunkuan trail (population G) and Tairoko (population H). For each treatment, 10–15 pollinated flowers were picked either 48 h or 96 h after pollination to monitor pollen germination on the stigma, pollen tube elongation inside the style, and pollen tubes entering the ovules. Moreover, we further monitored flowers pollinated after bulbil initiation to check if the latter had any effect on the fertilisation process.

Pollinated flowers were fixed in FAA (1:1:18, 40% formaldehyde: glacial acetic acid: 70% ethanol) for 24 h and stored in 70% ethanol. Prior to examination, styles were softened in 8 N NaOH for 2–4 h then stained with 0.1% aniline blue for not more than 10 min (Dafni 1992). After staining, excess stain was replaced by water to reduce background staining. The styles were then cut longitudinally and individually squashed under a cover-slip in a drop of 10% glycerol. The slide was then examined immediately under UV light with a fluorescence microscope (Axioscop; Zeiss, Welwyn Garden City, UK). The exposure time of slides under UV light was kept to a minimum to avoid bleaching effects.

To investigate fertilisation failure in more detail, we examined pollen tip growth inside the ovary near the ovules in flowers pollinated before and after bulbil initiation; 12 2-week old cross-pollinated flowers of the Wulai-Ronhua genotype combination were

Table 2 Results of field pollination and germination experiments on *T. oldhamii* population A (Kuanfu lo), including fruit/flower (Fr/FI) ratio (number of enlarged capsule/number of flowers studied), seed set per capsule, seed:ovule (S/O) ratio, abortion rate (AR), early germination rate and total germination counted after 14 days

Treatment	1	2	3	4	5	6
	Pollen exclusion	Within flower selfing	Geitonogamy	Open pollination	Within population crosses	Inter population crosses
Parameter						
Individual plants	12	14	16	14	19	15
<i>N</i> (Individual flowers)	25	27	28	105	32	25
Fr/FI ratio	0	0	0.32	0.32	0.63	0.68
Seed set per capsule	0	0	17.4±10.7 a	29.2±7.4 a	79.3±14.5 a	223.8±36.7 b
S/O ratio (%)	–	–	1.2±1 a	1.9±3 a	3.2±1 b	10.1±1 c
AR (%)	–	–	7±3	9±4	11±2	13±3
Early germination rate (%)	–	–	4±2 a	7±2 a	39±5 b	67±6 c
Total germination (%)	–	–	15±5 a	17±4 a	57±6 b	80±5 b
RE	–	–	0.0006	0.0010	0.0115	0.0549

analysed using scanning electron microscopy (SEM) (5 before and 7 after bulbil initiation). The SEM procedure, sample treatment and equipment used are identical to that of Wang and Cronk (2003).

Statistical analysis

Pollination and germination data were arcsine transformed and analysed by ANOVA to test for significant differences between treatments. The ANOVA test was implemented through the “VassarStats” package (<http://faculty.vassar.edu/lowry/VassarStats.html>). If ANOVA results indicated that results from treatments were significantly different, a Tukey HSD test (Tukey’s honestly significant difference) was performed to determine the pairwise differences. Values presented in the tables are means with their standard errors (SE).

Results

Floral phenology and pollinator observations

The flower of *T. oldhamii* has a distinctive deep yellow corolla tube with lobes blotched dark crimson-brown. The anthers dehisce as soon as the flower is open. Behind the anthers in the corolla tube, the style gradually elongates. The stigma becomes receptive about 2–3 days after flower opening. The corolla abscises about 5 days after opening, or as soon as it is pollinated. Flowering starts in June and continues until August. Late in the flowering season, bulbils start to initiate at the top of the inflorescence and from axillary meristems of every flower (Fig. 1).

Several generalist pollinators were observed visiting *Titanotrichum*. They were identified as the bees *Xylocopa appendiculata* and *Ceratina flavipes*, and the butterfly *Notocrypta curvifascia*. They tended to visit flowers within the same raceme before visiting another individual. The peak of their activity was at midday in sunny weather but their visits were not regular. Moreover, they were nonspecific pollinators, as they more frequently visited other species nearby (e.g. *Begonia* spp.).

and 30 days, respectively, and reproductive success (RE) resulting from different pollination treatments (year 1999). Values followed by the same letter indicate groups of means that do not differ statistically

Pollination studies

Field experiments

Among all treatments, artificial inter-population crosses resulted in the highest seed set per flower (223.8±36.7), which converted to an S/O ratio of 10.1% (Table 2). This ratio is over three times higher than that of within-population crosses (3.2%). Selfing within the same flower or pollen exclusion (a test for agamospermy) did not result in any enlarged capsules or seeds. Geitonogamous selfing and open pollination resulted in much reduced seed set per capsule (17.4 and 29.2, respectively), with only 1.2% and 1.9% of ovules developing into seeds in these treatments.

The early germination rate (a measure of germination vigour) and total germination (germinability) of geitonogamous seeds (4% and 15%) and seeds from natural pollination (7% and 17%) was also significantly lower ($F=21.99$, $P<0.001$) than that of seeds from within-population (39% and 57%) and between-population crosses (67% and 80%; Table 2).

The RE was also significantly higher in inter-population crosses (0.0549) ($P<0.001$) compared to that of the selfing and open pollination treatments (0.0006 and 0.001, respectively).

Glasshouse experiments

The glasshouse experiments confirmed the results of the field study. Among all individuals from the nine populations studied, interpopulation crosses resulted in significantly greater seed set (F ranges from 7.34 to 39.27, $P<0.001$), compared to within-population crosses and geitonogamous selfing (Table 3). Unlike the results of the field experiments, there were scarcely any seed set differences between the within-population crosses and the geitonogamous selfing treatments (RE =0.04 vs 0.049, Table 3). However, RE was much higher under glass-

Table 3 Results from glasshouse pollination experiments combined over two flowering seasons (year 2000 and 2001), including Fr/FI ratio, seed set per capsule, S/O ratio, AR and RE resulting from geitonogamous selfing, within-population crossing and inter-population crosses. The population codes follow Table 1. All the results from the inter-population crosses differ significantly from those of the geitonogamy and within-population treatments (year 2000 and 2001), including Fr/FI ratio, seed set per capsule, S/O ratio, AR and RE resulting from geitonogamous selfing, within-population crossing and inter-population crosses. The population codes follow Table 1. All the results from the inter-population crosses do not differ statistically between the three treatments in Tukey's HSD test

Population code	Geitonogamy						Within-population crosses						Inter-population crosses						
	N (Flowers used)	Fr/FI ratio	Seed set	S/O ratio (%)	AR (%)	RE	N (Flowers used)	Fr/FI ratio	Seed set	S/O ratio (%)	AR (%)	RE	N (Flowers used)	Fr/FI ratio	Seed set	S/O ratio (%)	AR (%)	RE	individual plants
A	20	0.55	48±13 a	3.1	39	0.012	17	0.59	116±24 a	8	19	0.036	31	0.58	281±15 b	19	11	0.083	5
B	26	0.73	256±64 a	12	63	0.068	15	0.53	211±66 a	10	54	0.042	53	0.79	1108±49 b	53	9	0.364	6
C	23	0.52	307±87 a	18	28	0.057	20	0.7	238±60 a	14	49	0.074	41	0.73	649±46 b	37	12	0.216	7
D	16	0.69	4±2 a	0.2	81	0.001	17	0.47	78±32 a	4	85	0.013	42	0.81	1112±89 b	55	5	0.361	6
E	21	0.62	546±99 a	24	32	0.113	15	0.53	242±66 a	11	49	0.045	46	0.80	1180±59 b	51	19	0.343	7
F	29	0.54	275±50 a	17	32	0.063	17	0.59	312±90 a	20	31	0.078	31	0.74	667±31 b	42	12	0.239	4
G	24	0.58	24±9 a	1	20	0.004	12	0.33	56±25 a	3	17	0.007	29	0.52	152±34 b	9	14	0.039	7
H	25	0.68	42±13 a	3	49	0.012	20	0.6	101±20 a	7	58	0.030	42	0.79	552±46 b	36	25	0.224	9
I	12	0.50	512±139 a	25	32	0.083	15	0.47	366±189 a	18	28	0.063	27	0.70	859±58 b	42	16	0.226	4
Sum	196						148						342						
Mean		0.63	210±25	11.5	42.5	0.049		0.55	242±32	10.6	44.5	0.040		0.73	816±28	38.2	13.5	0.229	

house conditions in comparison to the same treatments in the field (e.g. selfing success of population A increased from 0.0006 to 0.012, Tables 2, 3).

The resulting seed set, S/O ratio and AR of different treatments varied from population to population. In selfing experiments (geitonogamy), populations A, D, G, and H had a considerably lower level of seed set (seed sets were below 48 and S/O ratios below 3.1%). Inter-population crosses did not result in higher seed set in populations A and G (seed set below 281). The low RE (<0.083) of these populations, even when artificially crossed, indicates that they might have an intrinsically low reproductive fitness.

On the other hand, the Fr/FI ratio did not show much variation between different treatments and populations. This ratio seems have no relationship to seed set differences. Except for a low Fr/FI ratio of 0.33 in within-population crosses of population G, most treatments among all populations had a ratio ranging from 0.47 to 0.81.

Seeds from crosses between populations had a consistently higher early germination rate (70% after 14 days) and a higher total germination rate (81% after 30 days) than those from within-population crosses (57% and 73%, respectively) and selfings (48% and 68%, respectively) (Table 4). The differences in total germination between crosses (between-populations; 81%) and selfings (68%) were not as great as the difference in the early germination rate (70% vs 48%). Except for an unusually low early germination rate (26%) and total germination (52%) of the geitonogamy treatment in population D, variation in seed germination among populations is limited.

Seed set in relation to flower position

Seed set varied significantly according to flower position (Fig. 2). The average seed set per capsule dropped from 514 at flower position 1, farthest from the bulbils, to below 78 at position 22, then dropped to no enlarged capsule at the top of the inflorescence nearest to the bulbils.

Pollination and the fertilisation process in the style and ovary

Among all pollination treatments, flowers pollinated before bulbil initiation showed pollen germination on the stigma surface within 48 h. The pollen tubes then grew to the bottom of the style. Later, about 96 h after pollination, pollen tubes from these treatments usually penetrated the ovule through the micropyle. We observed that self pollen tubes had slightly slower pollen tube elongation, and some showed callose formation at the pollen tube tip in the style. However, the majority of self pollen tubes still penetrated the ovules (over 80%), a proportion almost exactly the same as in the outcross pollen tubes (data not shown).

Table 4 Seed germination results from glasshouse pollination experiments combined over two flowering seasons (year 2000 and 2001), including the early germination rate (14 days after sowing) and the total germination (30 days after sowing) between glasshouse treatments. The population codes follow Table 1. The inter-population germination rates are consistently higher than those of the other treatments

Seed early germination rate (germinating vigor)			
Population code	Geitonogamy (%)	Within-population crosses (%)	Inter-population crosses (%)
A	52	65	68
B	56	59	77
C	41	61	68
D	26	47	70
E	62	65	74
F	44	41	65
G	41	54	71
H	42	58	70
I	45	56	65
Mean	48	57	70
Seed total germinability			
Population code	Geitonogamy (%)	Within-population crosses (%)	Inter-population crosses (%)
A	71	76	75
B	78	79	87
C	61	75	80
D	52	68	81
E	76	77	84
F	69	66	77
G	64	71	83
H	61	72	79
I	66	75	77
Mean	68	73	81

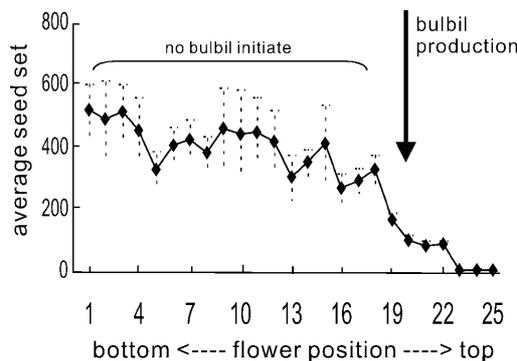


Fig. 2 Average seed set per capsule by flower position in the inflorescence of *T. oldhamii*. The seeds came from crosses between one individual from population Ronhua (E) (pollen donor), and an individual from population Wulai (C) (pollen recipient). Dashed lines Standard deviation, arrow initiation time of bulbils

However, a loss of pollen tube guidance in the ovary was found in pollinated flowers when bulbils developed at the top of the inflorescence (Fig. 3). The pollen grains showed no inhibition of germination and tube elongation in the styles of these flowers. However, after the pollen tubes reached the ovary, instead of penetrating the ovules through the micropyle, they exhibited a loss of direction

while approaching the ovules (Fig. 3c, d). The tips of these tubes were observed to grow in all directions around the ovules and were apparently unable to locate the position of the micropyle. The capsules from “bulbil-affected” pollinations occasionally enlarged after pollination but only a very few seeds developed (<5).

Discussion

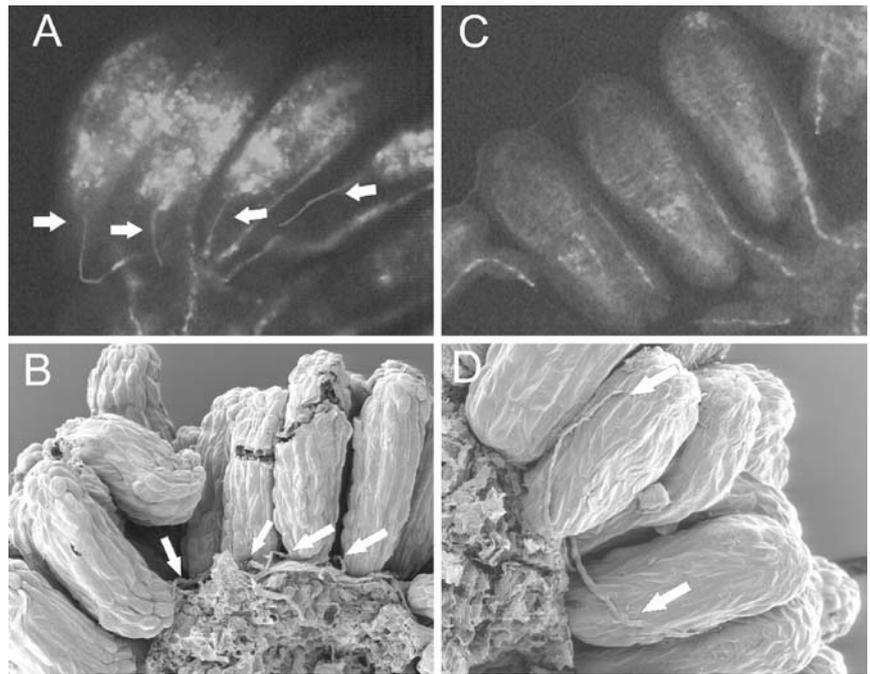
Vegetatively reproducing plants often show a reduced resource allocation for sexual reproduction and this appears to be true in *T. oldhamii*. Under optimal conditions and outcrossing, the S/O ratio in this species is only 38.2%, i.e. only just over one-third of the ovules will set seeds. In the field, *T. oldhamii* shows an even lower level of reproduction and has clear reproductive abnormalities.

Our results have identified four different levels of fertilisation failure in *T. oldhamii*: limited pollinator services (artificial cross pollination in the field increases seed set over open pollination), unfavourable natural environmental conditions (glasshouse seed set is much higher than the same treatments in the field), inbreeding effects (inter-population crosses yield significantly higher seed set), and physiological interference by the initiation of bulbil production (loss of pollen tube guidance in the ovary in flowers near bulbils).

Limited pollinator service in *T. oldhamii*

Seed set from open pollination is low (Table 2). This is consistent with results from the examination of many herbarium specimens (E; TAI; PE; KYU). Also, artificial geitonogamy, selfing and open pollination in natural populations all gave a similar low level of seed set. It is uncertain whether this low seed set is the result of inter-population outcrossing with pollen limitation, or intra-population crosses among clonal progenies showing inbreeding depression. However, from previous population genetic analyses (Wang et al. 2004b), and our preliminary field observations on pollinators, both appear to be probable. Our previous genetic study has shown that many natural populations of *T. oldhamii* are probably clonally maintained by bulbils and rhizomes (Wang et al. 2004b). A low genetic variation within studied populations is observed, with the majority of variation occurring between populations. We observed that general pollinators such as bumble bees (*Xylocopa* sp.) tended to visit flowers within the same raceme before visiting another individual and that they visited other plants nearby more frequently than they did *T. oldhamii*. Given this pollinator behaviour, it is likely that these generalist pollinators are not very efficient in promoting outcrossing between populations. This could be tested in future work by progeny analysis from genotyped parental individuals from adjacent populations.

Fig. 3 Fertilisation process inside the ovary of *T. oldhamii* observed by fluorescence microscopy and scanning electron microscopy. **A, B** Successful fertilisation with pollen tubes penetrating the ovules through the micropyle. **C, D** Loss of pollen tube guidance. *Arrows* Pollen tubes. Ovules were examined 2 weeks after pollination



Environmental stress

The low level of seed set in the field could be attributable to environmental conditions. The habitat niche required by *T. oldhamii* is very narrow and is limited to limestone cliffs, in well shaded positions with water dripping or flowing at all times (Wang and Cronk 2003); individuals are often crowded onto small patches of cliff rock. Under unpredictable natural environmental conditions (e.g. temporary water stress), resource limitation may adversely affect seed and fruit set. We have also observed that water stress causes the receptive stigmatic surface to shrivel (C.-N. Wang, unpublished observation). Differences in seed set between glasshouse (under optimal conditions) and field experiments demonstrates the severity of environmental effects. For geitonogamy, the S/O ratio decreased from 3.1% in the glasshouse to 1.2% under field conditions, while for inter-population crosses the ratio decreased from 19% to 10.1% (population A; Tables 2, 3). A similar effect was observed for germination success. In general, field-grown seeds had a lower early germination rate in within-population pollinations (39%), compared to glasshouse grown seeds (57%). The same was true of final germination rate (57% vs 73%). Self seeds particularly have markedly lower germination vigor when they develop under harsher conditions in the field (15%, field; 68%, glasshouse) (Tables 2, 4).

Inbreeding effects

From the pollination results in the field, it is clear that *T. oldhamii* benefits from outcrossing, as it resulted in not only ten times higher seed set (17.4 vs 223.8; Table 2), but seeds produced from outcrossings also exhibited sig-

nificantly higher germination vigor and total germination. Our observations of pollen tube growth did not indicate the presence of classical self-incompatibility (Seavey and Bawa 1986; Levin 1996). Instead, the results are consistent with inbreeding depression acting on reproductive traits (Charlesworth et al. 1990; Tsukamoto et al. 1999; Stephenson et al. 2000).

The level of lethal equivalents (B) is linearly dependent on the inbreeding coefficient (F) and thus has frequently been used to evaluate inbreeding effects (Levin 1984; Charlesworth and Charlesworth 1987). Using the relative survivorship of selfed and outcrossed embryos, we can calculate B as equal to $-2\ln S/S_1$, where S is the selfing S/O ratio at $F=0.5$ and S_1 is the crossing S/O ratio at $F=0$ (Sorensen 1969). This estimate reveals an average of 2.4 lethal equivalents in *T. oldhamii* (with an unusually high value in population D of 11.23, all other populations range from 1.03 to 4.96). These values are much lower than those in perennials with a high inbreeding genetic load such as *Vaccinium corymbosum* ($B=9.6$) or *Stylidium* species ($B=11$) (Levin 1984; Burbidge and James 1991; Krebs and Hancock 1991). This indicates that *T. oldhamii* does not suffer from an exceptionally strong selfing genetic load.

It has been suggested that inbreeding depression can play a significant role in shaping plant mating systems (Johnston 1992). In our case, the moderate inbreeding depression in the outcrosser *T. oldhamii* could allow selfing in clonal populations. Thus, it is tempting to speculate that selfing helps to retain the restricted sexual reproduction of *T. oldhamii*, in addition to the occasional outcrossing.

Asexual reproduction may have affected the sexual reproductive fitness of *T. oldhamii* over a long period, as it can release sexual reproduction from purifying selec-

tion; mutations are more easily established through asexual reproduction, and non-lethal mutations negatively affecting sexual reproduction can be retained in populations. In the clonal plant *Butomus umbellatus*, the overall seed set under optimal conditions does not exceed 1% (Fernando and Cass 1997a). Several developmental irregularities, such as abortion of embryo sacs, have been attributed to accumulated mutations in its clonal lineages. A genome-wide survey of somatic mutation of clonal bermudagrass (*Chrysanthemum morifolium* Ramat.) indicated an apparent rate of 1.05×10^{-8} per nucleotide per generation (Caetano-Anollés 1999). This mutation rate is about 100 times higher than the apparent rate in other, non-clonal grasses. Sexual traits can be lost by mutation in clonal plants because sexual reproduction can no longer increase fitness. This has been reported for the clonal plant *Decodon verticillatus* (Eckert et al. 1999).

Bulbil-seed conflict and fertilisation failure

Pollen tube guidance in the ovary appears to be strongly affected by the state of the inflorescence, particularly in relation to the conversion from flower to bulbil production in *T. oldhamii* (Figs. 2, 3). Applying pollen from a single donor to flowers sequentially along an inflorescence results in seed set dropping significantly near to the bulbils (Fig. 2). Although these very intriguing observations are preliminary, they are also corroborated by observations in the field and findings on herbarium sheets; mature capsules are frequently found near the base of the inflorescence, never close to bulbils.

A loss of pollen tube guidance has also been observed in *B. umbellatus* L. (Butomaceae), a clonal aquatic monocot (Fernando and Cass 1997b). Failure of the pollen tube to enter the micropyle was one among several major causes of sexual reproductive failure found. This particular kind of pollination failure may even prove to be a feature common to bulbiliferous plants. Effective guidance of pollen tubes in *Nicotiana* and *Prunus* involve several chemical substances and a sucrose gradient inside the ovary (Herrero 2000; Lush et al. 2000). If the gradient is inefficient or absent, pollen tubes will not be led to the micropyle (Lush et al. 2000). Resource competition might directly or indirectly affect secretion of these chemicals and sucrose concentration. In *T. oldhamii*, bulbil formation might thus interfere with the endogenous chemical balance by creating a strong source-sink gradient towards bulbil meristems away from floral tissue. This hypothesis could be tested by the application of exogenous sucrose or plant hormones to determine whether these chemicals can restore fertilisation success (Arathi et al. 1999).

Overall low reproductive success

We observed that, under optimal conditions (i.e. between-population crosses under glasshouse), the average S/O ratio (percentage of ovules developing into seeds) among

all nine populations was only 38.2%, with population D having a maximum of 55%. Ovule survivorship or seed abortion after cross pollination is often life history related (Wiens 1984, Sutherland 1986). Our results indicated that the S/O ratio of *T. oldhamii* is markedly below the average value (57.2%) for outcrossing perennial herbaceous species (Wiens 1984). It is believed that most of these plants either need to store enough resources for next season's growth or that there is a developmental selection towards aborting the least vigorous embryos by competition, through patterns of resource allocation (Wiens 1984; Wiens et al. 1987; Charlesworth 1989).

If the mutational load containing recessive lethal alleles contributes to the low success of sexual reproduction in *T. oldhamii*, we should observe strong inbreeding depression. However, inbreeding depression appears to be moderate in *T. oldhamii* (see discussion above). Thus, the strong sink of resource reallocation to bulbil and rhizome development may be the most plausible explanation of the generally low seed production in *T. oldhamii*. Karlsson and Pate (1992) studied resource reallocation from seed to asexual gemma in sundew (*Drosera*) species. They found that only 2.5% of resources (nitrogen, phosphorus and dry matter) are allocated to seed development in gemma-producing species, compared to 50–60% of resources allocated to seed production in non-gemma producing relatives. A similar phenomenon may be present in *T. oldhamii*.

Considerations for conservation

T. oldhamii populations are often small and scattered in their natural distribution. The range of *T. oldhamii* has become fragmented recently as a result of deforestation (Wang et al. 2004b). Our results indicate that its sexual reproduction, although still functional, is negatively affected by various factors. It is highly dependent on pollinators. Pollinator activity might be rarer in smaller and more scattered populations, leading to increased inbreeding with low seed set. The genetic divergence of *T. oldhamii* could thus be trapped in these fragmented patches. A similar study on the endangered clonal perennial *Coptis teeta* ssp. *lohitensis* (Ranunculaceae) in the Himalayas indicated that lack of sexual reproduction greatly contributes to its rarity (Pandit and Babu 2003). Reproductive capacity clearly needs to be given careful consideration in management decisions regarding rare and endangered species (Wiens et al. 1989), and this is particularly true in the case of *Titanotrichum*.

A conservation strategy for *T. oldhamii* requires the protection and maintenance of the existing habitats the plants occupy. However, artificial transplanting between nearby populations might also be required to enhance sexual reproductive success, genetic diversity and to prevent genetic drift.

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