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Author(s): Retha M. Edens-Meier, Nan Vance, Yi-Bo Luo, Peng Li, Eric Westhus, and Peter Bernhardt
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POLLEN-PISTIL INTERACTIONS IN NORTH AMERICAN AND CHINESE *CYPRIPEDIUM* L. (ORCHIDACEAE)

Retha M. Edens-Meier,[1]* Nan Vance,† Yi-Bo Luo,‡ Peng Li,‡ Eric Westhus,§ and Peter Bernhardt‖

*Department of Educational Studies, Saint Louis University, St. Louis, Missouri 63103, U.S.A.; †USDA Forest Service, Pacific Northwest Research Station, 3200 SW Jefferson Way, Corvallis, Oregon 97331, U.S.A.; ‡State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing, China; §Department of Integrated and Applied Sciences, Saint Louis University, St. Louis, Missouri 63103, U.S.A.; ‖Bernhardt/Edens-Meier Laboratory, Department of Biology, Saint Louis University, St. Louis, Missouri 63103, U.S.A.

Fluorescence microscopy is used to compare frequencies of pollen tube penetration in in situ populations of *Cypripedium bardolphianum* W.W. Smith et Farrer, *Cypripedium flavum* W.W. Smith, *Cypripedium montanum* Dougl. ex Lindl., *Cypripedium parviflorum* Salisbury var. *pubescens* (Wildenow) O.W. Knight, *Cypripedium reginae* Walter, and *Cypripedium tibeticum* Schltr. The average natural (insect-mediated) pollination rates measured over five seasons are wide ranging among the six species (0.08–0.74). However, the pollination rate of hand-manipulated populations (self and/or cross) is significantly greater than the rate of insect-mediated pollinations in all species studied. A few pollen tubes in both self- and cross-pollinations display aberrant growth in the styles and/or ovaries, but their numbers are too small to suggest evidence of self-incompatibility. Pollen tubes germinate and grow up to the bases of styles within 48 h in *C. bardolphianum*, *C. flavum*, and *C. tibeticum*. Pollen tubes remain at the bases of the styles in *C. montanum* for 5 d after pollination. In *C. parviflorum*, pollen tubes penetrate ovaries at 7 d. Pollen tube penetration of ovaries is observed within 15 d after hand pollination in all six species but remains incomplete at this time, with the greatest number of ovule penetrations observed in *C. reginae* (which has the shortest floral life span). Therefore, we suggest there are additional factors aside from low pollinator visitation for low conversion rates of flowers into fruits in these *Cypripedium* species. These include inadequate pollen loads deposited on receptive stigmas (pollen limitation), coupled with environmental stress and/or predation disrupting or destroying the slow processes of fertilization and/or fruit maturation.

Keywords: pollination, pollen, pistil, stigma, style, ovary, self-compatibility, *Cypripedium reginae*, pods.

Introduction

Botanists and conservationists attempting to increase and/or regenerate native populations of *Cypripedium* spp. and other terrestrial orchids must contend with a suite of recurrent problems. Habitat destruction and overcollection (exploitation) are generally well documented in the literature on orchid conservation (see Koopowitz and Kaye 1983; Sheviak 1990). In particular, anthropogenic activities have demographic implications leading to the decline and regional extinctions of populations of *Cypripedium* spp. (Bowles 1983; Case et al. 1998; Isawa et al. 2007).

Furthermore, all *Cypripedium* spp. studied to date fail to offer rewards to their pollinators (Cribb 1997) and are, therefore, pollinated by deceit sensu lato (see Dafni and Bernhardt 1989). Reviews of the literature (see Tremblay et al. 2005) show that orchid species lacking edible rewards usually have lower rates of fecundity than species that offer nectar. Low rates of conversion of orchid gynoecia into fruits filled with viable seeds in species with nectarless flowers are commonly interpreted as examples of pollinator-limited systems (sensu Committee on the Status of Pollinators in North America 2007). That means that the delivery of sperm to viable ovules in outcrossing *Cypripedium* spp. is often insufficient, presumably because their prospective pollinators lack floral constancy (Slaa and Blesmeijer 2005), where an insect either ignores flowers completely or visits one flower and then never visits a second member of the same species.

These interpretations are usually based on long-term studies on the reproductive phenology and pollination ecology of *Cypripedium* spp. in situ (Curtis 1954; Nilsson 1979; Gill 1989; Primack and Stacy 1998). However, low fruit set is documented in other angiosperms that are unrelated to orchids as there are other prezygotic factors that lower opportunities for seed set other than the low density of pollinators in a habitat and/or their putative foraging biases.

For example, in many cases fruit set fails because the gynoecium has one or more self-recognition mechanisms. It rejects its own pollen when self-pollinated by hand and also rejects pollen of other members in the same population presumably because they share the same S allele or alleles (Richards 1997; Vance et al. 2004; Sapir et al. 2005). Prezygotic self-incompatibility in orchids was first reported by Darwin (1868) and was based on his correspondence with hobbyists and horticulturists attempting to produce seeds by manually self-pollinating the flowers of potted specimens. More recent reports and reviews extend examples of self-incompatibility within additional spe-

[1] Author for correspondence; e-mail: rmeier3@gmail.com.

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cies and genera within the subfamily Epidendroideae (Agnew 1986; Johansen 1990; Tremblay et al. 2005). Fewer examples of self-incompatiblity are confirmed within genera now placed within subfamily Orchidoideae (Stoutamire 1975; Tremblay et al. 2005). However, prezygotic self-incompatibility has never been detected in any member of the subfamily Cypripedioideae (Cribb 1997, 1998). Hand self-pollinations of Asian *Cypripedium* and *Paphiopedilum* spp. produce fruits (Cribb 1997, 1998; Banziger et al. 2005; Li et al. 2006; Zheng et al., forthcoming).

Three factors remain untested in the genus *Cypripedium*. First, do pollen grains deposited via self-pollination germinate, penetrate style tissue, and enter ovules at the same rate as pollen grains deposited via cross-pollination? Second, are pollen tubes produced as a result of insect-pollination equal to the number and penetration rate of pollen tubes produced by hand-mediated pollinations? Third, there is an often overlooked factor that could also limit reproductive success in orchids in general and *Cypripedium* in particular. Specifically, the pistils of many orchid species take a long time to mature and set fruit following hand pollination. Past embryological studies show that the fertilization of embryo sacs in some cypripedioid orchids (*Cypripedium* and *Paphiopedilum*) is extremely slow and variable, occurring within 28–150 d after pollinia are deposited on viable stigmas (see review in Arditti 1992). While delayed fertilization is not unique to cypripedioid orchids or to members of the Orchidaceae in general (see review in Sogo and Tobe 2006), this delayed unification of sperm and egg should be regarded as a potentially vulnerable period in the life cycle because slow-maturing embryo sacs and/or fruits may be destroyed by specific predators, pathogens, trampling, or climatic stress.

Therefore, to compare the impact of pollinator activity to variation in compatibility systems and ovary/ovule penetration rates in *Cypripedium* populations, we recommend more documentation on pollen tube progress in natural (insect-mediated) versus hand-pollinated pistils of *Cypripedium* spp. This allows us to test three hypotheses: (1) If pollinators of *Cypripedium* flowers are efficient pollen vectors in situ, then these insects will pollinate the same number of pistils (or more) as can be hand pollinated in situ. Therefore, the number of pollen tubes found inside natural (insect-pollinated) pistils should be the same number and should grow the same distance through tissue as pollen tubes inside our hand-pollinated pistils. (2) If a *Cypripedium* sp. is self-compatible, then a pistil cross-pollinated by hand contains the same number of pollen tubes and grows the same distance through female tissue as a pistil self-pollinated by hand over the same period of time. (3) If fertilization is an equally slow process in all *Cypripedium* sp. (see above), then pollen tubes growing through pistil tissue should grow at the same rate in all species regardless of pistil length.

Methods

Taxonomy and Study Sites in North American and Chinese Populations

Plant taxonomy for three North American *Cypripedium* species followed Sheviak (2002), so all references to *C. parviflorum* were to var. *pubescens*. Field studies on *C. montanum*, *C. parviflorum*, and *C. reginae* represented a combination of five seasons of fieldwork (May in each year 2004–2008). The taxonomy of three Chinese *Cypripedium* species followed Chen et al. (1999) and Perner and Luo (2007) as all populations studied were found in the Huanglong Reserve. *Cypripedium bardolphianum*, *C. flavum*, and *C. tibeticum* were studied over two seasons (May–June in 2005 and 2006). General locations of all six species are presented in table 1. American and Chinese authorities requested that precise locations be kept confidential to protect existing population from poaching.

Floral Life Span

To determine whether there was a relationship between floral life span and the rate at which pollen tubes penetrated

Table 1

Approximate Locations of the Six *Cypripedium* Species Used in Study Including the Site's Elevation and Mean Annual Precipitation

Species	Site location and estimated no. flowering stems at site over 1–2 seasons	Study season	Elevation (m)	Precipitation (mm)
C. bardolphianum	Huanglong Valley, Songpan County, Sichuan Province, China; $n = 3000$	May 2005	3200	759
C. flavum	Huanglong Valley Songpan County Sichuan Province, China; $n = 5000$	June 2005	3200	759
C. montanum	GROWISER Reserve, Union County, OR; $n = 120$	June 2004	1000	617
C. montanum	Deschutes National Forest, Jefferson County, OR	June 2005	1000	540
C. parviflorum	St. Francois State Park, St. Francois County, MO; $n = 23$	May 2005, May 2006	244	1000
C. parviflorum	Hawn State Park, St. Genevieve County, MO; $n = 29$ (May 2006)	May 2005, May 2006	230	1000
C. parviflorum	Meramec State Park, Franklin County, MO; data not recorded	May 2005, May 2006	244	1000
C. parviflorum	Cuivre River State Park, Lincoln County, MO; data not recorded	May 2005, May 2006	168	1000
C. reginae	Angeline Conservation Area (Lick Log Hollow), Shannon County, MO; $n = 36$	May 2005, May 2008	198	1100
C. tibeticum	Huanglong Valley, Songpan County, Sichuan Province, China; $n = 4000$	May 2005	3200	759

Table 2

Comparative Lengths of Styles, Ovaries, and Floral Life Spans

Cypripedium species	No. pistils measured	Mean style SD (length mm)	Mean ovary SD (length mm)	Floral life span (d)
C. bardolphianum	62	2	12	22–25
C. flavum	72	13	23	18–28
C. montanum	31	5	22	7–21
C. parviflorum	111	8	25	13–17
C. reginae	23	13	32	8–12
C. tibeticum	72	13	22	19–30

ovules in the ovaries, we selected and tagged specimens at random while they were in the full bud stage. The life span of an individual flower was counted in days starting from the day that the dorsal sepal separated from the labellum and the labellum inflated exposing the central dorsal orifice through which insects entered the floral chamber (see Lipow et al. 2002). Floral life span was recorded as over when the labellum senesced, (i.e., shows brown blotches and deflation). However, we did not record the life span of any flower in which the labella showed signs of predation by herbivorous insects as any physical damage to the labellum invariably resulted in premature senescence of this organ (P. B. Bernhardt, R. M. Edens-Meier, Y.-B. Luo, P. Li, and N. Vance, personal observations).

Natural Rates of Insect-Mediated Pollination

Previous fieldwork at the same sites showed that all six species failed to self-pollinate when flowers were isolated in bags or their labella was removed preventing contact between a receptive stigma and an insect's dorsum (Li et al. 2006; Herring 2007; Banziger et al. 2008; Zheng et al., forthcoming). Within a population, flower buds (one bud on each peduncle) were tagged at random (see above), but they were not bagged or manipulated. The flower was harvested following the browning and collapse of the undamaged labellum for each species 15 d after the labellum expanded (see above). The perianth segments and staminodia were removed, and the remainder of the gynostemium (two anthers plus pistil) was fixed in 3 : 1 0.95 ethanol : glacial acetic acid for a minimum of 2–24 h depending on the physical size of the organs. The fixative was decanted, and the specimen was preserved in a solution of 0.70 ethanol (Bernhardt and Edens 2004). To observe pollen tubes in each pistil, the specimens were softened and cleared by incubating each one separately in a glass vial in which it was submerged in a 0.10 solution of sodium sulfite at 45°C for up to 12 h. Incubation periods varied because small pistils (e.g., *C. bardolphianum*) required only half the softening time of large pistils (e.g., *C. reginae*). Softened specimens were washed in deionized water, the anthers were removed, and the remaining pistils were split longitudinally with a razor blade (mirror images) and mounted on glass slides. Split specimens were stained with drops of decolorized aniline blue and spread under a glass coverslip by tapping the coverslip with the tip of a dissecting needle to separate the tissues. Mounted and spread specimens were stored in the refrigerator a minimum of 24 h before viewing under epifluorescence (see Lipow et al. 2002). However, due to the massive and tangled quantities of bundled skeins of pollen tubes, it was not possible to accurately count >100 tubes in each stigma, style, and ovary. Therefore, the pollen tube content within each pistil (pooling the contents of both halves) was rated nonparametrically: 1 = 0–50 pollen tubes, 2 = 51–100 pollen tubes, and 3 = >100 pollen tubes.

Hand-Pollination Experiments

To compare rates of self-compatibility and the time it took for a pollen tube to grow from the stigma to the ovary, a second series of flowers was selected at random, isolated, and subdivided into two experimental categories—self-pollinated and cross-pollinated. To isolate flowers from prospective pollinators, we selected mature flower buds and bagged them in tulle fabric for the floral life span, or we waited for the morning when the dorsal sepal separated from the labellum, and then we removed the labellum and the staminode. Both techniques proved equally effective as we observed that insects could not receive pollen from dehiscent anthers or contact the nodding, receptive stigmas in either case.

Pollen was transferred to the stigmas using wooden toothpicks (each toothpick was used only once) and applied to the receptive undersurface until it was visible to the naked eye. Five species were hand pollinated the first day that the flower opened and the labellum inflated. Flowers of *C. parviflorum* could not be pollinated until the second day after the labellum inflated because the pollinia would not detach itself from the anther on the first day. Self-pollinated flowers received pollen from one or both anthers in the same flower. Cross-pollinated flowers received pollen from the anther of a second flower that had an inflated, intact labellum. As some *Cypripedium*

Table 3

Stigmas of *Cypripedium* spp. Bearing Pollinia Deposited by Insects

Species	No. pistils	No. stigmas bearing pollen and germinating tubes	Ratio
C. bardolphianum	13	6	.46
C. flavum	12	4	.33
C. montanum	16	15	.94
C. parviflorum	51	38	.74
C. reginae	9	1	.11
C. tibeticum	12	1	.08

Table 4

Pollen Tube Penetration in Insect-Pollinated *Cypripedium* spp.

Species	*n*	Mean no. pollen tubes penetrating[a]		
		Stigma	Style	Ovary
C. bardolphianum	13	1.1	.9	.3
C. flavum	12	.7	.6	.2
C. montanum	16	2.2	1.7	.4
C. parviflorum	51	1.4	1	.3
C. reginae	9	1	1	1
C. tibeticum	12	.25	.25	.25

[a] The mean number of tubes penetrating the stigma, style, and ovary based on nonparametric rating of tube counts with 1 = 0–50 pollen tubes, 2 = 51–100 pollen tubes, and 3 = >100 pollen tubes.

spp. are rhizomatous (Chen et al. 1999; Sheviak 2002), the flower selected for its cross-pollen donation had to be blooming a minimum of 1 m away from the flower receiving cross-pollen. Flowers were harvested at three different periods to determine how long it took for pollen tubes to reach the ovary: (1) Flowers of all three Chinese species were fixed 48 h after pollen deposition on the stigma; flowers of *C. parviflorum* were harvested and fixed 7 d following deposition on the stigma; flowers of *C. montanum* were harvested and fixed 5 d following deposition on the stigma. (2) Flowers of all six species were harvested and fixed 15 d following pollen deposition on the stigma to compare with the series exposed to insect pollinators (see above). Pistil collection, fixation, and preparation and analyses of pollen tubes followed the same protocol as for the naturally pollinated, insect-visited flowers, as above (see also Lipow et al. 2002).

Floral Measurements

To determine whether there was any correlation between the time period during which pollen tubes reached the ovaries and penetrated ovules and the sheer length of the pistil, we measured the length of preserved pistils with digital calipers before pollen tube analyses (see below). We made two measurements for each pistil in five species following methods used by Herring (2007) for *C. reginae*: (1) length from the tip of the receptive surface of the stigma to the juncture connecting the base of the style to the ovary and (2) length of the ovary, as measured from the juncture where it connects to the base of the style to the apex of its pedicel.

Data Analysis

Analysis of data was performed using software from the R Project, a free, open-source, GNU-licensed programming environment for statistical computing (http://www.r-project.org). A series of Kruskal-Wallis tests was used to test for differences in the number of tubes in the stigma, style, and ovary among treatment groups (natural insect–pollinated, hand-manipulated cross-pollinated, and hand-manipulated self-pollinated) for each species. When significant differences were detected by the Kruskal-Wallis tests, post hoc comparisons consisting of pairwise Wilcoxon contrasts were used to determine the nature of those differences. A series of Wilcoxon tests was also used to detect differences in the number of tubes in ovary after short time intervals from pollination (2, 5, or 7 d) and after a long period (15 d). A series of Spearman correlations was used to test for a relationship between the number of tubes in ovary and pistil length for each species.

Results

Floral Life Span and Floral Size

Floral life span varied with season and site (table 2). *Cypripedium montanum* had the most variable life span combining

Table 5

Pollen Tube Analyses of Three Hand-Pollinated North American Species of the Genus *Cypripedium*

Species and pollination type	Time (d)	*n*	Mean no. pollen tubes penetrating[a]		
			Stigma	Style	Ovary
C. parviflorum:					
Self-pollination	7	16	3	2.9	1.2
Self-pollination	15	19	3	3	2.7
Cross-pollination	7	15	3	3	1.6
Cross-pollination	15	12	3	3	1.8
C. montanum:					
Self-pollination	5	7	3	3	0
Self-pollination	15	8	3	3	1.6
Cross-pollination	5	7	3	2.6	.14
Cross-pollination	15	6	3	2.8	1
C. reginae:					
Self-pollination	5	NA	NA	NA	NA
Self-pollination	15	4	2.75	2.75	2.75
Cross-pollination	5	NA	NA	NA	NA
Cross-pollination	15	2	3	3	3

Note. *n* = number of pistils analyzed; NA = not assessed.

[a] The mean number of tubes penetrating the stigma, style, and ovary based on nonparametric rating of tube counts with 1 = 0–50 pollen tubes, 2 = 51–100 pollen tubes, and 3 = >100 pollen tubes.

the two sites over two seasons. *Cypripedium tibeticum* had the longest floral life span, followed by *C. flavum*, *C. bardolphianum*, *C. montanum*, and *C. parviflorum*. *Cypripedium reginae* had the shortest life span but it also had the longest pistil according to Herring (2007). In descending order, the remaining pistil lengths were in *C. flavum*, *C. tibeticum*, *C. parviflorum*, *C. montanum*, and *C. bardolphianum*.

Natural Rates of Insect-Pollination

Pollinia deposited on stigmas by native pollinators also varied broadly between species with the highest rate of pollination for *C. montanum* (0.94) and the lowest for *C. tibeticum* (0.08; table 3). The rate of insects depositing pollinia on the stigmas was <0.50 in four species—*C. bardolphianum*, *C. flavum*, *C. reginae*, and *C. tibeticum*. The number and penetration

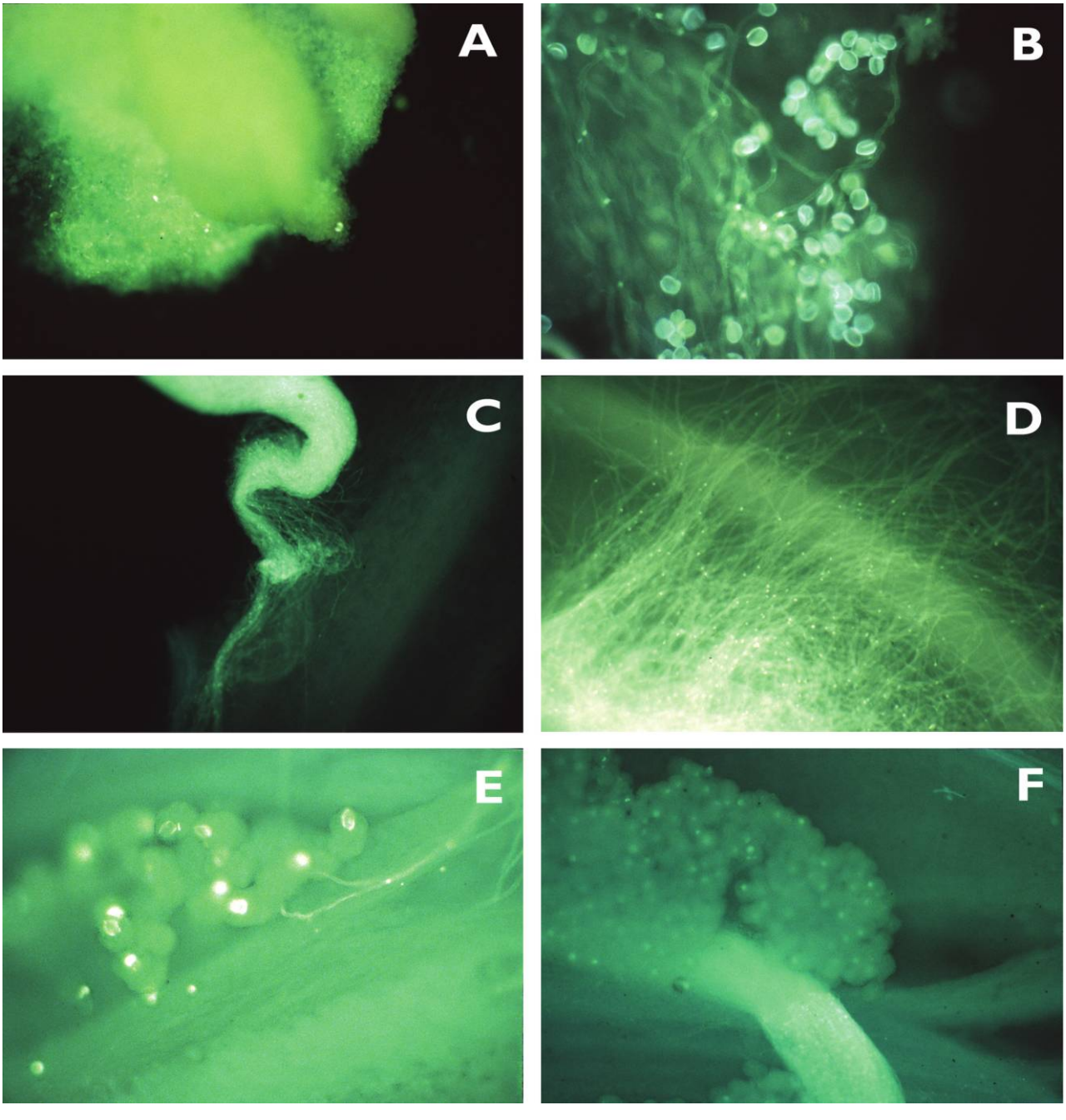

Fig. 1 Pollen tubes in pistils of *Cypripedium* spp. *A*, Pollinium mass and germinating tubes on stigma of *C. parviflorum*. *B*, Close-up of germinating pollen grains in *C. parviflorum*. *C*, Growth of pollen tubes through style in self-pollinated *C. parviflorum* after 7 d. Note how the tubes taper off in number toward the base of the style (where it interconnects with the ovary). *D*, Tubes growing through style in *C. parviflorum*. *E*, A few pollen tubes enter ovules of self-pollinated *C. montanum* at 15 d. *F*, A thick skein of pollen tubes penetrates ovary and grows toward ovules after 15 d in *C. parviflorum*. (Photographs by R. M. Edens-Meier.)

length of pollen tubes found in the pistils of insect-pollinated flowers was lower than the number and length of tubes found in hand-cross-pollinations and/or self-pollinations collected and fixed after 15 d for all species (tables 4, 5). A solitary ovary of *C. reginae* was the only insect-pollinated pistil containing pollen tubes penetrating ovules from the top to the bottom of the ovary. In all the pistils of the remaining species pollinated by insects, pollen tube penetration of the ovary, when it occurred at all, was confined to the top of the ovary, with penetration of those ovules located closest to the base of the style at the time they were collected (fig. 1*E*). Kruskal-Wallis tests show that the number of tubes that reach the stigma, style, and ovary generally differs between insect-pollinated flowers and hand-manipulated pollinations (table 7).

Hand-Manipulated Self- versus Cross-Pollinated Pistils

There was no difference between hand-manipulated self- and cross-pollinations in the two North American (tables 5, 7) and the three Chinese species (tables 6, 7) when pistils were harvested after 15 d. Pollen grains applied to stigmas in self-pollinated crosses adhered to the conical papillae, hydrated, germinated, and produced pollen tubes that penetrated ovaries at the same rate as grains applied in cross-pollinations (fig. 1*A*).

We report the presence of abnormal pollen tubes (sensu Tangmitcharoen and Owens 1997) in the pistils of both cross-pollinated and self-pollinated pistils of all species. Abnormal tubes were always <0.01 of the total number of tubes in style and ovary tissue, but they always fluoresced more brightly than normal tubes appearing to be "thicker" in width than normal tubes. Abnormal tubes in five species showed kinked growth (fig. 2*A*) that sometimes terminated in bloated tips. In *C. reginae* the tubes were coiled dramatically (fig. 2*B*). We noted in one ovary of *C. reginae* that a few coiled tubes attempted entry into ovules but left bloated tips in the micropyles.

Comparative Pollen Tube Growth in Hand-Pollinated Pistils versus Time

In all species, pollen grains deposited on the stigmas germinated and penetrated stigmatic tissue within 2–7 d (tables 5, 6). In all species, pollen tubes that failed to enter the ovary within 2–7 d appeared arrested within transmission tissue located at the bases of their styles (fig. 1*C*), where they formed thick yellow bundles (fig. 1*C*). After 48 h none of the pollen tubes in the three Chinese species penetrated their respective ovaries (table 6). After 5 d pollen tubes reached the bases of the styles in *C. montanum* but proceeded no further. Some pollen tube penetration of the ovary occurred at 7 d in *C. parvflorum* (table 5).

We were not able to perform the pollination and collection of pistils of *C. reginae* after 5 d. However, in the remaining five species tested, Wilcoxon tests showed a significant difference in the number of tubes penetrating the ovary between the short (2, 5, 7 d) time period and the long (15 d) time period (table 8). Penetration of ovary tissue was always highest at 15 d regardless of whether pollinia application was based on insect-pollination, self-pollination, or cross-pollination, and at 15 d all six species tested showed evidence of pollen tubes contacting some ovules (tables 5, 6). While the number of pollen tubes in the ovaries of *C. flavum* and *C. tibeticum* and self-pollinations of *C. parviflorum* was comparable to the number of tubes in their respective styles at 15 d, the tubes in their ovaries were always concentrated at the ovary tops, directly below the bases of their respective styles. Only in the six pistils

Table 6

Pollen Tube Analyses of Three Hand-Pollinated Chinese Species of the Genus *Cypripedium*

Species and pollination type	Time (d)	*n*	Mean no. pollen tubes penetrating[a]		
			Stigma	Style	Ovary
C. bardolphianum:					
Self-pollination	2	11	2.4	1.4	0
Self-pollination	15	9	2.7	2.9	.7
Cross-pollination	2	11	1.5	0	0
Cross-pollination	15	5	2.4	2.2	.8
C. flavum:					
Self-pollination	2	12	.2	0	0
Self-pollination	15	12	2.8	2.8	2.5
Cross-pollination	2	12	.8	.1	0
Cross-pollination	15	12	3	3	2.9
C. tibeticum:					
Self-pollination	2	12	2.8	0	0
Self-pollination	15	12	3	3	3
Cross-pollination	2	12	2.8	0	0
Cross-pollination	15	12	3	3	2.9

Note. *n* = number of pistils analyzed.

[a] The mean number of tubes penetrating the stigma, style, and ovary based on nonparametric rating of tube counts with 1 = 0–50 pollen tubes, 2 = 51–100 pollen tubes, and 3 = >100 pollen tubes.

Table 7

Kruskal-Wallis Tests of Tube Count (in Stigma, Style, and Ovary) among Treatments with Wilcoxon Post Hoc Contrasts

Cypripedium spp., parameters	χ^2	df	*P*	Post hoc contrasts (*P*)		
				Treatment	HMSP	NI
C. bardolphianum:						
Stigma	7.2935	2	.02608			
				HMCP	.742	.231
				HMSP	...	.044
Style	11.0182	2	.00405			
				HMCP	.2269	.1906
				HMSP	...	.0048
Ovary	2.2719	2	.3211			
				HMCP	...	...
				HMSP	...	...
C. parviflorum:						
Stigma	29.5387	2	3.853E−7			
				HMCP	...	.00023
				HMSP	...	1.8E−5
Style	35.2349	2	2.233E−8			
				HMCP	...	3.7E−5
				HMSP	...	1.8E−6
Ovary	32.4204	2	9.12E−8			
				HMCP	.00223	.00083
				HMSP	...	4.1E−7
C. flavum:						
Stigma	24.0863	2	5.885E−6			
				HMCP	.35932	.00026
				HMSP	...	.00043
Style	22.7209	2	1.165E−5			
				HMCP	.35932	.00022
				HMSP	...	.00098
Ovary	26.5827	2	1.689E−6			
				HMCP	.26620	1.8E−5
				HMSP	...	.00014
C. montanum:						
Stigma	5.3189	2	.06999			
				HMCP	...	...
				HMSP	...	...
Style	7.0439	2	.02954			
				HMCP	.312	.277
				HMSP	...	.058
Ovary	3.2141	2	.2005			
				HMCP	...	...
				HMSP	...	...
C. reginae:						
Stigma	12.4803	2	.001950			
				HMCP	.7237	.0211
				HMSP	...	.0058
Style	12.4803	2	.001950			
				HMCP	.7237	.0211
				HMSP	...	.0058
Ovary	12.4803	2	.001950			
				HMCP	.7237	.0211
				HMSP	...	.0058
C. tibeticum:						
Stigma	30.8	2	2.051E−7			
				HMCP	...	2.4E−5
				HMSP	...	2.4E−5
Style	30.8	2	2.051E−7			
				HMCP	...	2.4E−5
				HMSP	...	2.4E−5
Ovary	28.9072	2	5.283E−7			
				HMCP	.36	4.3E−5
				HMSP	...	3.6E−5

Note. NI = natural insect–pollinated, HMCP = hand-manipulated cross-pollinated, HMSP = hand-manipulated self-pollinated.

of *C. reginae*, harvested at 15 d, did we observe pollen tubes penetrating ovules from the top to the bottom of the ovary.

Pistil Length versus Pollen Tube Growth

Tables 2, 5, and 6 suggest a lack of consistent relationships between pistil length and the number of pollen tubes in the ovaries of six species at 15 d. Spearman correlations detected significant correlation between pistil length and pollen tube presence in the ovary only for *C. bardolphianum*, *C. parviflorum*, and *C. tibeticum* (table 9). Pollen tubes penetrated ovaries of *C. reginae* (combined pistil length 45 mm) in greater numbers (+3.0; table 5) within 15 d than pollen tubes in the ovary of *C. bardolphianum* (+0.8; table 6), which had the shortest pistils (combined pistil length 14 mm; table 2).

Discussion

Floral Life Span versus Pistil Size versus Pollen Tube Growth

Floral life span in *Cypripedium* spp. appears to vary at the interspecific and intraspecific level (table 2). Flowers of the three Chinese species at the Huanglong Reserve grow at higher elevations than the three North American species and appear to have a longer floral life span. We suggest this may be due to elevation (table 1) leading to cooler night temperatures and other environmental factors. Note also the 10-d variation in the floral life span of two isolated populations of *C. montanum*, both found at 1000 m. The longer-lived (21 d) population at the private wildflower refuge GROWISER grew in shady gaps and galleries in forest humus, while the population from the Deschutes National Forest (7–10 d) primarily grew on a fast-draining road cut exposed to full sun for 4 or 5 h each day (P. B. Bernhardt and N. Vance, personal observations). Pistil size does not appear to correlate with the floral life span of *Cypripedium* flowers either, even though *C. flavum* has the second-longest floral life span (maximum 18–28 d) and the second-longest pistil (maximum 36 mm). Unfortunately, *C. reginae* has the longest pistil but the shortest floral life span, while *C. bardolphianum* has the shortest pistil but only the third-longest floral life span. To summarize, we are unable to generalize that the *Cypripedium* spp. in this study that have the longest pistil also have the longest floral life span and vice versa. Floral life span in *Cypripedium* species appears to be determined by factors (e.g., physical climate, degree of coevolution with pollinators, genetics) other than the physical parameters of the pistil. This correlates with the classic review and observations made by Primack (1985), who found that there was no correlation between the physical size of a flower and its life span even though floral size varied within the same genus. Instead, Primack (1985) presented evidence that floral life span showed a positive correlation with increasing elevation and precipitation.

Interestingly, there is some evidence that pollen tubes penetrating a pistil of a *Cypripedium* spp. grow more rapidly through tissues in short-lived flowers. Note that hand-pollinated pistils of *C. reginae* were the only ovaries that contained pollen tubes penetrating ovules from top to bottom within 15 d. *Cypripedium reginae* had the shortest floral life span of the six species studied. These results concur with Primack (1985) as pollen tubes must penetrate and grow through the gynoecium, releasing their sperm in ovules before stigmas and styles dehydrate and senesce.

Natural Rates of Insect-Pollination

As anticipated for all six species, hand-pollinated pistils are more likely to contain pollen tubes that grow longer distances through the pistil than are pistils exposed to their true pollinators. In all six species, studied pollinators (Bernhardt and Edens-Meier, forthcoming) fail to deposit pollinia on stigmas of all members of the same population, and this is indicative of most orchid species bearing flowers that produce no rewards (Tremblay et al. 2005). The penetration length of pollen tubes in a hand-pollinated flower could be found further down the length of the pistil than in an insect-pollinated flower because we always hand-pollinated the flower within the first 24 h of its opening. In contrast, the pollinia-laden insects may not have visited their flowers until several days after their flowers opened (if they ever visited at all).

Since pollination rates (pollinia detected on stigmas) in *C. tibeticum* are comparable to fruit set rates in the same species over several seasons at the same sites (Zheng et al., forthcoming), it appears that the annual, relatively low fruit set ratio (9.57%–26.0%) in this species is due primarily to inattentive/inactive pollinators. However, is poor fruit set in a *Cypripedium* sp. always due to a primary lack of pollinator activity? Note that Lipow et al. (2002) found that pollen tubes in pistils of wasp-pollinated *C. fasciculatum* were higher than the actual fruit set ratio. This discrepancy also appears to occur in two species at the Huanglong Reserve. *Cypripedium bardolphianum* has a fruit set ratio between 10.8% and 13.2%, while the ratio for *C. flavum* hovers between 7.1% and 9.2% (Zheng et al., forthcoming). These low fruit set frequencies stand in stark contrast to our pistil squashes at the same sites that show 46.0% rates of pollination for *C. bardolphianum* and 33.0% for *C. flavum*. Yes, many *Cypripedium* spp. are pollinator limited (Bernhardt and Edens-Meier, forthcoming), but there must be other genetic-based and/or environmental stress factors such as fruit predators and various climactic conditions all preventing fruit or seed maturation following successful pollination. As evidence of detrimental environmental stress factors on fruit set, buds of *C. parviflorum* were aborted following flooding during the spring 2007, while 75% of *C. reginae* capsules were destroyed by fruit predators in 2008 (R. M. Edens-Meier, personal observations). Additionally, predation of pollinated pistils is recorded in eudicotyledons. For example, Bernhardt and Dafni (2000) noted that a proportion of bee-pollinated pistils of *Mandragora officinarum* L. (Solanaceae) were consumed by land snails before the pistils matured into a berry.

Hand-Manipulated Self- versus Cross-Pollinated Pistils

In any case, our results show that prezygotic self-incompatibility may be discounted as a cause of low fruit set in these six *Cypripedium* spp. With the rather unconvincing exception of *C. passerinum* (Catling 1983), there is still no evidence that

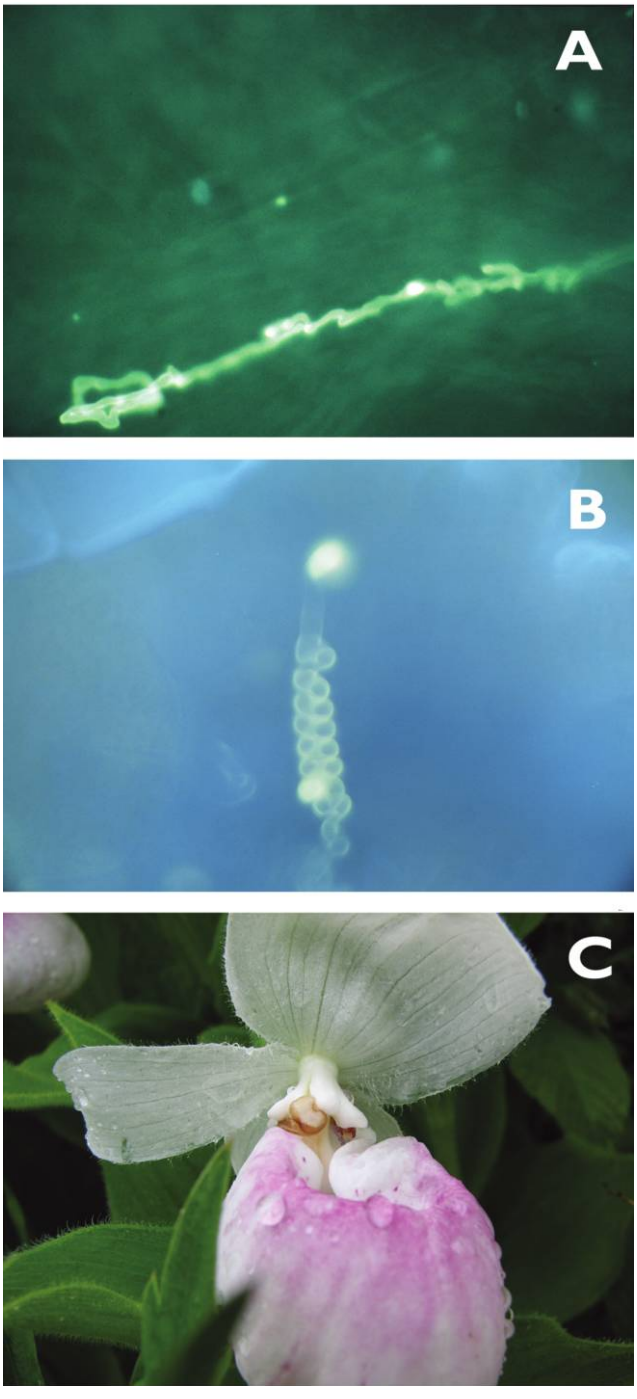

Fig. 2 Aberrant tubes. *A*, Jagged tube in style of *Cypripedium montanum*. *B*, Coiled tube in ovary of *Cypripedium reginae* with bloated tip. *C*, *Cypripedium reginae* flower with missing lateral petal, distorted labellum, and extra anther. (Photographs by R. M. Edens-Meier.)

mechanical self-pollination (autogamy) serves as a "fail-safe mechanism" (Schemske et al. 1978) in the genus *Cypripedium*, as it does in many other vernal flowering herbaceous perennials when pollinators are absent. On the other hand, in the absence of a prezygotic self-isolation mechanism, pollinator-mediated self-pollination could occur in any of these six species if the same pollinator returned to the same flower or flowers on the same plant more than once. Although no observations were made on repeated visits of potential pollinators during this study, the possibility of repeat visits is possible.

Aberrant, erratic, or distorted pollen tubes are described in styles and ovaries following breeding experiments in a number of unrelated angiosperm species. They are usually interpreted as early or late expression of an incompatibility response that is monomorphic (Lush and Clarke 1997; Mazzucato et al. 2003; Vance et al. 2004), heteromorphic (e.g., *Primula*; Richards 1986), or interspecific (Lefol et al. 1996; Hayes et al. 2005). Consequently, there are two ways of interpreting the small but consistent number of aberrant tubes in pistils of *Cypripedium* spp. They may represent a latent system inherited from a self-incompatibile ancestor. This is unlikely as there is no evidence for self-incompatibility within the Cypripedioideae and the review by Tremblay et al. (2005) indicates that self-incompatibility has evolved independently and secondarily within several clades within the family Orchidaceae. We speculate instead that since we are living in a time of habitat fragmentation (Koopowitz and Kay 1983), it is more likely that aberrant tubes found in both hand-mediated cross- and self-pollinations are symptomatic of an early expression of inbreeding depression as these orchid populations decline in density. Aberrant tubes in cross-pollinated flowers may reflect exchanges of gametes between closely related individuals. Later generations may suffer the consequences of inbreeding depression through physical deformities (fig. 2*C*) as well as compromised breeding systems.

Comparative Pollen Tube Growth in Hand-Pollinated Pistils versus Time and Pollen Tube Length

Pollen tube growth in hand-pollinated pistils was a slow process in all six *Cypripedium* spp. compared with many other angiosperm taxa in which pollen tubes are delivered to the micropyle in 24–48 h (see Vance et al. 2004). Based on the above results, there is no obvious correlation between the rates at which pollen tubes of *Cypripedium* spp. are found in ovaries and the sheer length of the pistil. Note that at 15 d the number of pollen tubes in ovaries remained lower than the number of pollen tubes in the styles (tables 5, 6). This suggests that the delivery of pollen tubes to ovules in *Cypri-*

Table 8

Wilcoxon Tests of Tubes in Ovary by Days after Treatment, Short (2, 5, or 7 d) versus Long (15 d)

Cypripedium species	*W*	*P*
C. bardolphianum	187	.001732
C. parviflorum	1051	.000783
C. flavum	132	2.359E−7
C. montanum	94.5	2.654E−5
C. reginae	...	...
C. tibeticum	132	1.427E−7

Table 9

Spearman Correlations of Tubes in Ovary with Pistil Length

Cypripedium species	*S*	*P*	*ρ*
C. bardolphianum	1888.307	.02768	.4235936
C. parviflorum	8521.918	4.001E−7	.6362197
C. flavum	6811.792	.4736	.1233215
C. montanum	1827.728	.6598	.09697214
C. reginae	...	...	...
C. tibeticum	4097.693	.003606	.4726263

pedium spp. appears to be continuous, over a series of days, instead of synchronous, even though tubes show initial signs of arrest at the base of styles. As *Cypripedium* fruits contain hundreds or thousands of seeds, it seems more likely that pollen tubes continue to pass into the ovaries after 15 d. Also, we note that, in the majority of ovaries containing pollen tubes, those tubes remained at the top of the ovary in all species except for *C. reginae*.

Comparative Rates of Pollen Tube Growth in the Pistils of Cypripedioideae versus Other Angiosperms

We note that delayed and or arrested pollen tubes in pistils and slow rates of ovule fertilization are not unique to the Orchidaceae. These developments have evolved independently in at least a dozen orders of eudicotyledons. Sogo and Tobe (2006) interpreted this trend as indicative of ovaries in which megasporogenesis is incomplete at the time of pollination, and there is also an older body of literature confirming late ovule development within the Orchidaceae (Wirth and Withner 1959; Arditti 1992). While this study does not contrast pollen tube growth rates with the development of the embryo sac in *Cypripedium* spp., late megasporogensis probably explains our repeated and stereotyped results. In five of the six *Cypripedium* spp., pollen tube growth through the pistil showed signs that they stopped specifically at the bases of styles after 2–5 d following deposition of pollen on stigmas. This arrest at the base of the styles occurred regardless of whether tubes were produced by hand-mediated cross- or self-pollinations. One wonders whether the entry to the ovary also serves as a site of maternal selection, since all tubes appear to stop at the same point, but not all tubes enter the ovary at the same time.

Conclusion

In this study, we examined six *Cypripedium* spp. to compare the impact of pollinator activity and variation in compatibility systems and ovary/ovule penetration rates. Based on results, we reject hypothesis 1, which states, "If pollinators of *Cypripedium* flowers are efficient pollen vectors in situ, then these insects will pollinate the same or greater number of pistils as can be hand pollinated in situ. Therefore, pollen tubes in natural (insect-pollinated) flowers should be the same number and grow through female tissue the same distance as in the pollen tubes of hand-pollinated pistils." Insects do not visit as many flowers as we do or leave as much pollen on the receptive stigma. Both pollinator-limited and pollen-limited systems are evident in the *Cypripedium* spp., as has been found in other orchid species that offer no rewards (Tremblay et al. 2005).

Our results support hypothesis 2: "If a *Cypripedium* sp. is self-compatible, then a pistil cross-pollinated by hand contains the same number of pollen tubes and grows the same distance through female tissue as a pistil self-pollinated by hand over the same period of time." Self-compatibility in *Cypripedium* appears more common than self-incompatibility in orchids (Tremblay et al. 2005).

We reject hypothesis 3: "If fertilization is an equally slow process in all *Cypripedium* spp., then pollen tubes growing through pistil tissue should grow at the same rate in all species regardless of pistil length." Yes, it is a slow process, but tissue penetration does not occur at the same rate. Pollen tube growth through pistils does not occur at the same rate in all *Cypripedium* spp. More analyses are required to compare rates of penetration in all *Cypripedium* spp. We found that pollen tube growth through the pistil occurs at a faster rate in *C. reginae* than in any of the other *Cypripedium* spp. investigated. Six out of an estimated 40 species of *Cypripedium* (Cribb 1998) were included in this research project. Are there other *Cypripedium* species that have a more rapid pollen tube growth than the six species included in this study?

Consequently, there are two weak links in the conservation biology of several, if not most, *Cypripedium* spp. First, it is obvious that most *Cypripedium* spp. have one or more pollinator-limited populations, as do most orchids with mimetic flowers (Tremblay et al. 2005) during their life histories. This leads to low rates of fertilization in many, if not most, populations. Second, as in most orchids, the act of fertilization is a slow process (Arditti 1992) compared to the speed in a majority of angiosperms. As the pollen tubes become arrested at the base of the style, both fertilization and fructification is at the mercy of changes in the environment. This may include predation and fluctuations in climactic factors. In conclusion, conservationists must pay extra attention to the identification, diversity, and behavior of pollinators of *Cypripedium* spp. as well as less studied factors that may destroy pollinated gynoecia.

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Literature Cited

Agnew JD 1986 Self-compatibility/incompatibility in some orchids of the subfamily Vandoideae. Plant Breed 97:183–186.

Arditti J 1992 Fundamentals of orchid biology. Wiley, New York.

Banziger H, HQ Sun, Y-B Luo 2005 Pollination of a slippery lady slipper orchid in southwest China: *C. guttatum* (Orchidaceae). Bot J Linn Soc 148:251–264.

——— 2008 Pollination of wild lady slipper orchids *Cypripedium yunnanense* and *Cypripedium flavum* (Orchidaceae) in south-west China: why are there no hybrids? Bot J Linn Soc 156:51–64.

Bernhardt P, A Dafni 2000 Breeding system and pollination biology of *Mandragora officinarum* L. (Solanaceae) in northern Israel. Pages 215–224 *in* O Totland, ed. The Scandinavian Association for Pollination Ecology honours Knut Faegri. Series 29. Det Norske Videnskaps-Akademi, Oslo.

Bernhardt P, R Edens 2004 A fertility clinic for flora. Plant Talk 36: 27–31.

Bernhardt P, R Edens-Meier Forthcoming What we think we know vs. what we need to know about orchid pollination and conservation: *Cypripedium* L. as a model lineage. Bot Rev.

Bowles ML 1983 The tallgrass prairie orchids *Platanthera leucophaea* (Nutt.) Lindl. and *Cypripedium candidum* Muhl. ex Willd: some aspects of their status, biology, and ecology and implications toward management. Nat Areas J 3:14–37.

Case MA, HT Mlodozeniec, LE Wallace, TW Eldy 1998 Conservation genetics and taxonomic status of the rare Kentucky lady's slipper: *Cypripedium kentuckiense* (Orchidaceae). Am J Bot 85: 1779–1786.

Catling PB 1983 Autogamy in eastern Canadian Orchidaceae: a review of current knowledge and some new observations. Nat Can 110:1983.

Chen SC, ZH Tsi, Y-B Luo 1999 Native orchids of China in colour. Science, New York.

Committee on the Status of Pollinators in North America 2007 Status of pollinators in North America. National Research Council. National Academies, Washington, DC.

Cribb P 1997 The genus *Cypripedium*. Timber, Portland, OR.

——— 1998 The genus *Paphiopedilum*. Timber, Portland, OR.

Curtis JT 1954 Annual fluctuations in rate of flower production by native *Cypripedium* during two decades. Bull Torrey Bot Club 81: 340–352.

Dafni A, P Bernhardt 1989 Pollination of terrestrial orchids of southern Australia and the Mediterranean region: systematic, ecological and evolutionary implications. Pages 193–252 *in* MK Hecht, B Wallace, J Macintyre, eds. Evolutionary biology. Vol 24. Plenum, New York.

Darwin C 1868 The variation of animals and plants under domestication. J Murray, London.

Gill D 1989 Fruiting failure, pollinator inefficiency and speciation in orchids. Pages 458–481 *in* D Ottee, JA Endler, eds. Speciation and its consequences. Sinauer, Sunderland, MA.

Hayes RJ, II Dinn, CS Thil 2005 Unilateral and bilateral hybridization barriers in inter-series crosses of 4x 2EBN *Solanum stoloniferum*, *S. pinatisectum*, *S. cardiophyllum*, and 2x 2EBN *S. tuberosum* haploids and haploid-species hybrids. Sex Plant Reprod 17:303–311.

Herring T 2007 A study of the reproductive systems of selected North American and Chinese *Cypripedium* species (Orchidaceae). PhD diss. Saint Louis University.

Isawa T, T Kawahara, H Takahashi 2007 Genetic diversity of an endangered plant, *Cypripedium macranthos* var. *rebunse* (Orchidaceae): background genetic research for future conservation. Conserv Genet 8:1369–1376.

Johansen B 1990 Incompatibility in *Dendrobium* (Orchidaceae). Bot J Linn Soc 103:165–196.

Koopowitz H, H Kaye 1983 Plant extinction: a global crisis. Stonewall, Washington, DC.

Lefol E, A Fleury, H Darmency 1996 Gene dispersal from transgenic crops. II. Hybridization between oilseed rape and the wild hoary mustard. Sex Plant Reprod 9:189–198.

Li P, Y-B Luo, P Bernhardt, XQ Yang, Y Kay 2006 Deceptive pollination of the lady's slipper *Cypripedium tibeticum* (Orchidaceae). Plant Syst Evol 262:53–63.

Lipow SR, P Bernhardt, N Vance 2002 Comparative rates of pollination and fruit set in widely separated populations of a rare orchid (*Cypripedium fasciculatum*). Int J Plant Sci 163:775–782.

Lush M, AE Clarke 1997 Observations of pollen tube growth in *Nicotiana alata* and their implications for the mechanism of self-incompatibility. Sex Plant Reprod 10:27–35.

Mazzucato A, I Olimpieri, F Clampolini, M Cresti, GP Soressi 2003 A defective pollen-pistil interaction contributes to hamper seed set in the parthenocarpic fruit tomato mutant. Sex Plant Reprod 16: 157–164.

Nilsson LA 1979 Anthecological studies on the lady's slipper, *Cypripedium calceolus* (Orchidaceae). Nord J Bot 1:461–480.

Perner H, Y-B Luo 2007 Orchids of Huanglong. Haunglong National Park. Sichuan.

Primack R, E Stacy 1998 Cost of reproduction in the pink lady's slipper orchid (*Cypripedium acaule*, Orchidaceae): an eleven-year experimental study of three populations. Am J Bot 85:1672–1679.

Primack RB 1985 Longevity of individual flowers. Annu Rev Ecol Syst 16:15–37.

Richards AJ 1986 Plant breeding systems. Allen & Unwin, London.

——— 1997 Plant breeding systems. 2nd ed. Chapman & Hall, London.

Sapir Y, A Shmida, G Ne'eman 2005 Pollination of *Oncocyclus irises* (*Iris*: Iridaceae) by night-sheltering male bees. Plant Biol 7: 417–424.

Schemske DW, MF Willson, MN Melampy, LJ Miller, L Verner, KM Schemske, LB Best 1978 Flowering ecology of some woodland herbs. Ecology 59:351–366.

Sheviak CJ 1990 Biological considerations in the management of temperate terrestrial orchid habitats. N Y State Mus Bull 471:194–196.

——— 2002 *Cypripedium*. Pages 499–507 *in* GW Agus, K Ghandi, P Golfblatt, WJ Hess, RW Kiger, JL Strother, FH Utech, JL Zarycchi, eds. Flora of North America. Vol 26. Oxford University Press, New York.

Slaa J, K Blesmeijer 2005 Flower constancy. Pages 381–400 *in* A Dafni, PG Kevan, BC Husband, eds. Practical pollination biology. Enviroquest, Cambridge, Ontario.

Sogo A, H Tobe 2006 Delayed fertilization and pollen-tube growth in pistils of *Fagus japonica* (Fagaceae). Am J Bot 93:1748–1756.

Stoutamire WP 1975 Pseudocopulation in Australian terrestrial orchids. Am Orchid Soc Bull 44:226–233.

Tangmitcharoen S, JN Owens 1997 Floral biology, pollination, pistil receptivity, and pollen tube growth of teak (*Tectona grandis* Linn f.). Ann Bot 79:227–241.

Tremblay RL, JD Ackerman, JK Zimmerman, RC Calvo 2005 Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. Biol J Linn Soc 84:1–54.

Vance N, P Bernhardt, R Edens 2004 Pollination and seed production in *Xerophyllum tenax* (Melanthiaceae) in the Cascade Range of central Oregon. Am J Bot 91:2060–2068.

Wirth M, CL Withner 1959 Embryology and development in the Orchidaceae. Pages 155–188 *in* CL Withner, ed. The orchids, a scientific survey. Ronald, New York.

Zheng GL, P Li, YD Tai, DJ An, Y Kou, Y-B Luo Forthcoming Flowering and fruit set dynamics in *Cypripedium*. Acta Ecol Sin.