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What We Think We Know vs. What We Need to Know About Orchid Pollination and Conservation: *Cypripedium* L. as a Model Lineage

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Abstract While Darwin (1862, 1877) showed that reproductive success in orchid populations depended on adaptive floral morphology coupled with pollinator visitation a more recent review of the literature (Tremblay et al., 2005) confirmed that many out-breeding species are pollinator-limited because most orchid species showing low fecundity also lack rewards. The absence of rewards depresses both pollinator fidelity and the frequency of pollinator visits to an orchid population even though orchid flowers that lack rewards retain the same interlocking floral structures for precise pollinia removal and deposition found in related species that offer rewards. Using the genus, *Cypripedium*, as a model lineage of non-rewarding flowers this study also shows that the correlation between low fruit set in a *Cypripedium* sp. and its specific pollinator(s) is insufficient to predict specific frequencies of low fecundity. Annual rates of fruit set often vary broadly between populations of the same species and within the same population over several seasons. We speculate that fruit-set rates also decline when orchid demography and additional biotic and abiotic factors interrupt rates of pollinator activity (pre-zygotic) and fertilization/fruit maturation (post-zygotic). We suggest that that traditional field studies on pollination ecology and breeding systems be combined with data sets recording genetic variation and orchid flower demography in relation to seasonal variation in climate. We also propose that the same information be collected in regard to genetic variation, demography and phenology of populations of known orchid pollinators and co-blooming angiosperm species native to orchid habitats.

Keywords *Cypripedium* · Conservation · Fruit Set · Predation · Pollinator-Limited

Introduction: Darwin's Orchids

The recent bicentennial of Charles Darwin's birthday (2009) should remind all plant conservationists that the modern study of orchid pollination begins with Darwin's two editions of a book that concentrated on the role of adaptive floral morphology in the evolution of orchid flowers (Darwin 1862, 1877). While Darwin was provided with living plants and/or the fresh flowers of tropical species most of the text in both books was devoted to his analyses of prospective insect-mediated pollination in 25–26 orchid species distributed through the British Isles (Allen, 1977). Darwin's text also indicates that, while he had access to populations of several native species growing near his country home, his work was much supplemented by additional specimens of native orchids and insects provided by correspondents resident in other parts of Britain. Such a rapid and seasonal exchange of now threatened and/or endangered species is not permitted today in most countries in Western Europe, Australia, the United States or Canada even if specimens come from wild populations growing on private property. State or federal permits to collect, dissect and/or experiment on populations of indigenous orchid species, when available, often run many pages and permission is seldom guaranteed. Many governments now accept the warnings of Koopowitz and Kaye (1982) restricting the collection of native orchids as we live during a time of global crisis in plant extinction and orchid species are particularly endangered. However, as Dixon (2009) argues convincingly that the restoration of a plant species also requires additional studies on the identification and restoration of its pollinator population(s), basic studies on the reproductive ecology of threatened/endangered orchid populations must continue even when it means harvesting some flowers and capturing and sacrificing some pollen vectors.

In fact, our knowledge of orchid pollination continued and grew over the past 150 years because Darwin's basic program of investigations (i.e. direct observation of flower-insect interactions in situ combined with manipulative studies back in a laboratory or study) proved so easy to emulate regardless of geographical location (see reviews in Dafni & Bernhardt, 1990, Cady & Rotherham, 1970; Erickson, 1965). These "students of Darwin" often made observations important to future programs in orchid conservation. For example, as early as the second half of the 19th century some naturalists recorded that many orchid species showed a low rate of conversion of flowers into fruits containing viable seeds (e.g. Fitzgerald, 1875–1895).

Unsurprisingly, low rates of fruit and seed set remain a major issue in orchid conservation in temperate regions (see Coates & Dixon, 2007) but they are particularly important in tropical conservation programs because orchid diversity usually peaks at middle elevations (*sensu* Dressler, 1981) while population densities of the same species tend to remain low. We offer the following review of flower-pollinator interactions because it has such an immediate and obvious effect on population fecundity and that is intrinsic to the conservation of species in the Orchidaceae. To bring this review up to date we also recognize that demographic, phenological and environmental factors ultimately influence natural rates of pollination and fruit set. To better illustrate variation of rates of pollination and fruit set within a threatened/endangered lineage we propose to use species within the much-studied temperate genus, *Cypripedium*, as a model system.

Orchids as Pollinator-Limited Species?

Orchid taxa under study are often regarded as model examples of pollinator-limited species (Tremblay et al., 2005). That is, low rates of fruit set in a population are interpreted typically, but not exclusively, as the result of infrequent or negligible visits to orchid flowers by their primary pollinators. Within a pollinator-limited population or species, the recognized pollen vectors, if present in the habitat at all, fail to remove the viable pollen during the flowering season of the species, in question, and/or fail to deposit viable pollen grains on receptive stigmas. Of course, this problem is not unique to orchid species (see Committee on Status of Pollinators in North America, 2007). Are low rates of pollination in orchids due to the fact that so many orchid species have specialized pollination systems in which the flowers can be pollinated only by relatively few, congeneric animal species (see Pemberton, this volume)? Some orchid species are pollinated by only a single insect genus or species and the pollinia vector(s) often belongs to only one gender (e.g. male euglossine bees, female fungus gnats, male wasps etc.; Dressler, 1993; Johnson et al., 1998).

These evolutionary trends in orchid reproductive ecology were reviewed thoroughly by Tremblay et al. (2005) and the authors came to the mutual conclusion that specialized pollination systems in the orchid family, in association with a number of other factors, reflects both the low rate of fecundity and the high rate of diversification/speciation. We do not challenge their conclusions. However, we do note that specialized pollination systems also evolved independently in other families of herbaceous angiosperms without reports of low rates of fruit or seed set based on infrequent visits by the few, legitimate pollen vectors. In particular, the diverse family Iridaceae, as distributed through southern Africa, shows a range of pollination systems as specialized as any orchid flora, on any continent. However there were no comparable reports of low fruit set in the Iridaceae of southern Africa (Bernhardt & Goldblatt, 2006; Goldblatt & Manning, 2008). Therefore, why should conservationists expect a dependable correlation between low fruit set in an orchid species and a narrow "pollinator vector spectrum" (*sensu* Van der Pijl & Dodson, 1969)?

Correlation Between Pollinator(s) and Fruit Set in *Cypripedium?*

We argue that blaming low fruit set on the performance of a discrete lineage of anthophilous insects as poor pollinia vectors must be approached with caution for two reasons. First, there is an impressive body of literature that shows that many orchid species self-pollinate when pollinators are rare or absent (Burns-Balogh & Bernhardt, 1988; Catling, 1990; Neiland & Wilcock, 1998). Second, if we use the genus *Cypripedium* and compare pollination systems we find there are a number of extenuating circumstances at both interspecific and intraspecific levels (Table 1).

All *Cypripedium* spp. lack edible rewards so we do not expect that the pollinator(s) of any *Cypripedium* sp. will show a high degree of faithfulness to the flowers once it recognizes and rejects deceptive pigmentation and scent cues. As 15 *Cypripedium* spp. failed to self-pollinate in the absence of pollinators (see references in Table 1) it should be possible to correlate low fruit set with those pollinators that occur at low frequencies within the orchids' habitats and/or are quickest to recognize and reject *Cypripedium* floral cues. Consequently, we might conclude that, *Cinetus* wasps and/or small-bodied, solitary bees were more dependable and frequent pollinators of *Cypripedium* spp. than were bumblebee gynes (*Bombus*), medium-large solitary bees, and tiny drosophilid flies. Unfortunately, there were too many exceptions to this rule to make such a sweeping generalization. At certain sites and in certain seasons,

Table 1 Pollinators vs. Fruit set Rates in *Cypripedium* spp.

Cyrpipedium spp. populations/study sites	Pollinator(s)	% Conversion Flowers/ Fruit	References
C. acaule	*Bombus* spp. (gynes)		(Stoutamire, 1967)
Broadmore		1.3–25.0	(Primack & Stacy, 1998)
Case Estates		0.0–25.0	(Primack & Stacy, 1998)
Hadley		4.3–09.4	(Davis, 1986)
Hammond Woods		0.0–09.8	(Primack & Stacy, 1998)
C. bardolphianum	*Drosophila* spp.	10.8–13.2	(Zheng et al., 2010)
C. calceolus s.s.	MSB[a]		(Nilsson, 1979)
Byelorussia		33.0–57.0	(Kull, 2008)
Estonia		10.5	(Kull, 2008)
Moscow		04.0–14.0	(Kull, 2008)
Sweden		25.0	(Nilsson, 1979)
C. fasciculatum	*Cinetus* spp. (females)		(Ferguson & Donham, 1999)
Colorado		18.0	(Lipow et al., 2002)
Oregon		69.2	(Lipow et al., 2002)
Idaho		28.9	(Lipow et al., 2002)
C. flavum	*Andrena* spp.	07.1–09.2	(Banziger et al., 2008)
C. guttatum	*Lasioglossum* spp.		(Banziger et al., 2005)
C. henryi	*Lasioglossum* spp.	17.0–22.2	(Li et al., 2008b)
C. japonicum	*Bombus* (gynes)	05.2–07.7	(Sun et al., 2009)
C. macranthos	*Bombus* spp. (gynes)		(Sugiura et al., 2002)
Site A		08.3–16.8	(Sugiura et al., 2002)
Site B		01.2–14.5	(Sugiura et al., 2002)
C. montanum	MSB[a]	75.0–85.0	(Vance, in prep)
C. parviflorum	MSB[a]		(Herring, 2007)
Big Springs		15.0	(Meir, in progress)
Cuivre River		40.0	(Herring, 2007)
Hawn		10.5	(Meier, in progress)
Merramac Park Site		22.3	(Herring, 2007)
St. Francois		15.0	(Meier, in progress)
C. plectrochilum	*Lasioglossum* spp.	38.7–45.9	(Li et al. 2008a)
C. reginae	*MLB*[b],		(Meier & Bernhardt, in progress)
	Syrphus, Trichiotinus spp.		(Vogt, 1990)
Angeline		04.6–23.0	(Herring, 2007)
Medley		33.0	(Herring, 2007)
C. tibeticum	*Bombus* spp.(gynes)	09.57–26.0	(Li et al., 2006)
C. yunnanense	*Lasioglossum* spp.	21.0	(Banziger et al., 2008)

[a] MSB = Mixed, small-medium, solitary bees (5–10 mm in length) representing genera in two or more families (Andrenidae, Apidae, Colletidae, Halictidae)

[b] MLB = Mixed Medium-Large Solitary or Eusocial bees 10–>10 mm in length (no *Bombus*) representing genera in one or two families (Apidae, Megachilidae)

bumblebee-pollinated *C. acaule, C. macranthos* and *C. tibeticum* had similar or higher rates of fruit set than some populations of *C. calceolus, C. flavum, C. henryi* and *C. yunnanense* (Table 1), all pollinated by small, solitary bees.

Table 1 strongly suggests that fruit set rates were far too variable between isolated populations of five *Cypripedium* spp. to conclude that some insect taxa or insect body types were always more dependable as primary pollinia vectors compared to others. The comparative fruit set ratio between isolated populations of *C. acaule* varied >0.15 in the most successful seasons. Variation between fruit set ratios in populations of *C. calceolus* was 0.43. It was 0.57 for *C. fasciculatum* populations, >0.39 for *C. parviflorum* populations and *0.29* between two populations of *C. reginae*. Consequently, there are seasons and sites in which bumblebee pollinated *C. acaule, C. macranthos* and *C. tibeticum* produced as much fruit as some small bee pollinated populations of *C. calceolus*, *C. parviflorum* and wasp-pollinated *C. fasciculatum* (Table 1).

Fruit set ratios were not always parallel with each other even when two different *Cypripedium* spp. shared the same genus of pollinators and flowered at the same site. Zheng et al. (2010) studied fruit set in populations of *C. henryi* and *C. plectorchilum* over three seasons in the Huang Long reserve (Sichuan, China), Both species were pollinated by *Lasioglossum spp*. but fruit set in *C. plectrochilum* was consistently higher than in *C. henryi* (Table 1).

Why Should Co-adaptation Between Orchid Flowers and Pollinators Reduce Reproductive Success?

Ironically, while pollinators of the same orchid species tend to belong to the same genus and/or share similar physical dimensions, this trend towards a canalized and restrictive pollinator vector spectrum never guarantees a high conversion rate (i.e. >0.25) of flowers into fruits in *C. bardolphianum*, *C. flavum* and *C. japonicum* (Table 1). The major review written by Tremblay et al. (2005) addressed low rates of reproductive success in orchids by considering several, interlocking factors that ultimately limited pollinator-orchid flower interactions leading to fruit set. We list these major points below in conjunction with the classic work of Van der Pijl and Dodson (1966) and Dressler (1981).

(1) The androecium and gynoecium of all orchids fuse together forming a column and the majority of species release their pollen as pollinia.
(2) The majority of orchid flowers attach pollinia indirectly to an adhesive plug (the viscidium) using stalk-like, connective structures produced by the anthers (caudicles) or the rostellum (stipes). Upon removal of whole pollinarium by a pollinator the pollinia may change their angle of orientation as the caudicles and/or stipes dry out and change positions (see also, Darwin, 1877).
(3) Almost all orchid flowers show bilateral symmetry canalizing successful entrance and exit of pollinators carrying and delivering pollinia.
(4) Almost all orchid species lack granular pollen as an edible reward. Many (most) species in the family offer no nectar or any edible rewards at all expressing some specialized mode of pollination-by-deceit or they secrete volatile odors collected exclusively by male, euglossine bees (Dressler, 1981).

However, none of the floral characters, listed above, are unique to the Orchidaceae. Columns and column-like structures evolved repeatedly within the angiosperms. Second, complicated pollinaria bearing adhesive and connective structures evolved independently in the family, Asclepiadaceae providing another excellent example of convergent evolution (see review, Burns-Balogh & Bernhardt, 1985). Third, bilateral floral symmetry is the rule, not the exception, in many other moncocot and eudicot families. Pollination-by-deceit mechanisms have been recorded, since the 19th century, in some species within the families, Araceae, Aristolochiaceae, Begoniaceae, Saxifragaceae etc. (Proctor et al., 1996). Furthermore Pemberton (this volume) notes that flowers belonging to species in other angiosperms families also provide male euglossines with terpene and terpenoid molecules.

Therefore, it's probably the combination of **all** four factors in the **same** flower that make so many orchid species pollinator-limited. This explains the comparative absence of reports of low reproductive success in members of the Iridaceae of southern Africa (see above). While the flowers of different species in this family have bilaterally symmetrical flowers, bear column-like structures (Goldblatt & Bernhardt, 1998), may lack edible rewards and/or are pollinated by a few specialized animals, **no** extant member of the Iridaceae releases pollen as pollinia (Bernhardt & Goldblatt, 2006).

It's this interlocking, often inseparable, suite of four characters that is responsible for both pollinator-limitation and low reproductive success in so many orchid species over time. Floral architecture and floral dimensions limited the number of visiting animals that functioned as legitimate pollinators as the pollinium or pollinarium was fixed specifically to the part of the vector's body that contacted the receptive stigma when it visited a second flower on a second plant of the same species. This was particularly obvious in recent studies of pollination of *Cypripedium spp.* in which the floral architecture and insect dimensions were measured and compared (Li et al., 2006, 2008a, b). In fact, *C. plectrochilum* (Li et al., 2008a) and *C. reginae* (Edens-Meier and Bernhardt, in preparation) are visited by a wide variety of local insects in the Orders Hymenoptera, Lepidoptera and Coleoptera but only ay few visiting species had the correct dimensions required to consistently remove the pollinia upon exiting the flower and to deposit the same pollinia on the stigma as they entered a second flower. If pollinator-floral dimensions are usually this precise in the Orchidaceae it is no surprise that we were unable to provide an obvious correlation between pollinator group and a consistently high fruit set in Table 1 (see above). Even flower visitors with similar body dimensions must vary by increments (see Li et al., 2008a).

Cypripedium reginae was the only *Cypripedium* sp. found to have two entirely different sets of pollinators at different sites (Table 1; Van der Cingel, 2001). Three seasons of observations and collections by Edens-Meier and Bernhardt (in preparation) showed that members of the Orders Diptera, Lepidoptera, Hymenoptera, and Coleoptera enter the labellum but only flies and bees crawled under the receptive stigma and escaped via the legitimate exit holes at the back of the flower of *C. reginae*. However, only medium-sized bees (at least 10 mm long with a thorax depth >3 mm) carried dorsal deposition of this orchid's pollinia in a population in southern Missouri. While this parallels observations of pollination by

C. reginae in Canada dating to the 19th century (Van der Cingel, 2001) Vogt (1990) found that small beetles and syprhid flies were the only pollinia vectors of *C. reginae* in a Vermont population. Removing pollinia from *Cypripedium* anthers does not make an insect a true pollinator of a *Cypripedium* sp. Unless the prospective vector is of a sufficient height it will not contact the receptive stigma and transfer pollinia when it re-enters a second labellum sac (see Banziger et al., 2008; Li et al., 2006, 2008a, b).

This self-consistent and precise deposition of pollinia on the same part of a vector's may be of even greater importance to reproductive success in monandrous orchids as they produce true pollinaria. As their connective stalks (caudicles and/or stipes) usually change position as they dry out this reorients the height and angle of the pollinia so they are more likely to contact the stigma(s) as the pollinator enters a second flower, preferably on a second plant (Darwin, 1877; Faegri & van der Pijl, 1966). In most orchid species studied, rates of successful pollinaria removals by floral visitors exceeded the rates of successful pollinia depositions on receptive stigmas (Tremblay et al., 2005). Unless the adhesive plug (viscidium) was deposited on the part of the vector's body that ultimately positioned the dried connective stalks the pollinia failed to contact stigmas of other flowers of the same species. Consequently, hand-mediated pollinations of flowers typically produced more fruits than did the true pollinators of orchid populations in situ (see Tremblay et al., 2005). In one population of *Caladenia gracilis s.l.*, insects removed a large proportion of pollinaria without leaving pollinia on stigmas (Tremblay, 2005) and no fruit was produced for an entire season. The parameters of floral structure within *Platanthera leucophaea* and the proboscis length and foraging modes of moths ultimately determined which moth species actually pollinated one of two variants (or cryptic species) within the *P. leucophaea* complex (Sheviak & Bowles, 1986).

While floral parameters, floral symmetry and pollinia presentation obviously limit both the diversity and efficiency of prospective pollinators, the absence of edible rewards in many orchid species limits rates of frequency of visitations by the same legitimate pollinators. The potential vector stops visiting the "rewardless" (*sensu* Smithson, 2002) flowers after a few unsatisfying/stressful visits. Ackerman (1986) estimated that up to a third of all orchid species don't secrete nectar. A review of the literature by Neiland & Wilcock (1998) showed that fruit set was higher in orchid species that secreted nectar compared to species that offered other rewards (e.g. scents), or produced no rewards. Regardless of the presence of rewards, fruit set tended to be lower in tropical species. Smithson (2002) and Smithson & Gigord (2001) hypothesized that the absence of edible rewards in orchids evolved repeatedly because it contributed selectively to the male fitness of a bisexual plant. Fathering offspring without spending energy and resources on the production of calorific nectar spreads a plant's genes far more economically compared to accepting sperm and producing fruits and seeds that require water and building materials, often taking months to mature. Unfortunately, as so many orchid species are long-lived, herbaceous plants, that may take years to reach sexual maturity, but form consistently small populations (Tremblay et al., 2005), this has become a poor survival strategy in an era of habitat destruction, over-collection and global warming.

Additional Factors Influencing Successful Orchid Pollination

Therefore, as we consider establishing additional reserves and parks to protect remaining populations of orchid species we must consider two problems. First, what other factors within reserves encourages or depresses fecundity in association with natural rates of pollination? Second, when considering those factors in association with natural rates of pollination how do we prioritize which orchid populations stand the greatest chance for producing and expanding successive generations?

1) Biotic Factors. As pollinators are necessary to maintain both levels of sexual recombination (heterozygosity) and the subsequent quality of recruitment in most orchid populations, a plant conservationist must become more familiar with the demography and phenology of both pollinators and those co-blooming angiosperms that offer nectar and/edible pollen (Sheviak, 1990). So-called specialized pollination within an orchid species may be a matter of degree and dependent, in large part on plant or animal distribution and demography (Dixon & Tremblay, 2008). For example, in the case of the eastern Australian, nectarless, pea blossom mimic, *Diuris maculata*, a southern population had access to more pollinator species than a northeastern population. Only males of *Trichocolletes venustus* pollinated *C. maculata* in its northeastern location (Indsto et al., 2006). This "localized trend towards specialization" within the broad distribution of an orchid species may be far more common than anticipated. While small bees belonging to three genera may pollinate *Cypripedium calceolus* (Table 1) there are sites in Europe where this orchid is pollinated exclusively by bees in a single genus (Kull, 2008). Consequently, maintaining pollinator diversity and density within a protected site is further complicated by the fact that an insect pollinator has different environmental and nutritional requirements at different stages its life cycle. The reader, therefore, is referred to Dafni and Pemberton (this volume) as they treat these problems in greater depth.

We will, however, make the additional point that, as many threatened and endangered orchid species lack edible rewards the survival of pollinators, and the provisioning of their offspring, are dependent on the nectar and/or pollen production of sympatric angiosperm species with flowering peaks overlapping with the flowering of mimetic orchids. Unfortunately, few field studies of orchid pollination observe and collect pollinators when they forage on flowers of species other than orchids even though such collections are not new (e.g. *Diuris*, see Rayment, 1935). Some recent studies offer this useful information (e.g. Banziger et al., 2008; Bernhardt & Burns-Balogh, 1983, 1986, 1987; Dafni & Calder, 1987; Indsto et al., 2006) as techniques for removing, staining, mounting and identifying pollen grains found on pollinator bodies are comparatively simple and inexpensive (Bernhardt, 2005). This is important for three reasons. First, if the orchid species actually secretes nectar then the identification of pollen grains of other nectariferous species on the orchid pollinator allows us to determine if the orchid species is in competition with co-blooming plants (Bernhardt & Burns-Balogh, 1987; Indsto et al., 2007). Second, this could help to explain why some "rewardless" orchids are ignored consistently by their pollinators at certain sites (Neiland & Wilcock, 1998). Third, if the flowers of the orchid offer no edible rewards, then one or more co-blooming species may nourish or provision the orchid pollinators or serve as models for orchid flowers pollinated-by-deceit (Dafni & Calder, 1987; Indsto et al., 2006). While many Australian, terrestrial orchids are pollinated exclusively by male wasps that attempt to copulate with labellum sculptures (Alcock, 1988; Gaskett & Heberstein, 2006; Dixon & Tremblay, 2008), we have few published profiles of the plants that nourish these males. This is critical for conservationists seeking to protect the many Australian species pollinated by male wasps in the family, Tiphiidae, as females are wingless and males carry them off in a nuptial flight ending with male and female taking nectar from local plants (Alcock & Gwynne, 1987). For some of these wasps the extra-floral nectar glands of Australian *Acacia* spp. may represent critical sources of nourishment (Bernhardt, 1987) at a vulnerable stage in their life cycles. Likewise, female parasitoid wasps in the genus, *Cinetus*, appear to be the only pollinators of *Cypripedium fasciculatum* (Table 1; Ferguson et al., 2005). The feeding habits of these insects remain unknown, but Tooker and Hanks (2001) suggest that many parasitoid wasps need the nectar of flowering plants in temperate sites.

2) Self-Incompatibility. While Darwin (1877) emphasized the role of self-pollination in *Ophrys apifera* he also corresponded with British collectors of tropical orchids and noted their pollination experiments on potted specimens. Certain species rejected their own pollen and the flowers dropped off without setting fruit (Darwin, 1868). The most recent and thorough review of pre- and post-zygotic incompatibility in the Orchidaceae was provided by Tremblay et al. (2005). While most species show some degree of self-compatibility (fruit and seed set occurs when self-pollinated by hand) the number of genera that contain at least one species that rejects self-pollinated pollinia has risen since Darwin's original correspondence. As in other angiosperm lineages (Richards, 1997), the degree of self-incompatibility, expressed by members of the same orchid population (e.g. *Galearis spectabilis*) may vary through the distribution of the species (see unpublished data by Zimmerman as cited by Tremblay et al., 2005). Self-pollination in some orchid species may result in fruit set but fewer embryos develop inside seeds (post-zygotic fatalities). Tremblay et al. (2005) reviewed seed set in 75 orchid species and found that 49 species produced fewer embryos (0.20–>0.50) when self-pollinated vs. when they were cross-pollinated.

This is a most important consideration for orchid conservation in the absence of information on breeding systems in populations. In particular, pre-zygotic self-incompatibility has the capacity to limit fecundity in a small population if it is based on the Founder Effect of seeds successfully dispersed by the same parent stock because the number of S alleles in most plant populations tend to be limited (Richards, 1997). Mature, flowering orchids sharing one, or more, of the same S alleles may be unable to set fruit no matter how closely they are located to each other and no matter how often the same pollinator(s) cross-pollinates more than one plant. This was well illustrated in self-incompatible *Coelogyne fimbriata* (Cheng et al., 2009), pollinated exclusively by worker wasps (*Vespula* sp.). The ability of the wasps to remove and deposit pollinia on the stigmas of these orchids varied from site to site but a maximum of 0.69 stigmas received pollinia from foraging wasps. Unfortunately, the rate of fruit set ranged from 0.01 to 0.06 regardless of site or study season. While the authors correctly blamed the majority of wasps for not visiting flowers on more than one multi-flowering clone the reason for fruit set failures needs expansion. Some wasps may have visited more than one orchid genotype, in or ex situ, but the plants exchanging pollinia could have shared one or more of the same S alleles and summarily rejected each other's sperm.

3) Fruit and Seed Predation. When do the fructifying ovaries of orchids in natural populations become targets of predation? Most field workers and green house managers have observed orchid flowers in partial states of consumption by insects, or other animals, but there is relatively little information on fruit predators at field sites. One might assume that most orchid fruits are unattractive to animals as most members of the Orchidaceae, produce dry, capsular fruits, their seeds lack endosperm and their embryos consist of only a single layer of cells (Dressler, 1981). However, Gaskett & Heberstein (2006) showed that the ovaries of *Cryptostylis* spp. became targets of predation following fertilization. To date, Ackerman & Montalvo (1990) published the most complete study on limitations to fruit production using *Epidendrum ciliare* as the model species. Predation reduced the number of developing fruit in both Control (insect-pollinated) and hand-pollinated fruits of this species. Fruit loss by predation was 0.28–0.29 in the Controls and 0.33–0.49 in the hand-pollinated pistils. As hand-pollination produced 0.23–0.34 more fruits than natural (insect-mediated) pollination that's a potentially serious problem for future conservation efforts. If hand-pollination rates produce far higher fruit sets compared to natural insect-pollination, in most wild populations of orchids (Tremblay et al. 2005), then unusually heavy accumulations of fruit on stems may become far greater targets of predation. Obviously, far more field-based studies are needed and the subject of fruit predation remains pivotal to the protection of some orchid species.
4) Fertilization/Fruit Set and Climate Fluctuation. The act of fertilization in orchid ovaries is a delayed process in most species studied because the flower bud opens and its stigma is receptive to pollination before macrosporogenesis is completed. The act of fertilization, in most orchid species, may not actually occur until weeks after the act of pollination. In fact, fertilization appeared to occur more slowly in tropical species compared to temperate ones (Arditti, 1992). Our own work on pollen tube-pistil interactions in North American and Chinese *Cypripedium* spp. (Edens-Meier et al., in press) indicated that, pollen grains deposited on receptive stigmas germinated and penetrated style tissue within 48 h but the same pollen tubes did not even begin to enter the ovaries, until 7–15 days following hand pollination. We speculate that this delayed process may become maladaptive in an age of global warming as orchids may be prevented from maturing and releasing seeds as climatic changes occur earlier and more rapidly. All buds within a population of *C. parviflorum* were aborted in 2007 following a spring flood (Edens-Meier et al., in press). Both sudden, or long-term, changes in ambient temperature and seasonal rates of precipitation must effect moisture levels in soils or epiphytic humus important to the survival and maturation of fruits produced by species native to mesic-temperate (Pimack & Hall, 1990; Primack & Stacy, 1998) and monsoon forest biomes. Surely, most readers involved in the protection of tropical orchids have

been frustrated by the sight of the fruiting remains of twig epiphytes strewn over the ground to rot following devastating storms. We must also consider the possibility that if climatic change disrupts or destroys orchid flowers and/or fruits they will probably disrupt the seasonal activities of the sympatric nectar-pollen flora (see above) and orchid pollinators

What Additional Information Is Required to Monitor and Expand Rates of Sexual Recombination in Conserved Populations of Endangered/Threatened Orchids?

While techniques and protocols for studying pollination and fruit set in the Orchidaceae have been employed and refined since the second half of the 19th century only a fraction of known species have been studied, to date, as it is one of the largest angiosperm families (Dressler, 1993). With more populations conserved in situ we are in a position to commission fieldwork on previously unstudied species and to initiate additional and novel analyses on the reproductive ecology of understudied species. Considering the four factors discussed above, we suggest that additional information be collected beyond the typical information provided by standard field studies in pollination ecology (e.g. identification of pollinators, mechanisms of pollinia transfer, breeding systems, fruit set ratios etc.; Dafni et al., 2005). Orchid conservationists and reproductive botanists should consider collecting one, or more, of the following data sets from one, or more, populations of orchid species protected in reserves or national parks.

1) Demography and natural history of pollinators. Once the pollinators of an orchid species are confirmed and identified to species field research should attempt to answer the question, are the pollinators' visits to orchid flowers infrequent because they are unattractive to their pollinators or because their pollinators are rare in situ? We suggest a program of pollinator capture and release coupled with attempts to locate nesting and/or oviposition sites for immature pollinators over several flowering seasons (see 2).
2) Identification, Phenology, Demography and Biochemistry of Sympatric Flora. Are pollinator visits to orchid populations infrequent because rewarding plants of other species are rare in situ and fail to feed orchid pollinators or too common and depress pollinator visits to orchid species? Fieldwork should include a program of collection, identification and deposition of vouchers of plant species blooming in the same site as the orchid species. Floristic analyses, including the demographics of rewarding plants in bloom may be considered. If pollen load analyses of pollinators caught on orchid flowers are not feasible the field worker should consider collecting floral foragers on other plant species found blooming at the same time in the same site. Do the foragers collected on pollen or nectar rich species match the insects found exiting orchid flowers? Nectar secreted by non-orchid species should be collected and measured for dissolved solutes and the quantity and proportions of sugars and amino acids (Dafni et al., 2005).
3) Genetic Variability Within Orchid Populations. Pre-zygotic self-incompatibility may be uncommon in orchid species but post-zygotic incompatibility, based on

the absence of viable embryos following self-pollination, appears to be common (see Tremblay et al.; 2005). Consequently, cross-pollination within a population consisting primarily of individuals sharing one, or more, parents may depress fecundity as effectively as repeated self-pollination due to shared S alleles or lethal combinations. This may be particularly important in orchid conservation as populations may arise from a single, Founder event (Tremblay et al., 2005). Genetic variation within the population should be recorded before an attempt is made to augment fruit set and seed set by transplanting different genotypes into the population and/or hand-pollinating plants using pollinia from a plant that came from an isolated site. This includes recording the karyology of sympatric, congeneric species as different chromosome numbers may limit the role of gene migration/filtration between closely related species sharing the same pollinators (Cozzolino et al., 2004).

4) Number of Genotypes That Have Reached Flowering Size vs. the Number of Plants That Bloom Annually. Table 1 shows that reproductive success (fruit set) varies from site to site within the same *Cypripedium* sp. This is not surprising as O'Connell and Johnston (998) showed that microhabitats within the populations of *C. acaule* had a greater effect on female and male rates of reproductive success than did floral traits. However, we presume that natural rates of pollination and fruit set are dependent, in part, on the actual number of flowering genotypes in an orchid population and when these genotypes bloom over the flowering period (Sun et al., 2009). Annual observations on pollination should, therefore, include annual counts of inflorescences in bloom and, when appropriate, the number of flowers/inflorescence (Tremblay, 2005). This is particularly important in a number of terrestrial species, as some mature individuals within a population do not bloom annually, staying dormant the year after they bloom (see Discussion in Tremblay, 2005). As woody sclerophyll vegetation invades and matures within open woodlands and heaths in temperate Australia, the flowering rates of bulbous-tuberous herbs often declines. Cyclical fires destroy or depress shrub growth and remove forest litter stimulating flowering in mature but dormant terrestrial orchids and other perennial herbs (Gill et al., 1981). Indeed some Australian species in the genera *Burnettia* (Bishop, 1996), *Leptoceras* (Erickson, 1965) and *Lyperanthus* (Woolcock & Woolcock, 1984) may not bloom prolifically until the season after a bush fire. By counting flowering stems each season following a bush fire we could test the hypothesis that pollination and fruit set rates are highest within the first few years following a fire. An additional virtue of counting flowering stems each year is that it may help us record the loss of mature plants by herbivores or human collectors, as orchids are most obvious and attractive when in bloom.
5) Pollination Rates and Fruit Set vs. the Impact of Climatic Cycles. Table 1 shows that reproductive success (fruit set) in *Cypripedium* spp. varies at the same site from year to year. This variation shows significant fluctuations in some populations of *C. acaule* (0.25), *C. calceolus* (0.24*), C. macranthos* (0.13), *C. montanum* (0.10) and *C. reginae* (0.18). Which environmental conditions increase or suppress the efficacy of resident/migratory pollinators and the ability of pollinated pistils to develop into fertilized fruits? We presume, for example, that insect pollinators are less active under low temperatures and continuous

> precipitation. Likewise, pollinated pistils are less likely to become fertilized and fructify during early and long droughts. We presume that levels of soil moisture ultimately influence competitive vegetation. Male and female pollination success in shade-tolerant, *C. acaule* is influenced, in part, by canopy density and the presence of ericaceous shrubs (O'Connell & Johnston, 1998). Therefore, some field studies on the reproductive ecology of orchids populations in reserves should consider recording such seasonally variable, abiotic features as light levels (cloud and leaf canopy cover), precipitation, soil moisture, rates of erosion etc. during the actual flowering season of the orchid species and the often protracted period in which pollinated pistils reach mature size.

In conclusion, at no time do we suggest abandoning traditional techniques or equipment for interpreting the pollination ecology of orchid species. In addition to such studies, the collection and quantification of data sets over several seasons, as described above, may allow orchid conservationists to monitor the fertility of populations and identify factors leading to boom or bust cycles in fruit set. Surely, reserve populations, once protected from collection and vandalism, are ideal for long-term studies.

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