

## Breeding system and pollination of selected orchids of the genus *Chloraea* (Orchidaceae) from central Chile

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### Abstract

The breeding system determines different ways whereby seeds will be produced, and the degree of dependency of plants on pollinators for seed set. The genus *Chloraea* (Orchidaceae) has its main center of diversity in southern South America. There is only poor knowledge concerning its breeding system and pollination. We determined the breeding system of *C. crispa*, *C. chrysantha*, *C. galeata*, and two color forms of *C. blettioides* (yellow- and white-flowered forms). None of the species in this study produced fruits through apomixis or autogamy, thereby indicating a complete dependency on pollen and pollinators. Geitogamy did not differ significantly with respect to xenogamy excepting in the yellow-flowered form of *C. blettioides*. Thus, the indexes of self-incompatibility for the white- and yellow-flowered forms of *C. blettioides*, *C. galeata*, *C. crispa*, and *C. chrysantha*, were 1.00, 0.56, 0.82, 1.09, and 0.81, respectively; indicating that, excluding the yellow-flowered form of *C. blettioides* which must be regarded as partially self-incompatible, all orchids assessed are totally self-compatible plants. Natural fruiting in the yellow-flowered *C. blettioides*, *C. chrysantha* and *C. galeata* was high, in spite of being nectarless orchids, since the availability of pollinators under natural conditions seemingly resulted unlimited. However, no pollinator was observed visiting *C. chrysantha* and *C. galeata*, whereas the yellow-flowered form of *C. blettioides* was visited by hymenopterans and coleopterans. At contrast, reproductive success of the white-flowered form of *C. blettioides* and *C. crispa* was pollen limited, the former being visited by hymenopterans, dipterans, and colepterans; and the latter by two hymenopterans.

**Keywords:** *Chloraea*; Orchidaceae; Self-compatibility; Pollinator-dependency

### Introduction

The extent to which flowering plants depend on external vectors for outcross pollen transportation is a central question in plant reproductive ecology. The

breeding system of a species determines the way whereby seeds will be produced, and the degree of dependency of plants on pollinators for seed set (Lovett-Doust and Lovett-Doust, 1988; Richards, 1997). Reproductive failure in plants can often be attributed to either pollination limitation where the movement of viable pollen between flowers through the absence of pollinators is insufficient (Bierzychudek, 1981; but see Zimmerman and Pyke, 1988), or to resource limitation where insufficient resources are available to allow the

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maximum fruit set to take place (Primack and Hall, 1990; Primack and Stacy, 1998). Among the Orchidaceae, nectariferous orchids produce more successfully fruits and seeds as compared with nectarless orchids (Neiland and Wilcock, 1998; Proctor et al., 1996). During the origin of Orchidaceae evolution in the Cretaceous, most angiosperm flowers would have been visited by unspecialized insects like beetles and flies, which pollinated them while feeding on both floral and vegetative tissues (Neiland and Wilcock, 1998; Proctor et al., 1996). The most likely origin of the highly adapted mechanisms among nectarless orchids is that they arose from primitive orchids, which had even lower levels of fruit set than seen today, probably owing to the existence of unspecialized and largely unrewarding flowering environments (Neiland and Wilcock, 1998). In this sense, a small adaptation that improved pollinator attraction would have had a reproductive advantage and would have increased in frequency.

The most likely shortcut to break away from the ecological and evolutionary limitations imposed by consistent sexual reproductive failures, is the adoption of pollinator-independent fruit production (i.e., autogamy and/or agamospermy), or the provision of rewards in order to entice pollinators (i.e., allogamy) (Neiland and Wilcock, 1998). Nevertheless, agamospermy is infrequent in orchids because it may be inhibited since embryo-sac formation in the family is unusual in requiring the presence of pollen on the stigma as a stimulus (Neiland and Wilcock, 1998). Automatic self-pollination has been reported to be more widespread but is probably morphologically prevented in most orchids by the herkogamous structure of flowers (Proctor et al., 1996). Therefore, the way adopted by orchids in order to ensure a successful fruit set is to produce nectar for pollinator enticing, thus precluding autogamy or agamospermy as possible pathways for seed set (Neiland and Wilcock, 1998; Proctor et al., 1996).

The genus *Chloraea* has its center of diversity in southern South America (Correa, 1969). Its taxonomy and systematics is poorly known (but see Correa, 1969; Lehnebach, 2003) equally than its breeding system and pollination (but see Clayton and Aizen, 1996; Lehnebach and Riveros, 2003). The aim of this work is to determine the breeding system and natural pollination of selected species of the genus *Chloraea* in order to increase our knowledge about the breeding strategies of a part of the endemic orchids of this region. If a great amount of orchids are self-compatible, we expected that self-compatibility in orchids of the genus *Chloraea* is widely represented. Because nectarless orchids are usually limited by pollinators for fruit setting, we also expected that all orchids herein assessed are limited by pollinators for fruit production regarding that all of them are thought to be deceit-pollinated on account of

bearing non-rewarding nectaries for attracting nectar-feeding pollinators (personal observations).

## Methods

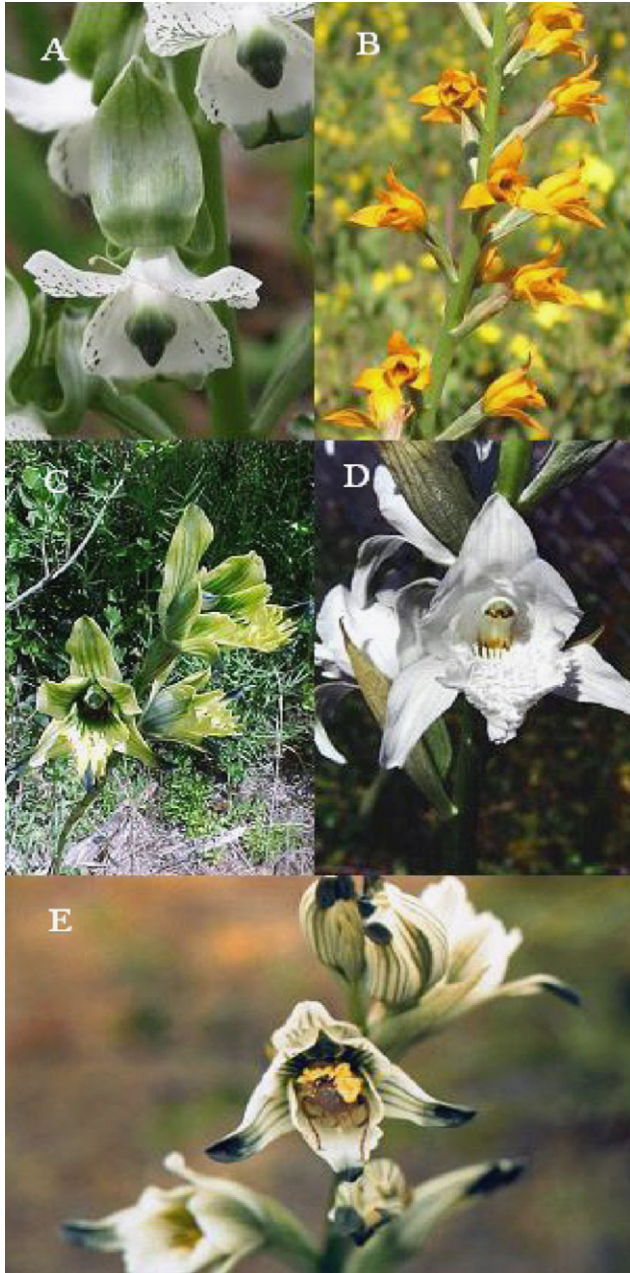
### Species and study site

The white- and yellow-flowered forms of *C. blettioides* Lindl., *C. crispa* Lindl., *C. chrysantha* Poepp., and *C. galeata* Lindl., are geophytic orchids that inhabit hillsides in central Chile (Fig. 1; Correa, 1969). All these species exhibit pauciflorous inflorescences with 3–10 flowers per plant in *C. blettioides* (both color morphs), 6–20 in *C. crispa*, 8–20 in *C. chrysantha*, and 10–20 in *C. galeata* (Correa, 1969; Novoa et al., 2006). Flowering and fruiting occur from October to January in the austral spring-time season (Novoa et al., 2006).

The white-flowered *C. blettioides* can be found from ca. 32°S to 38°S and was studied at the Río Clarillo National Reserve (33°S), from October to December 2003, specifically in equatorial-facing slopes neighboring the Clarillo river. *C. crispa* ranges from ca. 38°S to 41°S and was studied in a field placed 20 km away from Yumbel city (38°S approx.) in an area of stabilized sand dunes, which is strongly cultivated with plantations of *Pinus radiata*. Both *C. chrysantha* and *C. galeata* range from 33°S to 38°S, whereas the yellow-flowered *C. blettioides* has been observed only at the 33°S. These three species were studied at the Roblería del Cobre de Loncha National Reserve (34°S approx.) from October 2002 to January 2003, and from October 2003 to December 2003, in equatorial-facing slopes nearby El Chivato hill.

### Breeding system and natural pollination

To determine the breeding system of *C. blettioides* (two color forms), *C. crispa*, *C. chrysantha*, and *C. galeata*, four pollination trials and in addition a control for natural pollination were conducted (Kearns and Inouye, 1993). Each treatment was performed in 20, 25, 90, 19, and 22 individual plants in the white- and yellow-flowered forms of *Chloraea blettioides*, *C. crispa*, *C. chrysantha*, and *C. galeata*, respectively (all treatments equally distributed on each plant). To test whether these orchids are capable of fruit setting in the absence of pollen (i.e., agamospermy), an apomixis test was performed on at least 15 flower buds, which were emasculated (i.e., anther excision) and bagged until seed dispersal in order to prevent any flower–pollinator interaction. Similarly, to test whether these orchids are capable of fruit setting after receiving pollen from the same flower in the absence of pollinators carrying pollen within the flower (i.e., autogamy), an automatic



**Fig. 1.** South American terrestrial orchids of the genus *Chloraea* included in this study: (A) *C. galeata*, (B) *C. chrysantha*, (C) yellow-flowered form of *C. blettioides*, (D) *C. crispa*, and (E) white-flowered form of *C. blettioides* with a pollinator of the genus *Arctodium* carrying a pollinium.

self-pollination test was performed by bagging at least 23 non-emasculated flower buds until seed dispersal. To test whether these orchids are capable of fruit setting with pollen of the same individual carried by pollinators (i.e., geitonogamy), a test was performed by bagging at least 16 flower buds which were previously emasculated and hand cross-pollinated with endogenous pollen at the stigmatic receptivity time. Another test was performed by bagging at least 14 emasculated flower buds, which at

the stigmatic receptivity time were hand cross-pollinated with exogenous pollen in order to test whether these orchids are capable of fruit setting with pollen from other individuals (i.e., xenogamy). Natural pollination was registered in at least 13 always-open flowers on each plant, which were inconspicuously tagged at the bud stage.

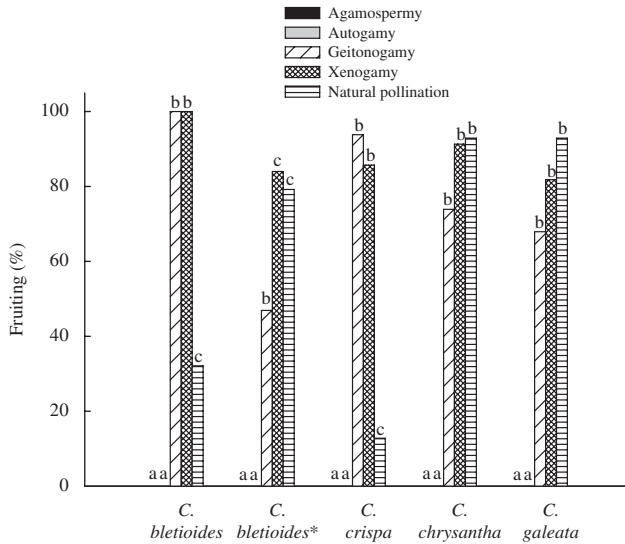
The results of these treatments were recorded by checking the ratio of fruits produced per crossed flower when the ovaries were ripe, prior to seed dispersal. Thereafter, to determine the degree of self-incompatibility, the index of self-incompatibility (ISI) was determined as the proportion of fruits produced by geitonogamy and by xenogamy (Ruiz-Zapata and Arroyo, 1978). The ISI ranges between 0 (self-incompatibility) and 1 (self-compatibility), albeit it is also possible to obtain values higher than one when fruit set attained by xenogamy is lower than that attained by geitonogamy (Ruiz-Zapata and Arroyo, 1978).

A survey was also carried out for determining the main floral visitors during the flowering season from October 2002 until mid-January 2003. While the white- and yellow-flowered form of *C. blettioides*, *C. chrysantha*, and *C. galeata* were observed for a total of 20 h in their respective study sites, *C. crispa* was observed during 36 h at Yumbel. Observations were performed by one observer at each site from 10:00 to 17:00 h, and during 4–5 days at the peak of flowering season. At each site, insects – the only floral visitors – were collected, identified, and classified depending on whether they did or did not carry pollinia attached on their bodies. Frequencies of pollinator visits were expressed as number of visits per plant per hour.

Each orchid species was assessed independently. Analyses of breeding systems were performed in a per-flower basis by using Tukey HSD tests for pair-wise comparisons between pollination treatments after evaluation of means of generalized linear models with a binomial error distribution and logit link functions. Because a zero pivot element was encountered during sweeping the Wald and related statistics cannot be calculated, the type I hypothesis was applied for obtaining a  $\chi^2$  value. All analyses were performed using the Statistica Software Package v. 6.0.

## Results

Fruit set differed significantly depending on the origin of pollen as well as on the presence of a pollen vector in the white-flowered form of *C. blettioides* ( $\chi^2 = 166.99$ ,  $P < 0.001$ ), yellow-flowered form of *C. blettioides* ( $\chi^2 = 155.34$ ,  $P < 0.001$ ), *C. crispa* ( $\chi^2 = 96.14$ ,  $P < 0.001$ ), *C. chrysantha* ( $\chi^2 = 94.37$ ,  $P < 0.001$ ), and *C. galeata* ( $\chi^2 = 89.92$ ,  $P < 0.001$ ) (Fig. 2). In fact, none of these



**Fig. 2.** Fruiting (%) in selected orchids of the genus *Chloraea* following four pollination treatments as well as a control for natural pollination (see text for details). Asterisk depicts the yellow-flowered form of *C. bletioides*. Dissimilar letters depict significant differences ( $P < 0.05$ ).

species produced seeds by agamospermy or autogamy. This indicates a complete dependency of seed production on pollen as well as on pollinators (Fig. 2). Geitonogamy did not differ significantly with respect to xenogamy except for the yellow-flowered form of *C. bletioides*. Thus, the ISI calculated for the white- and yellow-flowered forms of *C. bletioides*, *C. galeata*, *C. crispa*, and *C. chrysantha*, were 1.00, 0.56, 0.82, 1.09, and 0.81, respectively. Therefore, all orchids assessed, excluding the yellow-flowered form of *C. bletioides* which must be regarded as a partially self-incompatible orchid, are totally self-compatible (sensu Ruiz-Zapata and Arroyo, 1978).

Natural pollination was lower than xenogamy in the white-flowered form of *C. bletioides* and *C. crispa* indicating a pollinator-limited reproductive success, while in the other orchids it did not differ significantly, hence indicating a reproductive success non-limited by pollinators (Fig. 2). Dissimilar frequencies of visits by pollinators and foraging behaviors may account for such differences in the reproductive success by natural pollination. Accordingly, in *C. chrysantha*, and *C. galeata* no insect visitor was observed during the observation periods. By contrast, the white-flowered form of *C. bletioides* was visited by *Mycteromia conica* (Diptera, Tabanidae): 0.0025 visits/plant/h; *Bombus dahlbomii* (Hymenoptera, Apidae): 0.0025 visits/plant/h; and *Arctodidium vulpinus* (Coleoptera, Scarabaeidae): 0.0200 visits/plant/h. The yellow-flowered form of *C. bletioides* was visited by *B. dahlbomii*: 0.0020 visits/plant/h; and by unidentified coleopterans: 0.0060 visits/plant/h. *Chloraea crispa* was visited by *B. dahlbomii*: 0.0003 visits/plant/h; and an unidentified Halictidae

(Hymenoptera): 0.0012 visits/plant/h. In the white-flowered form of *C. bletioides*, only *M. conica* and *A. vulpinus* were observed carrying pollinia attached on their bodies. In the yellow-flowered form of *C. bletioides* only unidentified coleopterans were observed carrying pollinia. In *C. crispa*, *B. dahlbomii* was the only floral visitor observed transporting pollinia on its body.

## Discussion

Species of the genus *Chloraea* are self-compatible and pollinator-dependent for fruit set. Moreover, agamospermy and autogamy failed in setting fruits in all species studied. The fact that allogamic mating is the more frequent way of fruit set in the selected species of *Chloraea* genus is consistent with the breeding systems assessed in the majority of orchids around the world (Neiland and Wilcock, 1998; Proctor et al., 1996), and with two other species of the genus *Chloraea*: *C. alpina* (Clayton and Aizen, 1996) and *C. lamellata* (Lehnebach and Riveros, 2003), which are self-compatible and allogamous plants.

Only the yellow-flowered form of *C. bletioides* showed a tendency to follow cross-pollination when compared to self-pollination treatment, which may be regarded as a partial form of self-incompatibility (sensu Ruiz-Zapata and Arroyo, 1978). This fact may lead to a mixed-mating system which may promote outcrossing by mode of incompatibility (Stephenson et al., 2000), and may provide opportunities for selection of increased selfing under conditions where pollinators are unreliable and fertility is compromised (Sage et al., 2001). In addition, the fact that two forms of the same species (*C. bletioides*) presented partially contrasting breeding systems, points to consider the intra-specific variability that can be expressed in orchids of *Chloraea* genus.

Natural fruiting in the yellow-flowered *C. bletioides*, *C. chrysantha* and *C. galeata* was high because the availability of pollinators under natural conditions seemingly was not limited. These results are surprising taking into account that nectarless orchids are usually limited by pollinators for seed setting (Proctor et al., 1996). In this regard, Neiland and Wilcock (1998) suggested that to provide shelter places is one of the most successful alternatives to rewards for nectarless orchids, where natural fruit set may attain values higher than fifty percent. In fact, in *C. bletioides*, beetles of the *Arctodidium* genus were observed using the inflorescences as shelter and mating sites – and also transporting pollinia – which may account for the high values of fruiting herein reported. Unfortunately, no insect visitor was observed in *C. chrysantha* and *C. galeata* during the observation periods. This points to consider that these species were meagerly visited by

highly efficient unidentified pollinators. Certainly, further studies should be addressed for clarifying this aspect.

On the contrary, reproductive success of the white-flowered form of *C. blettioides* and *C. crispa* was pollen limited. Accordingly, the fruit set attained by experimental cross-pollination was significantly higher if compared with that obtained from natural pollination, especially in *C. crispa* whose natural fruit set was only 12.8%. The pollen limitation may be triggered by infrequent or ineffective pollinator visits, or may occur when incompatible pollen is received (Ackerman and Montalvo, 1990). Regarding that both species are totally self-compatible, infrequent and ineffective pollinator visits may explain such pollen limitations. Thus, taking into account that the lower reproductive success of the white-flowered form of *C. blettioides* was uncoupled to the higher frequency of pollinator visits received if compared with other orchids, a low pollinator efficiency resulting from differences in foraging behaviors might explain such a fruiting pattern. In *C. crispa*, the natural fruit set was low probably due to infrequent pollinator visits because we observed solitary bees entering to the floral bracts of flowers, but with no pollinia attached on their bodies. In addition, in Yumbel, where *C. crispa* was studied, the pesticide applications for the control of the European pine shoot moth (*Rhyacionia buoliana*) in pine plantations (*Pinus radiata*), could reduce the abundance and diversity of pollinators and, in turn, the orchid fruit production (e.g., Parra-Tabla et al., 2000). Taken together, the lower and ineffective frequency of pollinator visits and the likely reduction in pollinator abundances may explain the lowest fruit set of *C. crispa* with respect to all other orchids herein assessed.

The results highlight the dependency of orchids upon pollinators for seed set even though they are self-compatible. Furthermore, it emphasizes the variability in pollination and breeding systems that can be found even in different forms of the same species. Certainly, this fact strongly encourages further studies concerning this subject in the southern South American terrestrial orchids.

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