



## Pollination biology of the Chilean endemic orchid *Chloraea lamellata*

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**Abstract.** *Chloraea lamellata* Lindl. is one of the 50 taxa of terrestrial orchids occurring in Chile. In this paper we report the breeding system, phenology and pollinator activity in a population of the species located in the Province of Valdivia, X Región, Chile (39°28' S). *Chl. lamellata* flowers from November to January, forming a lax spike with ca. 13 flowers. The floral life-span is  $14 \pm 4$  days. The species is self-compatible. There was no statistical difference between the amount of seed produced after hand cross-pollination and hand self-pollination treatments. Neither direct autogamy nor agamospermy are involved in seed setting, thus pollinating agents are essential for the species' reproduction. Also, it has a high number of pollen grains (881733) and ovules (599833) per flower. The P/O ratio calculated is low (1.46). Probable pollinators belong to the orders Hymenoptera (*Corynura chloris* and *Ruizantheda proxima*) and Diptera (Sarcophagidae); these insects had a low visitation rate (0.00002 visits/spike/minute). The rate of pollination observed was low (28.6% flowers with pollinia deposited versus 71.3% flowers with pollinia removed). The reproductive features of *Chl. lamellata* suggest that the survival capability and long-term persistence of the species will be seriously threatened if the plant–pollinator interaction is disrupted.

### Introduction

The Orchidaceae is possibly one of the least studied group of flowering plants in Chile, and practically all the current information dates from the last century. Except for the studies of Reiche (1910), Gumprecht (1980), Riveros (1991), van Nieuwenhuizen (1993a, b) and Pridgeon (1997), all dealing to a certain extent with the biology of the species, orchids have been solely recorded in plant surveys. Their biology, ecology, and taxonomy still remain largely unknown. To date, there is no national legislation to protect native orchids, the precise number of species occurring in the country is still uncertain, and their conservation status is unknown (Lehnebach 1999). For instance, orchids were not included when the conservation status of the Chilean monocotyledonous geophytes was assessed, this because “they are a rather numerous group that still requires more study” (Hoffmann 1989).

In Chile, the Orchid family comprises seven genera and 50 taxa (Lehnebach 2003). *Chloraea* Lindl. contains the greater number of taxa (30). Endemic to temperate South America, it is believed that, along with other genera of the Chloraeinae subtribe (Dressler 1993), it originated in the southern Gondwanaland (Pridgeon and Chase 1995). *Chloraea* species are primarily terrestrial, herbaceous

perennials with fleshy roots and lanceolate-ovate leaves forming a rosette that wilts during the anthesis. The flowers are united in an inflorescence of 3 to 30 or more flowers, sometimes reaching over 1 m high. In Chile, *Chloraea* species occur in moist mountain environments and rocky or sandy soils of the Cordillera de la Costa, Cordillera de Los Andes and the Central Valley. *Chloraea lamellata* Lindl. occurs between 35° and 45° S in the Central Valley, occupying anthropogenic grasslands, secondary growth scrubs and forests of native or exotic species.

Recently, the threats to the survival of Chilean orchids and their conservation needs have been examined in the newsletter of the IUCN/SSC Orchid Specialist Group (Lehnebach 1999). One of the objectives proposed there was the compilation of information on the biology of the native orchids. Since then, as an effort to gradually accomplish this major goal, we have studied several native orchids, focusing mainly on their pollination ecology.

Pollination mechanisms in orchids are generally highly specialised, and species-specific pollination systems occur in many species (van der Pijl and Dodson 1966). Unfortunately, specialisation makes species more dependent and vulnerable to loss of the mutualistic partner (Bond 1994, 1995). Hence, information regarding the breeding system and plant–pollinator interactions is essential when aiming to maintain self-sustaining populations in the wild in the long term. Pollination studies on the orchids from the temperate zone of South America are lacking (see Neiland and Wilcock 1998) and only two records of insect visitation exist in the literature (Gumprecht 1980; Aizen et al. 2002). In this first article, we present results on the reproductive biology of the endemic species *Chl. lamellata*, including aspects of the breeding system, phenology and pollinators.

## Methods

### *Species description*

*Chloraea lamellata* is a species of 30–70 cm high with a lax inflorescence bearing from 3 up to 30 yellow flowers. Flowers are ca. 20–30 mm across. Sepal tips are generally fleshy; petals usually have a few greenish warts close to their base. The labellum is ca. 20 mm long, covered with yellow green-tipped falcate processes along the veins (for a detailed description see Correa 1969). Although the species bears two nectariferous channels along the ovary, they are not functional (Lehnebach and Riveros 1999).

### *Study site*

This study was conducted in a population of *Chl. lamellata* occurring in a pasture located at 50 km from the city of Valdivia, X Región de Los Lagos, Chile (39°28' S). The site, with an area of 0.5 ha, supports a large population ( $\geq 1000$  flowering plants). Although the area has been highly disturbed in the past (it is surrounded by a train line, the main highway, a *Pinus radiata* plantation and a gravel mine), a wire

fence has kept the population isolated and protected from grazing cattle. However, during 2000–2001, a considerable part of the population has been destroyed by a highway-widening project.

#### *Breeding system*

In order to determine the breeding system and presence of self-incompatibility barriers, the following hand-pollination treatments were performed during two flowering seasons: *Direct autogamy*: flowers were bagged before anthesis using paper bags until senescence, excluding pollinators. *Apomixis*: before anthesis, the entire pollinarium was removed with forceps, and the flowers were then bagged until senescence. The test allowed determination of agamospermy. *Self-compatibility*: the pollinarium was removed and flowers were bagged until stigma receptivity, flowers were then pollinated with the whole pollinarium (four pollinia) from another flower on the same inflorescence. Flowers were bagged again until wilting. *Cross-pollination*: the same as above, but flowers were pollinated with pollen from another individual. *Natural pollination*: floral buds were tagged and allowed to develop to fruit under natural pollination conditions.

All five treatments were done along the flowering spike of 36 plants; at least 30 flowers were used in each treatment. In order to diminish bi-parental inbreeding depression, a minimum distance of 10–20 m between individuals was considered (Kearns and Inouye 1993; Sipes and Tepedino 1995). Before dehiscence, capsules were collected and stored in paper bags.

#### *Seed-set and self-incompatibility index*

Seeds per capsule were estimated following Sipes and Tepedino (1995). The entire capsule was emptied into a Petri dish with a paper graph placed at the bottom. A square of  $6 \times 6$  cm was drawn and subdivided into 144 quadrants of  $5 \times 5$  mm. Under the microscope, all viable and non-viable seeds in 30 randomly chosen quadrants were counted. Viability was assessed by the presence of a well-developed embryo. The presence of self-incompatibility was determined using the index of self-incompatibility (ISI) proposed by Ruíz and Arroyo (1978) and Riveros (1991) (ISI: number of viable seeds produced by hand self-pollination/number of seeds produced by hand cross-pollination). A one-way ANOVA was applied to the seed-set produced after each pollination treatment. Tukey's test (95%) was applied to determine differences between treatments using Statgraphic Plus 2.0.

#### *Pollen stainability, pollen and ovule numbers, P/O ratio*

The entire pollinia from 10 flowers, each from a different plant, were collected. Pollinia were macerated and stained using Alexander's differential dye (Alexander 1980). Total number of tetrads was counted using a haemocytometer. Stained and non-stained pollen were counted. Since ovules in the Orchidaceae do not finish development until pollination has taken place (Neiland and Wilcock 1995), their

management and observation is rather difficult; for that reason capsules in the early ripening stage were used to determine ovule numbers (R. Cruden, personal communication). P/O ratio was obtained following Cruden (1977).

#### *Phenology and pollinator observation*

Phenological phases, i.e. growth period, dormancy, spike development and flower life-span and fruiting, were observed in 46 tagged plants both in the field and in the greenhouse. Flower longevity was observed in 29 flowers tagged before anthesis and visited daily until senescence. The remaining stages were assessed by weekly visits to the population.

Pollinator activity was observed in 208 individuals during 38 periods of 10 min, over 5 days during the flowering period of the species. Observation periods were conducted during the day from 10 A.M. to 6 P.M. Insects were collected and later identified. Visiting insects were classified according to their pollination effectiveness following Adams and Lawson (1993). Also, climatic conditions were recorded during pollinator activity.

#### *Pollination assessment*

In order to determine the efficiency of the pollination process in the population, the pollination status of 324 randomly selected flowers was recorded. Following Thien (1969), flowers were classified according to pollinia removal and deposition, into flowers with pollinia removed, pollinia deposited and not removed. The pollination status was evaluated at the peak of the flowering season (late December).

## **Results**

#### *Breeding system and genetic incompatibility*

Fruit-set under natural pollination conditions was low, only 15.6% of the 64 flowers tagged in the site set fruits; however, these capsules scored the highest number of viable seeds (Figure 1). Conversely, cross-pollinated flowers had the highest fruit-set (76%), but the lowest number of viable seeds per capsule (Figure 1). The number of viable seeds set through natural pollination and hand cross-pollination was significantly different ( $P$ -value  $< 0.01$ ). Neither direct autogamy nor apomixis were capable of producing fruits (Figure 1), thus pollinating agents are essential to achieve pollination. There was no significant difference between the number of viable seeds set after hand cross- and hand self-pollination. Furthermore, the ISI obtained for the species is 1.36, confirming the absence of self-incompatibility.

#### *Pollen stainability, pollen and ovule numbers, P/O ratio*

A total of  $881\,733 \pm 225$  grains per flower was recorded. The number of ovules per

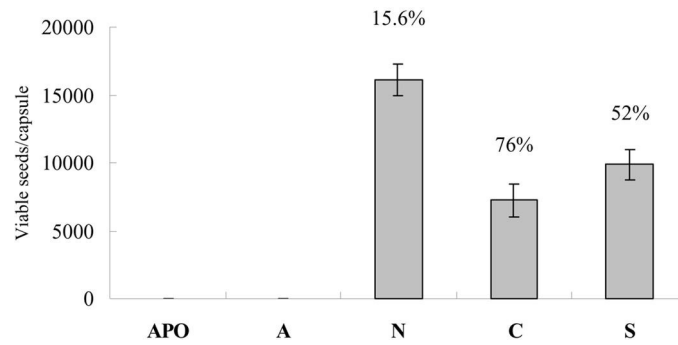


Figure 1. Viable seeds per capsule (mean  $\pm$  SD) and percent of fruit-set produced in each hand-pollination treatment (APO: apomixis, A: direct autogamy, N: natural pollination, C: cross-pollinations, S: self-compatibility).

carpel was 199 944, making a total of  $599\,833 \pm 167\,861$  ovules per flower (figures are expressed as mean  $\pm$  SD). Stainability of pollen is high, 97.7%. The P/O obtained was 1.46.

#### Phenology and pollinators

*Chl. lamellata* flowers from mid-November to late January. The single spike takes almost 6 months to complete its development. Flowering is acropetal, and the spike life-span fluctuates from 19 to 30 days (mean 20.5 days). The resupinated flowers have a life-span of  $14.3 \pm 4.2$  days (mean  $\pm$  SD). Once pollination has occurred, floral structures wilt rather immediately, so that spikes can be found simultaneously at the flowering and fruiting stages. The fruiting period goes from December to late February, lasting each capsule 20 to 30 days to complete its development. After fruiting, the spike wilts and the plant gets into a dormancy period for 6 weeks.

Insect visitation occurred only in 7 of the 38 periods of observation. The species recorded belong to the order Diptera (2 spp.), Hymenoptera (2 spp.), Lepidoptera (1 sp.) and Coleoptera (1 spp.). These insects were ranked into probable pollinators and visitors. Adams and Lawson (1993) described as 'probable pollinators' those insects that are observed taking up pollinia of the plant but later do not deposit them on a stigma of a co-specific flower. According to this, *Ruizantheda proxima* Spin. (Hymenoptera: Halictidae), a sarcophagid fly (Diptera: Sarcophagidae), and *Corynura chloris* Spin. (Hymenoptera: Halictidae) were classified as probable pollinators (when captured they were observed carrying the four pollinia attached to the thorax). *R. proxima* and the sarcophagid fly had a low visitation rate, both with 0.00002 visits per spike per minute. The weekly visitation rate of each of these insects was calculated as 0.1 visit per spike. The visitation rate of *C. chloris* is lacking, since it was captured during other field observations. The remaining insects, *Vanessa carye* (Hubn.) (Lepidoptera: Nymphalidae), *Allograpta* sp. (Diptera: Syrphidae) and one species of the order Coleoptera, were only considered as visitors.

### *Pollination assessment*

Surveyed flowers were grouped into three categories, flowers with the pollinia removed ( $n = 87$ ), not removed ( $n = 202$ ), and flowers with pollinia deposited on the stigma ( $n = 35$ ). Pollination in the population is low; only 10.8% of the flowers observed had, at least, one pollinium on the stigma, suggesting low pollination activity. Finally, pollinia donation was greater than pollinia deposition, 71.3 and 28.6% respectively (this considering only flowers with pollinia deposited and/or removed).

## **Discussion**

### *Reproductive biology*

*Chl. lamellata* is a self-compatible orchid that depends on pollinators to set fruits. Self-compatibility has been widely reported in terrestrial orchids of temperate regions and considered as an adaptation to a poor insect fauna and/or low insect visitation rates, and likely the first step towards mechanic self-pollination (Thien and Marcks 1972; Mehrhoff 1983; Beardsell et al. 1986; Sydes and Calder 1993; Sipes and Tepedino 1995; Clayton and Aizen 1996; Galetto et al. 1997). Although self-compatibility may favour fruit-set, successful pollination in cross-pollinating orchids is rare (Neiland and Wilcock 1995) and *Chl. lamellata* was not an exception to this feature with a fruit-set of 15.6%.

In orchids, reproductive success has often been described as pollen-limited (see Proctor and Harder 1994). Pollen limitation in *Chl. lamellata* is noticeable, with only 10% of 324 flowers surveyed in this study having pollinia deposited on the stigma. Similar figures have been reported for *Chloraea alpina* Poepp. (Clayton and Aizen 1996) and other terrestrial orchids (Thien 1969; Thien and Utech 1970; Firmage and Russell 1988; Johnson and Bond 1992; Sydes and Calder 1993; Proctor and Harder 1995). Generally, pollen limitation in the orchid family has been attributed to the poor fidelity (Beardsell et al. 1986) and the habitat preferences of pollinators (Johnson and Bond 1992). Besides pollinator availability, the reproductive success of orchids may be limited by the amount of resources allocated for seed production (Firmage and Russell 1988; Calvo 1990; Gregg 1991; Rasmussen 1995; Sabat and Ackerman 1996). In this study, despite the fact that natural fruit-set was increased by hand-pollination, the number of viable seeds produced was very low. This suggests that the reproductive success of this orchid is, to a certain extent, resource limited. Resource limitation is such an important factor in terrestrial orchids that when fruit-set is artificially increased, fecundity and the future growth of the individual can be dramatically affected (Snow and Whigham 1989; Ackerman and Montalvo 1990). Therefore, producing few capsules containing only several thousand seeds may be a good strategy to save the plant resources (Gregg 1991).

The insects associated to this orchid are numerous; six species in four orders. But only *Ruizantheda proxima*, *Corynura chloris* and the sarcophagid fly could be

considered as probable pollinators (*sensu* Adams and Lawson 1993). These insects are the appropriate size to achieve pollination and were captured carrying pollinia. Pollination of orchids by Hymenoptera and Diptera has been previously reported for some terrestrial orchids elsewhere (Hogan 1983; Mehrhoff 1983; Stoutamire 1983; and others). In Chile the only record of insect pollination in native orchids is by a small bee of the genus *Colletes* carrying the pollinia of *Chloraea cylindrostachya* Poepp. (Gumprecht 1980).

Contrary to Riveros (1991), who reported insect visitation rates from 0.006 to 10.5 visits per flower per 10-min period in native non-orchid species in a protected area of the same region, insects visiting *Chl. lamellata* have an extremely low visitation rate (0.0002 visits per spike per 10-min). Low visitation rates in terrestrial orchids have also been reported by Hogan (1983), 0.00012 visits per flower per 10-min period in *Aplectrum hyemale* (Orchidaceae), and by Firmage and Russell (1988), who suggested that the frequency of visits resulting in pollen deposition or pollinia removal in *Calopogon tuberosus* (Orchidaceae) may be as low as one visit per 3–5 days per plant.

Usually, nectar and pollen are the main rewards for the pollinators (Faegri and van der Pijl 1976), but *Chl. lamellata* is nectarless. This has also been observed in *Chl. alpina* (Clayton and Aizen 1996), and in closely related genera such as *Caladenia* and *Lyperanthus* (Dafni and Bernhardt 1990; Bernhardt 1995). With regard to the use of pollen as a reward, Dafni and Bernhardt (1990) state that pollen of monandric orchids (e.g. *Chl. lamellata*) is rarely consumed by pollinators. Hence, *Chl. lamellata* is considered a non-rewarding species, in which pollination relies on a system of unidirectional exploitation (Dafni 1984); in other words, pollinators are deceived. In every type of deception, olfactory and optical clues are important to the success of this system (Dafni 1984). Species of the genus *Chloraea* are notable for the presence of fleshy-thickened sepal tips, where scent glands have been observed (Gumprecht 1980; Vogel 1990; Lehnebach and Riveros 1999). Colour diversity in deceptive species is important, reducing the pollinator's chance of learning, and thus pollinator's avoidance (see Dafni (1984) for more references). Floral polymorphism has been previously reported in *Chl. lamellata* (Correa 1969) and was also observed in the population studied.

In *Chl. lamellata* the floral longevity is about 2 weeks, which is long if compared with more typical spans of 4 to 10 days anthesis measured for other orchid species (Hogan 1983; Mehrhoff 1983; Beardsell et al. 1986; Firmage and Russell 1988). The flower life-span of the cogenetic *Chl. alpina* has been estimated at ca. 3 weeks in South Argentina (Clayton and Aizen 1996). Neiland and Wilcock (1995) suggested that flower persistence in some European orchids has probably evolved in response to low visitation. This could also be the case for *Chl. lamellata*, with both low visitation rates and long flower longevity.

Overall, studies regarding the P/O ratios in the Orchidaceae are scarce (e.g. Mehrhoff 1983; Neiland and Wilcock 1995; Nazarov and Gerlach 1997). P/O ratios have been used as an alternative way to infer the reproductive strategies of some plants (Cruden 1977) and, in orchids, the group of pollinating insects (Neiland and Wilcock 1995). *Chl. lamellata* shows a P/O of 1.46, a very low ratio for out-

crossing species compared with P/O values reported by Cruden (1977) and Neiland and Wilcock (1995). Neiland and Wilcock (1995) suggested that low P/O values in xenogamous orchids are more likely to be found in orchids pollinated by highly efficient pollinators such as Hymenoptera. In *Chl. lamellata* two of the probable pollinators belong to this order.

#### *Conservation implications*

In orchids, survival can be limited by the normal functioning of at least three ecological interactions: plant–pollinator, plant–mycorrhiza and, in epiphytes, plant–host tree. These interactions, usually species-specific, make orchids extremely vulnerable to disturbances in the ecosystem (Wells 1981). The reproductive features of *Chl. lamellata* suggest that survival and long-term persistence of the species will be seriously threatened if the plant–pollinator interaction is disrupted. This orchid is an obligate xenogamous and depends on pollinators to produce fruits. Despite pollination not being species-specific, insect visitation is low and fruit-set scant. The latter is resource limited and does not occur every year. In addition, the survival capability of this orchid is reduced by the lack of compensatory mechanisms such as vegetative propagation.

Immediate threats facing *Chl. lamellata* populations are habitat destruction, fragmentation and the invasion of exotic plants, and are likely the causes of the poor reproductive success observed in this population. Habitat transformation and fragmentation have been considered one of the main threats to the appropriate functioning of plant–pollinator interactions, as they change the local insect community and the availability of resources for pollinators (Aizen and Feisinger 1994; Cunningham 2000). Exploitive activities such as agriculture, mining and urbanisation have dramatically fragmented and destroyed the habitat supporting this orchid population. Additionally, these activities have eased colonisation by exotic plants such as *Cytisus scoparius* (L.) Link (broom) and *Rubus constrictus* Muell. et Lef. (blackberry). These species are highly invasive in the south of Chile, and contrary to *Chl. lamellata*, their sexual reproduction and vegetative propagation is considerable. In addition, flowering of these exotic species overlaps with the flowering period of *Chl. lamellata*, increasing competition for the pollinator service in the community.

Currently, the destruction of native forests by processes such as urbanisation, logging and conversion to pine-tree plantations or agricultural land, is one of the major conservation problems in Chile (Lara et al. 1995). Although the effect of these threats on orchid populations may be readily detected, the effect of reproductive failure on species survival is not (Bond 1994, 1995). Loss of pollinators and reproductive failure can lead to a form of delayed extinction (Johnson and Steiner 2000) only detectable after several years (e.g. Parra-Tabla et al. 2000). Consequently, evaluating the reproductive success, recruitment and mortality rates is indispensable to forecast the population's future. Conducting such monitoring activities, along with autoecological studies, in this and other orchid populations is essential to develop future managing strategies, estimate population viability in



fragmented habitats and anticipate the threat pollinator loss may impose on the survival of these orchids in the temperate forests of South America.

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