REPRODUCTIVE BIOLOGY OF *PHRAGMIPEDIUM LONGIFOLIUM* AND FLORAL ANATOMY ASSOCIATED WITH POLLINATOR ATTRACTION IN THE GENUS

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Phragmipedium is a genus with 21 species restricted to the tropics and subtropics from Central and South America. As it is a genus capable of producing seeds from both self- and cross-pollinated flowers, it is an ideal group to investigate the components of its reproductive success. In this research some of those components are described in *P. longifolium* as well as anatomical floral structures possibly related to the attraction of pollinators. To study the reproductive system in *P. longifolium*, manual self- and cross-pollinations were carried out, and seed viability was measured by tetrazolium staining.

No significant differences in fruit production between self- and cross-pollinated flowers were found. Higher seed viability was found in the self-pollinated flowers, which indicates that this species is frequently self-pollinated. Sections of the petals of *P. humboldtii*, *P. longifolium*, and *P. warzewiczianum* were stained with toluidine blue in order to identify and characterize anatomical structures possibly involved in the attraction of pollinators. Three kinds of glandular trichomes were observed on the petals of all three species, which are possibly involved in the secretion of fragrances to attract pollinators.

Cypripedioideae is a subfamily of Orchidaceae that comprises about 180 species distributed in five genera. In spite of their wide geographic range, the diverse habitats and variable sizes of plants, almost all the species known in the subfamily share the same basic floral structure and (for those in which it has been documented) the same pollination mechanism. Some species in Cypripedioideae are self-compatible; however, in most cases the flower needs an insect that transfers the pollen from an anther to the stigma (Pemberton 2013).

To date, no pollinator-rewarding species are known in Cypripedioideae, and deceit-pollination is uniformly assumed (except for the few consistently self-pollinated species, e.g., *Phragmipedium lindenii* (Lindl.) Dressler & N.H.Williams subsp. *lindenii*). The orchids in this group have a peculiar floral morphology with a saccate lip. At the point of union of the base of the lip with the column, two lateral openings on each side of the lip occur, each one located right next to one anther on the side of the column (Burns-Balogh and Hesse 1986; Pemberton 2013).

The lip works like a trap for potential pollinators. The insects that are attracted to

Table 1. Results of the binary logistic regression with categorical variables to evaluate the effect of different pollination treatments in fruit production of *Phragmipedium longifolium* (*= p< 0.001).

Coefficient	Estimates	Error	Z Value	P > (|Z|)	OR	95% CI
Cross-pollination (intercept)	0.8001	0.2162	3.700	0.0002*	2.2258	1.4712 − 3.4453
Self-pollination	0.5862	0.3305	1.773	0.0762	1.7971	0.9462 − 3.4751

the flowers enter by the main opening and get trapped inside the bag. To make their way out, the insects must climb on the internal surface of the lip up to one of the lateral openings between the lip and the column. If the insect is carrying pollen on its body, passing through the lateral openings will force contact with the stigma, which is covered with small papillae that help collect pollen grains. After passing the stigma, the insect will contact one of the anthers, collect more pollen on its body, and then exit the flower through the lateral opening (Pemberton 2013).

Despite the fact that pollination in Cypripedioideae has been moderately documented (Pemberton 2013), few studies have focused on the reproductive biology of the species and even fewer on the floral anatomy involved in pollinator attraction. Most of the papers published for the group are limited to systematic and evolutionary studies (Cox *et al.* 1997; Li *et al.* 2011; Chochai 2014). However, ecological studies that analyze the reproductive aspects from attraction of pollinators to the development of seeds on pollinated flowers are scarce. The genus *Phragmipedium* comprises about 21 species (Cribb and Purver 2017), but pollinator reports exist for only four of these, in addition to the three species that are known to be autogamous (Pemberton 2013; Edens-Meier and Bernhardt 2014). As described in *Paphiopedilum* (Atwood 1985), the flowers of *Phragmipedium* may also be mimicking aphids, which are known to serve as a food resource for larvae of many species of syrphid flies, their most known pollinators

(Dodson 1966; Pemberton 2011). The species of *Phragmipedium* pollinated by syrphid flies usually have opaque colors and small dots on the lip that are apparently mimicking the aphids (Pemberton 2011).

Osmophores are glands that produce scents, some of which can be easily distinguished anatomically and morphologically from adjacent tissues, but others cannot (Pridgeon and Stern 1983; Vogel 1990). Vogel (1990) described glandular trichomes on the petals of *Phragmipedium* (as "*Phragmopedilum*"), which he interpreted as possible osmophores (he actually called them "fragrance hairs" to distinguish them from his own strict definition of osmophore, even when they putatively have the same function). Stern *et al.* (1986) used the stains neutral red and Sudan Black B to detect fragrance glands in different orchid species including *Paphiopedilum concolor* (Bateman) Pfitzer and *Phragmipedium caricinum* (Lindl. & Paxton) Rolfe.

Those are the only references that we could find in the literature about anatomical structures that may be playing a role in the attraction of pollinators in Cypripedioideae. Documenting and describing these structures may provide a better understanding of the pollination system in these species.

The aim of this study is to present information about the reproductive biology of *P. longifolium* and to describe the glandular trichomes on the petals of *P. longifolium*, *P. humboldtii*, and *P. warzewiczianum*, possibly associated with the production of pollinator-attracting scents.

Material and methods

Site and species under study

The study was carried out at Bosque de Paz Biological Reserve (10°12'17.3"N 84°18'59.1"W, 1534 m), located in Zarcero county, Alajuela province, Costa Rica.

The site has nearly 110 plants of *Phragmipedium longifolium* cultivated close to the border of a montane mature forest. These plants bloom throughout the year and produce fruits constantly, evidencing the presence of natural pollinators. *Phragmipedium longifolium* is characterized by a terrestrial habit, an inflorescence with sequentially produced, green to purple flowers (one or occasionally two open at a time), and linear petals slightly longer than the lip. This species is distributed from Costa Rica to Ecuador (Dressler 2003). The identification of anatomical structures was made on cultivated plants of *P. humboldtii* (Warsz.) J.T.Atwood & Dressler and *P. lindenii* subsp. *warzewiczianum* (Rchb.f.) Schltr. Results of the binary logistic regression with categorical variables to evaluate the effect of different pollinatio treatments in fruit production of *Phragmipedium longifolium* (*= p< 0.001). (Rchb.f.) Dressler. The former is distributed from southern Mexico to western Panama and is recognized by its epiphytic habit and the long and pendent petals (Dressler 2003). The latter is distinguished by a well-developed lip (in contrast with *P. lindenii* subsp. *lindenii*) with a narrow keel around the border of the main opening and is only known from Ecuador and Peru.

Reproductive biology

To identify the possible effects of self- and cross-pollination in the reproduction of *P. longifolium*, four treatments were applied: 1) manual self-pollination, 2) manual cross-pollination, 3) exclusion of pollinators, and 4) open pollination (control treatment). Each treatment was applied monthly to 10 marked flowers from different plants during 10 months
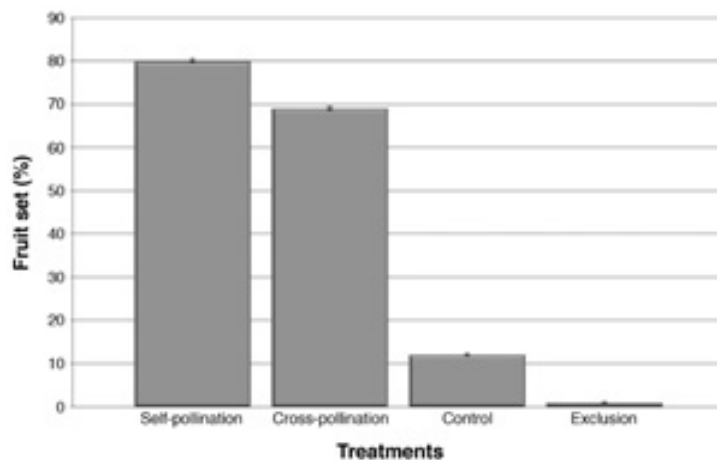

Fig. 1. Percentage of fruits produced in the self-pollination, cross-pollination, open pollination (control), and exclusion of pollinators treatments in a population of *Phragmipedium longifolium* in Costa Rica (n= 100 flowers per treatment). Vertical lines represent standard deviation.

for a total of 100 flowers per treatment. Only flowers in early anthesis and with both pollen masses visibly intact were used to rule out the influence of any previous visits.

Self-pollination consisted in transferring both pollen masses (those of both anthers) of a flower to its own stigma. For cross-pollination, the pollen masses were placed on the stigma of a flower from another plant in the same population. For exclusion of pollinators, floral buds were enclosed in mesh bags to prevent visits of insects large enough to be potential pollinators. For open pollination, the marked flower remained in its natural condition. A month after each treatment was performed, the number of any resulting fruits was counted; at the end of the 10-month study period, the proportion of fruits (fruit set) was calculated per treatment. The results were analyzed with a binary logistic regression with categorical variables in order to detect differences in the fruit set between cross-pollination treatment vs. self-pollination, exclusion and open pollination treatments. Cross-pollination treatment was used as intercept in the model because it was expected to be the treatment with the largest fruit set. From the regression coefficients estimates, the odds ratio (OR) and the 95% confidence

intervals (CI) were calculated to obtain the probability of producing fruits for each treatment compared with the cross-pollination treatment. The analyses were made using the statistical environment RStudio 1.0.153 (R Development Core Team 2017).

Floral anatomy and histology

To identify and describe anatomical structures possibly involved in the attraction of pollinators in *P. longifolium*, *P. humboldtii,* and *P. warzewiczianum*, plants in cultivation at Jardín Botánico Lankester were used. Since Vogel (1990) had previously reported what he called "fragrance hairs" on the petals of *P. Grande* (a hybrid), these structures were looked for in transverse sections of the petals prepared using the Paraplast® fixation technique described by Sandoval *et al.* (2005), which were cut with Leica RM2125 RTS and American Optical 820 microtomes. The sections were stained with toluidine blue 1% and acidified in a saturated solution of boric acid using the indirect method described by Sandoval *et al.* (2005). Photographs of the stained sections were obtained with a Leica ICC50 camera attached to a Leica DM500 microscope and a Sony Exwave HAD camera attached to a Carl Zeiss Axioskop microscope. Anatomical structures in the petals were identified and described from the photographs.

Results

Reproductive biology

Eighty percent of the self-pollinated flowers and 69% of the cross-pollinated flowers produced fruits. Only 1% and 12% of flowers produced fruits in the exclusion and open pollination treatments, respectively (Fig. 1). The fruit set of open pollination (p< 0.001) and exclusion (p< 0.001) significantly differs from the fruit set of cross-pollination (Table 1). No significant differences were found between the fruit set of self- and cross-pollination (p> 0.05) (Table 1).

It was not possible to determine the ratio in the probability of producing fruits between cross- and self-pollination because the 95% CI included the null value (OR=1) in a wide range, which indicates a low OR precision (Table 1). Open pollination (OR=0.0613) showed a lower probability of fruit set than cross-pollination, and that probability was even lower for the exclusion treatment (OR=0.045) (Table 1).

Floral anatomy and histology

Three different kinds of glandular trichomes were found, denoted GT1, GT2, and GT3. Compared with the epidermal cells, the cells of the glandular trichomes frequently have larger nuclei that can comprise about half of the cell volume. GT1 trichomes are uniseriate with 2-6 cells that are usually cylindrical and of similar size except the apical cell, which is longer and conical. GT2 trichomes are uniseriate with 5-8 cylindrical cells, the apical cell slightly smaller and rounded. GT3 trichomes have variable
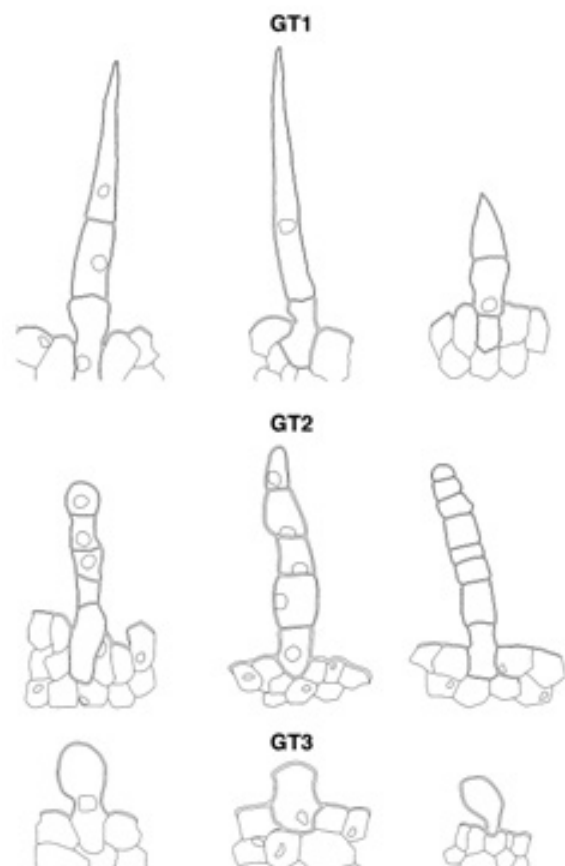

Fig. 2. The three different kinds of glandular trichomes (GT1, GT2, and GT3) observed on P. *humboldtii*, P. *longifolium*, and P. *wallisii* and their possible varieties. (Illustrations: M. Díaz-Morales)

shapes but are easily distinguished by being unicellular (Fig. 2).

Discussion

Reproductive biology

Phragmipedium longifolium is a species capable of cross- and self-pollination. However, in both cases it requires a pollinator to transfer the pollen to the stigma. Some authors (e.g., Muñoz *et al.* 2010; Walsh and Michaels 2017) suggested that pollination by deceit could reduce the probability of a plant being self-pollinated because the pollinator usually moves away from the flower to avoid being fooled again immediately or because the lack of a reward makes it disinterested in the flower. However, others affirm that self-compatibility might be the result of an adaptation to its pollination syndrome, because accepting its own pollen might raise the chances of being fertilized, although this could affect genetic structure and variation within and among populations (Liu *et al.* 2006).

A study on population genetics indicated that Costa Rican *P. longifolium* reproduces mainly by outcrossing, based on the lower genetic variation among populations than within populations (Muñoz *et al.* 2010). However, our study shows a tendency for self-pollination. The latter could represent a strategy in populations reduced by a bottleneck effect, in which self pollination is favored to ensure reproduction (Lande and Schemske 1985; Schoen *et al.* 1996).

Self-pollination may provide reproductive assurance in habitats where pollinator service may be unavailable or unpredictable (Lande and Schemske 1985; González-Díaz and Ackerman 1988). In Costa Rica, some populations of *P. longifolium* with several individuals have been identified but are relatively isolated from each other because this species colonizes disturbed habitats such as road-cuts and landslides (Muñoz and Warner 2007). Geitonogamy may be occurring in these populations because two-flowered inflorescences occasionally occur, and single plants may have two inflorescences blooming simultaneously.

Orchids with pollination by deceit usually have low visitation and pollination rates (Neiland and Wilcock 1998; Tremblay *et al.* 2005). *Phragmipedium longifolium* is not the exception; according to our results, only about a 12% of the flowers produced fruits. Nevertheless, low pollination rates are also frequent in tropical orchids dependent on a pollinator, regardless of what mechanism is employed to attract them (Neiland and Wilcock 1998; Tremblay *et al.* 2005).

Floral anatomy and histology

The glandular trichomes detected by Vogel (1990) in *P. Grande* (here designated GT2) were found on the petals of the three *Phragmipedium* species studied. Two additional kinds of glandular trichomes were also found. The large nuclei of the GT2 trichomes are related to an increase of euchromatine (characteristic of glandular tissues) and a high metabolic activity. Large nuclei were also observed in the other two kinds of glandular trichomes observed in the three *Phragmipedium* species studied. Recent observations indicate that the petals of *P. longifolium* play the main role in chemical attraction of pollinators (Díaz-Morales *et al.* unpublished), probably by the emission of fragrances from these glandular trichomes.

Acknowledgements

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