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NECTARY STRUCTURE AND NECTAR IN *SOBRALIA* AND *ELLEANTHUS* (SOBRALIEAE: ORCHIDACEAE)

KURT M. NEUBIG[1,2,4], BARBARA S. CARLSWARD[3], W. MARK WHITTEN[2] & NORRIS H. WILLIAMS[2]

[1] Department of Plant Biology, Southern Illinois University of Carbondale, Carbondale, Illinois 62901
[2] Florida Museum of Natural History, University of Florida, Gainesville, Florida 32611
[3] Department of Biological Sciences, Eastern Illinois University, Charleston, Illinois 61920
[4] Corresponding author: kneubig@siu.edu

ABSTRACT. With approximately 200 species, the tribe Sobralieae is a dominant and common Neotropical group of orchids, yet little is known of variation in floral morphology as it relates to their pollination. As currently circumscribed, the tribe includes four genera that differ considerably in flower size and morphology: *Elleanthus*, *Epilyna*, *Sertifera*, and *Sobralia*. Although nectar-foraging pollinators are known for some species, the relationships of pollination to deceit and to nectar production are all poorly understood. We examined pollination-related of nectaries and nectar characteristics (presence/absence, volume, and concentration) for major clades of Sobralieae. Some species produce abundant nectar, but many species offer no reward. When present, nectar is secreted by thickened calli at the lip base. The cells of the nectariferous calli contain starch, which is rapidly converted to sugar during a brief anthesis (often lasting only one day). Most *Sobralia* flowers are relatively large, bee-pollinated, with a gullet-shaped lip, false nectary, large pollinia, and offer no reward. *Elleanthus* flowers are relatively small with a legitimate nectar reward, and most species are hummingbird-pollinated. Hummingbird-pollinated Sobralieae flowers are relatively small, brightly colored in the perianth and/or the subtending bracts, somewhat tubular, with a lip that forms a cup around the callus for storing nectar, and pollinia that are dark and relatively small.

KEY WORDS: Deceit, *Elleanthus*, Nectar, Nectary, Pollination, *Sobralia*, Sobralieae

Introduction. In Orchidaceae, floral rewards are extremely diverse (van der Cingel, 2001; van der Pijl and Dodson, 1966) and include nectar, oils, resin, wax, food bodies, and even fragrances (Davies and Stpiczyńska, 2008a; Whitten *et al*., 2007). By far, the most common reward is nectar, which is presented by flowers of varying morphology to many different pollinators: Diptera (flies), Hymenoptera (bees and wasps), Lepidoptera (moths and butterflies), Trochilidae (hummingbirds) in the New World (van der Cingel, 2001; van der Pijl and Dodson, 1966), Nectariniidae (sunbirds) (Johnson *et al*., 1998) and Zosteropidae (white-eyes) in the Old World (Micheneau *et al*., 2006).

However, it is estimated that one-third of all species of orchids use deceit strategies (Cozzolino and Widmer, 2005). This high percentage of such deceitful orchids is evidence that pollination by deceit is a successful adaptive strategy. The evolutionary forces driving deceit pollination are complex and not well understood (Jersáková *et al*., 2006).

In Sobralieae, known pollinators include various bees and hummingbirds. Some species produce nectar rewards, but others produce no apparent reward. Whereas food-foraging bees are attracted to flowers of diverse morphology, colors, fragrances, nectar guides, and nectar rewards, birds are attracted to nectariferous flowers with bright corollas and/or bracts of contrasting color, and that usually lack fragrances. Most investigated species of *Sobralia* Ruiz & Pav. are reported to be pollinated by a variety of large solitary bees, especially by euglossine bees, whereas hummingbird pollination is known in *Elleanthus* C. Presl and in a few *Sobralia* species (Braga, 1977; Dodson, 1962, 1965; Dressler, 1971, 1976, 2002; Ducke, 1902; Dziedzioch *et al*., 2003; Fogden and Fogden, 2006; Roubik, 2000;

Table 1. Observations of nectar secretion in this study. Although some species were observed and confirmed to have nectar, not all had measurable amounts of nectar. Only sucrose was directly measured. Volumes are in microliters (μL) and concentrations are in % sucrose (sometimes noted as °Bx). Additional species were sampled and produced nectar, but were too small to measure: *S. ciliata*, *E. lancifolius*, *E. graminifolius*, *E. fractiflexus*, and *E. robustus*.

Species	Sample size, flowers (n)	Mean (avg)	Standard deviation (σ)	Range	Vouchers	Syndrome
Elleanthus aurantiacus	5				none (population sampling)	hummingbird
volume		4.4	1.8	2-7		
concentration		22.8	1.9	21-26		
E. caravata	52				*Neubig 202*	hummingbird
volume		5.7	2.4	2-10.1		
concentration		24.3	6.9	12-40		
E. cynarocephalus	5				*Neubig 247*	hummingbird
volume		6	3.1	2-10		
concentration		9.2	6.6	5-21		
E. sodiroi	46				*Neubig 246*	hummingbird
volume		13.6	6.5	4-31.5		
concentration		15.9	6.4	7-25		
Sobralia bouchei	52				*Blanco 3009, Neubig 208*	bee
volume		14.1	7.9	2-43		
concentration		21.2	3.3	12-28		
S. callosa	27				*Blanco 3021, Neubig 224*	hummingbird
volume		6.3	2.4	1.5-12		
concentration		16.3	2	12-19.5		
S. macrophylla	6				*Blanco 3022*	bee
volume		4.9	2.4	1-8		
concentration		20.6	1.4	18-22		
S. rosea	46				none (population sampling)	bee
volume		8.4	8.6	3-35		
concentration		13.8	3.1	5-19.5		

Roubik and Ackerman, 1987; Singer, 2003; van der Pijl and Dodson, 1966). Molecular data demonstrate that *Sobralia* is not monophyletic (Neubig, 2012; Neubig *et al*., 2011), and so understanding relationships with pollinators within a phylogenetic context is critical to develop hypotheses of evolution in pollination.

The objectives of this study are to document traits of nectary structure and nectar production relative to other morphological features in *Sobralia* and *Elleanthus* and to relate these features with pollen vectors.

Materials and Methods. Observations were primarily made on cultivated plants in greenhouses of the Florida Museum of Natural History over the course of May 2007 through May 2011 and in Ecuagenera nurseries in Gualaceo, Ecuador, as well as on various natural populations in Ecuador, February 2009. Voucher specimens were deposited at FLAS and QCA herbaria. A list of taxa examined for nectar is presented in Table 1.

Nectar Volume and Quantity –. Flowers were examined for nectar presence/absence. If nectar was found, measurements were made of both volume and sucrose concentration. Sucrose concentration was measured with a 0-53 brix Atago refractometer at various times of the day, but primarily at midday and at approximately room temperature (Corbet, 2003). Concentrations are presented in percent sucrose (i.e., equivalent to Degrees Brix, g sucrose per 100 g solution), because

it is a common unit used in nectar and food science (Bolten *et al*., 1979; Corbet, 2003; Dafni, 1992). Nectar was pipetted and measured with a 0.5-20 µL Rainin micropipetter. Sugar composition and minor nectar constituents such as amino acids (Gottsberger *et al*., 1984) were not examined in this study.

Most plants were cultivated in a closed greenhouse, and were therefore not exposed to insects or other potential pollinators that might remove nectar. All plants of *Sobralia rosea* and *Elleanthus aurantiacus* were sampled in the wild and could therefore have had their nectar removed (thus modifying nectar volume) by visiting pollinators. Alternatively, rain could have modified nectar volume and concentration in these species. However, except for visitation by pollinators, the occurrence of such factors was the subject of careful inspection and, as far as was possible, controlled experimentation.

Floral Anatomy –. Flowers of selected species were fixed in FAA (9 parts 70% ethanol: 0.5 part glacial acetic acid: 0.5 part commercial formalin) for several days and stored in 70% ethanol. Floral tissues were dehydrated in a graded tertiary butanol:ethanol:water series (6 h for each of the following solutions 20:50:30, 35:50:15, 55:45:0, 75:25:0, and two changes of 100% tertiary butanol). Dehydrated tissues were embedded in Paraplast® tissue embedding medium (melting point 56° C) and sectioned with an American Optical 820 rotary microtome at 10 µm. Sections were attached to slides using Haupt's adhesive (1 g gelatin: 100 mL water: 2 g phenol: 15 mL glycerol) and allowed to dry at 30° C for 12 h. Tissues were treated in 3% ferric ammonium sulfate for 20 min, stained in 0.5% Heidenhain's iron-alum hematoxylin for 5-10 min, and counterstained with a 0.01% solution of safranin for 6 h. Stained tissues were dehydrated in a graded ethanol series (95%, 95%, 100%, 100%) for 5 min each and subsequently cleared in two changes of limonene. Coverslips were mounted onto slides using Permount. Observations and photographs were taken with a PixeraPro 150es digital camera attached to a Zeiss Axioskop 40 microscope. Additional hand-cut sections were made of flowers of various species of *Sobralia* and *Elleanthus* to demonstrate variation and the presence of nectaries and cavities. Entire flowers were cleared and/or hand-sectioned, then stained with Lugol's solution (I_2KI: iodine - potassium iodide) to test for starch. Labella of mature flowers were also hand sectioned in the morning (7 am), at noon, and in the evening (7 pm) and stained with I_2KI. Hand-cut sections of fresh floral tissues were also stained with methylene blue (1% dissolved in H_2O) for the purpose of indicting cavities and cellular contrast.

To examine cellular detail of the surface of calli, tissues were first pickled in FAA, then dehydrated in a graded ethanol series and dried in a critical point dryer using liquid CO_2. Dried samples were then mounted on clean aluminum stubs with double-sided adhesive graphite tabs. Mounted sections were coated with gold-palladium for approximately 60s in an argon vacuum. Sections were photographed digitally using a Hitachi S-4000 scanning electron microscope attached to a computer utilizing Spectrum Mono software.

Results. *Callus Structure* –. The labellar callus is probably not homologous throughout Orchidaceae but is apparently homologous within Sobralieae. The typical callus of most *Sobralia* species consists of two raised ridges borne opposite each other along the length of the labellum base. When seen from the distal end of the labellum, the space between the calli forms a narrow tube (Fig. 1C), which may guide the tongue of a visiting bee, channeling it to the double cuniculus (Fig. 1D-I, see later section for definition). The calli of *S. bouchei* and *S. callosa* differ from both of the previously mentioned types. They are fused and expanded to form a pad on the median portion of the base of the labellum (Figs. 2C, H-I and 3D-H).

In *Elleanthus*, the callus usually consists of two relatively large, globose masses at the base of the lip (Fig. 4D-F, 5 D-E). Exceptions include *E. caravata* and *E. robustus* in which the callus is approximately the same size, but is fused into a single structure (Fig. 4D-F). All investigated species of nectariferous Sobralieae produce nectar from large stores of starch present in the callus.

Starch –. All species contained at least some starch. However, the amount of starch and the thickness of the tissue containing the starch varied. In all of the nectar-secreting species, the pad-like or globosse callus contained abundant starch. In species that produced no nectar (*i.e.*, most species of *Sobralia*), the starch was less abundant, and often restricted to the epidermis of

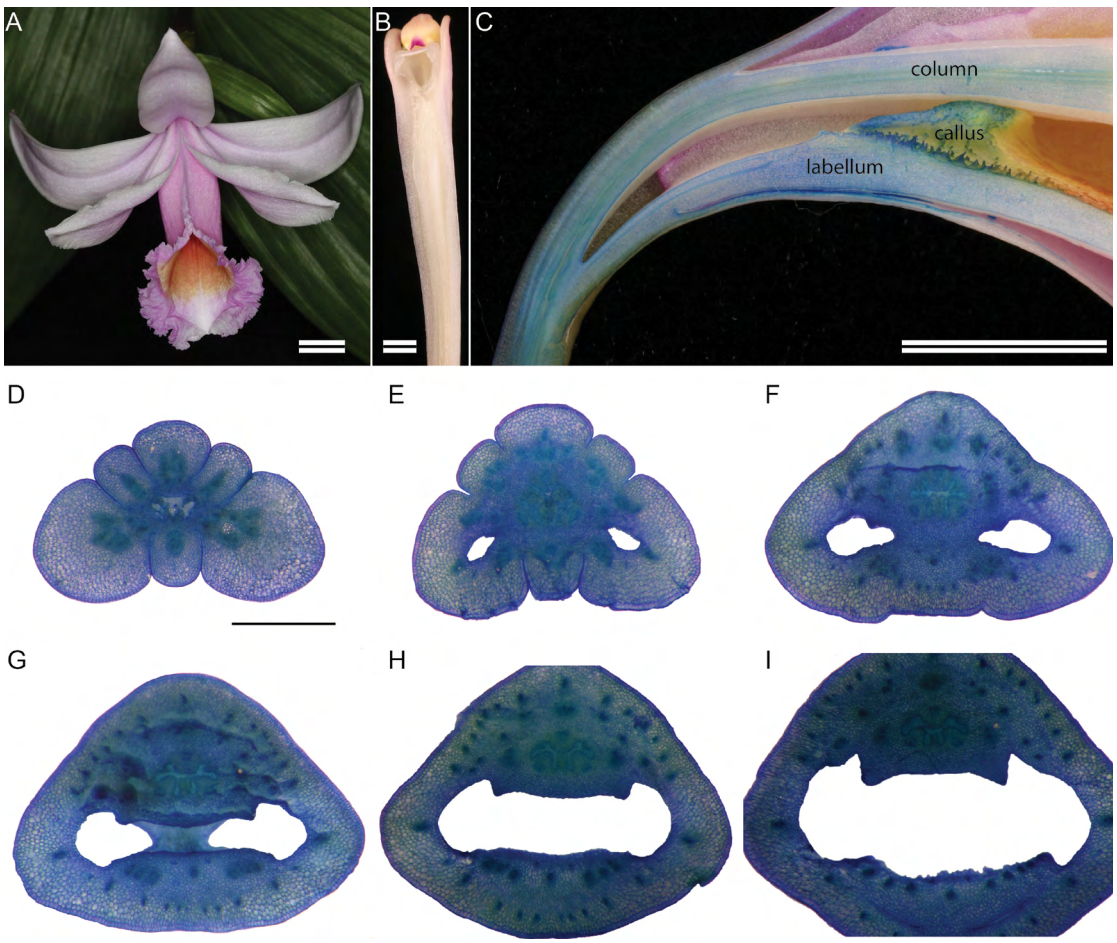

FIGURE 1. *Sobralia decora* (*Whitten 3280*) flower; a bee-pollinated flower with no nectar reward. All blue surfaces are stained with methylene blue. A. General floral morphology, scale bar = 1 cm. B. Column showing ventral surface with a common elastic rostellum which scrapes the pollinia from the scutellum as the bee exits a flower, scale bar = 3 mm. C. A longitudinal section of the flower, scale bar = 1 cm. Note the long ridged callus. D–I. Serial transverse sections of the pedicel, ovary, and perianth, scale bar = 1 mm. Note the two vacant spaces (double cuniculus) present between the sepals and the column fused to the lip; these form a pair of false nectar spurs.

the callus and epidermal trichomes. Sections of the callus made with a rotary microtome show amyloplasts that exhibited typical birefringent (cross-shaped) patterns when viewed with polarized light (Fig 5F).

Double Cuniculus –. The double cuniculus is a novel term used here to describe the paired tubes formed between the ovary and the lateral sepals. This paired, tubular, false nectary comprising a double cuniculus was found in *S. chrysostoma*, *S. decora*, *S. gloriana*, *S. macrophylla*, *S. helleri*, *S. klotzscheana*, *S. powellii*, *S. warszewiczii*, and *S.* sp. Species lacking a double cuniculus include *S. bouchei*, *S. callosa*, *S. crocea*, and *S. rosea*. No species of *Sobralia* sect. *Sobralia*, *Elleanthus*, *Epilyna,* or *Sertifera* examined has a double cuniculus.

Nectary and Nectar –. Nectar sucrose concentration and volume were measured for four species of *Sobralia* (*S. bouchei*, *S. callosa*, *S. macrophylla*, and *S. rosea*; Table 1) and four species of *Elleanthus* (*E. aurantiacus*, *E. caravata*, *E. cynarocephalus*, and *E. sodiroi*; Table 1). The following species were observed to produce nectar, but the volumes produced were

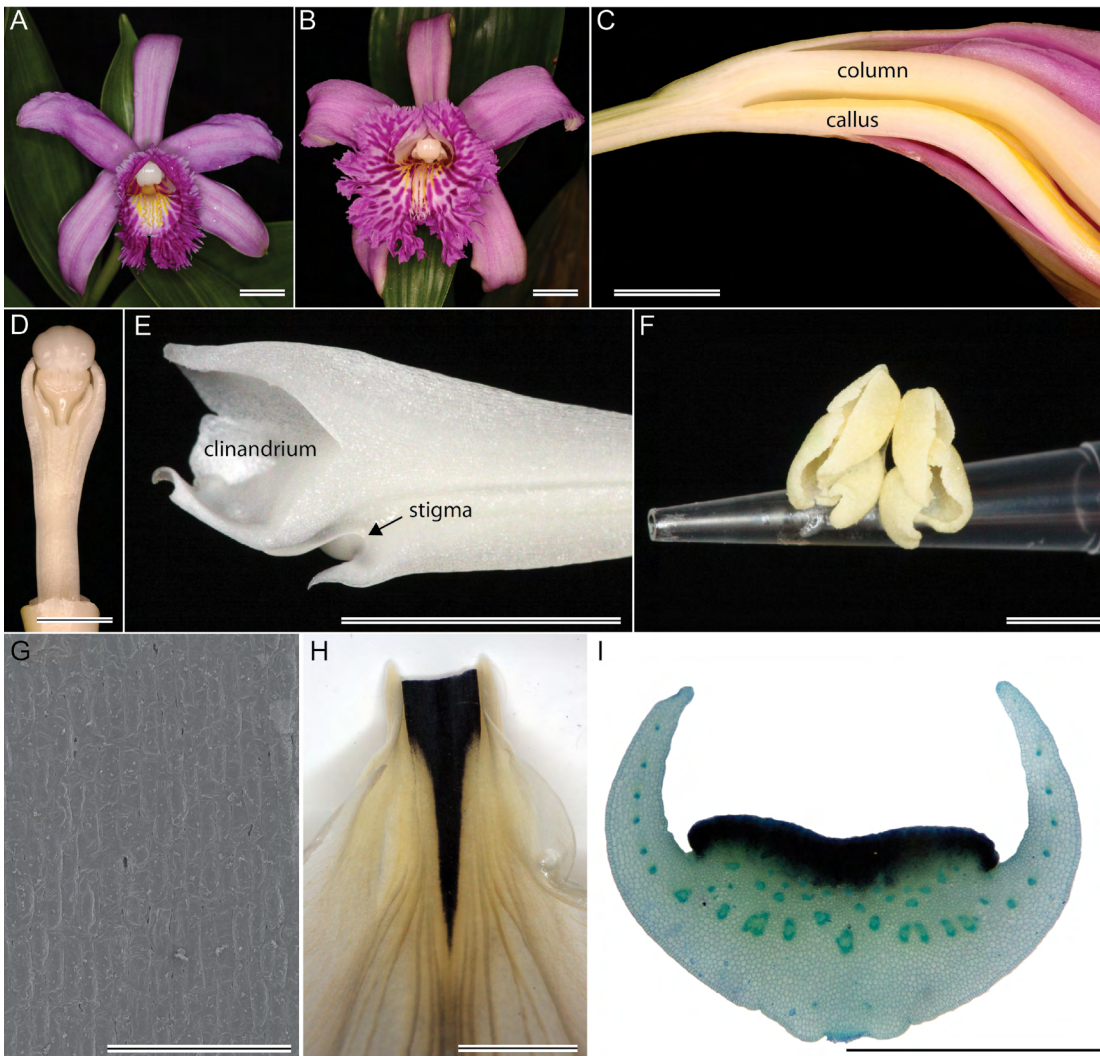

Figure 2. Flower of *Sobralia bouchei*, a bee-pollinated flower that produces nectar rewards. A. Frontal view of flower (*Blanco 3009*), scale bar = 1 cm. B. Frontal view of flower (*Neubig 208*), scale bar = 1 cm. C. A longitudinal section of the flower, scale bar = 1 cm. D. Ventral view of column showing the distinctive large anther cap and slit-like stigmatic surface differing from almost all other *Sobralia*, scale bar = 1 cm. E. The same column in longitudinal section with the anther removed, scale bar = 1 cm. F. Pollinia. G. SEM of the surface of the callus of lip, showing very different cellular surface texture compared to other *Sobralia* species, scale bar = 1 mm. Note the pores (intercellular spaces), which probably serve to increase surface area for nectar secretion. H. Basal portion of young lip, stained with I_2KI to indicate starch, precisely outlining the callus, scale bar = 1 cm. This starch is the putative carbohydrate source for nectar secretion. I. Transverse section of lip, showing the same callus with starch stained black from I_2KI while other tissues are stained with methylene blue solution, scale bar = 0.5 cm. This thick pad represents the fusion of the two distinct calli seen in most other members of the tribe.

too small to be measured: *S. ciliata*, *E. lancifolius*, *E. graminifolius*, *E. fractiflexus*, and *E. robustus*. The following species appear to lack nectar: *S. andreae*, *S. atropubescens*, *S. caloglossa*, *S. chrysostoma*, *S. citrea*, *S. crispissima*, *S. crocea*, *S. decora* (Fig. 1), *S. dichotoma*, *S. doremiliae*, *S. exigua*, *S. gloriana*, *S. helleri*, *S. kerryae*, *S. lacerata*, *S. leucoxantha*, *S. lindleyana*, *S. macrantha*, *S. mandonii*, *S. mucronata*,

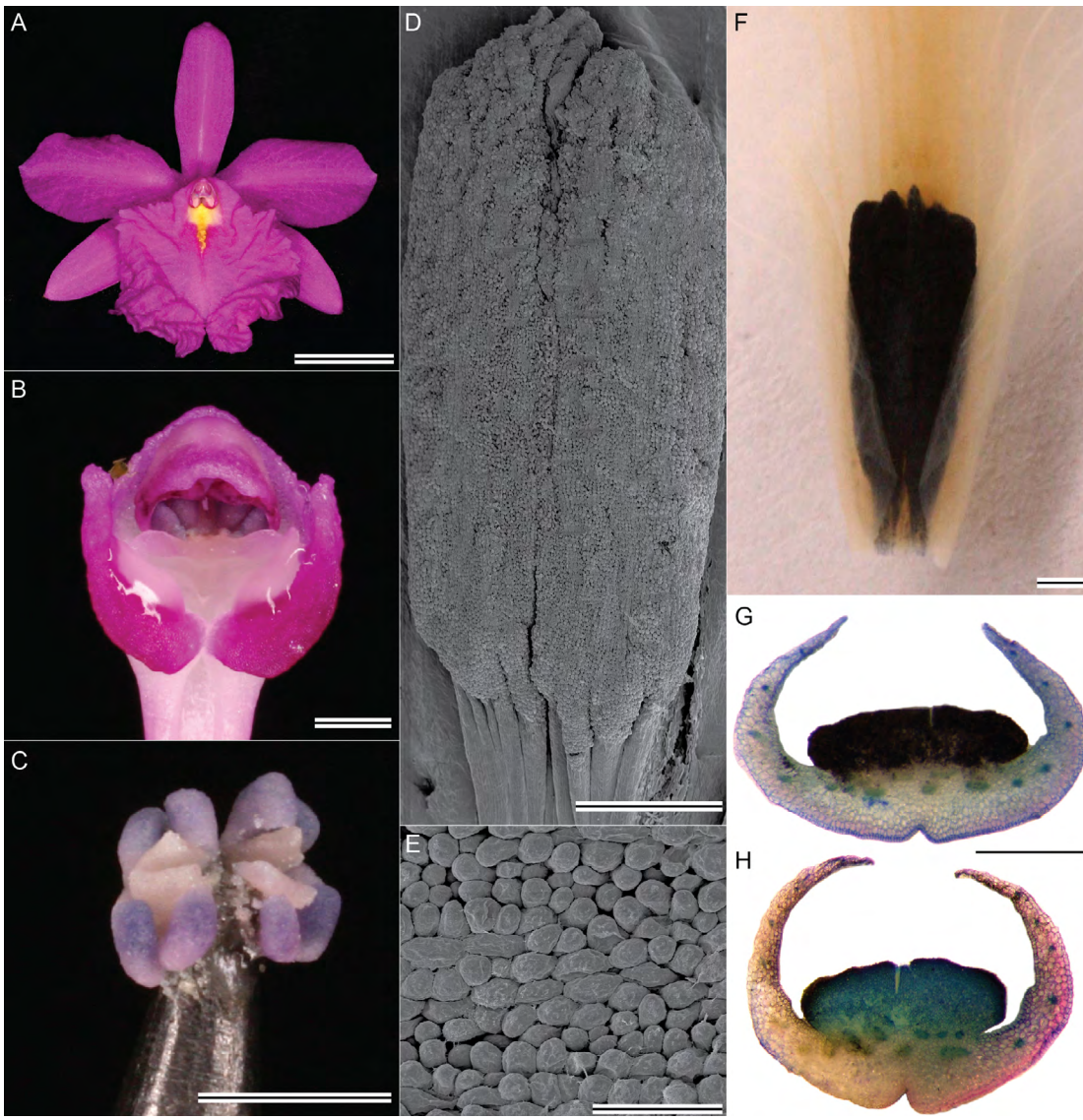

FIGURE 3. Flower of *Sobralia callosa* (*Blanco 3021*), a hummingbird-pollinated flower that produces nectar rewards. A. Frontal view of this flower, scale bar = 1 cm. B. Frontal view of the column, scale bar = 1 mm. Note the highly differentiated stigmatic orientation of anteriorly-facing surface which would require very different mechanical deposition during the pollination process; the pollinia would be scraped off during entry to the flower, and thus deposited on the stigma. C. Cryptic pollinia, scale bar = 1 mm. D. SEM of the whole callus, scale bar = 1 mm. E. Surface of the callus, showing very different cellular surface texture from *S. bouchei* (Fig. 2), scale bar = 100 µm. Note the extremely papillose surface texture which probably serves to increase surface area for nectar secretion. F. Basal portion of young lip, cleared, then stained with I_2KI to indicate starch, precisely outlining the callus, scale bar = 1 mm. G–H. Transverse sections of the lip and stained with I_2KI to reveal starch in a young flower (morning) and an old flower (evening), respectively, showing the gradual reduction in starch over time, scale bar = 1 mm.

S. quinata, *S. recta*, *S. theobromina*, *S. violacea*, *S. warszewiczii*, and *S. yauaperyensis*. The only other species of *Sobralia* reported to produce nectar, *S. amabilis*, was not investigated in this study.

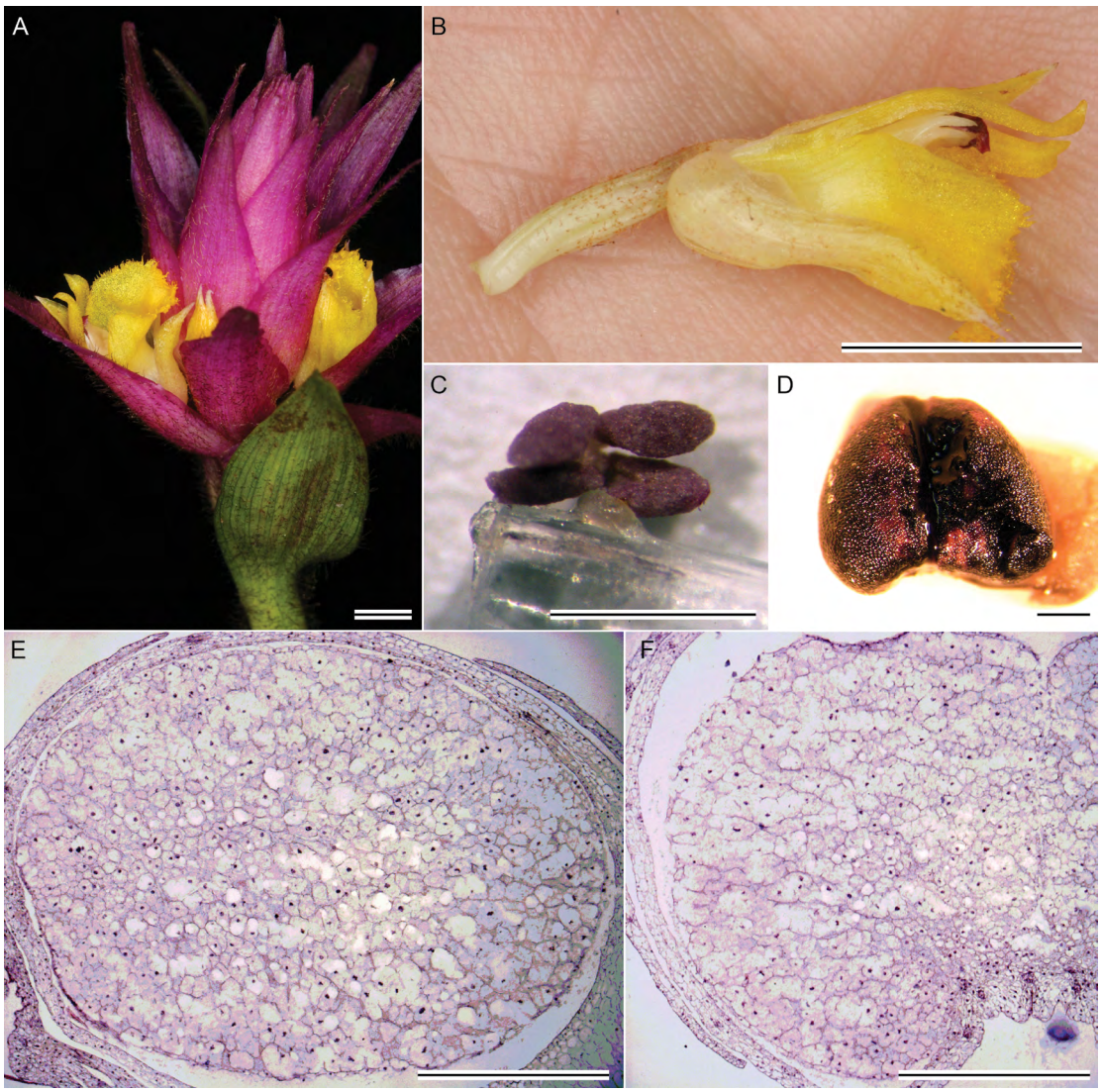

FIGURE 4. Flowers of *Elleanthus caravata* (*Neubig 202*), a hummingbird-pollinated flower that produces nectar rewards. A. Inflorescence showing the bright color contrast of bract and flower, typical of bird pollination, scale bar = 1 cm. B. Flower showing saccate base where nectar is secreted and stored, scale bar = 1 cm. C. Pollinia showing their relatively small size, dark color, and hard texture, scale bar = 1 mm. D. The callus of the lip in a young flower, stained with I_2KI indicating the presence of starch, scale bar = 1 mm. E. Longitudinal section of callus, scale bar = 1 mm. F. Transverse section of callus, scale bar = 1mm.

Discussion. *Anatomy of floral nectaries and starch* –. In orchids, nectar is produced in a variety of structures, including spurs or nectaries derived from the lip callus. The callus is a term given to any raised or sculptured portion of the lip. Although the callus is probably not homologous within Orchidaceae, the ability to produce thickened tissue on various floral parts may be an exaptation for secreting large amounts of a reward, either nectar or other compounds.

In all species of tribe Sobralieae, there are two calli at the base of the lip, but the calli vary in shape, size, and degree of fusion between species. Darwin (1862) first described the nectary structure of *Elleanthus* as large "balls", referring to the callus at the base of

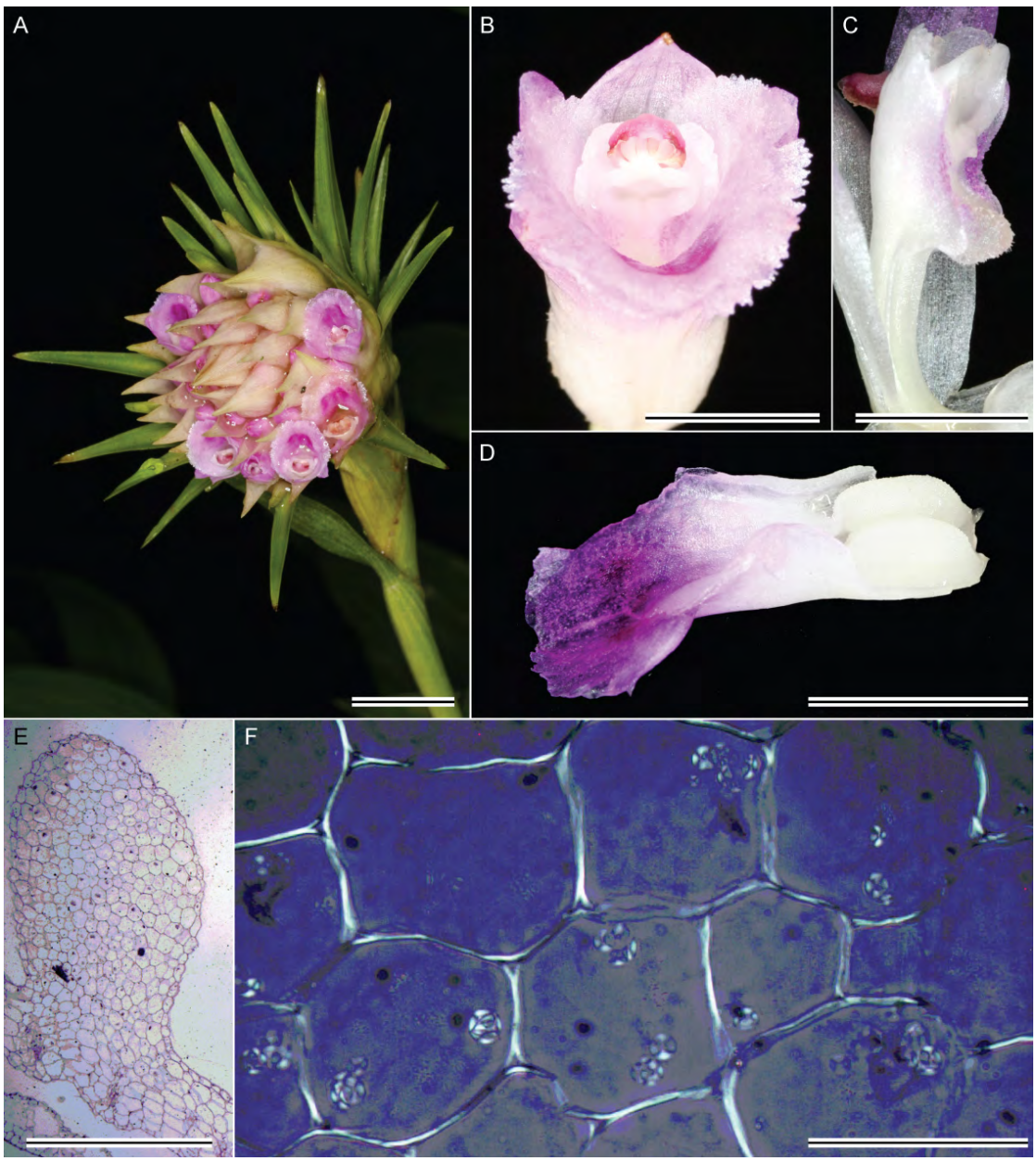

FIGURE 5. Flowers of *Elleanthus sodiroi* (*Neubig 246*) a hummingbird-pollinated flower that produces nectar rewards. A. Dense capitate inflorescence, scale bar = 1 cm. B. Frontal view of flower showing the entrance point for the pollinator, scale bar = 0.5 cm. C. Oblique view of ventral surface of the column, scale bar = 0.5 cm. Note the median ridge of the column which forms a "pocket" with the lip. D. Lip of flower showing the two spherical calli at the base, scale bar = 1 cm. E. Transverse section of one callus, scale bar = 1 mm. F. Transverse section of callus under polarized light, scale bar = 10 µm. Note the birefringent granules within each cell, indicating the presence of starch.

the labellum. Recently, the anatomy of the callus of *Elleanthus brasiliensis* (Lindl.) Rchb.f. was examined in detail (Nunes *et al*., 2013) and the callus was identified as the secretory structure. Veyret (1981), likewise, studied the floral and fruit morphology of *Elleanthus* and *Sobralia*. Veyret did not study the

nectary structure of *Elleanthus*, but did study the unusual fusion of floral parts in *Sobralia*. In that study, a novel structure, referred to as "éperon bifide," or bifid spur, was identified in *Sobralia sessilis*. Our data indicate that many other species of *Sobralia* also have this bifid spur, which we refer to here as a double cuniculus, in reference to the very similar structure found in Epidendreae, although these structures are not homologous. The double cuniculus probably functions as a pair of parallel false nectaries or pseudo-nectaries for long-tongued insect visitors (*e.g.,* nectar-foraging euglossine bees). No nectar was ever observed within the double cuniculus, nor is the anatomy consistent with metabolically active secretory cells.

Treatment of floral sections with I_2KI (Figs. 2H-I, 3F-H, & 4D), as well as visualization with polarized light (Fig. 5F), revealed the distribution of starch within floral tissues. The occurrence of starch in the callus is a constant feature among all flowers in the Sobralieae; however, the quantity of the starch is variable. Even species of *Sobralia* that do not secrete nectar will nonetheless accumulate small amounts of starch. Furthermore, the hydrolysis of floral starch might also provide the energy for fragrance production. Those species with the greatest accumulation of starch relative to flower size produce the most nectar. Nunes *et al*. (2013) did not report the presence of starch in *Elleanthus brasiliensis* calli, but these authors may have examined old flowers with exhausted starch reserves, or possibly starch was lost during tissue manipulation. Although their flowers are relatively small compared to those of *Sobralia*, the calli of *Elleanthus* are large compared with the size of their flowers (each being approximately 2-3 mm long) and densely packed with starch, at least prior to anthesis. *Sobralia* calli were more variable both in structure and in terms of starch content. *Sobralia* species that produce nectar (*e.g.*, *S. bouchei*, *S. callosa*, *S. macrophylla*, and *S. rosea*) have two calli that fuse together resulting in the formation of a thickened pad, which prior to anthesis, is densely packed with starch. This starch is no longer present later in the day (Fig. 3G-H) and thus, presumably, acts as a substrate both for nectar sugar production and as a source of metabolic energy for nectar secretion.

We conclude that the callus is the probable source of nectar in Sobralieae based on four observations: 1) In early stages of anthesis, droplets of nectar can be seen to form directly on the surface of the callus (and not on any other tissues); 2) All nectariferous species have starch-filled calli during the first stages of anthesis; by the onset of floral senescence, the starch is largely exhausted; 3) These calli have a dense cytoplasm that is consistent with cells that move nectar directly though the cell wall; 4) No pores or stomata with underlying vascular tissue (typical of phloem-fed nectaries) were observed on the epidermis of the callus.

A nectariferous callus has been reported in other orchids, but the frequency and distribution of such a structure within the family is poorly documented. A callus that secretes nectar has been demonstrated for *Maxillariella anceps* (Ames & C. Schweinf.) M.A. Blanco & Carnevali (Davies *et al*., 2005), *Stenorrhynchos* Rich. ex Spreng. (Galetto *et al*., 1997), and in some other orchid groups (Davies and Stpiczyńska, 2008a). Many orchids are known to accumulate starch for various secretory purposes relating to pollination (*e.g*., fragrance production in Stanhopeinae). Starch accumulation followed by depletion associated with nectar secretion also has been found in other orchids such as *Scaphyglottis* Poepp. & Endl. (Stpiczyńska *et al*., 2005a), *Acianthera* Scheidw. (de Melo *et al*., 2010), *Limodorum* L. (Figueiredo and Pais, 1992), *Epipactis* Zinn (Pais and Figueiredo, 1994), in multiple species in subfamily Orchidoideae (Galetto *et al*., 1997; Stpiczyńska *et al*., 2005b), and among other plant families (Durkee, 1983). Based on its ubiquity, it would appear that having a fixed reserve of starch is advantageous for the rapid production of floral secretions, whether they are fragrance (Curry *et al*., 1991) or nectar.

The ultrastructure of floral nectaries (Fahn, 1979; Vassilyev, 2010), together with the transport and secretion of nectar (Pacini and Nepi, 2007) is generally well understood. In orchids, the anatomy of structures that secrete floral rewards (including nectaries, osmophores, elaiophores, and resin-secreting structures) has only been studied recently, and for only a small number of orchid species (Davies and Stpiczyńska, 2008a; Davies and Stpiczyńska, 2008b; Davies *et al*., 2005; Stpiczyńska, 2003; Stpiczyńska *et al*., 2003, 2005a; Stpiczyńska *et al*., 2010). Based on these studies, floral secretions are produced by diverse anatomical structures. The secretion of nectar onto the surface of the flower can be achieved in two

main ways. The first is via stomata in the epidermis overlying the nectary. The second is via the cell walls of the epidermis. It is this latter method that seems to predominate in Sobralieae, because there are virtually no stomata or hairs on the nectar-secreting surface of the callus (Figs. 2G & 3D-E). The epidermis of most species that we examined (except for *S. bouchei*) were highly papillose, with no intercellular spaces to increase the surface area through which secretion could occur. In *S. bouchei*, the callus surface, as viewed using SEM, was relatively glabrous comprising brick-shaped cells with narrow intercellular spaces (Fig. 2G).

Some orchids (*Aerangis* Rchb.f. and *Platanthera* Rich.) and non-orchids (*Brassica napus* L.) have the ability to reabsorb the sugars secreted in unconsumed nectar (Burquez and Corbet, 1991; Koopowitz and Marchant, 1998; Stpiczyńska, 2003). There is no evidence to support this type of reabsorption in Sobralieae.

Nectar concentration and volume –. Nectar concentration and volume are two traits that are thought to be linked to the class of pollinator (Baker and Baker, 1983). Hummingbird-pollinated flowers are thought to produce relatively large volumes of dilute nectar, whereas bee-pollinated taxa produce comparatively smaller volumes of more concentrated nectar (Bolten and Feinsinger, 1978; Hainsworth and Wolf, 1972, 1976; Pyke and Waser, 1981).

The sugar concentrations of nectar have been studied extensively for various angiosperm groups, but not for Sobralieae. Many studies have demonstrated that there are differences between the floral nectar of flowers having different pollinators. For example, the range of sucrose concentrations for solitary bee nectar is 16-50%, whereas that for hummingbirds is 13-30% (Baker, 1975; Baker and Baker, 1983). These ranges tend to overlap by a considerable margin and the immediate difference occurs only in the upper range of concentrations for bees. The largest difference between pollinators is the relative ratio of sucrose-glucose and fructose, but again, there is considerable overlap.

It has been suggested that hummingbird-pollinated flowers "never" have high ratios of glucose and fructose (*i.e.*, their nectar contains relatively high concentrations of sucrose; Baker and Baker, 1983). A relatively high ratio of sucrose was found in a broad sampling of hummingbird-pollinated plants in Costa Rica (Stiles and Freeman, 1993). Therefore, there is a substantial degree of overlap in nectar volume and its sugar concentration relative to the type of pollinator. As hummingbird pollination is often a relatively derived condition within predominantly insect-pollinated groups (Beardsley *et al*., 2003; Kay *et al*., 2005), it is reasonable to assume that hummingbirds select for a specific type of nectar. More recently, the hypothesis of nectar preferences in hummingbirds has been challenged by more recent studies (Johnson and Nicolson, 2007). Similar trends in sugar ratios, as they relate to pollinators, have also been reported for *Ipomoea* (Galetto and Bernardello, 2004), as well as in other plant groups (Burke *et al*., 2000; Galetto *et al*., 1998), however, these studies found no significant differences in nectar composition between plants having different pollinators. Other surveys involving many unrelated plants have shown variable nectar concentrations for hummingbird-pollinated taxa (McDade and Weeks, 2004). Similarly, our observations show that sucrose concentration in Sobralieae is highly variable and were not related to pollination syndrome (Table 1; Figs. 6). We did not analyze the sugar composition of nectar nor the ratios of the individual sugars. Nevertheless, our data revealed differences in nectar volume between pollinator classes. Many of the hummingbird-pollinated species produced smaller volumes (perhaps because each inflorescence bears numerous small flowers), generally approximately 6 µL per flower (except for *E. sodiroi*, which produced as much as 32 µL nectar per flower). Bee-pollinated flowers, such as those of *S. bouchei* and *S. rosea*, produced more nectar, an average yield of 8.4-14.1 µL per flower. Conversely, *S. macrophylla* produced very little nectar, and although we examined approximately 50 flowers from several different plants (n=6), only rarely was nectar observed.

The majority of documented pollinators of *Sobralia* are nectar-foraging euglossine bees (Apidae: Euglossini). The nectar viscosity of some euglossine bee-pollinated plants other than orchids has been studied relative to the length of the proboscis of the pollinating bee (Borrell 2005, 2006). Borrell (2007) also measured sugar concentrations from euglossine

bee crops and from various euglossine nectar sources and found that orchid bees harvest nectars with 34%-42% sucrose, independent of body size. Borrell (2006) speculated that long nectar spurs may be a mechanism by which flowers conserve nectar while remaining attractive to traplining bee visitors. Our analyses of *Sobralia* nectar produced lower values than those conducted by Borrell.

Bee-pollinated species of Sobralieae produced relatively low-viscosity nectar (*i.e.*, *S. bouchei*, *S. macrophylla*, and *S. rosea*), whereas species of *Sobralia* having deceit strategies produced no observable nectar. A larger sampling of *Sobralia* species that are bee-pollinated, yet produce nectar, would be difficult, since so few species of the genus produce rewards. Nevertheless, it is likely that at least a few, hitherto unexamined species, produce nectar, and Romero (1998) has reported the occurrence of pseudopollen in *S. liliastrum* Lindl., suggesting that both mimicry and rewards other than nectar may occur in certain species of this genus.

Nectar Deceit –. Many orchids have "gullet flowers" that produce no nectar, *e.g.*, *Cattleya* (Dressler, 1981) and *Cochleanthes* (Ackerman, 1983). *Sobralia*, like many food-deceit orchids, probably takes advantage of a general floral bauplan that is attractive to a wide variety of pollinators. This is termed generalized food deception (Jersáková *et al*., 2006), and the mechanism is apparently frequent and sometimes referred to as pollinator naiveté (Ackerman, 1986). Most *Sobralia* species exhibit generalized food deception. Food deception based on generalized foraging behavior has been demonstrated for many orchids (see Jersáková, Johnson, & Kindlmann (2006) for a detailed list of such groups) and most *Sobralia* species exhibit this strategy. Narrow pollinator specificity also exists in many orchids (Schiestl and Schluter, 2009), but is not known for any pollination system found in Sobralieae.

Whether pollination is achieved by rewards or deceit, floral structural adaptation is necessary for effective pollination. Orchids have a plethora of structures for presenting nectar to pollinators, especially long-tongued insects. Some members of tribe Vandeae (especially *Angraecum*) have long tubular spurs (formed from an invagination of the lip) that are associated with hawk moth pollination (van der Cingel, 2001). In some orchids, a cuniculus is formed by the fusion of a hypanthium-like structure, as in the Laeliinae (*e.g.*, *Brassavola* R.Br.), and forms a single tube serving much the same function as the spurs in *Angraecum* (Stpiczyńska *et al*., 2010). In several genera of Zygopetalinae, a gap at the base of the lip leads into a rolled, tubular backswept sepal that forms a false spur (Ackerman, 1983). Even though these structures may not be homologous, they all have a similar function, namely to facilitate pollination, either by deceit or through the production of a legitimate rewards. Most species of *Sobralia* deceive the pollinator in that they have a ridged callus that forms a tube that serves as a funnel and guides the proboscis of the pollinator deep into the "double cuniculus" embedded within the ovary (Fig. 1).

The double cuniculus is unusual among orchids and is found only in part of the core group of *Sobralia* (Neubig, 2012; Neubig *et al*., 2011). It comprises an open channel that runs between the lateral sepals and the ovary and can extend up to several centimeters into the latter (Fig. 1). This is perhaps the most significant feature of the double cuniculus. All the flowers having a double cuniculus that we examined offered no nectar reward, neither at the callus, as is typical of other Sobralieae, nor within this cunicular region, deep inside the ovary. Because *Sobralia* usually has a typical gullet-shaped flower (zygomorphic, with a tubular lip and nectar guides), and because it produces no nectar, this double cuniculus is interpreted as being a pair of false nectaries. This probably contributes to the effectiveness of the deceit, especially in the case of long-tongued bees, and in particular, Apidae (Danforth *et al*., 2006). This interpretation is supported by the fact that the width of the individual tubes of the double cuniculus exceeds the width of the proboscis of known bee pollinators (*e.g.*, euglossines). We speculate that the deep double cuniculus induces the bee to probe further into the throat of the flower, thereby increasing the likelihood of effective pollination (Nilsson, 1988). Because long-tongued euglossine bees are the most commonly observed pollinators of *Sobralia*, this length-mediated deceit probably contributes significantly to pollinator selection.

Based on the fact that euglossines have the longest proboscises of any Neotropical bee subtribe, we hypothesize that any *Sobralia* species that possesses

cunicular tubes that penetrate deeply into the ovary is likely to be pollinated by nectar-foraging euglossine bees (male or female). Even nectariferous species, such as *Sobralia rosea,* have very large flowers with a particularly long, tubular throat (~5 cm), at the base of which occurs a true nectary favoring pollination by long-tongued bees.

Future directions –. Detailed observations of floral morphology, anatomy, and secretions cannot substitute for careful field studies of pollination biology, but they may contribute to a hypothesis that can inform and prioritize fieldwork. The most glaring gaps in our knowledge relate to plant-pollinator relationships at the species level, especially for the white-flowered species of *Elleanthus* sect. *Elleanthus*, sect. *Chloidelyna*, and *Epilyna*. Verification of hummingbird pollination in other taxa, such as *Sertifera*, *Sobralia ciliata*, *S. callosa*, and *S. crocea*, is also critical for accurate interpretation of the number of modifications to this derived pollination syndrome.

The pollinators of the small, white-flowered species of *Elleanthus* and *Epilyna* are still not known. These species include *E. lancifolius* (sect. *Elleanthus*), all of sect. *Chloidelyna* (*e.g.*, *E. fractiflexus*, *E. graminifolius*, *E. linifolius*, *E. poiformis*, and *E. stolonifer*), *E. caricoides*, and all of *Epilyna*. These flowers are even smaller than those of typical hummingbird-pollinated species, and have no bright colors, and therefore, it is highly unlikely that they attract or can be pollinated effectively by hummingbirds. These species have yellow pollinia and very small quantities of nectar (<1 μL), and it has been speculated that they are pollinated by small, nectar-seeking moths, such as Noctuidae (C. Dodson, pers. comm.).

Most intriguing is the species *S. rarae-avis* (and the putatively closely related *S. madisonii* and *S. infundibuligera*, neither of which were examined morphologically in this study); their nocturnal fragrance is suggestive of pollination by hawkmoths or crepuscular bees, pollinator classes hitherto unknown for Sobralieae. The advent of increasingly cheap and portable digital video cameras should prove useful in documenting visits by pollinators.

By elucidating a greater number of specific plant-pollinator interactions for selected clades of Sobralieae, a more fine-tuned appreciation of the evolution of pollination-related floral features might be obtained, and recent molecular phylogenetic studies can be used to provide the evolutionary context for mapping such features (Neubig, 2012; Neubig *et al*., 2011).

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POLLINATION ECOLOGY OF *RODRIGUEZIA GRANADENSIS* (ORCHIDACEAE)

NHORA H. OSPINA-CALDERÓN[1,2,7], CAROL A. DUQUE-BUITRAGO[3], RAYMOND L. TREMBLAY[4] & J. TUPAC OTERO[5,6]

[1]Departamento de Biología, Maestría en Biología-Ecología, Universidad Nacional de Colombia, Bogotá, Colombia

[2]Departamento de Ingeniería, Carrera de Biología, Pontificia Universidad Javeriana Cali, Colombia

[3]Programa de Biología. Universidad de Caldas, Manizales, Colombia

[4]Center for Applied Tropical Ecology and Conservation , PO Box 23360, University of Puerto Rico, Río Piedras, Puerto Rico, USA

[5]Departamento de Ciencias Biológicas, Universidad Nacional de Colombia, Palmira, Colombia

[6]Instituto de Estudios Ambientales IDEA, Palmira. Universidad Nacional de Colombia, Palmira, Colombia

[7]Author for correspondence: nhora_helena@yahoo.com

ABSTRACT. In this paper we describe the phenotypic variation and pollination ecology of the twig orchid epiphyte *Rodriguezia granadensis*. The species presents flower color polymorphism (pink to white), suggesting that different color forms might be pollinated by different pollinators. To evaluate this hypothesis, one hundred plants were monitored in the field and their flowering phenology and color polymorphism was noted, two peaks of flowering were noted over the year. We evaluated the reproductive success (pollinaria removal and fruit set) and the visit of potential pollinators to both morphs. Fruit production by autogamy, geitonogamy, xenogamy, and emasculation were compared. Sugar concentration in the nectar was measured with a refractometer. Potential pollinators, euglossini bees, were attracted using methyl salicylate and eugenol. We evidenced that *R. granadensis* is pollinated by nectar-foraging euglossine bees. The fluctuation in nectar production and the scarce reproductive success among individuals suggests that the orchids may employ an attraction-deceit system as a self-mimetic or a diffuse rewarding phenomenon.

RESUMEN. En éste artículo describimos la variación fenotípica y ecología de la polinización de la epífita de ramita *Rodriguezia granadensis*. La especie presenta polimorfismo para el color de las flores (blanco a rosa), lo que lo llevó a hipotetizar que las diferentes formas de color pueden ser polinizadas por diferentes polinizadores. Para evaluar esta hipótesis, se monitorearon cien plantas en campo y se anotó su fenología de floración y polimorfismo en color. Dos picos de floración se presentan en el año. Evaluamos el éxito reproductivo (*fitness* masculino y femenino) y la visita de los polinizadores potenciales en ambos morfos. Se puso a prueba la producción de frutos por autogamia, geitonogamia, xenogamia y emasculación. La producción de néctar se midió con un refractómetro. Adicionalmente, usamos trampas de fragancia con salicilato de metilo y eugenol para atraer polinizadores potenciales (abejas euglosinas). Presentamos evidencia de la polinización de *R. granadensis* por abejas euglosinas que buscan néctar. La fluctuación en la producción de néctar y el escaso éxito reproductivo entre los individuos sugiere que la orquídea puede emplear un sistema de atracción/engaño como auto-mimetismo o un fenómeno de recompensas difusas.

KEY WORDS: self-mimetism, floral baits, deceit pollination, Euglossine bee, melitophily, reproductive success

Introduction. Floral characteristics likely arose as a consequence of natural selection and therefore are thought to be adaptive. Within species and populations, floral phenotypic traits can vary and are often perceived to vary as a consequence of pollinator-mediated selection (Ackerman 1986a, Gravendeel *et al*. 2004, Tremblay *et al*. 2005, Cuartas-Domínguez & Medel 2010). Several studies have shown that pollinators

can influence floral characters through selection on floral morphology (e.g. Medel *et al*. 2009), including orchids (Jersáková & Kindlmann 2004, Tremblay *et al*. 2005). Comprehension of the pollinator interaction with floral characters and their evolutionary potential is key in understanding the origin and evolution of Angiosperms. Across the Angiosperms, certain floral characteristics are associated with particular pollinator syndromes (van der Pijl 1961). Melittophily or pollination by bees is a pollination syndrome characterized by zygomorphic flowers with contrasting colors such as yellow, white or purple, the color red is generally absent. Bee pollinated flowers present diurnal anthesis, fragrance, and nectar, although this reward is often scarce compared to other pollination syndromes (van der Pijl 1961).

It is estimated that ten percent of the orchid species, with distribution in the neotropical regions, are pollinated by euglossine bees (Roubik & Hanson 2004). These so-called orchid-bees, are corbiculate bees of the Euglossini tribe. Both, male and females visit and pollinate a diversity of species in search of nectar, pollen, fragrances, and resins (Roubik & Hanson 2004). Male euglossine bees are known to visit orchids to obtain fragrance rewards (androeuglossophily), but they may also seek nectar from orchids and other flowers (Roubik & Hanson 2004). When either male or female euglossine bees visit flowers to search for nectar, they extend their proboscis and introduce it in the nectar cavity of the flower. The flowers with this pollination mechanisms are classified as melittophily (Roubik & Hanson 2004).

Intra-specific variation is frequent in orchid flowers (van der Pijl & Dodson 1966). *Oncidium abortivum* Rchb.f. has heteromorphic flowers, including both functional and non-functional flowers in the same inflorescence (van der Pijl & Dodson 1966, Garay 1970). Male and female flowers of *Catasetum* sp. and *Cycnoches* sp. are very different in form, size and color, sometimes having distinct male and female flowers (Romero 1991).

High morphological variation is associated with deceptive pollination systems (Ackerman & Galarza-Pérez 1991, Sabat & Ackerman 1996, Ackerman & Carromero 2005, Ackerman *et al*. 2011). Non-rewarding orchids rely on visits by naïve pollinators who are fooled due to the variation presented among individuals within the same populations (*Psychilis monensis* Sauleda; Aragon & Ackerman 2003), and form and fragrances (*Tolumnia variegata* (Sw.) Braem; Ackerman & Galarza-Pérez 1991). This intra-specific variation is the raw material for natural selection (Endler 1986, Tremblay *et al*. 2010). Without variation (at the genetic and phenotypic level) evolution cannot proceed.

We studied the pollination ecology of the neotropical epiphytic orchid *Rodriguezia granadensis* (Lindl.) Rchb.f. and how variation in floral traits might affect pollinator attraction. Previous studies of morphological traits (Ortiz *et al*. 1991) suggested and hypothesized that pink forms of *R. granadensis* should be pollinated by hummingbird while bees would pollinate the white forms. To test this hypothesis we surveyed a population of this species of orchids in their natural habitat over three flowering periods. We monitored the visit of potential pollinators, nectar production and reproductive success (pollinaria removal and fruit set) of the white and pink forms of the orchids.

Materials and Methods. *Species and study site –*. *Rodriguezia granadensis* is a twig epiphyte orchid distributed from Panama to Peru. In Colombia, it occurs on the three main mountain chains between 700-1900 m of altitude. It is common on cultivated fruit trees and blooms from March to September in sub-Andean forests (Ortiz *et al*. 1991, Calderón-Sáenz 2007). The species is polymorphic in flower color, with white and pink flowers; has a spur that originates from the labellum tissue that surrounds the lateral sepals, which often produce nectar (Ortiz *et al*. 1994).

The interactions between *R. granadensis* and its pollinators, within the Yotoco Nature Forest Reserve (YNFR) and surrounding protected buffer zone (3º52’51.56’’N, 76º25’53.53’’W), in the Yotoco district (Valle del Cauca, Colombia), were studied. The Reserve in Cauca Valley (559 ha) represents one of the lasts remnants of the subtropical transitional forest (1200-1950 m elevation) in the eastern section of the western mountain range of the Andes (Escobar 2001).

Individuals of *R. granadensis* at YNFR are 12-15 cm in height, and form small modular colonies of one to 20 pseudobulbs ($\bar{x}$= 5, SE= 0.45, N=100) in a

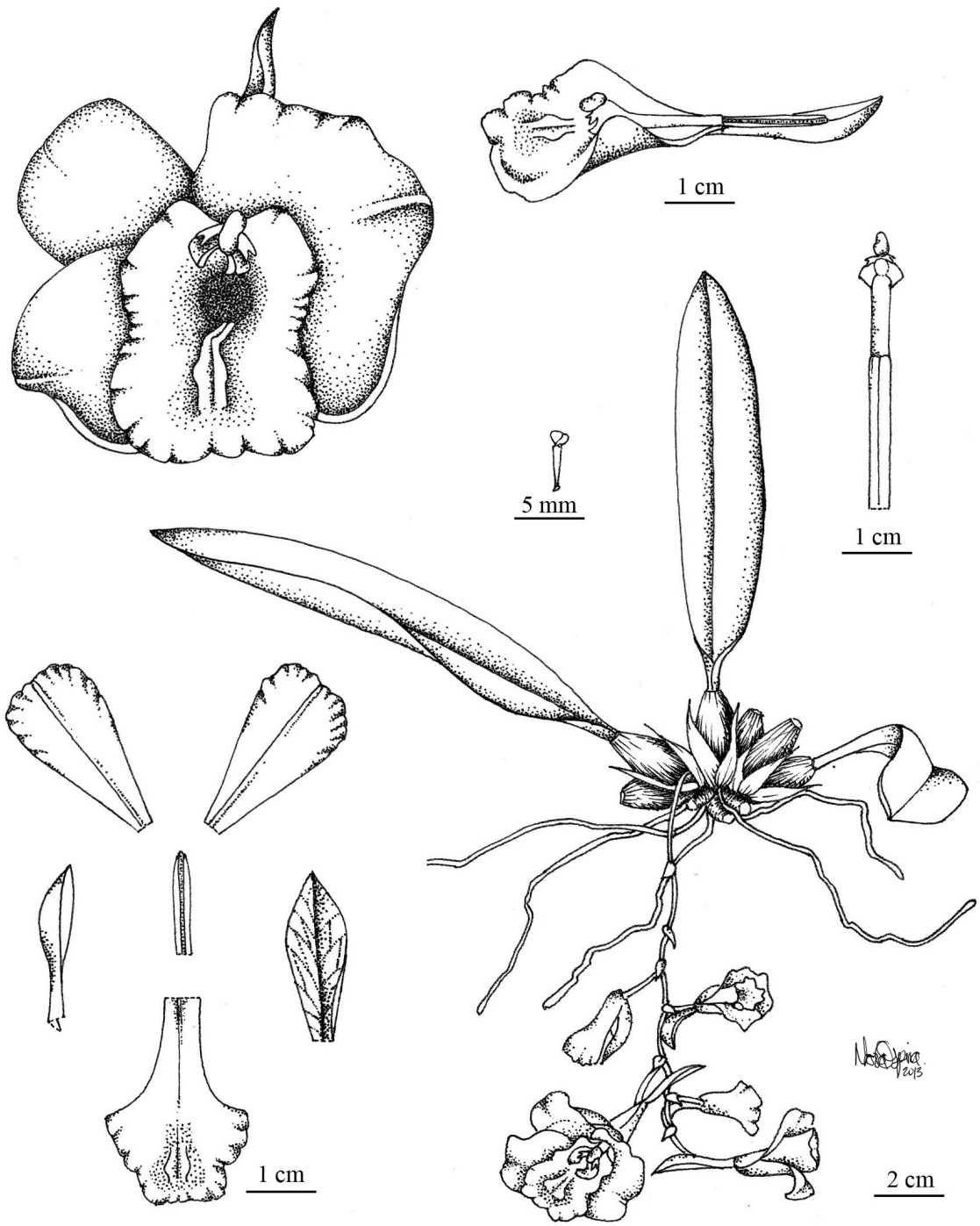

FIGURE 1. *Rodriguezia granadensis* plant and flower.

phalanx growing strategy (Gibson 2002). The plants have one or two leaves on each pseudobulb, lateral pendulous inflorescences, usually with one or two inflorescences on each pseudobulb, with an average of four flowers on each inflorescence.

The flowers have a bilobed labellum, callus with

FIGURE 2. Photo of A. the flower and B. the habit of *R. granadensis*.

two or three lamellas, and a synsepalous spur with nectar. There is continuous variation among plants in flower color (pink to white), and flowers usually have a delicate sweet pleasant perfume (Fig. 1, 2).

Reproductive phenology and success –. During 2008 –2009, 100 plants of *R. granadensis* were surveyed in order to describe the reproductive phenology and success. Surveys were performed monthly prior to the flowering period and weekly once flowering had initiated. Information was collected on the life stages of the plant, (flowering or not, number of flowers and fruit). Indirect evidence of reproductive potential was evaluated in open flowers as pollinaria removal for male fitness and stigma state (open or closed) for female fitness.

Flower color variation –. To evaluate floral coloration variation, digital photographs were taken of 50 flowers, 38 pink and 12 white (from 32 plants, 25 pink and 7 white), from the living orchid collection at YNFR, with *in situ* white balance. Four zones of the flower were chosen for the colorimetric analysis (Fig. 3) in order to characterize the different tonalities of the color in the flowers. A colorimetric analysis of the different parts of the orchid flower (zones) was performed using ACA System (2008). The color composition of each zone in the photographed flowers was standardized using the video methodology measuring base on the amount of red, green, and blue (RGB) and the printing methodology using cyan, magenta, yellow and key (CMYK) (Galer & Horvat 2003).

Floral morphology –. The variation of the species was evaluated using basic morphometric analysis (Dafni 1992) by measuring all flowers of the first season in 209, 42 flowers from 15 plants. The number of plants that flowered in the population was limited. The characters were measured in the field with a caliper and included floral width wingspread (frontal view), the length of the spur, and the length of the column. To compare the wingspread and the length of the spur between the color morphs (pink and white) a Mann-Whitney U test was performed because data did not have a normal distribution according to a Shapiro-Wilk test.

The nectar production was measured from 15 flowers (8 white, 7 pink) bagged before anthesis (March 2009, 1100-1400 hr) with a 1.15 mm diameter capillary. The sugar concentration was measured using a portable refractometer (Reichert BRIX 35HP). The concentration was measured as the weight of sugar/ weight of solution (ºBx), that represents the percent sugar in the aliquot (Dafni 1992).

Pollination –. Ten plants were surveyed continuously for 15 days between 0900-1600 hours (more than

100 hours in 2008) during the flowering peak; a photographic and video record was made of the visitors and pollinators landing on the flowers. We recorded the number of flowers visitors per plant, pollinators' behavior, pollinaria removal, and pollinaria deposition.

Euglossine baiting traps –. Euglossine bees were captured with fragrance traps (Vélez & Pulido-Barrios 2005). Three odor baiting traps were set up in the field with the following attractants, methyl salicylate, eugenol, and a mixture of both in the equal proportions (Otero & Sallenave 2003, Otero & Sandino 2003). The traps were located near the orchid population during flowering time (March to April 2010) and were checked (daily) during the day between 0800-1400 hours for 25 days. Captured bees with pollinaria were identified with the Bonilla-Gómez & Nates-Parra (1992) and Roubik & Hanson (2004) keys, and were deposited at the Entomological Collection Biology Program at the University of Caldas (CEBUC), Manizales, Colombia. To evaluate whether the pollinaria came from *R. granadensis*, their structure and placement on the bees were analyzed (Dressler 1976, Roubik & Hanson 2004).

Reproductive system –. During (March to April) 2009, the reproductive biology of *R. granadensis* was evaluated using 30 additional cultivated plants with a total of 40 flowers. Four treatments of manual pollination (autogamy, geitonogamy, xenogamy, and emasculation) were assigned randomly across flowers, and 10 flowers were assigned to each treatment (Dafni 1992). To test for autogamous pollination, flowers were self-pollinated using pollen from the same flower and bagged; to test for geitonogamous pollination, pollen from the same plant but from different flowers were used; to test for xenogamous pollination, pollen came from a different plant; and to test for apomixis, the emasculation treatment, pollen was removed from the flower and bagged without pollination. Treatments of self-fertilization were not performed because previous research has shown that plants with bagged flowers without pollination did not produced fruits (Ospina-Calderón 2009).

Results. *Rodriguezia granadensis* formed small clusters from one to more than 20 pseudobulbs ($\bar{x}$ =5, sd=5, n=100 plants). The plants have one leaf per pseudobulb, with pendant lateral inflorescences, usually one or two inflorescences per pseudobulb, with an average of five flowers per inflorescence (sd = 1.54, n = 42).

Color variation –. Flower color varied among plants from white to pink, but over six flowering seasons, no color variation was seen within an individual. Flower color did not change with the age of the flower or the result of pollination.

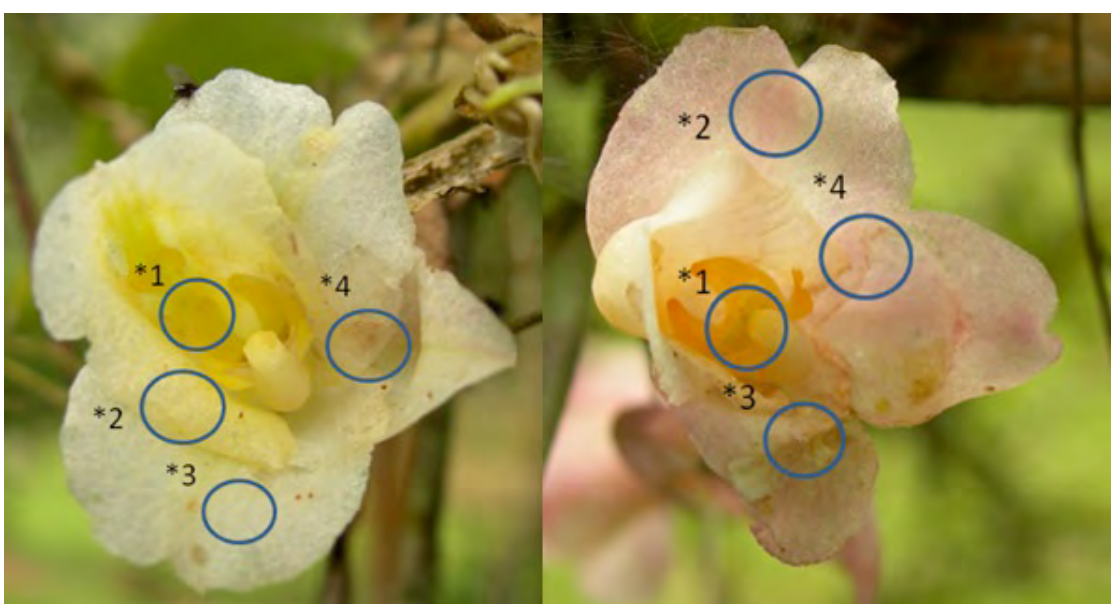

Figure 3. Flowers of *R. granadensis*, analysis of zones, with delimitation of zones for colorimetic analysis.

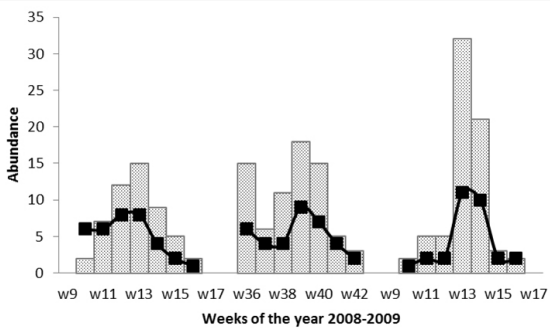

FIGURE 4. Reproductive phenology of *R. granadensis* by weeks in the Yotoco Nature Forest Reserve 2008-2009, Number of open flowers (bar), number of flowering plants (line).

Reproductive phenology and reproductive success –. *Rodriguezia granadensis* in the YNFR bloomed synchronously, twice a year, the first period starting at week ten (March-April) and the other starting at week 36 (September-October). In March 2008 eight of the 100 plants studied produced 15 flowers; in September nine plants produced 19 flowers. During March 2009 15 plants produced 42 flowers (Fig. 4). In the March 2009 flowering, the average number of flowers per plant was 3.6 (Fig. 4). Two plants flowered in both 2008 and 2009. Each flower remained open for seven days on average (sd = 0.27, n = 42) and the population had open flowers during over a period of seven weeks. The flowering peak was the third week after the beginning of blooming, and the fruit set 11.3% (sd=1.98, n=3).

The observations for male and female fitness in the population were the following: in March 2008, five flowers donated pollen and two of them received it; on September 2008 five flowers donated pollen and, two received; and during March 2009, six flowers donated with three flowers receiving pollen (with closed stigmas). There were no significant differences among flower color morphs on reproductive success (Mann Whitney, W=2.00, p=0.772).

Floral morphology –. The average flower wingspread was 24.5 mm (sd = 6.79, n= 42), and spur length was 26.5 mm (sd = 3.17, n = 42). There were no significant difference among floral color morphs in wingspread (Mann Whitney, W=260.5 p=0.12), or spur length (Mann Whitney, W=192 p=0.79).

The mean nectar volume per plant was 1.96 µl (sd = 2.96, n= 14) and mean sugar concentration was 31.32º Brix (mass percentage of sucrose) (sd = 2.87, n= 5). Not all bagged flowers had nectar, and only 15 of 42 (36%) wild flowers contained nectar. No significant differences was noted in nectar volume among flower color morphs (Mann Whitney, W=149.5, p=0.10). Additionally, a pleasant smell was detected in the all flowers. The spur was formed by two channels, which produce sugar secretions into a cavity formed by the lateral fused sepals (Fig. 5).

Pollination –. Observations of pollinators visiting flowers of *R. granadensis* were scarce. The behavior of the insects in the vicinity of nine plants and 19 flowers over more than 105 hours (seven hours a day, for 15 days) was recorded and *Eulaema meriana* Olivier was seen visiting two pink flowers of the same plant. The bee was observed landing on the labellum of the flower with the proboscis extended in a visit of five to ten seconds, looking for nectar, removing the pollinaria, that stick to the labrum of the bee and depositing those carried previously. Both flowers visited produced a fruit (Fig. 6a, b). These two fruits were later damaged by herbivores.

Baiting traps –. A total of 11 male euglossinae bees were captured using the odor baiting traps: with a mix of eugenol and methyl salicylate one *Exaerete smaragdina* Guerin-Meneville, and one *Eulaema cingulata* Fabricius were collected; with only methyl salicylate, five *Eulaema meriana* were captured (Fig. 6c); with only eugenol an additional four *Eulaema cingulata* were captured. From the 11 collected bees, 5 had *R. granadensis* pollinaria attached to the labrum. The one individual of *Exaerete smaracdina* had four pollinaria, two of them with stipe and pollinia and two stipes without pollinia. One *El. meriana* had two stipes and another had a complete pollinarium. Two *El. cingulata* had each one complete pollinarium.

Reproductive system –. *Rodriguezia granadensis* is self-incompatible with obligated xenogamy as none of the self-pollination and geitonogamy treatments produced any fruits. Nevertheless, the self-pollination induced swollen reaction and closure of the stigma showing that the self-incompatibility is gametophytic rather than sporophytic. The emasculation treatment did not produce any fruits, suggesting that there is no apomixis in *R. granadensis*. The xenogamy treatment had 100% fruit production.

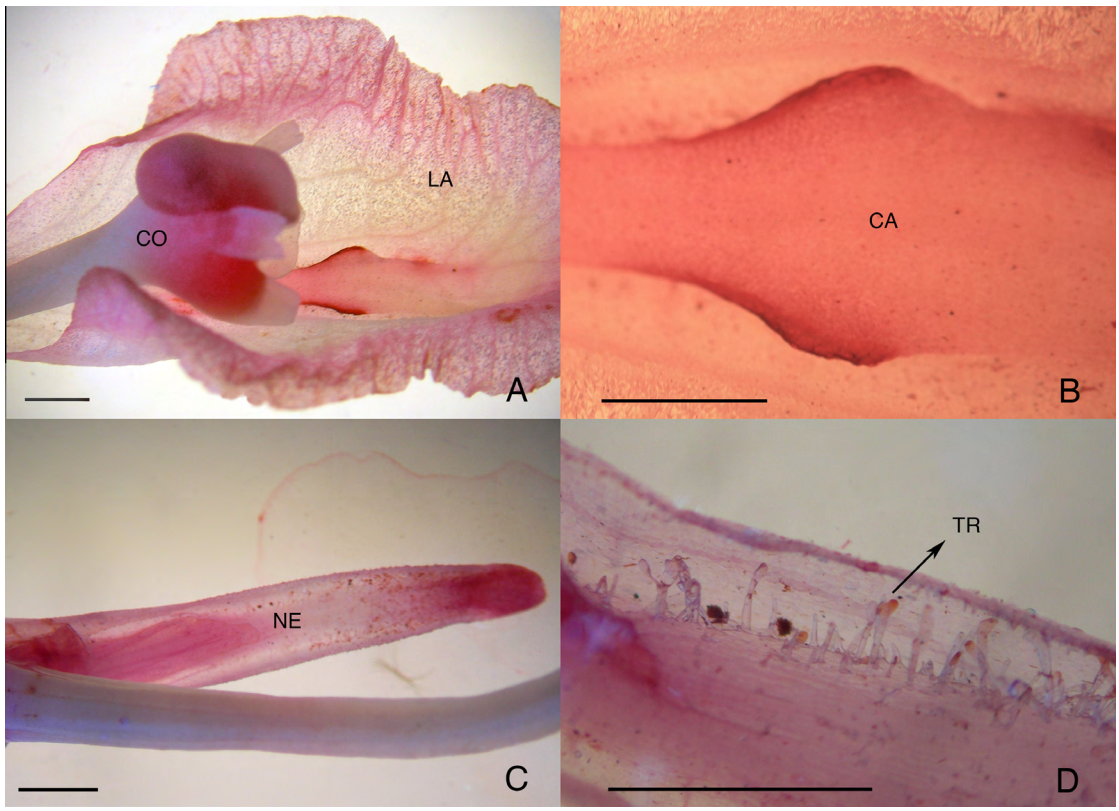

Figure 5. A floral morphology of *R. granadensis* with neutral red dye, A. General view, column and labellum. B. Close-up of the callus of the labellum, colored laminas; C. Nectary after removing the synsepal; D. Nectary detail column tissue enveloped in the labellum tissue, partially colored trichomas. Escale bar= 1mm; CO= column; LA= labellum; CA= callus of the labellum; NE= nectary; TR= trichomes.

Colorimetry –. The studied population had 15 white and 27 pink flowers; the colors were consistent within an individual and did not change with the maturity of the flowers or the reproductive state. According to the RGB analysis, there are no differences between the color phenotypes among the plants in the population (Fig. 7), with similar values of reflection for each zone and color (Table 1). However, in the color space of CMYK there was a difference in the quantity of magenta absorbed in the region 2 (Fig. 7) analyzed from the different color morphs of *R. granadensis*. Intermediate morphs were also observed in nature in the same population and in other sites.

Discussion. *Rodriguezia granadensis* plants flowered synchronously twice a year at the YNFR (Fig. 4) in a massive blooming strategy (Dafni 1992, Gentry 1978). It coincides with the peak of precipitation at YNFR during the first week of April and October (CVC 2006). This synchrony between the floral phenology of the orchids and the local precipitation may be a consequence of the plant physiology and the abundance of resources of the time (Zimmerman *et al*. 1989). Epiphytic environments are very dry as little water is stored after rains, suggesting that water could be a limiting resource for epiphytic plants (Zotz & Hietz 2001). Ample evidence suggests that, most tropical epiphytic species synchronize their reproduction with rain patterns (Ospina-Calderón *et al*. 2007, Sahagún-Godinez 1996, Zimmerman *et al*. 1989)

Throughout the observation period, we recorded only two effective visits of *Eulaema meriana* to two pink flowers of *R. granadensis* on the same plant. Both flowers were pollinated as a consequence of these visits (Fig. 6). The bee had a typical feeding behavior of extending its proboscis (Barth 1991). It did not

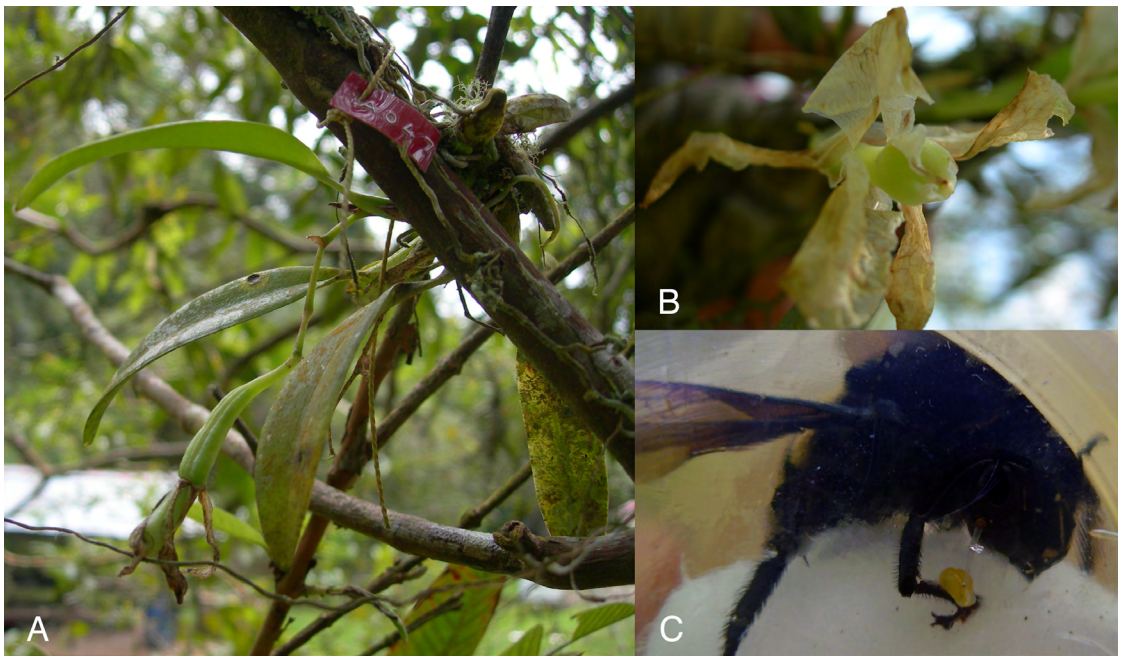

FIGURE 6. Pollination of *R. granadensis* in the Yotoco Nature Forest Reserve. A. developing fruit. B. Closed (pollinated) stigma. C. Male *Eulaema meriana* bearing a pollinarium of *R. granadensis* attached to the labrum.

exhibit the behavior of fragrance collecting (scratching the floral tissue) followed by hovering to put the fragrance in its hind tibia (Roubik & Hanson 2004). Additionally, we captured males of *Eulaema meriana*, *Eulaema cingulata* and *Exaerete smaracdina* with pollinaria of *R. granadensis* on their labrum. These are the first observations of effective pollination in *R. granadensis*; previously, it was speculated that yellow or white flowers were pollinated by bees, while pink flowers were pollinated by birds (Ortiz *et al*. 1991, Ortiz *et al*. 1994), nevertheless, we found no evidence of bird visitation.

The lack of red coloration or contrasting colors, in the form of a tunnel or bell, zygomorphic with a nectar guide, landing platform, diurnal anthesis and odors nectar or deceptive would suggest that flowers of *R. granadensis* are melittophilic (van der Pijl 1961).

TABLE 1. Reflected color Wilcoxon tests between white and pink flowers for four colorimetric analyses zones.

Zone B/R	W	z	p
1	13,000	0,524	0,600
2	18,000	0,676	0,499
3	15,500	0,255	0,799
4	18,000	0,676	0,499

Rodriguezia granadensis is an obligated xenogamic species with gametophytic self-incompatibility (none of the plants fertilized by autogamy developed fruits). Self-pollen treatment deposition results in flower wilting and the stigma closing around the pollinia (Dafni 1992), but the fruit does not develop (Lovett-Doust & Lovett-Doust 1988).

The spur of *R. granadensis* presents an average length of 26.5mm while the *E. meriana* can have a proboscis up to 40 mm, and it is known that euglossine bees forage a wide diversity of tubular flowers of different depth (Roubik & Hanson 2004, Otero & Sandino 2003).

Thirty six percent of the *R. granadensis* flowers possessed nectar, at a high sugar concentration (33%). Nectar volumes were greater than those observed in *Rodriguezia bahiensis* with only 0.4µl and 16.5 % of sugar (Carvalho & Machado 2006). *R. bahiensis* is known to be pollinated by flies and visited by bees. The quantity and quality of nectar found in *R. granadensis* is in the reported range foraged by euglossines (0.003µl - 120µl), with similar high variation in sugar concentration, 5 - 75% w/w. However, these bees are not known to consume highly concentrated nectar (Borrell 2005, Borrell 2006).

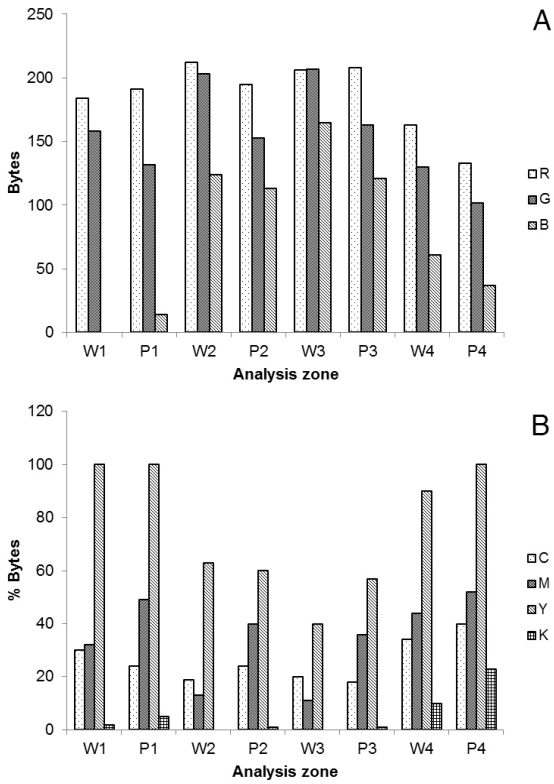

FIGURE 7. A. Flowers of *R. granadensis*, analysis of four zones for color morph, white to pink (W1, P1; W2, P2; W3, P3; W4, P4) RGB space, additive, spectrum intensity of the reflected light. R, Red, G, Green, B, Blue. B. CMYK space C, Cyan, M, Magenta, Y, Yellow, K, Key or black.

Sixty four percent of the flowers of *R. granadensis* studied had no nectar. Thus, most of the flowers of this population are deceptive. Given this variation in nectar presence, it is possible that this species has an attraction system that is ´self-mimetic´ or a diffuse rewarding phenomenon (Jersáková *et al*. 2006, Ackerman 1986b). This would assume that pollinators that follow planned routes to forage and do probe deceptive flowers do occasionally find rewards (Jersáková *et al*. 2006, Ackerman 1986b), at least sufficiently frequently to merit continuous exploration. Other studies focused on determining if there is frequency dependent selection for the polymorphic condition in deceit pollination systems, showed that pollinators can learn, and the presence of deceitful flowers may influence positively reproductive success in mix rewarding system (Ospina-Calderón *et al*. 2007, Ackerman & Carromero 2005, Aragon & Ackerman 2003, Ackerman *et al*. 1997, Sabat & Ackerman 1996, Ackerman & Galarza-Pérez 1991).

In *Comparettia falcata*, another nectariferous orchid, the production of nectar is not necessary a good indicator of reproductive success (Ackerman *et al*. 1994, Rodríguez-Robles *et al*. 1992). In this case, the pollinators, a hummingbird, is apparently sensitive to lack of nectar (Ackerman *et al*. 1994), however, does not respond to nectar supplementation (Salguero-Faría & Ackerman 1999). The lack of nectar in some individuals of *R. granadensis* suggest that individuals with or without nectar may have the same reproductive success and consequently, fitness of individuals with nectar may not differ from those that lack nectar. If there is a high cost to producing nectar then it is likely that there could be a disadvantage, and these individuals would result in a lower lifetime reproductive success. It is possible that they only bloom during the rainy season because water as is a limiting resource, and nectar may only be produced when sufficient water is available. Additionally, the production of too many fruits may attract seed predators (Ackerman & Montalvo 1990), making a limited fruit set a more effective reproductive strategy.

The fragrance of *R. granadensis* is one of the features that suggests melittophily (van der Pijl 1961) and it is also the floral attractive agent for other orchids, including polymorphic *Tolumnia variegata*, a food deceptive, twig epiphytic orchid pollinated by the bee *Centris versicolor* (Ackerman *et al*. 1997).

Our pollination data are too few to adequately test the hypothesis that euglossine bees pollinate both colors morphs (pink and white) of *R. granadensis*, based on the quality and quantity of the reward offered. Our data do not agree with the published hypothesis suggesting the pink morphs of *R. granadensis* are pollinated by hummingbirds (Ortiz *et al*. 1991). However, because of the widespread geographic distribution of this species, the possibility of other pollinators for this species cannot be discounted. Variation in nectar volume suggests the species may be a useful model to evaluate further evolutionary questions relating to the transition between rewarding and deceitful pollination systems.

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THREE NEW SPECIES OF *MASDEVALLIA* (ORCHIDACEAE: PLEUROTHALLIDINAE) FROM THE AYACUCHO AND PUNO REGIONS IN PERU

STIG DALSTRÖM[1,3] & SAUL RUÍZ PÉREZ[2]

[1] 2304 Ringling Boulevard, unit 119, Sarasota FL 34237, U.S.A.
Lankester Botanical Garden, University of Costa Rica, Cartago, Costa Rica
National Biodiversity Centre, Serbithang, Bhutan

[2] Allamanda 142, Surco, Lima 33, Peru

[3] Corresponding author: stigdalstrom@gmail.com

ABSTRACT. Three new species of *Masdevallia*, subgenus *Masdevallia* are described, illustrated with line drawings and color photographs, one dwarf species in section *Coriaceae* and two attractive and rather large species in section *Masdevallia*. The former section is also treated as the genus *Byrsella* by Luer (2006). All three new species are distinguished by unique combinations of features that separate them from all other species in the large genus *Masdevallia*.

KEY WORDS: New *Masdevallia*, Pleurothallidinae, Epidendreae, Epidendroideae, Peru

In 2006 the popular genus *Masdevallia* Ruíz & Pav. had become expansive with over 500 species, classed into numerous subdivisions (Luer, 2000a, 2000b, 2001, 2002, 2003). This vast number of species, in combination with molecular investigations by various authors (Abele *et al.*, 2005; Pridgeon & Chase, 2001), encouraged Luer to split the genus into 16 new genera, in addition to the 4 already existing, which included *Masdevallia* (Luer, 2006). Although splitting large genera into smaller units may sometimes be helpful in order to make them more easily surveyed from a taxonomic point of view, the authors do not recognize the advantages of the 2006 division of *Masdevallia*. We find it more difficult to identify which genus many of the morphologically similar species really belong to, and believe it to be more user-friendly and practical to maintain the previous and more conservative taxonomic treatment of the genus, as circumscribed by Luer (2000a, 2000b, 2001, 2002, 2003).

TAXONOMIC TREATMENT

Masdevallia goettfertiana Dalström & Ruíz-Pérez, *sp. nov*.

TYPE: Peru. Puno, Cerro Marrón, 2000 m, S 14° 12.351'; W 69° 13.408', flowered in cultivation by Perúflora in December 2013, *S. Dalström et al. 3743* (holotype: USM). Figs. 1-3.

Diagnosis. *Masdevallia goettfertiana* belongs to the subgenus *Masdevallia*, section *Coriaceae* which is mainly characterized by the thick leaf, the fleshy sepals, petals without any descending process, and a fleshy and verrucose lip. The dwarf plant habit with fleshy, pale glaucous leaves in combination with the fleshy flower with apically very narrow petals readily separate this species from all others in the genus.

Epiphytic *herb*. *Plant* dwarf for the subgenus, caespitose. *Ramicauls* erect, slender, to *ca*. 7 mm long, enclosed basally by 3 to 4 tubular sheaths. *Leaf* glaucous on both sides, erect to arching, coriaceous and fleshy, petiolate, blade basally conduplicate and cuneate, elliptic, obtuse, 35–55 × 7–8 mm, including the 5–15 mm long petiole. *Inflorescence* purple mottled, erect, terete, uniflowered, with a to *ca*. 15 mm long peduncle; *peduncular bract* 1, basal, tubular, *ca*. 4.5 mm long; *floral bract* appressed, tubular, *ca*. 6 mm long; *pedicel* to *ca*. 6 mm long; *ovary* deeply sulcate, indistinctly rugose, *ca*. 2.5 mm long. *Flower* shallowly cupulate and fleshy; *dorsal sepal* pale yellow, carinate externally, glabrous, connate to the lateral sepals for ca. 3–4 mm, then broadly acuminate and turning into an indistinct fleshy cauda, *ca*. 15 × 5 mm, including the tail; *lateral sepals* similar in texture, dark brown, except for the pale translucent base covered by dark purple

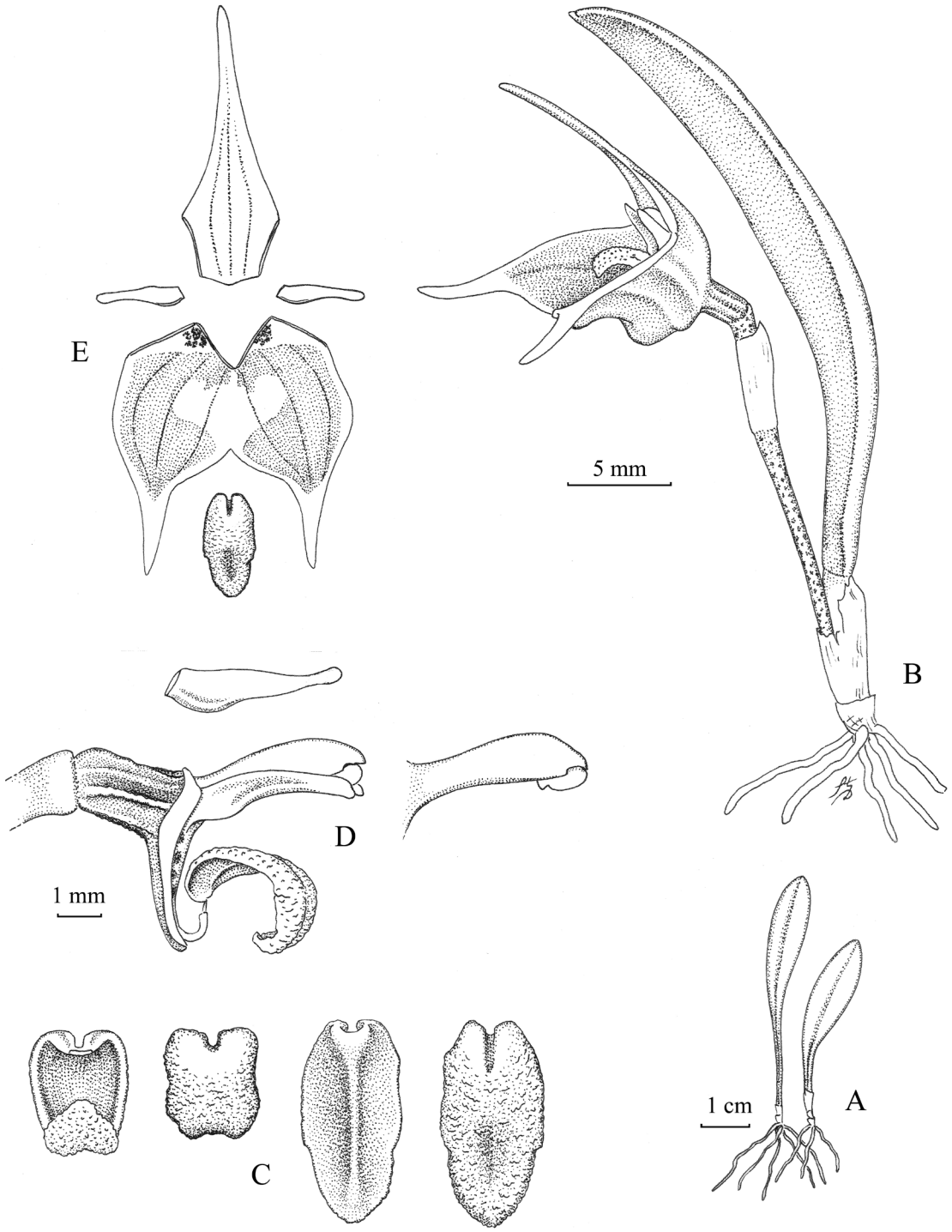

FIGURE 1. *Masdevallia goettfertiana.* A. Leaves. B. Plant habit with flower. C. Lip dorsal and ventral views in natural state and flattened. D. Column, lip and petals, lateral views. E. Dissected flower. Drawn from the holotype by Stig Dalström.

Figure 2. *Masdevallia goettfertiana*, flower in cultivation, front view. Photo of the plant that provided the holotype by S. Dalström.

Figure 3. *Masdevallia goettfertiana*, flower in cultivation, lateral view. Photo of the plant that provided the holotype by S. Dalström.

Figure 4. Former habitat of *Masdevallia goettfertiana* and many other species. Photo by S. Dalström.

spots, and the yellow indistinct and short tails, indistinctly carinate externally, *ca.* 12–13 × 12–13 mm combined, including the *ca*. 2–3 mm long tails, connate to each other for ca. 4–5 mm; *petals* whitish, cartilaginous, obliquely and narrowly ovate and with a short fleshy, ventral keel, then with a narrow and elongate midsection with a rounded apex, *ca*. 4 × 1 mm; *lip* dorsally dark purple, ventrally white, fleshy and minutely erose, hinged on the column foot by a minute strap-like tissue, lamina ovate, obtuse, with indistinct angles above the middle, carnose and sub-verrucose, basally distinctly channeled, which turns into a shallow, longitudinal groove above the middle, and with a recurved apex, *ca.* 4 × 2 mm when flattened; *column* pale yellowish green with purple lateral stripes, almost straight, *ca*. 4.5 mm long, with an equally long, curved foot with a hook-shaped apex; *anther cap* whitish and campanulate; *pollinia* not seen.

The first plant of *Masdevallia goettfertiana* was found growing epiphytically at eye level on a dead branch along an old and well frequented trail. The habitat is characterized by secondary and very dense brush vegetation. The original plant was without any flower at the time of collection, but flowered the following year in cultivation. The dwarf plant with fleshy and glaucous leaves immediately gave rise to the notion that it probably represented something different and probably a new species. It came as a surprise, however, to realize that this small but charming orchid belongs to the section *Coriaceae* Rchb.f. a group of usually much larger species.

Additional material seen: Peru. Only a small population of plants has been observed in the same location as the holotype. No other collections known.

Distribution and habitat: *Masdevallia goettfertiana* has only been found in a single location in scrubby and dense cloud forest on Cerro Marrón, between San Juan del Oro and Pilcopata, where the habitat is severely threatened by deforestation. Fig. 4.

Eponymy: This species is named in honor of Peter Göttfert, of Västerås, Sweden, an avid orchid enthusiast and well known Swedish nursery man, who is also a great supporter of orchid research.

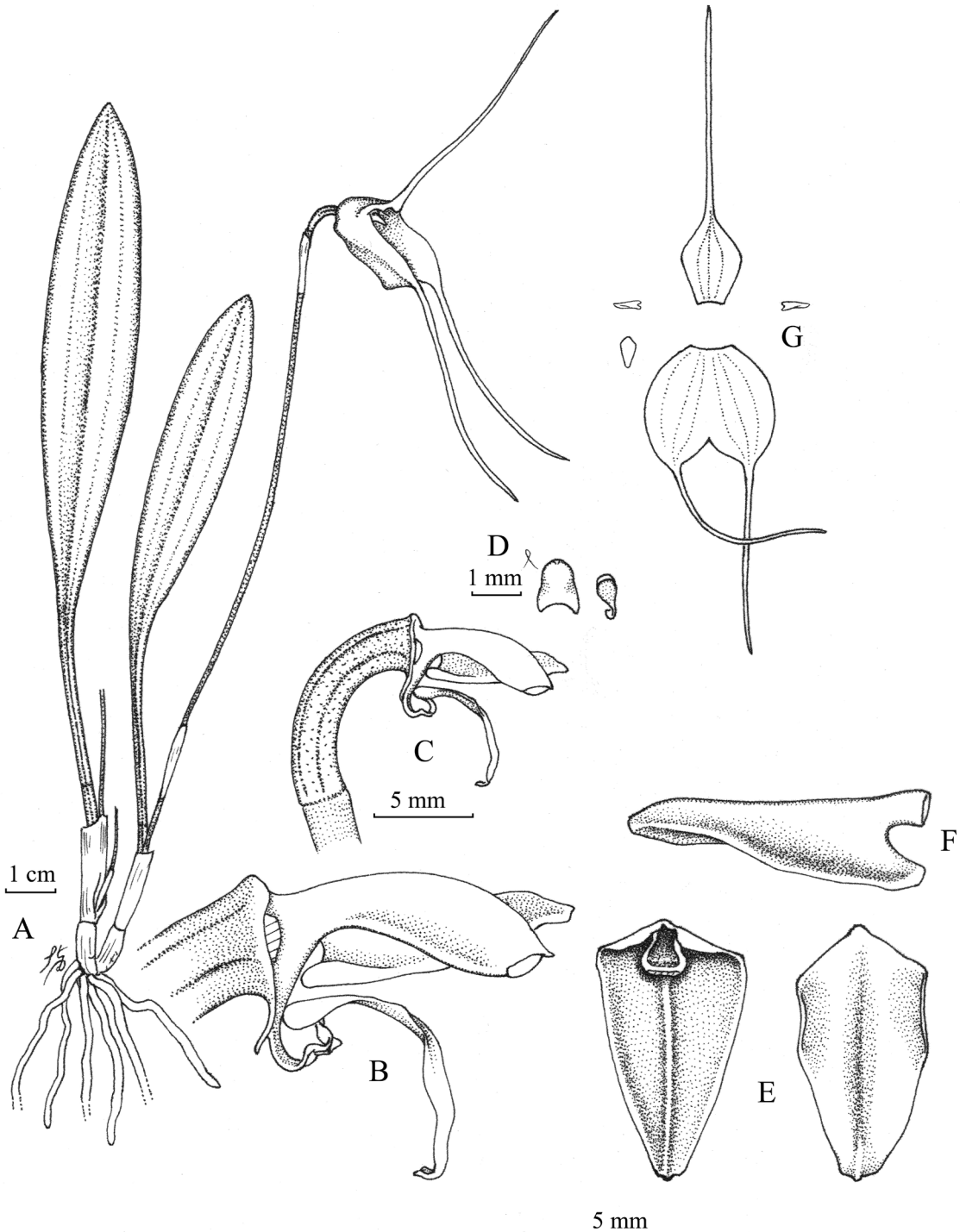

FIGURE 5. *Masdevallia robineae.* A. Plant habit. B. Column, lip and petal, lateral view. C. Ovary, column, lip and petal view. D. Anther cap and pollinia. E. Lip dorsal and ventral views. F. Petal internal lateral view. G. Flower dissected. Drawn from the holotype by Stig Dalström.

Masdevallia robineae Dalström & Ruíz-Pérez, *sp. nov*.

TYPE: Peru. Puno, east of Ollachea, Camatani, La Villa, epiphytic in mossy cloud forest at 2115 – 2200 m elevation, flowered in cultivation Nov. 2014, *S. Dalström* 3774 (holotype: USM). Fig. 5.

Diagnosis. *Masdevallia robineae* belongs to the subgenus *Masdevallia*, section *Masdevallia*, subsection *Masdevallia*, and is distinguished by the white and basally pale yellow flower, with a triangular and rather flat lip with a truncate apex with an indistinct apiculum.

Lithophytic or epiphytic *herb*. *Plant* medium sized to large for the genus, caespitose. *Ramicauls* erect, slender, to *ca*. 4 cm long, enclosed basally by 2 or 3 tubular sheaths. *Leaf* erect to arching, coriaceous, petiolate, blade basally conduplicate and cuneate, elliptic, obtuse, to *ca*. 14 × 2 cm, including the *ca*. 4 cm long petiole. *Inflorescence* erect, terete and slender, single flowered, with a to *ca*. 13 cm long peduncle; *peduncular bracts* 2 (one hidden inside basal bract), tubular, below the middle of the peduncle, to *ca*. 16 mm long; *floral bract* appressed, tubular, to *ca*. 12 mm long; *pedicel* to *ca*. 13 mm long; *ovary* smooth, indistinctly sulcate, with scattered minute 'fungal-pits' (tiny pits where some fungi appear to establish in the wild), *ca*. 11 mm long. *Flower* attractive, cupulate; *dorsal sepal* basally pale lemon yellow and apically whitish, indistinctly carinate externally, glabrous, basally cuneate and connate to the lateral sepals for *ca*. 10 mm, then obtuse and acuminate into a narrow, suberect to arching yellow tail, *ca*. 60 × 12 mm, including the *ca*. 45 mm long tail; *lateral sepals* similar in coloration but with pale orange basally, indistinctly carinate externally, glabrous, connate to each other for ca. 20 mm, broadly and slightly obliquely elliptic, obtuse, acuminate with pale greenish yellow tails, *ca*. 65 × 27 mm combined, including the *ca*. 40 mm long tails; *petals* white, cartilaginous and slightly oblique, unguiculate, with a distinct ventral lobe, extending longitudinally and ending in a fleshy ridge near the bluntly obtuse apex, *ca*. 7 × 2.5 mm; *lip* white, hinged on the apex of the hook-shaped column foot via a canaliculated, strap-like structure, cuneate with involute basal margins in a fresh state (cordate basally when flattened), lamina almost triangular with a bluntly truncate and minutely apiculate apex, almost flat but with a shallow longitudinal groove and indistinctly erect lateral lobes, *ca*. 5.5–6.0 × 3 mm when flattened; *column* white with a purple base, curved downwards, *ca*. 6 mm long, with an slightly shorter, apically hook-shaped foot ; *anther cap* white and campanulate; *pollinia* 2, minute and pyriform.

Masdevallia robineae (Figs. 5-7) is an attractive species and is sympatric with the equally attractive and rare *M. leonii* (Fig. 8) of subsection *Caudatae*.

Figure 6. *Masdevallia robineae*. Flowering plant in natural habitat. Photo by Stig Dalström.

Figure 7. *Masdevallia robineae in situ*. Photo by S. Dalström.

Generally, the former species has a much longer leaf of a more slender shape, versus the much shorter, broadly ovate and paddle-shaped leaf of the latter species. The flowers are also quite different in shape. However, a plant with longer and slender leaves was found without flowers previous to the discovery of the type plant of *M. robineae*. It was believed to be something 'different' from *M. leonii*, which had previously been observed in flower. When the 'long-leaved' plant later flowered, however, it turned out to be a perfect *M. leonii*. Whether this was the result of natural hybridization or just some freak anomaly is unknown.

Additional material seen: Peru. Puno, east of Ollachea, Camatani, La Villa, lithophytic in shade on mossy boulder along the road, at 2200 m elevation, S 013° 43.832'; W 70° 27.602. Digital photo (Dalström archives). Only a few plants have been observed in the type area, growing epiphytically or lithophytically on mossy boulders. No other material seen.

Figure 8. *Masdevallia leonii in situ*. Photo by S. Dalström.

Figure 9. *Masdevallia robineae* habitat along the Ollachea – San Gaban road. Photo by S. Dalström.

Distribution and habitat: *Masdevallia robineae* is only known from the steep and locally densely forested valley between Ollachea and San Gaban, Puno, at the altitude of *ca.* 2100–2200 m. Fig. 9.

Eponymy: This species is named in honor of Robine Coppens by the request of her grandfather, Guido Deburghgraeve of Liedekerke, Belgium, who discovered the type plant.

Masdevallia roseogena Dalström & Ruíz-Pérez, *sp. nov*.

TYPE: Peru. Ayacucho, Calicanto, collected by a team lead by Saúl Ruíz on the ridge above the village, in wet scrubby cloud forest at ca. 2500 – 2600 m elevation, 5 Dec. 2010, flowered in cultivation by Perúflora in December 2013, *S. Dalström et al 3722* (holotype: USM). Figs. 10, 11.

Diagnosis. *Masdevallia roseogena* belongs to subgenus *Masdevallia*, section *Masdevallia*, subsection *Masdevallia*, and is distinguished by the attractive snowy white flowers covered by rosy magenta spots and flush on the petals and lip, and internally and externally on the lateral sepals, and with a broadly ovate, minutely apiculate lip without any visible dorsal callus.

Epiphytic or terrestrial *herb*. *Plant* medium to large for the genus, caespitose. *Ramicauls* erect, slender, *ca*. 3–4 cm long, enclosed basally by 3 to 4 tubular sheaths. *Leaf* erect to arching, coriaceous, petiolate, blade basally conduplicate and cuneate, more or less elliptic, obtuse, *ca*. 12.5 × 2.5 cm, including the *ca*. 4 cm long petiole. *Inflorescence* erect, terete and slender, single-flowered, with a to *ca*. 10 cm long peduncle; *peduncular bracts* 2, tubular, below the middle of the peduncle, to *ca*. 1 cm long; *floral bracts* appressed, tubular, to *ca*. 1.2 cm long; *pedicel* to *ca*. 2.5 cm long; *ovary* smooth and indistinctly sulcate, with scattered minute 'fungal-pits', *ca*. 6 mm long. *Flower* attractive, cupulate; *dorsal sepal* snowy white with rose-magenta flush externally and richly spotted internally, glabrous and carinate externally,

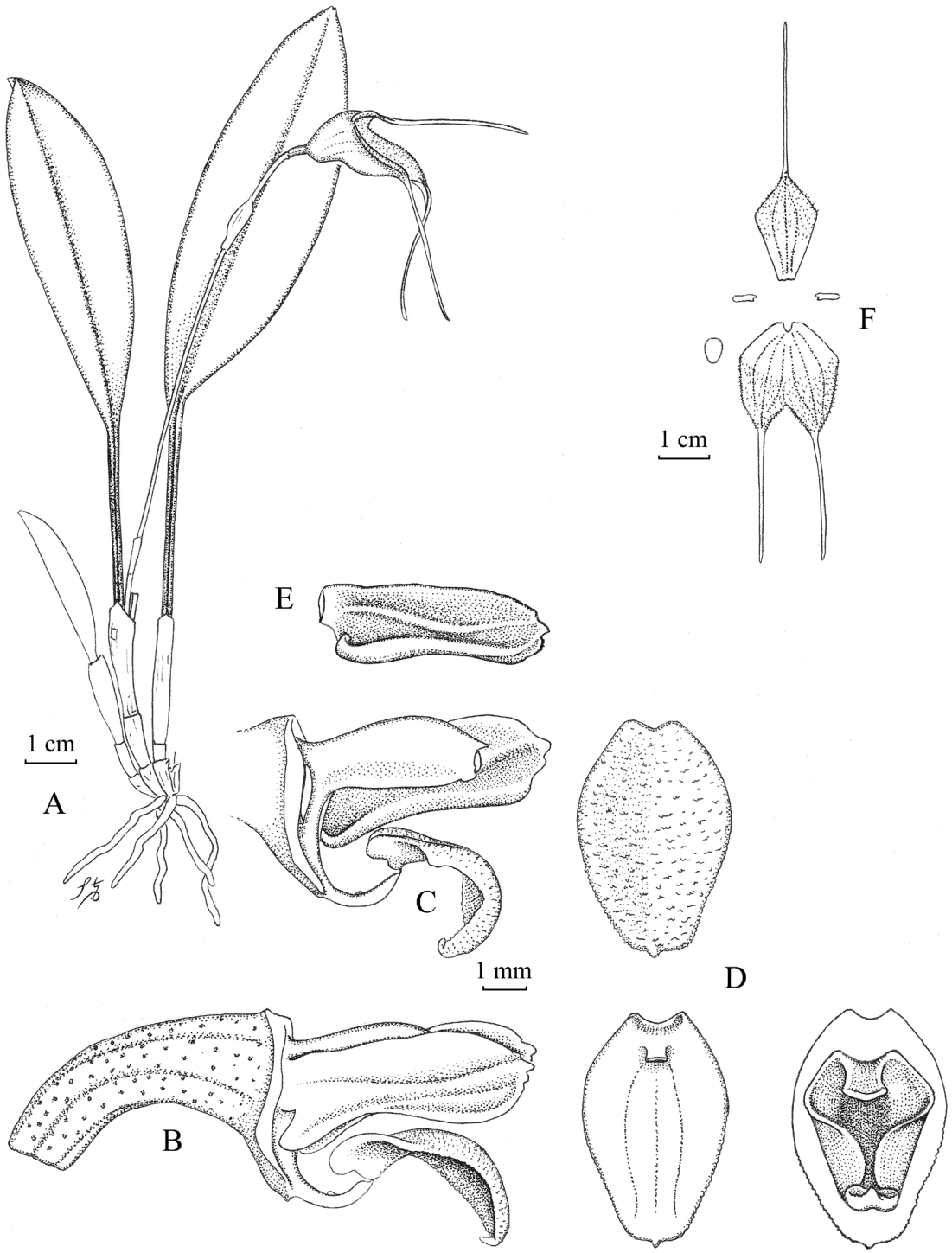

Figure 10. *Masdevallia roseogena.* A. Plant habit. B. Ovary, column, lip and petals, lateral view. C. Column, lip and petal lateral view. D. Lip dorsal and ventral views (flattened and in natural state). E. Petal internal lateral view. F. Flower dissected. Drawn from the holotype by Stig Dalström.

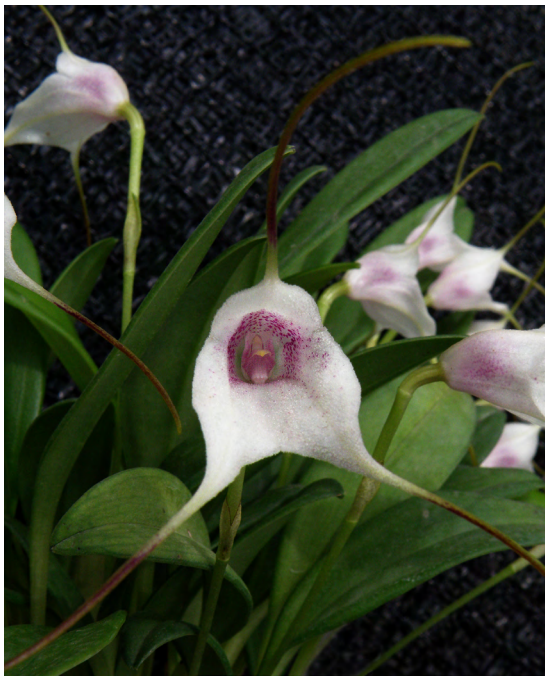

FIGURE 11. *Masdevallia roseogena*, flowered in cultivation by Perúflora. Photo by S. Dalström.

densely microscopically glandular internally, connate to the lateral sepals for ca. 13–15 mm, then acute and apically extended into an elongate and narrow tail, ca. 13 × 50 mm, including the 30 mm long, basally white, then purple, gradually turning olive green tail; *lateral sepals* similar in texture and coloration, glabrous and carinate externally, microscopically glandular internally, connate for *ca*. 15 mm, then slightly and obliquely obtuse, extending in a slender tail, *ca*.50 × 20 mm combined, including the *ca*. 25 mm long tails; *petals* white with rose-magenta markings, cartilaginous and slightly oblique, indistinctly unguiculate with a distinct, curved lateral lobe, forming a fleshy, longitudinal ridge ending near the truncate and apiculate apex, *ca*. 5.5 × 2 mm; *lip* white suffused with rose-magenta and a pale yellow apex, hinged on the hook-shaped column foot via a minute strap-like structure, lamina ovate, minutely sub-verrucose dorsally, obtuse and apiculate when flattened, but revolute and ventrally concave in the natural state, with a shallow notch at the base, *ca.*4.5–5.0 × 3.2–3.5 mm when flattened; *column* white with purple edges, straight, *ca*. 4 mm long, with an equally long, curved, apically hook-shaped foot ; *anther cap* white and campanulate; *pollinia* not seen.

FIGURE 12. *Masdevallia roseogena* habitat in the Ayacucho region. Photo by S. Dalström.

FIGURE 13. *Masdevallia roseogena* habitat in the Ayacucho region. Photo by S. Ruíz.

ADDITIONAL MATERIAL SEEN: Peru. Only a few scattered plants have been discovered in the same area as the holotype. No other collections known. This region is subject to intense deforestation and habitat destruction, particularly at higher elevations.

DISTRIBUTION AND HABITAT: *Masdevallia roseogena* has only been found in a single location along the densely forested ridge above the village of Calicanto, Ayacucho. Figs. 12, 13.

ETYMOLOGY: This species is named in reference to the white flowers with a rosy 'blush' on the lateral sides of the sepals (the 'cheeks'); Latin for rosy-red producing, and 'gena' referring to the cheeks.

Masdevallia roseogena was originally discovered by a team lead by Saúl Ruíz in extremely wet and dense, scrubby cloud forest along the ridge above

the small town of Calicanto in the Ayacucho region. This is a dangerous area, teeming with military presence and subject to occasional violence by the Shining Path movement. This in turn renders the local population extremely suspicious of foreigners and their whereabouts.

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PROTOCORM-LIKE BODIES AND PLANT REGENERATION FROM FOLIAR EXPLANTS OF *COELOGYNE FLACCIDA*, A HORTICULTURALLY AND MEDICINALLY IMPORTANT ENDANGERED ORCHID OF EASTERN HIMALAYA

KALYAN K. DE[1] & SUJIT SIL

Post Graduate Department of Botany, Hooghly Mohsin College
Chinsurah -712 101, Hooghly, West Bengal, India
[1] Author for correspondence: kalyannet2003@yahoo.co.in

ABSTRACT. An efficient induction of protocrom-like bodies (PLBs) and plantlet regeneration from the young leaves of *in vitro* grown seedlings of *Coelogyne flaccida*, an horticulturally and medicinally important endangered epiphytic orchid, was accomplished in order to develop mass-scale propagation. The young leaves (1.5 cm in length) from 110 days old aseptically germinated seedling were grown *in vitro* in Murashige and Skoog's (MS) medium supplemented with different concentrations and combinations of NAA (0.5-2 mg/L), BAP (0.5-2 mg/L) and Kn (0.5-2 mg/L). The explants produced protocorm-like bodies directly from the epidermal cells of leaf without the formation of intervening callus tissue within four weeks of culture. The highest number of plantlets regenerated through PLBs per explant after 15 weeks was 35-36 in presence of NAA (2mg/l) and Kn (2mg/l). Within 20-25 weeks individual plantlets produced 2-3 leaves and 2-3 roots. Chromosome number from all plants regenerated from leaf explants showed the same chromosome number as the mother plant as 2n = 40. During acclimatization, 80% of the plantlets survived after one month of transplantation.

KEY WORDS: *Coelogyne flaccida*, foliar explant, micropropagation

Introduction. *Coelogyne flaccida* Lindl. is one of the most beautiful horticulturally important orchids native to Nepal, India, Myanmar, West China and Laos. It occurs as an epiphytic herb in the sub-tropical regions at the elevations of 900-2300 m (Clayton 2002). The pseudobulbs of this orchid bear a pair of linear, lanceolate leaves at the apex. The inflorescence is of a pendulous recemose type arising from the base of pseudobulb. The mildly scented long lasting flowers (3-5 cm across) are white with yellow on the middle of the lip, stripped red in the side lobes and spotted red at the base of the middle lobe. The beautiful flowers of this orchid have high ornamental value as a cut flower which has made it popular. It has resulted in over collecting. This factor along with a shrinking natural habitat is putting pressures on the survival of the species. In addition to this, orchids are very slow growing plants which has added to their being rare and endangered (Bailes 1985, Wu *et al.* 2009). Besides their horticultural importance, the genus *Coelogyne* in general, has some therapeutic value. It is mainly used for the treatment of tuberculosis, but different species of *Coelogyne* have some other uses in herbal medicine for example the pseudobulb and leaf of *C. flaccida* are used to treat headache and indigestion (Rajbhandari & Bhattarai 2001). The active principle isolated from this orchid is a new type of stilbenoides designated as callosin whose chemical structure was established as 2, 6 dihydroxy-4, 7 dimethoxy-9, 10 dihydrophenanthrene (Majumdar *et al.* 1995). Therefore, to overcome the danger of extinction of such a horticulturally and medicinally important orchid and to prevent illegal collection from wild, it is urgently needed to develop rapid clonal propagation method for their resurrection in terms of conservation in their wild habitat.

Commonly, shoot tip or apical meristem is used for *in vitro* clonal propagation of orchids. Leaves are also preferable as a source of explants for clonal propagation (Arditti & Ernst 1993, Nayak *et al.* 1997, Chen *et al.*1999, 2004, 2006, Park *et al.* 2002, Pathak & Vij 2001, Seeni & Latha 2000, Vij & Aggarwal 2003, Vij & Pathak 1990, Vij *et al.* 2000, 2002, Kai *et al.* 2008, Gow et al. 2009, Mayer *et al.* 2010, Naing

et al. 2011). The shoot tip culture of orchids for clonal propagation entails the sacrifice of whole plants or the entire new growth. However, the use of leaf tissue has the advantage of not endangering or even seriously damaging a plant. In this study, the authors report the development of an efficient simple and reproducible one step protocol for getting large scale *in vitro* clonal plantlets of *C. flaccida* from young leaf explants via induction of PLBs directly without the formation of intervening callus tissue and the successful transplantation of plantlets to the *ex vitro* condition.

Materials and methods. The green undehised capsules (60-63 days after pollination) were collected from their natural habitat of mother plant from Kalimpong area (1247 m above sea level, Latitude 27.06°, Longitude 88.47°) of Darjeeling hill which is a part of Eastern Himalaya. The immature seeds were scooped out from the surface-sterilized and dissected capsule and germinated aseptically in Orchimax medium (Duchefa Biochemie BV product no. 0 0257) supplemented with 1mg/L NAA and 15% coconut water for the preparation of aseptic plantlets. The seeds were incubated under dark condition at the temperature of 20 °C ± 2 °C.

Immature leaves (1.5 cm in length) of 110 days old aseptic plantlets maintained in sterile culture were taken as explants for *in vitro* culture in Murashige & Skoog's (MS) medium (1962) supplemented with different concentrations and combinations of NAA (0.5-2 mg/L), BAP (0.5-2 mg/L) and Kn (0.5-2 mg/L) as shown in Table 1 and a control set was maintained in parallel in the basal medium which is free from plant growth regulators. The pH of the media was adjusted to 5.65. The young leaves taken from the axenic plantlets were aseptically inoculated (one or two leaves per culture tube containing 20 ml medium) and cultures of 380 tubes (25 mm diameter x 150 mm length) were incubated at 20 °C ± 2 °C under 16 hours photoperiod from cool white light giving 2659 µmol m^{-1} s^{-1} at culture level. The culture of one tube constitutes one replication and for each combination and concentration of plant hormones as shown in Table 1, twenty tubes of culture were made. The experiments were repeated twice following the same methodology and keeping the same culture condition. The response of each explant in each culture tube were carefully examined every day and frequently by microscopic observation. For counting the number of PLBs or number of plantlets when it was needed, the responding leaf explant was conveniently dissected into a number of pieces and the total number of PLBs or all regenerated plantlets per explants was examined and counted physically under dissecting microscope. Every 30 days interval each culture was sub-cultured into fresh medium keeping the same respective hormone combination and concentration. Experimental data was collected quantitatively or qualitatively on the basis of initiation of response or nature of response of explants in terms of time period of culture. After regeneration of plantlets with roots, they were finally sub-cultured in 250 ml glass bottles containing ½ strength MS medium without supplementation of any hormone and sugar. On reaching a height 60-65 mm, the plantlets with three or four well-developed roots were taken out of the culture, washed thoroughly to remove all remnants of agar gel under running tap water and were finally potted in wetted coconut husk for acclimatization. The pots were maintained under mist and 50% shade for 2 months and after that they were moved to standard green house conditions.

The fresh roots of the donor plants and regenerated plants were pretreated with the mixture of saturated Paradichlorobenzene (p-DB) and 8-hydroxyquinolene (1:1) for 4 hours at 14-16 °C temperature, followed by washing and fixation in acetic-ethanol (1:3) for overnight. The root tips were kept in 45% acetic acid solution for 1 minute and stained with 2% aceto-orcein stain and 1 (N) HCL (9:1). Finally root tips were squashed in 45% acetic acid and the chromosome number of mitotic metaphase was counted.

Results. About 90% of the seeds of *C. flaccida* were successfully germinated under *in vitro* condition in the Orchimax medium supplemented with 1mg/L NAA and 15% coconut water. Typically a seed gave rise to a PLB, which in turn developed into a seedling after 45 days of culture and then they were moved to light condition and under 16 hours photoperiod.

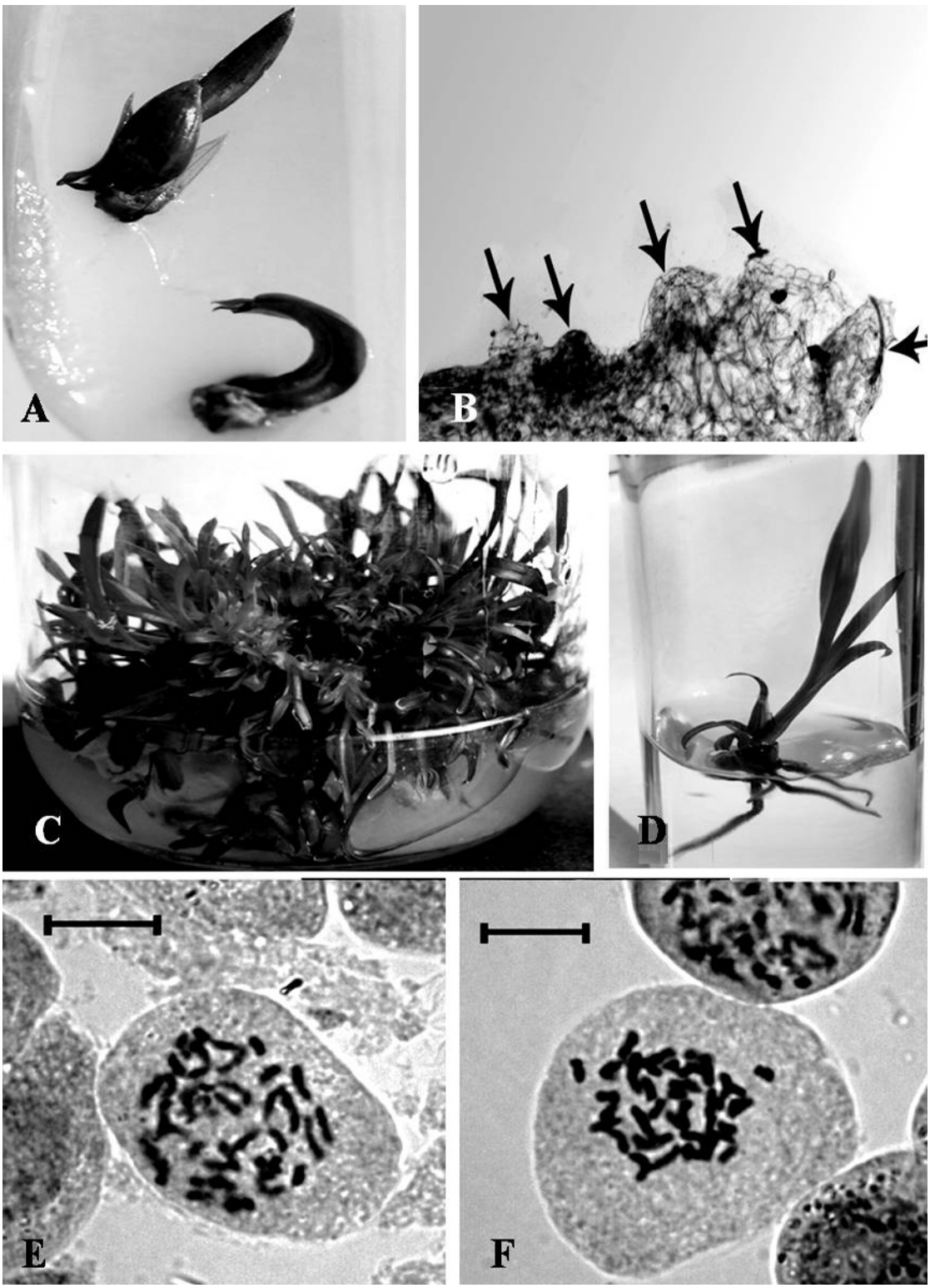
Since the plantlets of *C. flaccida* were obtained from the aseptically germinated seeds and grown under controlled *in vitro* condition, the plantlets were contamination free and physiologically uniform and stable. So, the explants were ideally procured from *in vitro* grown plantlets for the present investigation. In

Table 1. *In vitro* plant regeneration response of *C. flaccida* foliar explants on MS medium.

MS Basal medium supplemented with Hormones	No. of PLB loci during initiation of response ± SE after 6 weeks of inoculation of leaf explant	No. of plantlets/explant obtained from PLBs after 15 weeks ±SE
NAA (0.5 mg/L) +BAP (0.5mg/L)	1.80±0.28	3.20±0.96
NAA (0.5 mg/L) + BAP (1 mg/L)	2.30±0.29	12.50±2.09
NAA (0.5 mg/L) + BAP (2 mg/L)	3.60±0.078	20.80±2.39
NAA (1 mg/L) + BAP (0.5 mg/L)	1.20±0.28	3.10±2.29
NAA (1 mg/L) + BAP (1 mg/L)	3.90±0.79	20.90± 2.8
NAA (1 mg/L) + BAP (2 mg/L)	3.10±0.69	24.70±2.68
NAA (2 mg/L) + BAP (0.5 mg/L)	2.20±0.29	3.60±0.79
NAA (2 mg/L) + BAP (1 mg/L)	3.60±0.078	22.70±1.76
NAA (2 mg/L) + BAP (2 mg/L)	3.90±0.79	27.80±2.39
NAA (0.5 mg/L) + Kn (0.5 mg/L)	1.60±0.28	3.70±0.78
NAA (0.5 mg/L) + Kn (1 mg/L)	2.20±0.29	11.30±1.8
NAA (0.5 mg/L) + Kn (2 mg/L)	3.70±0.49	23.70±1.39
NAA (1 mg/L) + Kn (0.5 mg/L)	2.10±0.39	6.60±1.09
NAA (1 mg/L) + Kn (1 mg/L)	3.80±0.78	28.80±1.68
NAA (1 mg/L) + Kn (2 mg/L)	3.90±0.79	29.40±2.09
NAA (2 mg/L) + Kn (0.5 mg/L)	2.20±0.29	8.60±1.19
NAA (2 mg/L) + Kn (1 mg/L)	4.10±0.89	32.10±2.47
NAA (2 mg/L) + Kn (2 mg/L)	4.20±0.89	35.30±2.51

the basal medium i.e. medium without supplemented any plant growth regulators, the explants exuded, turned brown and perished within two months. In case of hormone added media, the first noticeable change of the explants under *in vitro* condition was the swelling and enlargement of the basal portion of the leaf within four weeks (Fig. 1A). After one month, the hand-made thin anatomical sections of the swelled portion of the explants microscopically revealed the appearance of small green protuberances (Fig. 1B) from the epidermal region. Such protuberances were ultimately transformed directly into protocorm like bodies (PLBs) in course of growth and development after six weeks without the formation of intervening callus tissue. In all cases, the PLBs were formed initially at the basal region of the foliar explants. However, in some cases depending on higher concentration of hormones (NAA 1-2 mg/l plus BAP 1-2 mg/l or Kn 1- 2 mg/l), more PLBs formation were induced and found to grow gradually towards apical part from basal region along both margins of the foliar explant.

Table 1 shows the number of PLB formation after 6 weeks and regeneration of plantlets per explants after 15 weeks in different hormone concentration and combination. The maximum number of plants were yielded after 15 weeks was 24-27 in presence of NAA (1mg/l) plus BAP (2mg/l) and NAA (2mg/l) plus BAP (2mg/L) combinations respectively and the lowest number from plant regeneration after 15 weeks was 3-4 in presence of NAA (0.5 mg/l) and BAP (0.5mg/l) combination. On the other hand, NAA (0.5 mg/l) in combination with Kn (0.5 mg/l) supported very low number of plantlets (3-4) regeneration per explants through a PLB mediated response, but when keeping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the same concentration of NAA i.e. 0.5 mg/l, the concentrations of Kn combination was changed and increased from 0.5 mg/l to either 1 mg/l or 2 mg/l in the media, the regeneration response was also significantly enhanced. The maximum regeneration response and the highest number of plantlets regeneration mediated through PLBs per explants (35-36) after 15 weeks were obtained in the medium supplemented with NAA (2 mg/l) and Kn (2 mg/l). Therefore, the efficiency of Kn at the concentration of 2 mg/l in combination with NAA (2mg/l) in terms of regeneration response and the number of plantlets regeneration per foliar explants of *Coelogyne flaccida* after 15 weeks were found better than that of same concentration of NAA and BAP used for the same purpose. When a clump of approximately 50 PLBs were separated and sub-cultured monthly upto 40 weeks in 250ml glass bottles containing the same medium i.e. supplemented with NAA (2 mg/l) and Kn (2 mg/l), the number of plantlets was further increasing by multiplication of PLBs and ultimately became uncountable number (Fig. 1C). However, in case of other media supplemented with different combination and concentration of NAA and BAP or Kn, further subculture upto 40 weeks also increased the number of plantlets comparatively at a slower rate. Initially three to four roots per plantlet were emerged (Fig. 1D). Chromosome number from all plants regenerated from leaf explants were counted and revealed that indeed the plants showed the same chromosome number as mother plant as 2n = 40 (Fig. 1E, F). The plantlets showed 80% survival rate when subjected to a very careful treatment for acclimatization.

Discussion. In the present investigation, young leaves were selected as the young tissue is known to regenerate better owing to their less rigid cell walls. The regeneration and proliferation competence of the juvenile leaves is much more than the relatively older explants. Plant regeneration from young leaf tissue could be induced either indirectly through the formation of intervening callus tissue (Hong *et al.* 2008, Huang & Chung 2010, Ng & Saleh 2011) or directly through the formation of protocorm-like bodies (Luo *et al.* 2008, Mayer *et al.* 2010, Naing *et al.* 2011). Regeneration of PLBs is comparable to the somatic embryogenesis pathway in orchids (Morel 1974). So, propagation by direct formation of PLBs from the leaf tissue is a preferred option because of the large number of PLBs that can be obtained within a short period of time. PLBs can proliferate rapidly and can readily regenerate into complete plantlets; so they are also the most general target tissue for genetic transformation in orchids (Liau *et al.* 2003, Sreeramanan *et al.* 2008). Moreover, PLBs are well-differentiated tissues that are sometimes regarded as orchid embryos that can develop two distinct bipolar structures, namely, the shoot and root meristem. Thus, these structures are able to convert to plantlets easily (Ng & Saleh 2011).

The pathway of response of an explant in culture preferably depends on the exogenous level of plant growth regulators. Callus induction from leaf segment of orchids is more difficult than any other meristemed explant. However, juvenile leaves have the possibility to form the callus tissue because of its potential meristimatic nature and it also depends on the presence of growth stimulus in nutrient pool. Moreover, tissue cultures of orchids have not been focused on callus because of their slower growth rate and increased necrosis during culture (Zhao *et al.* 2008). In the present study, we succeeded in getting PLBs directly from the leaf tissue instead of callus stage. Similar result has been demonstrated for *Aerides crispa* Lindl. (Sheelavanthmath *et al.* 2005). Juvenile explants like young leaves were important for the efficient induction of PLBs and the subsequent regeneration of plants in *Aerides crispa*. Lee & Phillips (1988) attributed this point as being of major importance because plants produced by direct regeneration via PLB formation will exhibit greater genetic stability than those produced by callus. Seeni and Latha (1992) regenerated a large number of phenotypically uniform plants from the basal part of the young leaf of flowering Red *Vanda*.

The types and concentrations of plant growth

Left, Figure 1. Direct plant regeneration from foliar explants of *Coelogyne flaccida*. (A) Swelled and enlarged leaf explants in nutrient medium after four weeks of culture. (B) Appearance of some small protuberances from the leaf epidermal region. (C) Multiplication of PLBs and regeneration of large number of plantlets. (D) Showing the complete young plant (E) somatic chromosome 2n = 40 of the root tip cells of regenerants (F) somatic chromosome 2n = 40 of the root tip cells of mother plants. Bar = 50µM.

hormones play an important role *in vitro* propagation of many orchid species (Arditti & Ernst 1993). In the present experiment, for young leaf explants, NAA was used as only auxin at the concentration of 0.5 mg/l, 1 mg/l and 2 mg/l in combination with Kinetin or BAP used as cytokinin. Differences in the induction rate for PLBs were observed between the treatments with Kn and the BAP in combination with NAA. Comparatively, Kn gave a superior response to BAP for inducing PLBs in young leaf tissue of *C. flaccida*. In this study, Kn at a particular concentration (2 mg/l) along with 2 mg/l NAA in combination strongly stimulated the formation of more PLBs. Kn facilitated conversion of more than 90% PLBs to shoots in foliar explants of *Dendrobium* (Martin *et al.* 2006). Effective stimulation of NAA and Kn on PLB formation is also in agreement with the observations in *Rhynchostylis retusa* (L.) Blume (Vij *et al.* 1984), *Coelogyne punctulata* Lindl. (Sharma & Tandon 1986), *Acampe praemorsa* (Roxb.) Blatt. & McCann (Nayak *et al.* 1997), *Ascocenda* 'kangla'(Kishor *et al.* 2006), *Vanda testacea* (Lindl.) Rchb. f. (Kaur & Bhutani 2009), *Cymbidium mastersii* Griff. ex Lindl. (Mohanty *et al.* 2012), and *Coelogynae flaccida* (Kaur & Bhutani 2013).

In the present culture, regeneration response in most of the cases is restricted to the basal region of the leaf whereas in few cases the leaves regenerated all along the surface. Development of PLBs at the base of leaves was similar to that in *Cattleya*. In *Cattleya*, the meristematic area which forms PLBs, are in the epidermal cells of the basal region of the leaf (Arditti 1977a, 1977b, Pierik 1989).The same is true of *Aranda* (Loh *et al.* 1975). The restriction of such an activity in the leaf base may be associated with the genetic makeup and physiological age of the explant,and/or the medium being employed (Vij *et al.* 1984). *Rhynchostylis retusa*, the initiation of PLBs formation was in the upper and lower epidermal cells near the basal ends of the explants. The entire surface of the juvenile leaf is potentially meristematic in *Rhynchostylis retusa* and *Phalaenopsis amabilis* (L.) Blume (Vij *et al.*1984, Chen *et al.* 2006). The potential adventives meristematic cells undergo repeated mitotic cycles and subsequently develop into PLBs.

The cytological uniformity in the root cells of the regenerants and mother plant in terms of chromosome number in the present cultures can be correlated with their origin from the epidermal layers. According to Dulieu (1972), the plants regenerated from epidermal layers are cytologically more stable and generally remain diploid. This has been corroborated in subsequent studies (Loh *et al.* 1975; Vij *et al.* 1984; Vij & Pathak 1990).

In conclusion, an efficient and rapid *in vitro* protocol for direct plant regeneration has been achieved from the foliar explants of *C. flaccida*, an endangered orchid of high medicinal and horticultural value. Plant establishment can be successfully completed after 8 months following the development of aseptic plantlets derived from aseptically seed germination under *in vitro* condition. The efficiency of Kn at the concentration of 2 mg/l in combination with NAA (2 mg/l) in terms of regeneration response and the number of plantlets regeneration per foliar explants of *C. flaccida* after 15 weeks were found better than that of same concentration of NAA and BAP used for the same purpose. Healthy plantlets developed from PLBs of foliar explants survived well when transplanted in the greenhouse. This protocol is simple, easy to carry out without damaging or further endangering the existing natural plant population and can provide a large number of plants for mass propagation. and conservation in their wild habitat. We expect that this ability will also open up the prospect of using biotechnological approaches for *C. flaccida* improvement.

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ROOT ANATOMY OF *GALEANDRA LEPTOCERAS* (ORCHIDACEAE)

Leila E. Barretta-dos-Santos[1], Jéssica Sant'Ana[1], Adarilda Petini-Benelli[2] & Cristiano Pedroso-de-Moraes[1,3]

[1] Biological Science Department of Hermínio Ometto University Center – UNIARARAS. Rua Maximiliano Baruto, 500, Jd. Universitário. CEP: 13.607-339. Araras/SP. Brasil

[2] Federal University of Mato Grosso, Herbarium-Botany Department, P.O. Box 198, Centro, CEP: 78005-970. Cuiabá/MT. Brasil

[3] cpmoraes@gmail.com

Abstract. Due to the scarce information about the root organization of *Galeandra* genus representatives, this study aimed to describe the root anatomy of *Galeandra leptoceras,* describing adaptations related to hydric relations and characters of taxonomic interest. Five roots of three plants were fixed and preserved in 50% alcohol. These ones were cut in midline with the use of razors. The sections were stained with 0.05% Safrablau and mounted in glycerin. It was observed that the roots of species are structurally adapted to epiphytism; however, some anatomical features show that this species requires more frequent watering or environments with constant humidity. The anatomical characteristics described for roots support results reported by authors that include the genus in subtribe Catasetinae, Cymbidieae tribe. The anatomical characters have generic uniformity, serving as a tool for the genus's systematic**.**

Key words: Roots, orchid, morphoanatomy, adaptation

Introduction. Orchidaceae is one of the largest families of monocots and includes about 850 genera and 25,000 species (Atwood 1986) prevalent in the tropics (Pabst & Dungs 1975, 1977).

The cultivation of orchid species is considered very important commercially. This is valid for Brazilian floriculture agribusiness, mainly due to the large capacity for genetic recombination, beauty, shape, size and the durability of the flowers which are presented in several species (Zanenga-Godoy & Costa 2003).

The Neotropical genus *Galeandra* Lindl. includes about 18 species distributed from southern Florida to northwest Argentina (Monteiro *et al.* 2010). The majority of species are found in Brazil, which is why the country is considered a diversity center, owning 16 valid *taxa* and two national endemic species (Barros *et al.* 2014). All representatives of genus are herbaceous with cylindrical-fusiform or ovoid, short and thickened pseudobulbs, according to the habit which can be epiphytic or terrestrial. The most striking features to distinguish between species are the presence or absence of trichomes on the surface of the lip and column, the number of keels and the shape of the lip when spread (Monteiro *et al.* 2009).

Galeandra was included in subtribe Cyrtopodiinae by Dressler, however, this classification was based solely on morphological characters, especially: presenting pseudobulbs with several internodes, distichous leaves, resupinate flowers and complete pollinarium (Dressler 1993). On the other hand, several recent phylogenetic analyzes of the Catasetinae subtribe based on nuclear DNA sequences (ITS) and plastid (rps4) involving some species of *Galeandra* resulted in their inclusion in Catasetinae (Pridgeon & Chase 1998). Pridgeon *et al.* (2009) considering that there is not a single character that can connect *Galeandra* to Catasetinae subtribe other than homoblastics pseudobulbs. However, Freudenstein *et al.* (2004) obtained a very significant result in their studies. Using a combination of *rbcL* and *matK*, the relationship was evident, standing *Galeandra* as a brother clade supported on *jackknife* for 100%. Besides those already mentioned, other studies involving molecular analyzes, highlight the possible positioning of *Galeandra* in Catasetinae subtribe (Chase 2003, Pridgeon *et al.* 2009). Demonstrably it is a monophyletic group (Monteiro *et al.* 2010).

Among the species that comprise the genus, *Galeandra leptoceras* Schltr. is one of the most known. Originated in Colombia, it lives in tropical areas,

showing epiphytic habit, small size, and carrying up to five cylindrical pseudobulbs with lanceolate, acuminate, plicate and glabrous leaves possessing three to five ribs. The racemes type inflorescence is terminal, with approximately 5 cm in length and number of flowers ranging from five to seven. The flexuous, thin and upright inflorescence, presents elliptic-lanceolate floral bracts and acuminate format (Schlechter 1920).

Vegetatively, the roots of orchids in general spread over the surface of its phorophytes to absorb available nutrients. These are often present themselves infected with mycorrhizal fungi, which are responsible for facilitating the absorption of minerals by plants in a mutualistic and harmonious relationship. Such organs are anatomically defined by multistratified epidermis called velamen (Pridgeon 1987, Porembski & Barthlott 1988), which has the function to absorb and deliver water from the vicinity of the rhizosphere ceding it to other tissues during drought periods (Pridgeon 1987, Pedroso-de-Moraes 2000, Moraes & Almeida 2004).

Anatomical descriptions of roots are used as tool for taxonomic identifications (Porembski & Barthlott 1988) in correlation with molecular data (Pedroso-de-Moraes *et al.* 2012, 2013) and allow to find if in high humidity environments, constant irrigation can be dispensed so that the excess of water does not generate the decay of these organs (Pedroso-de-Moraes 2000).

Thus, besides the enrichment of systematic and anatomical knowledge, the description of the radicular anatomy characters in orchid species can be assisted in the development of efficient management techniques, aiming to improve phytotechnical aspects of plants for sale at the same time reducing production costs related to water use (Pedroso-de-Moraes *et al.* 2013).

This study aimed to describe the anatomical radicular organization in *Galeandra leptoceras* indicating hydric adaptations for epiphytism, which can be used in cultivation, and anatomical characters of taxonomic interest.

Materials and Methods

Botanical material –. The material comes from the Live Collection of Uniararas (greenhouse with 70% shading, subjected to daily irrigation) of Centro Universitário Hermínio Ometto - Uniararas, Araras (SP) (VHO) and corresponds to the following specifications: *Galeandra leptoceras* Schltr. (VHO: 63, 68, 72).

Anatomical evaluations –. To evaluate the root anatomy, five organs with an average length of 7 ± 1,5 cm of three adult plants (six years in culture) were fixed in FAA 50% and preserved in 50% alcohol (Johansen 1940). Each one was sectioned in the midline, with the aid of razors. Transverse sections were subjected to double staining with 0.05% Safrablau (Bukatsh 1972) and mounted in glycerin. For starch identification Lugol solution was used (Bücherl 1962); for lignin staining with hydrochloric phloroglucin (Jansen 1962); for lipids with Sudan III (Johansen 1940); and flavonoids with potassium hydroxide (Costa 1982). The most important aspects were recorded with a digital camera attached to a Olympus microscope (Model BX51).

Results. The cross section in roots of *Galeandra leptoceras* Schltr. f. & Warm, showed three distinct regions: velamen, parenchymatous cortex and vascular cylinder (Fig. 1A-C).

In cross section the velamen shows polygonal and elliptical cells and consists of 4-5 layers (Fig. 1A-B). The more external layer in velamen, referred as epivelamen, is formed by flat periclinally cells and the underlying layers are composed of larger and radially elongated cells.

The epivelamen features smaller cells than the inner layers. The endovelamen is formed by isodiametric cells possessing narrow and spiral thickenings (Fig. 1B).

The cortex has three distinct regions: exodermis, the layer below velamen, cortex and endodermis (Fig. 1A-B). The exodermic cells are isodiametric and bigger in relation to the other cortical layers. These present walls with little thickness except for the periclinal external wall, in which, histochemical tests with hydrochloric acid plus phloroglucin and Sudan III revealed the presence of lignin and suberin (Fig. 1B). Internally the exodermis, the cortical parenchyma, relatively developed, consists of 11-12 layers of parenchyma cells. These layers are generally formed by isodiametric, rounded and cells of different sizes, defining small triangular intercellular spaces (Fig. 1A-B). Common observations are mycorrhizal arbuscules near the exodermis (Fig. 1D) and crystals of calcium oxalate raphides type in idioblasts distributed throughout the cortex (Fig. 1F). Unistratified endodermis presents isodiametric cells with thickening

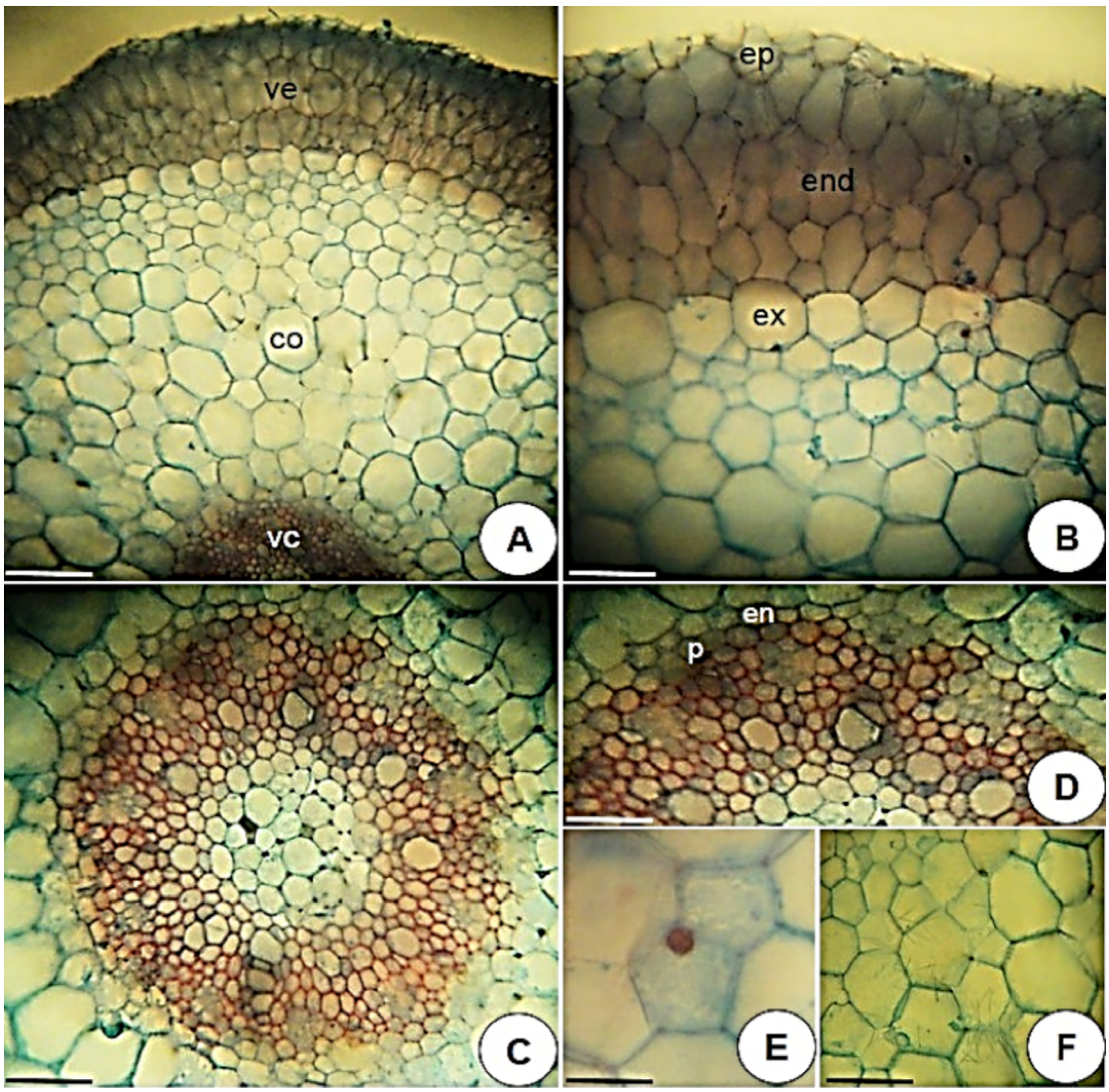

Figure 1 - Cross sections in roots of *Galeandraleptoceras*Schltr.f. A) general appearance of the epidermis and cortex; B) exoderm detail; C) vascular cylinder; D) endodermis and pericycle; E) fungal arbuscule; F) calcium oxalate raphids. co = cortex; n = endoderm; end = endovelamen; p = epivelamen; ex = exoderm; p = pericycle; ve = velamen; vc = vascular cylinder. Bars = A and D = 100 µm; E and F = 50 µm.

in the internal periclinal and anticlinal walls, except for the passage cells, which have thin walls (Fig. 1C-D). The root of *G. leptoceras* is poliarc and shows ten protoxylem poles. The medulla is formed by non-lignified parenchyma cells possessing thin walls and the presence of triangular cell spaces (Fig. 1C).

Discussion. The velamen is comprised by a specialized epidermis that consists of multiple layers of cells with thin walls, being bounded internally by the cortex. Ontogenetic studies in roots of orchids proved the origin of such tissue from periclinal divisions of protodermic cells and defined it as originating from dead cells, bearing secondarily thickened walls and filled with air when not hydrated (Pridgeon 1987).

The velamen, besides described for Orchidaceae, is also recognized in other plant families, such as: Araceae, Liliaceae, Dioscoreaceae, Taccaceae, Ama-

ryllidaceae and Commelinaceae (Dahlgren *et al.* 1985). This tissue protects the interior of the root avoiding heating and consequent water loss; and also preventing the excessive accumulation of this element (Pridgeon 1987, Gonzaga & Gonzaga 1996). The existence of the canopy is related to epiphytism (Engard 1944, Dycus & Knudson1957), however, this tissue may also occur less frequently in terrestrial orchids (Porembski & Barthlott 1988, Stern *et al.* 1993a, 1993b, Kurzweil *et al.* 1995).

In some Catasetinae, the tissue presents itself well-developed, for example, in *Mormodes tapoayensis* F.E.L. Miranda & K.G. Lacerda, where the velamen is constituted by eight layers (Stern & Judd 2001). However, for many species of *Catasetum* L. C. Rich. and *Cycnoches* Lindl. genera, an average number of layers between 4-6 is reported (Stern & Judd 2001; Pedroso-de-Moraes *et al.* 2012).

The velamen is usually divided into two parts: the epivelamen and endovelamen. The epivelamen arises from deeper tissue layers and its cells do not present protoplasts in maturity (Shushan 1959, Sanford & Andalawo 1973, Noel 1974). As in other Catasetinae, particularly for most species of *Mormodes* Lindl. genus, epivelamen cells are smaller than the endovelamen ones (Stern & Judd 2001), having this character been clearly demonstrated for the species *Mormodes sinuate* Rchb.f. & Warm. (Pedroso-de-Moraes *et al.* 2013).

It was found in the examined roots that the composition of the cell walls in velamen is of cellulose with different degrees of impregnation for lignin and suberin, with a degree of lignification and suberization that can vary widely between species of orchids (Noel 1974, Benzing *et al.* 1983). Furthermore, one of the possible functions of the wall thickening in velamen is to provide support and prevent cell collapse during drying (Noel 1974).

It is common in the inner layer of velamen of the roots of orchids, the presence of specialized cells called tilossomes, which assist in condensation of water vapor and other gases (Pridgeon 1987). However, these cells are absent in Catasetinae, therefore they do not appear in *Galeandra leptoceras* nor in *Catasetum barbatum* (Lindl.) Lindl., *Catasetum fimbriatum* (C. Morren.) Lindl., *Catasetum gnomus* Linden & Rchb. f., *Catasetum viridiflavum* Hook., *Clowesia amazonica* K. G. Lacerda & V. P. Castro and *M. sinuata* (Stern &Judd 2001, Pedroso-de-Moraes *et al.* 2012; 2013).

In the analyzed root, the cortical region showed a unistratified exodermis and the existence of thickening in the outer tangential wall, which was also observed for *C. fimbriatum* (Oliveira & Sajo 1999), to *M. sinuata* (Pedroso-de-Moraes *et al.* 2013) and several representatives of the genus *Mormodes* (Stern & Judd 2001). The set exoderm-velamen functions as a system in which long suberized and lignified exodermic cells protect cortical parenchyma against desiccation (Haberlandt 1914).

The number of layers in velamen, in cortex and the poles of protoxylem are anatomical characteristics that may influence the radicular diameter (Moreira & Isaias 2008, Pedroso-de-Moraes *et al.* 2012). However, each one shall exercise in greater or lesser extent the overall diameter, the environment, in turn, influence the development of these tissues and radicular structures and hence in diameter (Pedroso-de-Moraes *et al.* 2013).

This relationship was confirmed in studies with different plants, in which it was found that in environments characterized by water scarcity, a reduced number of cortical layers are found in plant species, suggesting that the shortest distance between the substrate and the stele facilitate the absorption of water under these circumstances (Fahn 1982). Still, radicular anatomic differences were found in roots of cultivars of sugar cane developed in different soil conditions: dry, wet and irrigated. The relationship between the measurements of the thickness of the cortex and vascular cylinder were higher in irrigated or waterlogged soils than in dry. Also, the thickening in cell walls of parenchyma occurring between the poles of xylem and phloem throughout development, were higher for roots in the cultivar developed under dry conditions (Venkatraman & Thomas 1922).

There is a relationship between the root diameter and the number of poles of protoxylem (Rütter & Stern 1992) in which roots of larger diameter, usually present more poles (Rosso 1966). However, this number varies in genus, according to different roots of the same species and the same root in different

height levels (Rütter & Stern 1992).

Mycorrhizal fungi are always present in habitats of orchids because they are essential for germination, development and distribution of such plants (Piccoli *et al.* 2014). Thus, larger populations of fungi are found near mature orchids (Perkins & McGee 1995, Batty *et al.* 2001, Otero *et al.* 2004, Diez 2007), which directly leads to the observation of mycorrhizal arbuscules often present in the radicular cortices of orchids as Catasetinae. The presence of raphides in different plant organs is common in Orchidaceae (Metcalfe 1963). Idioblasts with raphides are formed by cells produced by unequal divisions in the meristem (Shushan 1959, Chiang 1970).

The endodermis is unistratified and their isodiametric cells present thickening in O, such as *M. sinuata* (Pedroso-de-Moraes *et al.* 2013) and most Catasetinae (Stern & Judd 2001, Pedroso-de-Moraes *et al.*2012).

The roots of orchids can be classified into 12 types according to the occurrence and combination of the following characters: epivelamen, number of layers of velamen, type of wall thickening in velamen and exodermis cells and number of cell layers in the cortex (Porembski & Barthlott, 1988). The root of the studied species matches the type *Cymbidium* by owning epivelamen, exodermis with external tangential thickening and over eight cortical cell layers. This uniformity among the roots corroborates the results of Chase *et al.* (2003), which recognize a single monophyletic tribe - Cymbidieae formed by Maxillareae, Cymbidiinae, Eulophiinae, Bromheadiinae and Catasetinae. Still, the anatomical features found in the roots of *Galeandra lepdoceras* corroborate and support the anatomical findings by Stern and Judd (2001) and highlighted by Pridgeon *et al.* (2009). Furthermore, the observed characters have found certain uniformity with Catasetinae species, which can serve as a tool for the subtribe systematic.

Conclusion. The roots of the studied species presents structural characters that represent adaptation to epiphytic habit, moreover, the presence of 4-5 layers in velamen and 11-12 layers in cortex, shows that such plants require more frequent watering or environment with constant humidity, as found in their natural habitat.

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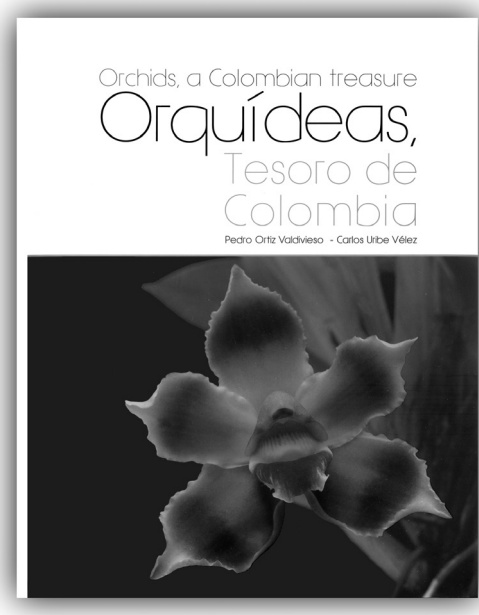
BOOK REVIEWS

Pedro Ortiz-Valdivieso† and Carlos Uribe Vélez. 2014. **Orquídeas, tesoro de Colombia / Orchids, a Colombian treasure. Tomo 1. A–D**. Da Vinci Publicidad y Medios, Bogotá. ISBN: 978-958-46-5329-1. Volume in 4to (29 x 23 cm), hardcover with dust jacket. 398 pp., 888 colour photographs and 499 line drawings. Bilingual, Spanish and English. 210.00 US$ / 178 €.



This impressive, large, solid and heavy hardcover book made of 398 pages printed on high-quality, semi-matte paper, is announced on the cover and the frontispiece as a work on the orchids of Colombia. One has to refer to the spine of the book, both on dust jacket and on the hardcover, to know that the volume is just the first release of a series including other two volumes to follow, to complete the monograph. This one, "*Tomo 1*", covers the genera between A (*Aa*) and D (*Dukeella*), dealing with 3848 species. The second volume is expected for the summer of 2015.

Colombia has not been stingy in terms of orchid books. Among the general works we have to cite at least the gorgeous volumes on microspermae from Mutis' "Flora de la Real Expedición Botánica del Nuevo Reino de Granada" (1963–1995), "Orquídeas colombianas / Colombian orchids" by Mariano Ospina (1958), the multi-authored "Orquídeas ornamentales de Colombia" (1980), the renown series "Native Colombian orchids", superbly edited by Rodrigo Escobar in four volumes plus two supplements (1991–1994), "Orquideas de Colombia" by Pedro Ortiz Valdivieso (1976) and his recent, mostly photographic "Orquídeas - Especies de Colombia" (2010). Colombian orchidology has also been enlighten by splendid books on more specific geographic areas of the country, like Guillermo Misas Urreta's "Orchids from the Serrania del Baudó, Chocó - Colombia" (2006), "Orchids in the mist: orchids of the cloud forests of southwestern Colombia", edited by Jorge E. Orejuela (2011), and the new "Orquídeas del valle de Aburrá" (2014) – a volume that we also review on this same issue of the journal –, with text by several authors and great photographs by Sebastian Vieira and other photographers, as well as on specific orchid groups, like Ortiz's "Las orquídeas del género *Masdevallia* en Colombia" (2000) and Óscar Duque's "Orchidaceae *Stelis* Swartz: Compendium" (2008), and on more specific topics, as Ospina's "Orchids and ecology in Colombia" (1996) and the "Libro rojo

de plantas de Colombia. Vol. 6: Orquídeas, primera parte", edited by E. Calderón-Sáenz (2006).

The new volume presented here represents the improved, hard printed version of a work previously presented in digital format with the title "*A gallery of Colombian orchids*". It is the combination of a book originally envisioned and partly written by the late Father Pedro Ortiz-Valdivieso (1926–2012), author of a large number of scientific papers and books on the orchid flora of Colombia, and a detailed introduction on morphology, ecology, pollination, and natural habitats of Colombian orchids (plus a final glossary of Spanish and English terms) by surgeon orthopedist Carlos Uribe Vélez, who – according to the information provided on the book jacket – devoted his last twenty years to the discovery, study and cultivation of orchids. Uribe Vélez also gathered all the available photographic records (including a large number of his own photographs), complementing them with schematic and partially coloured sketches of most of the species of each genus.

The introductory chapters, which offer some general information on orchid morphology and ecology (including reproductive biology), are illustrated with good and useful photographs depicting orchid habitats, variation in flower shapes and sizes, and enlarged flower details to explain some of the terms used troughout the texts. Even though most of the arguments are not specific to the orchids of Colombia, the photographs invariably illustrate Colombian specimens, and the images of pollinators in action will be of some interest to the student of pollination biology.

According to the original plan of the book by Father Ortiz, and on the basis of the texts that he wrote for the second edition of his "Orquídeas de Colombia" (1995), for each genus the original authorship, indication of the type species, etymology, distribution, a botanical description and an essential bibliography are provided.

The illustrations consist in one or more full page photographs and other, smaller photographs of other recorded species. Ink illustrations of the flower, mostly partially coloured and sometimes accompanied by a sketch of some diagnostic details, are provided for most of the species. The drawings of the flowers are pretty crude and highly schematic, and they are reproduced so small to preclude any real utility for species identification. The quality of the photographs is variable, but nonetheless it is adequate in most of the cases. Several of the photographs printed full page suffer however of an insufficient resolution and/or sharpness and they look quite blurred at the actual size. Close to each photograph and drawing a coloured dot indicates the climate (or perhaps the general range of temperature) where the plants grow.

A noteworthy point is that the photographs, even when they were taken by different authors with different photographic skills, depict nonetheless true Colombian plants and are therefore of real utility to understand the taxonomic concepts used by the junior author. So, for example, whilst the *Brassia caudata* on p. 133 is probably not the same species as the Jamaican type, it is however a real species from Colombia and therefore truly informative. In the same way, whilst one can debate about the use of the name *Cattleya aurea* versus *dowiana* subsp. *aurea*, the specimens illustrated on pages 180–181 do show some of the real variation exhibited by this taxon in Colombia.

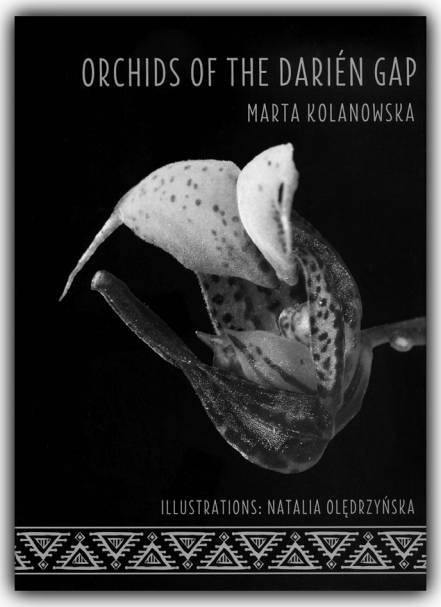
The classification follows a mix a old and new taxonomic "schools". So, for example, *Ada* is retained ad distinct from *Brassia*, *Bollea* as distinct from *Pescatoria, Cochlioda* from *Oncidium, Condylago* from *Stelis*, and *Diadenium* from *Comparettia*, while *Guarianthe* is treated under *Cattleya*, and the genus *Chondrohyncha* is treated in a broad sense, inlcuding *Aetheorhyncha, Benzingia, Daiotyla, Echinorhyncha, Euryblema, Ixyophora,* and *Stenotyla*, but not *Chondroscaphe* and *Cochleanthes*, which are treated independently instead. At the specific level, *Chondrorhyncha* no. 10 (p. 215) is correctely *Daiotyla xanthina*, while [*Chondrorhyncha*] *xanthina* (p. 216) is most probably a still undescribed species of *Daiotyla*. *Chondrorhyncha* sp. on p. 217 is almost surely a new *Euryblema* species. *Chaubardiella chasmatochila* is tretaed as different from *C. subquadrata*, so the true *C. subquadrata* is depicted under the synonymous name of *C. chasmatochila* (p. 208), while the photographs of *C. subquadarata* (p. 209) probably depicts an unnamed species. *Cischweinfia glicensteinii*, an invention by the late Eric A. Christenson from Costa Rica, is now sadly recorded also from Colombia. *Cochleanthes* sp. (p. 239, no. 3) is most surely a hybrid, albeit natural. *Dichaea pendula* is depicted under the synonymous

(and illegitimate name) *D. swartzii*, the congested form of *D. hystricina* is pictured as *D. selaginella*, and the infaustous name *D. muricata* is used for what it is probably the true *D. robusta*.

Apart from these few and very understandably mistakes, the book is a useful resource for the student of the Andean orchid flora. With an estimate diversity of around six thousand species, the Orchidaceae of Colombia requires any honest effort to disclose its true composition. With the first step of their three-volumes work, Ortiz-Valdivieso y Uribe Vélez surely did a genuine contribution to this aim.

Franco Pupulin
Lankester Botanical Garden, University of Costa Rica

Marta Kolanowska. 2014. **Orchids of the Darien Gap**. Koeltz Scientific Books, Koenigstein, Germany. ISBN 978-3-87429-475-1. Hardcover, 348 pages, 259 line figures, 162 color photographs. English. 141.60 US$ / 120 € approx.



The Pan-American Highway is a road interconnecting about 48000 km from Prudhoe Bay, USA to Ushuaia, Argentina. This road is continuous except at one point, the Darien Gap. This term refers to the interruption of the Pan-American Highway between Panama and Colombia. Large extensions of primary forest in the Panamanian province of Darién and the marshlands areas of Atrato river in the Department of Chocó in Colombia prevent the construction of a road link between the two countries. This region, still one of the most attractive for botanists because of its inestimable biodiversity has remained little explored until nowadays. The difficult terrain and the lack of roads added to the insecurity due to drug trafficking and guerrillas make the area intriguing and mysterious. Biologically this region has been treated as Chocó-Darién-Western Ecuador hotspot while the term Darien Gap is mostly associated with the ground interruption of the Pan-American Highway.

Marta Kolanowska presents a compilation of 270 orchid species for the Darien Gap, defined by the author as the political division of the province of Darién in Panama plus the Colombian regions from Sapzurro to Brazon Supiqui [?] in the Caribbean of Colombia, the Pacific coast of Chocó from Punta La Tortuguera to the Bahía Colombia between Choco and Antioquia excluding Serranía del Baudo. A map showing author's delimitation of the Darien Gap is included.

This compilation is entirely based upon 1700 herbarium specimens and digitized records in

databases on internet from about 20 herbaria. The book did not include fieldwork results except for the few photographs of the author ostensibly taken in the study area. A foreword of the Darien Gap with notes on natural conditions and wildlife conservation as well as a review of the orchid records are presented. The book is organized alphabetically for taxa above genus, genera and species. A key to the genera and dichotomous keys for species identification are provided. Information on generitypes and type specimens and their associated synonymy are cited. All genera and species have morphological descriptions, accompanied by 258 line drawings by Natalia Olędrzyńska mostly from perianth parts or just the lip. Most of the drawings are not strictly based on plants collected in Darien. The book includes illustrations copied or redrawn from taxonomic treatments published elsewhere and based on specimens collected outside the study area. Exsiccatae from Darien Gap are cited apart from the other specimens studied. Each morphological description is complemented by information on geographical distribution, habitat, ecology and brief taxonomic notes, sometimes avoiding discussions of recently published works that do not agree with the data presented by the author. One new species is described in the book as *Sobralia dariensis*. It is illustrated by a floral diagram and compared to *S. amabilis*.

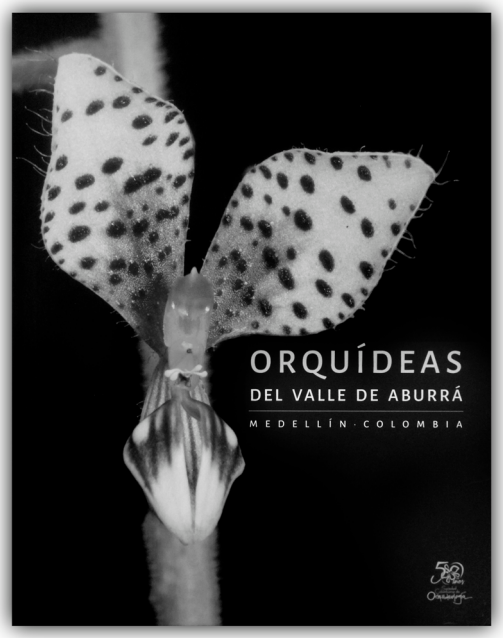
The classification system mostly follows *Systema Orchidalium* by Dariusz Szlachetko and it is similar to that adopted by the same author in the two volumes of "Orchids of the Department of Valle del Cauca". Kolanowska again recognized Cypripediaceae as a separate family from Orchidaceae (then it should not be strictly included in a book on orchids). Six subfamilies are recognized among them Spiranthoideae, Tropidioideae and Vandoideae are not currently accepted by the majority of orchidologists. The same issue is present with the adoption of the subtribes Comparettinae, Cryptarrheninae, Dichaeinae, Ionopsidinae, Ornithocephaliinae, Trichocentrinae and Trichopiliinae among others. The inclusion of *Hexisea, Nidema* and *Scaphyglottis* in Ponerinae also opposes the phylogenetic evidence available.

The book is complimented by 162 pictures that make the work colorful. However, as with the illustrations, most of the photographs are from specimens out of the Darien Gap. Despite being a significant effort, this book must be taken with caution especially for students entering in the taxonomy of orchids. The fact of using pictures or illustrations of specimens that are no native to Darien makes the taxonomic work less accurate and might introduce confusion. It is known that many species of orchids do not have such wide distributions. On the other hand the Darien region is biogeographically less akin to western Panama and Costa Rica and the north of Mesoamerica. A clear example is the *Cattleya dowiana*, a species native to the Caribbean region of Costa Rica and western Panama. The debate over whether populations from the Darien region and northern Colombia should be treated as a distinct subspecies of *C. dowiana* or as a distinct species under *Cattleya aurea* is still unresolved. It would have been helpful to clarify this if the author included at least photographs based on Darien specimens, however the photographs presented are based on plants from Costa Rica. Other examples are the pictures of the species complexes of *Specklinia* (*Empusella*) *endotrachys* (now splited in 5 species with *S. endotrachys* endemic to Costa Rica), *Stelis* (*Unciferia*) segoviensis, *Sobralia powellii* and *Prosthechea vespa*, which likely correspond to other species that are not native to Darien.

This book is a good compilation of species to the regions of Darien and Choco. Especially the citation of herbarium specimens and localities can serve as a basis for future floristic projects in the area. It is definite that many species not yet recorded will be revealed, so this book can serve as a preliminary approach to the understanding of this highly diverse and challenging Neotropical region.

Diego Bogarín
Lankester Botanical Garden, University of Costa Rica

Orquídeas del Valle de Aburrá, Medellín, Colombia. Sociedad Colombiana de Orquideología. Panamericana Formas e Impresos S.A., Colombia, 2014. ISBN: 978-958-58263-1-1. 397 pages, 285 color plates. 56.00 US$.



Last year the Colombian Orchid Society (Sociedad Colombiana de Orquidelogía) celebrated 50 years of existence, and in the midst of their celebrations published "Orquídeas del Valle de Aburrá". This heavy production has a very carefully presented hardcover and is just shy of four hundred pages. However, it is it's almost 300 color photographs that really make it impressive, especially because most of them are extremely beautiful.

The book starts off with a one page preface in which the mission of the society is given, together with an explanation as to how and why the work was conceived. Aburrá Valley in the sense of this book is said to cover "from Barbosa to the North, towards Caldas in the South, and from the sharp mountain edges of the East and West up to the river". The orchid species included are those that are found in the area currently and in the past. This is established using bona fide collection data from diverse collections and collectors, some of them from the time of the expeditions of Humboldt and Mutis.

The preface is followed by the generalities section, which presents a brief but useful historical account of botanical exploration in Colombia and morphological recognition and characterization of orchids. The taxonomy section shortly explains the authors´ line of thought behind the name usage in the book and gives an interesting description of the origin of plant classification and its use.

The rest of the book is dedicated to generously illustrating and describing each genus of the Orchidaceae that is or was to be found in Aburrá. Each genus includes brief discussions as to its taxonomy, generalities, distribution and habitat, and well as accounts on their conservation status and culture. The texts are useful for any orchid enthusiast, and help to understand the origin of many names and are rich in their details about the orchids themselves. Nevertheless, it is the photographs of the species treated here that really makes the book. Every genus includes at least one photograph, but most include a few. The species shown are those reported from the area (unfortunately they lack a voucher), and thus many overall rare species are depicted. We can only hope that the specimens depicted are indeed from the region. The authors account for some 390 species of orchids in the valley, distributed in more than 92 genera, making the region's orchid flora quite rich.

Finally, a discussion as to the conservation status of

the orchids at Aburrá is presented, together with their worries about the future of this immensely diverse region. Useful maps of the regions and their protected areas are also given.

The use of generic names is mostly what we would call "old school" as none of more modern generic circumscriptions are followed. *Brachtia, Fernandezia, Odontoglossum, Ophidion* and *Stellilabium* are recognized as distinct genera. Meanwhile, the genera *Erythrodes, Masdevallia, Maxillaria, Oncidium, Pleurothallis, Stelis* and *Trichosalpinx* are all kept in their "traditional sense". The controversial *Ida* and *Neooreophilus* are notable exceptions, being the only recently proposed genera that made the cut. Species-level taxonomy has been done with due care, and the authors have made little mistakes. The most unfortunate mixups are perhaps the photographs of *Pleurothallis* [*Apoda-prorepentia*] *kateora* under *Jacquiniella* sp. and of a species of *Psilochilus* under *Sauroglossum* sp. Finally, the photographs labelled *Pleurothallis* [*Specklinia*] *costaricensis* are certainly not that and are most probably of an undescribed species, while the species labeled *Epidendrum difforme,* a species endemic to the Antilles, is more likely to be *Epidendrum chlorocorymbos*.

The book is a must have for all students of the Colombian flora, be it professionally or not. For those that love orchids in general and especially like little known ones depicted in high quality photography this book is quite a delight.

Orquídeas del Valle de Aburrá is presented as a book authored by the Sociedad Colombiana de Orquidelogía, and thus seems authorless. Nevertheless, credits should be given to 5 editorial committee members, 7 text authors, 1 reviewer, 14 photographers and 1 designer.

Adam P. Karremans
Lankester Botanical Garden, University of Costa Rica

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- Prof. Franco Pupulin
  Editor-in-Chief, LANKESTERIANA
  Universidad de Costa Rica
  Jardín Botánico Lankester
  P.O. Box 302-7050 Cartago
  Costa Rica
  E-mail: franco.pupulin@ucr.ac.cr
  Phone number (+506) 2511-7949, 2511-7931
- Prof. Adam P. Karremans
  Managing editor, LANKESTERIANA
  Universidad de Costa Rica
  Jardín Botánico Lankester
  P.O. Box 302-7050 Cartago
  Costa Rica
  E-mail: adam.karremans@ucr.ac.cr
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  Editorial assistant, LANKESTERIANA
  Universidad de Costa Rica
  Jardín Botánico Lankester
  P.O. Box 302-7050 Cartago
  Costa Rica
  E-mail:melissa.diaz_m@ucr.ac.cr

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