Gynostemium structure and development in subtribe Spiranthinae (Cranichideae, Orchidaceae)

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We analysed gynostemium development and morphology of 13 species of Spiranthinae to understand the structure and homology of this organ. Flowers and flower buds in different developmental stages were examined with scanning electron and light microscopy. Gynostemium ontogeny is similar in the early stages among the analysed species, and most of the differences arose at later developmental stages. Gynostemium development starts with the appearance of the anther primordium, followed by the median carpel and finally by the lateral carpel apices. The last emerges as two congenitally united, crescent-shaped prominences located between the median carpel apex and the labellum, eventually forming a rim of tissue on the proximal margin of the stigma. The rim can be receptive or not. The base of the median carpel apex contributes mostly to the receptive stigmatic zone and its apical region develops into the viscidium. The entrance of the stylar canal is located between the two partially non-receptive lateral carpel apices and the receptive base of the median carpel apex, refuting the idea that in *Sarcoglottis* it is located above the stigmatic area. There are no staminodal primordia, and the membranaceous appendages at each side of the column apex represent extensions of the clinandrium margins.

ADDITIONAL KEYWORDS: flower – organogenesis – rostellum – rostellum remnant – staminodes – stigma – viscidium.

INTRODUCTION

Orchidaceae subtribe Spiranthinae (Orchidoideae, Cranichideae) are the most diverse lineage of terrestrial orchids in the Neotropics, including *c*. 40 genera and > 500 species (Salazar *et al*., 2018). They differ from their relatives by having tubular flowers (Fig. 1A–C) and a labellum that is adhered to the sides of the gynostemium, forming a basal nectar chamber together with the bases of the lateral sepals (Fig. 1D–F).

In Spiranthinae, the gynostemium is clavate, with the style and filament usually fully fused into a column part; the anther is dorsal and erect, and its base is held in a shallow cavity, the clinandrium, which often has lateral membranaceous margins. The receptive stigmatic area is located on the ventral surface (Fig. 1E, G). The pollinarium usually ends into an apical viscidium (Fig. 1H–L), which on removal leaves a variously shaped rostellar remnant (e.g. Salazar, 2003; Figueroa *et al*., 2012: fig. 1E–I; Salazar, Cabrera & Figueroa, 2011a: fig. 4; Salazar *et al*., 2018: fig. 2C–D, G–H, O, Q–T).

Traditionally, the generic classification of Spiranthinae relied mainly on attributes of floral morphology, especially features of the gynostemium, including the rostellum. The various classifications of Spiranthinae published during the 20th century, all focusing on the same set

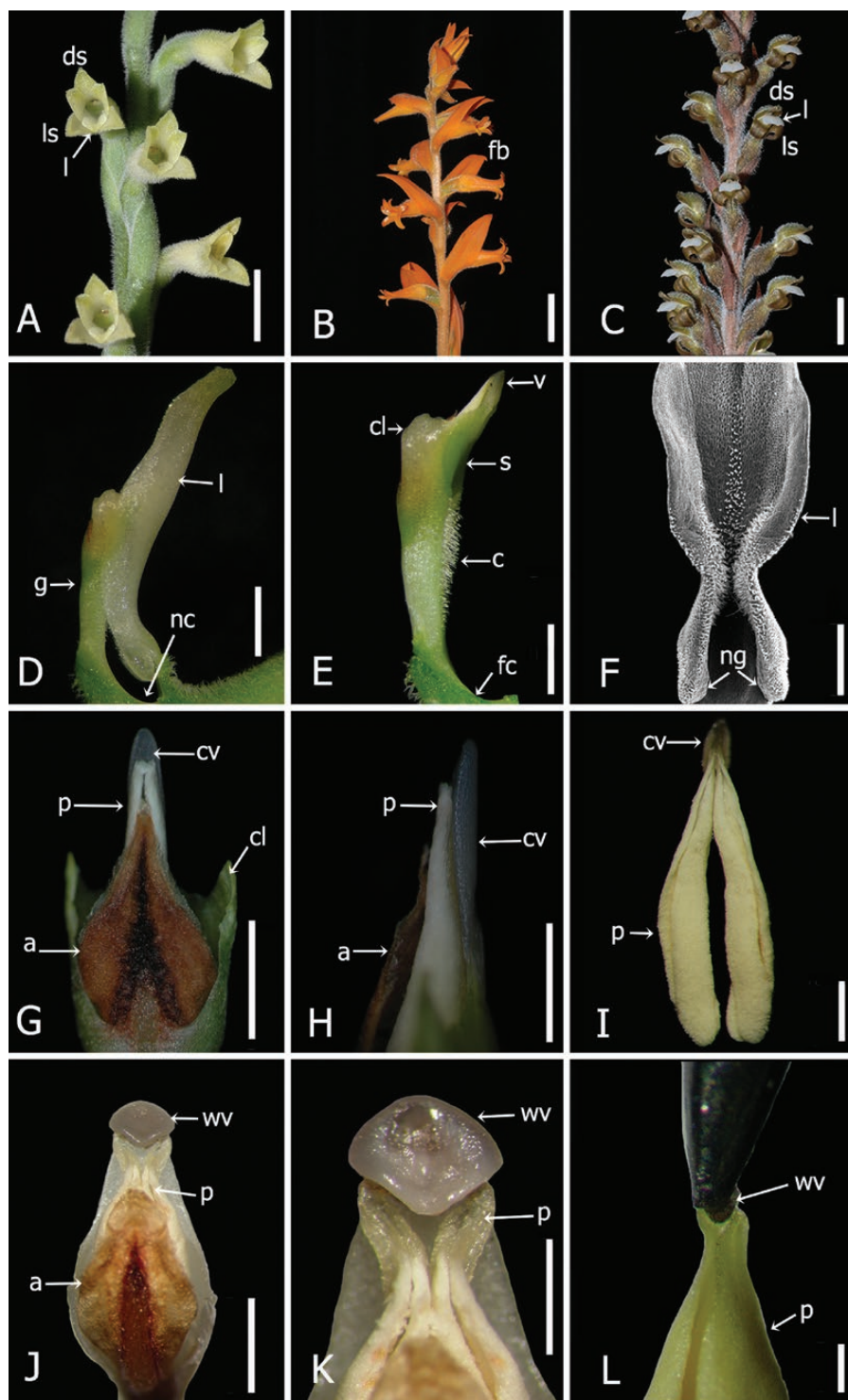*Corresponding authors. E-mails: cfigueroa@uacj.mx; gasc@ib.unam.mx

Figure 1. General morphology of selected members Spiranthinae. A, D–F, I, *Kionophyton sawyeri* (GAS 7252). B, *Dichromanthus aurantiacus* (s.n.). C, J, K, *Sarcoglottis schaffneri* (GAS 6584). L, *Cyclopogon luteoalbus* (GAS 7500). G–H, *Deiregyne albovaginata* (GAS 6560). A–C, Inflorescences, notice tubular flowers. D, Labellum adhered to the sides of the gynostemium. E, Gynostemium lateral view, notice stigma, columnar portion and apical viscidium. F, Labellum, nectarium. G–I, Cushion-type viscidium. G, Gynostemium dorsal view, see anther and clinandrium. H, Gynostemium lateral view, notice viscidium and pollinium. I, Pollinarium. J–L, Wedge-type viscidium. J, Gynostemium with wedge-type viscidium, dorsal

of floral characters, differed from each other regarding the number of genera accepted and the assignment of species to such genera (Schlechter, 1920; Balogh, 1982; Garay, 1982; Burns-Balogh, 1986; Szlachetko, 1995).

Recently published molecular phylogenetic analyses of Spiranthinae based on DNA sequences have provided an explicit evolutionary framework for comparative assessment of vegetative and floral morphology (Salazar *et al*., 2003, 2009, 2011a, 2011b, 2016, 2018, 2019; Figueroa *et al*., 2008, 2012; Salazar & Ballesteros-Barrera, 2010; Batista *et al*., 2011; Salazar & Dressler, 2011; Salazar & Jost, 2012; Borba *et al*., 2014). Such studies have revealed incongruence between some clades recovered based on DNA analyses and previous taxonomical approaches using intuitive assessment of floral morphological characters. Since many of the floral attributes used by taxonomists to define generic limits are directly involved in pollination, it is likely that such incongruences result from distantly related species evolving a similar floral structure because of adaptation to the same type of pollinator (e.g. Salazar *et al*., 2003, 2011a, 2019). Alternatively, the molecular phylogenetic trees have uncovered instances of closely related groups (i.e. sister clades) that exhibit notoriously divergent floral morphology (Batista *et al*., 2011; Salazar & Dressler, 2011).

Few studies have investigated comparatively the structure of the gynostemium in Spiranthinae. Rasmussen (1982) carried out a survey of gynostemium morphology and ontogeny of the 'neottioid orchids', a polyphyletic assemblage of taxa with an erect anther and soft pollinia now known to include members of Vanilloideae, 'lower' Epidendroideae and Orchidoideae (Cameron *et al*., 1999; Chase *et al*., 2003, 2015). Rasmussen (1982) provided brief descriptions of post-anthetic gynostemia of six species belonging to six genera of Spiranthinae, whereas Greenwood (1982) described the structure of the fully developed viscidium (the detachable portion of the rostellum that produces the viscid matter that sticks the pollinarium to the pollinator) of Mexican representatives of ten genera of Spiranthinae. In addition, Kurzweil (1988) conducted a comparative study of flower development in the neottioid orchids, including one species each of *Cyclopogon* C.Presl and *Sarcoglottis* C.Presl (Spiranthinae), for which he described some ontogenetic stages of the gynostemium. Szlachetko & Rutkowski (2000) published general descriptions of the post-anthetic gynostemia of the 51 genera that they recognized in Spiranthinae. More recently,

Figueroa *et al*. (2012) studied floral morphology and gynostemium development of *Dichromanthus michuacanus* (Lex.) Salazar & Soto Arenas, providing the first detailed description of gynostemium ontogeny for a member of Spiranthinae.

A better understanding of the structure and development of floral organs would establish a firm ground for homology assessments and resolution of controversies regarding the nature of some floral parts. For instance, Szlachetko & Rutkowski (2000) interpreted the usually pointed, membranaceous margins or appendages at each side of the clinandrium of many Spiranthinae as staminodes, whereas other authors have stated that staminodes are missing in all developmental stages in Spiranthinae (Kurzweil, 1988 for *Cyclopogon* sp.; Figueroa *et al*., 2012 for *Dichromanthus michuacanus*). Moreover, Burns-Balogh & Robinson, (1983) and Szlachetko & Rutkowski (2000) claimed that in most orchids, including Spiranthinae, the receptive surface of the stigma is formed by the lateral carpel apices, but the few ontogenetic studies published so far suggest that the receptive stigmatic area(s) usually are a part of the median carpel apex, the non-receptive portion of which corresponds to the rostellum (Rasmussen, 1982; Kurzweil, 1988, 1998; Figueroa *et al*., 2012). Burns-Balogh & Robinson (1983) also stated that, in *Pelexia* Poit. ex Lindl. and *Sarcoglottis*, the entrance of the stylar canal is located 'above the stigmatic area' instead of 'an area between the two functional lobes', an interpretation that was questioned by Salazar *et al*. (2018).

In this work, we conducted a comparative survey of the gynostemium morphology and its development in 13 species belonging to eight genera of Spiranthinae. The aims of this study are to: (1) provide a detailed description of gynostemium structure and ontogeny of representative species of Spiranthinae and (2) discuss several characters of the gynostemium that have been controversial, including the clinandrium appendages, the receptive part of the stigma, the entrance of the stylar canal and features of the rostellum/viscidium.

MATERIAL AND METHODS

TAXONOMIC SAMPLING

Specimens of 13 species of eight genera of Spiranthinae were studied. These species are representatives of three lineages of the subtribe: (1) the *Pelexia* clade:

view. K, Close up of viscidium and pollinium, notice de adhesive portion and lateral insertion of pollinium. L, Pollinarium with detachable viscidium. a, anther; c, column; cl, clinandrium; cv, cushion viscidium; ds, dorsal sepal; fb, floral bract; fc, foot column; g, gynostemium; l, labellum; ls, lateral sepal; nc, nectar chamber; ng, nectar gland; p, pollinium; s, stigma; v, viscidium; wv, wedge viscidium. Scale bars: 2 cm (B); 1 cm (A, C); 2 mm (F); 1 mm (D, E, G, H, J); 0.5 mm (I, K); 100 μm (L).

Cyclopogon (two species) and *Sarcoglottis* (two species); (2) the *Spiranthes* Rich. clade: *Aulosepalum* Garay (one species), *Deiregyne* Schltr. (one species), *Dichromanthus* Garay (three species), *Kionophyton* Garay (one species) and *Mesadenus* Schltr. (one species) and (3) the *Stenorrhynchos* Rich. ex Spreng. clade: *Sacoila* Raf. (one species) and *Stenorrhynchos* (one species). A list of voucher specimens used for undertaking the morphological and developmental observations is provided in Table 1.

MORPHOLOGICAL OBSERVATIONS

Fresh flowers at anthesis were dissected and photographed with a digital camera (Nikon D7000, Nikon, Tokyo, Japan) provided with a 60 mm AF Micro Nikkor lens (Nikon) or under a stereomicroscope (Stemi SV 6, Carl Zeiss) with a digital camera (Coolpix 5200, Nikon). Inflorescences at different stages of development were collected in the field or from plants cultivated in a greenhouse in Mexico City.

SCANNING ELECTRON MICROSCOPY

Flowers and floral buds were fixed in FAA (5% formaldehyde, 5% acetic acid, 50% ethanol; Sass, 1958), rinsed in water and stored in ethanol 50%. Samples were dehydrated in an ethanol series from 50 to 100% in increments of 10%, keeping the samples in each concentration for at least 48 h; then the samples were critical point dried, coated with gold and examined using a scanning electron microscope (SEM) Hitachi S-2460 N (Hitachi, Tokyo, Japan) operating at 15 kV.

Micrographs were taken with a camera (Pentax Z10, Japan) using 35-mm Kodak 100 TMAX film and the negatives were subsequently digitized using a scanner (Nikon Super Coolscan 5000, Nikon).

LIGHT MICROSCOPY

Floral buds were dehydrated using isopropyl alcohol (50, 70, 80, 96, 100, 100%) for 2 h at each concentration (Figueroa *et al*., 2012). Then the tissue was infiltrated in isopropyl alcohol:xylene (3:1, 1:1, 1:3) and xylene (two changes) for 4 h each. After this, the tissue was embedded in paraffin with two changes for 12 h each. Transverse and longitudinal sections (10 µm thick) were cut using a rotatory microtome (American Optical Company 820, New York, USA). Paraffin sections were mounted on glass slides with chromium gelatine adhesive. Staining was performed with Safranin O-fast green (Johansen, 1940). Sections were mounted in synthetic resin and dried on a hot plate at 60 °C. Slides were examined using a light microscope (Olympus BX51, Tokyo, Japan) and photographed with a digital camera (Evolution LC Color digital, Maryland, USA).

RESULTS

EARLY ONTOGENY

Early floral organogenesis is similar in the 13 species that were studied. The flowers develop acropetally in a spiral (Fig. 2A). Development starts with a floral primordium located in the axil of the floral bract (Fig. 2A, B). The floral primordium is a transversely

Table 1. Taxa studied and voucher information. Vouchers was deposited at Herbario Nacional de México (MEXU)

| Taxa	Origin	Voucher Anatomy
Aulosepalum pyramidale (Lindl.)M.A.Dix & M.W. Dix	Mexico, Distrito Federal	*Figueroa 251*; *Salazar 6334, 7381*
Deiregyne albovaginata (C.Schweinf.) Garay	Mexico, Distrito Federal	*Figueroa 252, 270, 281*
Cyclopogon luteoalbus Schltr.	Mexico	*Salazar 5896*
Cyclopogon ovalifolius C.Presl.	Ecuador	*Salazar s/n* Nov. 2007
Dichromanthus aurantiacus (Lex.) Salazar & Soto Arenas	Mexico Distrito Federal	*Figueroa 183, 249*
Dichromanthus cinnabarinus (Lex.) Garay	Mexico Distrito Federal	*Figueroa 181, 250*; *Salazar 6493*
Dichromanthus michuacanus (Lex.) Salazar & Soto Arenas	Mexico Distrito Federal	*Figueroa 192*; *Soto 10166*
Kionophyton sawyeri (Standl. & L.O.Williams) Garay	Mexico Edo. De México	*Salazar 7252*
Mesadenus lucayanus Schltr.	Mexico	*Salazar 6714, 6687*
Sacoila lanceolata (Aubl.) Garay	Mexico	*Kew 200–1578*
Sarcoglottis assurgens Schltr.	Mexico, Guerrero	*Figueroa 302*
Sarcoglottis schaffneri Ames.	Mexico, Distrito Federal	*Figueroa 293, 253*; *Salazar 7720, 6584, 7105*
Stenorrhynchos glicensteinii Christenson	El Salvador	*Salazar & Linares 7532*
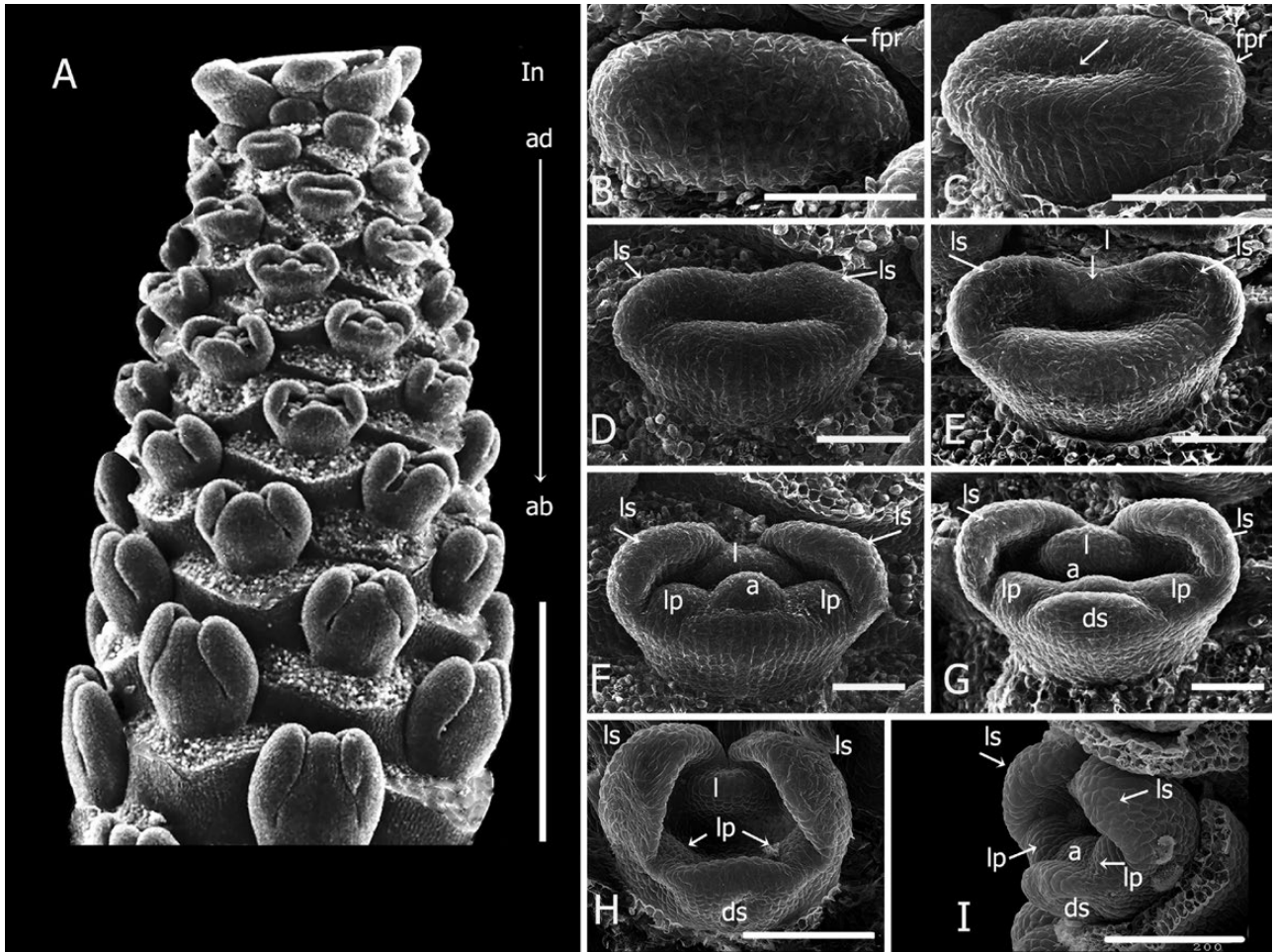
Figure 2. Organogenesis in Spiranthinae. A–G, *Aulosepalum pyramidale*, see anther prior dorsal sepal. H, *Stenorhynchos gliscensteinii*. I, *Cyclopogon ovalifolius*. A, development of inflorescence. B, C, Ellipsoidal floral primordium. D, E, Early development of lateral sepals. F, Petals and anther primordia. G, Dorsal sepal, the last primordium. H, I, See dorsal sepal prior anther. a, anther; ab, abaxial; ad, adaxial; ds, dorsal sepal; fpr, floral primordium; In, inflorescence; l, labellum; lp, lateral petals; ls, lateral sepals. Scale bars: 1 mm (A); 100 µm (B–G); 200 µm (H, I).

ellipsoid protuberance, which develops a transverse depression due to proliferation of the peripheral tissue (Fig. 2C). Organ differentiation follows in an adaxial–abaxial direction, beginning with the lateral sepals, followed by the labellum, the petals, the anther and the dorsal sepal in *Aulosepalum pyramidale* (Lindl.) M.A.Dix & M.W.Dix, *Deiregyne albovaginata* (C.Schweinf.) Garay, the three species of *Dichromanthus* and *Mesadenus lucayanus* Schltr. (all belonging to the *Spiranthes* clade) (Fig. 2D–G). On the other hand, in the representatives of the *Pelexia* clade (*Cyclopogon luteoalbus* Schltr., *Cyclopogon ovalifolius* C.Presl., *Sarcoglottis assurgens* Schltr., *Sarcoglottis schaffneri* Ames) and in *Stenorrhynchos glicensteinii* Christenson (*Stenorrhynchos* clade), the dorsal sepal primordium differentiates before the anther (Fig. 2H–I). Such

early stages could not be observed for *Kionophyton sawyeri* (Standl. & L.O.Williams) Garay and *Sacoila lanceolata* (Aubl.) Garay, because at the time of collecting only inflorescences in intermediate stages of development and anthesis were found; hence we could not determine whether the anther or the dorsal sepal develops first in these species. The perianth segments subsequently acquire their laminar shape due to periclinal and anticlinal cell divisions and the anther rapidly increases in size (Fig. 2A).

GYNOSTEMIUM DEVELOPMENT

The development of the gynostemium starts with the appearance of the anther primordium, followed by the median carpel apex and finally by the lateral carpel apices (Fig. 3A, E). The full sequence of development
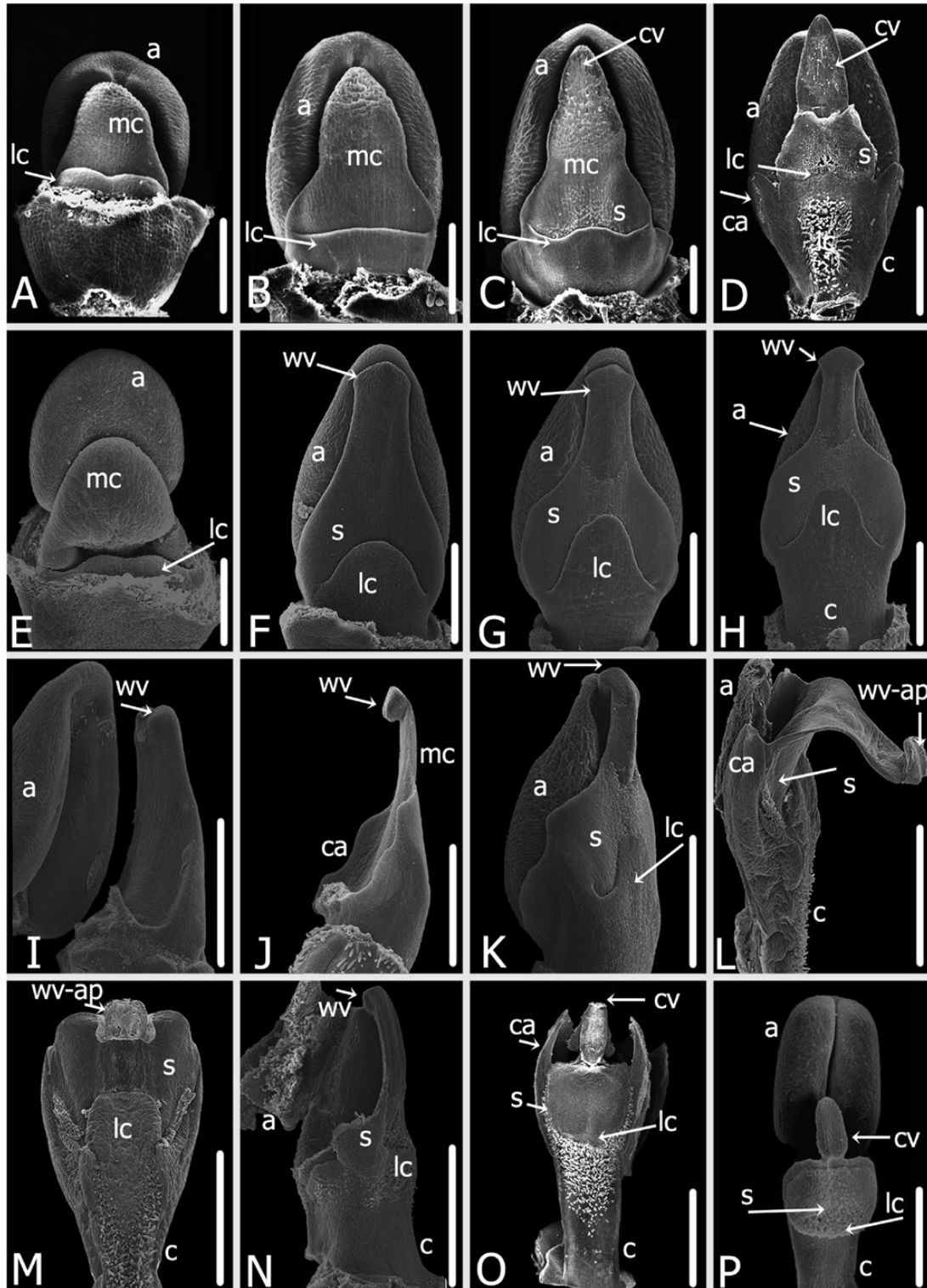
Figure 3. Gynostemium development. A–D, *Aulosepalum pyramidale*. E–M, *Sarcoglottis assurgens*. N, *Cyclopogon luteoalbus*. O, *Deiregyne albovaginata*. P, *Mesadenus lucayanus*. A–D, Development of gynostemium with cushion-type viscidium. A, early gynostemium with anther, median carpel and lateral carpels. B, Viscidium differentiation. C, Stigma differentiation. D, Pre-anthetic gynostemium. E–M, Development of gynostemium with wedge-type viscidium. E, Early gynostemium with anther, median carpel and lateral carpels. F, Viscidium differentiation. G, Stigma differentiation. H,

of the anther and derived structures and the gynoecial structures (including the rostellum and viscidium), is described in the following.

Anther

The anther primordium arises as a bulge opposite to the labellum, either before or after the dorsal sepal primordium (see earlier). Differentiation of the thecae begins soon after, as the anther starts elongating (Fig. 3B–C). In none of the studied species was there an indication of primordia that may give rise to staminodes. Instead, the membranaceous appendages at each side of the column apex that are evident at anthesis in some of the species seem to represent mere extensions of the clinandrium margins (Fig. 3D, O). The mature anther is erect and opens adaxially, and it has two thecae, each partially divided into two cavities by sterile tissue. Thus, each of the two pollinia is deeply cleft. In most cases the filament is fully adnate to the columnar part, but in *Mesadenus* it is partially free (Fig. 4H). The viscidium, produced at the apex of the rostellum (see later), usually is attached to the apex of the pollinia, except in *Mesadenus*, in which it is placed near the middle of the adaxial side of the pollinia (Fig. 3P; see Salazar *et al.*, 2019).

Stigma and rostellum

The median carpel apex first emerges as a rounded bulge opposite to the anther and quickly elongates until it equals the length of the anther (Fig. 3A–H). The only exception is *M. lucayanus*, in which the median carpel apex stops elongating when it has reached about one-half of the anther length (Fig. 3P; see Salazar *et al.*, 2019). As the median carpel apex elongates, the lateral carpel apices emerge as two congenitally united, crescent-shaped prominences located between the median carpel apex and the labellum (Fig. 3A–H). As development proceeds, the median carpel apex gives rise to both the rostellum and most or all the receptive stigmatic surface (Fig. 3I–L). Frequently, the lateral carpel apices constitute a rim at the proximal (basalmost) margin of the mature stigma, without contributing to the receptive surface (Figs 3D–H, K, M–P, 4A–F). However, in *M. lucayanus*, *K. sawyeri*, *Sacoila lanceolata* and *Stenorrhynchos glicensteinii*, the rim is papillate, as the receptive part of the median carpel apex, and all three carpel apices appear to contribute, in different proportions,

to the mature stigma (Fig. 4G–L). In *S. lanceolata*, the receptive papillose rim is prominent and somewhat recurved (Fig. 4K, L). The stigmatic papillae reach their maximum size as anthesis approaches, and the stigmatic area develops a viscous film, probably secreted by dissolving papillae (Fig. 4D, E); this film gives a shiny appearance to the mature stigma in fresh condition. The stigmatic papillae are not evident in SEM micrographs, but they are clearly seen in anatomical sections, most probably because in the latter the previously mentioned film dissolves during sample processing.

Viscidium differentiation involves an increase in protoplast density and, sometimes, changes in cell organization and orientation at the respective portion of the rostellum. In most of the studied species, there are tangentially elongated cells on the ventral surface of the rostellum (i.e. toward the surface on which the stigma is located), but in some species (*A. pyramidale*, *M. lucayanus*) the cells remain isodiametric throughout development (Fig. 5A–H). Both the 'cushion-type' and the 'wedge-type' viscidia, first described by Greenwood (1982) as occurring in Spiranthinae, are present among the examined species. The most common type is the cushion-type, which is present in all the species of the *Spiranthes* and *Stenorrhynchos* clades; in these, the adhesive portion is located on the ventral side of the rostellum, with the pollinia being attached to its dorsal surface (Fig. 5A–H). In contrast, in the wedge-type viscidium, present in all members of the *Pelexia* clade examined (*Cyclopogon* spp. and *Sarcoglottis* spp.), the adhesive surface of the viscidium is located on the dorsal side of the rostellum (Fig. 5I–K); the viscidium is situated between the divergent apices of the pollinia.

As anthesis approaches, the viscidia show a 'rupture zone' that enables its separation from the rest of the rostellum, during the removal of the pollinarium by a pollinator (Fig. 5D, H, L, T). Near anthesis, the cells that form the rupture zone show degradated cell walls, and at anthesis such cells are dissolved (Fig. 5N, P, R, T). Such a rupture zone enables the formation of a distinctive portion of tissue named 'rostellum remnant', which is variable in shape and length, between the stigma and the viscidium. The rostellar remnant is particularly developed and evident in the species of *Pelexia* and *Stenorrhynchos* clades, being oblong-triangular in *C. luteoalbus* and *Sarcoglottis* spp. (Fig. 5L) and

Pre-anthetic gynostemium. I–L, Similar phases, lateral positions. M, Anthetic gynostemium. N–P, Three different gynostemia at anthesis. a, anther; c, column; ca, clinandrium appendages; cv, cushion viscidium; lc, lateral carpel; mc, median carpel; s, stigma; wv, wedge viscidium; wv-ap, adhesive part in wedge viscidium. Scale bars: 2 mm (L, M, O); 1 mm (D, H, J, K, N, P); 500 μm (C, F, G, I); 200 μm (A, B, E).

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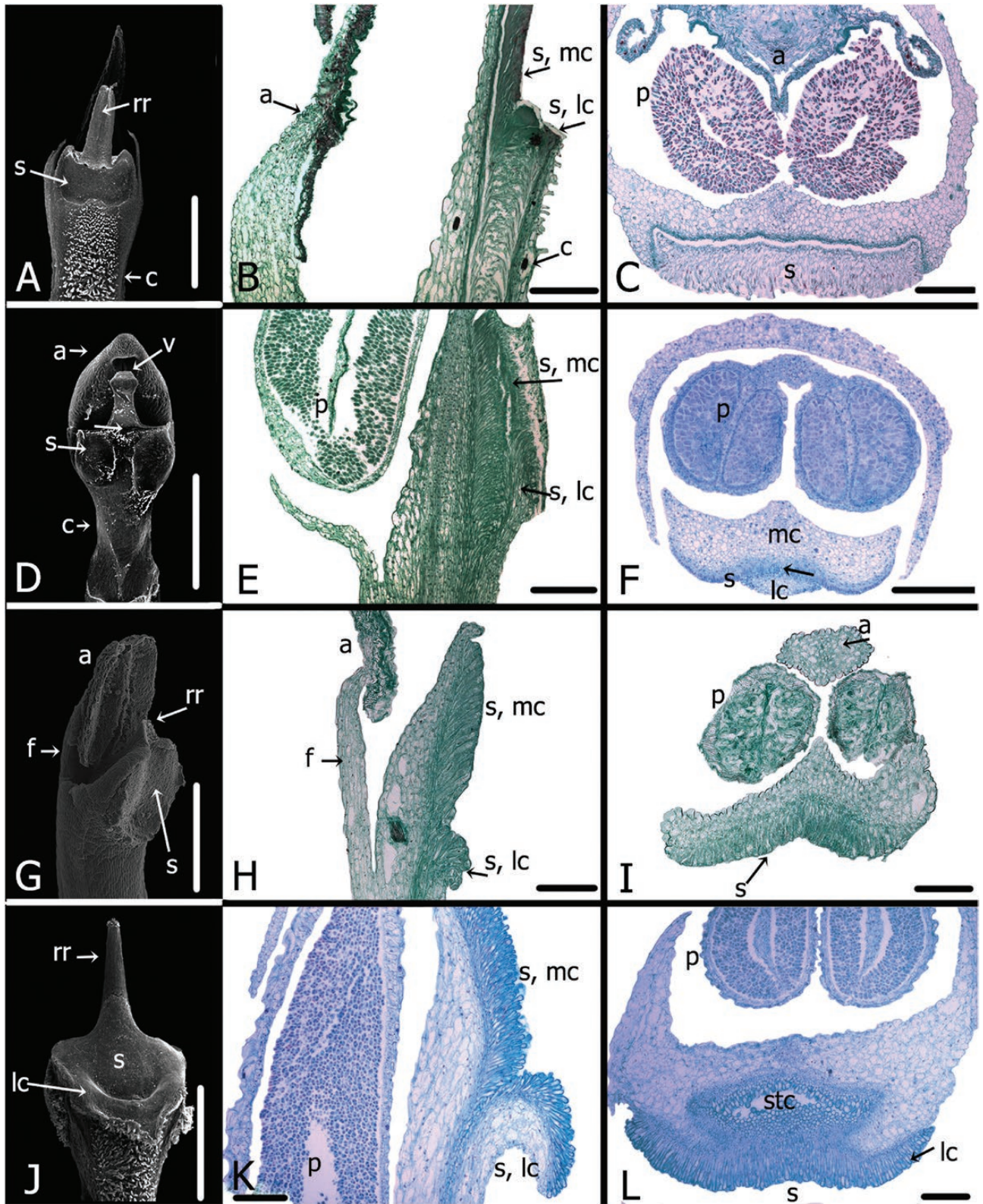
Figure 4. Stigma receptive zone in anthetic gynostemia. A–C, *Dichromanthus cinnabarinus*. D–F, *Sarcoglottis schaffneri*. G–I, *Mesadenus lucayanus*. J–L, *Sacoila lanceolata*. A, Gynostemium ventral view. B, Stigmatic area formed by median

narrowly triangular in *Sacoila* and *Stenorrhynchos* (Figs 4J, 5T). The position of the rupture zone varies among genera and results in differences in the shape of the respective rostellum remnant, depending on whether a cushion-type or a wedge-type viscidium is present. Among the species with a cushion-type viscidium, the rostellar remant may be merely an inconspicuous transverse band of tissue, as in *Aulosepalum* (Fig. 5D), a short apicule as in *Mesadenus* (Fig. 5H) or a long point, which can be flexible in the case of *D. albovaginata* (Fig. 5P) and *K. sawyeri* or stiff in *Sacoila* and *Stenorrhynchos* (Fig. 5S, T). In taxa with a wedge-type viscidium, rupture always occus transversely at the base of the viscidium, leaving an oblong-triangular, truncate to shallowly emarginate rostellum remnant, which is characteristic of most genera of the *Pelexia* clade (Fig. 5L; Salazar *et al.*, 2018: Fig. 2R, T).

Columnar portion

Once the stigma, rostellum and viscidium have developed, the columnar part of the gynostemium (i.e. the main body of the column below the stigma, rostellum and anther) elongates to reach different lenghts, depending on the species (Fig. 1D, E, 3L–P). The columnar part of the gynostemium has a ventral extension at the base that is decurrent on the ovary. It is known as the column foot, which in *Sacoila* spp. and *Sarcoglottis* spp. is as long or longer than the gynostemium proper, although in the remainder taxa studied in this work it is shorter. In Spiranthinae, the column foot usually constitutes the 'roof' of the nectar chamber, which is formed by the fused bases of the lateral sepals and the labellum at the base of the floral tube.

DISCUSSION

EARLY ONTOGENY

In Spiranthinae, as in other orchids, the order of differentiation of the organs is directional, i.e. the floral organ development follows an adaxial–abaxial direction. Such directionality is related to the acquisition and maintenance of zygomorphy (Endress, 1990, 1994, 1999). Our observations of the initial organogenesis of the 13 species investigated in this study agree with what has been described previously for other members of Spiranthinae (Kurzweil, 1988, 1998). Zygomorphy is evident from the beginning in the transversely ellipsoidal floral primordium, as in other monandrous orchids (Epidendroideae, Vandoideae and Orchidoideae). In contrast, the non-monandrous subfamilies Apostasioideae (Kocyan & Endress, 2001) and Cypripedioideae (Kurzweil, 1993) show a rounded floral promordium. Some of the species examined here follow the usual order of floral organ appearance in Orchidaceae (Kurzweil, 1988, 1998): lateral sepals, labellum, lateral petals, dorsal sepal, anther, median carpel and lateral carpels, including *C. luteoalbus*, *S. schaffneri*, *S. assurgens* and *Stenorrhynchos glicensteinii*. However, in six species (*A. pyramidale*, *Deiregyne albovaginata*, *Dichromanthus aurantiacus* (Lex.) Salazar & Soto Arenas, *Dichromanthus cinnabarinus* (Lex.) Garay, *D. michuacanus* and *M. lucayanus*), we found that the anther begins to differentiate before the dorsal sepal, which had not been previously reported in Spiranthinae, but it occurs in *Dactylorhiza maculata* (L.) Soó (subtribe Orchidinae; Kurzweil, 1987). All the species showing the 'atypical' developmental order belong to the *Spiranthes* clade (Fig. 6A), but beyond noticing that this may be a potential morphological synapomorphy for that clade, we have not observed any functional or structural difference associated with it. Some development studies have shown that a delay or acceleration of the timing of differentiation of the floral organs could explain variations in perianth symmetry (Endress, 1999; Pabón & González, 2008), but we are unaware of similar studies focused on the gynostemium.

GYNOSTEMIUM DEVELOPMENT

Anther

Gynostemium ontogeny of the species included in this work is similar to that described for other representatives of Cranichideae, such as *Goodyera repens* (L.) R.Br., *Ludisia discolor* (Ker Gawl.) Blume (Goodyerinae; Rasmussen, 1982), *Ponthieva parvilabris* (Lindl.) Rchb.f. (Rasmussen, 1982), *Prescottia plantaginea* Lindl. (Cranichidinae; Kurzweil, 1988) and *Sarcoglottis* sp. and *Cyclopogon* sp. (Spiranthinae; Kurzweil, 1988). The first organ that differentiates is the anther, followed by the median carpel apex and then the lateral carpel apices.

carpel. C, Stigma papillae. D, Gynostemium ventral view. E, Stigma and no receptive lateral carpels. F, No receptive lateral carpels. G, Gynostemium with short apiculate rostellum remnant. H, Stigma with three receptive carpels. I, Stigma papillae. J, Gynostemium ventral view. K, Stigma, see three receptive carpels and recurved lateral carpels. L, Stigma, see the stylar canal entrance and prominent papillae. Transversal section (C, F, I, L). Longitudinal section (B, E, H, K). a, anther; c, column; f, filament; lc, lateral carpel; mc, median carpel; p, pollinium; rr, rostellum remnant; s, stigma; stc, stylar canal; v, viscidium. Scale bars: 2 mm (A, G, J); 500 μm (D); 300 μm (B, C, H, I, K, L); 100 μm (E, F).
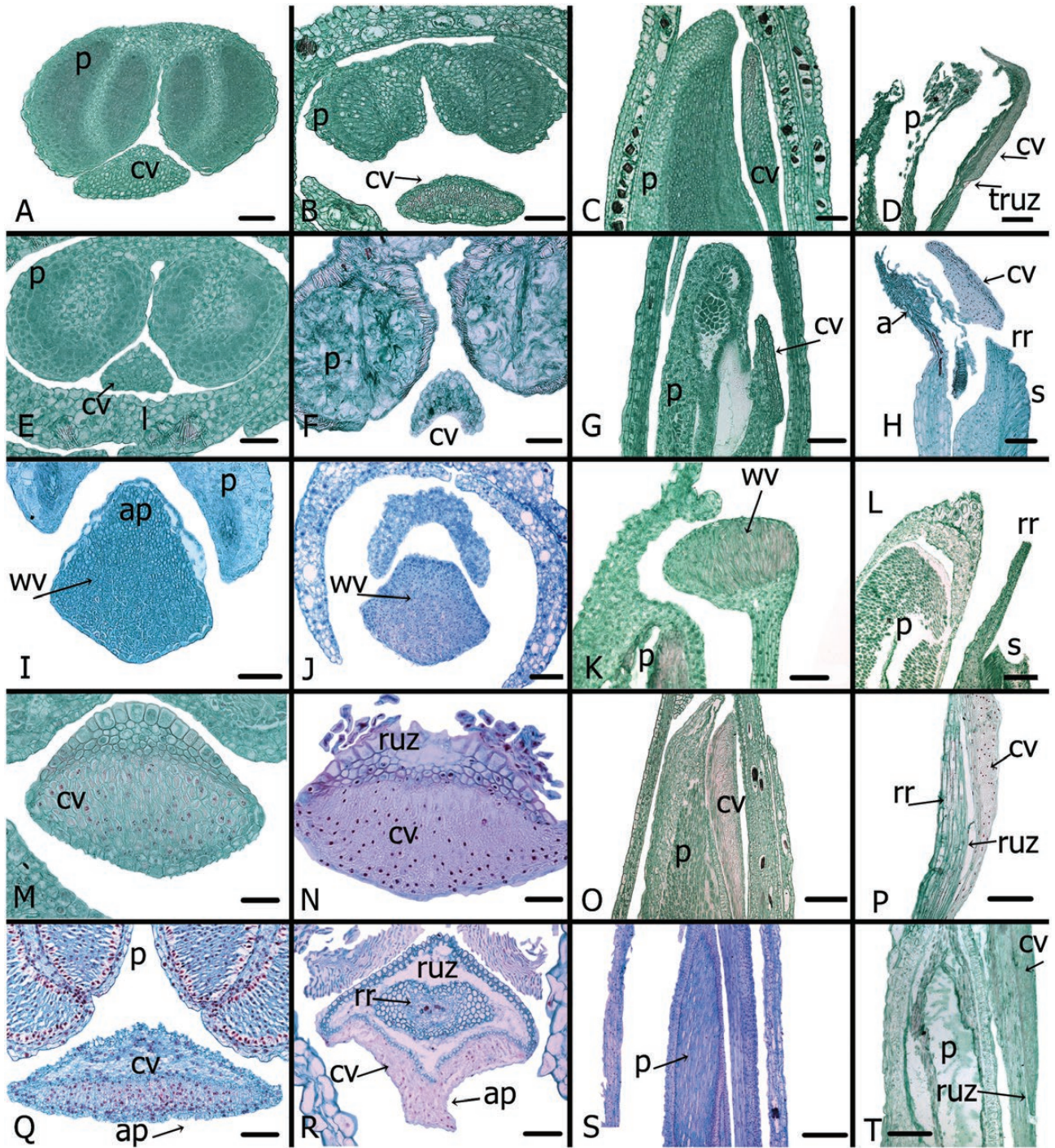
Figure 5. Rostellum, viscidium and rostellum remnant. A–D, *Aulosepalum pyramidale*. E–H, *Mesadenus lucayanus*. I–L, *Sarcoglottis schaffneri*. M–P, *Deiregyne albovaginata*. Q–S, *Dichromanthus aurantiacus*. T, *Sacoila lanceolata*. A, C, Gynostemium at pre-anthesis, differentiation of viscidium. B, D, Gynostemium at anthesis, mature viscidium. E, G, Gynostemium at pre-anthesis. F, H, Gynostemium at post-anthesis, see channelled viscidium and rostellum remnant. I–K, Differentiation of wedge-type viscidium. L, Gynostemium at post-anthesis, see truncate rostellum remnant. M, N, Differentiation of cushion-type viscidium, pre and post-anthesis, see dissolving cells in N. O, P, Rupture zone, pre- and post-anthesis, see a space between viscidium and rostellum remnant. Q, R, Viscidium at pre and post-anthesis, see rupture zone in R. S, T, Viscidium and stiff rostellum remnant. Transversal section (A, B, E, F, I, J, M, N, Q, R); longitudinal section (C, D,

We did not find evidence of staminodes. In other Orchidaceae in which they are present, such as in some members of Diurideae (e.g. *Caleana major* R.Br., *Orthoceras strictum* R.Br., *Prasophyllum fimbriatum* R.Br. and *Diuris longifolia* R.Br.), staminodes arise in the early stages of development, when the median carpel apex starts to differentiate (Kurzweil, 1998). Some authors (Szlachetko & Rutkowski, 2000; Szlachetko *et al.*, 2005; Rutkowski, Szlachetko & Górniak, 2008) have assumed that the membranaceous appendages present at each side of the column apex of some members of Spiranthinae represent staminodes. However, we did not observe any prominence that may represent staminodal primordia, and we did not find vestiges of vascular bundles other than that of the anther (Wilson, 1982). Our results are in agreement with previous findings of Figueroa *et al.* (2012) for *D. michuacanus*, which suggest that the lateral prominences of the column apex are merely projections of the membranaceous margins of the clinadrium (Fig. 3L, O).

Stigma

The overall anatomical traits of the stigma in the species studied here are similar to those described by Dannenbaum *et al.* (1989) for their type IIA, in which they included for several species of Spiranthinae; this consists of a wet stigma with convex surface and finger-shaped papillae.

Although the stigma in most orchids derives from the three carpels (Endress, 1994), Orchidoideae, to which Spiranthinae belong, show the greatest variation in stigma origin (Kurzweil, 1998). For instance, three variations have been reported for Diseae: (1) from the median carpel only (*Huttonaea pulchra* Harv.: Kurzweil, 1989; Coryciinae: Kurzweil, 1991; *Brownleea coerulea* Harv. ex Lindl. and *B. maculata* P.J.Cribb: Linder & Kurzweil, 1995); (2) from the lateral carpels only (Satyriinae: Kurzweil, 1996) and (3) from all three carpel apices (*Brownleea parviflora* Harv. ex Lindl. and species of *Disa* P.J.Bergius *s.l.* (including some as *Monadenia* Lindl. and *Schizodium bifidum* Rchb.f.): Kurzweil, 1990, 1998).

The receptive stigmatic surface of the species of Spiranthinae examined here is most commonly derived only from the base of the median carpel apex, but there are several instances in which the lateral carpel apices contribute partially to the receptive surface. These include *S. glicensteinii*, *S. lanceolata*, *M. lucayanus* and *K. sawyeri*. There are previous reports of other genera of Spiranthinae with three-carpellary stigma, in which the median carpel contributes the greater portion of the receptive surface (*Spiranthes*, *Eurystyles* Wawra, *Pelexia* and *Lankesterella* Ames: Rasmussen, 1982; *Cyclopogon* and *Sarcoglottis*: Kurzweil, 1998).

Szlachetko & Rutkowski (2000) stated that the receptive area of the stigma of various genera of Spiranthinae, namely *Gracielanthus* R.González & Szlach. (= *Aulosepalum*), *Greenwoodia* Burns-Bal. (= *Kionophyton*), *Stenorrhynchos*, *Cyclopogon* and *Sarcoglottis*, is formed by the apices of the lateral carpels, whereas the median carpel apex is not receptive and all of it constitutes the rostellum. In contrast, our observations demonstrate that the stigmas of the species of *Aulosepalum*, *Cyclopogon*, *Deiregyne* and *Sarcoglottis* examined here originate only from the median carpel apex, supporting the earlier interpretation of Kurzweil (1988). It is worth noting that Szlachetko (1995), Szlachetko & Rutkowski (2000), Szlachetko *et al.* (2005) and Rutkowski *et al.* (2008) based their interpretations on comparison of anthetic gynostemia, without anatomical or ontogenetic studies.

In *S. assurgens* there are two receptive zones (Fig. 3H, L), which originate jointly from the base of median carpel apex and are later separated by sterile tissue derived from the lateral carpel apices (Fig. 3F–H); the lateral carpel apices do not participate in the receptive area. Other species of Cranichideae are known to have two receptive stigmatic areas partially or completely separated by sterile tissue, such as *Discyphus scopulariae* (Rchb.f.) Schltr. (Discyphinae; Salazar, van den Berg & Popovkin, 2014), species of *Galeoglossum* A.Rich & Galeotti (Cranichidinae; Salazar, 2009; Salazar *et al.*, 2011b), species of *Vrydagzynea* Blume, *Anoectochilus* Blume, *Myrmechis* Blume and *Hetaeria* Blume and *Zeuxine affinis* Benth. ex Hook.f. (Goodyerinae; Rasmussen, 1982), and some species of *Sauroglossum* Lindl. (Szlachetko & Rutkowski, 2000; Salazar, 2003). Rutkowski *et al.* (2008) assumed that the separate receptive areas represent the lateral carpel apices, but their interpretation is based on comparison of mature flowers, not backed by developmental studies. An ongoing study of floral ontogeny of *Galeoglossum* (including *Pseudocranichis* Garay) shows that the two receptive areas, and the sterile tissue separating them, are all derived solely from the median carpel apex (G. A. Salazar, C. Figueroa & F. Rizo Patrón, unpubl. data). Given the variation in the carpellar origin of the receptive stigmatic area(s)

G, H, K, L, O, P, S, T). a, anther; ap, adhesive portion; cv, cushion viscidium; l, labellum; p, pollinium; rr, rostellum remnant; ruz, rupture zone; s, stigma; truz, transversal rupture zone; wv, wedge viscidium. Scale bars = 300 μm (O, S, T); 200 μm (D); 100 μm (A–C, G, H, J–L, P–R); 50 μm (E, F, I, M, N).
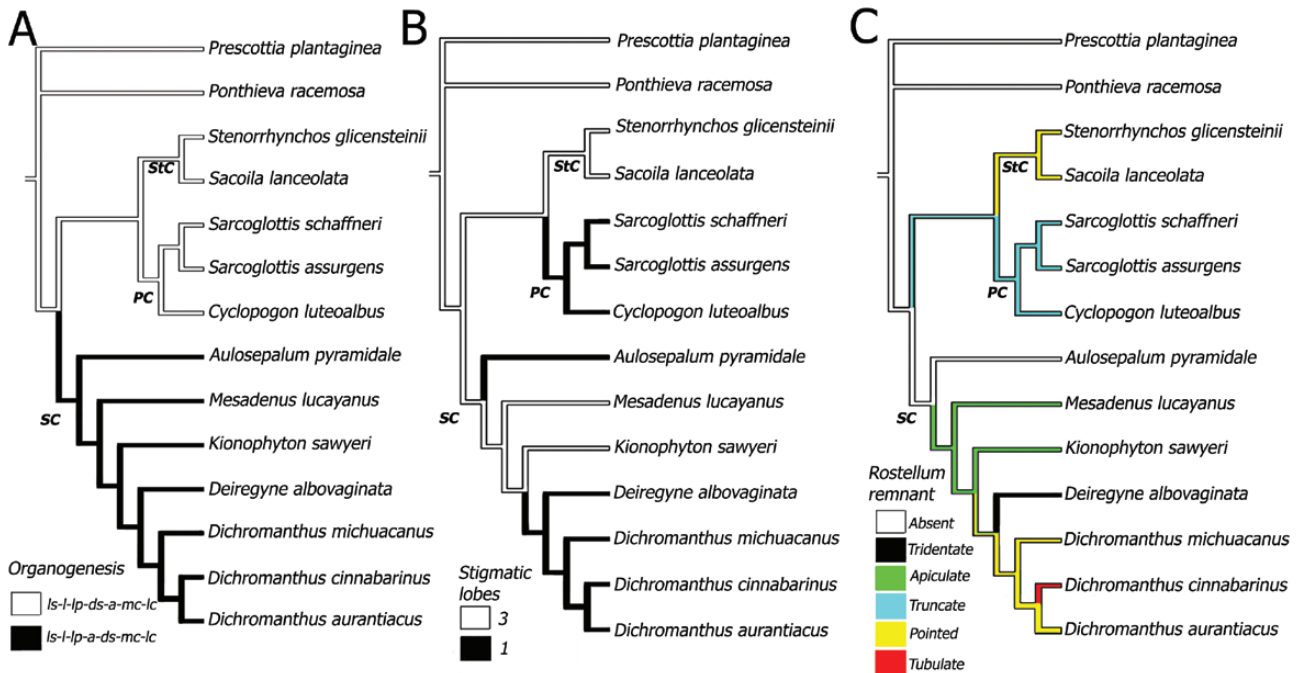
Figure 6. Optimization of three characters: A, organogenesis; B, stigmatic lobes and C, rostellum remnant on one of the six most parsimonious trees obtained for the combined parsimony analysis of the two DNA regions combined (*ITS* and *trnL-trnF*), (length = 660 steps, CI = 0.77, RI = 0.71). See details about the analysis in Figueroa (2014). SC, *Spiranthes* clade; PC, *Pelexia* clade; StC, *Stenorrhynchos* clade.

mentioned previously, any discussion of the issue not backed by ontogenetic data is moot. The optimization of the conditions observed here for stigmatic lobe characters indicates that the unicarpellar stigma evolved in parallel in the *Spiranthes* and *Pelexia* clades (Fig. 6B).

A last feature related to the stigma that has been the subject of contention in Spiranthinae is the position of the stylar canal. Burns-Balogh & Robinson (1983) stated the entrance of the stylar canal is located 'above the stigmatic area' instead of in 'an area between the two functional lobes.' Salazar *et al*. (2018) argued that such view was based on an incorrect interpretation of the homology of the structures stemming from their misunderstanding of the structure and developmental origin of the stigma: Burns-Balogh & Robinson (1983) assumed that the often bilobed or bipartite receptive stigmatic surface of Spiranthinae represents the lateral (carpel) lobes of the stigma, with the 'sterile rostellum' representing the median (carpel) lobe. However, as shown earlier, in all the species of Spiranthinae examined by us so far the entrance of the stylar canal is located at the intersection of the three stigmatic carpel apices, i.e. between the two partially non-receptive lateral carpel apices and the base of fertile median carpel apex (Fig. 4E, F).

Rostellum and viscidium

The most variable morphological traits of the gynostemium in the studied species include the structure of the rostellum, the rostellum remnant and the viscidium, as previously shown to be common in Spiranthinae (Greenwood, 1982; Szlachetko & Rutkowski, 2000). The morphological variation in these structures seems to be related to pollination, probably representing mechanical adjustments for adapting to different pollinators. It has been demonstrated for other clades of Orchidoideae that rostellum morphology determines the part of the body of the pollinator where the pollinarium will be adhered (Johnson, 1997; van der Niet, Liltved & Johnson *et al*., 2011). Such conclusions are supported by the studies on natural pollination of Spiranthinae conducted to date (see Salazar *et al*., 2018, and references therein).

In particular, the cushion-type viscidium exhibits a variable detachment position, resulting in particular rostellum remnant shapes, e.g. pointed in most *Dichromanthus*, apiculate in *Mesadenus*, tridentate in *Deiregyne* and truncate in *Aulosepalum*. Moreover, the characteristics of the rostellum remnant can show variation within a genus, as among *Dichromanthus* spp., or evolve convergently in unrelated genera, e.g. between *Dichromanthus*, *Stenorrhynchos* and *Sacoila* (Salazar *et al*., 2011a, 2018). The common

theme among all those variations of the cushion-type viscidium is that the ventral surface of the viscidium adheres to the dorsal surface of some portion of the antherior part of the pollinator, such as the beak of a hummingbird (Singer & Sazima, 2000; Siegel, 2011) or the feeding parts or face (clypeus) of a bee (e.g. Catling, 1983; Figueroa *et al.*, 2012).

In contrast, the wedge-type viscidium is more uniform among the species of *Cyclopogon*, *Sarcoglottis* and *Pelexia* we analysed. In this type of viscidium, the adhesive portion is dorsal, with the divergent pollinium apices laterally united to it and a transverse rupture from the rostellum that results in either a truncate or a shallowly emarginate rostellum remnant (Greenwood, 1982; Burns-Balogh & Robinson, 1983; Salazar *et al.*, 2018). This viscidium structure involves a pollination mechanism in which it attaches to the underside of the labrum of several kinds of bees as they extend their mouthparts to probe a flower for nectar (Singer & Sazima, 1999; Salazar *et al.*, 2018). The dorsal location of the sticky portion of the wedge-type viscidium (Fig. 5I–L) is reminiscent of 'hamular' viscidia like those observed by Rasmussen (1982) in *Ponthieva* R.Br. (Cranichidinae), but determining whether the cushion-type viscidium of some Spiranthinae represents a hamulus will require clarification from further anatomical and developmental studies.

Rutkowski *et al.* (2008) conducted phenetic and cladistic analyses of six vegetative and 43 floral features, the latter including rostellum characters that, as noted by Salazar *et al.* (2018), were over-weighted by coding as different presence/absence characters the different character states displayed by the rostellum/rostellum remnant. Previously, Burns-Balogh & Robinson (1983) had considered the oblong, truncate or shallowly emarginate rostellum remnant and the apical viscidium held between the apices of the pollinia and located on the dorsal side of the rostellum as two synapomorphies of their version of the *Pelexia* alliance. However, as noted earlier, these two features always occur together in wedge-type viscidia because of functional constraints: the diverging apices of the pollinia, attached to the sides of the viscidium, do not interfere with the contact between the pollinator's labrum and the glue, as would be the case if the pollinia were attached dorsally to the viscidium as in the cushion-type viscidium.

The optimization of the rostellum remnant character showed that it is highly variable and does not reflect phylogenetic signal (Fig. 6C). Hence, its value as taxonomic and phylogenetic marker defining genera or monophyletic groups of genera based on cursory comparisons and biased character coding is questionable.

The developmental data in this work reflects that most morphological differences in the rostellum arise at late stages of development, whereas the early stages are relatively uniform between all analysed species. The growth of structures in different directions determined the general shape of different gynostemia, such as column elongation, viscidium enlargement, growth of stigmatic lobes and clinandrium, as found in previous reports (Kurzweil, 1998). Also, in anthetic flowers, the rupture zone is structured by the degradation of some viscidium cells. Therefore, the rupture zone could be defined as a pollinium accessory, similar to the 'pollinium stalks' that arose late in flower development (Rasmussen, 1982, 1986; Kurzweil, 1988).

Although in previous articles that included representatives of Spiranthinae, the 'detachable' viscidium is mentioned (Rasmussen, 1982; Kurzweil, 1988), only brief comments were made about the rupture mechanism and the morphological variation of the rostellar remnant. In compound organs such as the orchid gynostemium, the boundaries of the contributing organs have never been fully specified across the full process of floral ontogeny (Bateman & Rudall, 2019).

CONCLUSIONS AND FUTURE DIRECTIONS

Gynostemium development in the members of Spiranthinae studied exhibits three general features: (1) early development following an adaxial-abaxial direction, with sequential differentiation of lateral sepals, labellum, petals, dorsal sepal and anther (with some exceptions in which the anther appears before the dorsal sepal such as the species of *Spiranthes* clade analysed here); (2) gynostemium development begins with the anther, followed by the median carpel apex and then the lateral carpel apices and (3) development is for the most part homogeneous, and differential growth, e.g. in column elongation, rostellum enlargement and different growth proportions of stigmatic lobes, occurs only at late stages and determines the main differences between the gynostemia of the species analysed, as found previously by Kurzweil (1998). The structure and location of the rupture zone of the viscidium also accounts for some of the morphological (and likely functional) differences.

The most variable portion of the gynostemium is the rostellum, including the viscidium and the rostellum remnant that remains after removal of the latter. Such variation is directly related to its functional role in pollination, and thus its value as taxonomic marker must be critically assessed by means of unbiased phylogenetic analyses that avoid the ovewheight that results from treating correlated characters as independent ones. However, only a small fraction of the diversity of Spiranthinae has been surveyed morphologically and ontogenetically. On the other

hand, studies of natural pollination documenting the functional dimension of floral structure, are lacking for most of the genera. Therefore, much work remains to be done to achieve a clearer picture of the structural variation existing in this clade and the causes underlying it.

ACKNOWLEDGEMENTS

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