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FLORAL ANATOMY AND EVOLUTION OF POLLINATION SYNDROMES IN *LEPANTHES* AND CLOSE RELATIVES

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Pleurothallidinae is the largest Neotropical orchid subtribe encompassing >5100 species that are mainly dipteran-pollinated. Various pollination syndromes, targeting hump-backed flies (Phoridae), fungal gnats (Sciaridae), and vinegar flies (Drosophilidae) have been documented in detail for *Acianthera, Dracula, Lepanthes, Octomeria, Pleurothallis,* and *Specklinia*, all belonging to different clades. Among them, the highly diverse genus *Lepanthes*, including about 25% of the species of the Pleurothallidinae, is most closely related to *Anathallis, Draconanthes, Frondaria, Lankesteriana, Lepanthopsis, Trichosalpinx,* and *Zootrophion*. Members of this "*Lepanthes* clade" display high floral divergence and are likely adapted to different pollinators . However, only two pollination studies have been published for the group: one for *Lepanthes*, pollinated by *Bradysia* (fungal gnats) and another for *Trichosalpinx*, pollinated by *Forcipomyia* (biting midges). Floral traits present in *Trichosalpinx*, such as purple flowers and a mobile, pubescent lip, evolved to accommodate pollination by biting midges. These traits are also found in other plant species pollinated by biting midges such as *Bulbophyllum*, *Ceropegia* spp. (Asclepiadaceae), and *Theobroma cacao* (Malvaceae). Because some members of the *Lepanthes* clade exhibit similar floral traits, we hypothesize that pollination by biting midges evolved in these orchids as well. In this study, we discuss the micromorphological and histochemical features of the flowers among some of the members of the *Lepanthes* clade in order to test the hypothesis on floral convergence in plants pollinated by biting midges and also which other pollination strategies can be inferred from flower anatomy in the sister genera. Based on histochemistry, LM, and SEM we found similar floral secretions such as carbohydrates, proteins, and lipids in different organs of *Anathallis*, *Lankesteriana*, and *Trichosalpinx*, supporting the hypothesis of floral parallelism in these genera. Lepanthopsis with a papillose lip and secretory glenion and *Zootrophion* with closing flowers and verrucose-papillose inner surface of sepals might employ different pollination systems. This study provides additional micromorphological and histochemical data to support future pollination studies of other members of the *Lepanthes* clade.

Keywords: *Bulbophyllum*, histochemistry, glenion, *Lepanthes*, micromorphology, myophily, Pleurothallidinae, *Trichosalpinx*

With more than 5100 species, Pleurothallidinae (Epidendreae) is the largest Neotropical orchid subtribe (Pridgeon *et al.* 2005). The species diversity of the group is concentrated in few genera. One of these is *Lepanthes* Sw., containing over 1200 species (about 25% of the species of Pleurothallidinae). The genus is phylogenetically most closely related to *Anathallis* Barb.Rodr., *Draconanthes* (Luer) Luer, *Frondaria* Luer, *Lankesteriana* Karremans, *Lepanthopsis* (Cogn.) Ames, *Trichosalpinx* Luer and *Zootrophion* Luer, all considered members of the *Lepanthes* clade (Chase *et al.* 2015; Karremans 2016; Fig. 1). These genera are much less diverse than *Lepanthes* and account for only 1% of the species of the clade (Bogarín *et al.* 2016). Studies of the evolution of the Pleurothallidinae showed that the most speciose lineages of the subtribe diversified recently (within the last 15 Mya), and the *Lepanthes* clade underwent the highest rate of species diversification (Pérez-Escobar *et al.* 2017). However, the factors that have shaped this incredible species diversity in the Pleurothallidinae such as the role of plant-pollinator interactions are still largely unknown because of the lack of knowledge of pollination strategies. Shifts in pollination strategies or adaptations to new pollinators exert evolutionary forces that enhance rapid speciation in angiosperms (Johnson 2010; Kay and Schemske 2008; Smith 2010). In other plant groups, however, species radiations without changes in pollinator specialization have been documented (Ollerton *et al.* 2009), and other studies pointed out that certain pollination systems may increase species diversification independently of the pollination shift (Valente *et al.* 2012).

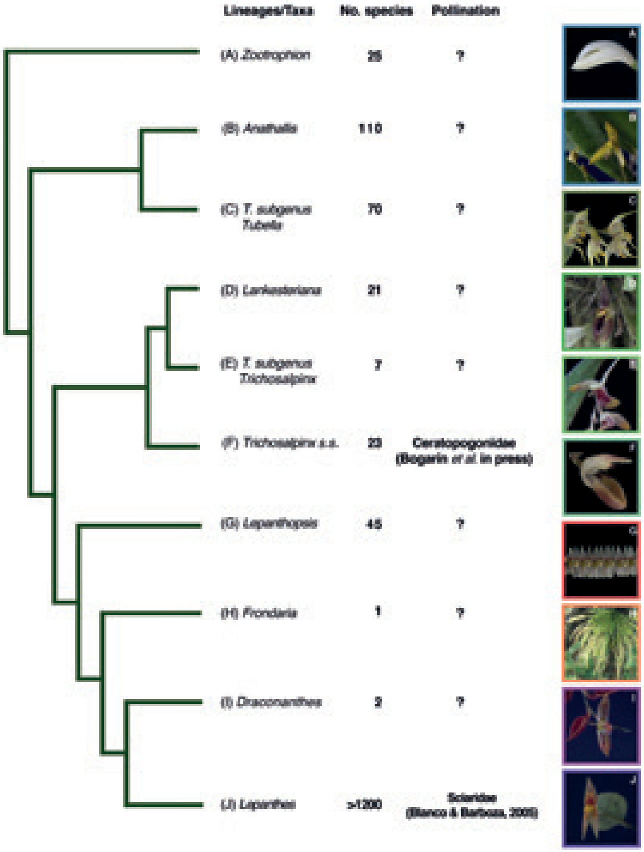

Fig. 1. Phylogeny summary of the *Lepanthes* clade showing the main lineages/taxa, the number of species in each taxa, and pollinator information. Phylogeny based on our unpublished data using nrITS and *matK*.

In the *Lepanthes* clade, nothing is known about the pollination strategies of the members of the clade besides the pollination studies of a few species of *Lepanthes* and *Trichosalpinx*, and this hampers the understanding of evolutionary relationships within this group and the associated pollination shifts. *Lepanthes* flowers, which are mostly characterized by a bilaminate lip with a central appendix, exhibit a highly specialized pollination system involving sexual deception. Flowers are specifically pollinated by male fungus gnats of the genus *Bradysia* (Diptera, Sciaridae) probably attracted by a pheromone-mimicking strategy (Blanco and Barboza 2005). On the other hand, the closely related *Trichosalpinx s.s.* (further referred to simply as *Trichosalpinx*), exhibits different floral traits compared to *Lepanthes* and consequently a different pollination mechanism. *Trichosalpinx* targets exclusively females of the genus *Forcipomyia* (Diptera, Ceratopogonidae). Flowers attract the insects with the motile, pubescent, papillose surface of the lip blade, which secretes proteins and carbohydrates. The presence of females, the absolute absence of males, and secretion of protein rewards indicate that *Trichosalpinx* imitates a model aimed at stimulating the protein collection behavior of females for egg production through a complex deceptive system likely related to kleptomyophyly (Bogarín *et al.*, i2018).

Some members of the clade exhibit similar floral traits, suggesting they may share similar pollination syndromes. For example, purple flowers with motile lips attached to the column foot by a thin ligament, as found in

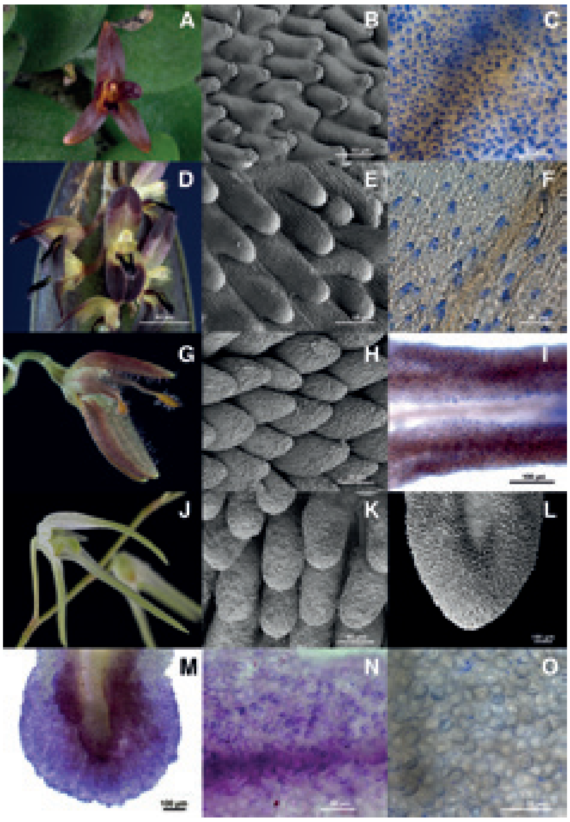

Fig. 2. Flower morphology of some representatives of *Anathallis*, *Lankesteriana*, *Trichosalpinx*, and *Tubella*. A-C: *Anathallis lewisiae*. A. Flower; B. Papillose surface of the lip; C. Detection of proteins with CBB in the epidermis of the lip; D-F: *Trichosalpinx reflexa*. D. Flower; E. Papillose surface of the lip; F. Detection of proteins with CBB in the epidermis of the lip; G-I: *Lankesteriana fractiflexa*. G. Flower; H. Papillose surface of the lip; I. Detection of proteins with CBB in the epidermis of the lip; J-O: *Tubella arbuscula*. J. Flower; K. Papillose surface of the lip; L. Papillose apex of the lip with the median groove; M. Apex of the lip with positive detection of carbohydrates (PAS); N. Papillose side of the lip with positive detection of carbohydrates (PAS); O. Apex of the lip with negative detection of proteins (CBB). (Photos: D. Bogarín)

Trichosalpinx, are present in both *Anathallis* and *Lankesteriana*. These traits most likely evolved to accommodate pollination by biting midges because these features are also present in *Trichosalpinx* and other angiosperms pollinated by similar strategies such as *Bulbophyllum* Thouars, *Ceropegia* L. spp. (Asclepiadaceae) and *Theobroma cacao* L. (Malvaceae) (Bartareau 1994; O'Doherty and Zoll 2012; Ollerton *et al.* 2009; Bogarín *et al.* 2018).

However, other genera of the *Lepanthes* clade display floral traits that suggest pollination through different syndromes. For example, *Lepanthopsis* flowers exhibit reduced petals, a glenion at the base of the lip, and a short column with bilobed stigma, much more similar to what is found in many *Pleurothallis* R.Br. and *Stelis* Sw. species rather than any of its closest relatives. In *Zootrophion*, the flowers are fleshy, with the sepals fused at the base and apex and leaving only a window-like aperture at each side of the flower, features that are unique among its close relatives but found as well in a few unrelated genera, including *Acianthera* Scheidw., *Phloeophila* Hoehne & Schltr., *Specklinia* Lindl., and *Stelis*. In *Trichosalpinx* subgenus *Tubella* (further referred to simply as *Tubella* Archila), flowers are mostly white with an entire lip blade, which contrast with the purple pubescent lips of *Trichosalpinx* (Luer 1997, 2004, 2006).

To make inferences about the pollination strategies of the remaining groups of the *Lepanthes* clade and test the hypothesis of floral parallelism/divergence, we investigated the anatomy, micromorphology, and possible rewards of flowers of some members of the clade by combining histochemistry, light microscopy (LM), and scanning electron microscopy (SEM) techniques. In this study, we discuss the anatomical features of the flowers among some of the members of the *Lepanthes* clade in order to assess 1) the characters shared among *Anathallis*, *Lankesteriana*, and *Trichosalpinx* that indicate adaptation to pollination by biting midges and thus parallelism, 2) pollination syndromes in the *Lepanthes* clade that can be inferred from floral anatomy, and 3) micromorphological characters that are useful in distinguishing the different groups within the *Lepanthes* clade.

Material and methods

Study site and sample collection

Plant samples were collected in the wild and cultivated in the greenhouses of the Lankester Botanical Garden (JBL) of the University of

Costa Rica (Cartago, Costa Rica) and the Hortus botanicus of Leiden University (Leiden, The Netherlands). We studied species of *Anathallis*, *Lankesteriana*, *Lepanthes*, *Lepanthopsis*, *Trichosalpinx* subgen. *Trichosalpinx*, *Tubella*, and *Zootrophion*. Due to unavailability of material, we did not include specimens of *Draconanthes*, *Frondaria*, *Trichosalpinx* subgenus *Pseudolepanthes* Luer, and *T.* subgenus *Xenia* Luer. Vouchers of plant specimens were deposited at CR, JBL (spirit), L, and USJ.

Digital imaging and microscopy

Photographs were taken with a Nikon D7100 and AF-S VR Micro-Nikkor 105mm f/2.8G IF-ED lens and PB-6 bellows with a Nikon AF Nikkor 50mm f/1.8D lens and Broncolor® Siros 800 S flashes. Stacking was performed with Zeiss Stereo Discovery V20 and AxioCam MRc 5 Zeiss camera. Digital images of light microscopy were taken with a Zeiss® AXIO Imager.M2 motorized microscope with an AxioCam MRc 5 Zeiss camera. Final digital images and composite figures were processed in Adobe Photoshop CS6®.

Microscopy fixation

Samples were stored in FAA (ethanol 50%, acetic acid, and formalin at 18:1:1 v/v) or 70% ethanol for at least a week or more. For Epon and LR White embedding, dissected fresh flowers were fixed for 3 hr in a modified Karnovsky fixative (2.5% glutaraldehyde, 2% formaldehyde, pH 7.2) and rinsed three times in 0.1 M sodium cacodylate buffer (pH 7.4) prior to embedding. Staining was performed for 2 hr in 2% osmium tetroxide and rinsed in 0.1 M sodium cacodylate buffer (pH 7.4).

Light microscopy (LM) and histochemistry

Entire fresh flowers and hand-cut sections of flowers were stained to detect lipids, polysaccharides, and proteins. Flowers fixed in 70% ethanol for several days were also used for staining. Heavily pigmented tissue areas of fresh flowers were cleared for 10-60 min in 10% (v/v) commercial solution of sodium hypochlorite and rinsed in 30% ethanol for 1 hr before staining to avoid the interference of tissue coloration in staining results (Ruzin 1999). Neutral or acidic lipids, phospholipids, and fatty acids were detected with a solution of Nile blue A 1% (NBA) (w/v, demi water) at 37 C for 1 min and differentiated in 1% acetic acid for 30 sec at 37 C and rinsed in demi water (Ruzin 1999). Sudan IV 0.5% (SIV) (w/v, 70% ethanol) and Sudan Black B (SBB) 0.07% (w/v, ethanol 70%) were used to detect lipids (fats, oils, and waxes) (Bronner 1975; Ruzin 1999). Insoluble polysaccharides and starch were detected with a periodic acid–Schiff reaction (PAS) by oxidizing the samples in aqueous solution of periodic acid (HIO4) 5%

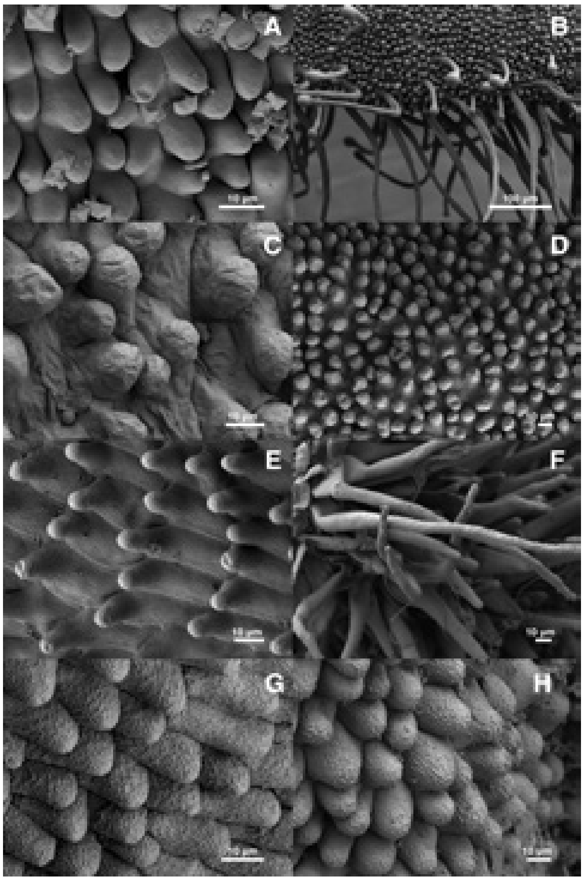

Fig. 3. SEM micrographs of the epidermis of the lip of: A. *Anathallis lewisiae*; B. *Lankesteriana barbulata*; C. *Trichosalpinx chamaelepanthes*; D. *T. pergrata*; E. *T. reflexa*; F. *T. ringens*; G. *T.* cf. *patula*; H. *Tubella dura*. (Photos: D. Bogarín)

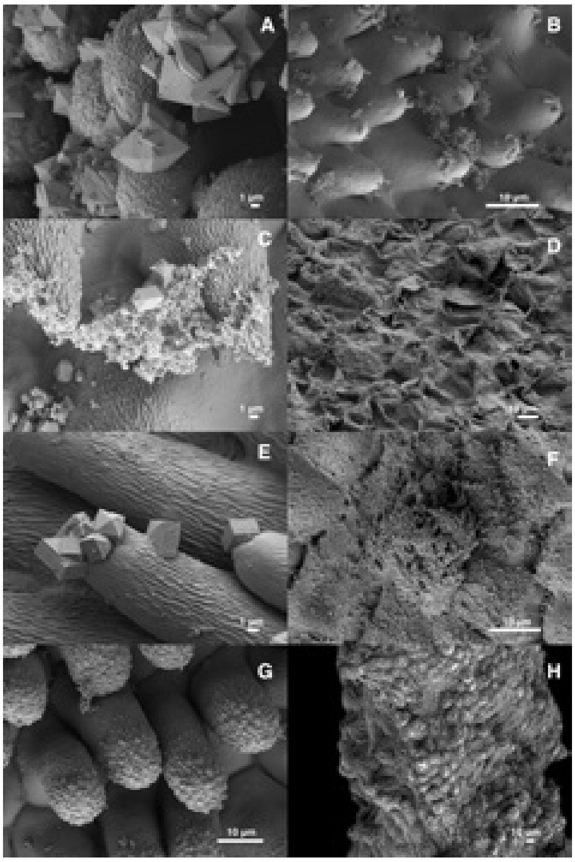

Fig. 4. SEM micrographs of the epicuticular secretions in the epidermis of the lip of: A. *Anathallis funerea*; B. *Lankesteriana barbulata*; C. *Lepanthes chameleon*; D. *Trichosalpinx pergrata*; E. *T. reflexa*; F. *T. ringens*; G. *Tubella dura*; H. *Z. endresianum*. (Photos: D. Bogarín)

(m/v) for 10 min, rinsing 3 times in distilled water for 2 min and submerging for 15 min in Schiff's reagent, and finally submerging in tap water at 50-60 C for 5 min (Ruzin 1999). Mucilage-secreting areas with acidic compounds, pectic acids or hexuronic acids were detected with a solution of ruthenium red 0.05% (RR) (w/v, tap water) for 15-20 min (Southworth 1973). Proteins were detected with Aniline Blue-Black (ABB) 1% in 7% acetic acid for 10 min at 50-60 C (Jensen, 1962; Fisher, 1968) and Coomassie Brilliant Blue R-250 (CBB) in a solution of 0.25% CBB, 50% ethanol, and 7% acetic acid for 3 min and rinsed in tap water (Jensen 1962; Fisher 1968). Areas of fragrance emission were detected by submerging the samples in a solution of neutral red 0.1% (NR) (w/v, tap water) for 15-20 min and differentiated with tap water (Ruzin 1999).

Scanning electron microscopy (SEM)

Fixed flowers were dehydrated for 20 min in a series of ethanol solutions (70%–96%–≥99.9%) and twice in fresh ≥99.8% acetone. Critical-point-drying using ≥99.8% acetone and liquid CO^2 as exchange fluids was performed in Automated Critical Point Dryer Leica EM CPD300 (Leica Microsystems, Wetzlar, Germany). The drying protocol included a cooling step at 15 C, 50% stirrer speed with auto version, slow CO^2 influx in the pressure chamber, with a delay of 120 sec after influx CO^2 and before starting the exchange process, 18 exchange cycles (CO^2: 99.8% acetone) at a speed of 5, a fast heating speed, and medium gas-out speed. Dried samples were mounted in stubs with adhesive carbon conductive tabs and sputter-coated with 20 nm of Pt/Pd in a Quorum Q150TS sputter-coater. Resulting samples were observed with a JEOL JSM-7600F field emission scanning electron microscope at an accelerating voltage of 10 kV.

Results

Anathallis

As in *Trichosalpinx* and *Lankesteriana*, the lip of the members of this genus is motile because it is hinged to the column foot by a thin membranous ligament. The papillose lip shows slightly striate cuticles and various secretions such as lipids (SIV) and insoluble polysaccharides (PAS) but most notably proteins (ABB, CBB) on the apex of the papillae and occasionally prismatic crystals of calcium oxalate, indicating a secretory function similar to *Trichosalpinx* (Fig. 1A-C, 3A, 4A). Differences with *Trichosalpinx* and *Lankesteriana* include the absence of elongated cells toward the margins and the raised callus at the base of the lip. In *A. lewisiae* (Ames) Solano & Soto Arenas, the petals are also papillose, with striate cuticles and secretions at the apices unlike the petals of *Trichosalpinx* (Fig. 5E). Flowers of some *Anathallis* species have purple colors, but other species have white or yellowish flowers.

Lankesteriana

Species belonging to the genus also show similarities with the lip of *Trichosalpinx* and *Anathallis* species, including the papillose surface of the lip with striate cuticle and secretory activity (Fig. 1G-I, 3B, 4B). We detected lipids (SIV), insoluble polysaccharides (PAS), and again proteins on top of the papillae (ABB, CBB; Fig. 1I). Also, the lip shows elongated cells with widened apices scattered towards the lip apex unlike the elongated cells of *Trichosalpinx* that are restricted to the margins and not widened at the apex (Fig. 3B). The combination of brown-purple with white in the flowers of some *Lankesteriana* is similar to the flowers of *Trichosalpinx* (Fig. 1D, G). The lip of *Lankesteriana* is grooved unlike that of *Trichosalpinx* and *Anathallis*. Like *Anathallis*, the apices of the petals are papillose and secretory with striate cuticles but sometimes with elongated cells (Fig. 5A-B). Some *Lankesteriana* species have elongated, pubescent or papillose petals, ending in widened apices such as *L. fractiflexa* (Ames & C.Schweinf.) Karremans. Other species such as *L. barbulata* (Lindl.) Karremans have *Trichosalpinx*-like petals without hairs but with irregular margins.

Trichosalpinx subgen. *Trichosalpinx*

In *Trichosalpinx* species [including the relatives of *T. berlineri* (Luer) Luer], the base of the lip is attached to the column foot by a membranous ligament. The lip blade is papillose with elongated cells appearing towards the margins. The papillae of the lip blade exhibit a noticeable striate cuticle, and toward the apices the cuticle is smooth and secretory. We detected lipophilic compounds (SBB), polysaccharides (PAS), and proteins (CBB and ABB) within and on the apex of the papillae, indicating a secretory function (Fig. 1D-F, 3C, E, F, G, 4D, E, F). Tests with (NR) were positive on the apices of the papillae. In particular, the lip of *T. ringens* Luer has elongated hairs with some striations in the cuticle unlike the short papillae of most *Trichosalpinx* species (Fig. 3F). Crystals of calcium oxalate were exuded on the apices of the papillae but were not present in all the specimens analyzed (Fig. 4E). The petals are oriented parallel to the column, and they do not have papillose surfaces (Fig. 5E); no secretory activities were detected because histochemical tests yielded no positive results. The column is arcuate with a rounded foot where the lip is attached. At the base of the column foot, there are similar papillae to those observed on the sepals and lip.

Lepanthes

Flowers of *Lepanthes* are characterized in general by a bilobed lip with an appendix at the base and an elongated column with apical anther and stigma (Fig. 6A, B, F-H). Our studies indicate a generalized pattern in the epidermal surface of the sepals and petals. The sepals have flattened, smooth cells and, in contrast, the petals and lip are always papillose (Fig. 5C, 6B, C). The sepals did not react to histochemical stains, but the petals reacted to all the stains applied for carbohydrates (PAS, RR), lipids (SBB, SIV), proteins (ABB, CBB), and scents (NR; Fig. 6D, E, I). The appendix of the lip is pubescent, hirsute or with a combination of elongated and flattened cells (Fig. 6F-H). Prismatic crystals and compounds on the surface of the cells were observed (Fig. 4C).

Lepanthopsis

This genus is easily recognized by the flattened flowers with reduced petals and the presence of a glenion at the base of the papillose lip, a feature not found in any of the sister genera (Fig. 7). In *L. floripecten* (Rchb.f.) Ames, the glenion is made up of an aggregation of

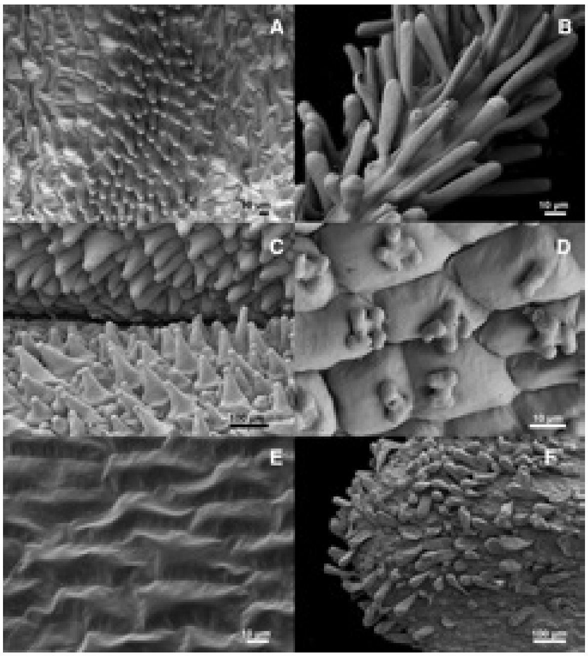

Fig. 5. SEM micrographs of the epidermis of the petals of: A. *Anathallis lewisiae*; B. *Lankesteriana fractiflexa*; C. *Lepanthes chameleon*; D. *Lepanthopsis prolifera*; E. *T. reflexa*; F. *T. pergrata*. (Photos: D. Bogarín)

papillose and secretory cells just in front of the reduced column. Flowers of some species resemble those of *Platystele* Schltr. (Fig. 7B-C, 8A-D). In *L. astrophora*, the glenion is also papillose but sunken (Fig. 8B). The papillose lip reacts for proteins on top of the papillae over the lip (ABB, CBB) but most notably in the glenion (Fig. 7D). SEM images show several compounds in the surface of the glenion that also react positively for lipids (SIV) and insoluble polysaccharides (PAS; Fig. 7 D, E). Petals and sepals have flattened cells and do not react for proteins and carbohydrates but probably contain epicuticular waxes (SIV). However, other species such as *L. prolifera* Garay have epidermal cells with characteristic projections (Fig. 5D). We observed prismatic crystals on the surface of the cells but mostly concentrated on the glenion (Fig. 7G).

Tubella

Flowers of this group are generally white-greenish to yellowish (Fig. 1, 2J). The lip is papillose with striate cuticles as in *Anathallis*, *Trichosalpinx*, and *Lankesteriana* but with a median groove of flattened cells and without hairs along the margins (Fig. 2K-M). Some areas of the lip at the sides and toward the apex contain insoluble polysaccharides (PAS), but the blade does not react for proteins (ABB, CBB) and lipids (SIV; Fig. M-O). Some species such as *T. arbuscula* (Lindl.) Luer are fragrant. The petals are flattened, not pubescent and without papillae. The sepals are elongated and also entire. Some species classified have smooth papillose surfaces on the lip, such as *T. chamaelepanthes* (Rchb.f.) Luer and *T. pergrata* (Ames) Luer (Fig. 3C, D). Also, the apex of the petals of *T. pergrata* are papillose in contrast of those of *T. arbuscula* and *T. dura* (Lindl.) Luer (Fig. 5E).

Zootrophion

Flowers of this genus are unique within the group. The basally and apically fused sepals form a closed flower with only two open sides, resembling windows. The color of the flower varies from white and yellow to pinkish and purple, sometimes with spotted sepals and petals (Fig. 9A). The inner surface of the synsepal is made up of papillose or rugose surfaces that react to carbohydrates (PAS) and lipids (SB). In *Z. vulturiceps* (Luer) Luer, a species with white flowers, we did not detect proteins (CBB) on the rugose surface (Fig. 9B-F). However, the papillose area of the synsepal of the purple-spotted *Zootrophion* sp. shows a positive reaction for proteins (CBB). The dorsal sepal is smooth and does not react with the stains. The lip is reduced and motile and is attached to the column foot by a membranose tissue, securing mobility. The surface is papillose and rugose with various evident secretions on the epidermal cells (Fig. 4H, 9G-H). The column is footed and elongated as in *Anathallis*, *Trichosalpinx*, and *Lankesteriana*. The petals are parallel to the column and enclose the column and lip.

Discussion

Floral micromorphology and histochemistry

The epidermal secretory papillae of the lip of *Anathallis*, *Lankesteriana*, and *Trichosalpinx* is also present in other myophilous species of *Bulbophyllum* and fly-pollinated Pleurothallidinae (De Pádua Teixeira *at al.* 2004; Nunes *at al.* 2014; Nunes *et al.* 2015; Pridgeon and Stern 1985). Secretion of proteins on the lip occurs in species of *Bulbophyllum* sect. *Racemosae* Benth. & Hook.f., possibly as floral rewards for female flies (Davies and Stpiczyńska 2014). In addition, flowers of *B. wendlandianum* contained protein secretions in the epithelium (Kowalkowska *et al.* 2014), as observed previously in *Trichosalpinx* and the species of *Anathallis* and *Lankesteriana* studied here. Positive reaction with NR and lipophilic compounds also indicate scent synthesis in the papillose epidermis. Vogel (1990) documented fragrance emission in the epithelium of the distal lobed ends of the corolla of *Ceropegia*, which is mostly pollinated by biting midges. These structures are purple, papillose or hairy, like the lip of some species of *Anathallis*, *Lankesteriana*, and *Trichosalpinx*. The striate cuticle of the papillae is another feature shared by species of these genera and also with some myophilous *Bulbophyllum* species (Davies and Stpiczy´ska 2014; Kowalkowska *et al.* 2014; Nunes *et al.* 2014, 2015; Stpiczyńska *et al.* 2015).

Striate cuticles have been associated with light diffraction, producing more intense "structural colors" acting as a visual effect on pollinators (Antoniou Kourounioti *et al.* 2012). Nunes *et al.* (2015) postulated that the striate cuticular patterns in *Bulbophyllum* sect. *Napellii* Rchb.f. are related to these visual cues. Our findings on *Anathallis* and *Lankesteriana* support previous observations in *Trichosalpinx* and *Bulbophyllum* in which the striate cuticles are present only in the purple-colored areas of the lip and petals, whereas the whitish or translucent areas have flattened and smooth epidermal cells.

The papillose epidermal areas of flowers might increase the area of emission of scents or “emission layer” (Vogel 1990). In *Lepanthes*, the active parts of the flower in terms of compound synthesis are the papillose epidermis of petals and scattered colleters of sepals. The role of this tissue in the production of pheromone-like odors that attract male fungus gnats as pollinators has to be tested experimentally, but our histochemical evidence indicates that the papillose petals are involved in scent synthesis. In addition, this papillose epidermis is mostly not striate and does not concentrate the secretions on the apex of the papillae as observed in *Anathallis*, *Lankesteriana*, and *Trichosalpinx*, indicating that *Lepanthes* flowers do not produce collectable rewards. This is consistent with the hypothesis of sexual deception and behavior of pollinators in the flowers that do not search for rewards. On the other hand, papillose or verrucose areas of the synsepal of *Zootrophion* are secretory, and the epicuticular compounds on the cells of the epidermis of the lip observed with SEM may indicate that *Zootrophion* flowers offer rewards.

The parallel position of the petals with respect to the column and the lack of rewards in the species with smooth epidermis indicate that the function of the petals is to keep the insects directed toward the base of the lip, preventing them from exiting at the sides. This is probably true for *Trichosalpinx* and some *Lankesteriana* and *Zootrophion*. However, in *Anathallis* and other species of *Lankesteriana*, some areas of the petals are papillose and secretory, probably acting as visual/olfactory attractants for pollinators as in some *Bulbophyllum* species (Pridgeon and Stern 1983; Vogel 1990; Kowalkowska *et al.* 2014; Nunes *et al.* 2014).

The detection of proteins and carbohydrates

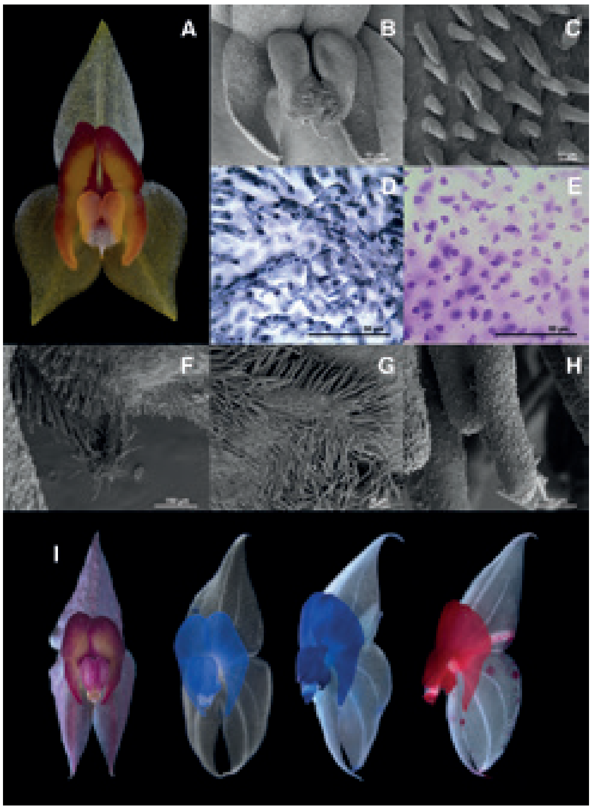

Fig. 6. Flower anatomy and histochemistry of: A-H: *Lepanthes horichii*. A. Flower; B. SEM of the lip and petals; C. Papillose surface of petals; D. LM of cells of the epidermis of the petal stained with SBB showing lipid droplets (black); E. LM of the epidermal cells the petal stained with PAS (carbohydrates); F. SEM micrographs of the hairy appendix of the lip; G. Detail of the appendix; H. Detail of the elongated cells (hairs) of the appendix; I. Histochemistry of *Lepanthes bradei* showing the positive reaction with stains, from left to right: flower with natural pigmentation, CBB, ABB, NR (Photos: D. Bogarín and M.M. Chabert)

on the apex of the papillae of the lip and particularly in the glenion of *Lepanthopsis* floripecten indicates that pollinators are guided toward this point. The glenion has been defined as a circular structure or callus at the base of the labellum, placed right in front of the reduced column; it occurs in several unrelated genera of Pleurothallidinae, namely *Brachionidium* Lindl., *Lepanthopsis*, *Platystele*, *Pleurothallis* R.Br.. *Stelis* Sw., and *Teagueia* (Luer) Luer (Pridgeon *et al.* 2005; Fig. 8). The function of the glenion in the pollination of species of these genera is discussed in further detail by Karremans and Díaz elsewhere in these *Proceedings*. Initial evidence indicates that this structure is an aggregation of papillose or flattened cells (sometimes sunken) of secretory activity. The anatomy of the glenion varies across these genera, and more ultrastructural and histochemical comparative studies are needed to characterize the micromorphology and its role in pollination (Bogarín *et al.*, unpubl.).

Crystals occur in the sepals, petals, and lip of many Pleurothallidinae (pers. obs.). The function of these non-protoplasmic inclusions is not entirely clear, and little is known about their role (if any) in pollination. Chase and Peacor (1987) proposed that the refractile properties of crystals in *Stelis* might mimic nectar droplets (or pseudonectar), which act as visual attractants that lure pollinators. Nunes *et al.* (2015) attributed to them a possible function as a visual signal, enhancing the reflection of light emitted in conjunction with the vacuoles containing pigments. Other studies suggest that they may be involved in regulation of high levels of calcium ions and calcium oxalate that eventually precipitate in epicuticular crystals (Franceschi and Horner 1980).

Pollination syndromes in the *Lepanthes* clade

The recent discovery of the pollination of *Trichosalpinx* by biting midges allows us to make inferences about the pollination systems of other members of the *Lepanthes* clade (Bogarín *et al.* in press). *Trichosalpinx*, *Lankesteriana*, and the *T. berlineri* group have a close affinity according to the latest phylogenetic analyses of the Pleurothallidinae and unpublished data (Karremans 2016; Karremans 2014; Pérez-Escobar *et al.* 2017; Rykaczewski *et al.* 2017; Bogarín *et al.*, unpubl.; Fig. 1). Species of *Lankesteriana* and the *T. berlineri* group have a mobile, pubescent lip that is almost indistinguishable from that of *Trichosalpinx* and some *Bulbophyllum* (Bartareau 1994; Luer 2006). Although no data on pollination of *Lankesteriana* and *T. berlineri* group are available, our findings indicate pollination by biting midges. The papillose epidermis with a striate cuticle and secretion of proteins are consistent with the anatomical features found previously in the *Trichosalpinx* species pollinated by females of *Forcipomyia* that search for proteins (Bogarín *et al.*, 2018). In addition, this hypothesis is strengthened by floral traits present in other angiosperm groups pollinated by biting midges such some *Aristolochia* L. and *Pararistolochia* Hutch. & Dalziel in *Aristolochia*ceae, Caralluma R.Br., *Ceropegia* L. in Apocynaceae, and *Abroma* Jacq., *Herrania* Goudot, and *Theobroma* L. in Malvaceae (Davies and Stpiczyńska 2014; Kowalkowska *et al.* 2014; Nunes *et al.* 2014, 2015; Stpiczyńska *et al.* 2015; Vogel 1990). In Orchidaceae, the flowers of the distantly related Australian *Bulbophyllum macphersonii* Rupp., a species pollinated by biting midges, are similar to those of *Trichosalpinx*, *Lankesteriana*, and some species of *Bulbophyllum* section *Hybochilus* Schltr., *B.* sect. *Oxysepalum* Schltr., and B. sect. *Polyblepharon* Schltr. (Bartareau 1994). Common features among these species are again the pubescent, purple, mobile lip with two basal auricles and the purple sepals and petals. *Trichosalpinx* and *B. macphersonii* are examples of evolutionary convergence toward a common mechanism of pollination, and this is likely occurring in *Lankesteriana* and the species of the *T. berlineri* group as well.

Similarity in floral traits are also present in some *Anathallis* species, such as *A. lewisiae* (Fig. 24A), *A. microgemma* (Schltr. ex Hoehne) Pridgeon & M.W. Chase, and *A. nanifolia* (Foldats) Luer as noted by Luer (1997). *Anathallis* appears to be related to species of *Tubella* in the phylogenetic analysis and are not embedded within the *Trichosalpinx* clade in the strict sense (Fig. 1). However, some species have purple flowers and a mobile lip (though not pubescent) hinged by a membrane at the bottom of the column foot. Pollinators of *Anathallis* are not yet known, but we hypothesize that some species showing striate papillae and secretion of proteins in the epidermis of the lip represent another case of evolutionary parallelism to attract the same type of pollinator guild as *Lankesteriana* and some *Bulbophyllum* and *Trichosalpinx* species.

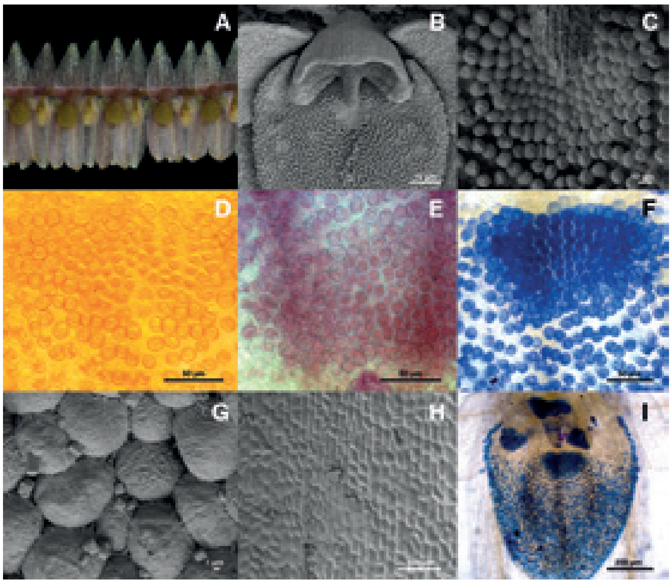

Fig. 7. Flower anatomy and histochemistry of: A-I: *Lepanthopsis floripecten*. A. Flowers; B. SEM of the petals, lip, and column; C. Papillose surface of the glenion; D. LM of cells of the glenion stained with SIV showing lipids surrounding the cell walls of the papillae; E. LM of cells of the glenion stained with PAS detecting carbohydrates; F. LM of cells of the glenion stained with CBB detecting proteins; G. Detail of the papillae of the glenion with some epicuticular secretions; H. Detail of the smooth surface of the petals; I. LM of the papillose lip stained with CBB detecting proteins (Photos: D. Bogarín and M. M. Chabert)

Floral morphology of *Trichosalpinx pergrata* (Ames) Luer, *T. ringens* Luer, and *T. sanctuarii* Mel.Fernández & Bogarín is different from the species of *Trichosalpinx* s.s. For example, the absence of a trembling lip with a flexible membrane and the *Acianthera*-like flowers of *T. ringens* and *T. sanctuarii* indicate that these species may be pollinated by different pollinator groups. The red flowers of *T. pergrata* with papillose apices of petals and two nectary-like structures in the column also indicate another, yet unknown, pollination mechanism (Luer 1997; Fernández and Bogarín 2011).

The species of *Tubella* are not strictly embedded within the *Trichosalpinx* clade (Pridgeon

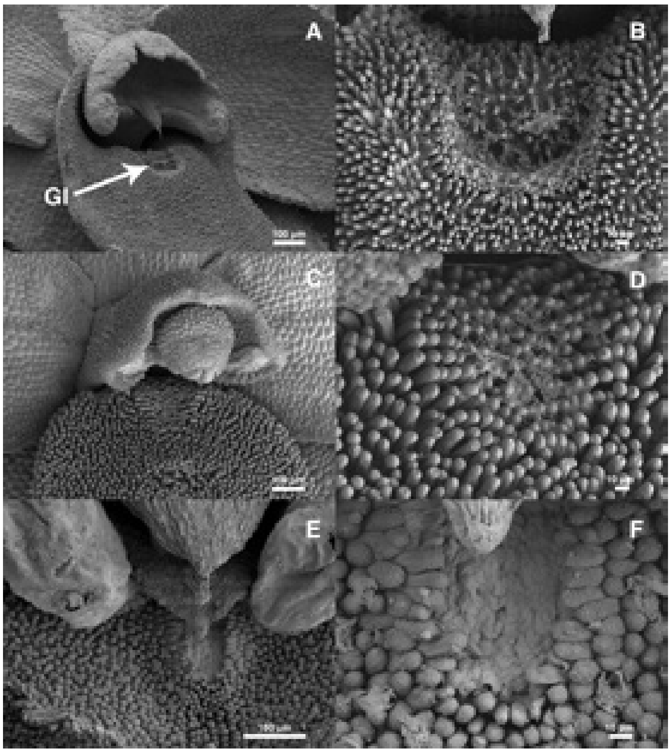

Fig. 8. SEM micrographs of the column and glenion at the base of the lip of: A. *Lepanthopsis astrophora*; B. Detail of the glenion of *Lepanthopsis astrophora*; C. *Platystele* sp.; D. Detail of the glenion of *Platystele* sp.; E. *Stelis* sp.; F. Detail of the glenion of *Stelis* sp. (Photos: D. Bogarín)

et al. 2001; Karremans 2016; Rykaczewski *et al.* 2017). Species of *Tubella* have white or yellowish flowers, and the lip is not pubescent. Besides the preference of biting midges for flowers with purple and hirsute structures, they have also been documented to visit plants such as *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg. (Euphorbiaceae) and *Mangifera indica* L. (Anacardiaceae) with white flowers (Borkent and Spinelli 2007). Males and females seek nectar in these small white flowers to meet their energy needs. Art Borkent (pers. comm.) observed biting midges of the genera *Atrichopogon* Kieffer and *Dasyhelea* Kieffer, a group with reduced mouthparts and without blood sucking behavior, visiting flowers of *Epidendrum piliferum* Rchb.f. in Monteverde, Costa Rica, an orchid with white flowers and purple nectar guides on the blade of the lip. Pedersen (1995) recorded biting midges of the genus *Forcipomyia* as a visitor to *Dendrochilum longibracteatum* Pfitzer, an orchid species with white flowers and a brownish lip. As already noted by Luer (1997), white flowers with caudate petals are present in species of *Specklinia* subgen. *Hymenodanthae*, such as *S. calyptrostele* (Schltr.) Pridgeon & M.W.Chase, which resemble flowers of *Tubella*. Karremans *et al.* (2016) recorded pollination of *S. calypstrotele* by a Ceratopogonidae species, possibly *Atrichopogon*. Therefore, the pollinators of *Tubella* may be biting midges as well, but the operating mechanism is probably different and similar to the anthophilous nectar-seeking flies pollinating the white flowers of *Hevea* or *M. indica*. Although more pollination observations and anatomical and histological studies are needed in this genus, the presence of carbohydrates found in the papillae of the lip instead of proteins supports these hypotheses.

Of the other genera belonging to the *Lepanthes* clade, there is no information available on pollinators yet. The floral morphology of these groups is unlike those already studied, indicating that at least two additional mechanisms may be in place. *Lepanthopsis* is unique among its close relatives in having a papillose secretory glenion of the lip. The sepals and petals are generally flat, and the sepals are caudate. The column is short, broad, and footless with an apical anther and bilobed stigma (except for a few species). This type of column indicates that the pollinarium is positioned either on the head, antenna or legs but most likely not on the dorsal part of the thorax or abdomen of the pollinator. Undoubtedly, this represents another pollination mechanism yet unknown but different from the currently documented cases in *Lepanthes* and *Trichosalpinx*. Unrelated genera such as *Brachionidium*, *Platystele*, *Pleurothallis*, *Stelis*, and *Teagueia* exhibit similar floral traits, specifically flat flowers with a short column with a bilobed stigma and glenion at the base of the lip (Luer 1990). Some of these groups are pollinated by Mycetophilidae and Sciaridae (Duque-Buitrago *et al.* 2014), and these families may be involved in the pollination of *Lepanthopsis* as well, as further discussed by Karremans and Díaz elsewhere in these Proceedings.

Some species of the aberrant group T. subgenus *Xenia* such as *Trichosalpinx ballatrix* Luer & Escobar, *T. escobarii* Luer, and *T. tenuiflora* (Schltr.) Luer are florally similar to some *Teagueia* (such as *T. barbeliana* L.Jost & A.Shepard and *T. puroana* L.Jost & A.Shepard). Unfortunately, we do not have any anatomical, phylogenetic or pollination data available for these groups yet (Luer 1997).

Because of the unique morphology of the flowers of *Zootrophion*, there is no doubt that a different pollinating mechanism operates in this genus. Flowers probably attract pollinators that enter through one of the so-called lateral "windows" in the synsepal. In other Pleurothallidinae such as *Dracula*, *Masdevallia*, and *Specklinia*, the papillose, warty sepals attract pollinators, which initially land on these surfaces and spend most of the time collecting floral rewards (Endara *et al.* 2010; Karremans *et al.* 2015). Later they are guided to the entrance of the tiny lip, initiating pollination. The combination of a footed column and motile lip that acts as a hinge in *Anathallis*, *Lankesteriana*, *Tubella*, and *Zootrophion* is similar to some *Bulbophyllum* species (Bartareau 1994; Borba and Semir 1998; Humeau *et al.* 2011). The mobility of the lip is crucial in the pollination mechanism, in which the insect normally walks toward the base of the lip, where its weight activates a lever movement. Consequently, the lip pushes the body of the insect onto the column, thereby sticking the pollinarium to the scutellum. This has been observed for *Trichosalpinx* and likely also occurs in *Anathallis*, *Lankesteriana*, *Tubella*, and *Zootrophion* (Bogarín *et al.* 2018)

In *Lepanthes* the combination of apical anthers and sticky viscidium are morphological traits linked to pollination by pseudocopulation in which the insect visits the flowers to mate with them but not to collect compounds. It is still unclear whether the pollination shift per se or the evolution toward a pseudocopulation system involving a diverse group of Diptera underpins the astonishing diversification of *Lepanthes* (Valente *et al.* 2012; Bogarín *et al.* 2016).

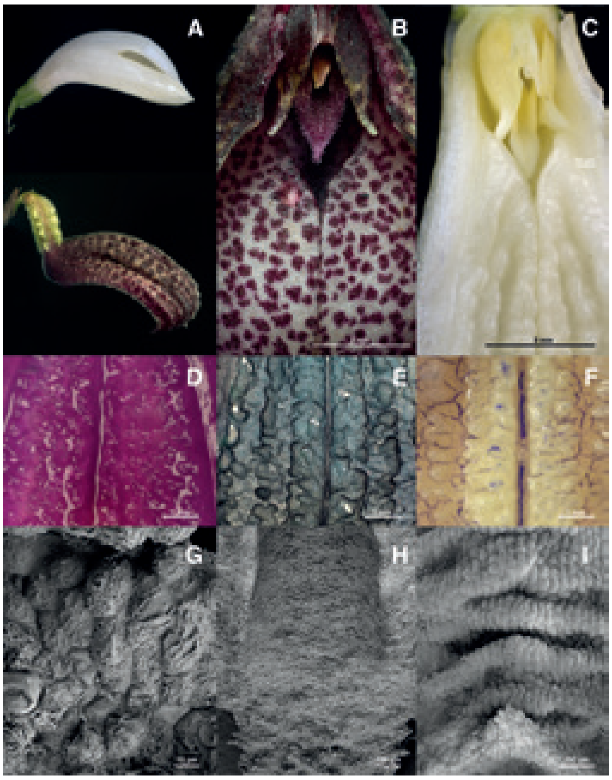

Fig. 9. Flower anatomy and histochemistry of *Zootrophion*. A. Flower of *Zootrophion vulturiceps* (upper), *Zootrophion* sp. (lower); B. Inner view of the flower of *Zootrophion* sp. showing the papillose surface of sepals and the shorter column, lip, and petals; C. Inner view of the flower of *Z. vulturiceps* showing the rugose surface of sepals and the shorter column, lip, and petals; D-F: Histochemistry of cells of the epidermis of the synsepal of *Z. vulturiceps*. D. Staining with PAS (carbohydrates); E. Staining with SBB showing lipids (black); F. Staining with CBB showing proteins (blue). G-I: SEM micrographs of the rugose surface of the lip of *Z. endresianum*. G. Epicuticular compounds on the epidermis; H. View of the papillose mid-part of the lip; I. Rugose surface of the lip.

Conclusions

In addition to macromorphological similarities of the flowers of *Lankesteriana* and *Trichosalpinx* and some *Anathallis*, the species of these genera share micromorphological and histological characters that support a hypothesis of pollination by biting midges and thus parallelism. One of the most important shared characters is the secretion of proteins in the papillae of the lip and the striate cuticle of their epidermis. Species of *Trichosalpinx* employ this strategy to attract females of *Forcipomyia* for pollination, and this might occur in *Lankesteriana* and some *Anathallis* as well.

Two different families of Diptera – Sciaridae and Ceratopogonidae – carry out the pollination of *Lepanthes* and *Trichosalpinx*, respectively. It is likely that other members of the group are pollinated by Diptera. In *Tubella*, *Lepanthopsis*, *Zootrophion*, and possibly other genera, the pollination systems are probably different from those already known. Apart from the pollination system, in *Anathallis*, *Frondaria*, *Lankesteriana*, *Tubella*, *Trichosalpinx* s.l., and *Zootrophion*, the pollinarium is deposited on the thorax of the pollinator because the columns are long and arcuate with incumbent anthers and pollinaria with sticky caudicles. In contrast, in *Lepanthopsis* the pollinarium is likely not deposited on the thorax of the pollinator because the column is short and bilobed, and the flower therefore does not allow for an entrance and exit as described for the genera mentioned above. Therefore, *Lepanthopsis* might employ a similar pollination strategy as *Platystele*, *Stelis* or *Pleurothallis*.

Among the most important micromorphological characters to characterize the groups in the *Lepanthes* clade are the location of papillose tissues, the striations of the cuticle of the lip, and the secretion of proteins or carbohydrates at the apex of the papillae. The presence of a papillose, secretory glenion is unique in *Lepanthopsis*; this feature does not occur in other members of the clade. The movable lip attached by a ligament to the column foot evolved several times in the clade and is probably linked to the pollination systems of *Anathallis*, *Frondaria*, *Lankesteriana*, *Tubella*, *Trichosalpinx* s.l., and *Zootrophion*.

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