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Pollination of *Trichosalpinx* (Orchidaceae: Pleurothallidinae) by biting midges (Diptera: Ceratopogonidae)

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Pleurothallidinae (Epidendreae) are a megadiverse Neotropical orchid subtribe comprising > 5100 species, most of which are probably pollinated by Diptera. The role of pollinators as drivers of species diversity is largely unknown because knowledge of pollination systems in Pleurothallidinae is still scarce. Here, we addressed the pollination of *Trichosalpinx s.s.* through study of floral anatomy, pollinator behaviour and floral traits shared with other angiosperms to elucidate its pollination mechanisms. We identified midge specimens with DNA barcoding and morphology, documented pollination with video recordings, studied the anatomy of flowers by combining microscopy (light microscopy, scanning electron microscopy and transmission electron microscopy) and histochemistry and analysed floral scents with gas chromatography–mass spectrometry. We found that two *Trichosalpinx* spp. are pollinated exclusively by female biting midges of a *Forcipomyia* (*Euprojoannisia*) sp. (Ceratopogonidae). The midges land on the motile lip and appear to suck substances from its papillose surface. We detected secretion of carbohydrates and proteins on the lip and sepals, and thus, *Trichosalpinx* might stimulate a protein collection instinct in female biting midges. The well-developed mandibles and poorly developed laciniae of the pollinators indicate that they mainly feed on invertebrate hosts from which they draw haemolymph. Thus, *Trichosalpinx* flowers offer small quantities of proteins and carbohydrates that may act as flavour teases and together with the colour, fragrances, trichomes and movement of the lip, they probably form part of a complex deceptive system. Some other angiosperms that are also pollinated by biting midges possess similar dark purple flowers with ciliate ornamentation and use myophily, sapromyophily or kleptomyiophily as strategies to exploit different families of Diptera as pollinators. One *Forcipomyia* sp. (*Euprojoannisia*) is kleptoparasitic, suggesting that kleptomyiophily may have evolved in *Trichosalpinx*. The similar floral morphology among members of *Trichosalpinx* and some species of the closely related genera *Anathallis* and *Lankesteriana* suggests that they are also pollinated by biting midges.

ADDITIONAL KEYWORDS: *Aristolochia* – *Bulbophyllum* – *Ceropegia* – *Forcipomyia* – histochemistry – kleptomyiophily – *Lepanthes* – micromorphology – myophily.

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INTRODUCTION

Floral evolution is closely linked to the attraction and behaviour of pollinators. Divergent floral morphologies among species of related genera can result from adaptation to different pollination systems. In contrast, floral similarity (convergence or parallelism) can result from the selection of floral traits due to adaptation to similar pollinators or mimicry of floral signals of co-occurring rewarding species (Borba & Semir, 2001; Smith, Ané & Baum, 2008; van der Niet & Johnson, 2012; Papadopulos *et al.*, 2013; Armbruster, 2014; Dirks-Mulder *et al.*, 2017). Shifts in pollination strategies or adaptations to new pollinators are often associated with plant diversifications (Kay & Schemske, 2008; Johnson, 2010; Smith, 2010), but these shifts or adaptations are not prerequisites for speciation because species radiations without changes in pollinator specialization have also occurred (Ollerton *et al.*, 2009). In addition, some specific pollination systems may increase species diversification independently of the pollination shift (Valente *et al.*, 2012). In Orchidaceae, however, knowledge of pollination systems of species-rich genera, including those in Pleurothallidinae (Epidendroideae: Epidendreae), is scarce. This precludes macroevolutionary studies combining phylogenetics, floral trait changes and pollinator shifts (Smith, 2010; van der Niet & Johnson, 2012; Forest *et al.*, 2014; Givnish *et al.*, 2015; Pérez-Escobar *et al.*, 2017).

Pleurothallidinae are the largest Neotropical orchid subtribe comprising > 5100 species, of which most are probably pollinated by Diptera (Pridgeon *et al.*, 2005). Various pollination strategies are known in detail for some species of *Acianthera* Scheidw. (Phoridae), *Dracula* Luer (Drosophilidae), *Lepanthes* Sw. (Sciaridae), *Octomeria* R.Br. (Sciaridae), *Pleurothallis* R.Br (Mycetophilidae) and *Specklinia* Lindl. (Drosophilidae), all belonging to phylogenetically unrelated groups in the subtribe and pollinated by unrelated Diptera (Duque, 1993; Blanco & Barboza, 2005; Barbosa, de Melo & Borba, 2009; Endara, Grimaldi & Roy, 2010; Duque-Buitrago, Alzate-Quintero & Otero, 2014; Karremans *et al.*, 2015; Pansarin, Pansarin & Martucci, 2016; Policha *et al.*, 2016). One of the most interesting pollination systems of these genera evolved in *Lepanthes*, in which species are pollinated by sexual deception through genitalic pseudocopulation with male fungus gnats of the genus *Bradysia* (Sciaridae), probably attracted by a pheromone-mimicking strategy (Blanco & Barboza, 2005). *Lepanthes* is the most species-rich genus of the subtribe (> 1200 spp.), with *Masdevallia* Ruiz & Pav., *Pleurothallis* and *Stelis* Sw. accounting for 60% of all species of Pleurothallidinae.

Lepanthes forms a monophyletic group with the much less diverse *Anathallis* Barb.Rodr., *Draconanthes* Luer, *Frondaria* Luer, *Lankesteriana* Karremans, *Lepanthopsis* (Cogn.) Ames, *Trichosalpinx* Luer *s.l.* and *Zootrophion* Luer (Pridgeon, Solano & Chase, 2001; Chiron, Guiard & van den Berg, 2012; Karremans, 2014). These genera display extraordinary divergent floral morphologies suggesting adaptation to different pollinators. In addition, this clade underwent rapid speciation in the highlands of the Andes and Central America and exhibits the highest rates of species diversification in Pleurothallidinae (Givnish *et al.*, 2015; Pérez-Escobar *et al.*, 2017). However, the role of pollinators as drivers of species diversity in the group is largely unknown. Apart from pollination of three *Lepanthes* spp., nothing is known about the pollination of the sister groups (Blanco & Barboza, 2005) (Fig. 1). To better understand the role of such biotic factors in the evolution of *Lepanthes* and close relatives, we investigated the pollination system of two *Trichosalpinx* spp.

Trichosalpinx comprises *c.* 110 species, ranging from Mexico and Central America to the Andean regions of Peru and Bolivia, Venezuela, French Guiana, southern Brazil and the Antilles (Luer, 1983). The genus is polyphyletic according to initial phylogenetic evidence (Pridgeon *et al.*, 2001; Chiron *et al.*, 2012; Karremans, 2014). Thus, in this study we focused on three species of *Trichosalpinx* subgenus *Trichosalpinx* or *Trichosalpinx s.s.* (herein referred to as *Trichosalpinx*) that belong to one of the subclades closely allied to *Lepanthes* (Fig. 1). *Trichosalpinx* spp. have non-prolific ramicauls, racemose inflorescences, which are usually shorter than the leaves, and produce purple, pinkish or reddish-vinaceous flowers that open simultaneously (Luer, 1997; Fernández & Bogarín, 2011).

One of the most visible features of *Trichosalpinx* flowers regarding pollination is the dark purple, ciliated lip, which is movable under the weight/momentum of the pollinators and which vibrates with the air due to the union of the lip base with the column foot through a flexible, thin labellar ligament (Luer, 1997). Motile lips also evolved independently in the closely related *Anathallis* and *Lankesteriana* (Pridgeon *et al.*, 2001; Luer, 2006; Karremans, 2014) and other Pleurothallidinae such as *Specklinia*, *Stelis s.l.* (*Condylago* Luer), *Masdevallia* and *Porroglossum* Schltr. (Pridgeon *et al.*, 2005). The pantropical *Bulbophyllum* Thouars (Dendrobiinae), which is another diverse but unrelated genus thought to contain many myophilous species, also exhibits a wide variety of motile lips and appendages (Bartareau, 1994; de Pádua Teixeira, Borba & Semir, 2004; Davies & Stpiczyńska, 2014; Kowalkowska, Kozieradzka-Kiszkurno & Turzyński, 2014; Phillips *et al.*, 2014; Stpiczyńska, Davies & Kamińska, 2015).

Vogel (2001) conducted extensive studies on the role of motile, vibrant structures called 'flickering bodies' that are mostly present on the sepals, petals or lip in some species of the *Bulbophyllum*, *Pleurothallis*, *Specklinia* Lindl. and *Trichosalpinx*. They are diverse in structure and comprise appendages, trichomes, cilia or vibratile hairs. These structures are associated with

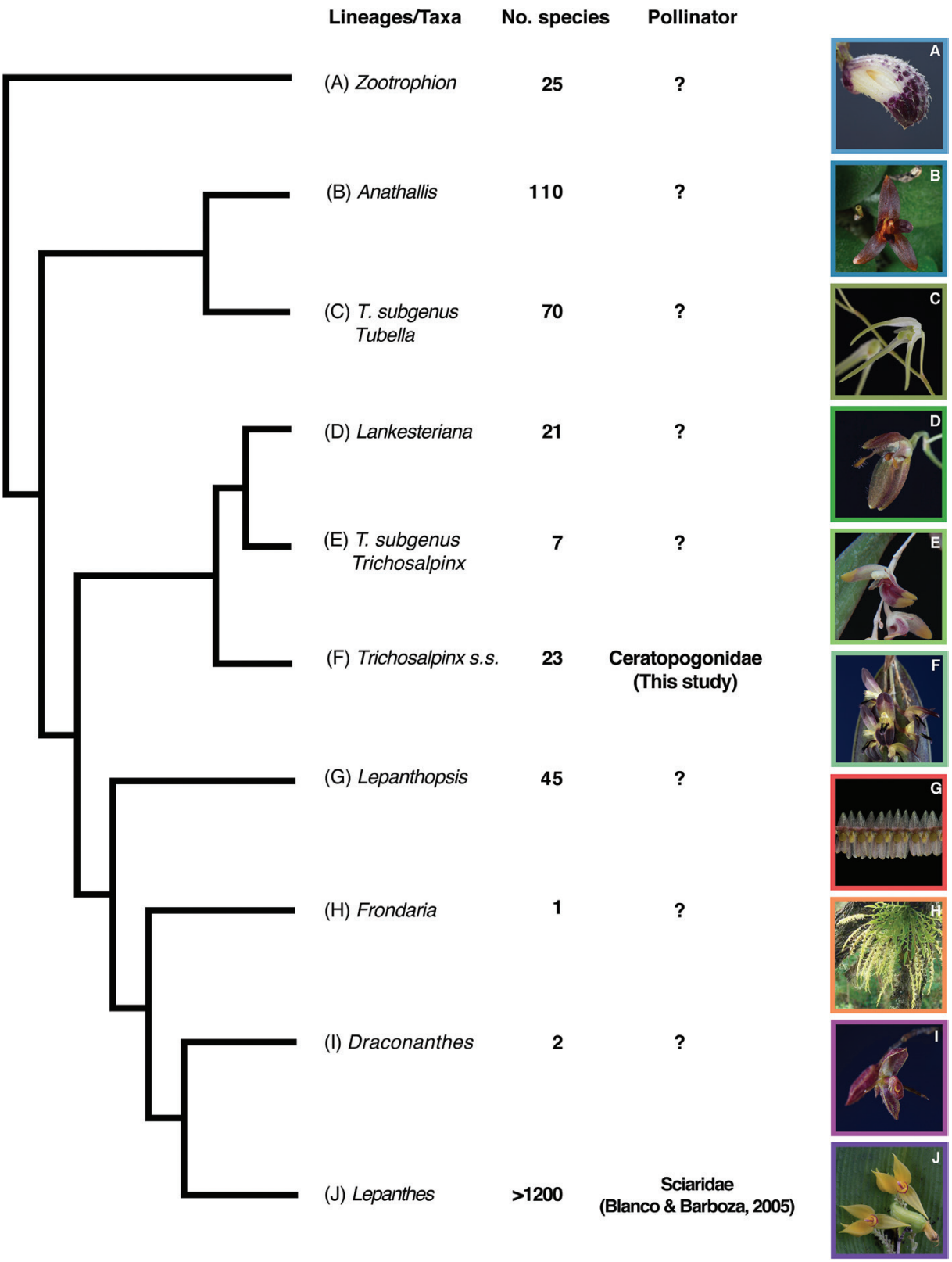

Figure 1. Phylogenetic summary of the *Lepanthes* clade showing the main lineages/taxa, the number of species in each taxa and pollinator information. *Lepanthes* accounts for 85% of the species of the clade. Only two cases of pollination have been documented for the entire clade. Phylogenetic tree based on our unpublished data using combined ITS and *matK* with Bayesian inference.

deceptive systems, which involve mimicry of insect prey models (Gibernau, Macquart & Przetak, 2004; Heiduk *et al.*, 2010, 2015, 2016; Oelschlägel *et al.*, 2015). Vogel (2001) considered flowers bearing flickering bodies as deceptive, even if they offer some nectar rewards. However, most of these pollination syndromes have not been tested experimentally.

Dark purple, motile floral appendages are present in several unrelated angiosperm families (magnoliids, monocots and eudicots) such as Aristolochiaceae (*Aristolochia* L. and *Pararistolochia* Hutch. & Dalziel), Apocynaceae (*Caralluma* R.Br., *Ceropegia* L., stapeliads), Malvaceae (*Abroma* Jacq., *Herrania* Goudot and *Theobroma* L.) and Orchidaceae (*Bulbophyllum*, *Caladenia* R.Br., *Disa* P.J.Bergius, *Genoplesium* R.Br., some Pleurothallidinae, *Pterostylis* R.Br. and *Telipogon* Kunth) (Meve & Liede, 1994; Young & Severson, 1994; Vogel, 2001; Jürgens, Dötterl & Meve, 2006; Ollerton *et al.*, 2009; Williams & Adam, 2010; Phillips *et al.*, 2014). Pollination strategies in some of these taxa involve dipterans belonging to different groups such as Ceratopogonidae (biting midges), Chloropidae (grass flies), Drosophilidae (vinegar flies), Milichiidae (filth flies), Cecidomyididae, Sciaridae and Phoridae (gall and fungal gnats), Sarcophagidae (flesh flies) and Calliphoridae (blowflies) (Bartareau, 1994; Borba & Semir, 1998; Ollerton *et al.*, 2009; Humeau *et al.*, 2011; Gamisch, Fischer & Comes, 2014; Woodcock *et al.*, 2014; Heiduk *et al.*, 2015; Stpiczyńska *et al.*, 2015).

In this study, we identified and documented the behaviour of pollinators of two *Trichosalpinx* spp., described the anatomy, ultrastructure and histochemistry of the flowers and compared these results with other angiosperms pollinated by similar insects to address the following questions: (1) What is the pollination mechanism of *Trichosalpinx*? (2) What is the function of the motile lip and how does the flower attract pollinators? (3) What are the anatomical features shared with other plants pollinated by similar insects? By answering these questions, we hope to improve understanding of the evolution of members of the *Lepanthes* clade.

MATERIAL AND METHODS

Study site and sample collection

We studied the pollination of *Trichosalpinx blaisdellii* (S.Watson) Luer and *Trichosalpinx reflexa* Mel. Fernández & Bogarín (Fig. 2) in semi-open greenhouses at Lankester Botanical Garden (JBL), University of Costa Rica, Cartago, and San Miguel de Santo Domingo, Heredia, Costa Rica. In addition, we studied a wild population of *T. reflexa* occurring along the shores of the Turrubaritos river, Turrubares, San José, Costa Rica between 2014 and 2016 (Supporting Information, Table S1). *Trichosalpinx reflexa* is endemic to the lowland areas of the northern and central Pacific of Costa Rica. Plants bloom during the rainy season from August to February and form large populations mostly on 'wild cashew' or 'espavel', *Anacardium excelsum* (Bertero & Balb. ex Kunth) Skeels (Anacardiaceae), and on species of *Ficus* L. (Moraceae). *Trichosalpinx blaisdellii* is found between 0 and 1800 m and blooms between June and March (Luer, 1997).

Midges were filmed, photographed and collected with a pooter between 7:00 and 17:00 and at night between 18:00 and 19:00 for a total of *c.* 36 h of observation. Samples were stored in absolute ethanol for DNA barcoding, and other samples were mounted on microscope slides for morphological identification following Borkent & Bissett (1990). Midge vouchers were deposited at JBL, L, Canadian National Collection (Ottawa, Canada) and Museo de Insectos of the University of Costa Rica (San José, Costa Rica). Plant vouchers were deposited at CR, JBL (spirit), L, and USJ (Supporting Information, Table S1).

Photography, video and digital imaging

Photographs of flowers and videos of flies were taken with a Nikon D7100 digital camera. Images of flies were taken with a Leica Z16 APO A macroscope and a DFC295 Leica camera and Zeiss Stereo Discovery V20 with an AxioCam MRc 5 camera. Digital images of light microscopy (LM) were taken with a Zeiss AXIO Imager. M2 with an AxioCam MRc 5 in bright field (H) and differential interference contrast (DIC). Final digital images and composite figures were processed in Adobe Photoshop CS6 and videos with Adobe Premiere CS6.

Fixation of flowers for microscopy

Samples were stored in FAA (ethanol 50%, acetic acid and formalin at 18:1:1 v/v) or 70% ethanol. For Epon (Electron Microscopy Sciences) and LR White (London Resin Company Ltd.) embedding, dissected fresh flowers were fixed for 3 h in a modified Karnovsky fixative (2.5% glutaraldehyde, 2% formaldehyde, pH 7.2), rinsed three times in 0.1 M sodium cacodylate buffer (pH 7.4), stained for 2 h in 2% osmium tetroxide and rinsed in 0.1 M sodium cacodylate buffer (pH 7.4).

Insect DNA barcoding identification

We extracted total genomic DNA from midge leg tissue with the Dneasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) and obtained a 666 bp DNA barcode fragment of the cytochrome *c* oxidase subunit I (*COI*) gene with the primers LCO1490 5I (Folmer *et al.*, 1994) and Lep-F1 5CO1490 5I (Hebert *et al.*, 2004). Polymerase chain reaction (PCR) followed Karremans *et al.* (2015). Sanger sequencing was conducted by BaseClear (http://www.baseclear.com), and sequences were deposited in NCBI GenBank (Supporting Information, Table S1).

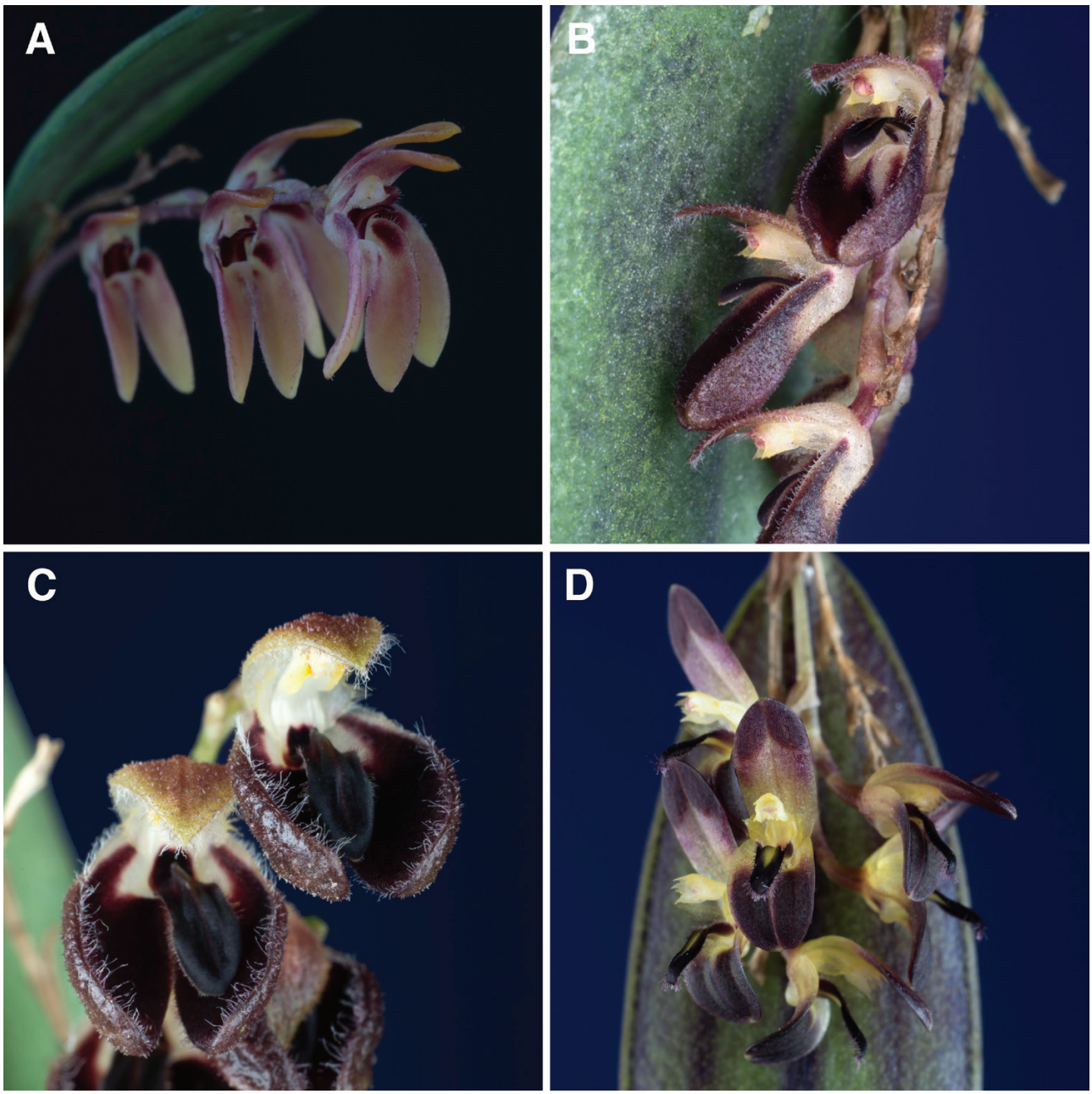

Figure 2. Some representatives of *Trichosalpinx* subgenus *Trichosalpinx*. A, *T. blaisdellii*. B, *T. memor*. C, *T. minutipetala*. D, *T. reflexa*. Photographs by D. Bogarín.

Fragrance sampling and gas chromatography–mass spectrometry

Floral volatiles were extracted by storing at least five flowers collected at anthesis in 4-mL amber glass vials with melamine cap and PTFE liner (Supelco–Sigma–Aldrich Co.) filled with 0.5 mL of chromatography grade hexane. We removed water and particles accumulated with a custom-made column filled with silica gel. Hexane was reduced down to 100 µL by evaporation using a stream of N_2 gas and subsequently analysed by gas chromatography–mass spectrometry (GC/MS). Analyses were carried out using a HP 6890/5973 system equipped with HP-5 fused silica capillary column (30 m × 0.25 mm × 0.25 µm). We identified the compounds using the NIST08 mass spectral database and the NIST MS Search software v2.0. Threshold detection was adjusted to exclude those peaks that were < 3% than the largest peak.

Histochemistry

Entire fresh flowers and hand-cut sections of flowers were stained to detect lipids, polysaccharides and proteins with appropriate positive controls. Pigmented areas of fresh flowers were cleared with 10% (v/v) sodium hypochlorite (Ruzin, 1999). Fresh unstained flowers and hand-cut sections were mounted in glycerine to observe the natural pigmentation. Calcium carbonate crystals were detected by a von Kossa reaction (VK), by treating fresh flowers for 1 h in a 5% (m/v) silver nitrate aqueous solution in the presence of light (60 W), then rinsed three times in distilled water and submerged in a 5% (m/v) sodium thiosulphate (hypo) aqueous solution for 5 min (Sheehan & Hrapchak, 1980; Crookham & Dapson, 1991). Crystals were also detected by birefringence under polarized LM and DIC. Neutral or acidic lipids, phospholipids and fatty acids were detected with a solution of Nile Blue A 1% (NBA) (w/v) (Ruzin, 1999). Sudan IV 0.5% (SIV) (w/v, ethanol 70%) and Sudan Black B (SBB) 0.07% (w/v, ethanol 70%) were used to detect lipids (fats, oils and waxes) (Bronner, 1975; Ruzin, 1999) and osmium tetroxide (OsO_4) for unsaturated fats (Southworth, 1973). Insoluble polysaccharides and starch were detected with a periodic acid–Schiff reaction (PAS) following Ruzin (1999). Mucilage-secreting areas with acidic compounds, pectic acids or hexuronic acids were detected with Ruthenium Red 0.05% (RR) (w/v) (Southworth, 1973). Proteins were detected with Aniline Blue-Black (ABB) 1% in 7% acetic acid (Jensen, 1962; Fisher, 1968) and Coomassie brilliant blue R-250 (CBB) in a solution of 0.25% CBB, 50% ethanol and 7% acetic acid (Jensen, 1962; Fisher, 1968). Areas of fragrance emission were detected with a solution of Neutral Red 0.1% (NR) (w/v, tap water) (Ruzin, 1999).

Light microscopy

Epoxy resin: fixed samples were dehydrated for 15 min in a series of ethanol and successive 1% UAR-EMS uranyl acetate replacement. The ≥ 99.9% ethanol was later replaced with propylene oxide. The samples were infiltrated in a mixture of propylene oxide and Epon. After overnight evaporation of the remaining propylene oxide, the samples were placed in fresh Epon for 3 h, polymerized at 60 °C for 48 h and sectioned at 1.5 µm with a Reichert Jung 2040 rotary microtome. Sections were mounted on microscope slides following Hamann, Smets & Lens (2011). Epon sections were observed with transmission electron microscopy (TEM) and LM. The Epon sections for LM were stained with toluidine blue O (TBO) 1% (w/v) in 1% (w/v) sodium borate and PAS as described above and mounted in Entellan. *LR White*: fixed samples were embedded following Hamann *et al.* (2011). Each sample was polymerized at 60 °C for 48 h, sectioned (4 µm thickness), mounted and stained as described above for Epon samples. *Paraffin-Paraplast:* fixed samples were rinsed in water and dehydrated in a series of ethanol:xylene solutions. Then, they were stored in xylene for 8 h, infiltrated in Leica Paraplast and placed in an oven at 60 °C for 1 day. Infiltrated samples were solidified and sectioned at 4–8 µm thickness. Deparaffination of samples was performed in a series of xylene:ethanol and later stained with TBO 1% (w/v) in 1% (w/v) sodium borate and PAS as described for Epon and LR White samples. Etzold's staining (Basic Fuchsin 10 mg, Safranin 40 mg, Astra Blue 150 mg, acetic acid 2 mL and distilled water to complete 100 mL) was performed by submerging the sections for 30 min in Etzold's, rinsed in tap water for 5 min and demineralized water for 1 min. Dehydration of samples for Etzold's, TBO and PAS was performed by a series of ethanol:xylene solutions.

Scanning electron microscopy

Fixed flowers were dehydrated in a series of ethanol solutions and twice in fresh ≥ 99.8% acetone. Critical-point drying was performed using ≥ 99.8% acetone and liquid CO_2 with an Automated Critical Point Dryer Leica EM CPD300 following the manufacturer's protocols (Leica Microsystems, Wetzlar, Germany). Samples were sputter-coated with 20 nm of Pt/Pd in a Quorum Q150TS sputter-coater and observed with a JEOL JSM-7600F field emission scanning electron microscope, at an accelerating voltage of 10 kV.

Transmission electron microscopy

Fresh dissected flowers were fixed in modified Karnovsky fixative and infiltrated in Epon blocks as described before. Sections of 95 nm were cut with a Leica EM UC7 ultratome with a diamond knife and mounted on film-coated copper slot grids that were later stained with uranyl acetate and lead citrate. Samples were observed and photographed with a JEM-1400 Plus TEM.

Reproductive biology

Cultivated plants of *T. reflexa* were pollinated by hand using pollinia of the same flower (n = 20), pollinia of different flowers of the same inflorescence (n = 26) and pollinia from flowers of different plants (n = 15).

RESULTS

Pollination biology and insect behaviour

Female biting midges of an undescribed species belonging to *Forcipomyia* subgenus *Euprojoannisia* (Diptera: Ceratopogonidae) exclusively visited and pollinated the flowers of the *Trichosalpinx* spp. studied (Figs 3, 4A, B). Formal description of the new species was not undertaken since males are unknown and

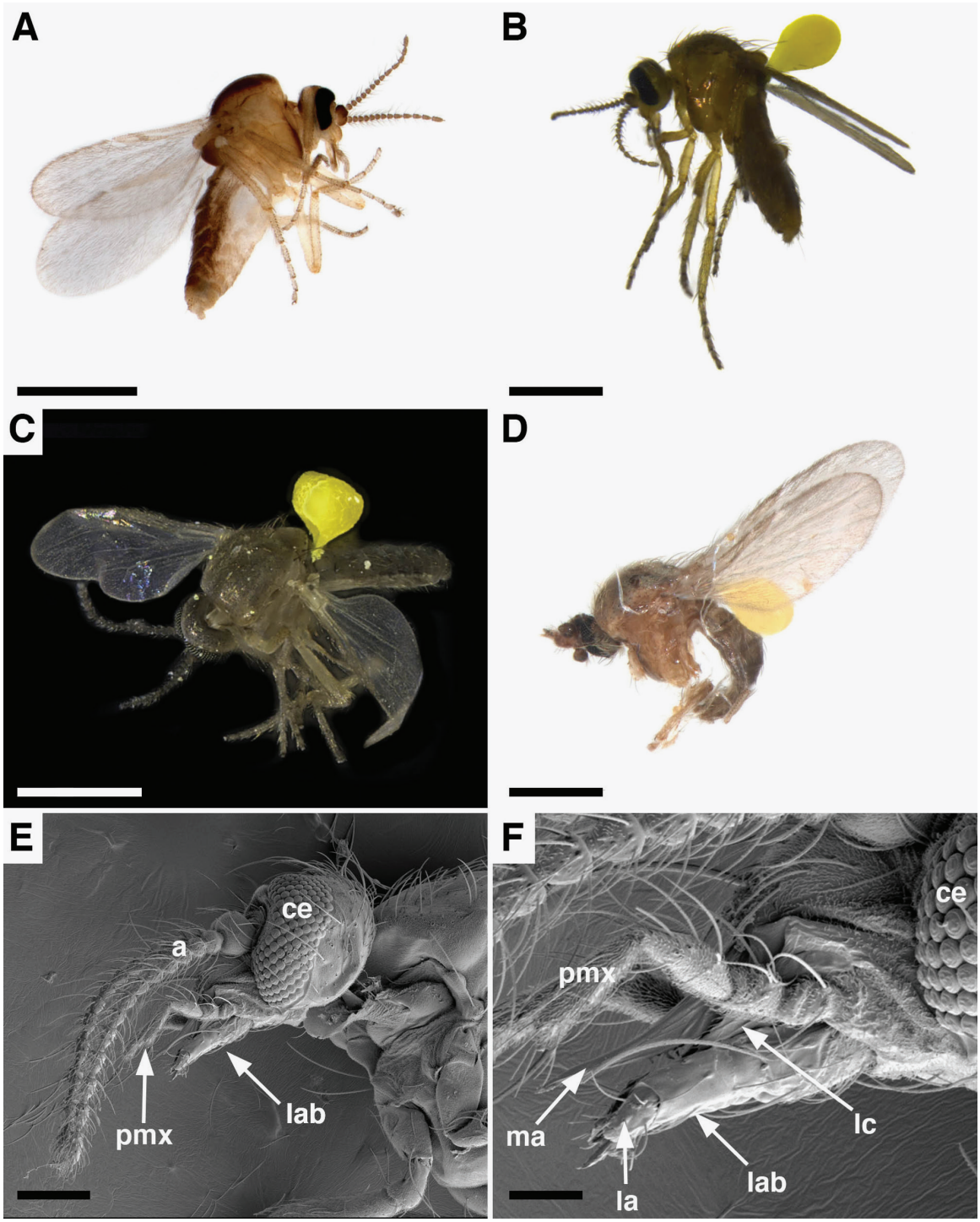

Figure 3. Females of *Forcipomyia* (*Euprojoannisia*) sp. collected visiting specimens of *Trichosalpinx*. A, female (*D. Bogarín 12058, JBL*) visiting *T. reflexa* (*D. Bogarín 7879, JBL*). B, *Forcipomyia* sp. (*D. Bogarín 11420, L*) carrying pollinia of *T. reflexa* (*D. Bogarín 11415, JBL*). C, *Forcipomyia* sp. (*D. Bogarín 12060, JBL*) carrying pollinia of *T. reflexa* (*D. Bogarín 7879, JBL*). D, *Forcipomyia* sp. (*D. Bogarín 11421, L*) carrying pollinia of *T. blaisdellii* (*D. Bogarín 7250, JBL*). E, SEM image of *Forcipomyia* sp. showing the head, compound eyes, antenna and mouth parts. F, detail of mouth parts showing the antenna, mandibles, lacinia and maxillary palps. Scale bars = A–D, 0.5 mm. E, 100 µm and F, 30 µm. a, antenna; ce, compound eye; la, labella; lab, labium; lc, lacinia; ma, mandibles; pmx, maxillary palp. Photographs by D. Bogarín.

Figure 4. A, females of *Forcipomyia* sp. visiting *T. reflexa*. B, *Forcipomyia* female dead in *T. blaisdellii*. C, fruit of *T. blaisdellii*. D, fruit of *T. reflexa*. Both fruits developed under greenhouse conditions after midge visitation. Photographs by D. Bogarín.

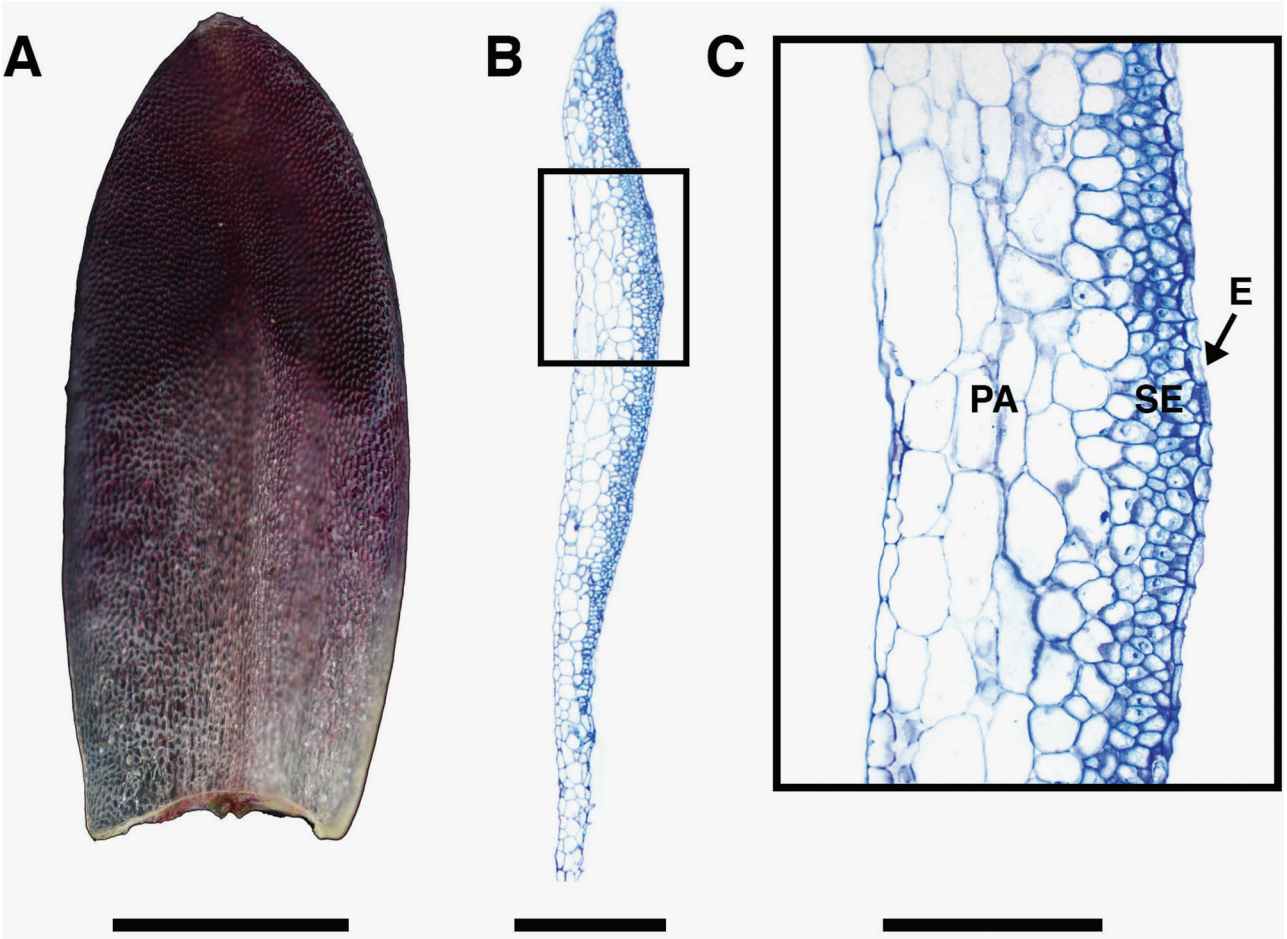

Figure 5. Anatomy of the dorsal sepal of *T. reflexa*. A, dorsal sepal of *T. reflexa* showing the colourless base and purple apex. B, longitudinal section of sepal showing anatomical differentiation of the cells of the epidermis and mesophyll. Stained with toluidine blue. C, detail of the longitudinal section of the sepal. Scale bars = 1 mm, 500 µm and 200 µm, respectively. E, epidermis; PA, ground parenchyma; SE, subepidermal layer. Photographs by D. Bogarín.

these generally exhibit diagnostic features. However, we obtained a *COI* barcode of five specimens to aid with future identification of males (Supporting Information, Table S1). We collected 21 midges (Supporting Information, Table S1) that visited the flowers mostly from 7:00 to 15:00 h, but we documented nocturnal visitation once (18:00). At least ten midges visited the five flowers of a single inflorescence and one to six individuals accessed a single flower simultaneously in *T. reflexa* (Supporting Information, Video S3). The latter was the most frequently visited species and four midges removed the pollinarium (Fig. 3; Supporting Information, Video S1). One midge removed the pollinarium of *T. reflexa* from the anther and deposited it on the stigma of another flower (Fig. 4A, B). We also observed six midges visiting *T. blaisdellii* (pollinaria removal and pollination only once; Supporting Information, Table S1 and Video S5) and *Trichosalpinx minutipetala* (visitation only). We did not observe visitation, pollination and fruit production in other *Trichosalpinx* spp. cultivated in the same greenhouse. Five individuals of *T. reflexa* and two of *T. blaisdellii* developed fruits under greenhouse conditions (Fig. 4C, D).

The female *Forcipomyia* sp. approached the flowers in an irregular zigzag flight and landed on the lateral sepals. They immediately walked to the lip and began to inspect the papillose surface from the apex to the base. They were particularly interested in the apex of the ciliate margin of the lip and the short papillae on the surface where they sought and sucked exudates from the cuticular surface using the labella of their mouthparts. The midges did not pierce the lip with mouthparts (Supporting Information, Videos S1–S4). Occasionally they also walked to the sepals and sucked substances from the surface. Sometimes, the midges walked on the purple surface of the sepals and attempted

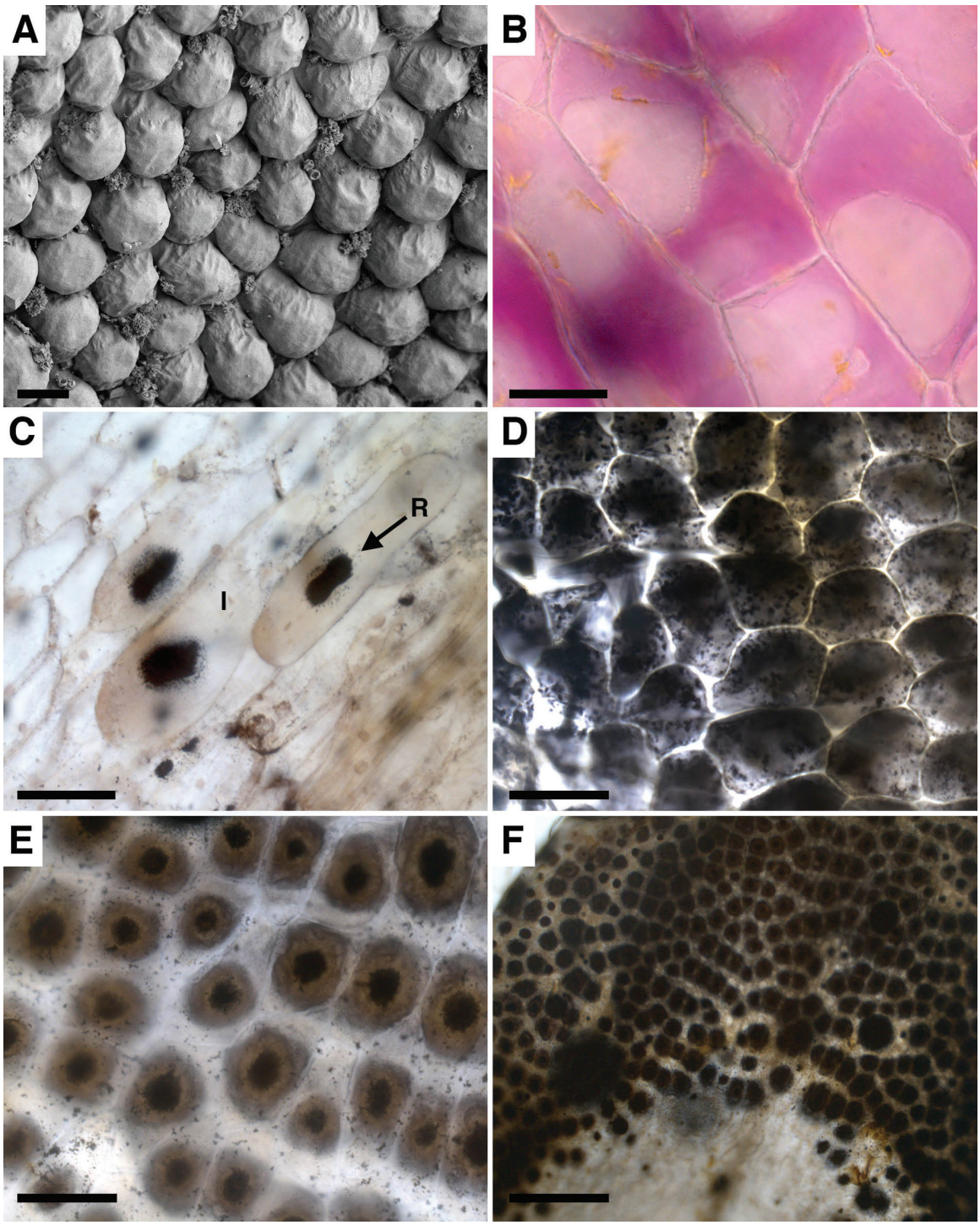

Figure 6. Anatomy and micromorphology of the sepals of *T. reflexa*. A, SEM of the surface of the apical part showing the dome-shaped cells of epidermis and epicuticular secretions. B, LM of unstained sepal showing pigmentation (anthocyanins). C, LM of raphides of parenchyma stained with VK (black). D, LM of cells of the epidermis stained with OsO_4 showing lipid droplets (black). E, LM of the epidermal cells of the dorsal sepal stained with VK (ions, phosphate, urates). F, LM of the apex of the dorsal sepal. Note that only the cells of the purple apical area react with VK. Scale bars = 10, 20, 50, 50, 50 and 200 µm, respectively. I, idioblast; R, raphides. Photographs by D. Bogarín and M. M. Chabert.

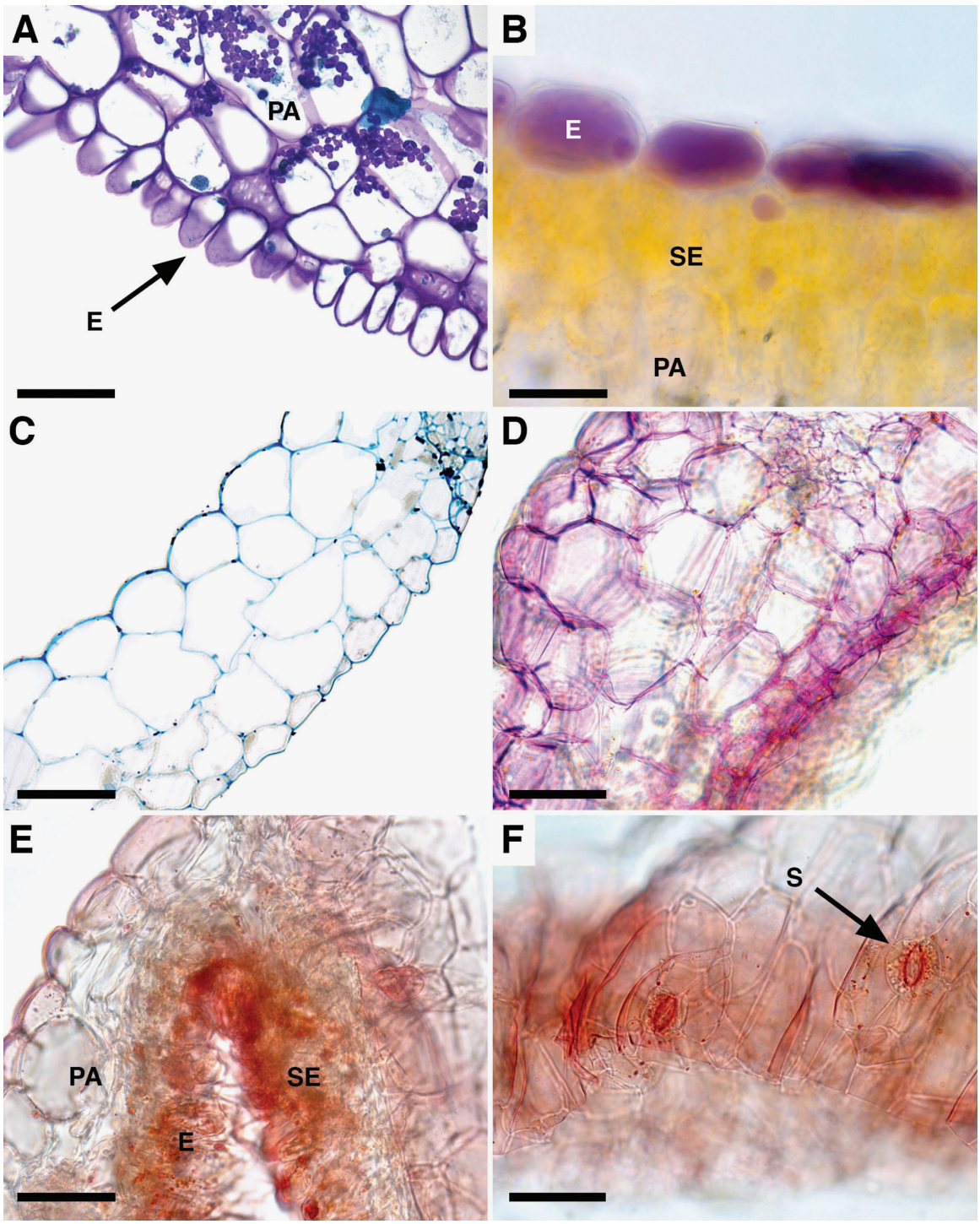

Figure 7. Anatomy and micromorphology of the sepals of *T. reflexa* under LM. A, ground parenchyma with starch grains (PAS) and ciliate epidermal cells with striate purple cell walls. B, hand-section of unstained sepal showing natural pigmentation in the epidermal cells (anthocyanins) and parenchymatous cells (carotenoids or xanthophylls). C, transverse section stained with NB. Cell walls contain acidic lipids, phospholipids and fatty acids. D, transverse section stained with RR (mucilage). E, transverse section stained with SIV showing lipid concentration in the subepidermal cells. F, transverse section stained with SIV showing stomata and lipid concentration in the guard cells. Scale bars = 50, 20, 50, 50, 50 and 50 µm, respectively. E, epidermis; PA, ground parenchyma; S, stomata; SE, subepidermal layer. Photographs by D. Bogarín and M. M. Chabert.

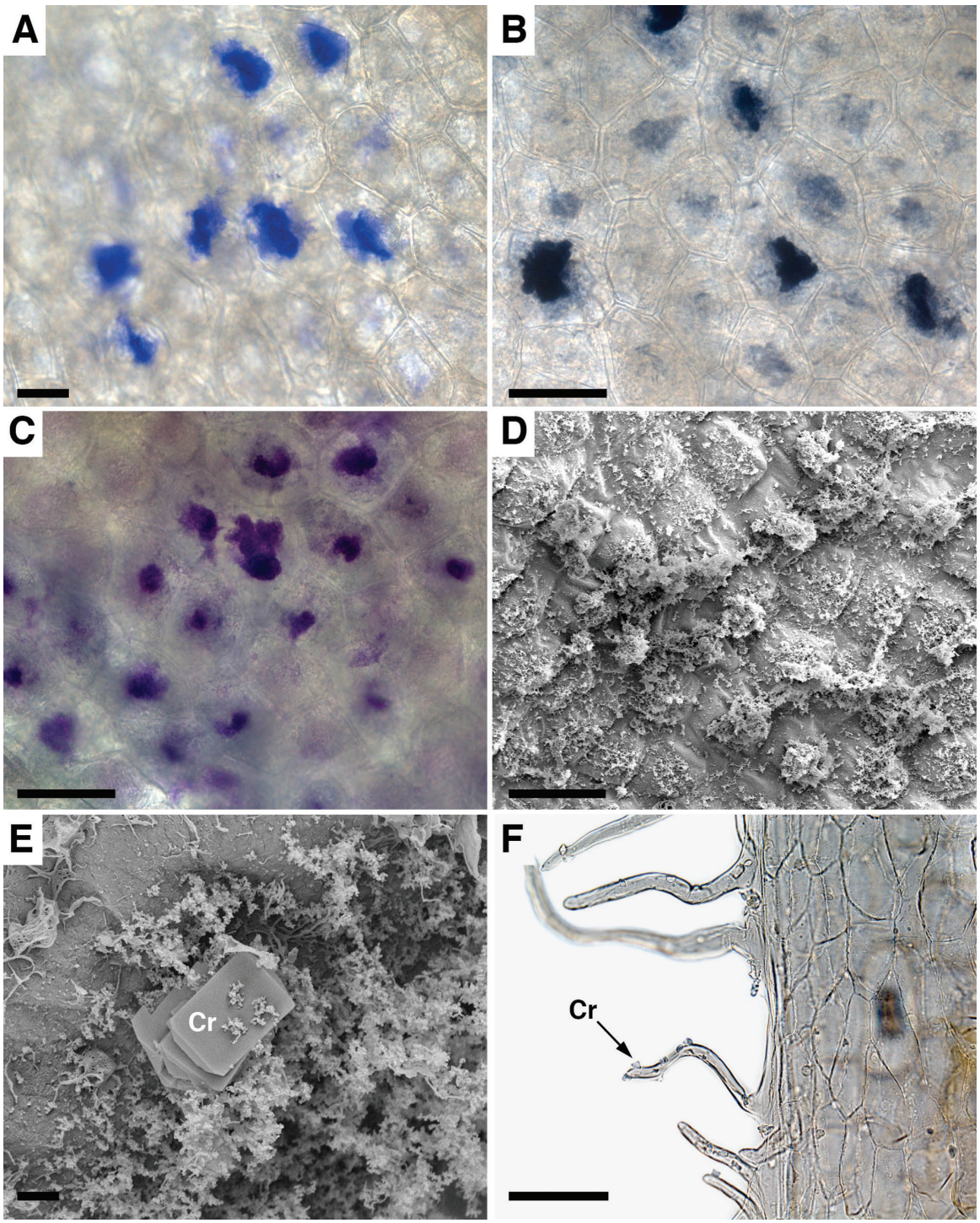

Figure 8. Histochemistry and micromorphology of the sepals of *T. reflexa* under LM and SEM. A, epicuticular proteins on surface of sepals detected with CBB (blue). B, epicuticular proteins on sepals detected with ABB (dark blue). C, epicuticular insoluble polysaccharides detected with PAS (purple). D, SEM of the surface of sepals where the epicuticular polysaccharides and proteins (seen as cotton-like substances) were detected. E, SEM of the surface of sepals showing crystals among the epicuticular compounds. F, LM of the margin of sepals of *T. memor* with calcium oxalate crystals on the surface of unicellular trichomes and epidermis. Scale bars = 50, 20, 50, 40, 10 and 50, respectively. Cr, crystal. Photographs by D. Bogarín and M. M. Chabert.

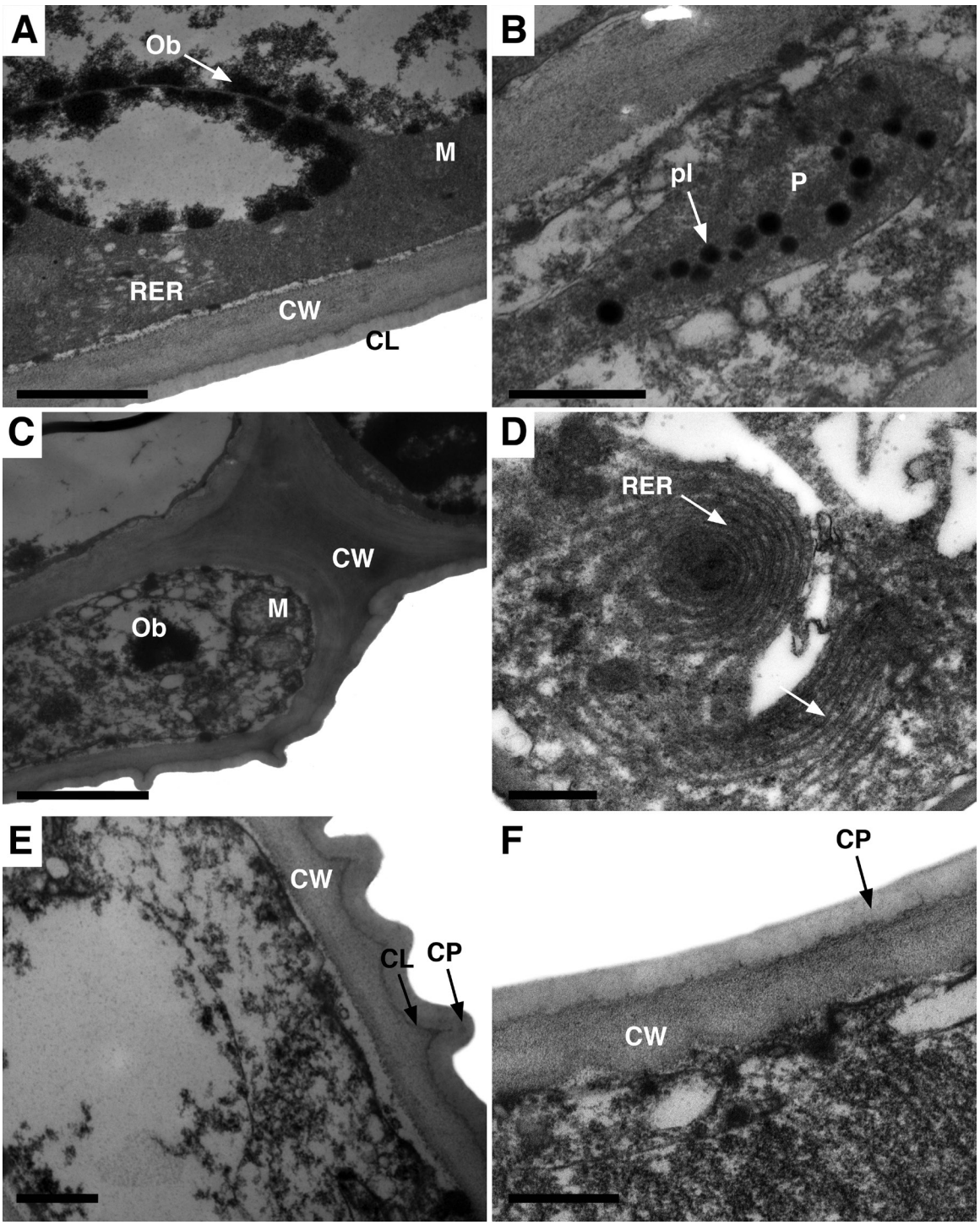

Figure 9. TEM images of the sepals of *T. blaisdellii*. A, osmiophilic bodies (black), mitochondrion and rough endoplasmic reticulum. B, plastid with plastoglobuli. C, epidermal cell with osmiophilic bodies and mitochondrion. D, rough endoplasmic reticulum in the epidermal cell. E, cell wall, cuticle layer and undulate cuticle proper of the epidermal cells. F, flat cuticle layer and osmiophilic bodies in the cytoplasm migrating towards the cell wall. Scale bars = 50, 20, 50, 40, 10 and 50 μm, respectively. CL, cuticle layer; CP, cuticle proper; CW, cell wall; M, mitochondrion; P, plastid; pl, plastoglobuli; RER, rough endoplasmic reticulum. Photographs by D. Bogarín and R. Langelaan.

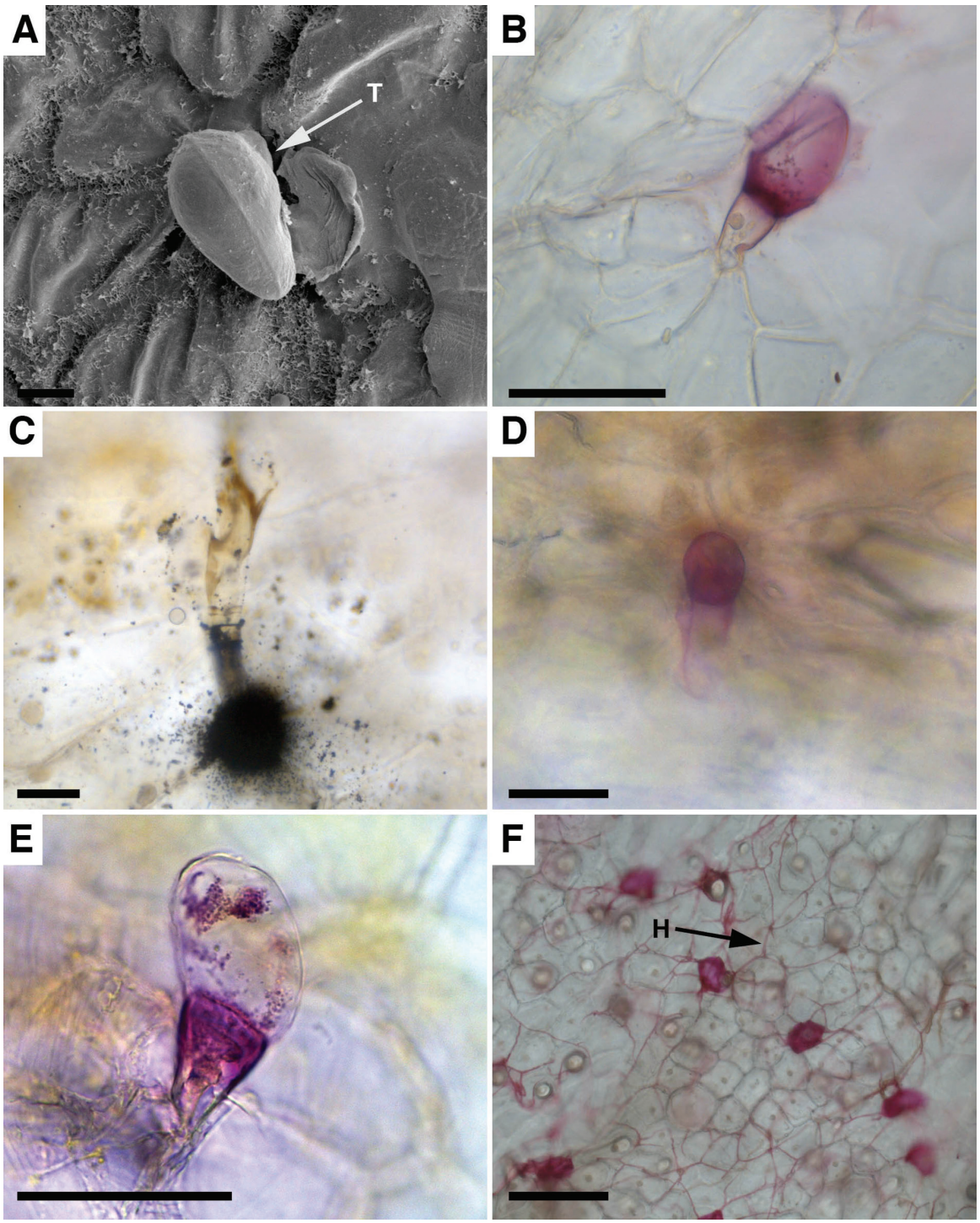

Figure 10. Histochemistry and micromorphology of the trichome-like colleters of sepals of *T. reflexa* and *T. memor* under LM and SEM. A, SEM of trichome-like colleter showing a globose apex among flat epidermal cells. B, LM of cylindrical base and globose apex of the trichome-like colleters showing fragrance emission and secretory activity detected with NR (red) concentrated at the apex. C, LM of the base of the colleter stained positively with VK (ions, phosphate, urates) (black). D, LM showing lipid concentration at the apex of the colleter (red). E, LM of the colleter showing insoluble polysaccharides at the base and some at the apex detected with PAS (pink). F, LM fungal hyphae network associated with colleters detected with PAS in *T. memor*. Scale bars = 50, 50, 20, 50, 50 and 100 μm, respectively. T, trichome-like colleter; H, fungal hyphae. Photographs by D. Bogarín and M. M. Chabert.

to suck substances or stopped to rest, but they paid most attention to the lip. They showed no interest in the petals (Supporting Information, Video S5). When they walked to the lip from the apex, torque forces initially kept the lip horizontally. When the *Forcipomyia* sp. female approached the base near the callus, at the balance point, the weight of the midge initiated a lever movement rapidly lifting the lip *c*. 30–40° upwards. In this movement, the midge was slammed against the column (Supporting Information, Videos S1, S2). When more than one midge was at the apex of the lip, the lever mechanism did not work, apparently because the weight of several individuals did not trigger the lip movement (Supporting Information, Video S3). While they were on the flower, they were occasionally observed cleaning their antennae and mouthparts with their front legs or rubbing their hind legs.

In the struggle to get free, the dorsal part of the midge scraped the apex of the column in the area of the caudicles and removed the pollinarium (or deposited the pollinarium on the stigma if it already carried one). The lip returned to the original horizontal position allowing the midge to fly to another flower or remain on the same. The lateral sepals also served as landing surface when the midge managed to get free from the column after capture. The pollinarium was attached to either the postnotum or first abdominal segment of the midge (Figs 3B–D, 4B). The midge was more easily

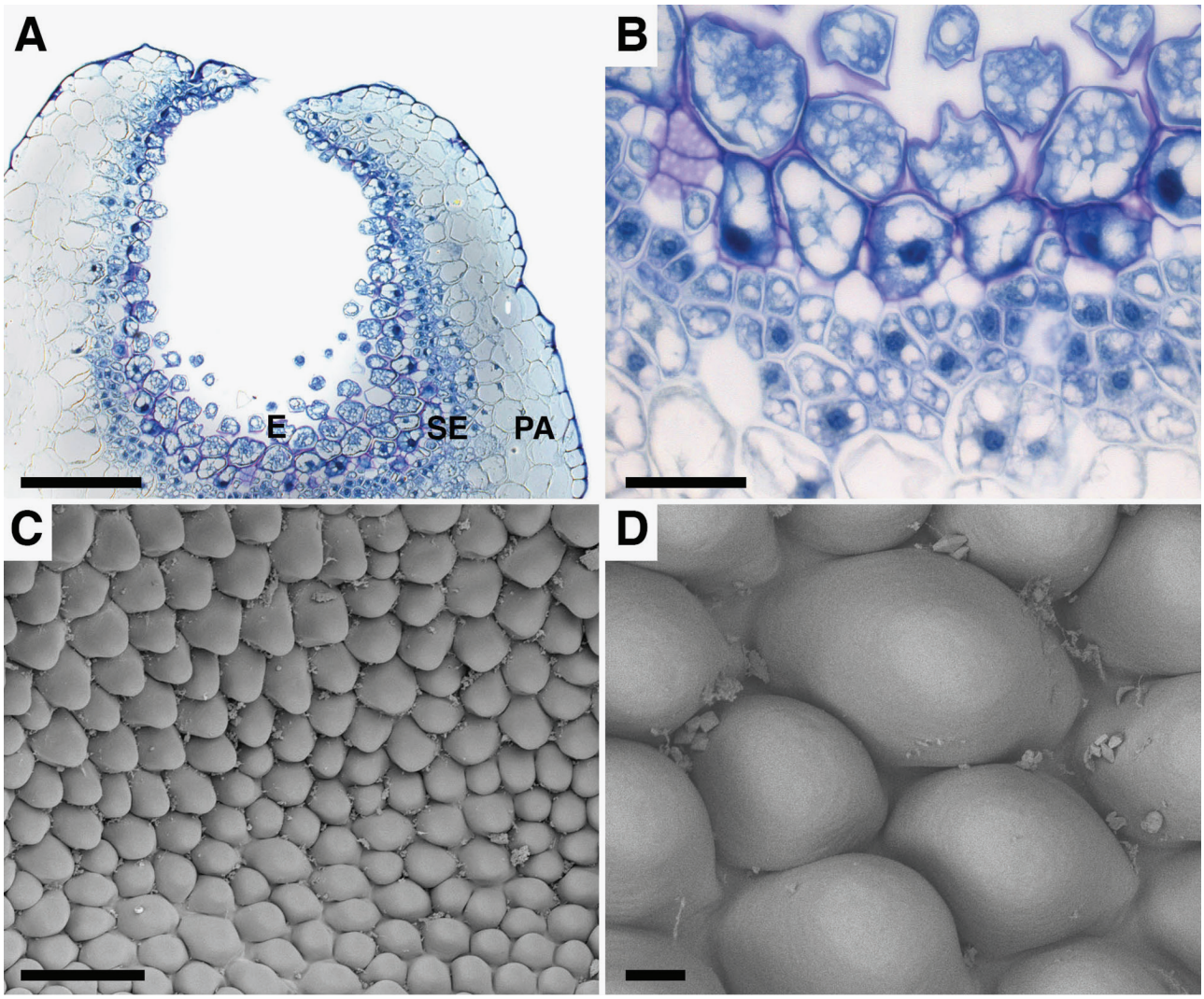

Figure 11. Anatomy and micromorphology of the lateral sepals of *T. memor* under LM and SEM. A, LM of the synsepal showing the anatomical differentiation of the cells of the epidermis and mesophyll. B, LM detail of the epidermal tissue. C, SEM of the epidermal surface showing the papillose surface and epicuticular secretions. D, SEM of the detail of epidermal surface. Scale bars = 200, 50, 100 and 10 µm, respectively. E, epidermis; PA, ground parenchyma; SE, subepidermal layer. Photographs by D. Bogarín and M. M. Chabert.

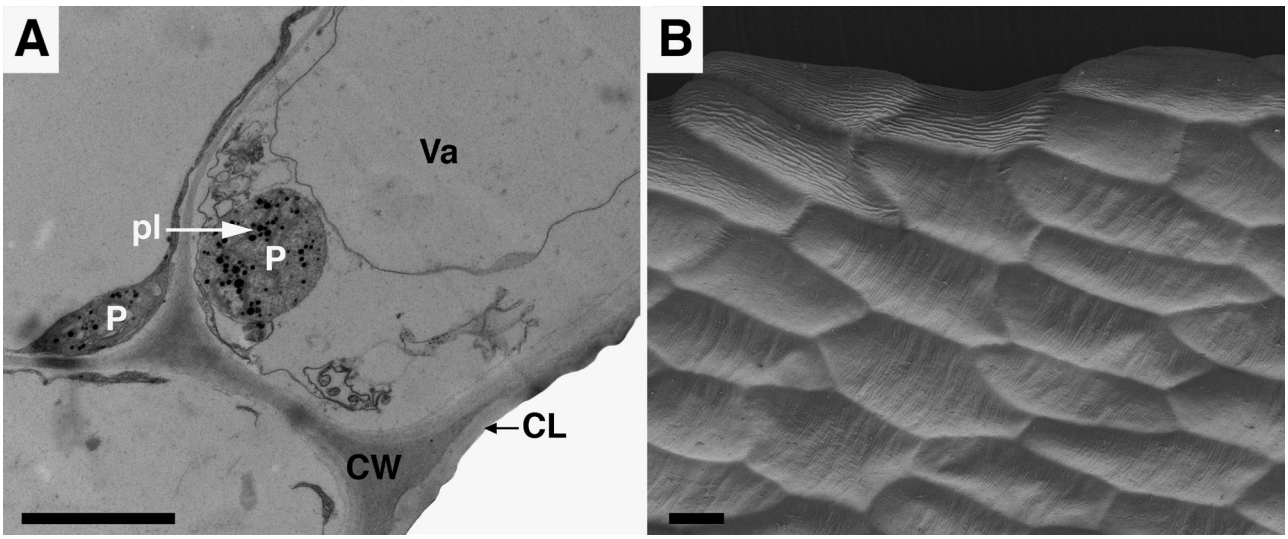

Figure 12. A, TEM image of the epidermal cells of petals of *T. blaisdellii* showing simple cytoplasm containing few plastids rich on plastoglobuli with a system of internal membranes sometimes elongated next to the cell wall, vacuoles, cell wall and cuticle layer. B, SEM image of the epidermal surface of *T. blaisdellii* showing smooth cuticles of the internal cells and the striate patter of the cells along the margin. Scale bars = 2 and 10 µm, respectively. CL, cuticle layer; CW, cell wall; P, plastid; pl, plastoglobuli; Va, vacuole. Photographs by D. Bogarín and R. Langelaan.

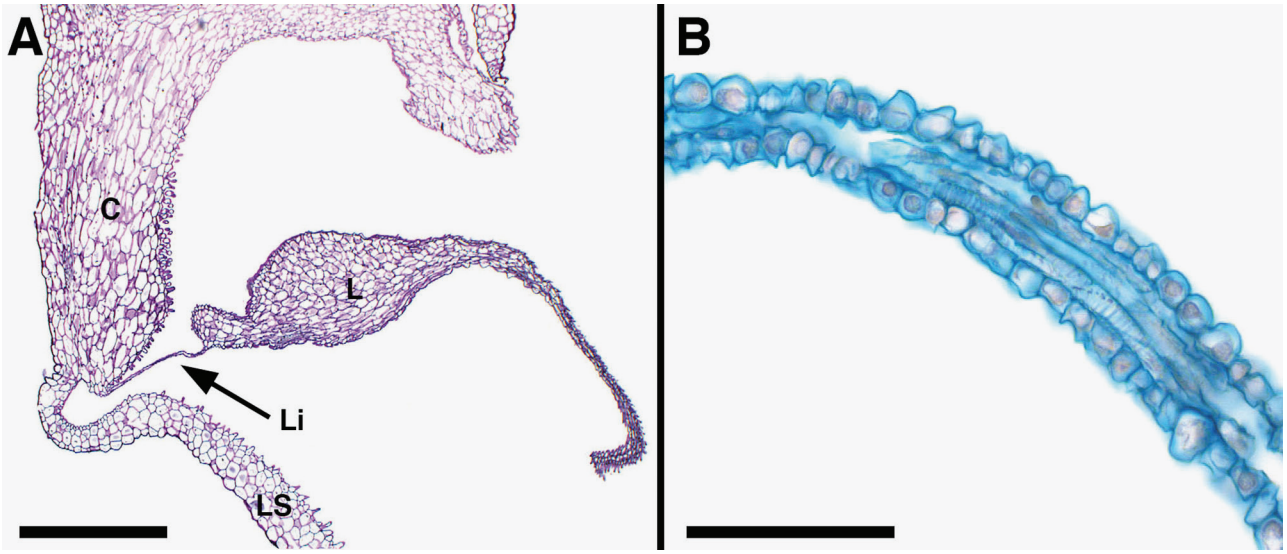

Figure 13. Section of a flower of *T. blaisdellii* (LM). A, LM transverse section showing column foot, labellar ligament with the hinged lip and part of the lateral sepal (stained with PAS). B, longitudinal section of the labellar ligament showing two layers of quadrate cells crossed in the middle by vascular bundles (stained with Etzold's). C, column; L, lip; Li, labellar ligament; LS, lateral sepal. Scale bars = 500 and 500 µm, respectively. Photographs by D. Bogarín and M. M. Chabert.

released if it tried to make a turn to the side after touching the caudicles. On a few occasions, the insect was not able to release the pollinarium and get free; thus, it was trapped and subsequently died in the flower (Fig. 4B). In addition, we did not observe any oviposition behaviour and the flowers observed in scanning electron microscopy (SEM) did not reveal any (traces of) eggs or larvae. Moreover, we did not observe any males visiting the flowers and consequently no sexual behaviour.

Anatomy and ultrastructure of the flowers

Floral morphology of *Trichosalpinx* was described by Luer (1983, 1997) and vegetative anatomy by Pridgeon (1982)

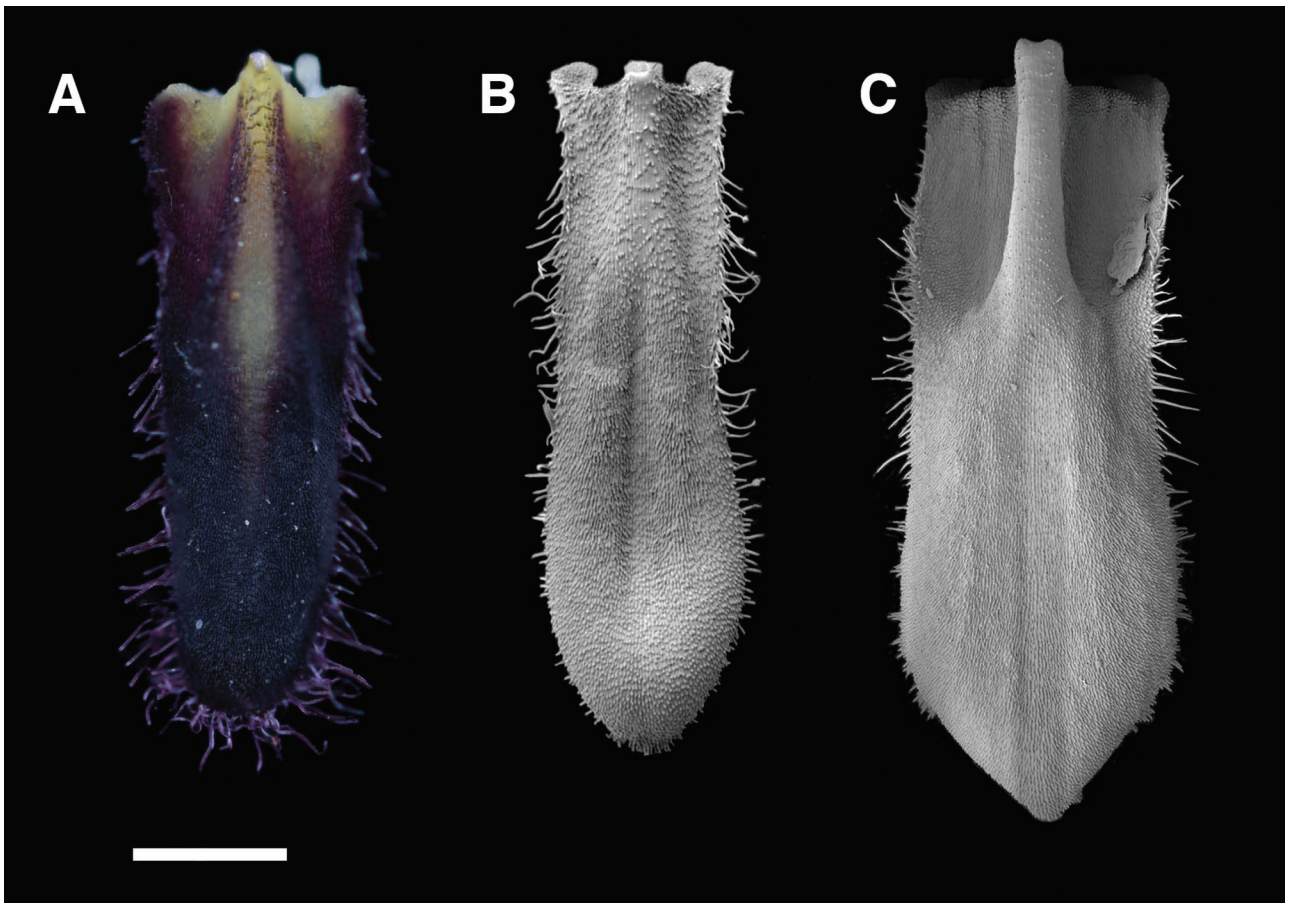

Figure 14. Morphology of the lip of *Trichosalpinx* subgenus *Trichosalpinx*. A, macrophotography of the lip of *T. reflexa* showing natural pigmentation. B, SEM image of *T. blaisdellii*. C, lip of *T. minutipetala* all showing ciliate margins, papillose surfaces, a basal raised callus and a pair of auricles at the base. Scale bar = 500 μm.

and Pridgeon *et al.* (2005). However, the ultrastructure and histochemistry of the flowers has not been previously studied. Here, we describe the ultrastructure of flowers of *T. blaisdellii* and *T. reflexa* focusing on their adaptations to pollination. Histochemical tests and results are consistent between the studied species (unless specified) and summarized in Supporting Information (Table S2).

The sepals have flattened epidermal cells at the base that lack anthocyanins, whereas at the apex, the cells are globose, with anthocyanins, thickened cell walls, lipids (OsO_4) and areas that react with VK (Figs 5A–C, 6A–F, 7B). The subepidermal parenchyma lacks pigments and contains idioblasts and starch grains (PAS) (Fig. 7A, B). The cell wall contains acidic lipids, phospholipids and fatty acids (NBA), insoluble polysaccharides (PAS) and mucilage (RR) (Fig. 7A–D). Stomata are uncommon and located near the midrib or the adaxial surface close to the margin (Fig. 7E, F). The cuticle is conspicuous, smooth, with a lipidic layer (SIV) and various epicuticular secretions such as crystals (VK), waxes (SIV), insoluble polysaccharides (PAS) and proteins (ABB, CBB) (Fig. 8A–F). Proteins and carbohydrates are seen as cotton-like substances, usually mixed with crystals (Fig. 8E). The base of the sepals shows unicellular trichomes (Fig. 8F). TEM observations revealed a reticulate cuticle, dense cytoplasm, plastids with plastoglobuli, osmiophilic droplets, mitochondria, rough endoplasmic reticulum (RER) and occasionally dictyosomes. An exchange of lipidic substances (OsO_4) occurs between the cytoplasm and cell wall of sepals and lip; these compounds migrate into the cuticle and accumulate in channels under the ridges of the cuticle (Fig. 9A–F). The sepals and the ovary have scattered secretory trichome-like colleters, which reacted with PAS, SIV, VK and NR (Fig. 10A–F). In *Trichosalpinx memor*, we detected fungal hyphae associated with colleters on the sepals (PAS) (Fig. 10F). Moreover, flowers of this species have a more obvious anatomical differentiation in the epidermal and subepidermal layers in the synsepal as compared with *T. blaisdellii* and *T. reflexa* (Fig. 11A–D). Furthermore, in *T. memor* (Rchb.f.) Luer and *T. minutipetala* (Ames & C.Schweinf.)

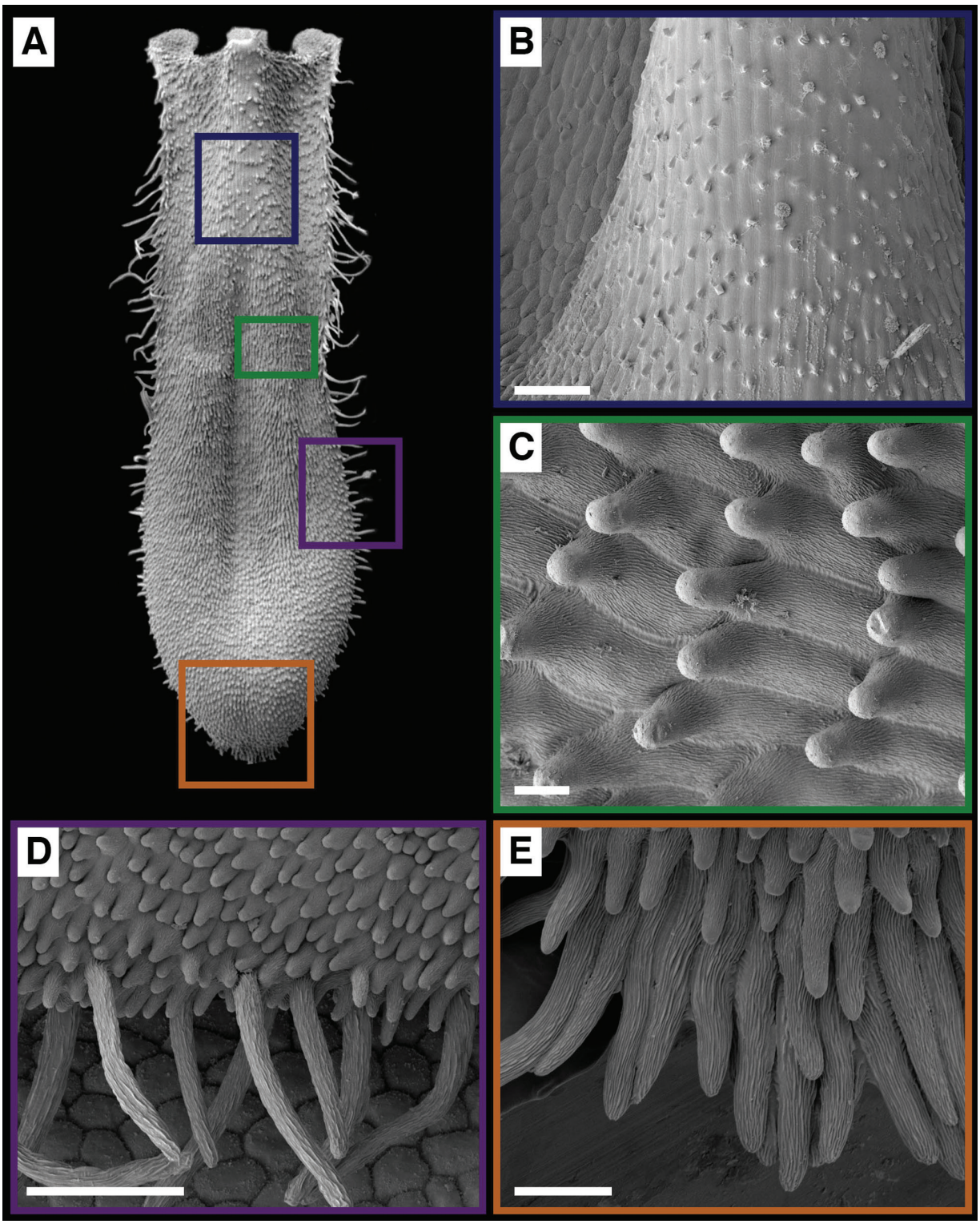

Figure 15. Anatomy of the lip of *T. blaisdellii* (SEM). A, morphology of the lip. B, detail of the callus surface showing scattered papillae and epicuticular substances. C, papillose surface of the lip with characteristic striated pattern of the cuticle and smooth apex of papillae. D, ciliate margin of the lip with elongated unicellular hairs and striate cuticle. E, apex of lip with elongated unicellular cells with striated pattern. Scale bars = 50, 50, 10 and 30 µm, respectively. Photographs by D. Bogarín, M. M. Chabert and F. Gardien.

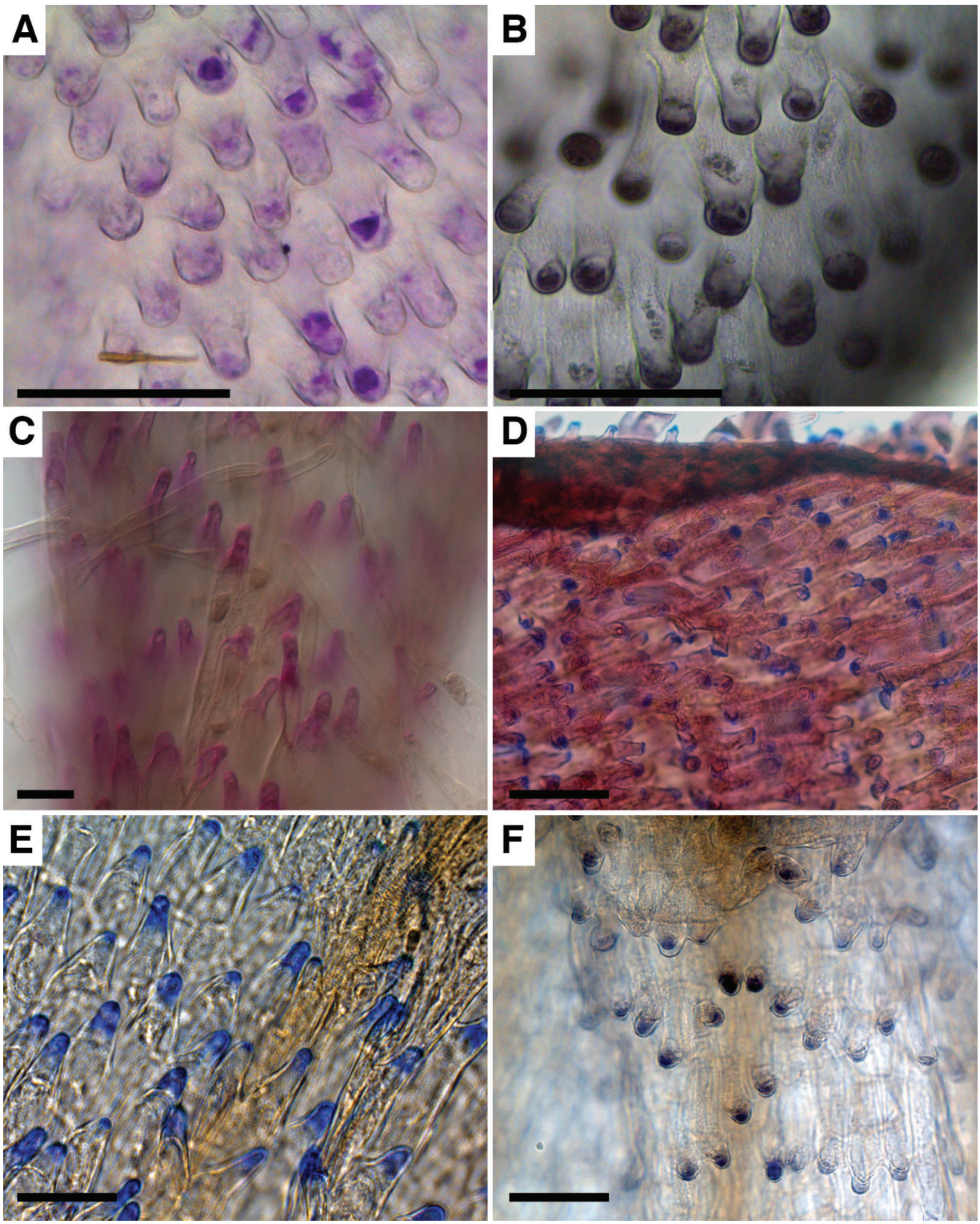

Figure 16. Histochemistry of the papillose surface of the lip of *T. reflexa* (LM). A, insoluble polysaccharides stain pink (PAS). B, lipids stain black (SBB). C, detection of scent emission with NR (red/pink). D, staining of fresh tissue lip with CBB yielded positive results for proteins (blue tips). E, decoloured lip also yielded positive results for proteins (CBB) blue tips. F, papillae of the callus showing proteins at the apices (ABB). Scale bars = 500 μm, 500 nm, 20 μm, 50 μm, 50 μm and 50 μm, respectively.

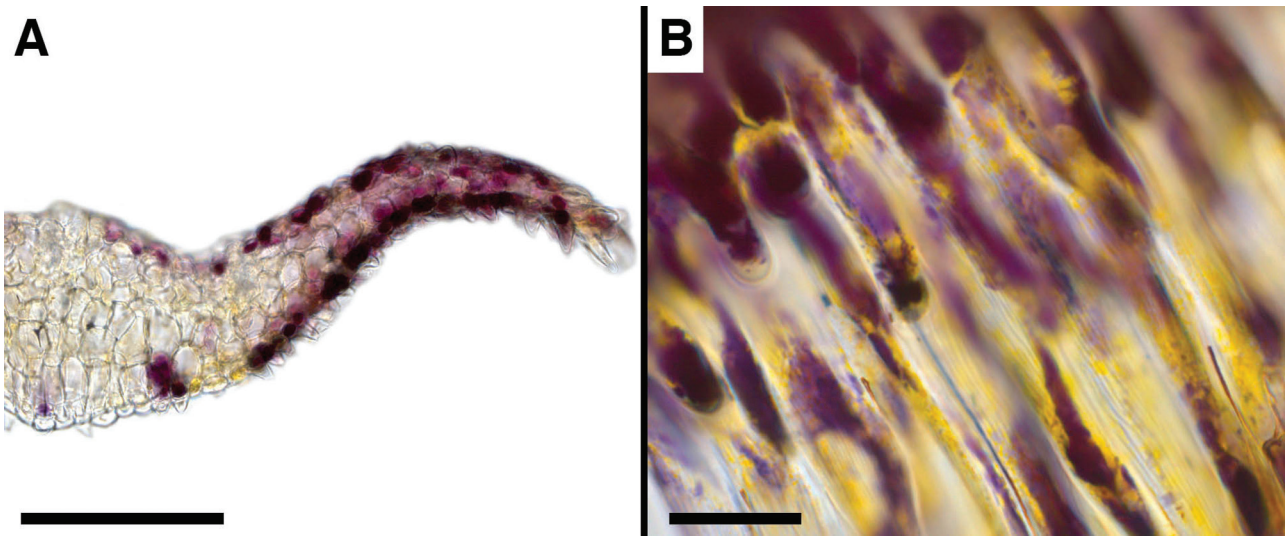

Figure 17. A, unstained transverse hand-section of the lip showing the purple epidermal cells containing anthocyanins, ciliate margins and the uncoloured ground parenchyma. B, detail of the unstained papillose surface of the lip showing the anthocyanins (purple) and carotenoids (yellow) and the striated cuticle. Scale bars = 50 and 20 µm, respectively. Photographs by D. Bogarín and M. M. Chabert.

Luer, the synsepal is concave unlike in *T. blaisdellii* and *T. reflexa*, which is convex or flattened (Fig. 2).

The petals are usually colourless at the base and the cells show few cytoplasmic contents. Histochemical tests yielded no positive results (Figs 2, 4, 12A, B). In contrast, the lip is the structure of interest for the insects, where they reside most of the time (Figs 13, 14, 15). The base of the lip is attached to the base of the column by a membranous tissue that provides the necessary flexibility for slamming a midge against the column (Fig. 13). The lip has two auricles at each side and a raised callus in the middle (Figs 14, 15A). The margins have unicellular elongated cells with a noticeably striated cuticle. The upper epidermis shows shorter cilia than the cells of the margin but with identical cuticle (Fig. 15A–E). We detected polysaccharides (PAS), lipophilic compounds (SBB) and osmiophilic bodies (OsO_4) (Fig. 16A, B) within the papillae, indicating a secretory function. The apices of the papillae along the entire blade reacted positively with NR (Fig. 16C), CBB (Fig. 16D–E) and ABB (Fig. 16F), indicating the presence of scents and proteins, respectively. Anthocyanins are restricted to the upper epidermis and chromoplasts are scattered in the epidermal and subepidermal cells (Fig. 17). In a few samples, we observed crystals of calcium oxalate (VK) exuded by the apices of the papillae (Fig. 18). SEM and TEM revealed a smooth cuticle with accumulations of compounds on the apices of papillae and exudates on the surface (and on crests of the reticulate cuticle at the base) (Figs 19–21). The cell content of the epidermal layer is remarkably dense and complex in comparison with the cells of the parenchyma (Fig. 20B). Osmiophilic substances (revealed by OsO_4) are present between the cytoplasm, plasmalemma and the cell wall and under the ridges of the cuticle (Fig. 21). The cytoplasm contains a dense protein matrix (CBB), an extensive network of RER and osmiophilic bodies (Fig. 20C, E). In addition, we did not detect nectaries or starch grains in the lip.

The arcuate, footed column has similar papillae to those observed on the sepals and lip. The apex is erose or ciliated with two small arms (Fig. 22A). The stigma is ventral, separated from the incumbent anther by a conspicuous membranous rostellum. The pollinarium consists of two globose pollinia with pollen grains arranged in triads or tetrads and gemmate ornamentation. At the base, the pollinarium has sticky caudicles that appear to be tetrads derived from immature pollen grains (Fig. 22).

Fragrance compounds detected by GC/MS

We detected acid chlorides, esters, fatty acids and long-chain aliphatic hydrocarbons with GC/MS. Methyl ester hexadecanoic acid and lactic acid were found in *T. blaisdellii* but not in *T. reflexa*, whereas tridecyl ester octanoic acid was found only in *T. reflexa*. Acid chlorides and aliphatic hydrocarbons were abundant in both species. Compounds detected with the NIST08 mass spectral database and the NIST MS Search software v2.0. are summarized in Supporting Information (Figure S1 and Table S3).

Breeding system

No flowers of *T. reflexa* that were hand pollinated with pollinia from the same (n = 20) or different flowers

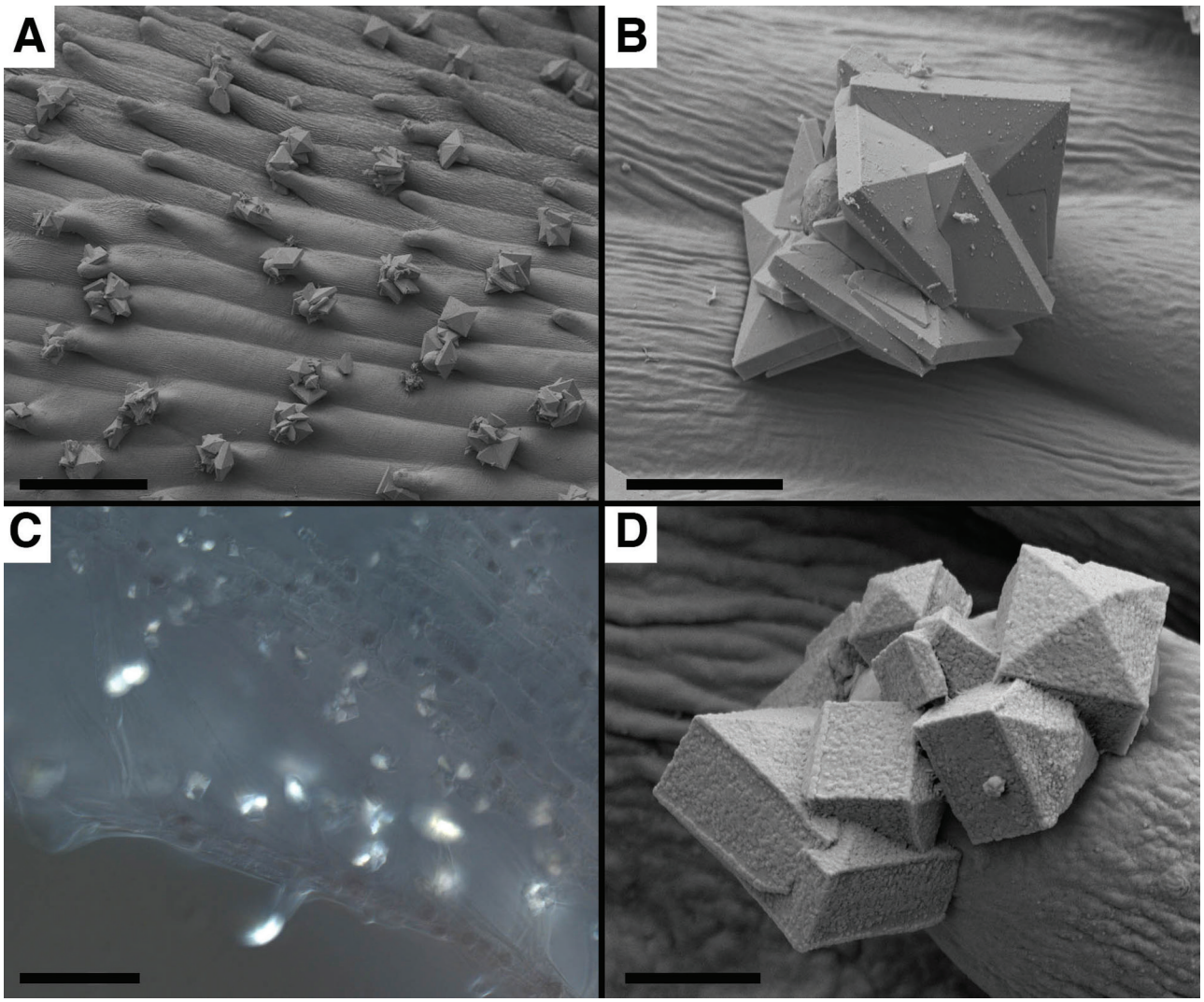

Figure 18. SEM and LM images of the epicuticular crystals of the lip of *T. blaisdellii*. A, crystals on the apices of the papillae. B, detail of the pyramid-shaped crystals with smooth surface. C, birefringence of crystals under LM DIC mode. D, crystals of the apex of papillae with rough surface. Scale bars = 30, 5, 50 and 2 μm, respectively. Photographs by D. Bogarín and M. M. Chabert.

from the same inflorescence (n = 26) developed fruits as all ovaries abscised 2 days after the flowers were pollinated. After hand pollination of flowers from different plants, although, 11 fruits developed (n = 15).

DISCUSSION

POLLINATION SYSTEM OF *TRICHOSALPINX*

Ceratopogonidae are a diverse group of Diptera with a worldwide distribution and 6267 named species (Borkent, 2016). The exclusive presence of females of one *Forcipomyia* (*Euprojoannisia*) sp., the absence of males as pollinators and the secretion of proteins on the lip suggest that *Trichosalpinx* might stimulate protein collection behaviour of females (for egg production) through prey related colours, odours and movement of flickering bodies (Vogel, 2001). Adult females of earliest-branching lineages in Ceratopogonidae are vertebrate blood feeders and, like other biting flies, require a protein meal to produce eggs (some are autogenous or facultatively autogenous) (Borkent, 2004). Males and females also require a source of nutrition to fuel flight, and this may be in the form of sugars from nectar or honeydew. Subfamily Forcipomyiinae, also an early lineage, includes two genera, *Forcipomyia* and *Atrichopogon*. The adult females are nearly all ectoparasites of other

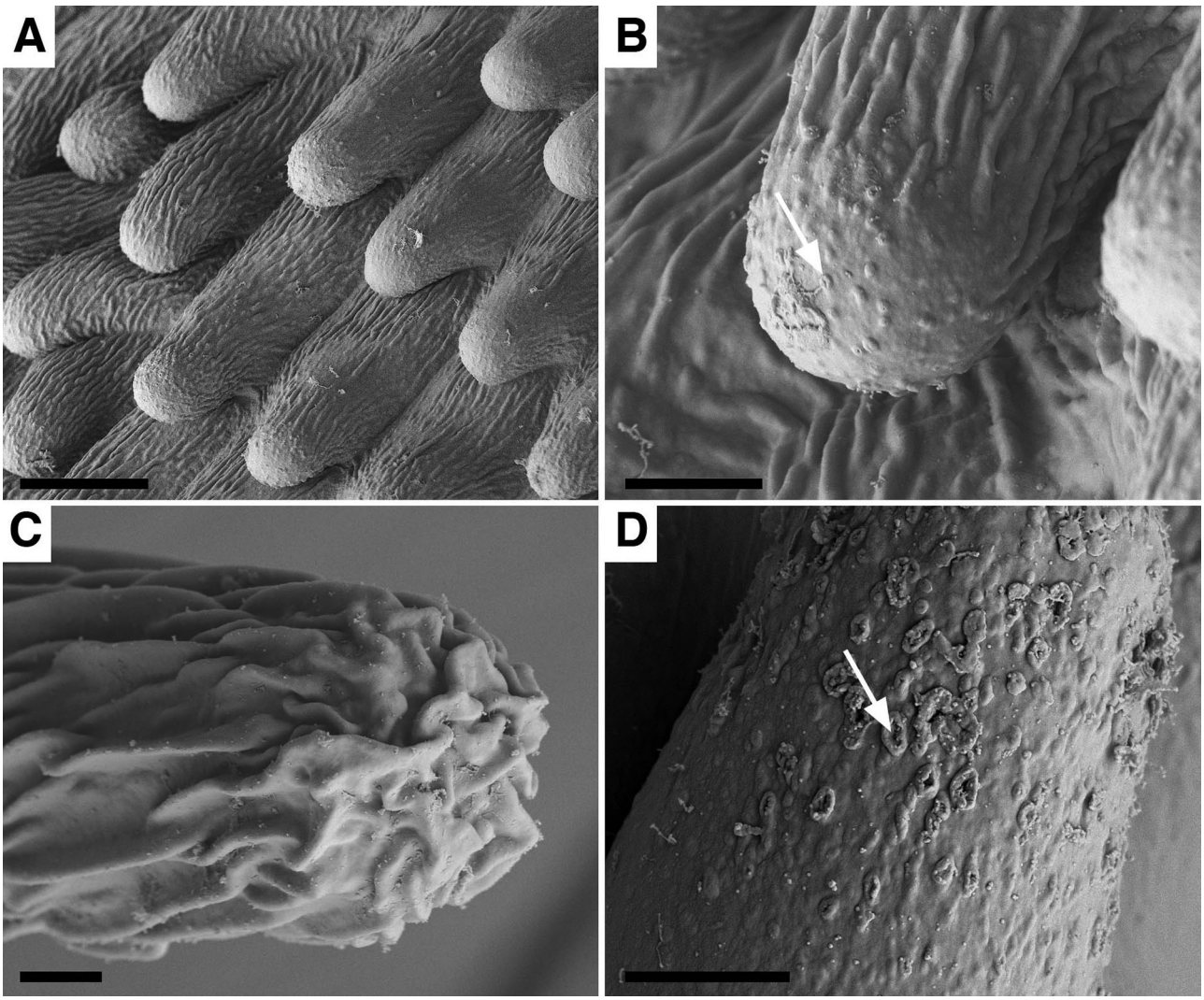

Figure 19. Anatomy of the papillae of the lip of *T. blaisdellii*. A, detail of the papillose surface with striated cuticle. B, apex of the papillae showing a smooth surface with pores and substances at the apex. C, striate cuticle of the elongated unicellular cells of the margin of the lip. D, magnification of the apex of the papillae showing pores and accumulation of substances (white arrow). Scale bars = 10 µm, 3 µm, 3 µm, 1 µm, 500 nm and 500 µm, respectively. Photographs by D. Bogarín and M. M. Chabert.

insects much larger than themselves and suck their haemolymph to obtain proteins for egg production. Species of *Forcipomyia* subgenus *Lasiohelea* are the only vertebrate blood feeders in the subfamily and, in the New World, are known to feed on the blood of frogs. The remaining *Forcipomyia* and all *Atrichopogon* have been recorded from a wide array of hosts, including spiders, phasmids, caterpillars, the wings of Odonata, Chrysopidae and Lepidoptera, Meloidae, Oedemeridae and adult Tipulidae and Culicidae (Borkent & Rocha Filho, 2006; Borkent & Spinelli, 2007; Marcus, 2016). A few records have involved the observation of *Atrichopogon* and *Forcipomyia* being kleptoparasitic on dead insects captured in spider webs (Borkent & Spinelli, 2007; Marshall *et al.*, 2015).

The exclusive presence of females is also associated with the imitation of brood or oviposition sites by the flowers. Females may be attracted by a long-distance fragrance that simulates an egg-laying substrate (mosses or decaying organic matter), but this would be inconsistent with the feeding behaviour of the midges. Another possible explanation for the single attraction of females is the imitation of sex pheromones. Christensen (1994) suggested that pollination via pseudocopulation operates in these orchids because of the insect-like moving lip. However, the exclusive pollination by females is strong evidence against the latter theory. Moreover, flowers are not a place for oviposition (no eggs or oviposition behaviour) or copulation (no males), discarding all other hypotheses.

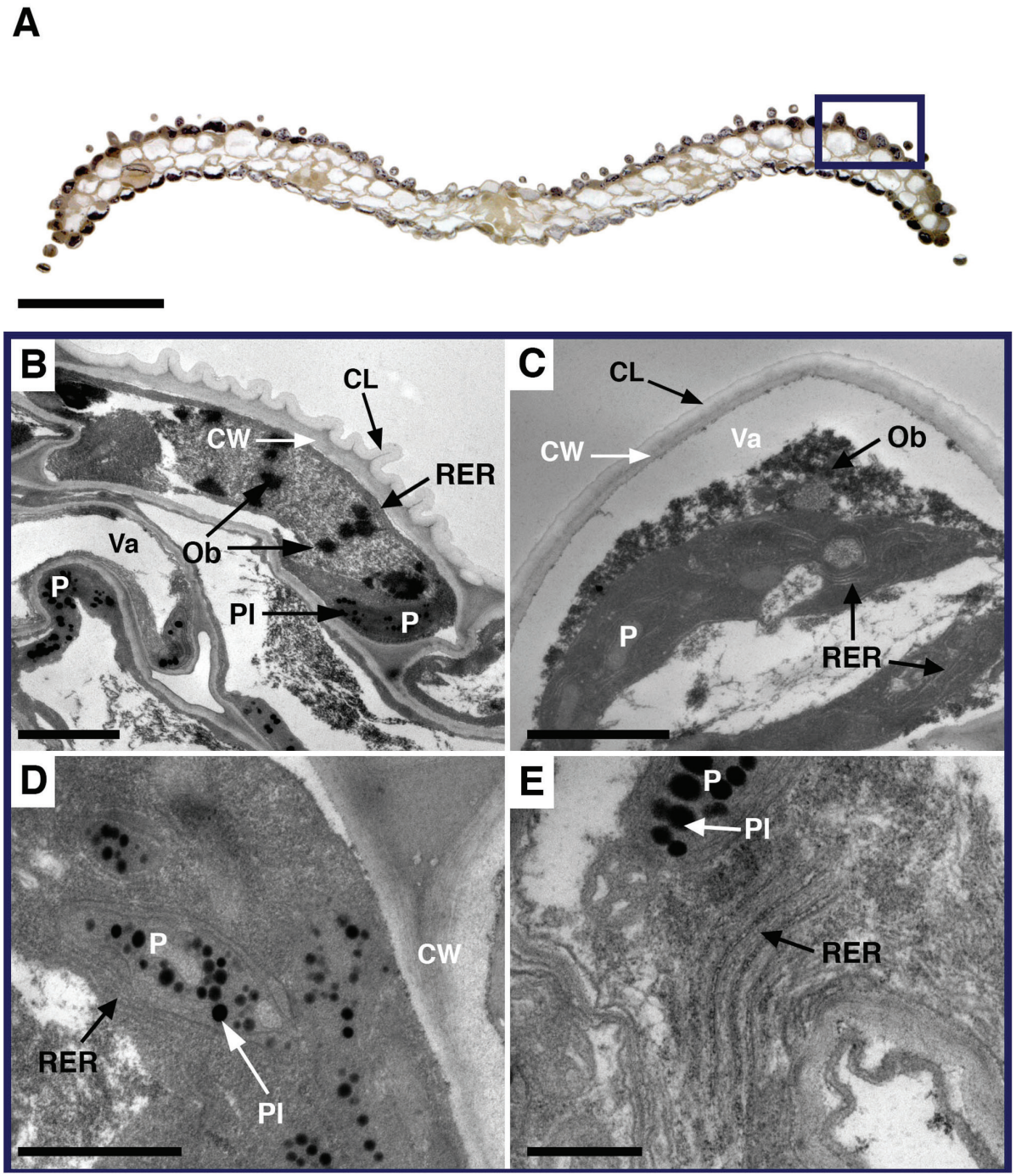

Figure 20. LM and TEM images of the lip of *T. reflexa*. A, transverse Epon section stained with OsO_4 showing osmiophilic contents restricted to the epidermal layers and the unstained ground parenchyma. B–E, TEM images of upper epidermal cells. B, epidermal cell showing the striate cuticle, parietal cytoplasm, endoplasmic reticulum profiles, plastids with plastoglobuli and osmiophilic bodies. Some plastids with plastoglobuli are observed in the sub-parenchyma. C, another epidermal cell showing dense cytoplasmic contents, with profiles of endoplasmic reticulum, vacuoles and plastids. D, plastids with plastoglobuli and endoplasmic reticulum. E, detail of rough endoplasmic reticulum with ribosomes. Scale bars = 10 µm, 2 µm, 2 µm, 1 µm and 500 nm, respectively. CL, cuticle layer; CW, cell wall; Ob, osmiophilic body; P, plastid; Pl, plastoglobuli; RER, rough endoplasmic reticulum; Va, vacuole. Photographs by D. Bogarín and R. Langelaan.

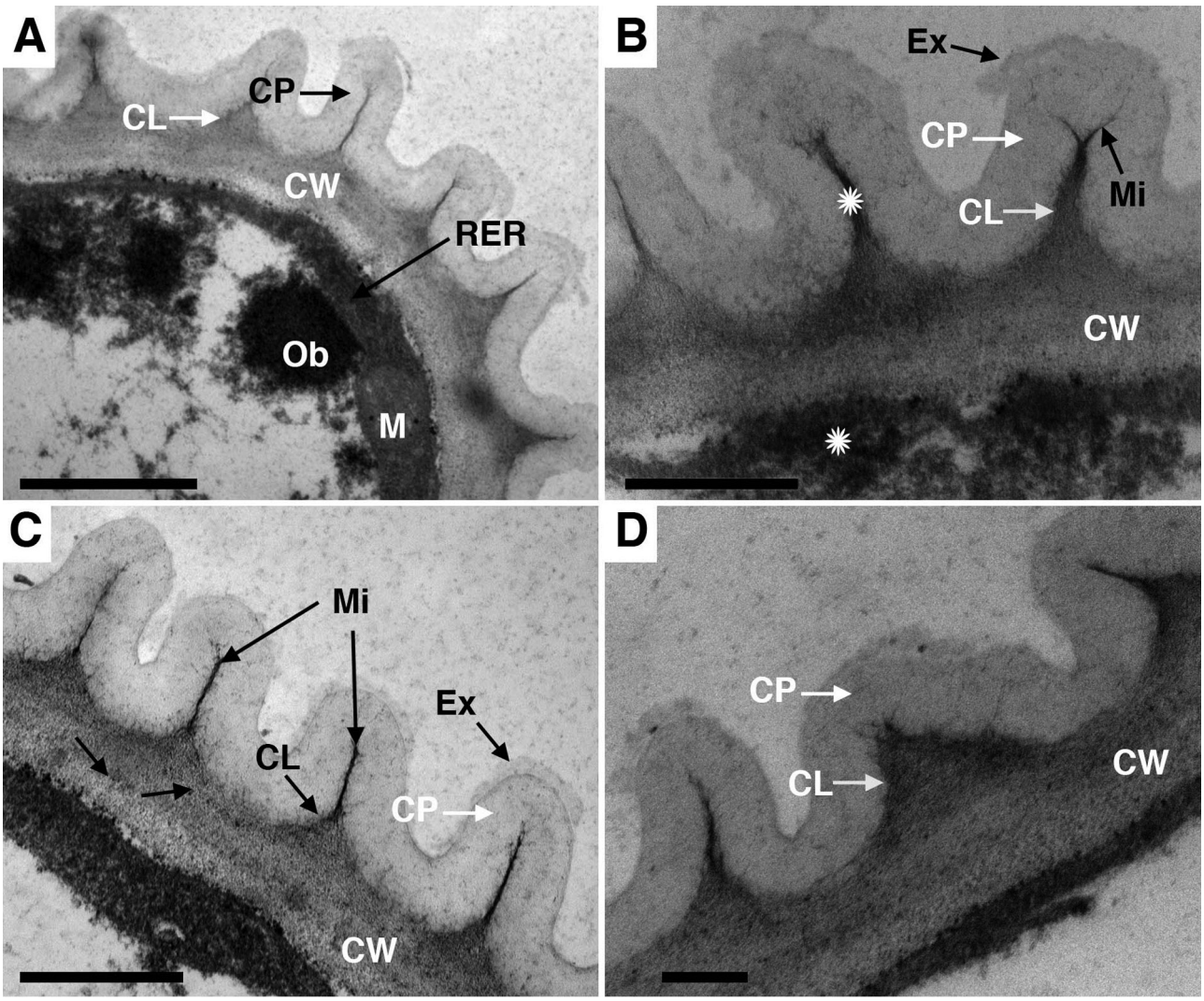

Figure 21. TEM images of the cuticle and cell wall of the lip of *T. reflexa* showing the migration of substances from the cytoplasm towards the cuticle. A, osmiophilic bodies close to the cell wall and profiles of rough endoplasmic reticulum. B, reticulate cuticle layer and cuticle proper with exudates penetrating the cell wall (asterisk) and bifurcated microchannels with osmiophilic substances. C, osmiophilic substances crossing the cell wall (black arrows) and accumulation of substances in the folds of the cuticle. C, detail of reticulate cuticle proper with osmiophilic substances accumulated along the plasmalemma and crossing the cell wall. CL, cuticle layer; CP, cuticle proper; CW, cell wall; Ex, exudates; M, mitochondrion; Mi, microchannels; Ob, osmiophilic body; RER, rough endoplasmic reticulum. Photographs by D. Bogarín and R. Langelaan. Scale bars = 1 µm, 500 nm, 500 nm, 1 µm and 500 nm, respectively.

The natural history and life cycles of Neotropical Ceratopogonidae are poorly understood. In temperate regions, males and females emerge nearly at the same time from the pupae, males form swarms and females fly through it to mate and then disperse (Borkent & Spinelli, 2007; Borkent, Spinelli & Grogan, 2009). The absence of males is noteworthy, but they are generally shorter lived (few days or a week), generally do not disperse and have a stronger seasonality, whereas females live for months. The absence of males after 3 years of sampling here suggests that the flowers are indeed selective or that males were not present in the area.

Borba & Semir (1998) found that females of *Pholeomyia* spp. (Diptera, Milichiidae) are the sole pollinators of three *Bulbophyllum* spp. in Brazil and they theorized that females are attracted by their oviposition instinct, although, they did not observe any flies ovipositing. Davies & Stpiczyńska (2014) suggested that offering proteins as a reward by a species of *Bulbophyllum* section *Racemosae* Benth. & Hook.f. is strong evidence that the attraction

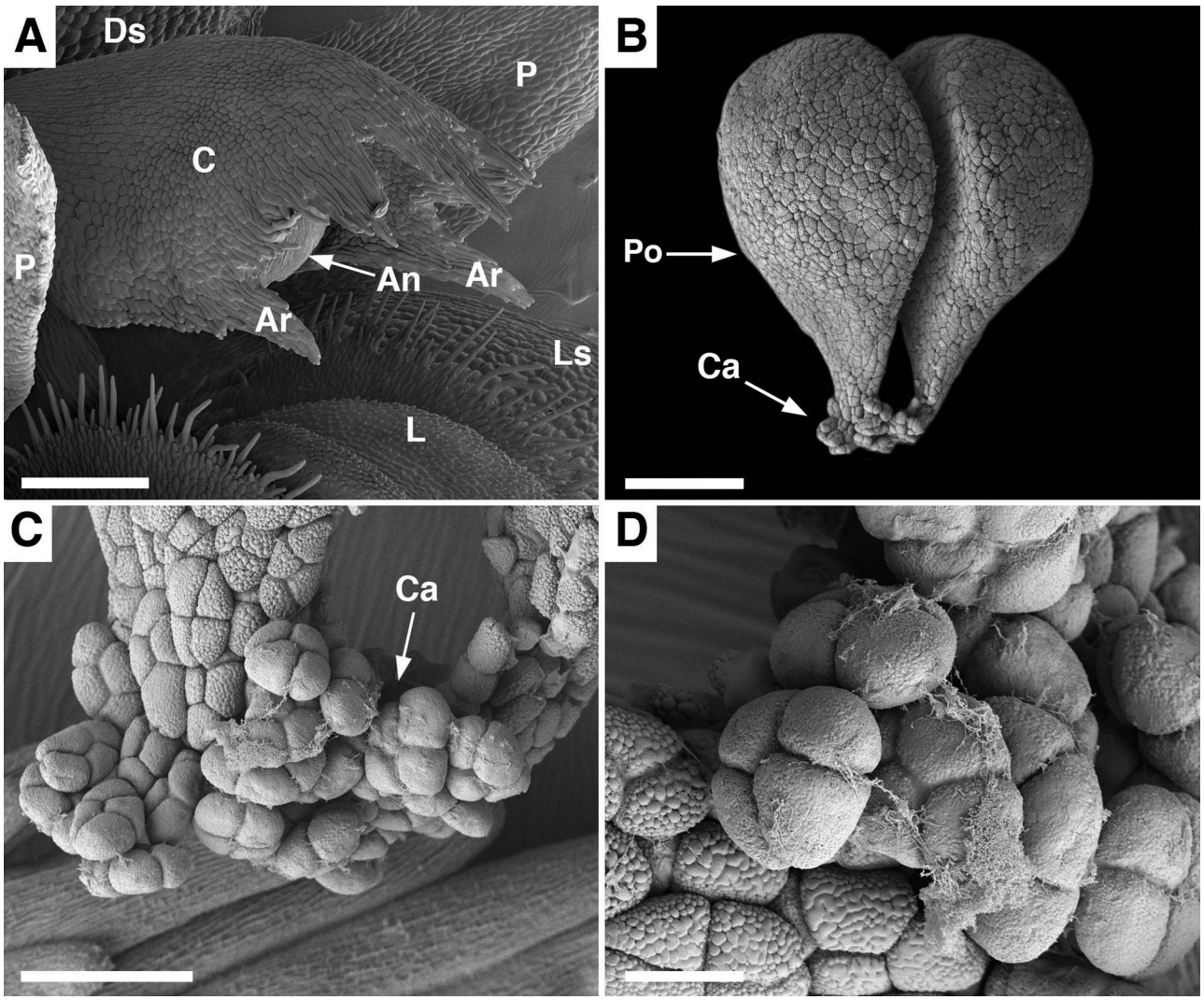

Figure 22. Floral morphology of *T. reflexa*: A, portion of the column showing the erose apex and column arms, anther cap, the papillose, ciliate lip and smooth surface of sepals and petals. B, pollinarium composed by two pollinia and sticky caudicles. C, detail of the base of the pollinia and caudicles showing gemmate ornamentation of cells of the pollinia and the smooth tetrads of the caudicle. D, magnification of the tetrads of the caudicle with a sticky substance. Scale bars = 300, 100, 30 and 10 μm, respectively. An, anther cap; Ar, column arms; Ca, caudicle; C, column; L, lip; P, petal; Ds, dorsal sepal; Ls, lateral sepal. Photographs by D. Bogarín and M. M. Chabert.

is due to the stimulation of the instinct of protein collection in the females. Our observations agree with the hypothesis of Davies & Stpiczyńska (2014), and we consider that *Trichosalpinx* might represent an analogous case. We could not identify the proteins synthesized by *Trichosalpinx* or demonstrate that these offered proteins are sufficient for females in terms of egg production. The females of *Forcipomyia* (*Euprojoannisia*) collected have well-developed mandibles and poorly developed laciniae, as do other species in this subgenus. This strongly indicates that the flies primarily feed on invertebrates (either live or dead) to draw protein-rich haemolymph for egg development, suggesting that the orchids are not probably their primary source of proteins.

Few documented observations of invertebrate prey of *Forcipomyia* (*Euprojoannisia*) are known. *Forcipomyia hardyi* feeds on caterpillars of Geometridae and Sphingidae (Lepidoptera) and one species is kleptoparasitic, attracted by immobilized wrapped termites captured by spiders (Bystrak & Wirth, 1978; Marshall *et al.*, 2015). Kleptoparasites use olfactory signals to locate their food sources including predator venoms, predator digestive

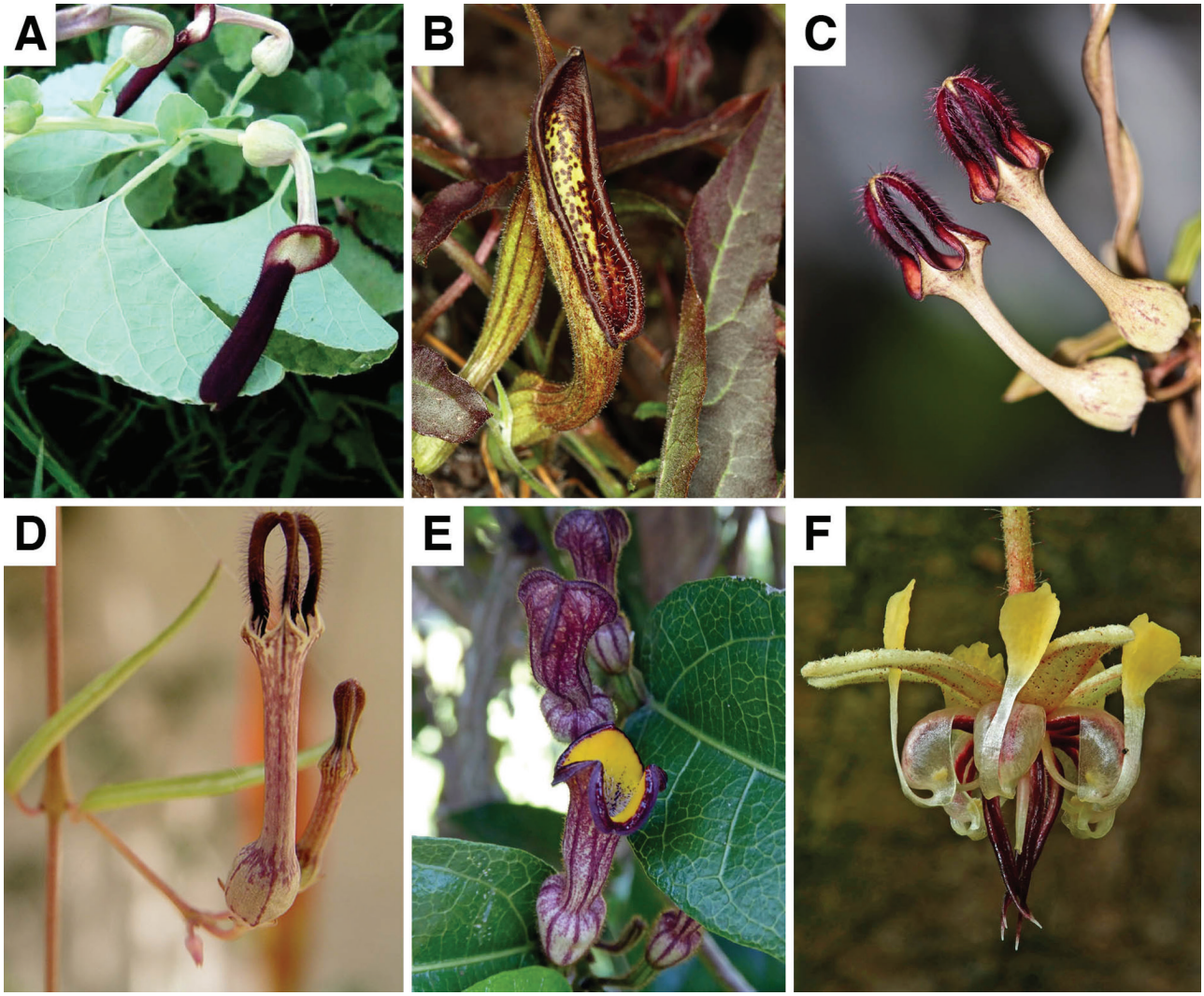

Figure 23. Floral morphology of some angiosperms pollinated by *Forcipomyia* spp. A, *Aristolochia bracteolata* from Asia showing the purple, hirsute limb. B, *Aristolochia watsonii* from North America with a purple-spotted, greenish limb. C, Asian *Ceropegia bulbosa* with the purple, hirsute apices of the corolla. D, *Ceropegia linearis* from Southern Africa showing the purple, hirsute apices of the corolla. E, *Pararistolochia praevenosa* from Australia showing the hirsute, spotted corolla with purple margin and the inner yellow surface. F, flower of the Neotropical *Theobroma cacao* with the purple, hairy staminodia. Photographs by A, ©Dr K. Sambandan. B, ©Russ Kleinman. C, ©Jason da Silva. D, ©David Midgley. E, ©Foam Bark Gully Gang. F, ©H. Zell.

secretions, odours of decaying insects, prey defence secretions released on disturbance or odours of freshly killed insects (Heiduk *et al.*, 2010, 2015, 2016; Oelschlägel *et al.*, 2015). Some angiosperms can mimic these fragrances luring kleptoparasites (Sivinski & Stowe, 1980).

Oelschlägel *et al.* (2015) and Heiduk *et al.* (2015, 2016) introduced the concept of kleptomyiophily, which is pollination by kleptoparasitic flies (food thieves which feed on prey of other predators) attracted to flowers that mimic insect related odours such as semiochemicals to stimulate food-seeking behaviour. *Aristolochia rotunda* L., *Ceropegia dolichophylla* and *Ceropegia sandersonii* use a kleptomyiophilous strategy to fool kleptoparasitic female flies of Chloropidae and Milichiidae. According to Oelschlägel *et al.* (2015), Ceratopogonidae are less important pollinators of *A. rotunda*, but they may be deceived in a similar way as the chloropids and milichiids because kleptoparasitism occurs in this group. Scents of these plants simulate those emitted by trapped prey or venoms injected by predators such as spiders.

The presence of kleptoparasitism in *Forcipomyia* (*Euprojoannisia*) raises the possibility that

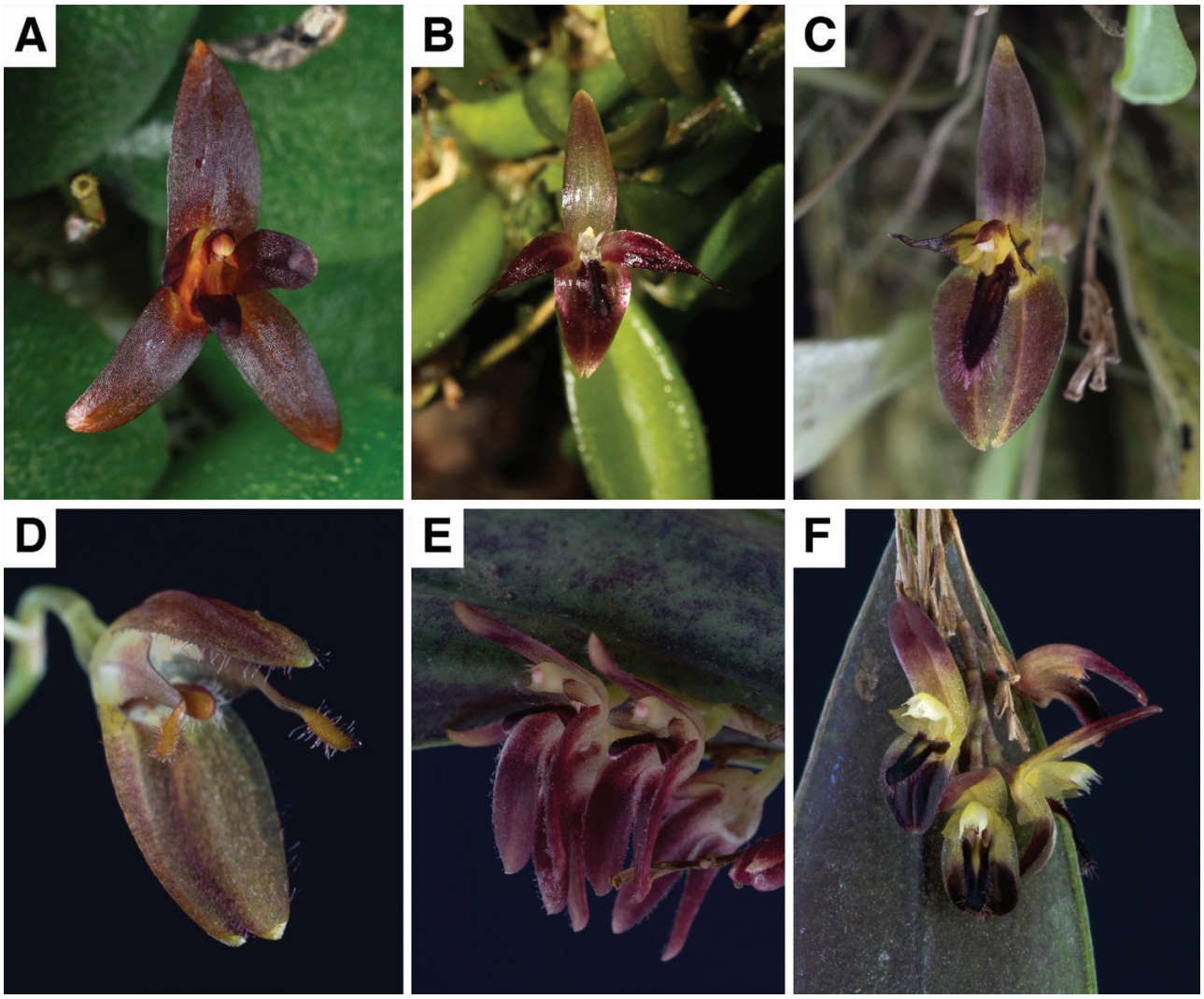

Figure 24. Flower morphology of some orchids pollinated by *Forcipomyia* spp. or probably adapted to a similar pollination system. A, Neotropical *Anathallis lewisiae* (Ames) Solano & Soto Arenas (*A. Karremans 6444*, JBL). B, *Bulbophyllum macphersonii* from Northern and North Eastern Queensland, Australia. C, *Lankesteriana barbulata* (Lindl.) Karremans (*D. Bogarín 12041*, JBL) from Costa Rica. D, *L. fractiflexa* (Ames & C.Schweinf.) Karremans (*E. Jiménez 2558*, JBL) from Costa Rica. E, Neotropical *T. blaisdellii* (*JBL-22846*, JBL). F, Costa Rican endemic *T. reflexa*. Photographs by A, C, D, E, F ©Diego Bogarín. B, ©John Varigos.

kleptomyiophily may have evolved in *Trichosalpinx*. The ciliated lip, which moves due to vibration or wind, might produce a visual effect similar to that of a prey trapped and immobilized in, for example, a spider web and could activate gregarious instinctual responses in predatory dipterans (Meve & Liede, 1994; Vogel, 2001) (Supporting Information, Video S3). The movement of the lip may also aid in dispersing attractive floral fragrances produced in the epithelium as observed in *Bulbophyllum* (da Silva *et al.*, 1999). The evolution of kleptomyiophily has not been fully documented in Orchidaceae. However, it probably occurs in the Australian orchid *Genoplesium littorale* D.L.Jones as suggested by Bower, Towle & Bickel (2015).

An alternative hypothesis could be an initial long-distance stimulus mediated by the imitation of aliphatic hydrocarbons and esters recorded in cuticles of insects and spiders (see 'Floral fragrances'). This would suggest that the flowers attract *Forcipomyia* females by mimicking an invertebrate host prey first by a long-distance fragrance and second by a short-distance tactile (hairy surfaces of the lip), visual (purple colour) and mechanical (movement of the lip) cues that mimic the body surface of caterpillars or possibly spiders (Bystrak & Wirth, 1978; Marcus,

2016). However, the hypothesis of kleptomyiophily or the alternative imitation of the cuticular hydrocarbons and body of an invertebrate host should be further tested, as we have little information about the feeding habits and biology of these pollinators.

The small quantities of proteins and insoluble carbohydrates on the surface of the lip suggest that *Trichosalpinx* is primarily food deceptive. Proteins could be a strong enough signal, a tease, to lure female biting midges into the flower and guide them to the point of balance of the lip (Borba & Semir, 1998; Vogel, 2001). Generally, both sexes of *Forcipomyia* feed on floral nectar to fuel flights. However, *T. blaisdellii* and *T. reflexa* lack nectaries and the absence of males suggests that the insoluble carbohydrates do not stimulate male visitation. Similar examples were observed in the nectar-producing flowers of *Ceropegia* L. (Apocynaceae). Vogel (1990, 2001) considered these flowers deceptive because the small amount of nectar offered is unlikely to be the primary award for the pollinators. In addition, the wind-assisted fly pollinated *Bulbophyllum ipanemense* Hoehne (a species that needs wind movement because the flies are unable to tilt the lip with their weight) is exclusively visited by female flies that are not attracted primarily to any nectar offered and males were instead observed feeding on extra-floral nectaries of sympatric *Epidendrum secundum* Jacq. (Borba & Semir, 1998). Thus, a partial reward deception may have evolved in these orchids. In addition, it is possible that a reward is provided to aid in correctly positioning the pollinator in the flower, but not enough to encourage fidelity. The advantage of offering a minimum reward or a tease over offering no reward might be that it extends the visitation time of insects and that the insects are not stimulated to learn to avoid the orchid as may occur in non-rewarding species where the pollination efficiency is lower (Tremblay *et al.*, 2005). Many flies, including Ceratopogonidae, are able to release saliva on dried sugars and suck up the resultant fluid. In *Trichosalpinx*, it takes longer for the midges to collect those meagre rewards, thus enhancing the possibility of pollination. Moreover, production of small rewards or teases might not require a lot of energy (Jersáková, Johnson & Kindlmann, 2006). Ackerman, Rodríguez-Robles & Melendez (1994) and Salguero-Faría & Ackerman (1999) studied the advantage of offering meagre rewards in *Comparettia falcata* Poepp. & Endl. They found that hummingbirds still perceive rewards despite the low quantities and concentration. In addition, an increase in the production of rewards does not necessarily imply an increase in pollinator visitation, pollen transfer or reproductive success. In the rewardless *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase, the artificial addition of nectar increased not only pollinator visitation but also geitonogamous pollination (Johnson, Peter & Agren, 2004).

Plant features

The sepals and lip are the most important structures for the attraction of pollinators. Some fly-pollinated Pleurothallidinae have anatomical and ultrastructural differences between the base and the apex of sepals and show osmophoric tissue at the apex (Pridgeon & Stern, 1983, 1985; Vogel, 1990; de Melo, Borba & Paiva, 2010; Pansarin *et al.*, 2016). In *Trichosalpinx*, the absence of osmophoric tissue suggests that the apices of the sepals act as visual rather than olfactory stimuli (Pridgeon & Stern, 1985).

The capitate trichome-like colleters of the sepals in several Pleurothallidinae secrete and synthesize fragrances (Mayer, Cardoso-Gustavson & Appezzato-da-Glória, 2011). In addition, some *Bulbophyllum* show glandular trichomes with similar histochemical features as *Trichosalpinx* (Nunes *et al.*, 2014, 2015; Stpiczyńska *et al.*, 2015). Cardoso-Gustavson *et al.* (2014) concluded that floral colleters in the Pleurothallidinae do not produce fragrances because secretion stopped before anthesis. However, our observations suggest that they secrete scents. Cardoso-Gustavson *et al.* (2014) also found fungal infections restricted to the colleters of the ovary. The fungal species producing these hyphae and their possible role in pollination are still unknown.

Crystals occur in the sepals, petals and lip of many other Pleurothallidinae (D. Bogarín, pers. observ.). However, little is known about their role in pollination. Chase & Peacor (1987) suggest that refractile properties of crystals in *Stelis* might mimic nectar droplets (pseudonectar) and are acting as visual attractants to lure pollinators. Crystals also occur in flowers of *Bulbophyllum* and other orchids (Prychid & Rudall, 1999; Davies & Stpiczyńska, 2014; Kowalkowska *et al.*, 2014; Nunes *et al.*, 2014, 2015; Stpiczyńska *et al.*, 2015).

Trichosalpinx, some fly-pollinated Pleurothallidinae and myophilous *Bulbophyllum* have unicellular papillae and secretory activity restricted to the adaxial epidermis of the lip (Pridgeon & Stern, 1985; de Pádua Teixeira *et al.*, 2004; Nunes *et al.*, 2014, 2015). Moreover, in some *Bulbophyllum*, *Restrepia* Kunth and *Scaphosepalum* Pfitzer, the synthesis of fragrances in the papillae is associated with starchless plastids rich in plastoglobuli, ER and osmiophilic droplets as found in *Trichosalpinx* (Pridgeon & Stern, 1983, 1985; Kowalkowska *et al.*, 2014). Lipophilic compounds and osmophoric tissue suggest a synthesis of fragrances on top of the papillae. Vogel (1990) documented the fragrance synthesis by the epithelium or 'emission

layer' in *Ceropegia*. Similar to the lip of *Trichosalpinx* and *Bulbophyllum* (Davies & Stpiczyńska, 2014), fragrance synthesis in *Ceropegia* takes place in the glandular epithelium of the distal lobar ends of the corolla (Vogel, 1990). *Trichosalpinx* flowers lack stomata on the lip indicating that the epithelium releases fragrances by diffusion through the cuticle (de Pádua Teixeira *et al.*, 2004; Kowalkowska *et al.*, 2014). The osmophoric papillae of the lip and trichomes of the sepals indicate that two types of olfactory signals might be used. Likewise, two heterogeneous centres of fragrance synthesis also occur in *Bulbophyllum ornatissimum* (Rchb.f.) J.J.Sm. (Vogel, 1990).

Dense cytoplasmic contents and an extensive network of RER are associated with the synthesis and secretion of proteins in the epithelium of the lip. This observation agrees with the ultrastructure and anatomy of the lip in some *Bulbophyllum*, which also produce abundant protein secretions probably as floral rewards or teases (Davies & Stpiczyńska, 2014). Similarly, Bulbophyllum *wendlandianum* (Kraenzl.) Dammer produces higher concentrations of protein in the epithelium rather than in the parenchyma as observed in *Trichosalpinx* (Kowalkowska *et al.*, 2014). The species of *Bulbophyllum* pollinated by an insect weight mechanism also lack nectaries but synthesize lipid droplets (de Pádua Teixeira *et al.*, 2004). The papillae of the lip lack starch granules, suggesting that no nectar is produced there.

The striated cuticle of epithelial cells of the lip is another feature shared by *Bulbophyllum* and *Trichosalpinx* (Davies & Stpiczyńska, 2014; Kowalkowska *et al.*, 2014; Nunes *et al.*, 2014, 2015; Stpiczyńska *et al.*, 2015). This cuticle pattern could be linked to a mechanism of light diffraction, producing iridescence or more intense 'structural colours' and thus acting as visual cues (Antoniou Kourounioti *et al.*, 2012; Nunes *et al.*, 2015). It can also increase the emitting surface area of the lip or function as a tactile stimulus to insects (Vogel, 1990).

The arcuate, incumbent column with a foot and the anatomy of the ligament of the lip that acts as a hinge evolved in several Pleurothallidinae and *Bulbophyllum* (Borba & Semir, 1998; Karremans *et al.*, 2015; Nunes *et al.*, 2015; Vogel, 2001). The pollinarium of *Trichosalpinx*, made up of two pollinia and sticky caudicles, occur in several Pleurothallidinae related to *Trichosalpinx* (Stenzel, 2000) and in some *Bulbophyllum* (Nunes *et al.*, 2015).

Pollination of angiosperms by biting midges

Biting midges of 13 genera have been recorded on the flowers of a wide array of angiosperms. Specifically, pollination by *Forcipomyia* spp. evolved independently in unrelated angiosperm families (Razzak, Ali & Ali, 1992; Gibernau *et al.*, 2004; Ollerton *et al.*, 2009). Although the mechanism of attraction used by these plants is largely unknown, the flowers adapted to the pollination of *Forcipomyia* often have dark, vinaceous, hirsute floral structures of different homology in combination with green, yellow or white structures (Figs 23, 24).

Biting midges pollinate *Aristolochia bracteolata* Lamk (Aristolochiaceae) (Razzak *et al.*, 1992) (Fig. 23A) and *Aristolochia watsonii* Wooton & Standl. (Aristolochiaceae) (Fig. 23B), possibly attracted by their hairy, dark purple limb and mouth of flowers (Crosswhite & Crosswhite, 1984; Woodcock *et al.*, 2014) (Fig. 23B). In addition, adult female biting midges are the main pollinators of at least 19 species of *Ceropegia* (Apocynaceae) and the vine *Pararistolochia praevenosa* (F.Muell.) Mich.J.Parsons (Aristolochiaceae) (Fig. 23E). (Fig. 23C–E). These species have tubular bristly, purple corolla apices with some white, green or yellow parts (Ollerton *et al.*, 2009; Williams & Adam, 2010). Two *Culicoides* spp. pollinate *Arum conophalloides* Kotschy ex Schott (Araceae), which has a purple spadix and spathe that produce scents that might mimic the vertebrate prey of the pollinator (Gibernau *et al.*, 2004). The pollination of cacao, *Theobroma cacao* L. (Malvaceae), has been studied intensively due to its economic importance. Some authors claim that several species of flies (mostly biting midges) are probably attracted to and feed on compounds secreted by the purple trichomes of the staminodia (Winder, 1978; O'Doherty & Zoll, 2012) (Fig. 23F). In Orchidaceae, *Forcipomyia sauteri* pollinates the Australian *Bulbophyllum macphersonii* Rupp (Fig. 24B) (Bartareau, 1994). *Trichosalpinx* spp. and *B. macphersonii* may be examples of evolutionary convergence towards a common mechanism of pollination (Figs 23, 24).

Floral fragrances

Some *Forcipomyia* spp. are attracted by aliphatic esters such as decyl hexanoate and hexyl hexanoate (Sugawara & Muto, 1974). However, the authors did not discuss any foraging behaviour of the flies in this experiment. The latter compound was also detected in the floral fragrance of kleptomyiophilous *A. rotunda* (Oelschlägel *et al.*, 2015). We detected lactic acid in *T. blaisdellii*, a compound which acts as long-distance attractant in *Forcipomyia taiwana*, a vertebrate feeder (Liu, Lee & Yang, 2009). Arsene, Schulz & Van Loon (2002) recorded *n*-alkenes such as heptacosene in the epicuticle of the cabbage white butterfly *Pieris rapae* (Lepidoptera). Other *n*-alkanes, alkenes and methyl-alkanes have been recorded as cuticular hydrocarbons in insects and spiders (Mant *et al.*, 2005). We detected alkanes in both *T. blaisdellii* and *T. reflexa*, suggesting

a correlation between insect epicuticles and floral fragrances of these orchids. Hexadecanoic acid (palmitic acid), octadecadienoic acid and 17-pentatriacontene, also detected in the floral fragrance of *Trichosalpinx*, are also wax components in spider webs (Prouvost *et al.*, 1999; Trabalon & Assi-Bessekon, 2008).

Heiduk *et al.* (2010) found mainly spiroacetals and aliphatic compounds in two *Ceropegia* spp. They argue that these species mimic a chemical signal that attracts kleptoparasites. Indeed, the milichiid flies that pollinate both *Ceropegia* spp. are kleptoparasites that feed on haemolymph of prey in spider webs (Heiduk *et al.*, 2010, 2015, 2016). Heiduk *et al.* (2010) detected mostly aliphatic esters in the fragrance of *C. dolichophylla*, which also attract kleptoparasitic flies. *Aristolochia rotunda* uses a similar strategy, but fools kleptoparasitic females of Chloropidae that feed on true bugs (Miridae) captured by spiders. The main attractants are also aliphatic esters, aliphatic hydrocarbons and aliphatic alcohols (Oelschlägel *et al.*, 2015). Further experimentation such as bioassays is needed to prove the role of these scents in the pollination of *Trichosalpinx* as has been performed in other deceptive systems (Phillips *et al.*, 2014; Oelschlägel *et al.*, 2015).

Aside from the aforementioned compounds, other fragrance compounds detected in the flowers of *T. blaisdellii* and *T. reflexa* are not shared with the fragrances of *A. rotunda*, *Bulbophyllum weddellii* Rchb.f., *Bulbophyllum involutum* Borba, Semir & F.Barros, *B. ipanemense*, *C. dolichophylla* and *T. cacao* (Young & Severson, 1994; da Silva *et al.*, 1999; Heiduk *et al.*, 2010; Oelschlägel *et al.*, 2015). However, aliphatic esters and aliphatic hydrocarbons are always present in the floral fragrances of both these plant species and the *Trichosalpinx* spp. Additional electro-antennography experiments are needed to investigate further the role of shared and species-specific floral fragrances in pollinator attraction.

Breeding system

Self-incompatibility is common in Pleurothallidinae (Borba *et al.*, 2011). In addition to *Trichosalpinx*, other members of the *Lepanthes* clade (Pridgeon *et al.*, 2001), such as *Anathallis* (Gontijo *et al.*, 2010; Borba *et al.*, 2011) and *Lepanthes* (Tremblay *et al.*, 2005), show a high degree of self-incompatibility. However, *Zootrophion* is self-compatible possible by reversal (Borba *et al.*, 2011). Caradonna & Ackerman (2010) hypothesized that *P. ruscifolia* (Jacq.) R.Br. produces cleistogamous flowers because of the absence or rarity of pollinators, which may be another case of reversal in Pleurothallidinae. Self-incompatibility in *Trichosalpinx* is probably a strategy to prevent autogamy or geitonogamy in response to the behaviour of the biting midges that generally visit several flowers on the same inflorescence or enter the same flower several times and thus initiate self-pollination (Pansarin *et al.*, 2016).

CONCLUSIONS

Trichosalpinx spp. might exclusively attract female midges by exploiting their protein collection instinct for egg production. The similar floral structures of other kleptomyophilous angiosperms compared to *Trichosalpinx* and the kleptoparasitic habits of *Forcipomyia* (*Euprojoannisia*) (only one report so far) suggest that kleptomyiophily may have evolved in *Trichosalpinx*. The well-developed mouthparts of the midges studied here indicate that they normally draw protein-rich haemolymph from animal hosts, so *Trichosalpinx* flowers are probably offering small quantities of proteins and sugars as meagre rewards. Although deception in pollination biology is usually equated with rewardlessness, it is possible that flowers use a deceit mimicking strategy, but they still provide rewards (Ackerman *et al.*, 1994; Salguero-Faría & Ackerman, 1999; Vogel, 2001).

In the phylogenetic context, at least two families of Diptera are involved in the pollination of species in the *Lepanthes* clade: Sciaridae males in *Lepanthes* and Ceratopogonidae females in *Trichosalpinx*. Most of the 25 *Trichosalpinx* spp. show similar floral traits and therefore we hypothesize that other *Trichosalpinx* spp. are pollinated via a similar system. The similarities among *Trichosalpinx* and the closely related *Anathallis* and *Lankesteriana* suggest that they also have similar pollination mechanisms. The pollination mechanisms of other related genera such as *Lepanthopsis*, *Tubella* and *Zootrophion* and the role of their pollinators as drivers of species diversification in the *Lepanthes* clade remain unknown.

Future research should investigate the natural history of the *Forcipomyia* sp. studied here, including the discovery of the males, their feeding and breeding sites, diets and prey. Dietary analysis, bioassays and behavioural studies of both this *Forcipomyia* sp. and their insect prey and GC/MS analyses of their pheromones and cuticular scents and the floral fragrance of other *Trichosalpinx* spp. are necessary to further test our hypotheses. Further biochemical characterization of the proteins, carbohydrates and crystals produced by the flowers and the detection of these compounds using stable isotopes to determine the extent of plant vs. animal food sources should also be conducted. Moreover, the fossil record of Ceratopogonidae is one of the best among Diptera. Research on amber fossils is key for the understanding of the evolution of pollination by biting midges and orchids (Borkent & Spinelli, 2007; Ramírez *et al.*, 2007).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. GenBank accessions and voucher information of *Forcipomyia* spp. and the species of *Trichosalpinx* studied.

Table S2. Histochemical tests performed on species of *Trichosalpinx*.

Table S3. Volatiles identified by GC-MS in flower extracts of *Trichosalpinx blaisdellii* and *T. reflexa*.

Figure S1. Chromatogram of flower extracts. A, *Trichosalpinx blaisdellii*. B, *T. reflexa*.

Video S1. *Forcipomyia* (*Euprojoannisia*) sp. females visiting and removing the pollinarium of *Trichosalpinx reflexa*.

Video S2. *Forcipomyia* sp. females visiting *Trichosalpinx reflexa*.

Video S3. Gregarious visitation of *Forcipomyia* sp. females on a single flower of *Trichosalpinx reflexa*.

Video S4. *Forcipomyia* sp. females visiting *Trichosalpinx reflexa*.

Video S5. *Forcipomyia* sp. females visiting and removing the pollinarium of *Trichosalpinx blaisdellii*. File:.mov, Codec: MPEG-4 Video.