"Evolution of Pollination Mechanisms in *Pleurothallis*"

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Abstract

The orchid genus *Pleurothallis* is believed to be predominantly fly-pollinated based on limited field studies. I hypothesized that both reward and deceit pollination syndromes occur in the genus and deceit pollination, more specifically, pseudocopulation has evolved more than once. Flowers were sampled from several infrageneric groups within *Pleurothallis* and examined by scanning electron microscopy. Morphological features of the labellum of the flower, such as the presence or absence of a glenion or other secretory tissue, or cavities possibly involved in pseudocopulation, were used to infer possible pollination mechanisms. Additionally, new nuclear ITS and plastid 3' *ycf1* sequences were added onto the phylogenies generated by previous students in Wilson Laboratory. Pollination mechanisms inferred from floral morphology was mapped onto the phylogenies. Preliminary data suggest that pseudocopulation may have evolved more than once in *Pleurothallis*.

Introduction

Pleurothallidinae is a large Neotropical subtribe with around 4000 species within the family Orchidaceae and the tribe Epidendreae. The high diversity of Pleurothallidinae and the orchid family is promoted by, among other things, the diverse pollination mechanisms (Cozzolino & Widmer 2005).

Pollination mechanisms of orchids can be divided into two categories, reward pollination and deceit pollination. In reward pollination, the orchid usually provides

nectar for the pollinator as a reward, with some species such as the genus *Disperis* providing oils as a reward (Nilsson 1992). Nectar production requires great amounts of energy, therefore the orchids evolved pollination mechanisms to defraud the pollinators (Jersáková 2005). Such an asymmetrical coevolution favoring the orchids lead to the presence of deceit pollination (Nilsson 1992). Further studies divide deceit pollination into smaller categories such as food deception and sexual deception (Jersáková 2005). In food deception the orchid can mimic flowers producing a nectar reward (Jersáková 2005), and in sexual deception the orchid imitates the movement and texture of the females of the pollinator species by releasing pheromone-like chemicals and developing specific morphology to lure the insects with opportunities of copulation (Blanco & Barboza 2005; Jersáková 2005; Schiestl 2005).

Pollination mechanisms are closely related to floral morphology and monocotyledons including Orchidaceae usually have trimerous floral merosity, with three or multiples of three sepals and petals (Decraene & Smets 1994). In a typical Pleurothallidinae flower such as *Pleurothallis rubrifolia* (Figure 1), there is a dorsal sepal (A) and the two lateral sepals are fused to be the synsepal (B). Apart from the two petals on the sides (C), the last petal is modified to be a labellum, also known as the lip (D), which plays an important role in pollinator attraction (Teixeira et al. 2004). In the middle of the flower is the sexual reproductive column (E) with both the male pollinarium (pollen sac) and female stigmatic surface at the tip.

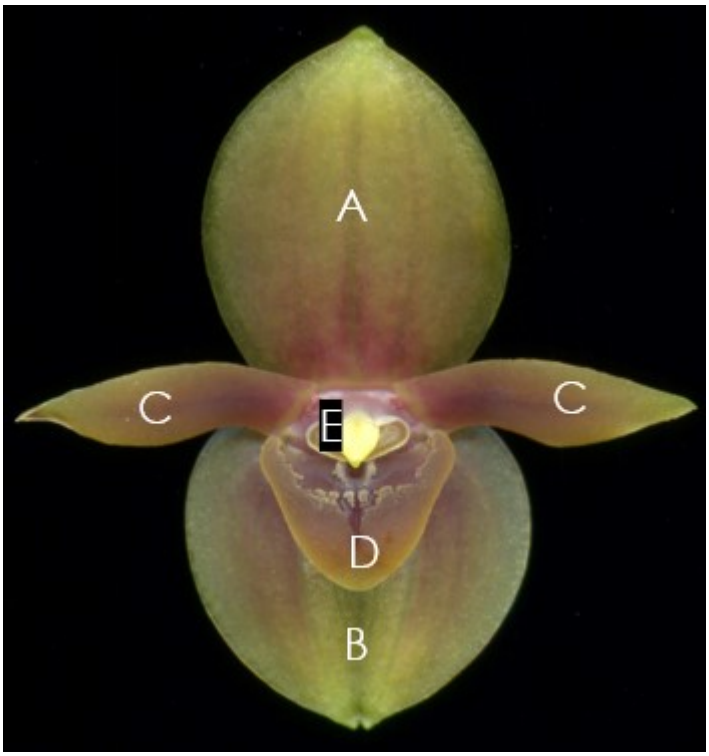

Figure 1. Flower anatomy of *Pleurothallis rubrifolia*

A. dorsal sepal B. synsepal C. petals D. lip E. column

There has been a lot of research on the pollination mechanisms of some genera within Pleurothallidinae. Genus *Acianthera* are mainly epiphytes or lithophytes in tropical areas and there are around 200 species in the genus (Cabral de Melo et al. 2010). These flowers are myophilous (pollinated by flies) and they usually have osmophores (structures releasing pheromone-like chemicals) on the sepals to release compounds containing nitrogen to attract saprophilous (feeding on decaying organic matters) flies. Both reward and deceit pollination mechanisms are observed in different species. Species such as *A. johannensis* have no nectar secretion and they rely on females of family *Chloropidae*, mainly by the genus *Tricimba*, to pollinate when these flies oviposit (Borba & Semir 2001). After landing on sepals or leaves, the females enter the flower and examine with their proboscis and touch the sepals with their ovipositors to lay eggs. When they land their

weight drops the labellum, which returns as the flies try to enter the flower further. Therefore when the flies try to leave they will be trapped in the narrow passage and their scutellum (a shield-shaped projection located on insect thorax) will get attached to the rostellum (a projection at column tip to separate pollinarium and stigmatic surface, preventing self-pollination) and remove the pollinaria.

Other species like *A. teres* produce a small amount of nectar-like liquid at the base of the labellum and are pollinated by family Phoridae (Borba & Semir 2001). The main pollinators, *Megaselia* females are attracted by the nectar-like liquid and they will be trapped with the rostellar glue (sticky liquid on rostellum tip) as they try to enter further into the labellum base for more nectar-like liquid. When they move backward to escape, they remove the pollinaria with the anther cap (a covering over the pollinaria) on the scutellum and the anther cap will remain there for a long period to avoid self-pollination as these species are self-incompatible and the flies tend to visit the same flower or inflorescence several times before they leave to the next plant.

Flowers of genus *Dracula* are unique for their mushroom-like labellum. The labellum mimics co-occurring mushrooms both visually and in odor to deceive mushroom flies into pollination. Policha et al. (2016) used artificial flowers from a 3D printer to investigate the visual and olfactory effects separately and they discovered that both attractants combine to attract pollinators. The labellum releases 1-octen-3-ol, which is also found in co-occurring mushrooms, as a main scent attractant. The dotted sepals resemble the pollinator wings and may trigger mating behavior. Courtship and mating behaviors were observed (Endara et al. 2010). Flies landing on sepals or the labellum will be guided by

the lamellae (a network of ridges branching out, located inside the labellum) radiated from the hypochile (basal part of the labellum) to enter the columnar chamber while the weight pushes the labellum away from the column. When the labellum returns the fly is pressed against the column and the scutellum will be coated with the sticky fluid from the rostellum and get stuck to the pollinia caudicles (the connecting stalk of pollinia). Thus when the fly leaves it pulls away the pollinia and at the same time pulls forward the rostellum to cover the stigmatic surface to avoid self-pollination (Endara et al. 2010).

Pseudocopulation could happen at either pre-copulatory behavioral phases or the actual copulation phase. Genus *Lepanthes* employs the latter, genitalic pseudocopulation is its pollination mechanism (Blanco & Barboza 2005). The main pollinators for this genus are fungus gnats, family Sciaridae of Diptera. Visually and structurally, the flowers mimic the female pollinator with the hairy motile labellum and the appendix similar to female abdominal terminalia (the place on female abdomen where the male holds during copulation). The flowers are also facing downward because female Diptera attract males under leaves. Besides the visual attraction, the osmophores at the papillae on the labellum blade surface release chemicals that mimic the sexual pheromones of female Diptera (Blanco & Barboza 2005). Male Diptera are attracted by the scent to the flower from the downwind direction and take copulation preparation movements such as fanning wings and open and closing gonostili (the organ male uses to hold the female terminalia during copulation) after landing. Then it will mount the lip and put the abdomen under the labellum with the gonostili open. After that the male grasps the labellum appendix (a tissue located on the *Lepanthes* labellum which mimics female abdominal terminalia) and

turns around 180° and that is when its abdomen touches the viscidium and gets the pollinarium stuck to its dorsal side of the 6th abdominal segment. Actual copulation takes place in this process and ejaculation and refractory period (a period following one ejaculation during which the male will not attempt to copulate with another flower) are observed in this study.

Genus *Specklinia* has a pollination syndrome of a combination of nectar reward and pheromone attraction and the main pollinators are *Drosophila* flies (Karremans et al. 2015). The flower releases aggregation pheromones with ethyl tiglate, methyl tiglate and isopropyl tiglate as the main compounds, which are very strong attractants for the *Drosophila* flies. The stomata on the sepals secrete nectar and the morphologically similar papillae on the lip and petals to those secretory papillae in *Acianthera* genus suggest that lip and petals may also secrete nectar. The nectar accumulates on the adaxial surface of the sepals and when the flies move around and lick the nectar after landing on the sepals, they will step on the motile lip and get attracted by the papillae there. At a certain angle the lip will get tilted and the fly will be pressed against the rostellum with the viscidium. When the fly leaves, the pollinaria will stick to the viscidium on the scutellum (Karremans et al. 2015).

Genus *Trichosalpinx* employs food deceit pollination mechanism. Instead of providing nectar as a reward, *Trichosalpinx* species produce a small amount of liquid with proteins and insoluble carbohydrates (Bogarin et al. 2018). Their main pollinator is female *Forcipomyia*, commonly referred to as midges, which require protein to produce eggs. The flowers release volatile compounds such as lactic acid as a long-distance attractant for the

female flies and the motile labellum mimics the host prey and help to release the fragrance at the same time. In a short distance, the female midges are attracted by the food and get closer to the labellum base when they suck the proteins but their weight lifts up the labellum and this presses the midges against the column. The caudicles get stuck to the back and then the pollinarium and then the labellum returns, allowing the midges to leave with the pollinarium (Bogarin et al. 2018). The labellum secretes some proteins as reward while since the amount is too small to be enough food to meets the need of a female for egg production, the mechanism is counted as a deceit pollination instead of reward pollination according to the authors.

The main focus of this study is genus *Pleurothallis* as circumscribed by Pridgeon et al. (2005). Previous students in the Wilson Laboratory have conducted phylogenetic research to examine the validity of the proposed circumscription of genus *Pleurothallis* in Pridgeon et al. (2005) (Wilson et al. 2015). These analyses support the recognition of clades within *Pleurothallis* previously described, including *Ancipitia/Scopula, Elongatia, Lalexia, Pleurothallis sensu stricto, Restrepioidia, Rhynchopera,* and *Talpinaria.*

Pleurothallis is the largest fly-pollinated genus in Pleurothallidinae (van der Pijl & Dodson 1966). However, despite the large number of species, there is little field data on their pollination mechanisms. From the limited data as shown in Table 1, Dipterans from the families Anthomyiidae, Bibionidae, Drosophilidae, Mycetophilidae and Sciaridae are the main pollinators. Mojica et al. (2018) examined the nectar-like liquid secreted by the lip of *P. coriacadia* and found a 13% sugar composition, suggesting that this liquid is a sugary nectar and thus is evidence of reward pollination in *Pleurothallis*. Duque Buitrago

et al. (2014) also described nectar secretion from the lip of *P. marthae*, again providing evidence for reward pollination in *Pleurothallis*.

Besides these limited field studies, the putative pollination mechanisms of most species in genus *Pleurothallis* are hypothesized from floral morphology. In the SEM photos, characteristics such as a glenion (an area with secretory cells located at lip base) or other possible secretory tissue suggest reward pollination. The absence of secretory tissue and long papillae and copulatory channels on the labellum would lead to a hypothesis of deceit population.

In subsection *Macrophyllae-Fasciculatae,* most species have lips with a glenion that secretes nectar-like liquid. The only exception is *P. minutilabia* which has no secretory tissues but instead has long papillae and a copulatory channel and is hypothesized to use pseudocopulation (Wilson et al. 2018). In subsection *Acroniae*, similarly, most species have lips with glenion and thus are hypothesized to be reward pollinated. In subsection *Macrophyllae-Racemosae*, there are species reported to be using reward pollination mechanism such as *P. colossus* (Calderon-Saenz 2011) while species with very unique lip structure and thus unknown pollination mechanisms such as *P. papillingua* also exist. In the *P. crocodiliceps* group, all species have similar lips marked by two horns, one apical cavity and long papillae, suggesting pseudocopulation (Wilson et al. 2017b). In the *P. talpinaria* group, all species have similar lips with a tri-lobed epichile (lip tip area) and a two-lobed hypochile and the absence of possible secretory tissues also suggests deceit pollination (Wilson et al. 2017a).

Species (or taxonomic group)	Putative pollinator -floral visitor	Reference
Pleurothallis rhopalocarpa (as *P. monocardia*)	*Sciara* sp. (as *Lycoria* sp.), Sciaridae, Diptera	Dodson (1962)
P. eumecocaulon	*Lacodrosophila* (?) sp., Drosophilidae, Diptera	Dodson (1965) [cited in van der Pijl & Dodson (1966)]
P. ruscifolia	*Lacodrosophila* (?) sp., Drosophilidae, Diptera	Dodson (1965) [cited in van der Pijl & Dodson (1966)]
P. xanthochlora	Drosophilidae, Diptera	
P. alvaroi	Bibionidae and Drosophilidae, Diptera; Curculionidae, Coleoptera	Duque (1993)
P. aves-seriales	Bibionidae, Drosophilidae, Diptera and Sciaridae, Diptera; Braconidae and Vespidae, Hymenoptera	Duque (1993)
P. chloroleuca	Drosophilidae and Empididae, Diptera	Duque (1993)
P. cordata (as *P. monocardia*)	Mycetophilidae, Diptera	Duque (1993)
P. divaricans	Bibionidae, Drosophilidae and Sciaridae, Diptera; Braconidae and Vespidae, Hymenoptera; Curculionidae, Coleoptera	Duque (1993)
P. grandiflora	Drosophilidae, Diptera	Duque (1993)
P. lindenii (as *P. secunda*)	Bibionidae and Drosophilidae, Diptera	Duque (1993)
P. phalangifera	Bibionidae and Drosophilidae, Diptera	
P. rhopalocarpa (as *P.cardiophylla*)	Tachinidae, Diptera	Duque (1993)
Pleurothallis sp*., Acroniae*	Chrysomelidae, Coleoptera	Duque (1993)
P. supervacanea (as *P. apoxys*)	Drosophilidae, Diptera	Duque (1993)
P. tetragona	Drosophilidae, Diptera; Curculionidae, Coleoptera	Duque (1993)
P. quadrifida	*Neocorynura* sp., Halicitidae, Hymenoptera	Damon & Salas-Roblero (2007)
P. rhopalocarpa	Halictidae, Hymenoptera	Costa (2009)
P. colossus	Anthomyiidae, Diptera	Calderón-Sáenz (2011)
P. marthae	*Mycetophila* sp., Mycetophilidae and *Bradysia* sp., Sciaridae, Diptera	Duque-Buitrago *et al.* (2014)
P. eumecocaulon	Drosphilidae, Diptera	Díaz-Morales & Karremans (2015)
P. ruscifolia	Ceratopogonidae, Diptera	Díaz-Morales & Karremans (2015)
Acroniae, Macrophyllae-Fasciculatae	*Linepithema* sp., Formicidae, Hymenoptera	Archila & Chiron (2015)
P. rhopalocarpa	*Megaselia* sp., Phoridae, Diptera	Mesa Londoño (unpubl. data)
P. millei	Diptera	Tobar (unpubl. data)

Table 1. Potential pollinators of species in *Pleurothallis* genus from published and unpublished observations (Wilson in preparation).

However, morphology alone is not enough to reveal the evolutionary pattern of pseudocopulation in *Pleurothallis*. Convergent evolution occurs across orchids sharing similar pollination mechanisms and species phylogenetically distant from each other could have evolved similar morphology to attract similar pollinators. Therefore phylogenetic analysis is also included to determine the evolution of pseudocopulation. *Pleurothallis* is originally a large genus with 32 subgenera (Luer 1999) such as *Ancipitia*, *Scopula*, *Pleurothallis*, and *Talpinaria* (Luer 1986). To determine how pseudocopulation evolved, morphological and phylogenetic analyses are combined.

The nuclear internal transcribed spacer (ITS) region is commonly used in phylogenetic analysis since it contains two highly variable portions, ITS1 and ITS2, generally yielding ample phylogenetic information for resolution of interspecific relationships. Between these is the less variable 5.8S region meeting the evolutionary conservative requirement (Baldwin 1992). An additional locus, the 3' hypothetical chloroplast open reading frame (*ycf1*), is the 3' end of the plastid *ycf1* gene, which is particularly phylogenetically informative at species level phylogenetic analysis since it is highly variable but still alignable in orchids (Neubig et al. 2009).

I hypothesize that species in genus *Pleurothallis* use both reward and deceit pollination and that pseudocopulation evolved at least once in *Pleurothallis*. In order to address this hypothesis, I conducted an extensive Scanning Electron Microscope (SEM) study of flowers from different subgenera within *Pleurothallis* and pollination mechanisms inferred from morphology were mapped onto phylogenies of the genus.

Materials and Methods

I. Plant Material

Plant material for this study was derived from the living collection of Colorado College, which were imported over several years from commercial nurseries from South America. All species, plant accession numbers, taxonomy information and whether they were analyzed by SEM and which genes or regions were sequenced are recorded in Table 2. All species in this study are vouchered with flowers in alcohol in the Colorado College herbarium (COCO).

Species	Plant ID Number	Taxonomic Clade	ITS	3' *ycf1*	SEM
P. acutipetala	PL0024	*Macrophyllae-Fasciculatae*	No	No	Yes
P. quadrifida	PL0294	*Lalexia*	No	No	Yes
P. aff. crocodiliceps	PL0505	*Ancipitia*	Yes	Yes	No
P. renieana	PL0512	*Ancipitia*	Yes	Yes	No
P. kelsoi	PL0594	*Ancipitia*	Yes	No	No
P. quadrifida	PL0706	*Lalexia*	Yes	Yes	No
P. correlii	PL0709	*Macrophyllae-Fasciculatae*	No	No	Yes
P. wielii	PL0713	*Ancipitia*	Yes	Yes	No
P. jostii	PL0782	*Talpinaria*	No	No	Yes
P. colossus	PL0787	*Macrophyllae-Racemosae*	No	No	Yes
P. onagriceps	PL0936	*Ancipitia*	No	No	Yes
P. gracilicolumna	PL0942	*Talpinaria*	No	No	Yes
P. talpinaria	PL0946	*Talpinaria*	No	No	Yes
P. papillingua	PL0951	*Macrophyllae-Racemosae*	No	No	Yes
P. gorgonaensis	PL0963	*Acroniae*	Yes	No	Yes
P. minutilabia	PL0996	*Macrophyllae-Fasciculatae*	No	No	Yes
P. fantastica	PL1006	*Macrophyllae-Fasciculatae*	No	No	Yes
P. luctuosa	PL1010	*Acroniae*	Yes	No	No
P. luctuosa	PL1011	*Acroniae*	Yes	No	No
P. sp. nov. "Lita"	PL1012	*Acroniae*	Yes	No	No
P. nelsonii	PL1013	*Ancipitia*	No	No	Yes
P. trimeroglossa	PL1025	*Talpinaria*	No	No	Yes
P. trimeroglossa	PL1026	*Talpinaria*	Yes	No	No
P. scabrata	PL1027	*Elongatia*	Yes	No	No
P. neossa	PL1034	*Acroniae*	Yes	No	Yes
P. gorgonaensis	PL1038	*Acroniae*	Yes	No	No
P. kaynagata	PL1039	*Abortivae*	Yes	No	No
P. aff. nelsonii "Orquideas Katia"	PL1045	*Ancipitia*	Yes	Yes	Yes
P. culpameae	PL1049	*Macrophyllae-Fasciculatae*	No	No	Yes
P. andreae	PL1051	*Ancipitia*	Yes	Yes	No
P. eumecocaulon	PL1066	*Ancipitia*	No	No	Yes
P. acutipetala	PL1069	*Macrophyllae-Fasciculatae*	Yes	No	No
P. luctuosa	PL1088	*Acroniae*	Yes	No	Yes
P. aff. nelsonii "Caqueta"	PL1090	*Ancipitia*	No	No	Yes
P. sp. nov. "Panama"	PL1091	*Macrophyllae-Fasciculatae*	No	No	Yes
P. sp. nov. "Romeral"	PL1098	*Ancipitia*	No	No	Yes
P. aff. restrepeoides	PL1110	Elongatia	Yes	No	No
P. sp. nov. "Romeral"	PL1117	*Ancipitia*	No	Yes	No

Table 2. Plant species analyzed in this study: plant accession numbers, taxonomic clades, regions or genes sequenced and those examined by SEM

II. Morphology

Scanning Electron Microscopy (SEM)

Flowers were taken from the plants in the Colorado College greenhouse and were fixed and stored in Kew mix (53% methanol, 37% water, 5% formaldehyde and 5% glycerol) until dehydration in an ethanol series. Species examined by SEM are listed in Table 2. For SEM preparation, flower samples were removed from the Kew mix and dehydrated with 70%, 90%, 100% and freshly opened 100% ethanol in order, each for 15 min. The dehydrated flowers were then dried in a critical point dryer and each mounted on an aluminum stub with a sticky carbon tab. Liquid carbon was applied around the specimen to improve electron conductivity. The stubs were left overnight to dry and sputter coated with gold and imaged with a Jeol JSM-6390LV scanning electron microscope at Colorado College with an accelerating voltage of 10-15 kV.

Light Microscopy

Sectioning

Flower specimens were washed in 100% toluene for three times, each for one hour. Specimens were then washed with melted paraffin three times, each for one hour and left overnight in the third round of melted paraffin. After two more washes with melted paraffin on the second day, the specimen and paraffin were poured into a preheated mold applied with glycerin and left to solidify. The solidified specimen was placed in cold running water for around 10 min and removed from the mold with a razor blade. Excess paraffin was trimmed off with a razor blade to fit the specimen on a small wooden block.

The paraffin specimen was trimmed into a pyramid shape and cut into 10 mm thick sections with a microtome and each 5 sections were made into a slide. The sections were relaxed in 50°C warm water bath for 3 s and placed on a dry, clean slide. Slides were left overnight to dry for staining.

Staining

The dried slides were deparaffinized in 100% xylene twice, with the first wash of 10 min and the second wash of 5 min. The slides were then hydrated in 50% ethanol for 2-3 min and stained in Safranin solution (1 g safranin and 100 ml 95% ethanol and the solution was 1:1 diluted with distilled water before use). After overnight stain in Safranin solution, the slides were rinsed in distilled water to remove excess stain and dipped in 70% acidified ethanol (9 drops of concentrated HCl in 200 ml 70% ethanol) twice to remove nonspecific stain. The slides were then washed in 95% ethanol for 15 s and washed in 100% ethanol twice, each for 15 s before putting in Fast Green solution (1 g Fast Green, 100 ml clove oil and 100 ml 100% ethanol). After a two-min staining in Fast Green solution, the slides were washed in Differentiating solution (100 ml clove oil, 50 ml 100% ethanol and 50 ml xylene) twice, each for 10 min. After three more washes in xylene, each for 15 min, slides were covered with cover slips with Canada balsam as a glue and viewed under a light microscope.

III. Phylogenetics

DNA extraction

Fresh or frozen leaf specimens were ground to fine powder with liquid nitrogen using a ceramic mortar and pestle. Genomic DNA was extracted from the ground powder using QIA DNeasy Plant Mini Kit or Epoch GenCatch Plant Genomic DNA Purification Kit following provided protocols and stored at -20°C. The extracted genomic DNA was run on a 0.8% agarose gel at 100 V for 15 min with a series of known quantities of λ DNA for comparison to determine concentration.

PCR amplification

ITS

ITS was amplified with forward primer 17SE (ACGAATTCATGGTCCGGTGAAGTGTTCG) and reverse primer 26SE (TAGAATTCCCCGGTTCGCTCGCCGTTAC) (Sun et al. 1994). For each reaction, a master mix was created with 1 µl of 17SE (25µM), 1 µl of 26SE (25µM), 1 µl of dimethyl sulfoxide (DMSO) and 12.5 µl of 2x Promega PCR Master Mix for a total volume of 15.5 µl. Extracted genomic DNA was diluted with molecular biology grade water to give a DNA template solution with around 5 ng of DNA in 9.5 µl per reaction. In each 0.2 ml PCR tube, 15.5 µl master mix and 9.5 µl DNA template were mixed thoroughly by pipetting and spinning. Four 25 µl ITS reactions per specimen were performed with a thermal cycler with the following program:

1 cycle: 94°C for 5 min

5 cycles: 94°C for 1 min, 60°C for 1 min, 72°C for 3 min

30 cycles: 94°C for 1 min, 58°C for 1 min, 72°C for 3 min

1 cycle: 72°C for 15 min

1 cycle: 4°C infinite hold

3' *ycf1*

3' *ycf1* is a long sequence and needs to be amplified with two pairs of primers: Pair A with forward primer 3720f (TACGTATGTAATGAACGAATG) and reverse primer IntR (TTTGATTGGGATGATCCAAGG), and Pair B with forward primer IntF (GATCTGGACCAATGCACATAT) and reverse primer 5500r (GCTGTTATTGGCATCAAACCA) (Neubig et al. 2009). The sequences amplified by Pairs A and B are aligned to give a final 3' *ycf* sequence for each specimen. For each reaction of Pair A, a master mix was created with 1 µl of 3720f (5µM), 1 µl of IntR (5µM), 1 µl of $MgCl_2$ (12.5 mM), 4.5 µl molecular biology grade water and 12.5 µl of 2x Promega PCR Master Mix for a total volume of 20 µl. For each reaction of Pair B, a master mix was created with 1 µl of IntF (5µM), 1 µl of 5500r (5µM), 1 µl of $MgCl_2$ (12.5 mM), 4.5 µl molecular biology grade water and 12.5 µl of 2x Promega PCR Master Mix for a total volume of 20 µl. Extracted genomic DNA was diluted with molecular biology grade water to give a DNA template solution with around 2.5 ng of DNA in 5 µl per reaction. In each 0.2 ml PCR tube, 20 µl master mix and 5 µl DNA template were mixed thoroughly by pipetting and spinning. Each plant specimen has three 25 µl 3' *ycf1* Pair A reactions and two 25 µl 3' *ycf1* Pair B reactions. Reactions were performed with a thermal cycler with the following program:

1 cycle: 94°C for 3 min

8 cycles: 94°C for 30 s, 60°C for 1 min (touchdown 1 degree, cycle to 52 °C), 72°C for 3 min

30 cycles: 94°C for 30s, 50°C for 1 min, 72°C for 3 min

1 cycle: 4°C infinite hold

PCR purification

Gel extraction

PCR products of ITS and 3' *ycf1* Pair A were purified by gel extraction. A 1.8% agarose gel was prepared with 50 ml 1X TAE buffer, 0.9 g agarose and 5 µl GelRed. Products of all four ITS PCR reactions were combined to give a total volume of 100 µl and mixed with 20 µl 6x Promega Blue/Orange loading dye and loaded into three wells, each with 40 µl. Products of all three 3' *ycf1* Pair A PCR reactions were combined to give a total volume of 75 µl and mixed with 15 µl 6x Promega Blue/Orange loading dye and loaded into three wells, each with 30 µl. Products were run alongside a 100 bp ladder in order to verify the desired product (ITS = ~875 bp, 3' *ycf1* A= ~850 bp). Gels were run at 100V for 90 min and photographed using a BioDoc-It Imaging System (UVP). The target bands were cut from the gel under UV light with a razor blade and excess gel was trimmed off. The DNA containing bands were weighed to estimate buffer volumes used in gel extraction. PCR products were extracted from excised gel cubes using a QIAquick Gel Extraction Kit (QIAGEN) according to the protocol provided. Concentration (ng/µL) and purity (A260/A280) of purified DNA were estimated on a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Spin purification

PCR products of 3' *ycf1* Pair B were purified by spin purification. Products of both reactions were combined to give a total volume of 50 µl. 20µl of the product was removed and combined with 4 µl of 6x Promega Blue/Orange loading dye and run alongside a 100 bp ladder in a 1.5% agarose gel as in gel extraction for 30 min to verify the desired product (3' *ycf1* B = ~1000bp). Once confirmed the desired product was successfully amplified, the rest of the 30 µl of PCR product was spin purified with QIAquick PCR Purification Kit (QIAGEN) according to the protocol provided. Concentration (ng/µL) and purity (A260/A280) of purified DNA were estimated on a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Preparation of DNA for sequencing

Purified PCR products were submitted to GeneWiz (South Plainfield, New Jersey) for sequencing. ITS PCR products were sent with the primers 17SE, 26SE, ITS1 (TCCGTAGGTGAACCTGCGG) (White et al. 1990), and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). 3' *ycf1* Pair A PCR products were sent with primers 3720f, IntR, and 3'ycf-for1 (ATGCCTAAAGAATGGCGAAA). 3' *ycf1* Pair B PCR products were sent with primers 5500r, IntF, IntF2 (CATTAACGTAAATCCAAAGAA) and 3'ycf-rev1 (TCATTCAAAAATTGCCCACA) (Neubig et al. 2009).

Sequence analysis and phylogeny construction

Trace files were downloaded from GeneWiz and viewed in Geneious to ensure

sequence viability and to confirm that peaks were called correctly, edited for accuracy when they were not, and truncated at the appropriate sites. Forward and reverse sequences were then aligned to create a consensus sequence for each specimen and exported as FASTA files. When sequences produced a poor consensus, with ambiguous nucleotides or a lack of corroboration between multiple sequences, samples were re-amplified, purified, and sent back to GeneWiz for another round of sequencing.

Consensus sequences were exported to MEGA 10.0 (Kumar et al. 2018) and aligned by ClustalW for phylogeny analysis and construction. The aligned sequences went through Maximum Parsimony (MP) analysis within MEGA 10.0 using all sites for data subset, Subtree-Pruning-Regrafting (Nei & Kumar 2000) as the MP Search Method and bootstrap method for test of phylogeny with 1000 replicates. The aligned sequences were also analyzed with the Maximum Likelihood (ML) analysis within MEGA 10.0, again using all sites for data subset and bootstrap method for phylogeny test with 1000 replicates. All the other settings remain default, with Jukes-Cantor model (Jukes & Cantor 1969), uniform rates, Nearest-Neighbor-Interchange (NNI) ML Heuristic Method and the initial tree automatically obtained with Neighbor-Join (NJ) and BioNJ algorithms (Kumar et al. 2018). All phylogenies were rooted with *Arpophyllum* and *Pabstiella* outgroup sequences from GenBank.

Results

I. Hypothesized Reward Pollination Species

To attract pollinators with reward pollination mechanism, an orchid flower needs to

have secretory tissues to produce nectar, usually located on its lip. Under SEM, most of these lips have a glenion, which is a small round area of cells different from surrounding cells, located at the base of the lip and usually under the column as shown in the *Pleurothallis gorgonaensis* lip in Figure 1a. Size and shape of the glenion differs in each species, but a common characteristic is the glenion cells have a larger space between each other, making them look different from other cells on the lip. More details about the glenion will be described for each species.

Pleurothallis gorgonaensis

SEM photos of *P. gorgonaensis* lip (Figure 2) were taken with the column removed to reveal the lip base area. Figure 2a shows the round glenion area at the lip base, a sulcus, which is a deep channel allowing "nectar" to flow and accumulate from the glenion area to middle of the lip. Figure 2b is a more detailed photo of the glenion only, showing the distinct glenion cells. Differing from the surrounding cells, glenion cells are smaller in size but have a larger space in between, creating the black space in the SEM photo.

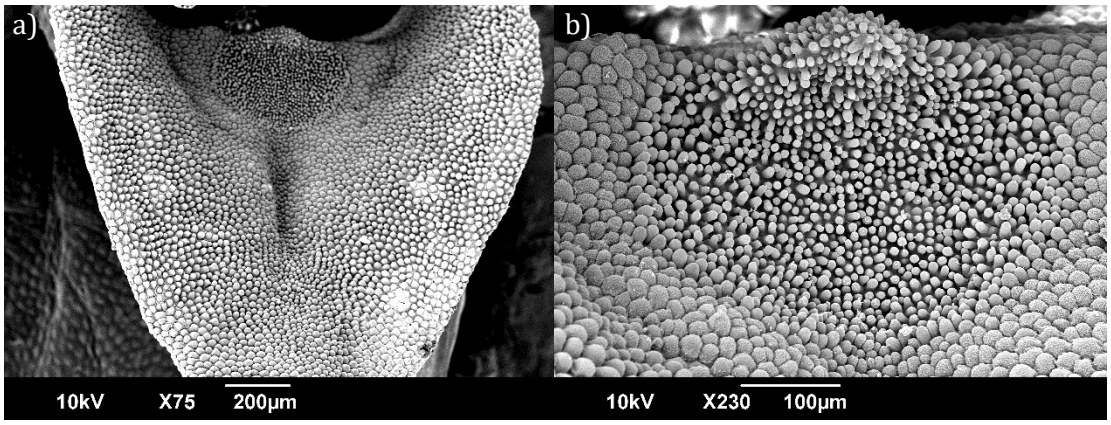

Figure 2. SEM photos of *Pleurothallis gorgonaensis*.
a) front view of lip with the glenion at lip base and a sulcus. b) glenion only.

Pleurothallis luctuosa

SEM photo of *P. luctuosa* (Figure 3) was taken from above to reveal the lip base – the viscidium and anther cap are right above the glenion. In the middle of the image is a round glenion with cells taller and more sparsely distributed. Around the glenion is a ring of callus with larger and taller cells which help the nectar-like liquid to accumulate between the glenion and the callus. The sulcus, which is the channel in the callus, allows the accumulated nectar-like liquid to flow out to attract pollinators.

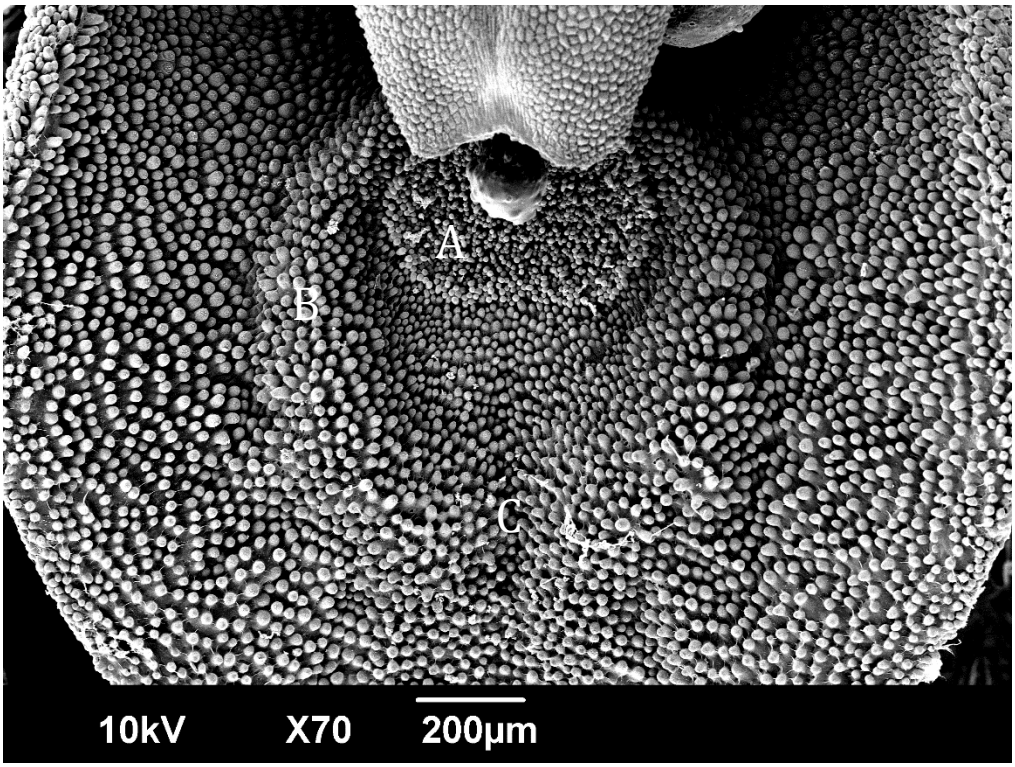

Figure 3. SEM photo of *Pleurothallis luctuosa*.

A. glenion B. callus. C. sulcus

Pleurothallis correllii

SEM image of *P. correllii* (Figure 4) was taken from a tilted angle to show both the column tip and the lip base. At the lip base there is a small oval-shaped area covered by a

layer of dried liquid hypothesized to be evidence for the nectar-like liquid. Under the liquid layer the cells have larger space in between than surrounding cells, suggesting glenions cells under the liquid and supporting the hypothesis that the glenion secretes nectar-like liquid to attract pollinators. On both sides of the glenion and liquid area are two elevated areas of cells. These calli help guide the secretions to flow in the correct direction toward lip tip to attract pollinator but not toward the two sides.

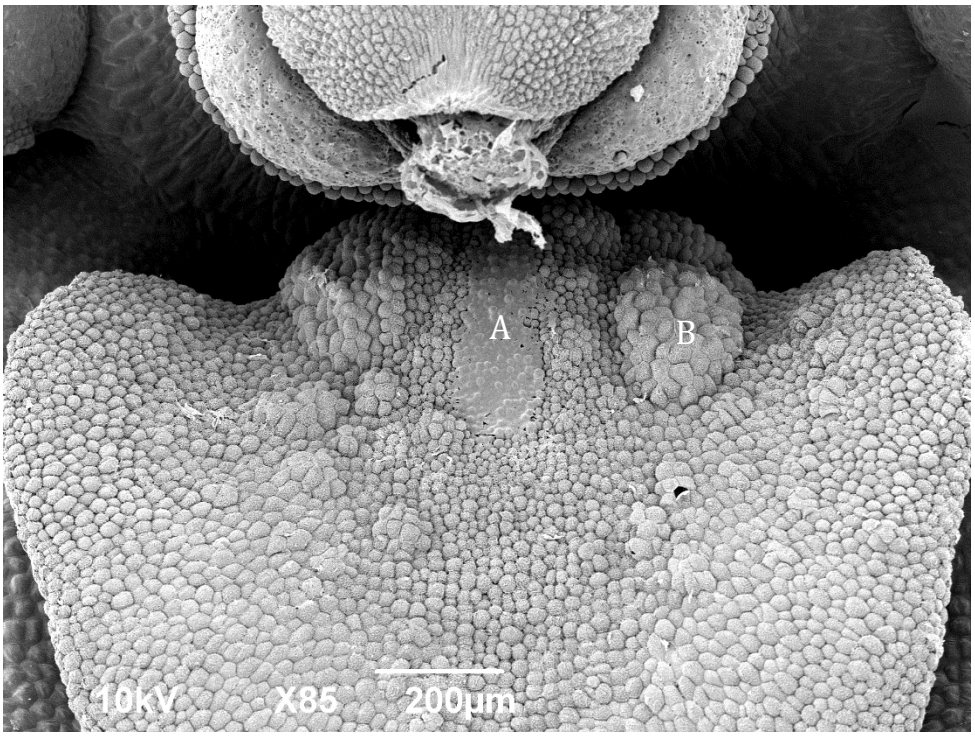

Figure 4. SEM photo of *Pleruothallis correllii*.

A. glenion covered by dried nectar-like liquid B. callus

Pleurothallis colossus

The SEM image of *P. colossus* (Figure 5) was also taken at a tilted angle to reveal the lip base since the lip is an arch shape and naturally the lip base is covered by the column. At the middle of the arch top area is a clear sulcus connecting lip base area and lip tip with callus around, suggesting liquid flow in this direction. In addition, at lip base there is an

area of cells (Figure 5b) similar to typical glenion cells. The back view better shows this part (Figure 5c&d) where there is an area of cells different from the surrounding, suggesting the existence of a glenion. This type of cells continues in the sulcus, suggesting that secretion happens not only in the glenion area but also happens in the sulcus.

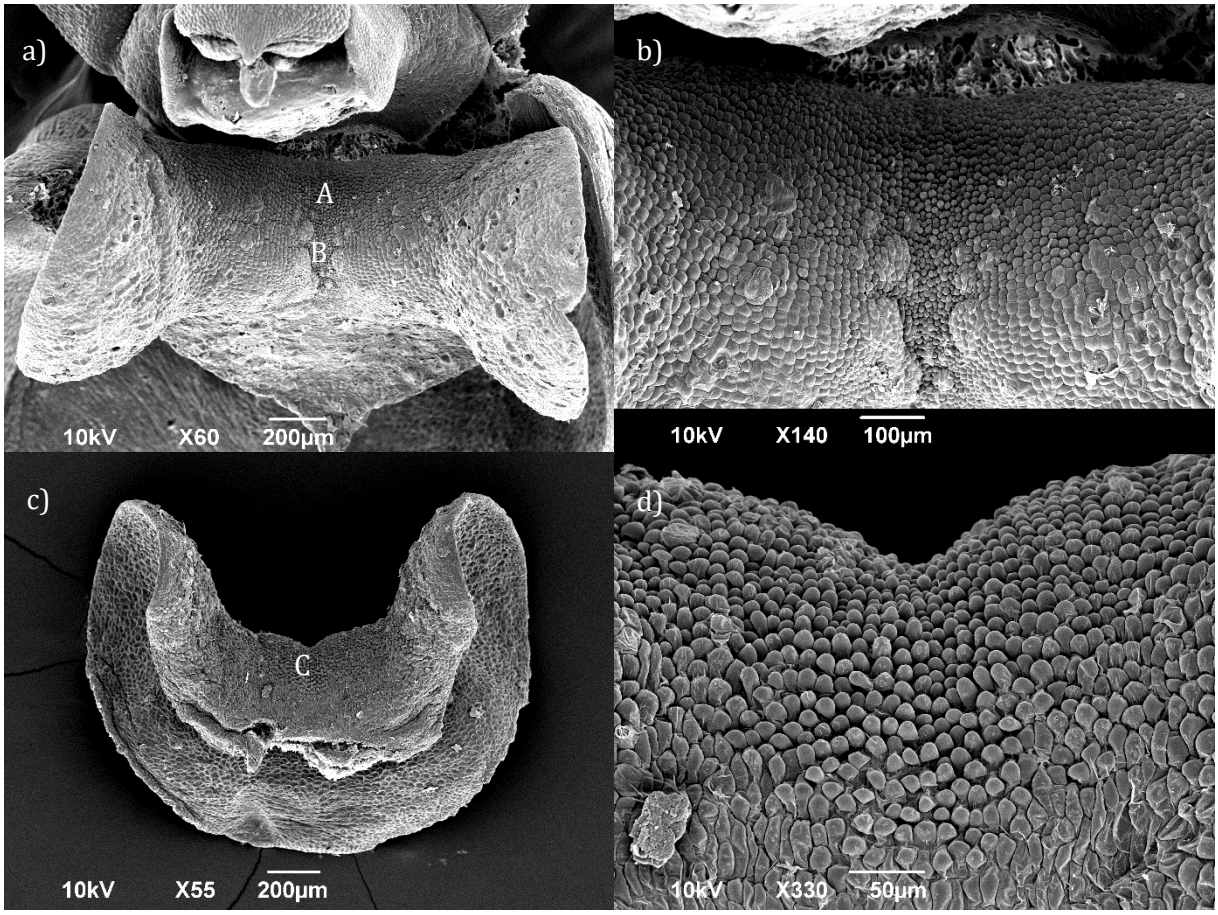

Figure 5. SEM photos of *Pleurothallis colossus*

a) front view of whole lip with A. glenion B. sulcus
b) a detailed photo of the glenion and sulcus area in a
c) back view of lip only with C. glenion
d) a detailed photo of the glenion from back in c

Pleurothallis quadrifida

SEM photos of *P. quadrifida* (Figure 6) were taken with column removed to reveal the lip base. From the above view, there is a deep sulcus at the mesochile area which is the middle part of the lip. A more detailed photo of the lip base area and part of sulcus shows

that compared to the surrounding cells, the cells in the sulcus and at middle of lip base are shorter and smoother, suggesting that these are different cells and are hypothesized to be secretory tissues.

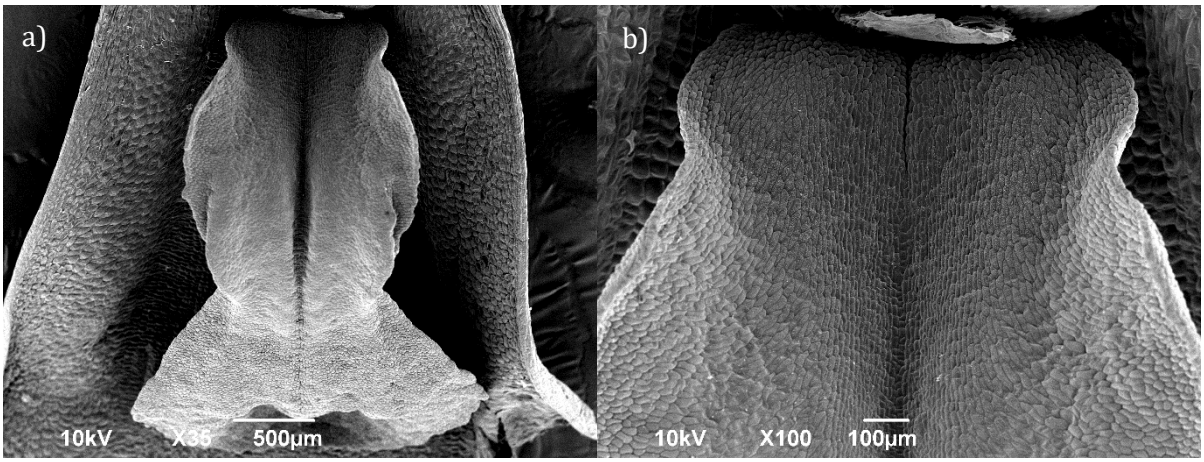

Figure 6. SEM photos of *Pleurothallis quadrifida*
a) whole lip with sulcus b) detailed lip base

Pleurothallis sp. nov. "Culpameae"

SEM of *Pleurothallis sp. nov.* "Culpameae" (Figure 7) shows a clear glenion at lip base under column. As shown in the detailed photo of Figure 7b) the glenion is covered by dried liquid, again supporting that the glenion secretes nectar-like liquid. The deep sulcus in the middle of the lip helps to guide the liquid flow towards lip tip. Surprisingly half of the lip is covered in dried liquid, as shown in Figure 7a) and the composition of this liquid is unknown.

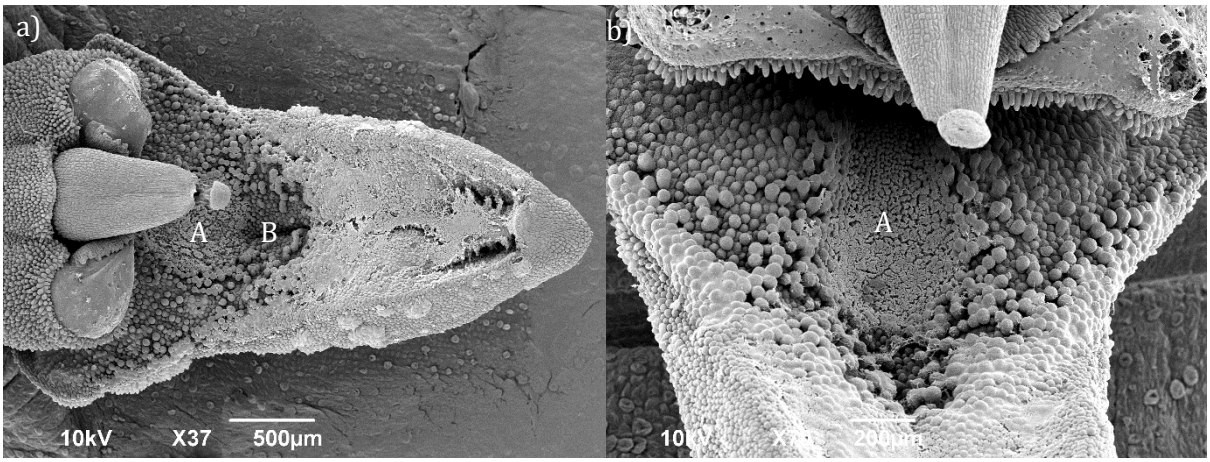

Figure 7. SEM photos of *Pleurothallis sp. nov.* "Culpameae"

a) above view of whole lip A. glenion B. sulcus

b) detailed photo of glenion in a A. glenion

Pleurothallis acutipetala

SEM photos of *P. acutipetala* (Figure 8) were taken with two specimens. From above view, the glenion is clearly shown at lip base, under the pollinarium tip (Figure 8a). Detailed photos of the glenion are shown in Figure 8b and 8c. Figure 8b shows a typical glenion with smaller cells and larger space in between. In addition, between the glenion cells and the normal lip cells are a layer of callus cells taller than both glenion cells and normal lip cells, creating a lower glenion area where the secreted nectar-like liquid could accumulate. Figure 8c shows a specimen with dried liquid at glenion area, supporting our hypothesis that the glenion secretes nectar-like liquid.

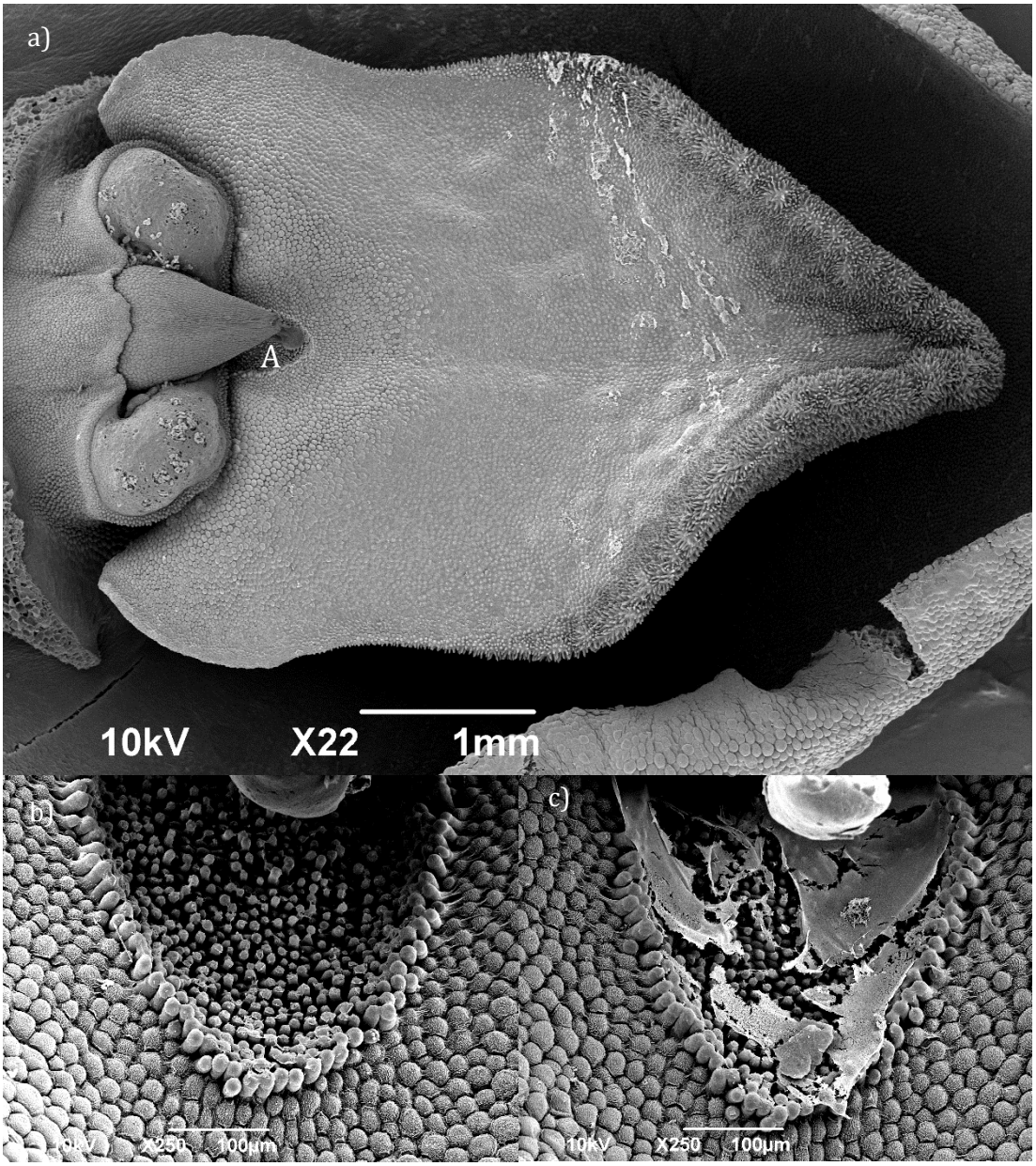

Figure 8. SEM photos of *Pleurothallis acutipetala*

a) above view of the whole lip A. glenion
b) detailed glenion
c) detailed glenion with dried liquid

Pleurothallis eumecocaulon

SEM images of *P. eumecocaulon* (Figure 9) was taken with column removed to reveal the whole lip. After examining, there are no different cell types on the adaxial lip base, suggesting that there is no glenion. However, there is a cavity with unknown depth located at mesochile with a very shallow sulcus (A) leading into it. It is hypothesized that this deep

cavity has evolved to accumulate nectar flowing in through the shallow sulcus and thus this species is a hypothetical reward pollinated species. From the sulcus to lip tip the cell type changes to have less space between each other. Under SEM the lighter color of these cells also differs from the other cells, suggesting that these cells are harder to get coated with gold and it is hypothesized that these cells secrete something that inhibits gold coating.

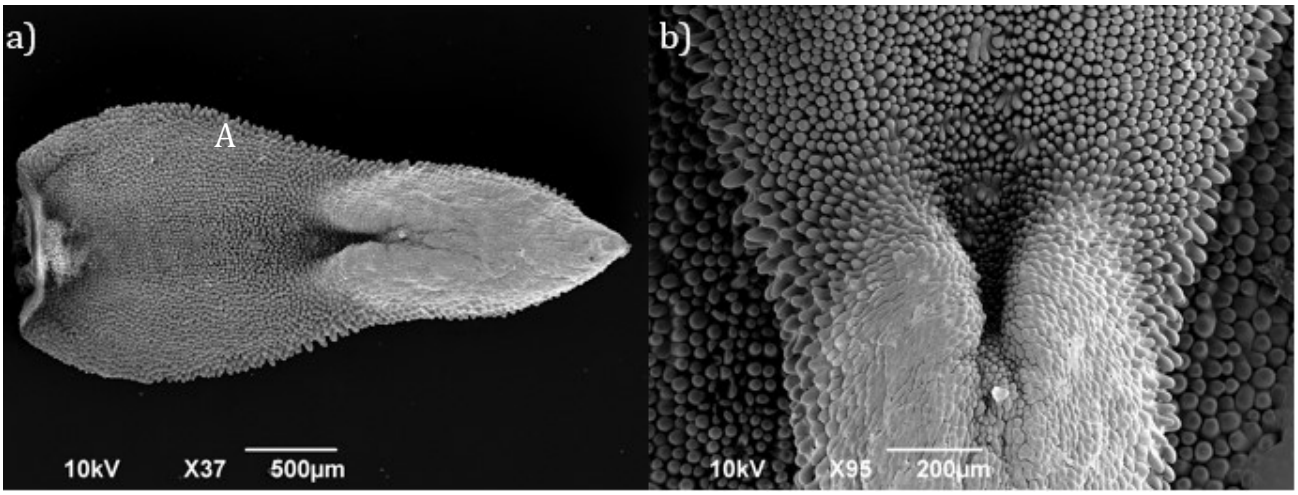

Figure 9. SEM photos of *Pleurothallis eumecocaulon*
a) whole lip only b) the cavity located at mesochile
A. shallow sulcus

II. Hypothesized Alternate Pollination Mechanisms Species

Unlike typical lips with a glenion or other secretory tissues suggesting reward pollination, these species have atypical lips that usually lack these rewarding secretions and thus these lip morphologies suggest alternate pollination methods. One hypothesized alternate pollination mechanism is deceit pollination by pseudocopulation, where the lip mimics the females of pollinator species by shape, texture, movement and sex pheromones. These lips usually have long papillae all over the lip and a cavity located at lip tip which imitates the female copulatory channel to attract male pollinators.

Pleurothallis minutilabia

SEM images of *P. minutilabia* (Figure 10) were taken at the front view since the tiny lip is completely covered by the column at the top view. Papillae appear all over the upper surface of the lip and especially long papillae exist at the lip tip around the apical cavity. The apical cavity points toward middle bottom of the lip and is hypothesized to mimic the female copulatory channel of the pollinator species and allow a male pollinator to insert its abdomen. The lip has a unique downward-pointing part (Figure 10b from the lip base acting as a support and it is hypothesized that the anchor part helps the tiny lip to remain horizontal.

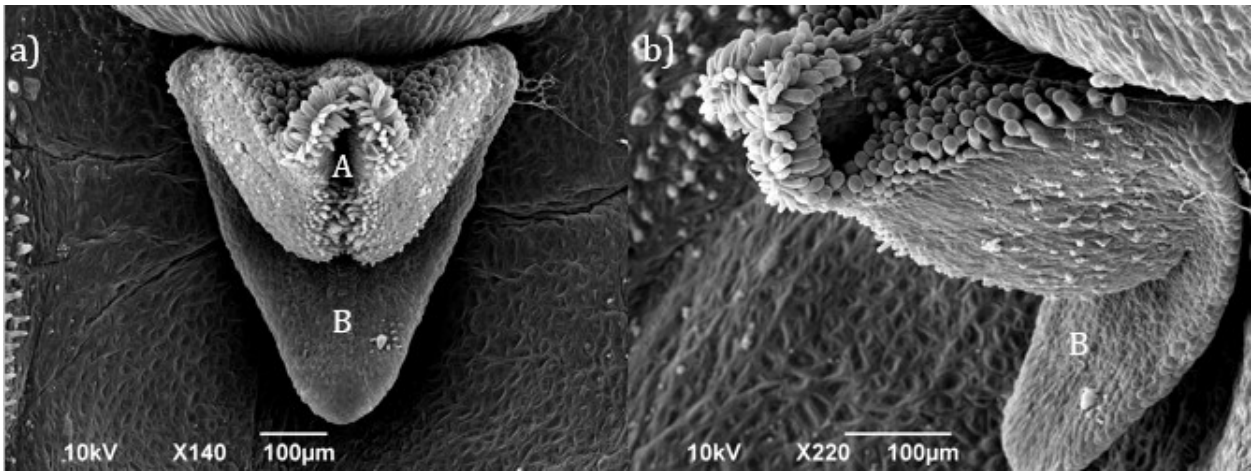

Figure 10. SEM photos of *Pleurothallis minutilabia*
A. apical cavity B. anchor part
a) front view b) side view

Pleurothallis papillingua

SEM images of *P. papillingua* (Figure 11) was taken with lip only. This lip has no glenion or other typical secretory tissues, so it is hypothesized to be pollinated by alternate pollination mechanism. There is no copulatory channel either and thus is not hypothesized to be pseudocopulation. Branch-like papillae appear all over the lip and

there is no data on whether these papillae have any osmophores so the possible pollination mechanism is still unknown.

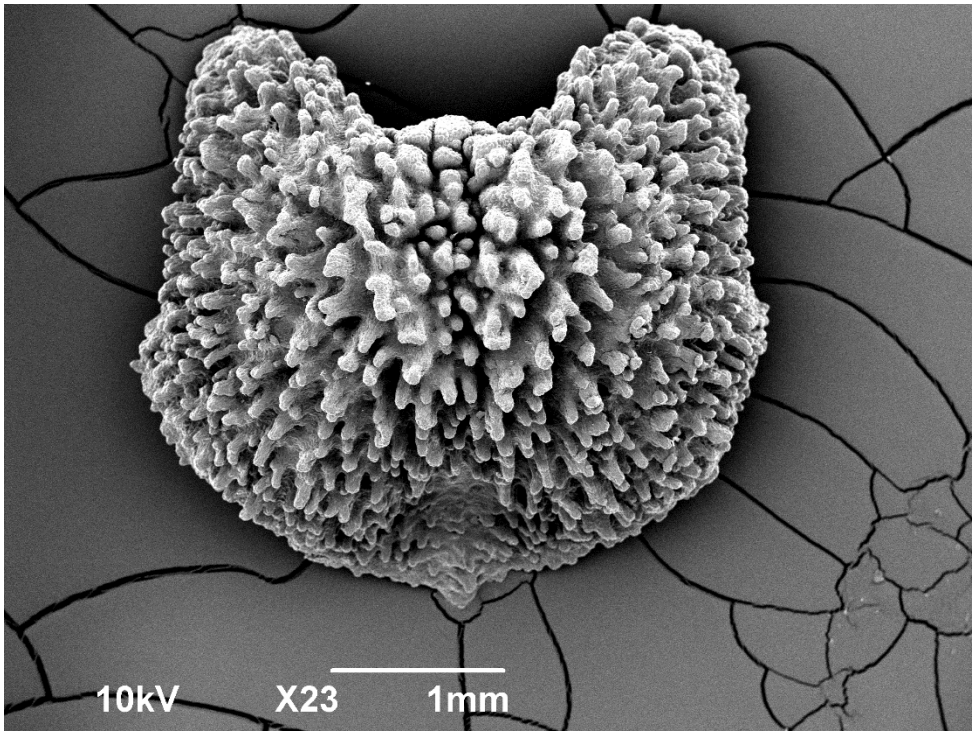

Figure 11. SEM photo of *Pleurothallis papillingua.*

Pleurothallis fantastica

SEM image of *P. fantastica* (Figure 12) shows a unique lip. The lip has two large lobes on the two sides, holding the column in between. Under the column there is one more lobe with a deep channel at tip, dividing into two smaller lobes. With column removed (Figure 12b), cells all over the lip were examined and no conspicuously different cell types were discovered, suggesting that there are no glenion or other secretory tissues.

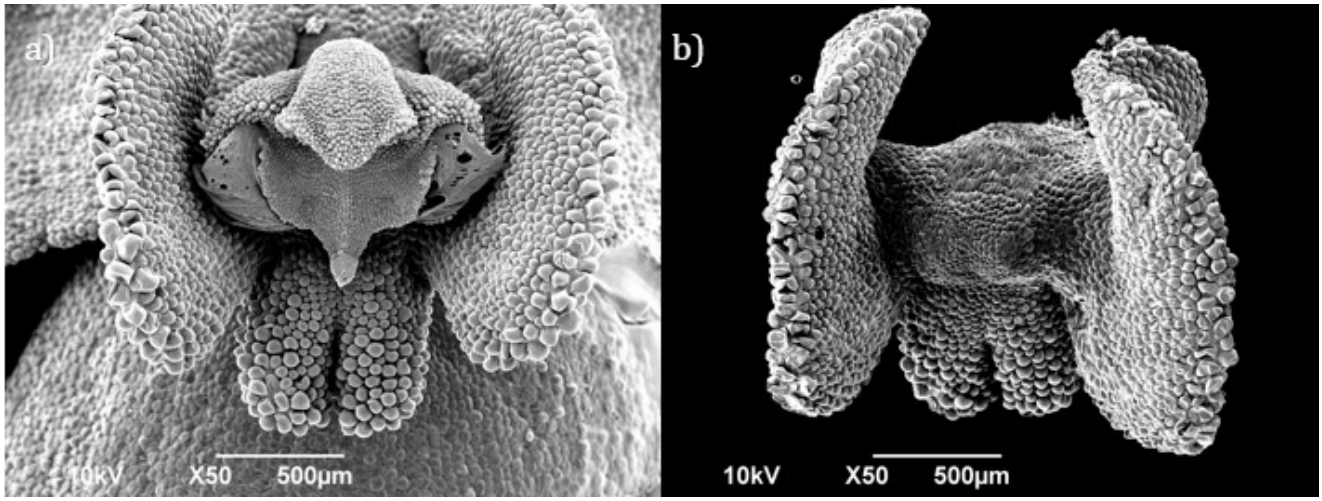

Figure 12. SEM photos of *Pleurothallis fantastica*

a) above view of lip with column b) lip only

Pleurothallis talpinaria group:

Pleurothallis talpinaria

SEM image of *P. talpinaria* (Figure 13) lip shows a typical lip structure of the *P. talpinaria* group, with a three-lobed epichile, a mesochile with hairy papillae on both sides and a hypochile with two lobes on the two sides. Unlike other species in this group, *P. talpinaria* has a thin and relatively shallow sulcus at mesochile though the cells types in the sulcus are the same as the surrounding cells, suggesting there is no secretion.

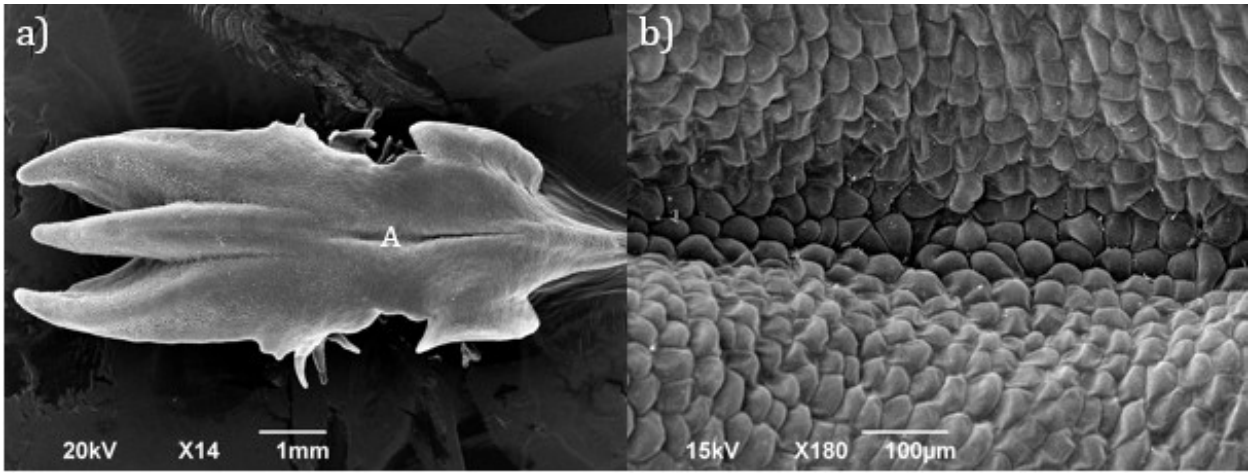

Figure 13. SEM photos of *Pleurothallis talpinaria*

a) whole lip A. sulcus b) a detailed photo of sulcus only

Pleurothallis jostii

SEM of *P. jostii* (Figure 14) shows a lip similar to *P. talpinaria* but shorter. Unlike *P. talpinaria*, there is no sulcus in the mesochile. As shown in the detailed mesochile photo (Figure 14b), there is a very shallow pit and there is no difference in cell types, suggesting no secretions.

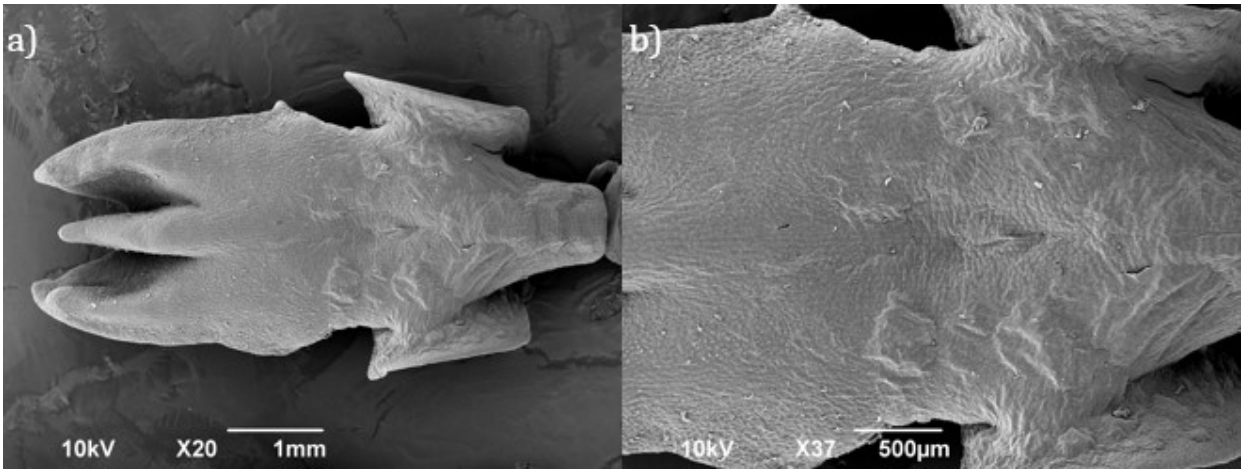

Figure 14. SEM photos of *Pleurothallis jostii.*
a) whole lip b) detailed mesochile

Pleurothallis trimeroglossa

SEM photo of *P. trimeroglossa* whole lip (Figure 15) is similar to other species in this *P. talpinaria* group and the most conspicuous difference is the mesochile. As shown in the detailed mesochile photo (Figure 15b), there is a shallow pit located at the middle of the mesochile. The location of the pit is similar to that on the *P. jostii* lip but much deeper and the mesochile of *P. trimeroglossa* is slimmer than that of *P. jostii*.

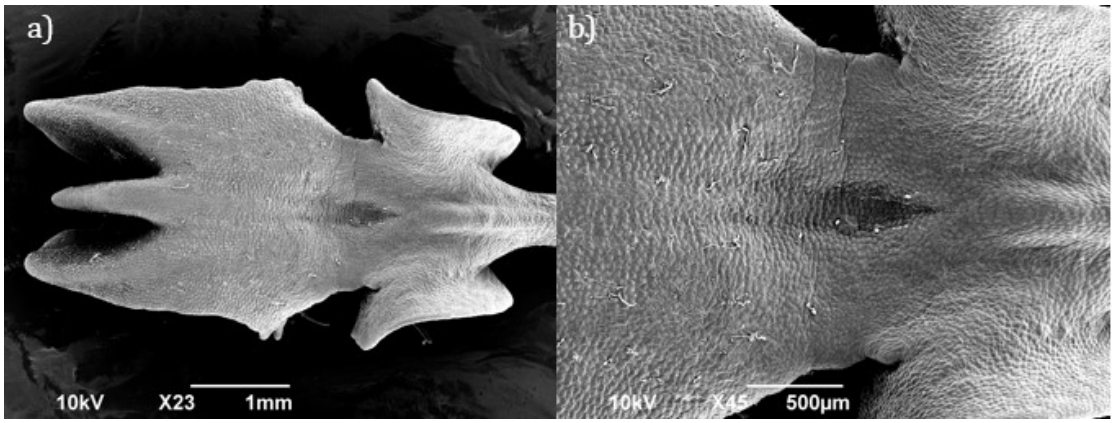

Figure 15. SEM photos of *Pleurothallis trimeroglossa*
a) whole lip b) detailed mesochile

Pleurothallis gracilicolumna

SEM of *P. gracilicolumna* (Figure 16) shows a lip different from the other three species in this group. Though it also has a typical three-lobed epichile, the mesochile and hypochile have edges folding up, unlike the relatively flat edges of the other species. *P. gracilicolumna* has a very deep cavity in the mesochile and the cell type at bottom could not be seen clearly so whether the cells are different from surrounding cells are still unknown. The hypochile has very thick calli on both sides, creating a deep sulcus in the middle and making it hard to determine whether the sulcus cells are different from surrounding cells. Therefore whether there is secretory cells in this sulcus is unknown, though the deep cavity may act as an area to accumulate secretion as in *P. eumecocaulon* (Figure 9). Without further evidence suggesting presence of secretion, *P. gracilicolumna* is listed here for now since it is a member in the *P. talpinaria* group.

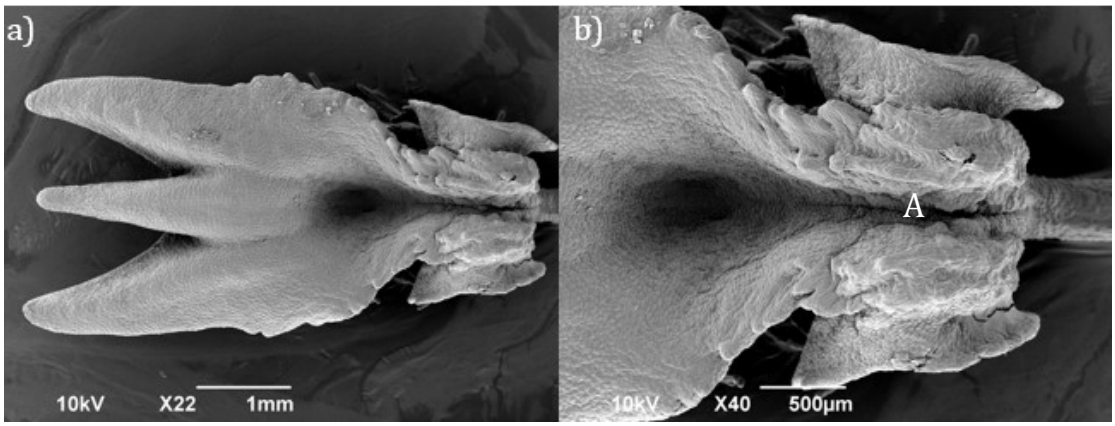

Figure 16. SEM photos of *Pleurothallis gracilicolumna*
a) whole lip b) detailed mesochile and hypochile A. sulcus between calli

Pleurothallis crocodiliceps group:

Pleurothallis nelsonii

SEM of *Pleurothallis nelsonii* whole lip (Figure 17a) was taken with lip only since the column covers most of the lip in the above view. Long papillae cover most of the lip, especially the lip tip and the two horns. The cavity at lip tip is partially covered by the long papillae and the two horns from lip base are pointing forward to the sides. After cutting the lip in half, the inside of cavity is also revealed (Figure 17b). The cavity is a channel deep down towards bottom middle of the lip. Papillae exist all over the inside wall of the channel and they are shorter and thicker than the outside papillae.

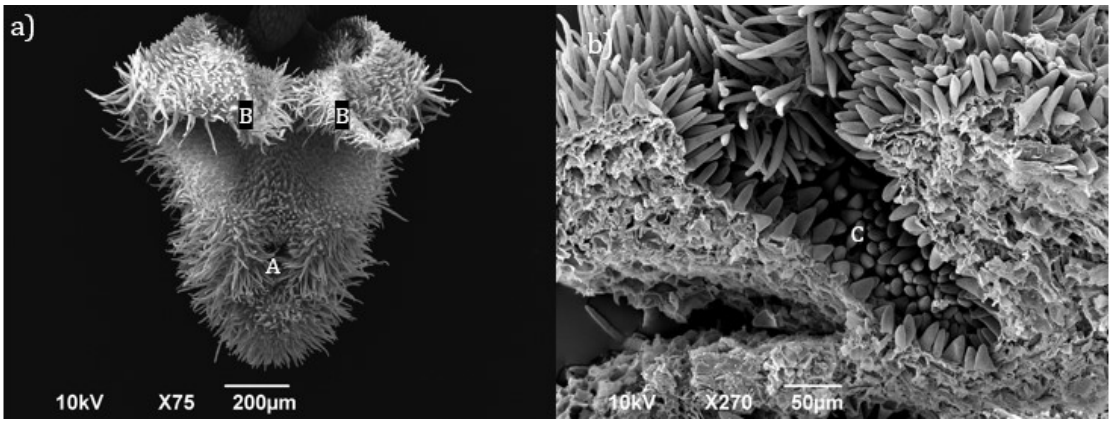

Figure 17. SEM photos of *Pleurothallis nelsonii*

a) above view of whole lip A. apical cavity B. horns

b) side view of cut apical cavity C. apical cavity

Pleurothallis wielii

Light microscope photos of *P. wielii* slides (Figure 18) show a side view of the inside of the apical cavity. Similar to the split lip SEM photo of *P. nelsonii*, the slide photo of *P. wielii* shows a deep channel towards bottom middle of the lip and there are papillae all over the inside of the channel which are shorter but thicker than the outside ones.

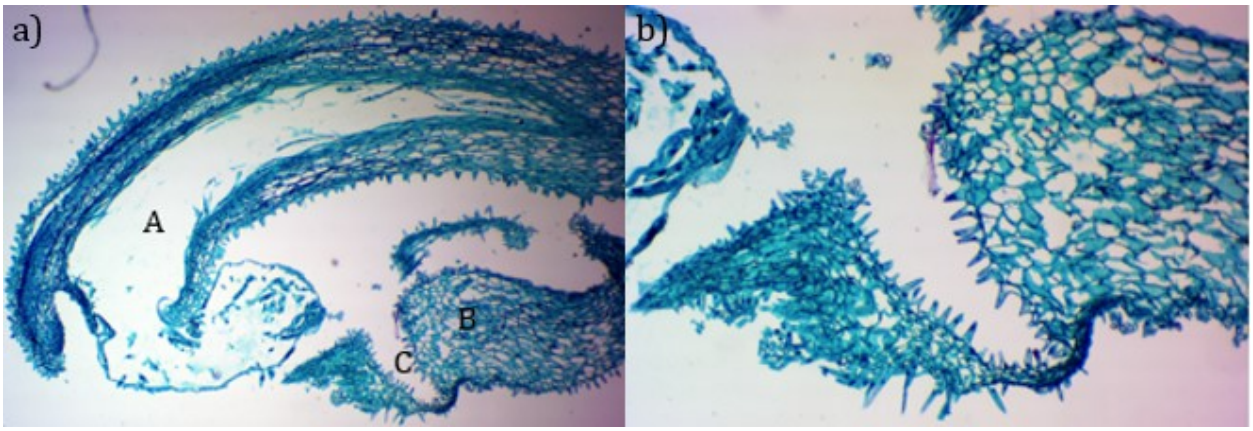

Figure 18. Light microscope photos of *Pleurothallis wielii* section slides

a) 40x A. column B. lip C. apical cavity

b) 100x detailed apical cavity

Pleurothallis aff. nelsonii "Orquideas Katia"

SEM of *P. aff. nelsonii* "Orquideas Katia" (Figure 19) shows a lip similar to that of *P.*

nelsonii, with two horns pointing forward to the sides, an apical cavity and long papillae at lip tip, around edges of the whole lip and on the two horns.

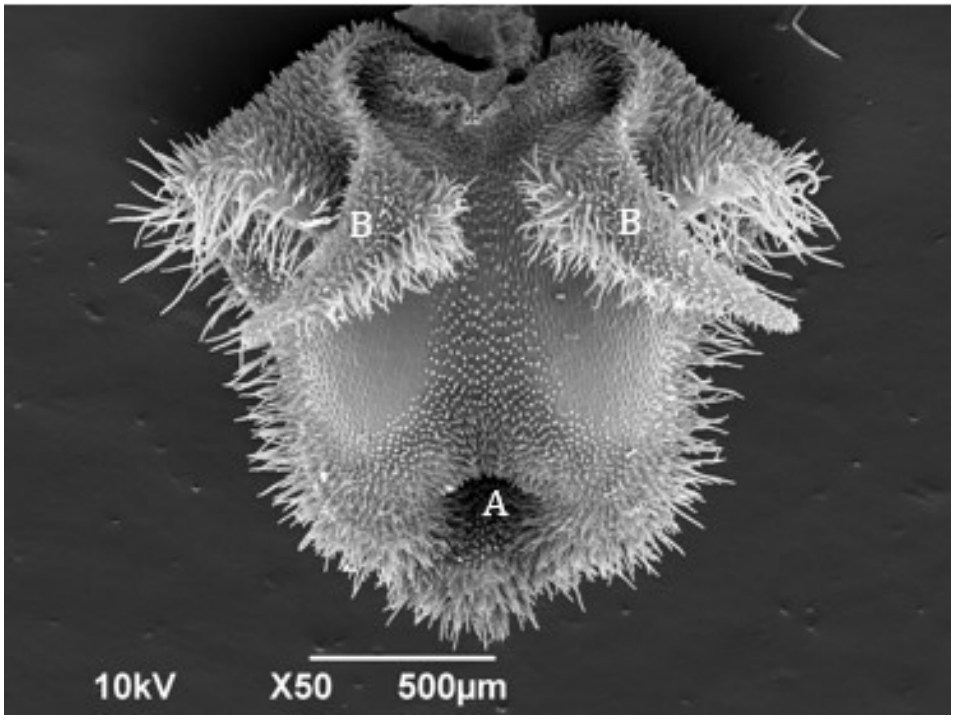

Figure 19. SEM photo of *Pleurothallis aff. nelsonii* "Orquideas Katia"
A. apical cavity B. horns

Pleurothallis aff. nelsonii "Caqueta"

The lip of *P. aff. nelsonii* "Caqueta" (Figure 20) is similar to those of *P. nelsonii* and *P. aff. nelsonii* "Orquideas Katia" marked by the two horns, one apical cavity and long papillae. The most conspicuous difference is the two horn tips point forward to the middle but not to the sides as in *P. nelsonii* and *P. aff. nelsonii* "Orquideas Katia". The horns are also wider and shorter comparing to the other two species. In addition, dried liquid was found on the inside of the petals, suggesting secretion by the petal cells.

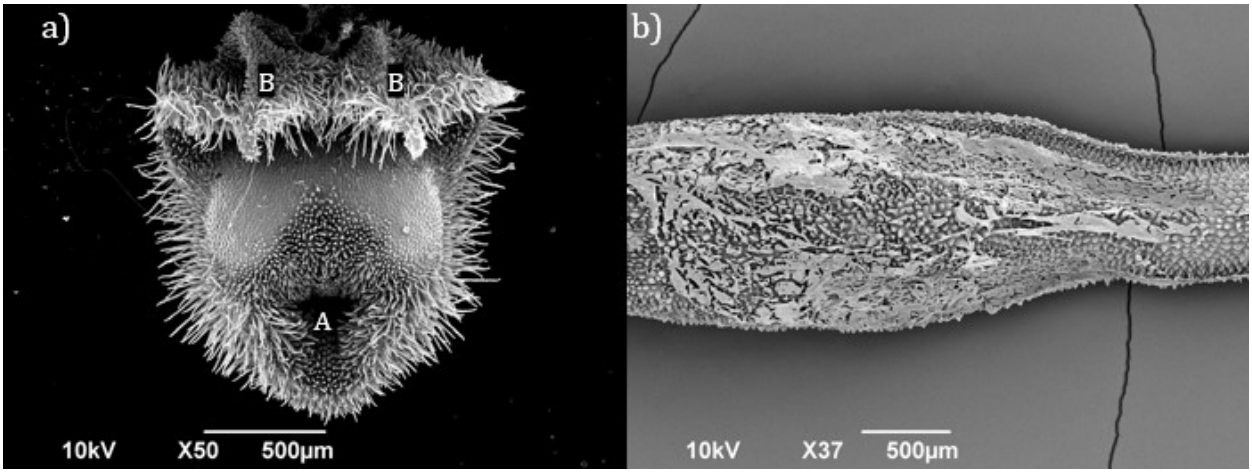

Figure 20. SEM photos of *Pleurothallis aff. nelsonii* "Caqueta"

a) whole lip A. apical cavity B. horns

b) dried liquid on petal

Pleurothallis onagriceps

SEM of *P. onagriceps* (Figure 21) shows a lip with a shape very different from the other species in the *P. crocodiliceps* group. Though it also has two horns, one apical cavity and long papillae as the other species, the lip tip and horn tips are round compared to the pointy lip tips and horn tips in the other species. The two forward pointing horns are much wider and longer than the species listed above, almost reaching lip tip. Long papillae also cover most of the lip but the papillae close to the apical cavity are shorter than other papillae, unlike in other species listed above there are usually more and longer papillae around the apical cavity.

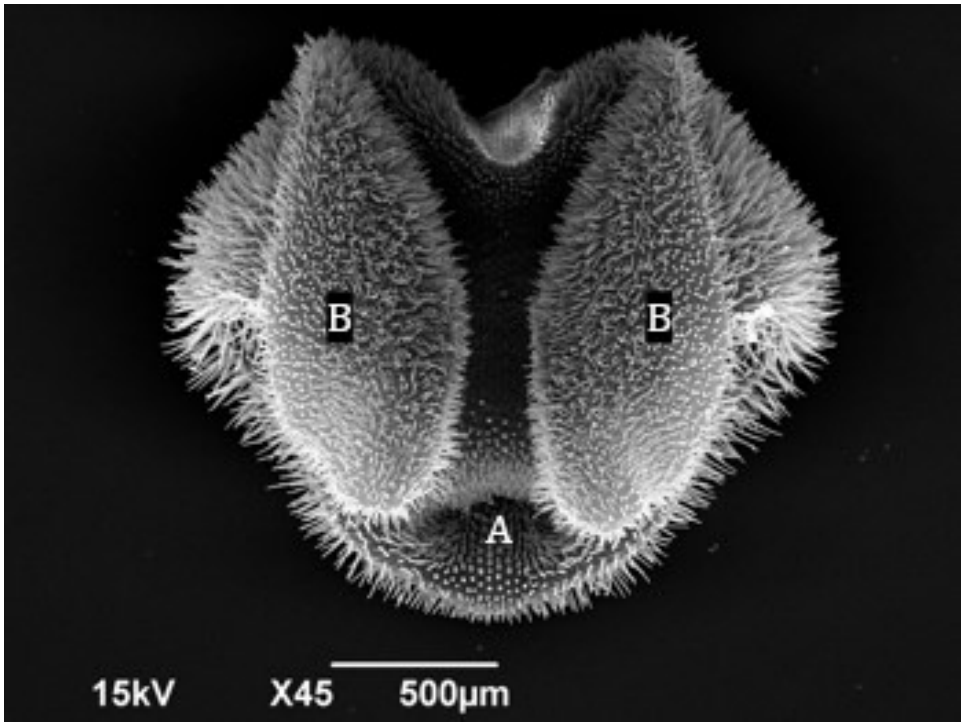

Figure 21. SEM photo of *Pleurothallis onagriceps*
A. apical cavity B. horns

Pleurothallis sp. nov. "Romeral"

SEM of *Pleurothallis sp. nov.* "Romeral" (Figure 22) also shows a unique lip shape. Despite the common characteristics of two horns, one apical cavity and long papillae, this lip differs from the other species by the bald lip tip. There are almost no long papillae around the whole lip tip and the apical cavity. Papillae only exist on the two horns and around the basal half of the lip. A detailed photo of lip tip part also shows no papillae inside the apical cavity, suggesting a very different mechanism to attract pollinators.

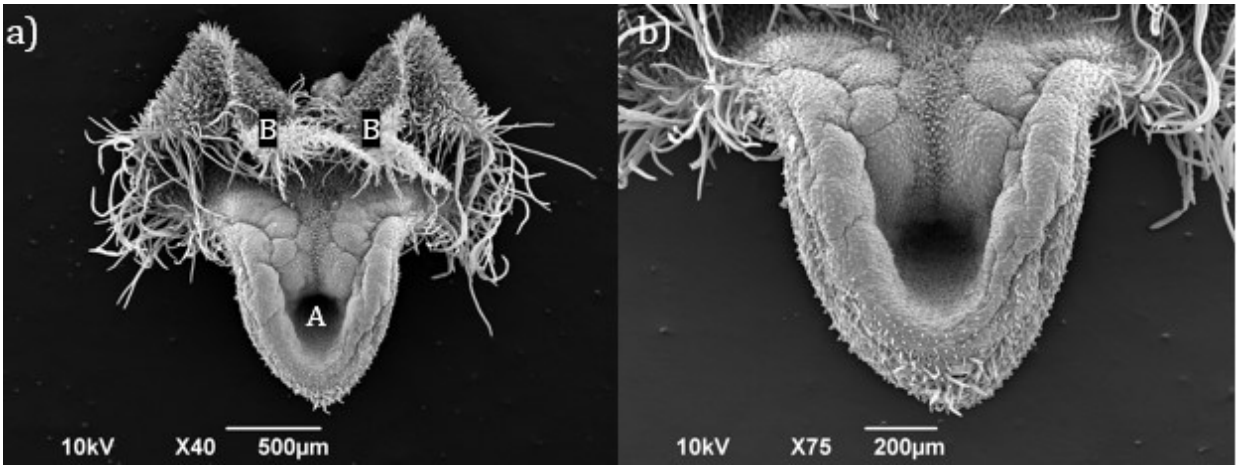

Figure 22. SEM photos of *Pleurothallis sp. nov.* "Romeral"

a) whole lip A. apical cavity B. horns

b) detailed lip tip with copulatory channel

Pleurothallis sp. nov. "Panama"

SEM photos of *Pleurothallis sp. nov.* "Panama" (Figures 23-25) were taken with five specimens picked at different stages after anthesis and two most different specimens were compared here.

Figure 23 shows a comparison of whole lip from above view of specimen 4 (Figure 23a) and specimen 3 (Figure 23b). The lip of specimen 4 has full cells while the lip of specimen 3 has cells with broken tips and there are several conglomerates stuck to the lip.

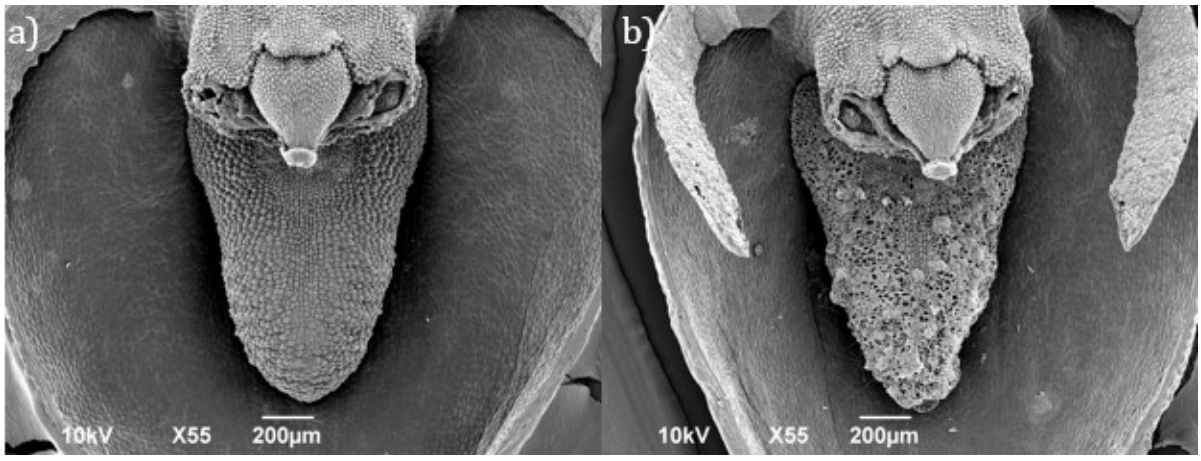

Figure 23. SEM photo comparison of *Pleurothallis sp. nov.* "Panama" whole lip

a) whole lip of specimen 4 b) whole lip of specimen 3

Figure 24 shows a comparison of the glenion area between specimens 4 and 3. Similarly, specimen 4 has a clear rectangular glenion area with a different type of cells while in Figure 24b specimen 3 has a lower glenion area with all the surface cells removed and an area with broken cells. Conglomerates and broken cells are also discovered outside the glenion area.

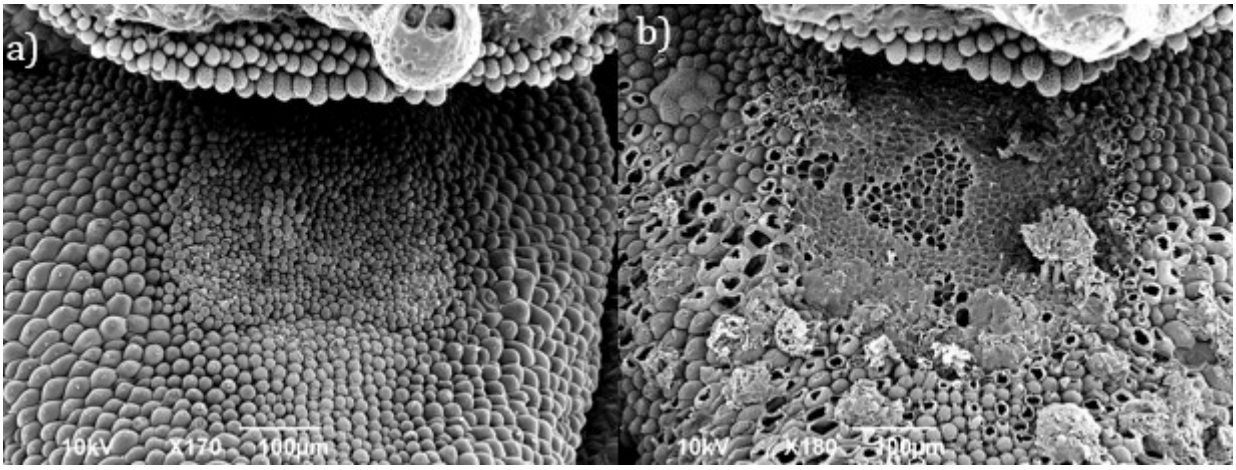

Figure 24. SEM photo comparison of *Pleurothallis sp. nov.* "Panama" glenion area
a) glenion of specimen 4 b) glenion of specimen 3

Figure 25 shows details of conglomerates and broken cells. As shown in Figure 25a, conglomerates are randomly distributed and broken cells are cluster distributed. Figure 25b shows a detailed photo of one conglomerate and it is debris of dried liquid mixed with cell tissues. It is hypothesized that these cell tissues are from the broken cells on the lip or from the glenion area and when the stuck conglomerates were removed in alcohol during transportation or during dehydration process, the conglomerates broke off the top of lip cells, leaving the broken cells uncovered.

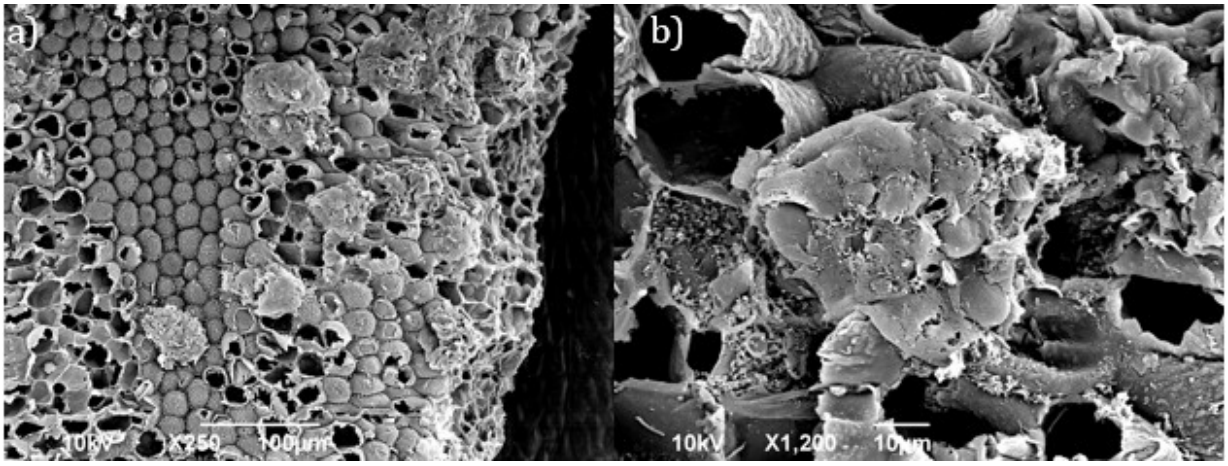

Figure 25. SEM photos of *Pleurothallis sp. nov.* "Panama"
a) part of lip with conglomerates and broken cells b) detailed conglomerate

III. Phylogenetics

ITS

Maximum Parsimony (MP) analysis (Figure 26) with 1000 bootstrap replicates was conducted with all the previously available ITS sequence data and new sequences generated in this study (Table 2) for species in taxonomic groups *Ancipitia* and *Scopula* (marked as *Colombiana* is Figures 26-28 due to name changing issues), a total of 62 taxa with 49 taxa in *Ancipitia* group and 13 in *Scopula* group. All *Scopula* species form a monophyletic group marked as clade 4 with a relatively low bootstrap support of 60%. Species in the *Ancipitia* group are grouped into three different monophyletic clades, clades 1, 2 and 3. Clades 2 and 3 are sister clades with a low bootstrap support of 36% and *Scopula* clade 4 is sister to *Ancipitia* clades 2 and 3 with a relatively high 93% bootstrap support. *Ancipitia* clade 1 is sister to *Scopula* and the rest of the *Ancipitia* group.

A Maximum Likelihood (ML) analysis (Figure 27) with 1000 bootstrap replicates was generated with the same data. Comparing to the MP phylogeny, the ML phylogeny has

more polytomies but the bootstrap support values are higher. Similar to the MP phylogeny, all species in *Scopula* form a monophyletic clade in the ML tree, also labeled as clade 4. The *Ancipitia* species in clade 1 on the MP phylogeny are again grouped as a monophyletic clade in the ML tree with a slightly higher bootstrap support of 77%. The rest of the *Ancipitia* group lie in a polytomy with the monophyletic *Scopula* clade 4. The monophyletic *Ancipitia* clade 1 is sister to the polytomy of *Scopula* and the rest of the *Ancipitia* group.

Species hypothesized to be pollinated by pseudocopulation are marked in green in Figures 26 and 27, and they are separately distributed in all three *Ancipitia* clades (clades 1, 2 and 3 in Figure 26 and clades 1 and 2&3 in Figure 27), suggesting that pseudocopulation evolved from ancestral reward pollination and that pseudocopulation evolved separately in *Ancipitia* more than once.

3' *ycf1*

Maximum Parsimony analysis (Figure 28) with 1000 bootstrap replicates was conducted with 3' *ycf*1sequence of 38 taxa, including species from subgenera *Ancipitia*, *Pleurothallis*, *Elongatia*, *Lalexia* and *Talpinaria*. Species from *P. crocodiliceps* group are included under subgenus *Ancipitia*. As shown in Figure 28, all *Ancipitia* species form a monophyletic clade A with a high bootstrap support of 98%. The *Ancipitia* clade is sister to the monophyletic clade of subgenus *Pleurothallis* and the two clades are further sister to subsection *Acroniae* with a high bootstrap support of 95%, in clade B.

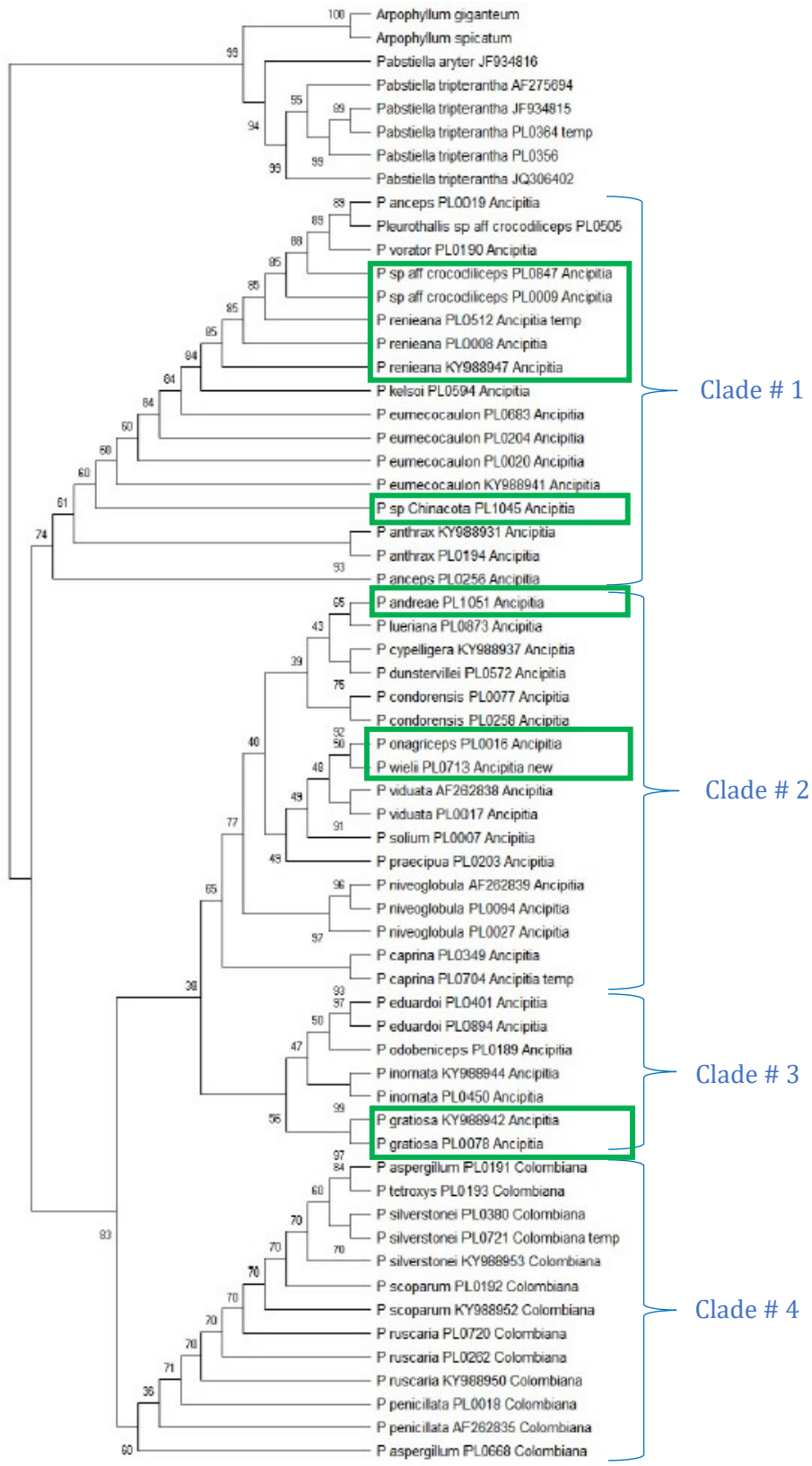

Figure 26. ITS Maximum Parsimony phylogeny of *Ancipitia* and *Scopula* groups at 1000 bootstrap

Species in subgenus *Ancipitia* are grouped into three monophyletic clades, clades 1-3. All species in subgenus *Scopula* are grouped into one monophyletic clade 4. Species marked in green are hypothesized to be pollinated by pseudocopulation.

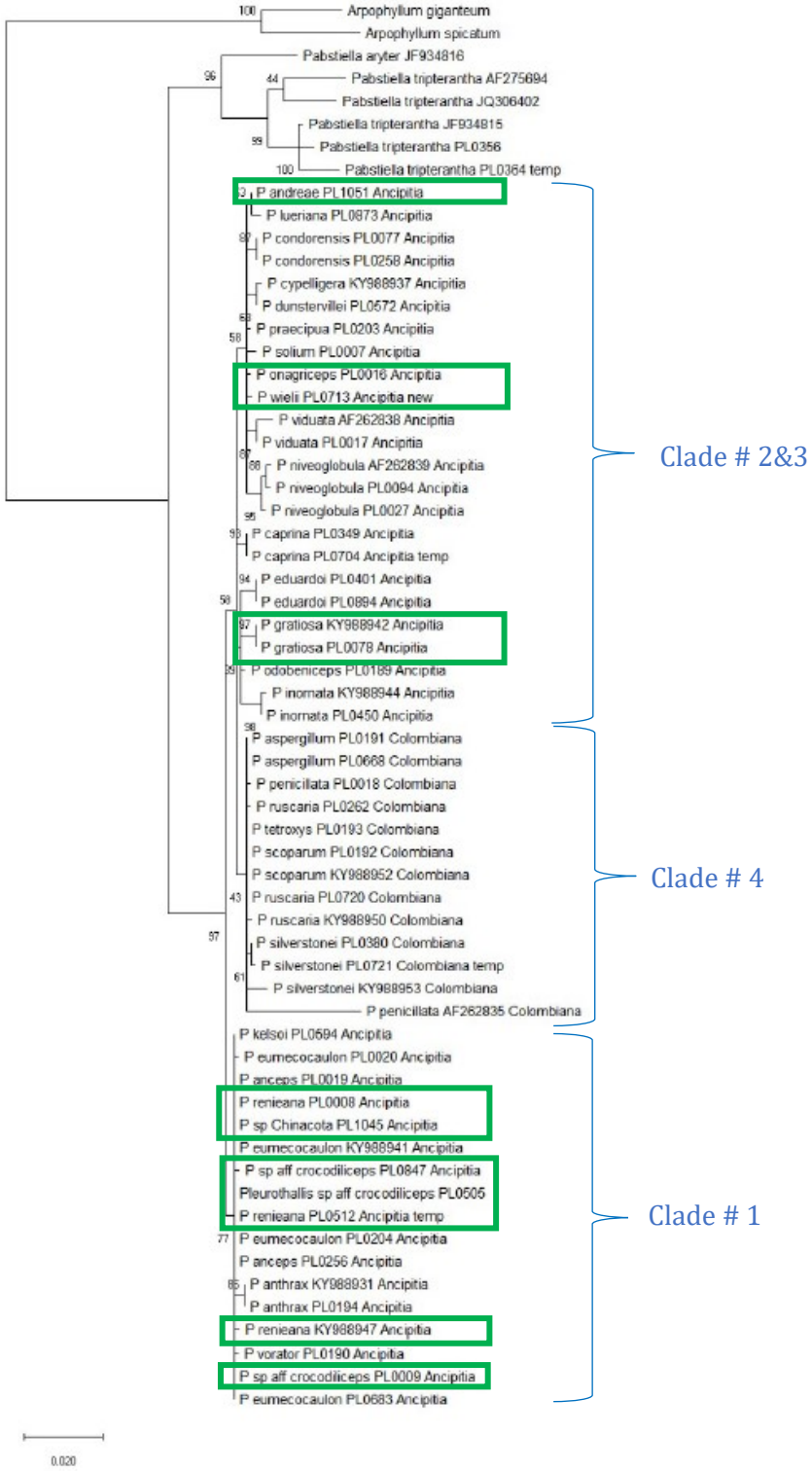

Figure 27. ITS Maximum Likelihood phylogeny of *Ancipitia* and *Scopula* groups at 1000 bootstrap

All species in subgenus *Scopula* are grouped into one monophyletic clade 4. Species in subgenus *Ancipitia* clade 1 in the MP phylogeny are again grouped in a monophyletic clade 1. The rest of the *Ancipitia* species in clades 2 and 3 in MP phylogeny lie in a polytomy with *Scopula* clade 4 and are marked as clade 2&3 in this ML phylogeny. Species marked in green are hypothesized to be pollinated by pseudocopulation.

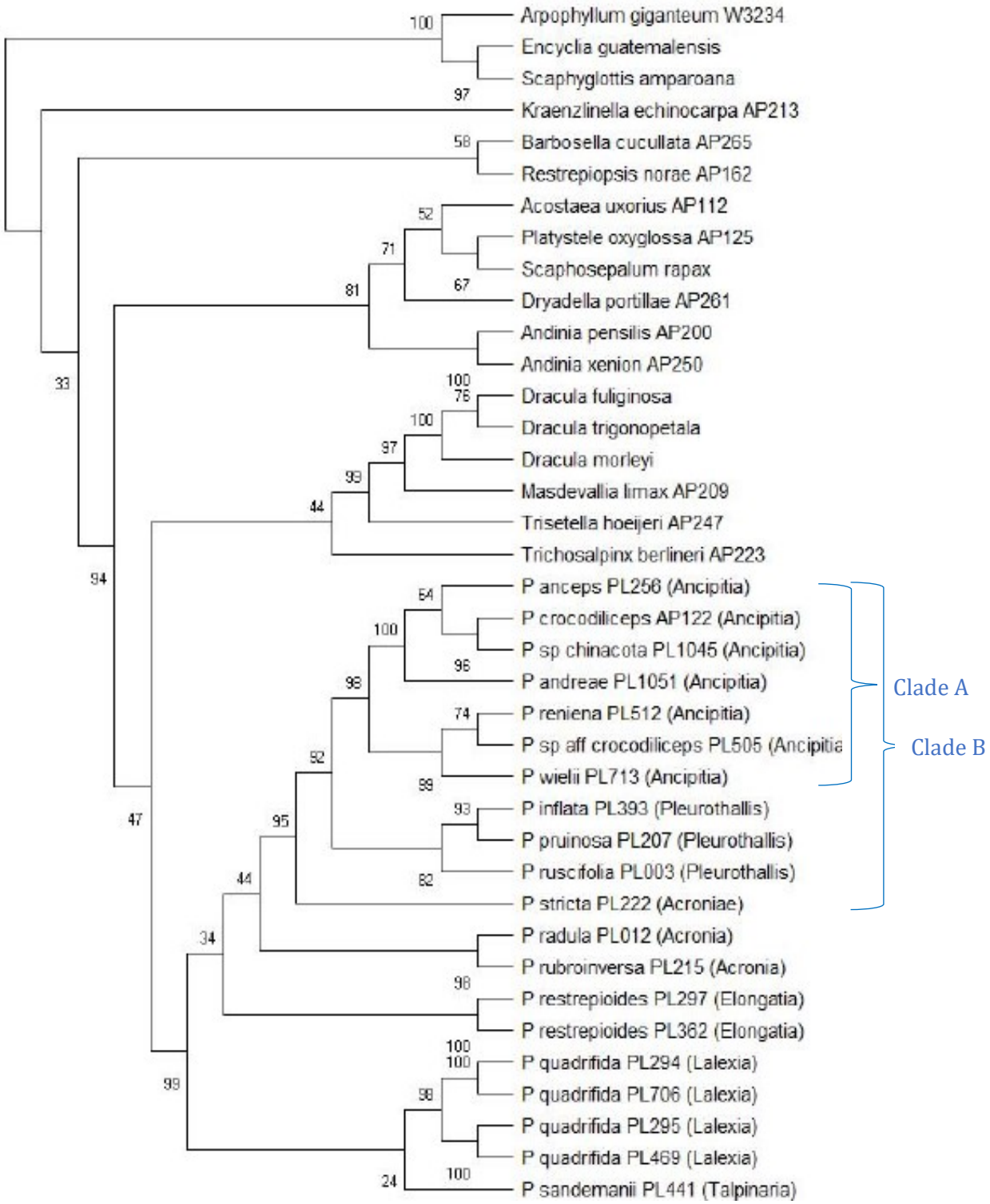

Figure 28. Maximum Parsimony phylogeny of 3' *ycf1* sequence for subgenera in genus *Pleurothallis* at 1000 bootstrap

Species from the *P. crocodiliceps* group in subgenus *Ancipitia* are grouped into a monophyletic clade A, sister to the other monophyletic clade of species from subgenus *Pleurothallis*, together forming a monophyletic clade B with one species *P. stricta* from subsection *Acroniae*. Clade B is further sister to other subgenera in genus *Pleurothallis*, with subgenera labeled for each species.

Discussion

I. Morphology

Hypothesized Reward Pollination

The data suggest that the glenion secretes a nectar-like liquid and plays an important role in reward pollination mechanism. The "nectar" produced from the glenion flows out toward the lip tip by gravity or capillary action and usually there is a sulcus where the "nectar" flows and accumulates. A pollinator is hypothesized to be attracted by the "nectar" and lands on the lip. It moves closer to the lip base as it follows where the "nectar" comes from and when it tries to reach the glenion, its back or thorax touches the sticky viscidium connected with pollinarium at the tip of the column. Thus when the pollinator leaves the flower it takes the pollinarium with it and when it lands on another flower and approaches the glenion again, the pollinarium on its back or thorax will get attached to the stigmatic surface on the column.

In this research dried liquid was discovered on many species with typical lips, such as *P. colossus* (Figure 5), *Pleurothallis sp. nov.* "Culpameae" (Figure 7b) and *P. acutipetala* (Figure 8c). These SEM photos are evidence for glenion secretion. In the other species with typical lips (*P. correllii, P. luctuosa, P. gorgonaensis* and *P. quadrifida*), though dried liquid was not found, a clear glenion with cells different from surrounding cells always existed. These glenion cells are usually smaller in size and have larger space between each other, creating space for secretion. Comparing the glenion photos of two *P. acutipetala* specimens (Figure 8b & c), the nectar-like liquid only covers the glenion cells, again supporting the hypothesis that glenion is the tissue that secretes the nectar-like liquid.

Though the composition of these liquids is still unknown, Mojica et al. (2018) detected a 13% sugar concentration in nectar of *P. coriacardia*, suggesting that the nectar-like liquid secreted by the glenion of *Pleurothallis* species also contains sugar. More composition analysis of other *Pleurothallis* species will be needed to support this hypothesis.

Hypothesized pseudocopulation

Long papillae and an apical cavity are suggested to be the two major characteristics of species hypothesized to be pollinated via pseudocopulation. The apical cavity mimics the copulatory channel of females of the pollinator species and the long papillae help to imitate female texture and may act as osmophores to release volatile chemicals (Blanco & Barboza 2005; Karremans et al. 2015; Policha et al. 2016) which resembles the sexual pheromones. When a male pollinator is attracted to the lip, thinking it is a female, it tries to copulate with the lip through the apical cavity. In this process the male pollinator's thorax or scutellum touches the viscidium and like reward pollination, it brings the viscidium and the connected pollinarium to the next flower where the pollinarium gets attached to the stigma surface.

In this research the apical cavities of *P. nelsonii* and *P. wielii* from the *P. crocodiliceps* group were further examined to reveal the inside. The lip of *P. wielii* was vertically sectioned to give a side view of the inside of the apical cavity (Figure 18) and the lip of *P. nelsonii* was split vertically in half with a scalpel blade (Figure 17b). Though the lips were treated differently, the photos give similar results: a deep channel with short papillae.

Further examination of the inner cavity is needed but with the current similarity between these two species it is hypothesized that other species in the *P. crocodiliceps* group also have such channels. In addition, the depth and the papillae all over the inside of the channel suggest that the channel plays an important role in the pseudocopulation mechanism. Instead of just being a shallow hole to mimic the morphology of females of the pollinator species, it is hypothesized that the channel also mimics the function of copulatory channel of the females and the male pollinator will insert its abdomen into the channel while pollinating. Field observations will be needed to support this hypothesis but if confirmed this will be the first case of genitalic pseudocopulation with an actual copulation phase in *Pleurothallis*, which in subtribe Pleurothallidinae has only been observed in genera *Lepanthes* and *Andinia* (Blanco and Barboza 2005).

Besides mimicking the appearance of female pollinator species, the long papillae are hypothesized to serve as osmophores. For example, in *Lepanthes*, the papillae osmophores located at the lip blade surface release volatile chemicals such as heptadecane and other long-chain saturated hydrocarbons which mimic the sexual pheromones of the pollinator species to attract male pollinators (Blanco & Barboza 2005). In this research, long papillae were discovered on lips of *P. minutilabia* (Figure 8), and all species in the *P. crocodiliceps* group (Figures 17-21). The papillae here are also hypothesized to release volatile chemicals and more analysis of the chemical composition will be needed to confirm this.

The osmophores are not limited to the lip but can also be located on sepals and petals and therefore flower tissues other than the lip also worth concerning. Despite the osmophores located at sepals and petals, *Specklinia* also have nectar secreted on sepals

with high sugar content (Karremans et al. 2015). The color and pattern of the calyx of *Dracula* influence pollinator landing (Policha et al. 2016), the sepals trigger aggregation and courtship and the petals trigger mating behaviors which will prompt the pollinators to approach the columnar chamber where they are trapped to pollinate (Endara et al. 2010). Droplets of nectar-like liquid have been discovered on petals of *P. colossus* to help attract pollinators (Calderon-Saenz 2011).

In addition to the lip, petals were also examined in this research to suggest possible pollination mechanisms. Dried liquid was discovered on the petals of *P. aff. nelsonii* "Caqueta" (Figure 29). The cross-section of the *P. aff. nelsonii* "Caqueta" petal has a triangular shape and with the current specimen liquid was found on more than one side. A detailed SEM photo of the dried liquid and the cells secreting it (Figure 29b) gives a unique cell type. These cells have a shape of "shark teeth", with clear edges and pointy tips. The petals were dehydrated and critical point dried with other flower tissues and the lip cells (Figure 20) do not have pointy tips, suggesting that the "shark teeth" cells on petals are not due to preservation but instead a new cell type. The liquid composition is not analyzed so whether this is sugar-containing nectar or other secretions is still unknown. Further analysis of the secretion composition will be needed to determine its function. *P. aff. nelsonii* "Caqueta" has atypical lips without a glenion that secretes "nectar" and therefore the presence of secretion at petals plays an important role in pollinator attraction. More specimens will be needed to determine whether such secretion only exists on one side of the petal or all three sides, which will help to reveal the specific pollination mechanism of this species.

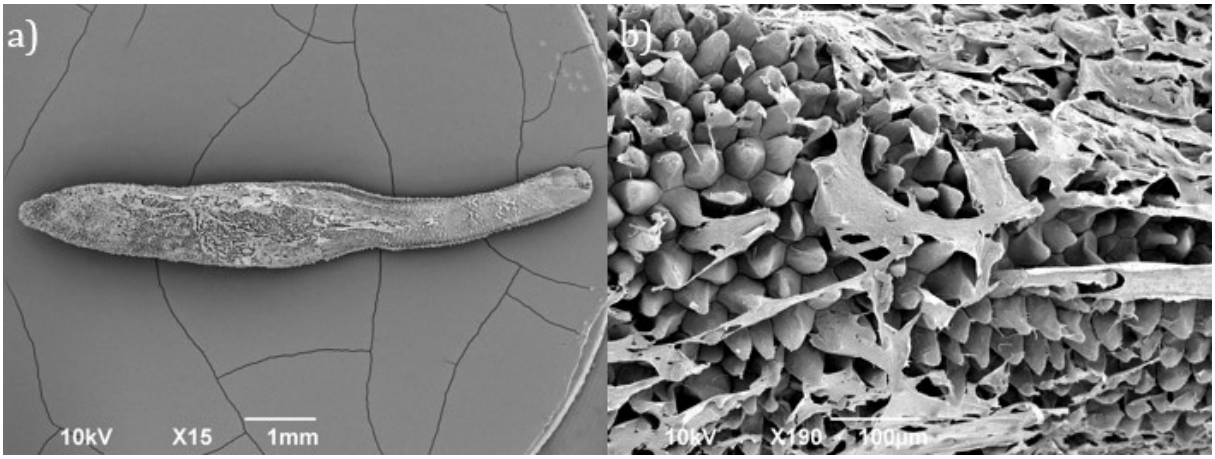

Figure 29. SEM photos of *Pleurothallis aff. nelsonii* "Caqueta" petals

a) Whole petal with inner side up b) detailed dried liquid and "shark teeth" cells

Besides *P. aff. nelsonii* "Caqueta", *P. colossus* also has unique petals. A close view of the lip tip (Figure 30a) shows three different types of cells, cells on the edge and cells on the two sides. Compared to the cells on side surfaces, the edge cells are smaller in size and a close examination of these edge cells (Figure 30c) shows evidence for dried liquid existence, suggesting liquid secretion by these edge cells. The cells on the underside are larger, round, full and smooth while the cells on upperside are rough and have more desiccation damage. This difference could be due to preservation so more specimens will be needed to check whether these two sides have different types of cells and their function if the difference does exist. From the current SEM photos there is no evidence for liquid presence on the side cells. *P. colossus* has a typical lip with a glenion secreting nectar-like liquid and thus the functions of the different cell types and secretion at petals are unknown. More specimens will be needed to confirm whether liquid secretion does exist and composition analysis will be helpful to determine its function in pollination.

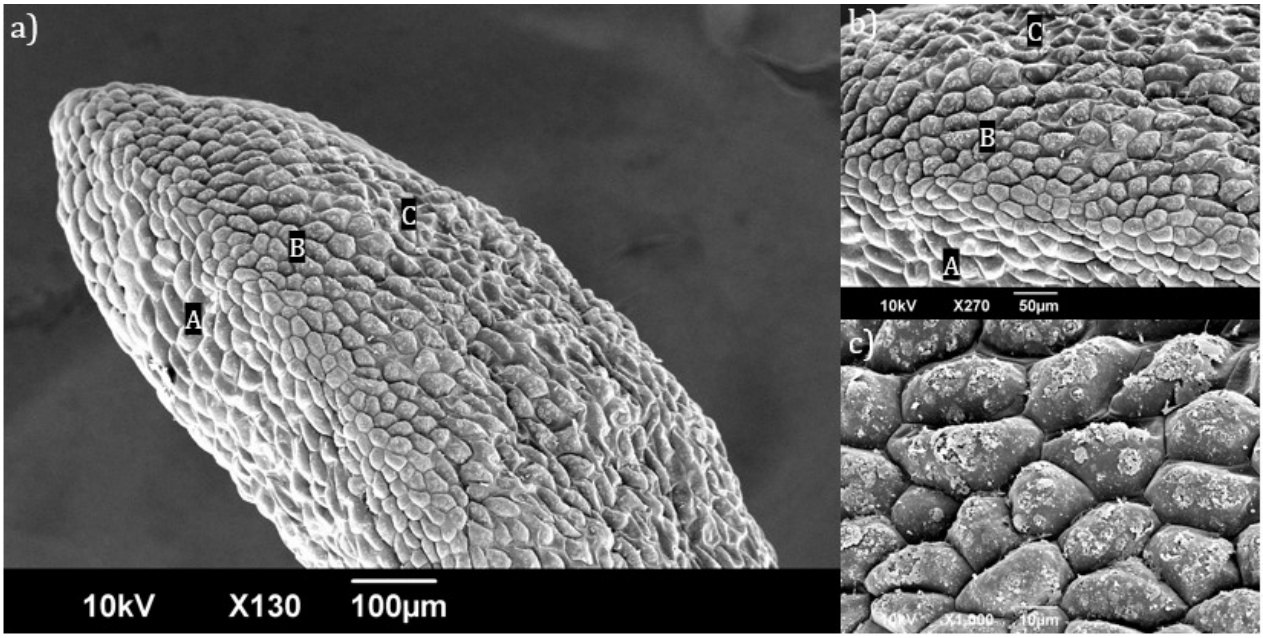

Figure 30. SEM photos of *Pleurothallis colossus* petal

a) detailed petal tip A. lower surface cells B. edge cells C. upper surface cells
b) detailed three types of cells c) detailed edge cells with hypothesized dried liquid

Species in the *P. crocodiliceps* group used to be described as one species but later discovered to be multiple different species (Wilson 2017). As described in results, species in this group all have two forward-pointing horns, one apical cavity and long papillae while details such as lip shape, papillae distribution and horn morphology differ between species. Figure 29 compiles all the lip-only SEM photos of the *P. crocodiliceps* group species. Regarding lip shape, *P. nelsonii* (Figure 31a), *P. aff. nelsonii* "Caqueta" (Figure 31b) and *P. aff. nelsonii* "Orquideas Katia" (Figure 31c) are more similar to each other than with the other two species. All three lips have a triangular shape with a wide lip base and a pointy lip tip where the apical cavity is located. The two horns are also wide at base and get pointier at tip. Long papillae exist at the edges of the lip, horns and especially around the apical cavity. According to the sectioned and detailed cavity photos, papillae continue to exist inside the cavity. These similarities suggest that these three species use similar pollination mechanisms.

In contrast, *Pleurothallis sp. nov.* "Romeral" (Figure 31d) has different papillae distribution. Though its lip also has a triangular shape, long papillae only exist around the edges of the wide lip base area and on the horns. Few papillae are found around and inside the apical cavity, suggesting that *P. sp. nov.* "Romeral" attracts a different pollinator species whose female is less hairy or *P. sp. nov.* "Romeral" employs a different pollination mechanism where the copulatory channel serves a different function.

Pleurothallis onagriceps (Figure 31e) also has long papillae all around the lip edges, the apical cavity and all over the two horns. It mainly differs from the other species with its round shape and especially wide and flat horns. The function of these horns in pollination is still unknown but such unique horns suggest a different pollinator species with different size or unique copulation habits.

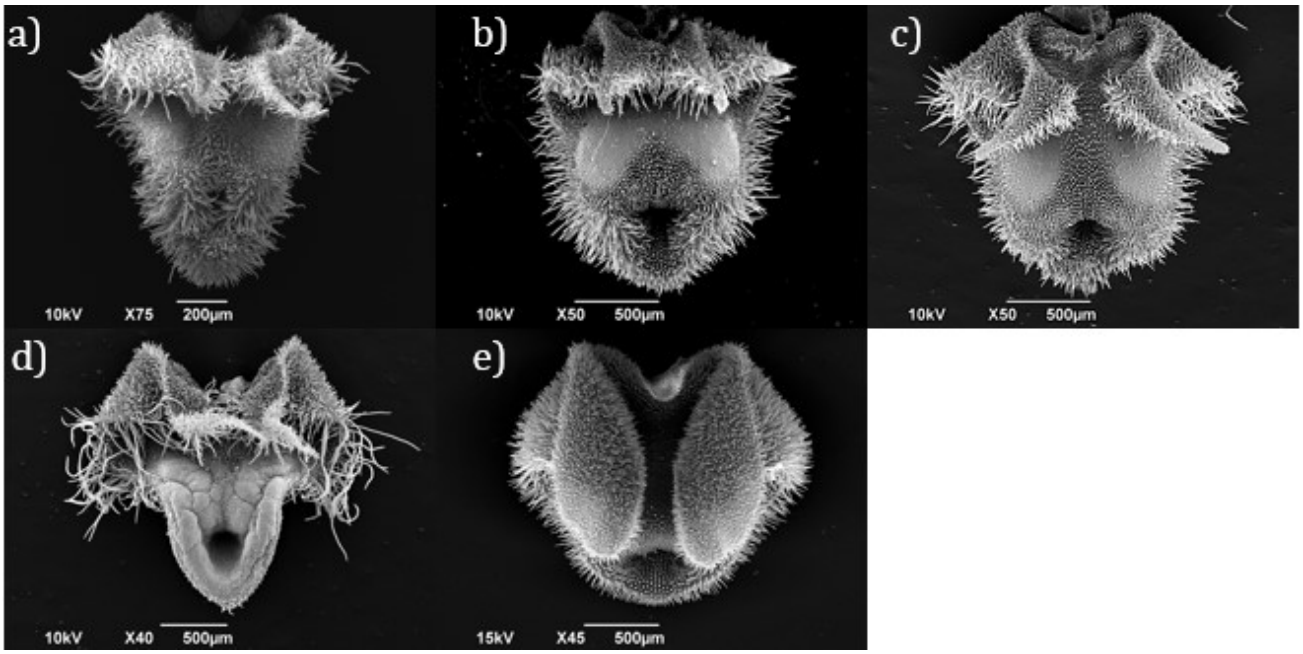

Figure 31. SEM photo comparison of lips in *Pleurothallis crocodiliceps* group
a) *P. nelsonii* b) *P. aff. nelsonii* "Caqueta" c) *P. aff. nelsonii* "Orquideas Katia"
d) *P. sp. nov.* "Romeral" e) *P. onagriceps*

Pleurothallis sp. nov. "Panama" is also a unique species with an atypical lip. Regarding the lip morphology, it should be treated as a typical lip since it has a glenion area (Figures

23a & 24a). However, specimens harvested at different stages of anthesis give different images under SEM (Figures 23-25). Under naked eyes, the *Pleurothallis sp. nov.* "Panama" lip has black secretions on the glenion area at early anthesis stage. The black secretion splits into small black balls and are randomly distributed all over the lip as time goes and the glenion has no black secretion left. With this discovery, it is hypothesized that the conglomerates in Figure 25 are the black balls. Since the conglomerates contain dried liquid and cell tissues, it is hypothesized that the glenion secretes a sticky liquid which brings off the top of glenion cells and splits into small black balls and spread over the lip when the flower gets more mature. Further evidence will be needed to support the correlation between anthesis stage and conditions of glenion and lip cells. Liquid composition also needs further analysis to determine if it is able to bring off cell tissues to form conglomerates. The purpose of splitting glenion secretion into small balls to spread over the lip also worth further research. If the glenion secretion is also a reward or it attracts pollinators in other ways such as color difference, then one further question is why there is no secretion left at the glenion area which means the pollinator will not be guided to the lip base area to touch the column.

II. Phylogenetics

While the phylogenetic analysis was not a focus of this study, in the process of updating phylogenetic trees some new observations were made following on the work of Wilson et al. (2015) and Dupree (2016).

Comparing the two ITS phylogenies generated with Maximum Parsimony and

Maximum Likelihood analyses, both have relatively low resolution shown as the low bootstrap support in the MP phylogeny (Figure 26) and the large number of polytomies in the ML phylogeny (Figure 27). According to the MP phylogeny (Figure 26) *Ancipitia* clades 2 and 3 are sister clades with low bootstrap support at most of the nodes while in the ML phylogeny (Figure 27) places the two clades into a polytomy with the monophyletic *Scopula* clade 4. Such a conflict could be explained by the more complex calculations of the Maximum Likelihood analysis which converts the low supported bifurcating phylogeny generated by the Maximum Parsimony analysis into the polytomy with higher bootstrap support.

According to the MP phylogeny (Figure 26), the 12 taxa hypothesized to be using pseudocopulation (marked in green) are separately distributed in three different clades, *Ancipitia* clades 1, 2 and 3. While in the ML phylogeny (Figure 27), these 12 taxa are distributed in two different clades, *Ancipitia* clades 1 and 2&3. Based on the difference in analysis complexity, both phylogenies suggest that peusodocopulation is evolved from ancestral reward pollination and evolved at least twice in subgenus *Ancipitia*. To determine exactly how many times pseudocopulation evolved separately, a phylogeny with higher resolution will be needed. To improve resolution, more complex analyses such as Bayesian Inference analysis and more Sanger sequencings of more gene loci for more species can be included in further research and Next Generation Sequencing can be a possible future direction.

Both MP and ML phylogenies place *Ancipitia* clade 1 as a monophyletic clade sister to the larger clade of monophyletic *Scopula* clade 4 and the rest of *Ancipitia* group (clades 2

and 3), suggesting that subgenus *Ancipitia* is paraphyletic and there should be a name change to remain monophyletic taxonomy. The sister relationship between *Scopula* clade 4 and *Ancipitia* clades 2 and 3 receives a high bootstrap support of 93% in the MP phylogeny (Figure 26) while the polytomy with *Scopula* clade 4 and *Ancipitia* clades 2&3 has a low support of 58% in the ML phylogeny (Figure 27). Therefore an improved phylogeny with higher resolution can also resolve the conflict and act as evidence for the name change of *Ancipitia* and *Scopula*.

Surprisingly, species in the *P. crocodiliceps* group are separately distributed in two clades, *Ancipitia* clades 1 and 2 in both MP and ML phylogenies. Species in this group used to be described as one species, *P. crocodiliceps*, due to their similar morphology but were later discovered to be different species (Wilson et al. 2017b). These morphologically highly similar species (Figure 31) are expected to be genetically closely related, but according to both ITS phylogenies, *Ancipitia* clade 2 is more closely related to *Scopula* clade 4 than to *Ancipitia* clade 1. Most speciation events in *Pleurothallis* genus occurred recently (Pérez-Escobar et al. 2017) and ITS sequence is not variable enough to differentiate these species.

To better differentiate these species, 3' *ycf1* gene was sequenced and a Maximum Parsimony phylogeny (Figure 28) was generated with species from subgenera *Pleurothallis*, *Elongatia*, *Lalexia* and *Talpinaria* where the species from *P. crocodiliceps* group are included in subgenus *Ancipitia*. All species from the *P. crocodiliceps* group are grouped in a monophyletic clade with a high 98% bootstrap support, supporting the close relationship between these species. The seven taxa are further grouped into two

subclades (Figure 28), with *P. wielii*, *P. renieana* and *P. sp. aff. crocodiliceps* PL505 as a subclade and the other species in the other subclade. There is a conflict with the grouping in ITS phylogenies where *P. wielii* is grouped with *P. andreae* and *P. onagriceps* in *Ancipitia* clade 2 (Figure 26). Such a conflict could arise due to different evolutionary histories of nuclear and plastid genes. To better determine the relationship between species in the *P. crocodiliceps* group, more samplings at more gene loci will be needed. Considering the difficulty in amplifying 3' *ycf1* in some species resulting in the sampling limitation, future projects could use other plastid regions such as *trnL-F* and *trnH-psbA* (Wilson 2017) and other more variable protein-coding sequences such as *rps*16, *ndh*C, *ndh*K, *rpl*22, *ndh*F, *rpl*32, and *ndh*I (Dong et al. 2018). More species in the *Ancipitia* subgenus should also be included in sampling to better reveal the evolutionary pattern of such a unique type of lip morphology.

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