Morphologically determining pollination strategies in Pleurothallis with an emphasis on rewards involving the glenion and other labellar secretory tissue

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Abstract

Pleurothallis is a large genus of myophilous Neotropical orchids with diverse pollination syndromes including both rewarding and deceptive species. SEM was used to analyze the micromorphology of the labellum of several species in Pleurothallis. Physical characteristics such as the size of the labellum, the presence or absence of dried liquid, a sulcus, an apical cavity, or hypochilar projections were used to infer pollination strategies. New ITS sequences were added to existing phylogenetic trees of Pleurothallis subsections to elucidate interspecific phylogenetic relationships. This study specifically documents species with a glenion, and phylogenetically catalogues the glenion in Pleurothallis subsections Ancipitia, Acroniae, and Macrophyllae-Fasciculatae. This study also introduces a previously undescribed tissue type, that we propose calling rugose secretory tissue, that occurs in Pleurothallis subsections Restrepioidia and Elongatia. Preliminary LC-MS data definitively identify large amounts of sugar on the flowers of several Pleurothallis species, strongly suggesting the presence of a nectar reward.

Introduction

Background on orchid pollination

Orchidaceae is one of the largest flowering plant families, including between 25,000 and 30,000 species, and is known for housing an incredible array of plants with intriguing morphologies and adaptive features (Siegel, 2013; Ray and Vendrame, 2015). Genetic evidence suggests orchids are an ancient family that diverged from other plant groups about 76-84 million years ago and began rapidly diversifying during the Cretaceous-Paleogene boundary 65 million years ago (Ramírez et al., 2007). While orchids exhibit an impressive range of morphologies, most can be distinguished by structures and pollination strategies unique among plant families (Nilsson, 1992).

Orchid flowers are zygomorphic (bilaterally symmetric) and generally resupinate (oriented so that the labellum is lowermost), so they can be visualized using the points of a compass. They have three sepals, one dorsal in the "northern" direction, and two lateral pointing "southeast" and "southwest". They have two identical petals in the "northeast" and "northwest" quadrants. The third southward petal is called the labellum, which is highly modified. At the center is a structure called the gynostemium or column, which consists of the combined male and female sexual organs. The column varies significantly in size and shape but typically protrudes in a rod-like manner. On the apex or underside of the column is the stigmatic surface, which takes the form of a shallow depression (Cozzolino and Widmer, 2005). At the tip of the column is a structure called the pollinarium. A pollinarium is constructed of two pollen sacs called pollinia that contain all of the pollen grains from a single flower. Extending from the pollinia is a filamentous structure called the stipe or caudicle that during pollination gets glued to a pollinator with an adhesive viscidium (Nilsson, 1992; Hapeman and Inoue, 1997).

The pollinarium as a botanical structure is nearly ubiquitous among orchids, and is only documented in one other plant family. The pollinarium is central to the pollination strategies of orchids because it facilitates efficient pollen transfer between flowers (Nilsson, 1992). The specific and consistent placement of the pollinia on the pollinator is a crucial function of the floral structures and can act as an important barrier to cross-species hybridization. When orchid pollinia are confined to distinct pollinator body parts, pollination between different orchid species becomes less likely or impossible (Ray and Vendrame, 2015). This is possible because many pollinaria have

evolved uniquely for pollinators such that the viscidium will only bind to the appropriate location on the pollinator. This creates a method of distinguishing between a pollinator's body surfaces. Common placements for pollinia on insects are the eyes, legs, scutellum (a triangular plate on the thorax), and proboscis (Hapeman and Inoue, 1997).

Orchid diversity and complexity are inextricably linked to the mechanisms by which they are pollinated, and the study of orchid pollination provides insight into the process of natural selection (Dodson, 1962; Hapeman and Inoue, 1997). Pollination in orchids is almost universally dependent on external pollen vectors (Papadopulos et al., 2013). In general, pollination by animals is a mutually beneficial arrangement, wherein the plants entice pollinators with beneficial substances, or rewards, and in the process of collecting these rewards in multiple flowers, pollen transfer occurs (Dodson, 1962). This process is known as reward pollination and is relatively straightforward, the more attractive the plants are to their pollinators, the more frequently they will be successfully pollinated. On an ecosystem level, pollinators and the species they pollinate support each other by mutually increasing the reproductive fitness of the other. Orchids, however, often exist in very low densities and even generously rewarding species may not be common enough to be the sole food source for a species of pollinator (Ackerman, 1986). One hypothesis postulates that this inequality between orchids and their pollinators is the key to understanding orchid variation. Orchids are often entirely reliant on their pollinators for reproductive success, their pollinators are generally not dependent on pollination rewards supplied by orchids to survive. This asymmetry could be partly responsible for a range of pollination strategies that do not supply a food reward and instead employ complicated mechanisms not observed in more abundant plant species (Nilsson, 1992; Scheistl, 2005).

Species of orchids that do not provide rewards, but are still pollinated by animals seeking a food source or reproductive advantage are considered to be deceit pollinated. Deceit pollination has evolved several times and is presumed to occur in one-third of all orchid species, which is much higher than in any other family of plants. There are several advantages and disadvantages to deceit pollination, and no one universal evolutionary strategy has been identified (Ackerman, 1986).

One hypothesis about the evolution of deceit pollination is called the resource-limitation hypothesis and is based on the assumption that reproductive success is sometimes limited by resource availability. In a situation where orchid reproduction is based on pollinator visits, it is more beneficial to provide a reward, as rewarding orchids almost always have a higher fruit set (a positive measure of reproductive success) (Neiland and Wilcock, 1998). However, if an orchid is resource-limited and the production of a reward is energetically costly and thus would decrease fitness, deception allows resources to be diverted into seed production. This hypothesis is subject to considerable debate, and one argument against it is that when deceitful orchids are pollinated experimentally, orchids do produce more fruits than in natural settings, meaning they have the resources to do so and can be considered pollinator-limited. However, this may not take into account the lifetime reproductive success of an organism, upon which natural selection acts. The seed viability, number of seeds, and reproductive success in subsequent years after a high fruiting season are all important variables that are not often analyzed in concert (Zimmerman and Aide, 1989).

Another hypothesized evolutionary benefit of deceit pollination is an increase in cross-pollination. Often, pollinating insects realize quickly there is no reward to be had on a deceitful flower and leave immediately. This is in stark contrast to rewarding flowers, where a pollinator may take several minutes collecting a reward, only succeeding in moving pollinaria between

different flowers on the same inflorescence, a behavior called geitonogamy. Self-pollination and geitonogamy predispose offspring to inbreeding depression and decrease viability. There are many ways deceptive and rewarding orchids attempt to prevent self-pollination or autogamy and the resulting inbreeding depression. The rostellum is a floral structure that reduces autogamy before fertilization by covering the stigmatic surface and blocking pollinarium deposition for a certain period of time after the pollinarium is discharged (Meser et al., 1980). The stipe of the pollinarium may also have a delayed bending effect, that would move the pollinia into a more ideal position for fertilization, but not until several minutes after being attached to a pollinator when it has likely moved onto another plant (Dodson, 1962). Additionally, some orchids are self-incompatible even after fertilization, and in these species the fruit may be aborted in the early stages of development (Gontijo et al., 2010). Regardless of the mechanisms of prevention, the less time a pollinator spends on an inflorescence, the lower the probability of geitonogamy occurring. This would mean a lower fruit set in deceitful species may not be a problem if the overall number of viable seeds is comparable (Peakall and Beattie, 1996; Scheistl, 2005).

The cross-pollination hypothesis for deceit in orchids also has its issues. While the incidence of cross-pollination is likely higher among deceitful orchids compared with rewarding orchids, the connection between cross-pollination and species success is not well defined. In an evolutionary sense an increase in cross-pollination seems beneficial, but several species of orchids are partially or entirely autogamous and still seem successful. Some species even partially or entirely reproduce through self-pollination in closed flowers, cleistogamy, and do not produce open flowers that can be pollinated externally. This may seem to be an evolutionary dead-end, but it can also be considered a unique adaptation permitting orchids to live in environments without insect pollinators, such as in very dry locations or at extreme elevations (Wang et al., 2019).

The evolution of deceit pollination in orchids may be difficult to explain because of problems in the mechanisms by which reproductive success is measured. High fruit set for example may not be entirely advantageous because fruit density could be correlated with fruit predation, lowering the effective reproductive success (Ackerman, 1986). Indeed, fruit set, which effectively measures successful pollinations, may be erroneously conflated with reproductive success. The ultimate goal of orchid reproduction is actually to maximize healthy seed production, which is typically much higher per fruit in deceptive species. When this is the case, if pollinator interest is equal in two deceptive orchids, the most represented genotypes are those that promote high viable seed content per capsule (Pérez-Hérnandez et al., 2011).

The balance of reward and deceit pollination in orchids becomes even more complicated on the ecosystem level because the propagation of deceitful orchids are themselves their own worst enemy. Deceitful orchid species are subject to negative frequency-dependent selection (increased reproductive success of rare individuals). As the deceit pollination strategy increases in prevalence, the reproductive success of those individuals decreases, because pollinators are less inclined to visit a flower if many like it have been rewardless (Schiestl, 2005). In most areas, orchids exist in such low densities this may not be a problem, but genetic evidence does show some species have evolved rewards whose ancestors have been historically deceitful, providing evidence that production of rewards might be more evolutionarily labile than previously thought (Johnson et al., 2013).

In light of the extreme complexity of orchid-pollinator interactions at the ecosystem level, many studies focus on describing and interpreting the pollination and lifecycle of individual orchid species. Considering the diversity of orchid pollination strategies and morphologies even within taxonomic subsections, generalization at the genus level is inappropriate and species-specific

accounts are still of great importance. Unfortunately, one of the major impediments to creating detailed accounts of orchid-pollinator interactions for a large number of species is that it is essentially impossible.

While the direct observation of pollination in nature is the only definitive way to identify a pollinator, field studies can take several years of observations only to witness and record pollination of a few species, and even then, the chance of missing important pollinators or misidentifying random visitors as pollinators is a possibility (Dodson, 1962). Especially in the Neotropics, with thousands of known orchid species, and many undescribed species, field observation of pollination would be tedious, expensive, and unrealistic. In these circumstances orchid flower morphology can be used to suggest pollination syndromes.

Reward and Deceit Pollination Strategies

By far the most common and most successful floral reward for pollinators is nectar. Nectar can be loosely classified as any floral secretion with a high sugar content (Castro and Singer, 2019). Sugar provides a vital energy source that pollinating insects require for flight. Nectar can be located in small secreted droplets around the labellum, or in higher quantities at the base of the labellum to properly position pollinators to collect pollinia (de Melo and Borba, 2010; Borba and Semir, 2001). Due to the ubiquity of nectar demand, nectar rewards are not highly specific attractants and may be plundered by non-pollinating insects. Orchids have also been documented rewarding their pollinators with a wide array of substances in addition to nectar, including resins (Krahl et al., 2019), oils (Cane et al., 1983; Cocucci et al., 2000; Gomiz et al., 2017), waxes (Bronstein et al., 2013), pseudopollen (Davies et al., 2000), proteinaceous secretions (Bogarín et al., 2018), and fragrance molecules (Eltz et al., 2005; Ramírez et al., 2011; Hetherington-Rauth and Ramírez, 2016).

Deceit pollination inherently relies on the mimicry of providing some form of fitness advantage to the pollinator, the mimicked fitness advantage could be in the form of a food source, or it could be more directly linked to reproduction where the perceived fitness advantage is a mate or a brooding site. In general, if there is an orchid that produces a reward, there are also orchids that mimic producing that same reward. In addition to mimicking rewarding species, deceptive orchids may also exhibit structures or coloration that mimics aphid colonies, decaying leaves, carrion, mushrooms, or females of the pollinator species. Even with so much variety, the most common deceit mechanism employs the mimicry of nectar-rewarding flowers (Siegel, 2013).

Nectar deceit is a recurring pollination strategy, essentially involving the visitation and pollination of flowers presumed by pollinators to provide nectar which do not. Two specific types of nectar deceit mimicry include Batesian and generalized mimicry, each of which have distinct evolutionary outcomes. In Batesian mimicry, deceitful species replicate the scent, shape, and color of a specific, often non-orchid, rewarding model that occupies the same area and blooms concurrently. Success for the deceitful party depends on it being indistinguishable to the pollinator from the rewarding species, and natural selection would drive the deceitful species towards increased similarity with the model. In generalized mimicry, nectarless flowers do not resemble a specific model organism, and instead promote their potential as a nectar source through attractive scents, empty nectar spurs, and bright colors. In this situation, deception relies on the diverse and abundant community of rewarding flowers and exploits forgetful or inexperienced and curious pollinators. Polymorphism is common in generalized mimics and is believed to promote more rapid differentiation to mitigate the learning curve of the pollinator from preventing pollination

(Scheistl, 2005). The mechanism responsible for the enormous number of species that are pollinated through generalized deceit in orchids has yet to be experimentally determined. One issue in this group of orchids specifically is the incidence of shared pollinators is particularly high, which could further complicate species boundaries in sympatric communities. One hypothesized mechanism to allow for sympatric speciation in nectarless orchids is the differential attraction of pollinators through volatile odors. In many cases, however, generalized food deceptive orchids lack strong odors that could be easily recognized by potential pollinators. It may be more likely speciation occurs through postzygotic barriers (Cozollino and Widmer, 2005; Cozollino et al., 2005).

Hundreds of orchid species are pollinated by a form of deception involving the mimicry of female pollinators. This form of deceit pollination is called sexual deception and is exclusive to the family Orchidaceae. In this pollination strategy orchids attract male pollinators by producing volatile chemical blends evocative of sexual pheromones produced by females of a pollinator species. These attractive scents draw male pollinators to the orchid which may have a labellum mimicking the female insect. In some species, sexual deceit is so highly developed the orchid will be a convincing enough potential mate that the insect will display precopulatory or even copulatory behavior, and in doing so pollinates the orchid in a phenomenon called pseudocopulation (Siegel, 2013).

Pollination in Pleurothallidinae and Pleurothallis

Pleurothallis, the genus to be investigated in this study, belongs to the subtribe Pleurothallidinae, which currently includes over 5,100 recognized species, a number which increases annually with the addition of around 85-90 new species every year. Pleurothallidinae is a Neotropical subtribe with a geographic range from Florida to Brazil. This group currently contains 37 genera with a diverse collection of floral morphologies. The subtribe is circumscribed phylogenetically, but can be physically characterized by a lack of pseudobulbs and a characteristic articulation between the pedicel and the ovary. Pleurothallidinae is generally considered myophilous (pollinated by Diptera), but observations of pollination only include a fourth of the genera and 2% of the species. Some accounts have described hummingbirds as a pollinator of a few species, but these orchids do not exhibit any of the common characteristics of ornithophilous species and the birds are more likely just curious visitors and not effective pollinators (Karremans and Díaz-Morales, 2019).

It is theorized that Pleurothallidinae is descended from mellitophilous (bee pollinated) orchids and that myophily is a derived trait within the subtribe (Borba et al., 2011). Myophily has many important implications for the evolution and morphology of the species in this subtribe. Flies are behaviorally very different from other insect pollinators in the orders Hymenoptera and Lepidoptera. Historically, flies have been considered inefficient or haphazard in their pollination, but copious research has indicated the contrary. Flies are often reliable and specific pollinators that make up for their lack of efficiency with their abundance (Mesler et al., 1980). Flies are also present in many locations that do not support bees, notably at high altitudes where many Pleurothallidinae species are exclusively located (Montiero et al., 2021). The order Diptera currently contains 125,000 described species, but is hypothesized to include upwards of 800,000 (Siegel, 2016). Due to the immense number of potential fly pollinator species, divergence in orchid species that attracts new species of flies may partly account for the explosive speciation in this orchid subtribe.

Fly pollination in Pleurothallidinae has undoubtedly exerted selective pressure on floral morphology, but the old evolutionary age of Diptera suggests this is likely not a case of coevolution. In the transition to a new pollinator, orchids are likely exploiting preexisting fly behavior related to nectar foraging, brood site exploration, and the search for mates. Evolution of floral characteristics towards pollination by dipteran species with similar characteristics and behavior patterns has created a high degree of morphological similarities between distantly related genera, so-called convergent evolution. However, evolution towards pollination by different dipteran species, or even different behaviors associated with specific life stages can also lead to a high degree of variation within genera. While there exists a large diversity of pollination mechanisms in the subtribe, three main morphological groups have been proposed by Karremans and Díaz-Morales (2019) to describe the essential differences.

For the most ubiquitous mechanism of pollination in Pleurothallidinae they propose the name “masdevalliform”, after the genus Masdevallia, which has been observationally identified in eight genera and hypothesized to occur in nine others. In this pollination mechanism, flies are initially attracted by volatile chemicals emitted by sepalar osmophores (epidermal scent glands). They are then guided by papillae on the delicately hinged labellum leading towards the column. After passing a balance point, the labellum tilts, bringing the fly towards the column and the viscidium. In the process of exiting backwards, the pollinia are attached to the scutellum of the fly (Karremans and Díaz-Morales, 2019). This pollination mechanism was recently observed in the pollination of two species of the genus Trichosalpinx by female biting midges (Ceratopogonidae) (Bogarín et al., 2018).

Karremans and Díaz-Morales propose calling the second pollination mechanism in Pleurothallidinae "steliform", after the genus Stelis. It has evolved independently in two genera, and is suspected to occur in five more. In steliform pollination, flies are attracted to flat flowers with a glenion (a round area of secretory cells) at the base of the labellum. The role of the glenion has not been experimentally confirmed, but is believed to secrete a reward to aid in the positioning of the pollinator at the base of the compact column. As a fly attempts to access the glenion, the viscidium droplet binds the pollinaria to its head or legs. Steliform may not be the ideal name for this pollination mechanism, since the genus Stelis is known to exhibit three different morphological pollination strategies (Karremans and Díaz-Morales, 2019).

Karremans and Díaz-Morales propose calling the third pollination mechanism in Pleurothallidinae “lepanthiform”, after Lepanthes, the genus in which it was first observed. In lepanthiform pollination, flies are attracted by sexual pheromones and attempt copulation with the labellum by grasping the appendix (the central lobe of a trilobed labellum). Many species in Lepanthes exhibit morphology consistent with this pollination mechanism, and pollination by pseudocopulation may be the ancestral state of this genus. Observing pseudocopulation is very rare, but has been confirmed in Lepanthes glicensteinii which is pollinated by male Sciaridae flies. In this species the pollinators emerged from the downwind direction indicating the role of floral scents in attraction. The pseudocopulation sometimes resulted in ejaculation, making this only the second observed orchid species to elicit such a response (Blanco and Barboza, 2005).

Pleurothallis specifically is a large genus that contains more than 500 species. Common traits in this genus include a synsepal, composed of the complete fusion of the two lateral sepals, attenuated petals, and a compact column. Observational accounts suggest that this genus is myophilous, though several other orders of insects have been reported visiting the flowers. These insect orders include Coleoptera, Hemiptera, and Hymenoptera. Thus far, these visitors have not been considered pollinators, even if pollinaria were occasionally removed. However, there is

speculation about wasps (Vespidae and Ichneumonidae) pollinating P. canaligera, P. divaricans, and P. quadrifida, due to the consistency of pollinarium deposition on the mouthparts (Karremans and Díaz-Morales, 2019).

Observational accounts of pollination in Pleurothallis have noted an array of fly species from families including Mycetophilidae (fungus gnats), Sciaridae (dark-winged fungus gnats), Drosophilidae (fruit flies), Anthomyiidae, Phoridae, Tephritidae (small fruit flies), Richardiinae, Calyptratae, Ceratopogonidae (biting midges), and Keroplatidae (also called fungus gnats). Although these observations have been made in sixteen species, as of 2019 only seven species had published accounts of their pollination, and in only four of these observations were pollinaria deposited on the pollinator (Karremans and Díaz-Morales, 2019). In one article only a single fly was observed visiting the flower and it made no attempt to approach the labellum, much less the pollinarium (Pupulin et al., 2017).

Several species of Pleurothallis are hypothesized to secrete a nectar reward as many visual accounts note liquid droplets on the labella and sometimes the petals and sepals of flowers. However, there has been very limited chemical analysis done to confirm the presence of sugars in the secretions. Some recent studies have been successful in testing for and confirming the presence of glucose in non-Pleurothallis species in Pleurothallidinae (Arévalo-Rodrigues et al., 2021). Previously, P. coriacardia was the only definitively nectar rewarding species in Pleurothallis, identified as such through chemical analyses that showed the liquid produced on the labellum contained 13% sugar (Sandoval, 2018).

Due to the extremely limited number of observational accounts of pollination, this study will use morphological evidence obtained by taking scanning electron micrographs of the orchid labellum to hypothesize pollination strategy. Pleurothallis species will be classified into generalized categories of either rewarding or deceptive. Rewarding species will be identified as those that exhibit signs of nectar production. These may include visible droplets of nectar-like liquid on fresh flowers, dried liquid in preserved flowers under SEM, a nectar channeling central sulcus, a detectable glenion, or those that show epithelial distinction between tissue that has been observed to produce liquid (viewed with a light microscope and/or in high resolution photographs), and the surrounding tissue. Regarding the glenion, the structure is hypothesized to be involved in the secretion of a nectar-like pollinator reward. The presence of the glenion on the hypochile indicates it is likely directly involved in positioning the pollinator in proper orientation for the attachment of the pollinarium to the fly's head or legs. However, the presence of a glenion may not be absolutely indicative of a rewarding species, as it could be a remnant structure in a species transitioning from rewarding into deceitful. Additionally, the presence of dried liquid, while suggestive of a nectar reward, cannot be used definitively to identify a species as nectar rewarding without the detection of high concentrations of sugars on the labellum. Preliminary LC-MS data (courtesy of Dr. Murphy Brasuel) will be used to identify species as producing nectar (those with high concentrations of sugar molecules on the lip or the whole flower). Without this chemical analysis dried liquid will be referred to as "nectar-like".

While this part of the study is essentially the identification of species in Pleurothallis exhibiting traits of the steliform pollination mechanism, the term "steliform" as coined by Karremans and Díaz-Morales (2019), will not be used as it overemphasizes the connection of this pollination strategy with the genus Stelis. As circumscribed by these authors, Stelis contains over 1000 species with several markedly different pollination syndromes. Therefore, the term "gleniform" will be used to replace the term "steliform" to describe the pollination syndrome

involving a glenion that is present in Stelis and several other genera that has evolved separately in both Pleurothallidinae and Bulbophyllum.

Potentially sexually deceptive species have been documented in Pleurothallis and possess a morphology very different from lepanthiform species. Species in the P. crocodiliceps complex have a characteristic reduced labellum with many identifiable features. The labellum is often covered in papillae and has two horn-like projections or lobes on either side extending from the base of the labellum forward and sometimes curving out to either side. The proposed pollination mechanism for this species is pseudocopulation by male flies (Wilson et al., 2017). This has also been proposed for the recently described species P. minutilabia, which also has a similarly bizarre miniscule labellum (Wilson et al., 2018). The labella of these species do not closely resemble the labella of species in Lepanthes that have been observed to be pollinated by pseudocopulation. There is no central appendage that would serve the function of the appendix to be grasped by the gonostili (male claspers that hold the female during copulation) of a fly during copulation. Instead, in the center of the epichile (the distal portion of the labellum) is a deep cavity, dubbed the "copulatorium", that could function to receive the abdomen of the male insect. Although pollination in these species has yet to be observed in situ, the morphological characteristics of these flowers provide interesting insight into the possible pollination mechanisms. In this study species with these features, the copulatorium (a deep apical cavity), the hypochilar "horns", and the dense and lengthy papillae, will be referred to as putatively sexually deceptive species.

Phylogenetics

The internal transcribed spacer (ITS) region of the nuclear genome is used in this study to elucidate phylogenetic relationships between species of Pleurothallis orchids. This region was used because there are two highly variable regions (ITS1 and ITS2) that have previously been sufficient in differentiating between Pleurothallis species (Frank, 2015; Dupree, 2017; Zhao, 2019). Phylogenetic trees of Pleurothallis species generated by previous Colorado College students are further developed in this study. The inclusion of undescribed species on the phylogenies will be used to identify the close relatives and appropriate subsection or subgenus of Pleurothallis for categorization. The constructed phylogenies will also be useful in analyzing the historical prevalence and convergent evolution of morphological microstructures, such as the glenion, labellar secretory tissue, and indicators of sexual deception. Previously, these phylogenies, produced collaboratively by several students at the Wilson laboratory, have been used to show that the inclusion of subgenera Ancipitia and what was known as Scopula into the subgenus Pleurothallis produces a single monophyletic subgenus (Dupree, 2017). They have also been used to map putatively rewarding and deceptive species in the Ancipitia/Scopula subsection to the conclusion that sexual deception has evolved in Ancipitia multiple times from an ancestral rewarding state (Zhao, 2019).

Materials and Methods

1. Phylogenetics

DNA Extraction

Leaf samples were collected from the Colorado College greenhouse and preserved in a freezer at -20°C prior to DNA extraction. At least 100 mg of wet leaf tissue was pulverized with a mortar and pestle while submerged in liquid nitrogen. Genomic DNA was extracted from the leaf

powder using a Qiagen DNeasy plant mini kit. DNA concentration in the extract was estimated by running 10 µL of sample DNA solution on a 0.8% agarose gel stained with GelRed along with DNA standards of 5 ng, 10 ng, 25 ng, 50 ng, 100 ng, and 150 ng per lane.

Polymerase Chain Reaction

The ITS region of nuclear DNA was amplified with forward 17SE primer (ACGAATTCATGGTCCGGTGAAGTGTTCG) and reverse 26SE primer (TAGAATTCCCCGGTTCGCTCGCCGTTAC) (Zhao, 2019). Three identical PCR reactions were performed for each plant DNA sample along with a single positive control PCR reaction for each PCR run. Master Mix was created with the following formula for each reaction; 0.4 µL 25 µM 17SE primer, 0.4 µL 25 µM 26SE primer, 0.4 µL 10 mM dNTPs, 5 µL 5x Platinum II buffer, 0.16 µL hot-start Platinum Taq polymerase, and 8.64 µL molecular biology grade water for a total volume of 15 µL. Approximately 10 ng of template DNA was added to each reaction diluted into 10 µL of molecular biology grade water for a total reaction volume of 25 µL. Thermocycler procedure was identical to the ITS program described in Zhao (2019).

Amplification of chloroplast DNA region 3' ycf1 was done with the same primers and thermocycling program as described in Zhao (2019) with a modified reaction formula. For each plant sample four reactions were created with primer pair A and four reactions were created for primer pair B. Master mix for each reaction was created with 1 µL 5 µM forward primer (IntF or 3720f), 1 µL 5 µM reverse primer (5500r or IntR), 1 µL 12.5 mM magnesium chloride ($MgCl_2$), 5 µL 5X Platinum II buffer, 0.4 µL 10 mM dNPTs, 0.16 µL hot-start Platinum Taq polymerase, and 11.44 µL molecular biology grade water for a total volume of 20 µL. Approximately 2.5 ng of sample DNA was added to each reaction diluted into 5 µL for a total reaction volume of 25 µL.

PCR Product Purification

Gel electrophoresis was used to separate the desired PCR product (either ITS or 3' ycf1) from non-specific amplification products and the other reaction components. Gels were prepared with 50 mL 1x TAE buffer, 0.75 g agarose, and 5 µL GelRed. PCR products were mixed with 5 µL blue/orange loading dye and run at 100 V for approximately 80 min along with a DNA ladder for size comparison. DNA ladders were made with 5 µL of Promega 100 bp ladder DNA, 5 µL molecular biology grade water, and 2 µL blue/orange loading dye. Gels were observed with UV light after 40 min to assess successful PCR amplification and imaged with an iPhone 5s. Once sufficient separation of bands was achieved, the desired band was excised under UV light with a new razor blade. If necessary, the gel band containing the PCR product was refrigerated until it could be further processed. PCR products were purified from the gel cube with a QIAquick Gel Extraction Kit and the purified DNA concentration was measured using an Eppendorf Biophotometer.

Sanger Sequencing

ITS and 3' ycf1 samples were mailed overnight to Genewiz for Sanger sequencing. For each ITS sample, four sequencing reactions were conducted. Each contained 10 µL of DNA solution with approximately 10 ng/µL DNA as well as 5 µL of a 5 mM primer either 17SE, 26SE, ITS1, or ITS4. For 3' ycf1 samples, two reactions were made with purified PCR product A, each with 10 µL of DNA solution containing 10 ng/µL DNA and 5 µL 5 mM primer, either IntR or 3720f. Two reactions were also made with purified PCR product B, with 10 µL DNA solution containing 10 ng/µL DNA and 5 µL 5 mM primer, either IntF or 5500r.

Sequence Processing

Sequence files were viewed and edited in Geneious 2020.1. ITS sequences were cut before the start sequence CGG GCG GTT and after the ending sequence CCA CCC G, or when the sequence became too low quality to be reliable. The 3' ycf1 sequences were cut before the start sequence TCT CGA TTA and after the ending sequence ATT AAA TTC. Heterozygous peaks were called on the individual sequences using the IUPAC nucleotide code. All four ITS sequences for one genetic sample were aligned by MUSCLE, using the reverse complement sequence for the sequences 26SE and ITS4. Sequence alignments were further analyzed to confirm heterozygous peaks and questionable nucleotides. Consensus sequences were exported in FASTA format and converted into MEGA format for incorporation into alignments.

Maximum likelihood (ML) and maximum parsimony (MP) phylogenetic trees for each subgenus were created in the MEGA-X program using all nucleotide bases with 1000 bootstrap replicates. Additional sequences were added to phylogenetic trees from previous students in the Wilson Laboratory and from GenBank.

2. Scanning Electron Microscopy (SEM)

The majority of flower samples were collected from the Colorado College greenhouse. Flowers were collected when observed and preserved in Kew mix containing 53% methanol, 37% water, 5% formaldehyde, and 5% glycerol, for long term storage. Individual samples to be imaged were dehydrated in a sequential ethanol series of 70%, 90%, 100%, and freshly opened 100%, for 15 min each. Dehydrated flowers were dried in a critical point dryer. During the critical point drying process each sample was soaked in liquid carbon dioxide three times, the duration varying depending on the mass of the flower with larger flowers requiring longer soaking periods. Generally, flowers were soaked twice for 6 min with stirring, and once for 5 min without stirring. Carbon dioxide purging between soaks was for 1 min to 1 min 30 s to ensure full carbon dioxide changeover. Alternative fixation methods included flowers from orchid-grower Kevin Holcomb preserved directly in 70% ethanol, and certain flowers preserved in 100% methanol in an attempt to decrease tissue damage during the drying process. Flowers preserved in 70% ethanol were dehydrated in the manner described above. Flowers preserved in methanol were transferred after at least 10 min into 100% ethanol for 30 min, and then 100% ethanol overnight as suggested by Talbot and White (2013).

Dried flowers were mounted on aluminum stubs with sticky carbon tabs. Before mounting, the dorsal sepal and the petals were removed with either a razor or a scalpel blade. Some lip samples were mounted while connected to the synsepal with the column and the anther cap intact, while other lips were removed and mounted separately. PLECO colloidal graphite was spread around the synsepal to reduce sample charging and left to dry for 24 h. Each specimen was sputter coated in gold at a slight angle four times, with a 90° rotation between rounds to ensure sufficient coverage.

Electron micrographs were obtained with a Jeol JSM-6390LV scanning electron microscope at Colorado College. Images were generally taken with a working distance of 20-25 mm, spot size of 30, and an accelerating voltage of 5-10 kV.

3. LC-MS methods

The ultra-performance liquid chromatography-mass spectrometry (LC-MS) analysis of floral washes was completed by Dr. Murphy Brasuel in the Colorado College chemistry department. Freshly picked flowers, harvested and prepared by Dr. Mark Wilson, were washed in ultra-pure water. The samples were gently agitated for approximately 10 min before the washate was decanted into clean 2 mL screw topped tubes. The decanted washate was stored at -70°C leading up to LC-MS analysis.

Table 1. Pleurothallis specimens examined in this study, provided for organizational purposes to document which species have been analyzed with each technique to date (January, 2021).

Species	Subgenus	Plant ID Number	ITS Sequenced	3'ycf Sequenced	SEM	Volatiles Analyzed	Nectar Observed	Glenion
P. tetragona	Ancipitia	1107 and 1103	Yes (Both)	No	No	No		No Data
P. odobeniceps	Ancipitia	1044 and 1052	Yes (Both)	No	No	Yes		No Data
P. duplex	Ancipitia	1161 and 1162	Yes (Both)	No	No	No		No Data
P. solium	Ancipitia	7 and 1145	Yes (1145)	No	Yes	Yes		Atypical
P. praecipua	Ancipitia	1078	Yes	No	Yes	Yes		Present
P. cypelligera	Ancipitia	682 and 1156	Yes (1156)	No	Yes	Yes		Absent
P. inornata	Ancipitia	775	No	No	Yes	No		Absent
P. vorator	Ancipitia	688	No	No	Yes	Yes		Absent
P. viduata	Ancipitia	1123	No	No	Yes	Yes		Atypical
P. atxite	Ancipitia	1117	Yes	No	Yes	No		Absent
P. andreae	Ancipitia	1060	Yes	No	No	No		No Data
P. penicillata	Ancipitia	1157	Yes	No	No	No		No Data
P. acinaciformis	Ancipitia	1101	Yes	No	No	Yes		No Data
P. aff. titan "strawberry lip"	Macrophyllae-fasciculatae	1140 and 1164	Yes (Both)	No	Yes	No	Yes	Present
P. aff titan "orange"	Macrophyllae-fasciculatae	1077	Yes	No	Yes	No	Yes	Present
P. titan	Macrophyllae-fasciculatae	1121 and 1122	Yes (Both)	No	No	No		No Data
P. aff. lilijae "culpameae"	Macrophyllae-fasciculatae	1049	No	No	Yes	Yes	Yes	Present
P. aff. lilijae "white"	Macrophyllae-fasciculatae	1142	Yes	No	Yes	Yes	Yes	Present
P. aff. lilijae "riksen"	Macrophyllae-fasciculatae	1174	No	No	Yes	No		Lip base not visible
P. aff. lilijae "podocarpus"	Macrophyllae-fasciculatae	1154	No	No	Yes	No		Present
P. lanigera	Macrophyllae-fasciculatae	1167	No	No	Yes	No	No	Present
P. aff. lilijae sp.	Macrophyllae-fasciculatae	KH006	No	No	Yes	No		Atypical
P. aff. lilijae sp.	Macrophyllae-fasciculatae	KH011	No	No	Yes	No		Atypical
P. aff. imperialis	Macrophyllae-fasciculatae	1138	No	No	Yes	No		Atypical
P. scurrula	Macrophyllae-fasciculatae	478	No	No	Yes	Yes	Yes	Present
P. pyelophora	Macrophyllae-fasciculatae	1151	No	No	Yes	Yes	Yes	Lip base not visible
P. sanchoi	Macrophyllae-fasciculatae	????	No	No	Yes	Yes	Yes	Present
P. anthrax	Macrophyllae-fasciculatae	587	No	No	Yes	No		Present
P. renieana	Macrophyllae-fasciculatae	8	No	No	Yes	Yes	No	Absent
P. nuda	Restrepioidia	1175	Yes	No	Yes	Yes	Yes	Absent
P. aff. nuda	Restrepioidia	854	No	No	Yes	Yes	Yes	Absent
P. restrepioides	Elongatia	362	No	No	Yes	No	Yes	Absent
P. aff. restrepioides	Elongatia	1110	No	No	Yes	No	Yes	Absent
P. sijmii	Elongatia	????	No	No	Yes	Yes	Yes	Absent
P. tenuisepala	Acroniae	963 and 1038	Yes (0963)	Yes	Yes	No	Yes	Present
P. aff. tenuisepala	Acroniae	1143	Yes	Yes	Yes	No	Yes	No Data
P. vieirae	Acroniae	1169	Yes	No	Yes	Yes	Yes	Present
P. sandemanii	Talpinaria	789	No	No	Yes	Yes	Yes	Absent

SEM Results

Subsection Ancipitia

Pleurothallis solium

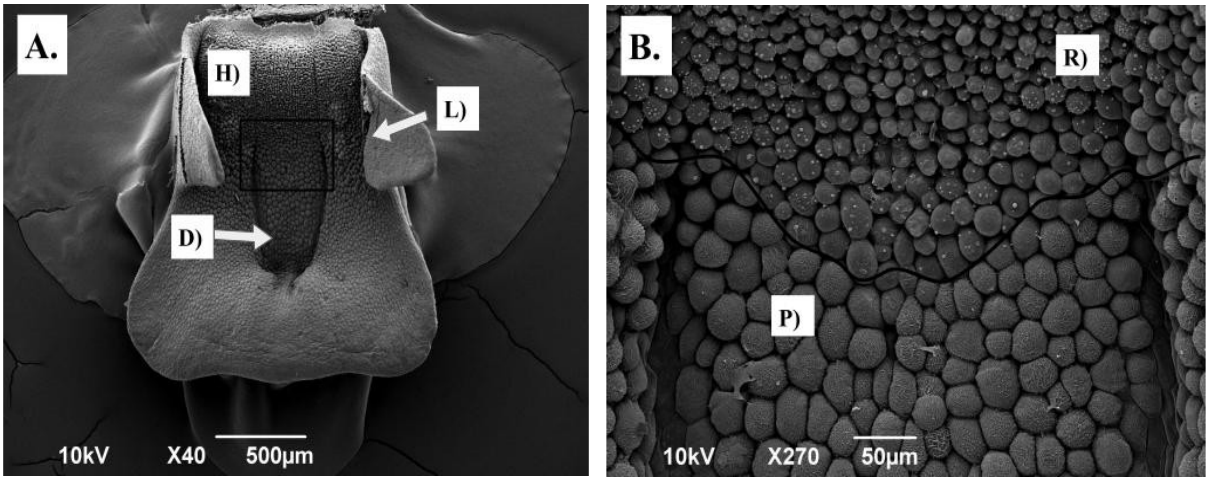

Figure 1.
Electron micrographs of Pleurothallis solium. A. whole lip from above showing the hypochile H), a central depression D), a lateral lobe L), and a box enclosing the enlarged area pictured in B.
B. higher magnification image of the hypochile region showing two cell types R) and P) separated by a black line.

The lip of P. solium was removed from the perianth and mounted alone. SEM micrographs were obtained at varying magnifications. The lip has a large central depression, possibly functioning as a nectar collection reservoir (Fig. 1A). The large lateral lobes visible on either side of the hypochile may restrict pollinator movement at the lip base thereby increasing the probability of pollinarium attachment and removal. While there is not a typical circular glenion at the lip base, there are two distinct cell types labelled R) and P) (Fig. 1B). Cell type R consists of smooth cells with globular epidermal protrusions, and cell type P are covered in a thick outer coating of wrinkled cuticle. Cell type R closely resembles glenion tissue present in P. viduata (Fig. 4) and P. praecipua (Fig. 6) and may be secretory.

Pleurothallis cypelligera

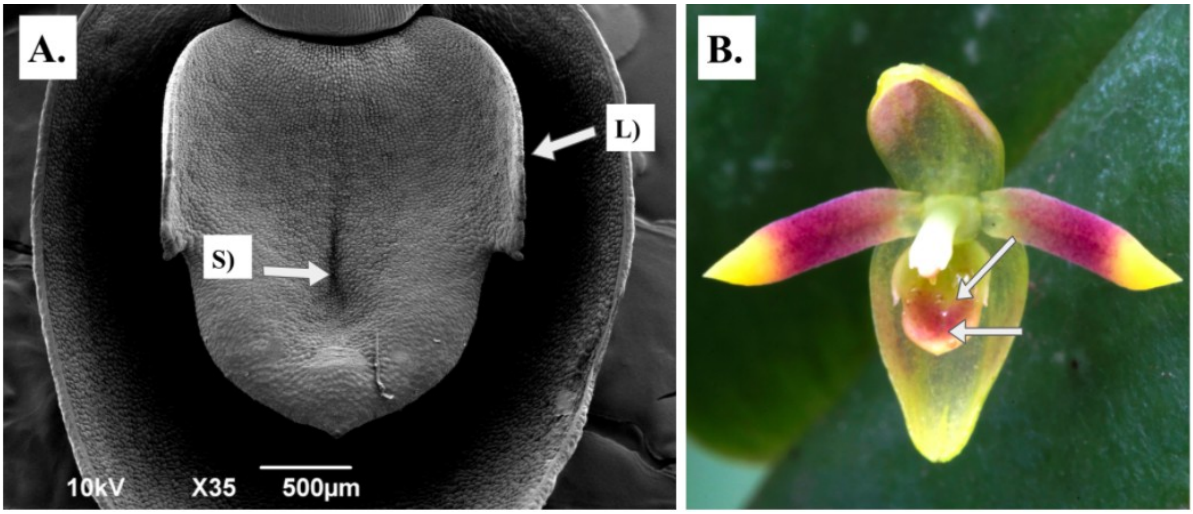

Figure 2.
A. Electron micrograph of Pleurothallis cypelligera showing whole lip with L) lateral lobes and S) sulcus. B. Photograph of whole P. cypelligera flower, arrows pointing to nectar-like liquid droplets on the labellum.

P. cypelligera was mounted on the synsepal with the column bent back to reveal the lip topography. Two lateral lobes L) extend vertically and may act as a pollinator-corralling system to properly position pollinators at the lip base to encounter the viscidium. The sulcus S) does not appear to be of a different cell type but could act as a nectar channel and may suggest this is a rewarding species (Fig. 2).

Pleurothallis inornata

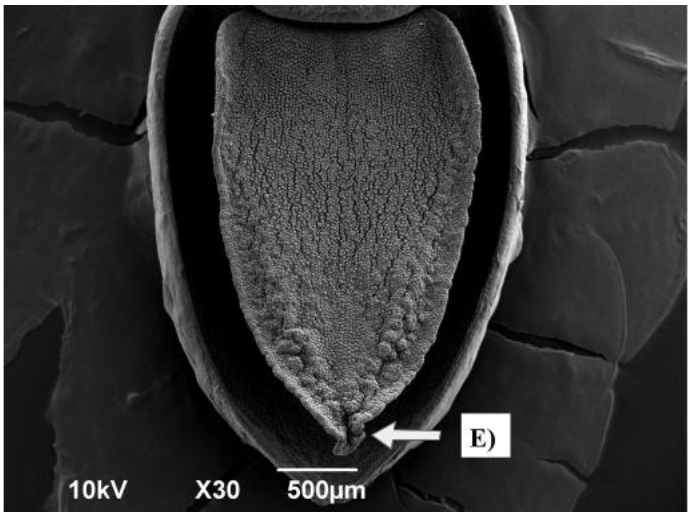

Figure 3.
Electron micrograph of Pleurothallis inornata with the epichile E) labelled.

P. inornata was mounted on the concave synsepal with the column bent away to reveal the surface of the lip. The surface appears to be of one cell type, but small lateral grooves along the majority of the lip could indicate secretory tissue. Additionally, the lumpy nature of the tissue along both sides of the lip could help liquid retention. The puckered epichile E) is not unique to P. inornata but is of unknown function (Fig. 3).

Pleurothallis viduata

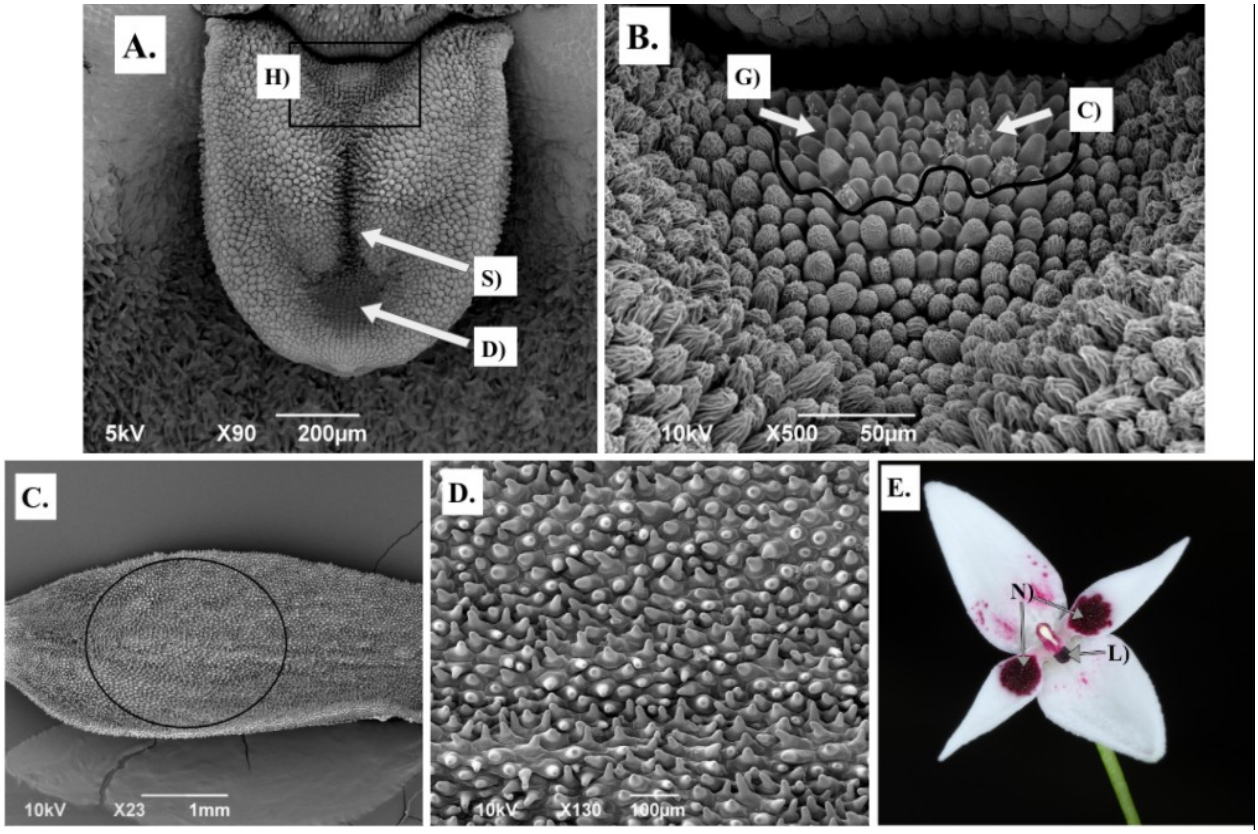

Figure 4.
Electron micrographs of Pleurothallis viduata. A. whole lip from above showing the hypochile H), sulcus S), depression D), and a box enclosing the enlarged area pictured in B.
B. higher magnification of lip base showing the glenion G) cells separated by a black line, some with crystalline protrusions C). C. Electron micrograph of a P. viduata petal, low magnification image from above, circle indicating the observed secretory area. D. Higher magnification image of petal epidermal cells. E. Photograph of a whole P. viduata flower with an arrow pointing to the small dark purple labellum L), and arrows pointing to the secretory areas N) on the petals.

P. viduata has a distinctively small lip not more than a millimeter long (Fig. 4E). Viewed without magnification the lip could appear to be vestigial, but several labellar features suggest nectar secretion and reward pollination. At the very base of the lip appears to be a small glenion G) identified by a distinct cell type with smooth surfaces. Some cells in the glenion area have crystals on the surface that are of unknown origin and function. Leading from the glenion area through the center of the lip is a sulcus S) that could direct nectar flow to the central depression D). This potential nectar flow from the hypochile to the epichile appears to be a guiding mechanism

directing the pollinator towards the lip base to be in position for pollinarium deposition (Fig. 4A and Fig. 4B). P. viduata petals have been observed to secrete large droplets of a nectar like substance in circular patches (circle). SEM images show a uniform epidermal surface and no apparent distinction between secretory and non-secretory cells (Fig. 4C and 4D).

Pleurothallis praecipua

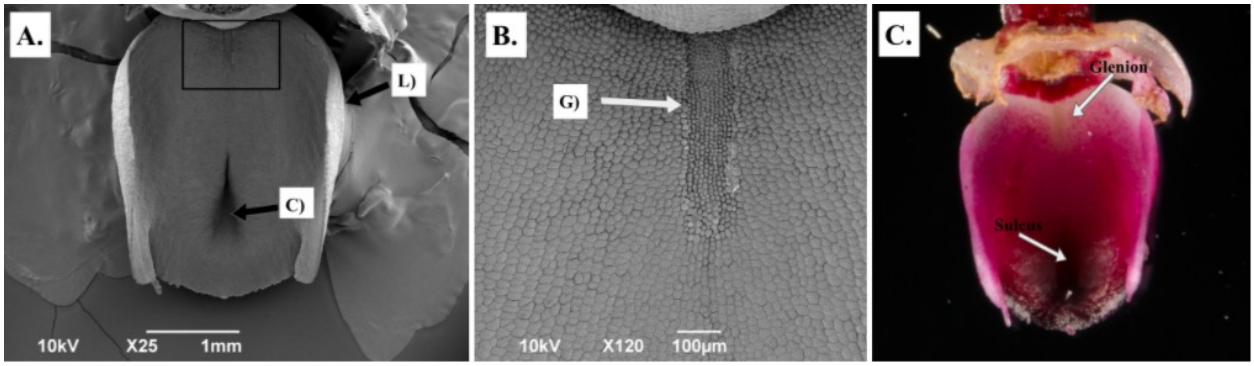

Figure 5.
Electron micrographs of Pleurothallis praecipua. A. whole lip from above with lateral lobes L), a central cavity C), and a box enclosing the enlarged area pictured in B.
B. Higher magnification of the lip base with a glenion G). C. Photograph of fresh P. praecipua labellum with petals, sepals, and column removed. Arrows identifying the central sulcus and the elongate glenion.

The lip of P. praecipua was removed from the perianth of the flower and the column was cut away to expose the whole lip surface (Fig. 5A). The large lateral lobes L) extend the whole length of the lip and likely restrict the size of insect pollinators that can access the reward as well as aid in pollinator positioning. This species has an elongated glenion G) (Fig. 5 B), and a central cavity C) that likely collects nectar secretions.

Pleurothallis vorator

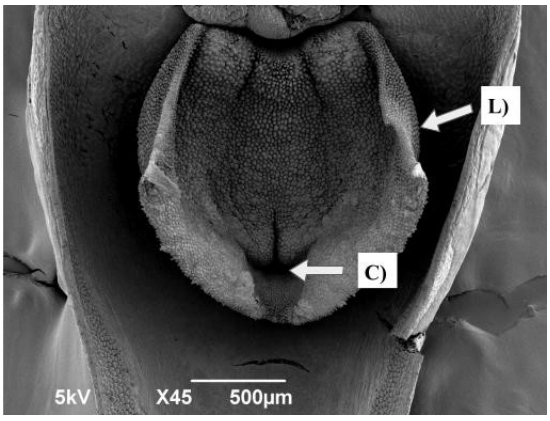

Figure 6.
Electron micrograph of Pleurothallis vorator whole lip from above showing lateral lobes L) and an apical cavity C).

P. vorator was mounted for SEM on the synsepal with the column bent away to reveal the lip base. The lip surface appears to be of a uniform cell type but there is a deep three-pronged apical depression C) that does not resemble those in putatively rewarding species such as P. solium (Fig. 1) or P. cypelligera (Fig. 2), and somewhat resembles a shallow version of a copulatorium as present in deceptive species such as P. aff. crocodiliceps “BL” (Fig. 9). This species may also represent an evolutionary stage in the acquisition of a copulatorium in a newly deceptive species.

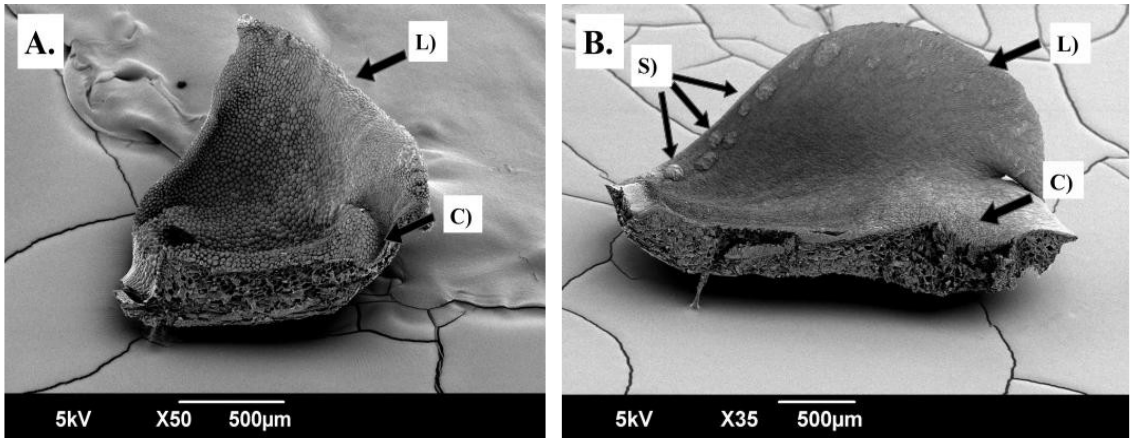

Figure 7.
Electron micrographs of A. P. vorator and B. P. praecipua after being cut down the center longitudinally.
L) lateral lobes C) cavities S) possible secretory areas

P. praecipua and P. vorator were cut down the center to view and compare the depth of the cavities for rewarding (P. praecipua) and potentially deceptive (P. vorator) species (Fig. 7). The cavity on P. vorator is shallow, but is more apparent than that of P. praecipua. Also visible on P. praecipua from the side angle are several raised areas of tissue S). These may be evidence of cellular damage that occurred during the drying process, but they could also indicate a difference in cell chemistry at a subepidermal level that could suggest secretory tissue.

Pleurothallis aff. crocdiliceps "atxite"

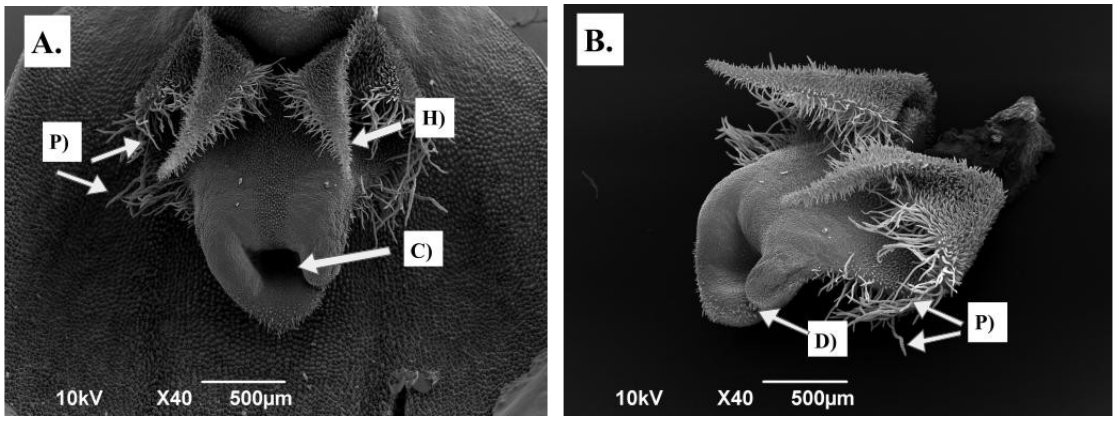

Figure 8.
Electron micrographs of Pleurothallis aff. crocodiliceps "atxite" A. whole lip photographed from above and B. whole lip photographs from the side. P) papillae C) Apical cavity H) horns D) deformity

P. aff. crocodilceps "atxite" was imaged while attached to the synsepal (Fig. 8A) and after it was removed and mounted alone (Fig. 8B). Similar to other Pleurothallis species presumed to be pollinated by sexual deception, P. "atxite" displays two horns branching of the hypochile and a distinct apical cavity. Dissimilarly to other presumed sexually deceptive Pleurothallis species, the apical cavity of P. "atxite" is not lined with long papillary cells and appears relatively smooth. The papillae around the horns P), however, are extremely long. This individual lip has a deformity D) on the right side of the epichile that is assumed to be unique to this flower.

Pleurothallis aff. crocodiliceps “BL”

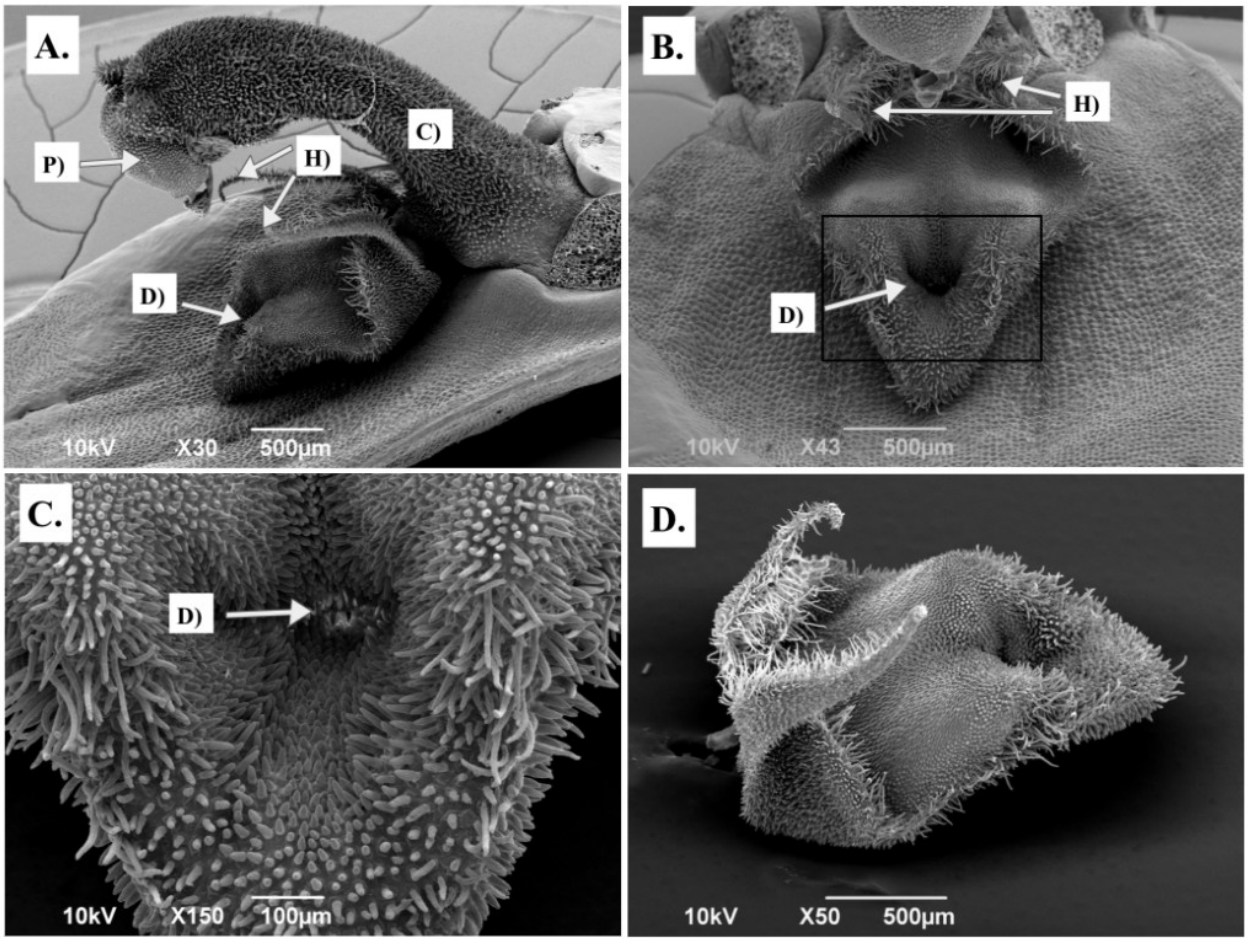

Figure 9.
Electron micrographs of Pleurothallis aff. crocodiliceps “BL”.
A. photographed from the side with the column C) intact. Showing horns H), pollinarium covered by the anther cap P), and the apical cavity D).
B. photographed from the front with the column intact. Showing horns H), apical cavity D), and a box enclosing the enlarged area pictured in C.
C. higher magnification image of the apical cavity D).
D. Side view of whole lip removed from synsepal and column.

P. aff. crocodiliceps "BL" was imaged while attached to the synsepal and column with only the dorsal sepal and the petals removed (Fig. 1A). This view of the lip in relation to the column,

and particularly the viscidium, provides information about spatial relationship between the morphological features of the lip with a potential pollinator. P. aff. crocodiliceps "BL" closely resembles the other species in the P. crocodiliceps complex with two protruding horns H) extending from either side of the hypochile, a deep apical cavity C), and papillae P) covering most of the horns, epichile, and underside of the lip. The apical cavity viewed in higher magnification clearly shows the papillae lining the inside of the putative copulatorium (Fig. 9C).

Pleurothallis renieana

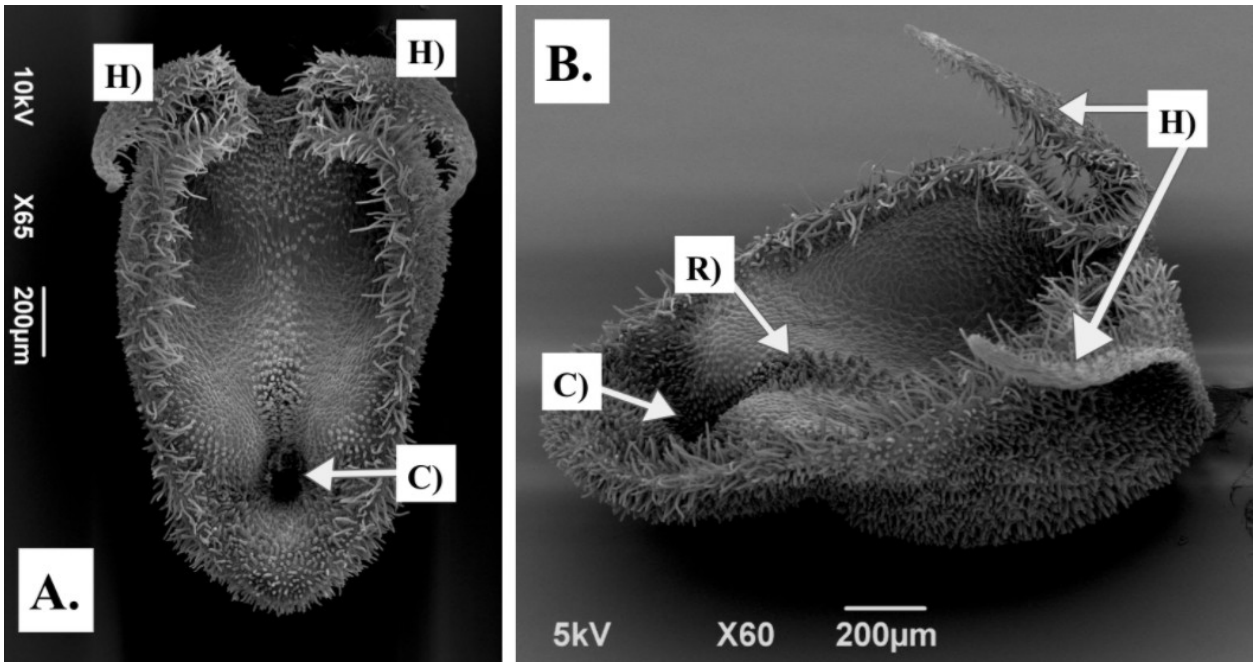

Figure 10.
Electron micrographs of Pleurothallis renieana. A. low magnification image of the whole lip mounted alone showing the two horns H) and the apical cavity C). B. low magnification image of the whole lip tilted sideways to show the horns H), the apical cavity C), and a papillate ridge R) in the center of the lip.

The lip of P. renieana appears visually similar to several of the deceptive species in Pleurothallis that are hypothesized to be pollinated by pseudocopulation. The lip was removed from the rest of the flower and mounted alone to be imaged from the front and side angles (Fig. 10). There are two long horns H) that extend from either side of the hypochile, there is an apical cavity C) that appears quite deep and is covered in short papillae inside. There are also long papillae covering the lip edges and horns, as well as forming a small papillate ridge R) from the apical cavity towards the hypochile.

Pleurothallis lueriana

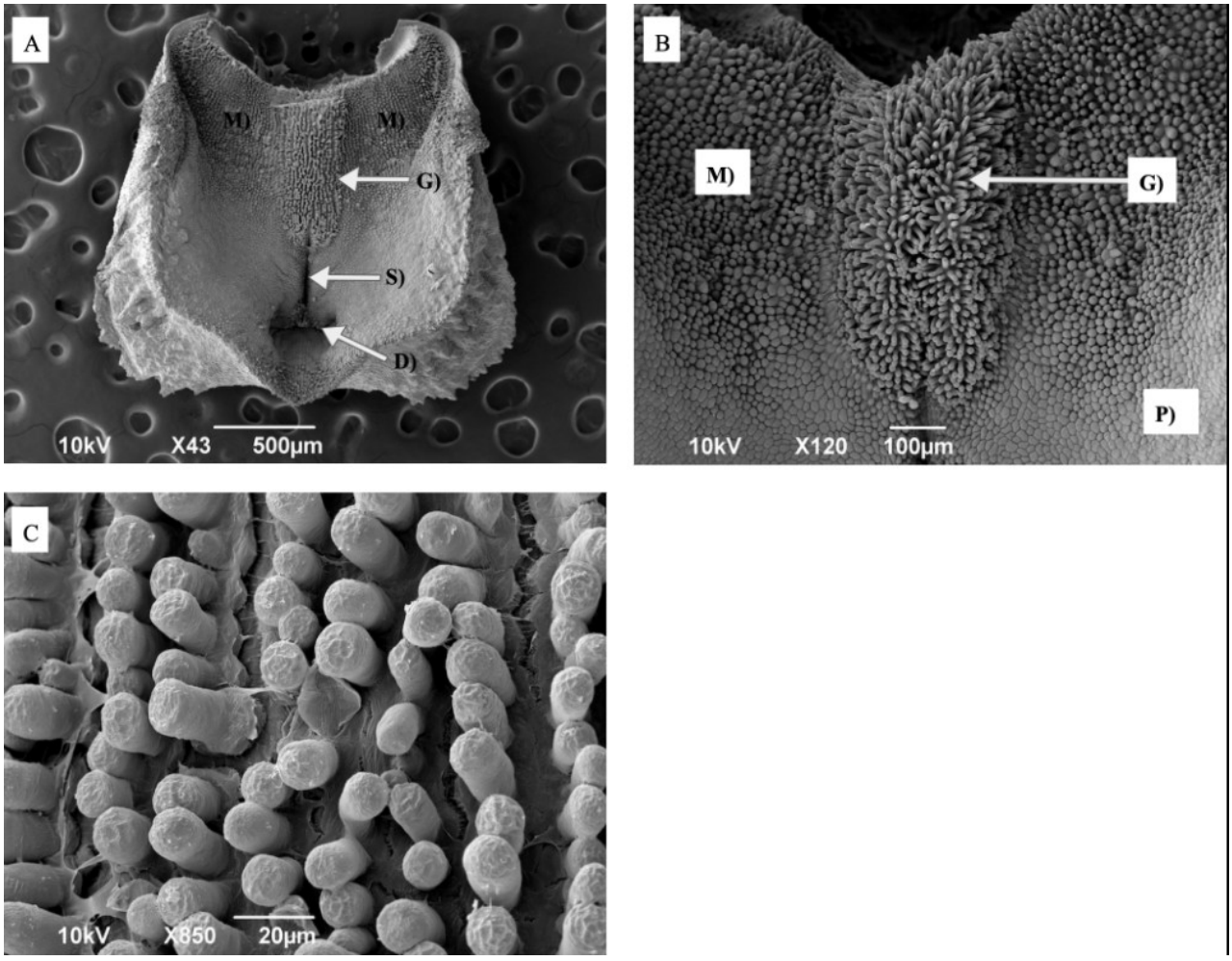

Figure 11.
Electron micrographs of Pleurothallis lueriana. A. low magnification image of the whole lip mounted on a carbon tab showing a glenion G), sulcus S), apical depression D), and hypochilar cell type M). B. higher magnification image of the glenion area G) showing cell types M) and P). C. high magnification image of glenion cells.

The lip of P. lueriana was prepared and mounted by a previous student in the Wilson Laboratory (Fig. 11). The lip has a large oblong glenion G), and a central sulcus S) leading to a shallow apical depression D). The glenion cells are significantly longer than the surrounding surface cells and appear to have dried liquid between them (Fig. 11C). The cells around the glenion on the hypochile M) appear slightly longer and more dispersed compared to the cells on the rest of the lip surface P). Although the apical depression is deeper than in other rewarding species, and resembles the three-pronged depression in P. vorator (Fig. 6), the liquid between the glenion cells indicates a rewarding pollination strategy.

Pleurothallis anthrax

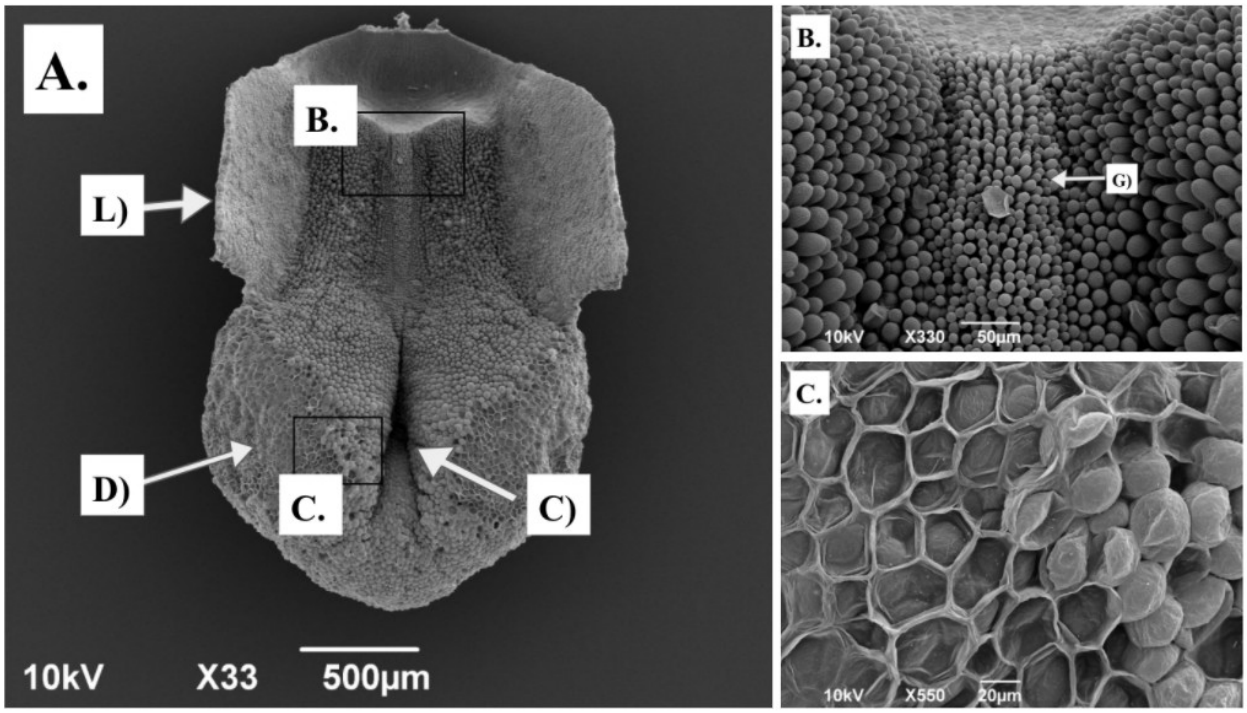

Figure 12.
Electron micrographs of Pleurothallis anthrax. A. low magnification image of the whole lip showing the two lateral lobes L), a central depression C), and two lobes of epichilar cell damage D). B. higher magnification image of the glenion area G) showing two cell types. C. higher magnification image of cellular damage affecting some cells and not others.

The lip of P. anthrax was removed from the flower and mounted alone to examine the glenion area (Fig. 12). Two lateral lobes on either side of the hypochile could function to restrict insect access to the lip base outside of a certain size range. The lip also has a central cavity that could function as a nectar reservoir, but does not resemble the nectar collecting sulcus extending from the glenion in other species. This specimen of P. anthrax sustained severe cellular damage in two patches on the lip D) that are positioned symmetrically about the central depression. This damage could indicate a subsurface compositional difference between the damaged cells and the surrounding cells. It is possible the damaged cells contained nectar to be secreted that caused them to react differently to the drying process (Fig. 12C). There is a clear elongated glenion G) located at the lip base extending as far down as the lateral lobes characterized by small and smooth cells that lack a cuticle layer (Fig. 12B.).

Subsection Macrophyllae-Fasciculatae

Pleurothallis aff. lilijae “white”

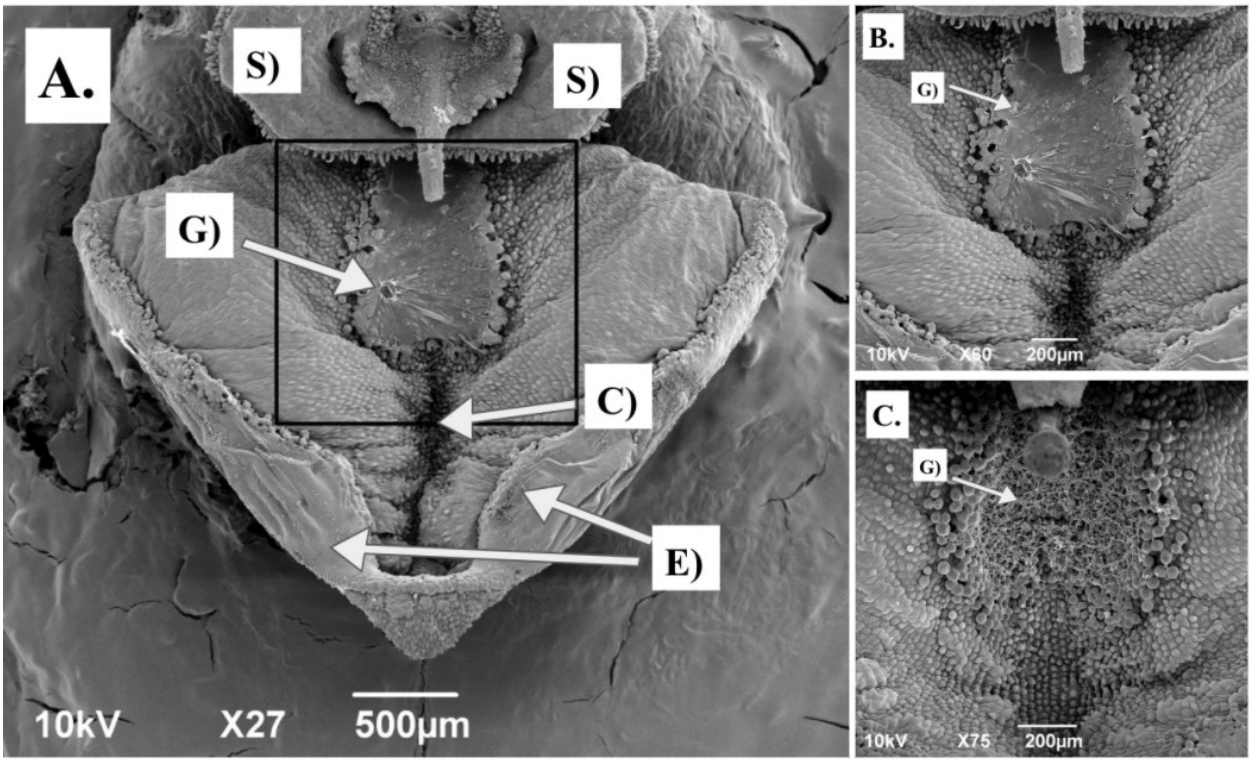

Figure 13.
Electron micrographs of Pleurothallis aff. lilijae “white”.
A. whole lip and column showing the bilobed stigmatic surface S), a glenion G), a central channel C), and two epichilar secretory regions E). B. higher magnification image of the glenion G) when preserved in methanol showing a liquid covering. C. higher magnification image of the glenion G) when preserved in ethanol showing a fibrous material covering.

The lip of P. aff. lilijae “white” was mounted whole with the column intact on the synsepal (Fig. 14). P. aff. lilijae “white” has a distinct circular glenion covered in a thick layer of secreted liquid. The glenion liquid is preserved when the flower is dehydrated in methanol (Fig. 13B), but dissolves or is otherwise removed when the flower is dehydrated in ethanol (Fig. 13C) leaving behind a fibrous covering. P. aff. lilijae “white” also produces a thick noticeably sticky secretion in two patches of the epichile. These epichilar secretions are of unknown chemical composition but appear mucosal and could function as a pollinator reward or alternatively as a trap for non-pollinating species. Additionally, this species has a deep channel leading from the glenion down towards the epichile.

Pleurothallis aff. lilijae “culpameae”

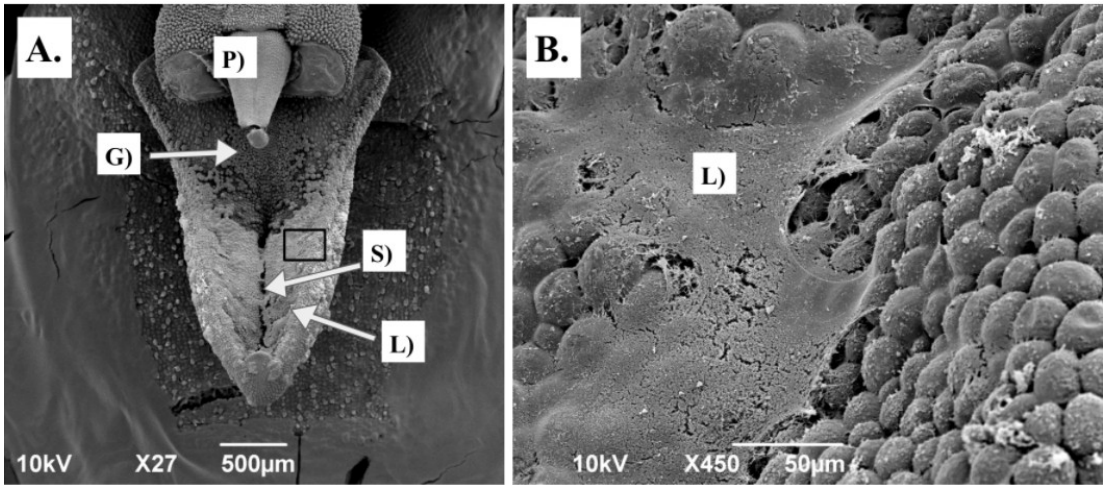

Figure 14.
Electron micrographs of Pleurothallis aff. lilijae “culpameae”. A. low magnification image of the whole lip and column showing anther cap P), glenion G), sulcus S), and dried liquid L). B. higher magnification showing dried liquid L) covering the cells on the lower half of the lip.

The images of P. aff. lilijae “culpameae” obtained by Zhao (2019) showed a significant amount of dried liquid on the mesochile and epichile, in an attempt to remove this liquid a specimen of P. aff. lilijae “culpameae” was washed in water for 1.5 h before ethanol dehydration. The water submersion did not remove the epichilar secretions and there remains a thick layer of dried liquid (Fig. 14B). As described by Zhao (2019), P. aff. lilijae has a clear glenion G) at the lip base and a sulcus S) leading towards to epichile.

Pleurothallis aff. lilijae “Riksen”

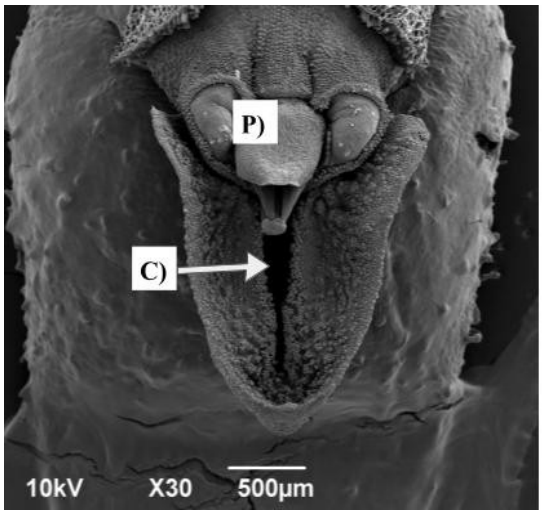

Figure 15.
Electron micrograph of Pleurothallis aff. lilijae “Riksen” showing the whole lip with the column intact and the anther cap P) covering the pollinia. At the center of the lip is a deep channel C).

P. aff. lilijae “Riksen” was mounted on the synsepal with the column intact (Fig. 15). Along the center of the lip is a deep channel C) the inside of which is obscured from view. The cells around the surface of the lip, while appearing bubbled or warty, do seem to be of one uniform cell type. Without cutting the lip longitudinally to view the cells inside the cavity it is unknown if P. aff. lilijae “culpameae” possesses a glenion or a different type of secretory cell inside the cavity. The outer edge of the epichile is turned upwards potentially to prevent nectar from flowing off the lip.

Pleurothallis aff. lilijae “Podocarpus”

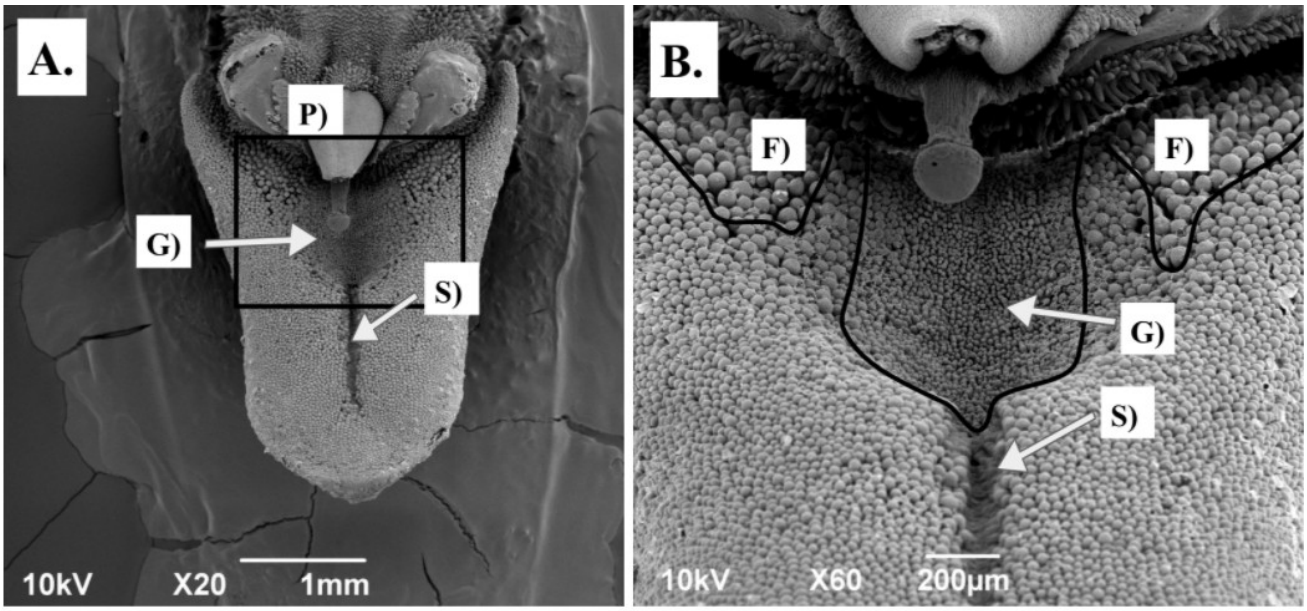

Figure 16.
Electron micrographs of Pleurothallis aff. lilijae “Podocarpus”. A. whole lip and column showing intact anther cap P) covering the pollinia, a glenion G), and a sulcus S).
B. higher magnification image of the glenion area showing three cell types, glenion cells G), another cell type F), and lip surface cells (unmarked), as well as a sulcus S).

The lip of P. aff. lilijae “Podocarpus” was mounted on the synsepal with the column intact (Fig. 16). A higher magnification of the glenion area (Fig. 16B) reveals three cell types on the lip surface. Most of the lip surface is composed of one uniform cell type that is unmarked in Figure 16. The glenion at the lip base has a second potentially secretory cell type characterized by smaller smoother cells with dried liquid at the edges. The third cell type F) appears in two patches on either side of the glenion and is made up of significantly larger cells that do not possess the same debris present on the rest of the lip surface cells. The lip also has a defined sulcus S) leading from the glenion towards the epichile.

Pleurothallis aff. lilijae KH006

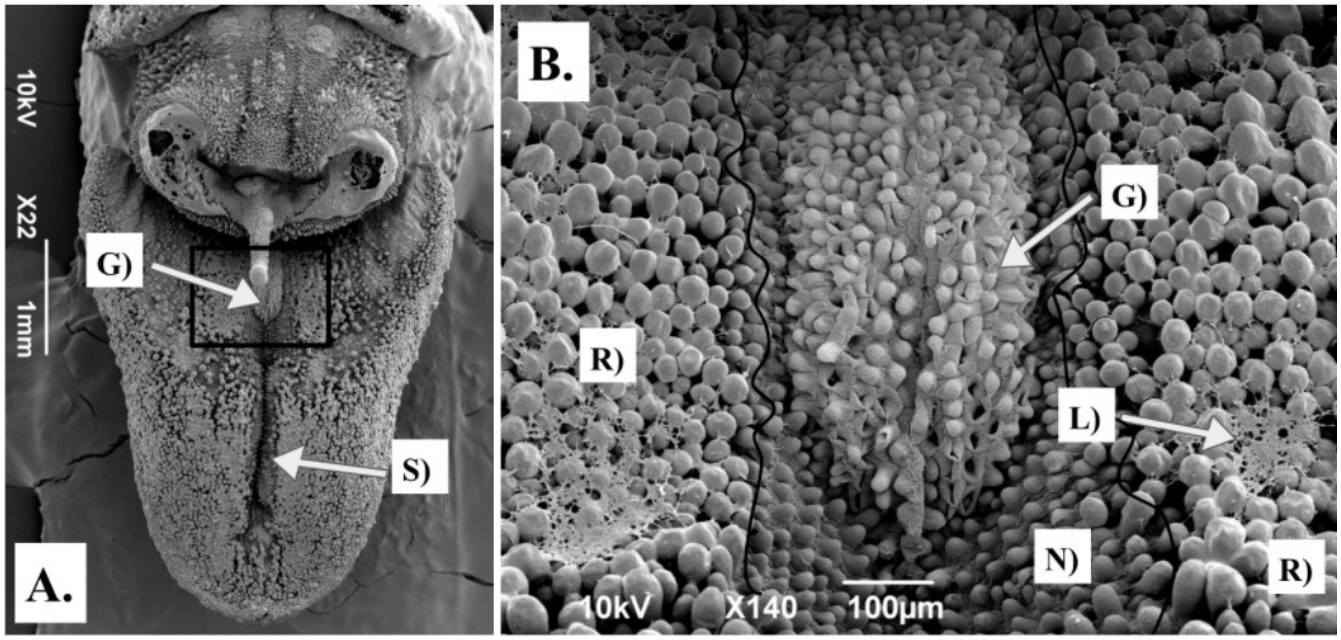

Figure 17.
Electron micrographs of Pleurothallis aff. lilijae sp. KH006.
A. whole lip and intact column showing atypical glenion G) and sulcus S).
B. higher magnification image of glenion area G) with cell types R) and N) and dried liquid on sides L).

The lip of P. aff. lilijae KH006 was mounted whole with the column intact on the synsepal. P. aff. lilijae KH006 has an atypical glenion area with three distinct cell types. The first cell type is present on the surface of the sulcus S) and are small round cells covered with a wrinkled cuticle layer. The raised oblong glenion area G) is made up of a second cell type, these cells are uncharacteristically covered in a wrinkled cuticle layer and do not resemble the glenion cells of any other species in the P. aff. lilijae complex. The third type of cells are much larger than the other two, they are round and appear to be covered in dried liquid L). Cells of the third type appear in patches on either side of the glenion area and cover the majority of the lip outside the sulcus and the glenion. The third cell type lack the wrinkled cuticle layer and appear to be nectar secreting.

Pleurothallis aff. lilijae KH011

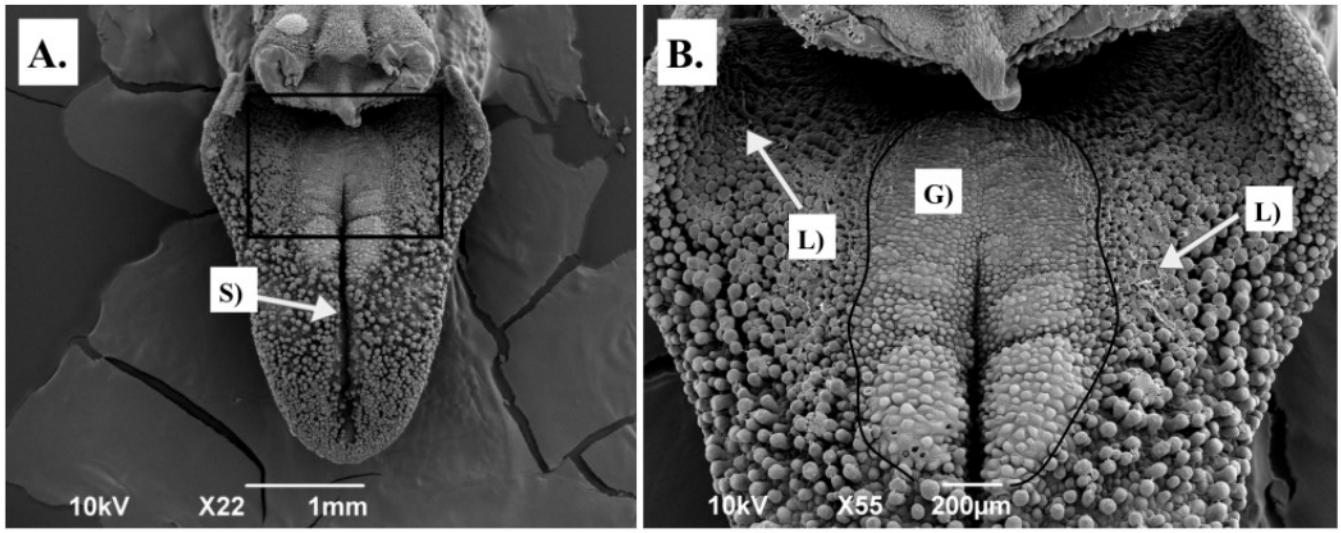

Figure 18.
Electron micrographs of Pleurothallis aff. lilijae sp. KH011.
A. whole lip and column showing the glenion area enclosed in a box and a sulcus S).
B. higher magnification image of the glenion area G) enclosed in a black line as well as dried liquid L) outside the glenion.

The lip of P. aff. lilijae KH011 was mounted whole on the synsepal with the column intact (Fig. 17). The glenion of this species is atypical because the distinct cell type in the glenion region G) extends past the base of the lip with the sulcus S) running through it. The cells in the glenion region are covered in a wrinkled cuticle and do not appear to secrete a liquid. However, lack of dried liquid could be attributable to the maturity of the individual flower when preserved. The cells composing the rest of the lip surface outside the glenion region have patches of dried liquid L) and closely resemble the presumably secretory non-glenial cells on P. aff. lilijae KH006. The sulcus S) of this species runs from inside the glenion region almost to the epichile and appears quite deep.

Pleurothallis lanigera

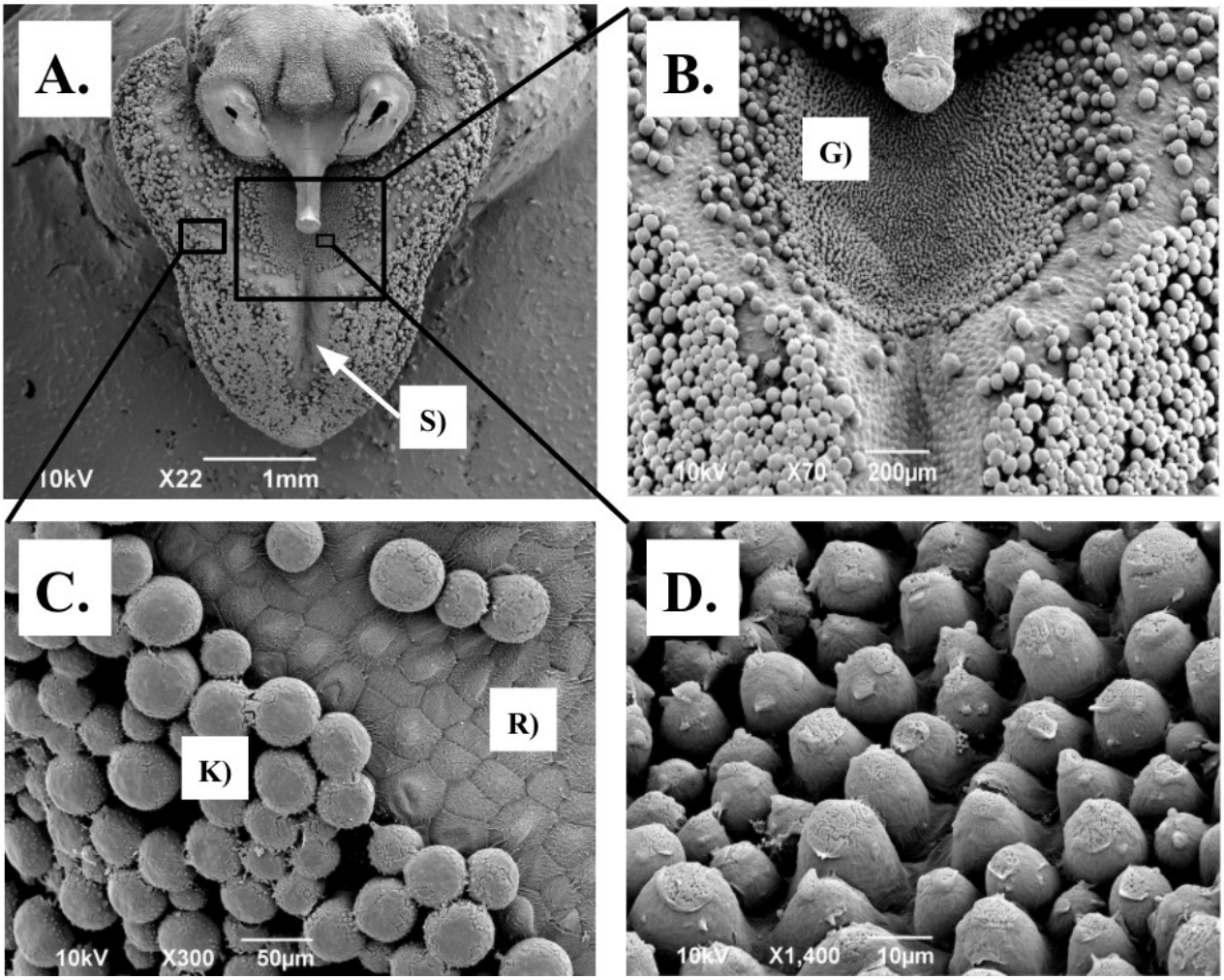

Figure 19.
Electron micrographs of Pleurothallis lanigera.
A. whole lip mounted with the column and stigmatic surfaces intact, showing the glenion, sulcus S) and three cell types. B. higher magnification image of the glenion G). C. higher magnification image of the two distinct surface cell types R) and K). D. higher magnification image of the glenion cells.

The lip of P.lanigera was mounted whole attached to the synsepal with the column and stigmatic surface intact (Fig. 19). The lip has a circular glenion G) with typical glenion cells that are smoother and smaller than the surrounding surface cells (Fig. 19D). There appears to be something on the surface of the glenion cells that is possibly a secreted reward. The surface of the lip has two distinct cell types (Fig. 19C), around the glenion are very flat cells R) with a thick wrinkled cuticle, covering the rest of the lip are large bubbly cells K) that appear to have a thin layer of dried liquid creating strings between them. The large glenion and the appearance of a dried liquid on the lip surface are evidence of this being a rewarding species.

Pleurothallis aff. titan “strawberry lip”

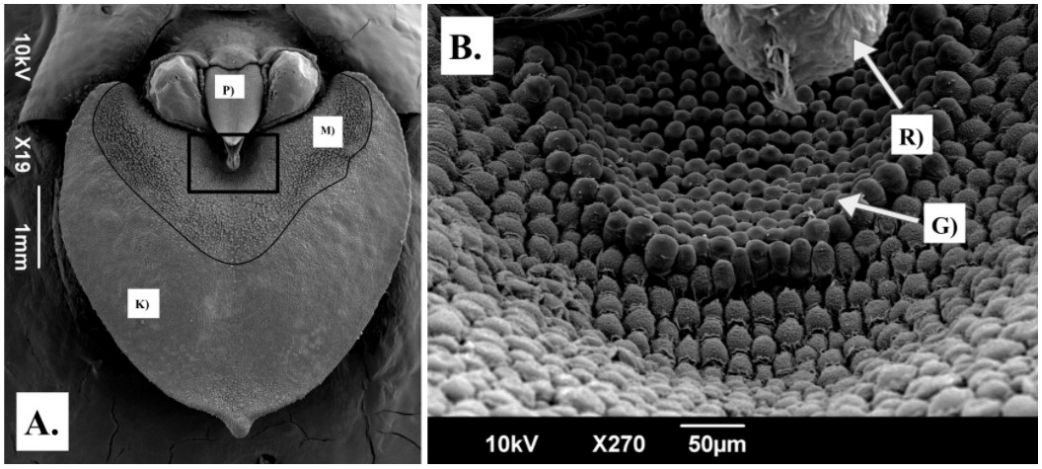

Figure 20.
Electron micrographs of Pleurothallis aff. titan “strawberry lip”. A. low magnification image of the whole lip and column showing anther cap P) and glenion area enclosed in box as well as two cell types K) and M). B. higher magnification image of the glenion G) below the rostellum R).

P. aff. titan “strawberry lip” has a flat circular lip that was mounted on the synsepal with the column intact (Fig. 20). The lip has three cell types, most of the lip surface outside of the lip base is one cell type characterized by flattened circular cells K). The glenion G) is located under the column and is made of raised smooth cells covered in dried liquid. Outside of the glenion is a third cell type M) that are larger than cell type K) and appear inflated like the glenion cells but with a wrinkled cuticle layer. P. aff. titan “strawberry lip” does not have a sulcus, but it does have a slightly puckered epichile that may aid in nectar retention.

Pleurothallis aff. titan “orange”

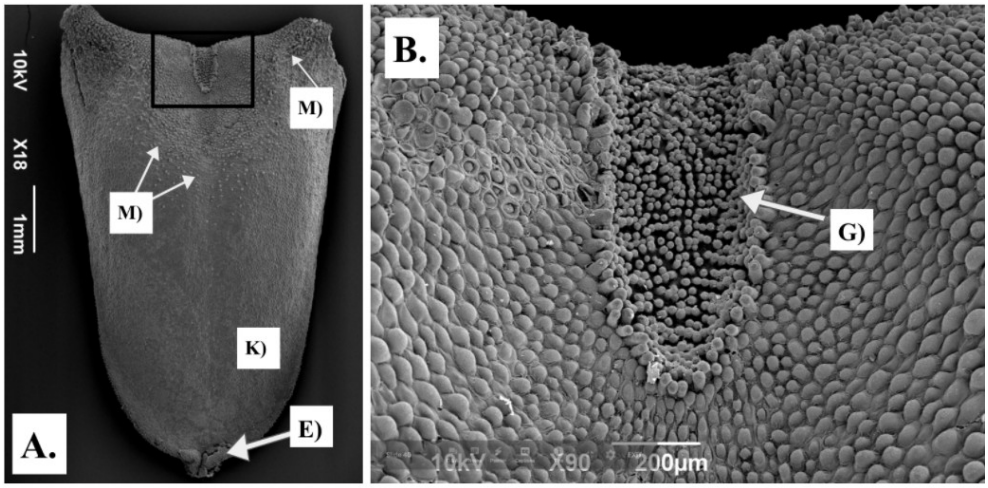

Figure 21.
Electron micrographs of Pleurothallis aff. titan “orange”. A. whole lip alone showing the glenion enclosed in a box, cell type K), cell type M), and the epichile. B. higher magnification image of the glenion area G).

The lip of P. aff. titan "orange" was removed from the rest of the flower to be mounted alone (Fig. 21). Compared to P. aff. titan "strawberry lip" (Fig. 20), this species has a more elongated lip and glenion G). Similar to P. aff. titan "strawberry lip", P. aff. titan "orange" has three cell types. Most of the lip is made up of cell type K) that appear flattened or slightly sunken in. The glenion G) is made up of small smooth cells. The third cell type M) appears in a high concentration in two patches on either side of the hypochile and sporadically around the upper half of the lip. Cell type M) have a cuticle layer, are larger than the glenion cells, and appear more inflated that cell type K). P. aff. titan "orange" does not have a defined sulcus but there is a shallow depression along the central longitude of the lip. The epichile E) of this individual sustained damage prior to SEM imaging, but the puckered look is notable in this species.

Pleurothallis aff. imperialis

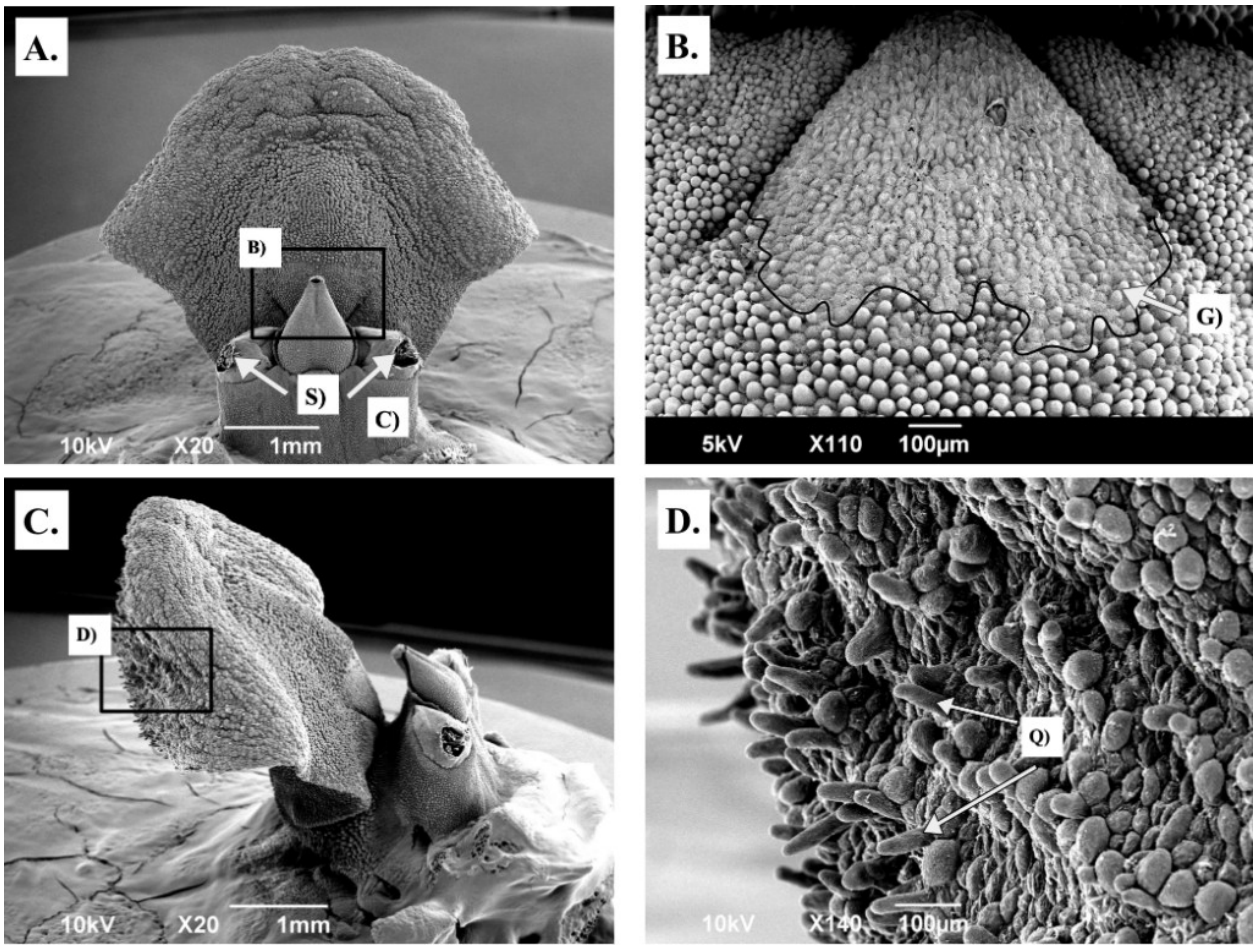

Figure 22.
Electron micrographs of Pleurothallis aff. imperialis. A. the whole lip and column oriented so the stigmatic surface S) and column C) are at the base. B. higher magnification image of the glenion area G) separated by a line and rotated so the column would be at the top of the image. C. low magnification image of the whole lip and column from the side. D) higher magnification image of the underside of the lip showing elongated projecting cells Q) and flatter epithelial cells.

The lip of P. aff. imperialis has several unique characteristics that may indicate a distinct pollination strategy. The lip is curved around into a cone shape and projects upwards from the synsepal (Fig. 22C). The adaxial surface of the lip is composed of at least two different cell types, one of which covers the majority of the lip surface and is characterized by round inflated cells that lack a wrinkled cuticle layer. The cells composing the glenion area G) are smaller and covered in a layer of dried liquid, the liquid obscures the cell surface so it is unclear if the glenion cells have a wrinkled cuticle (Fig. 22B). The abaxial surface of the lip also has at least two cell types, one of which is visually similar to the non-glenion cells on the lip surface (Fig. 22D). The final cell type Q) are elongated and possibly function to increase surface area for volatile chemical emission (Fig. 22D).

Pleurothallis scurrula

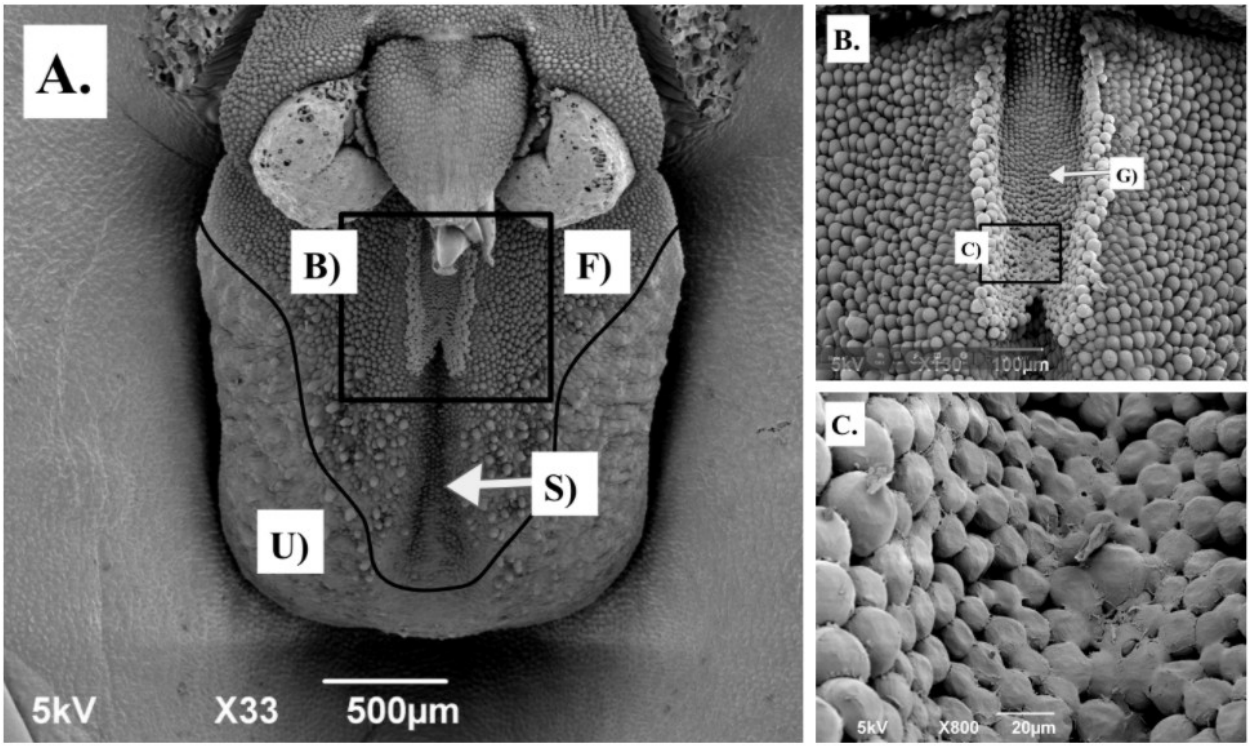

Figure 23.
Electron micrographs of Pleurothallis scurrula. A. low magnification image of the whole lip and column showing the sulcus S) and cell types U) and F). B. higher magnification image of the glenion area G) showing two cell types. C. high magnification image of the glenion area showing a dried liquid layer.

The lip of P. scurrula was mounted on the synsepal with the column intact (Fig. 23). P. scurrula has a clear elongated glenion G) at the base of the lip with distinct cells that are smaller and without a wrinkled cuticle layer (Fig. 23B), these cells are covered in a dried layer of liquid (Fig. 23C). Leading from the glenion area towards the epichile is a sulcus S) that ends in a well (Fig. 23A). A portion of the lip surface surrounding the hypochile outside the glenion is covered in round inflated cells F). Cell type U) appears in two patches on either side of the lip, in this type the cells appear flattened.

Pleurothallis pyelophora

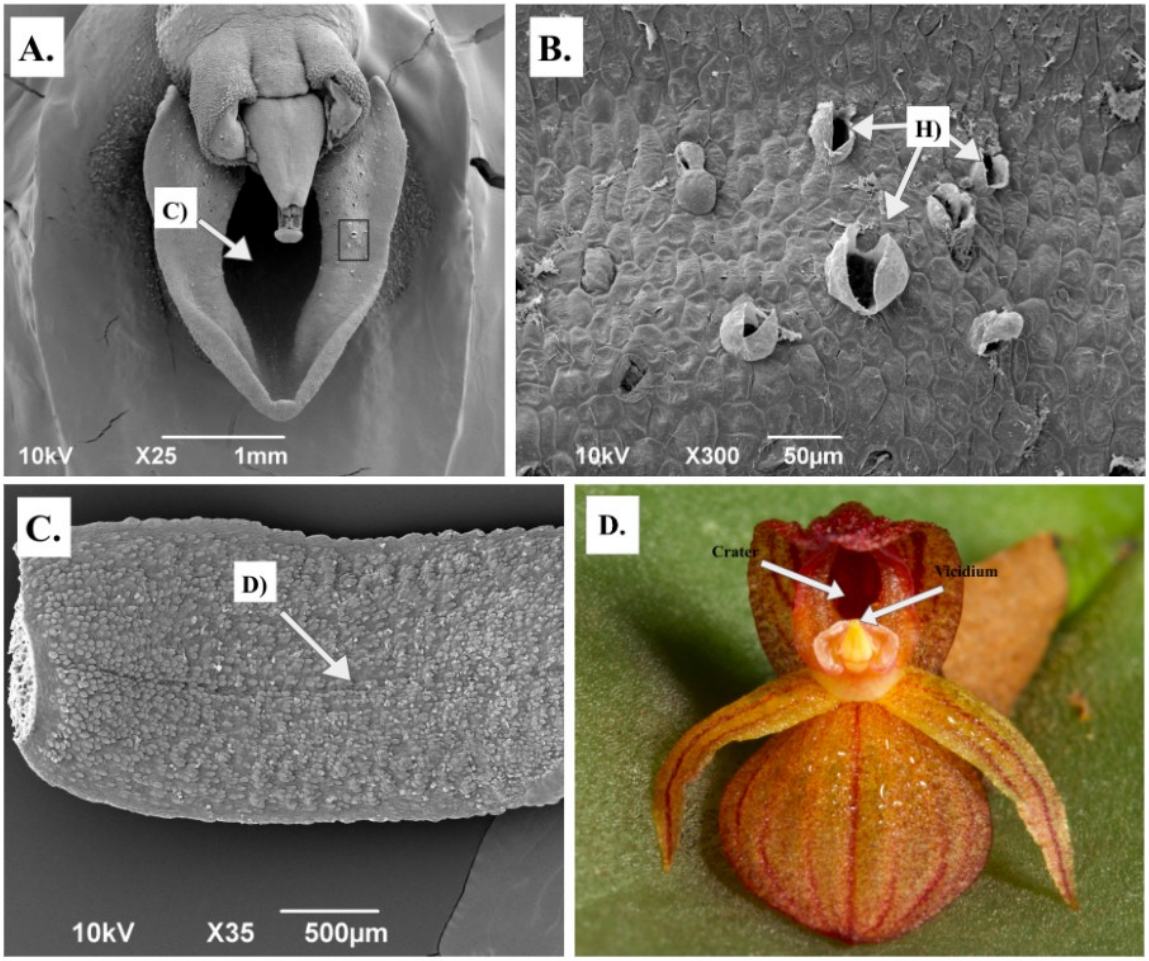

Figure 24.
Electron micrographs of Pleurothallis pyelophora, a crateriform species. A. low magnification image of the whole lip and column showing the deep crater C) the lip forms behind the viscidium. B. higher magnification image of surface structures H) on the outer edge of the lip. C. low magnification image of a P. pyelophora petal with a slight lateral depression D). D. Photograph of a whole P. pyelophora flower, with arrows identifying the crater shaped labellum and the viscidium at the tip of the pollinarium.

Unlike most Pleurothallis, P. pyelophora is non-resupinate species so the lip extends upwards behind the column instead of outward. The whole lip of P. pyelophora was mounted on the synsepal with the column intact (Fig. 24). The lip forms a large crater that secretes nectar likely to attract insect pollinators. Although pollination has not been observed, the lip morphology suggests insects that reach over the column to feed on the nectar may encounter the viscidium which could attach the pollinia to the underside of the thorax. On the outer edge of the lip are several holes H) that appear visually similar to burst epithelial cells. These holes are of unknown origin and function. The petals of P. pyelophora have been observed to secrete a nectar-like fluid (Fig. 24D), but SEM imaging showed a uniform cell type on the surface of the petal. The only distinguishable feature on the petal was a slight central depression D) running laterally that appears as a line (Fig. 24C).

Subsection Acroniae
Pleurothallis vieirae

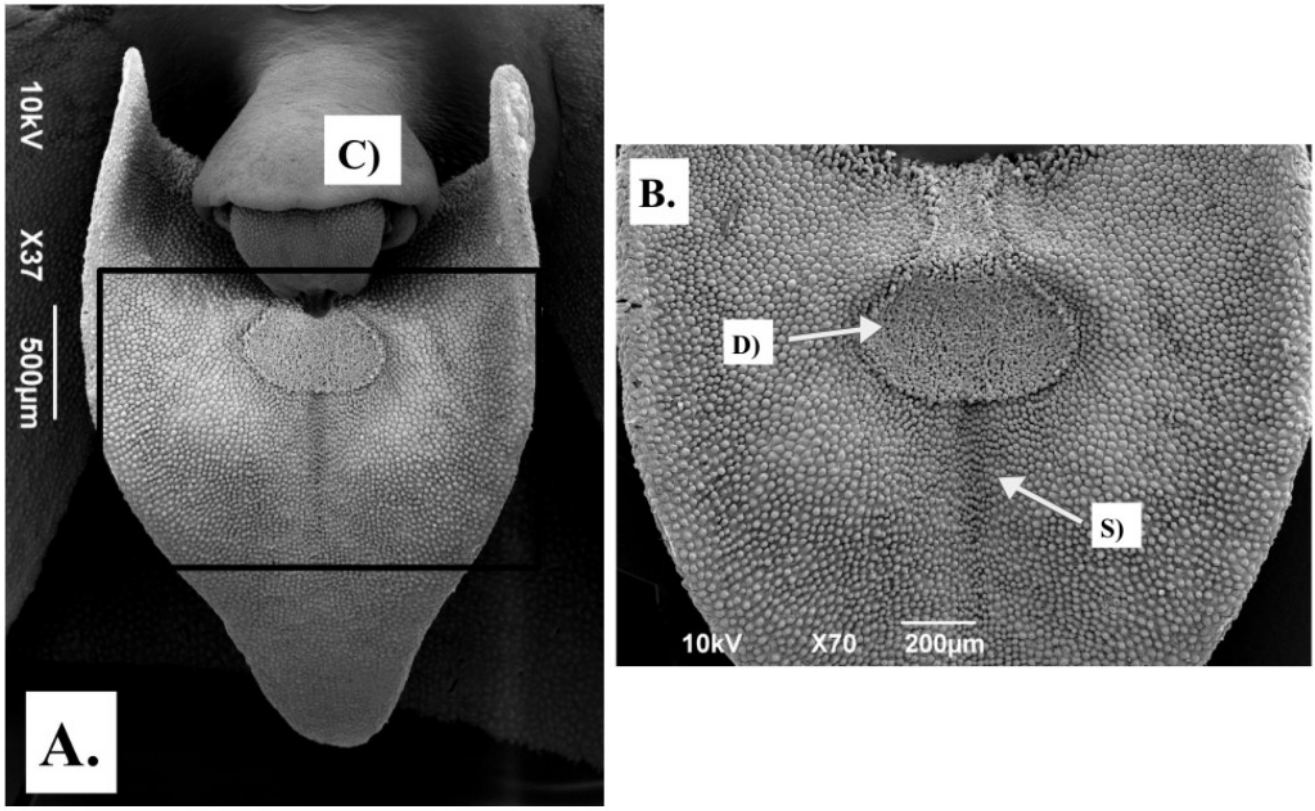

Figure 25.
Electron micrographs of Pleurothallis vieirae. A. showing whole lip and column C) and a box enclosing the enlarged area pictured in B.
B. higher magnification image of the glenion D) and the sulcus S).

P. vieirae was mounted with the petals, synsepal, and column intact and the dorsal sepal pulled back to reveal the lip (Fig. 25A), the lip was then removed and mounted alone (Fig. 25B). P. vieirae exhibits a clear circular glenion D) that is seated in a depression with a sulcus S) leading from the glenion towards the epichile. This system possibly functions to channel glenial nectar pooled at the base of the lip towards the epichile, thereby directing pollinators towards the hypochile for pollination.

Pleurothallis aff. tenuisepala

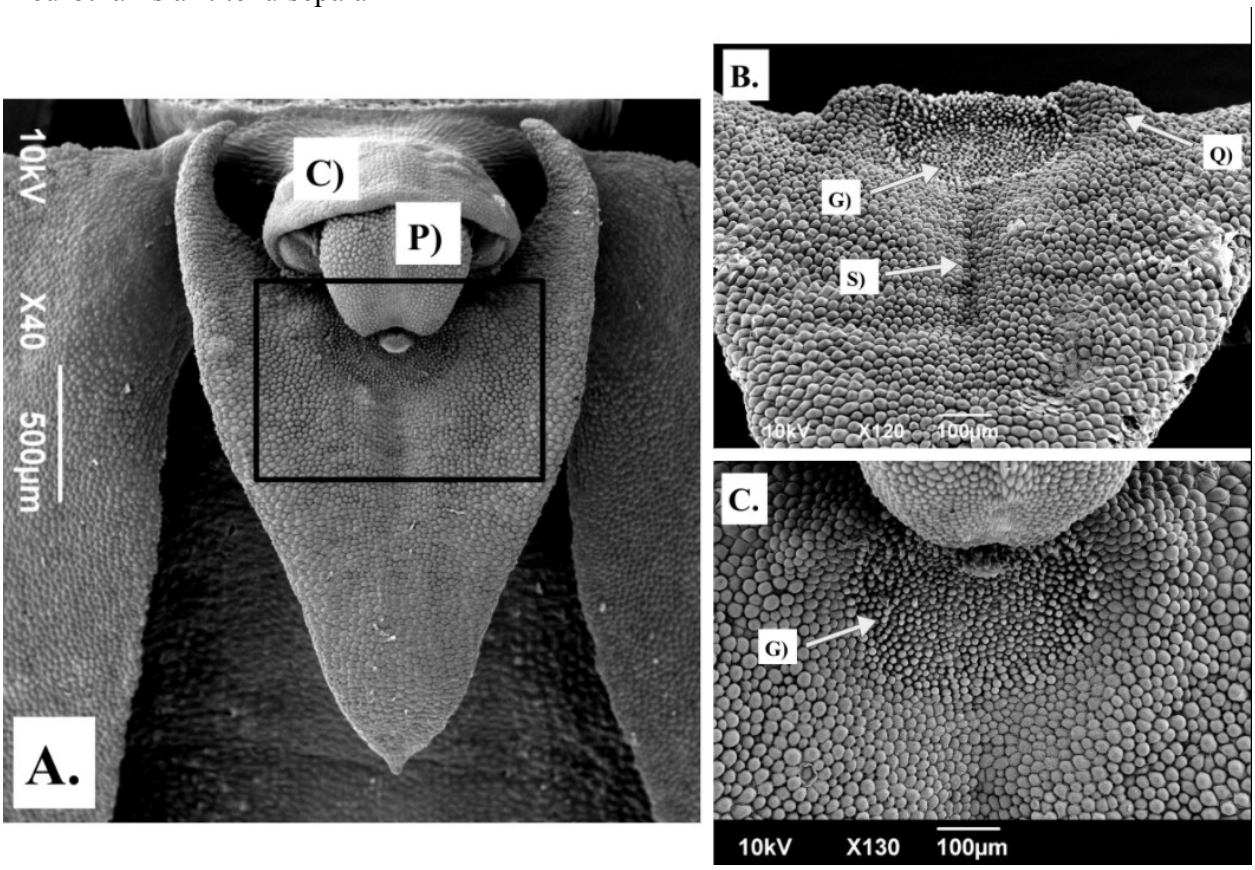

Figure 26.
Electron micrographs of Pleurothallis aff. tenuisepala. A. showing the whole lip, column C), anther cap P), and a box showing the enlarged area pictured in C.
B. the removed lip tilted to emphasize the calli Q) surrounding the glenion G) as well as the sulcus S).
C. Higher magnification image of the glenion G).

P. aff. tenuisepala was mounted with the whole with the dorsal sepal bent backwards to reveal the lip (Fig. 26A), the lip was then removed and mounted separately (Fig. 26C). P. aff. tenuisepala has a clear circular glenion at the base of the lip with calli Q) creating a well around it. As visible in P. vieirae (Fig. 26B), P. aff. tenuisepala also has a sulcus leading from the glenion area towards the epichile. The glenion of both species are characterized by smaller smoother cells.

Subgenus Restrepioidia

Pleurothallis nuda

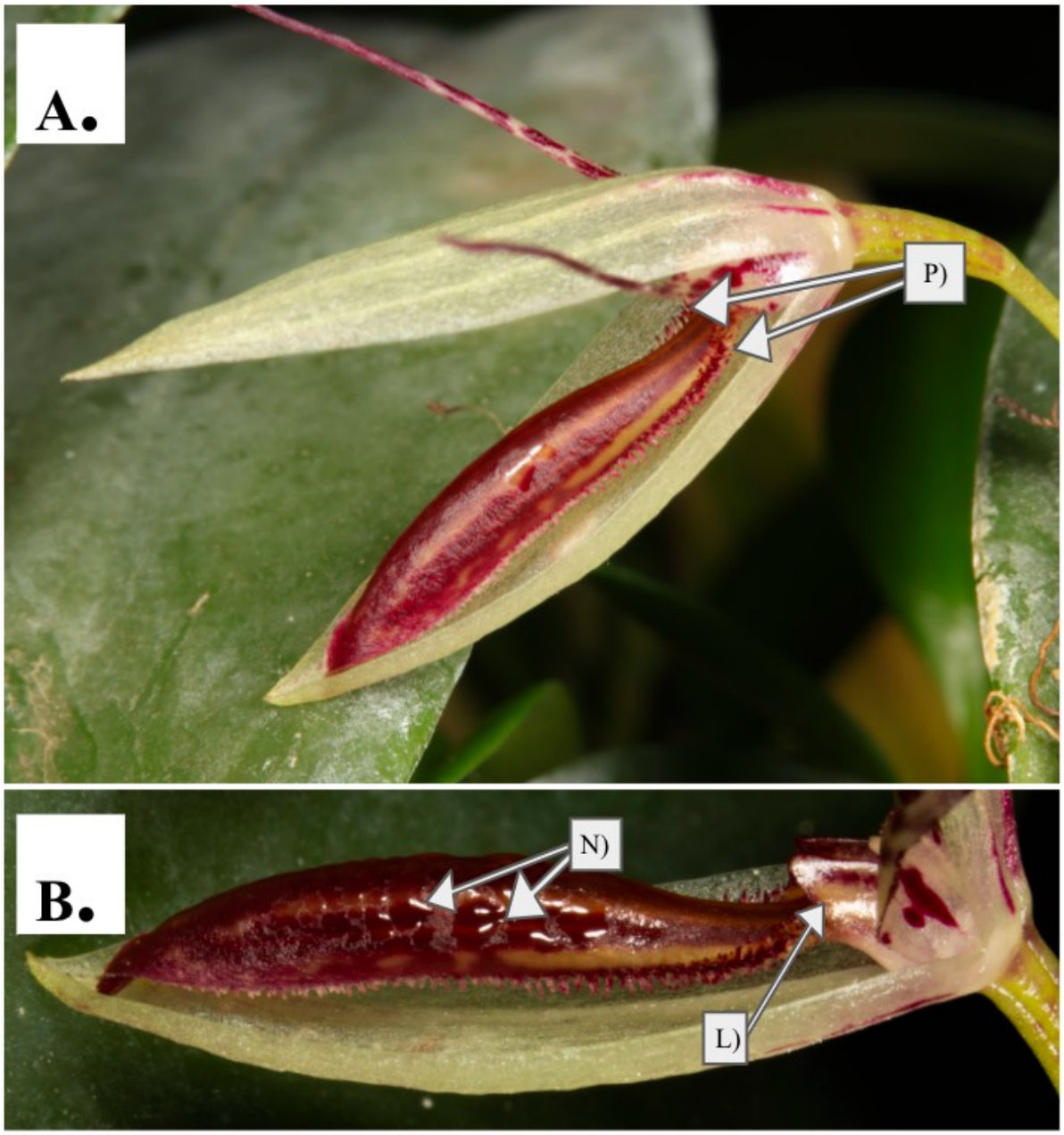

Figure 27.
Photographs of Pleurothallis nuda. A. Whole flower with arrows indicating papillate labellar protrusions. B. Whole lip with arrows identifying nectar-like liquid droplets N) and lateral lobes on the hypochile L). Photos by Mark Wilson.

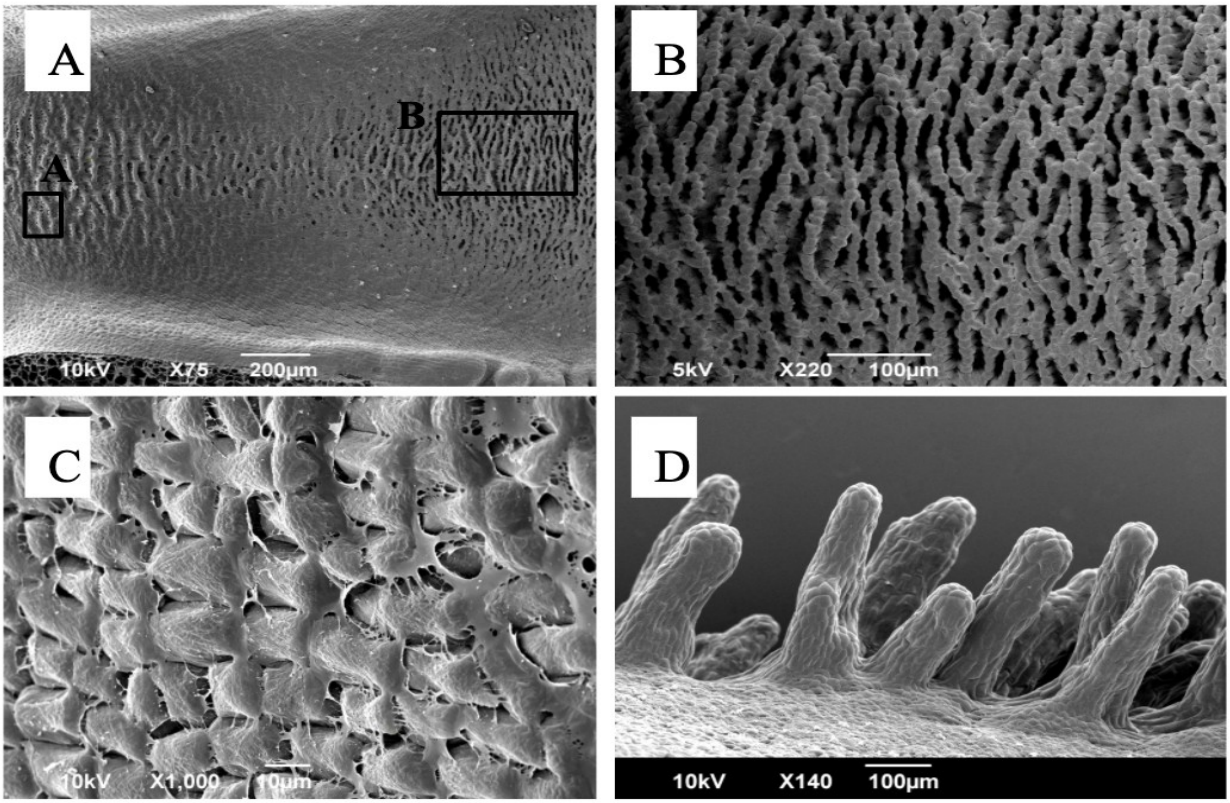

Figure 28.
Electron micrographs of Pleurothallis nuda. A. low magnification photograph of the hypochile and mesochile positioned laterally. B. higher magnification image of the mesochile secretory tissue. C. higher magnification image of the hypochile cells covered in dried liquid. D. multicellular projections on lip edges.

The lip of P. nuda is too large to be mounted on one stub and was therefore divided into two pieces, one being the lip from the hypochile transitioning into the mesochile, and the other being the mesochile and epichile. There appears to be a central path of secretory tissue along the lip (Fig. 28A) characterized by a unique tissue type (Fig. 28B). The potentially secreting tissue can be split into two regions along this central pathway, type one at the lip base (Fig. 28C for close up) and type two on the rest of the lip (Fig. 28B). Along the outer edges of the lip are short papillate protrusions (Fig. 28D) that may function in the release of volatiles chemicals as pollinator attractants.

Pleurothallis aff. nuda

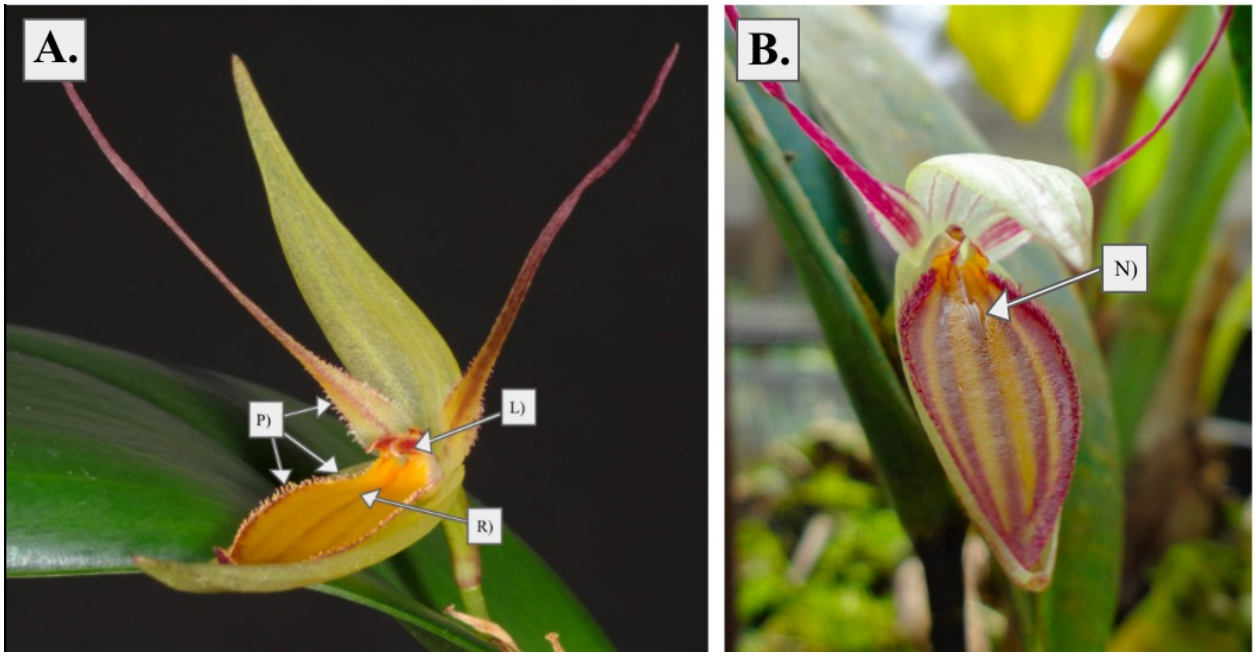

Figure 29.
Photographs of whole Pleurothallis aff. nuda flower. A. arrows pointing to papillate protrusions on the edges of the lip and petals P), the lateral lobes on the epichile L), and the central channel of secretory tissue R). B. arrow showing nectar-like secretion on lip N). Photo A. by Mark Wilson, photo B. by Marcos Romero.

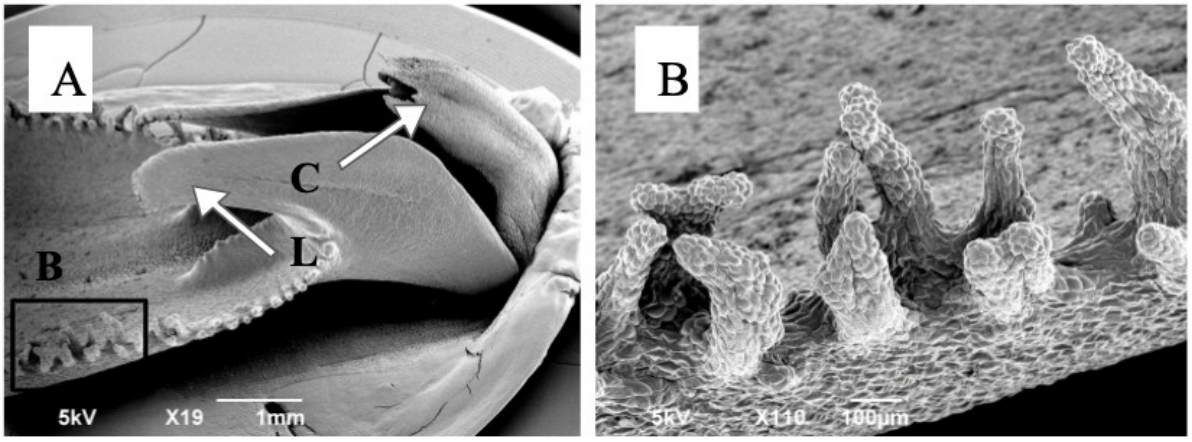

Figure 30.
Electron micrographs of Pleurothallis aff. nuda. A. whole lip and column C), lateral lobes L), and edge protuberances (box). B. higher magnification image of edge protuberances.

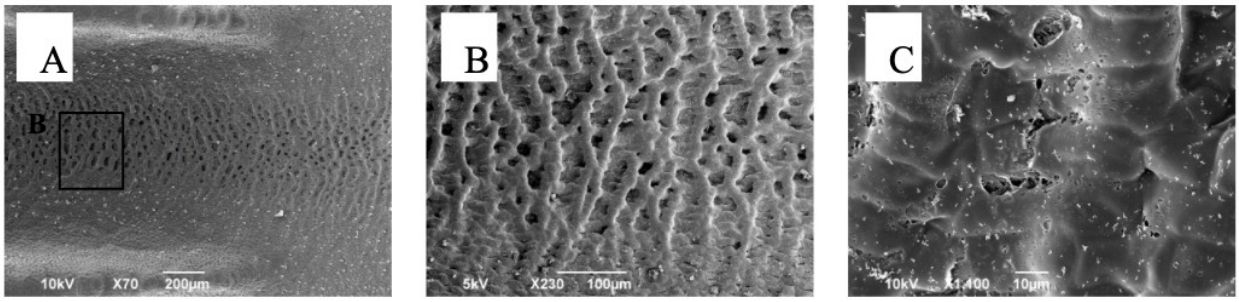

Figure 31.
Electron micrographs of Pleurothallis aff. nuda. A. low magnification image of P. aff. nuda hypochile. B. higher magnification image of secretory tissue on hypochile. C. high magnification of lip base secretory tissue showing dried liquid.

The whole lip, column, and synsepal of P. aff. nuda was mounted whole and photographed (Fig. 30A), after which the column was removed and the lateral lobes were removed to expose the lip base. The lip has two lateral lobes L) that effectively create a narrow space between them to limit insects outside a certain size range. Similar to P. nuda, P. aff. nuda has a path of anatomically similar secretory tissue along the length of the lip. Unlike P. nuda (Fig. 28), the path of secretory tissue is continuous and does not have a separate lip base area. Higher magnification images show a thick layer of dried liquid covering the secretory areas (Fig. 31C).

Subgenus Elongatia

Pleurothallis restrepioides

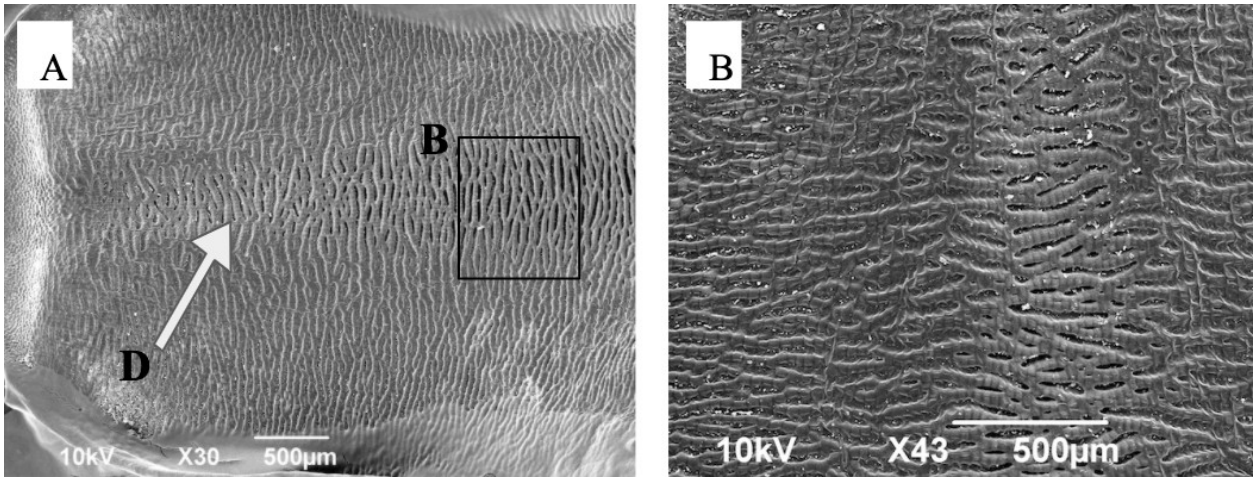

Figure 32.
Electron micrographs of Pleurothallis restrepioides showing A. the lip base and mesochile oriented horizontally, showing a central depression D). B. higher magnification image of secretory tissue.

The lip of P. restrepioides is too large to be imaged in one photograph and was therefore oriented horizontally to include as much surface area as possible (Fig. 32A). The lip has a secretory path along the length of the lip similar to P. nuda (Fig. 28) and P. aff. nuda (Fig. 30). At the base

of the lip there appears to be a slight elongated depression D) that extends into the secreting tissue (Fig. 32B for close up).

Pleurothallis aff. restrepioides

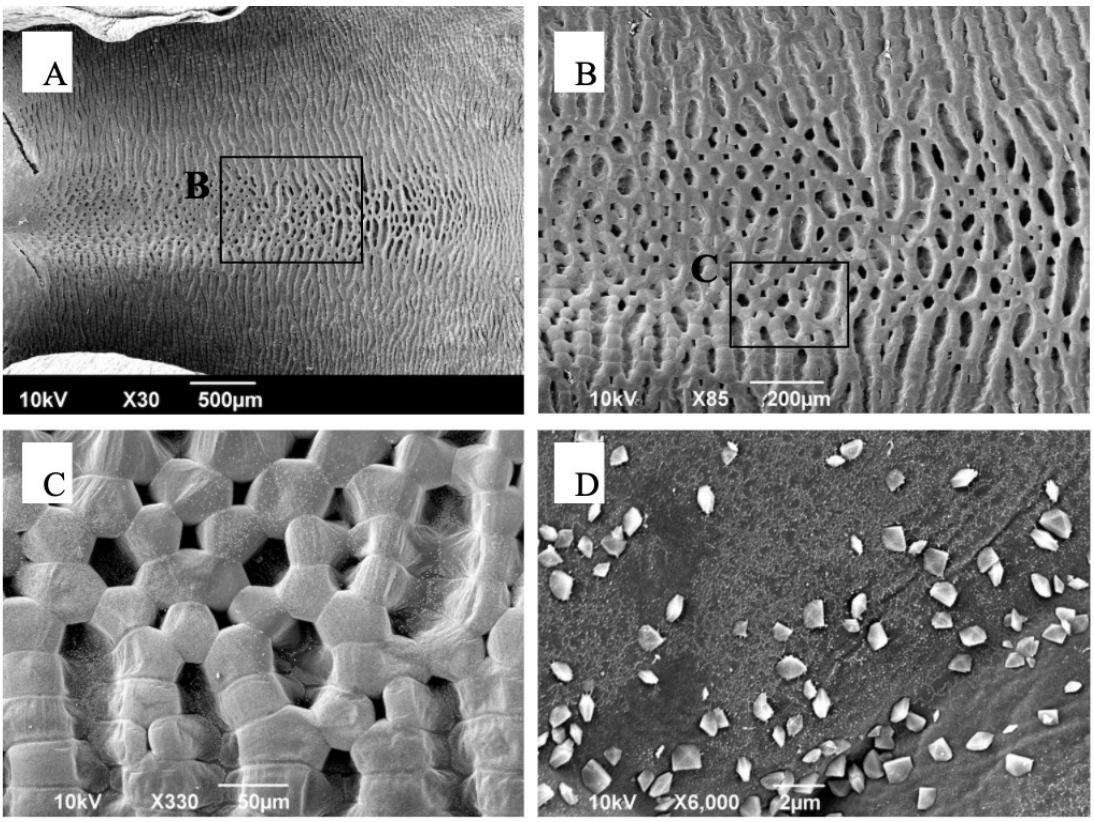

Figure 33.
Electron micrographs of Pleurothallis aff. restrepioides. A. low magnification image showing the lip base oriented horizontally. B. higher magnification image of central secreting tissue. C. high magnification image of secreting tissue. D. high magnification image of secretory cell surface showing crystalline structures.

The lip of P. aff. restrepioides was oriented horizontally to include as much surface area as possible (Fig. 33A). P. aff. restrepioides possesses a central path of secretory tissue extending from the lip base into the mesochile similar to P. restrepioides (Fig. 32), P. nuda (Fig. 28), and P. aff. nuda (Fig. 30 and 31). Unlike other species with this secretory tissue type, secretory cells on the lip of P. aff. restrepioides are covered in small crystal structures of unknown chemical makeup (Fig. 33D).

Pleurothallis sijmii

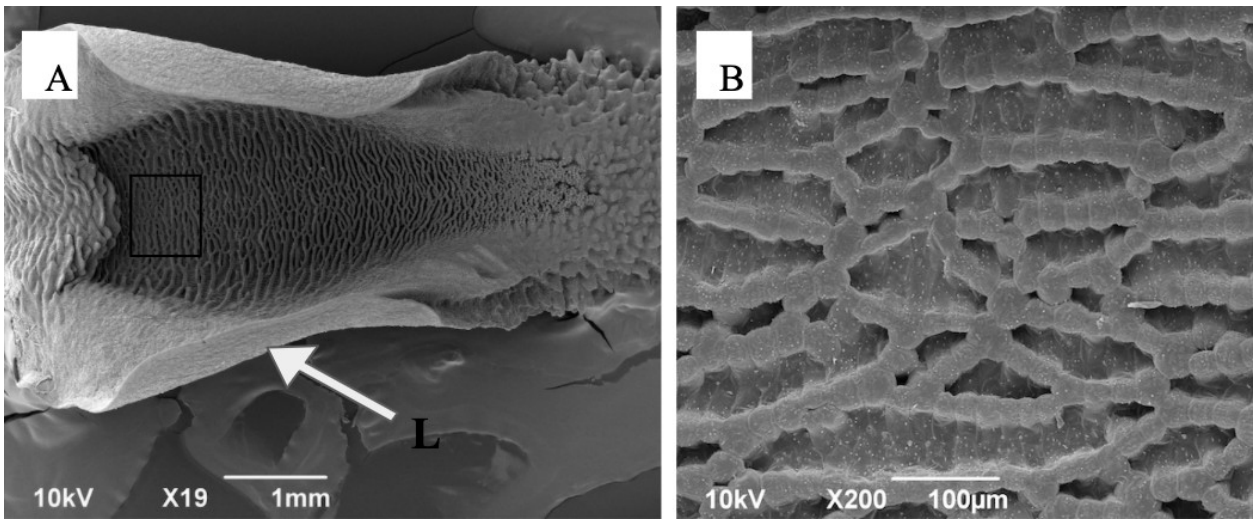

Figure 34.
Electron micrographs of Pleurothallis sijmii. A. low magnification of lip oriented horizontally showing lateral lobes L). B. higher magnification image of secretory tissue from the hypochile.

The lip of P. sijmii possesses a similar secretory cell tissue pattern as P. nuda (Fig. 28), P. aff. nuda (Fig. 30 and 31), P. restrepioides (Fig. 32), and P. aff. restrepioides (Fig. 33) even though P. sijmii is not closely related to these species (Fig. 34). Unlike other species with this cell pattern, P. sijmii does not have a channel of secretory tissue down the center of the lip and instead has secreting tissue covering the whole surface of the lip between the lateral lobes L.

Pleurothallis excelsa

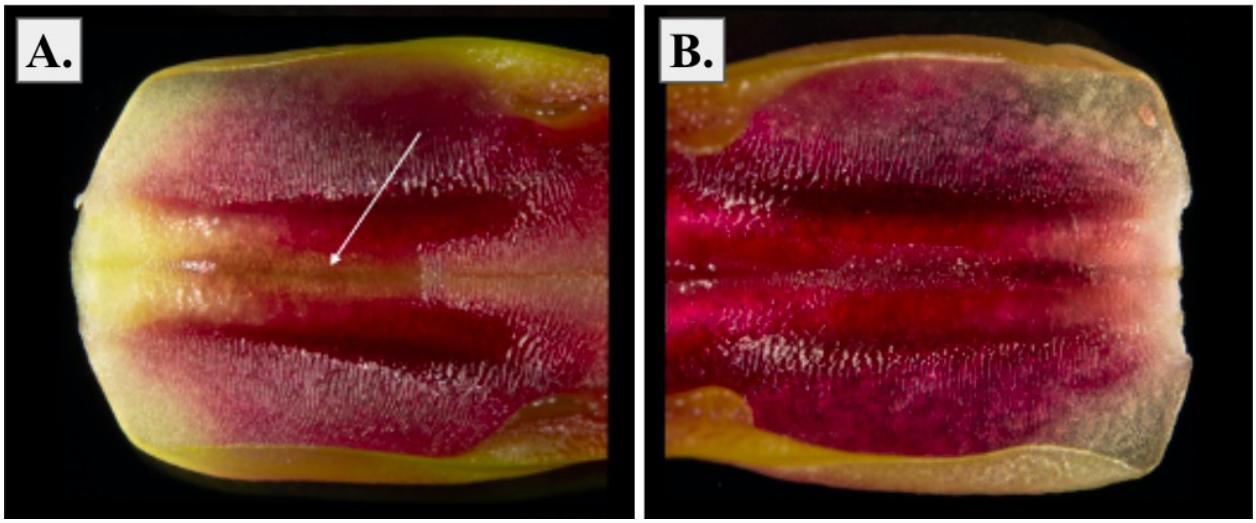

Figure 35.
Close up photographs of Pleurothallis excelsa lip. A. Lip epichile showing pooled nectar-like liquid, arrow identifying central path of secretory tissue. B. Lip hypochile showing secretory tissue wrinkles. Photos taken by Mark Wilson.

P. excelsa also exhibits similar morphologically distinct secretory labellar tissue (Fig. 35) similar to P. nuda (Fig. 28), P. aff. nuda (Fig. 30 and 31), P. restrepioides (Fig. 32), P. aff. restrepioides (Fig. 33), and P. sijmii (Fig. 34). Being a much larger flower than the other Pleurothallis species, SEM was unnecessary since the unique labellar secretory tissue was visible even under a dissecting microscope (Fig. 35A). Nectar-like liquid was observed to accumulate between two calli at the base of the lip (Fig. 36B) supporting the hypothesis that it is a rewarding species.

Subgenus Talpinaria

Pleurothallis sandemanii

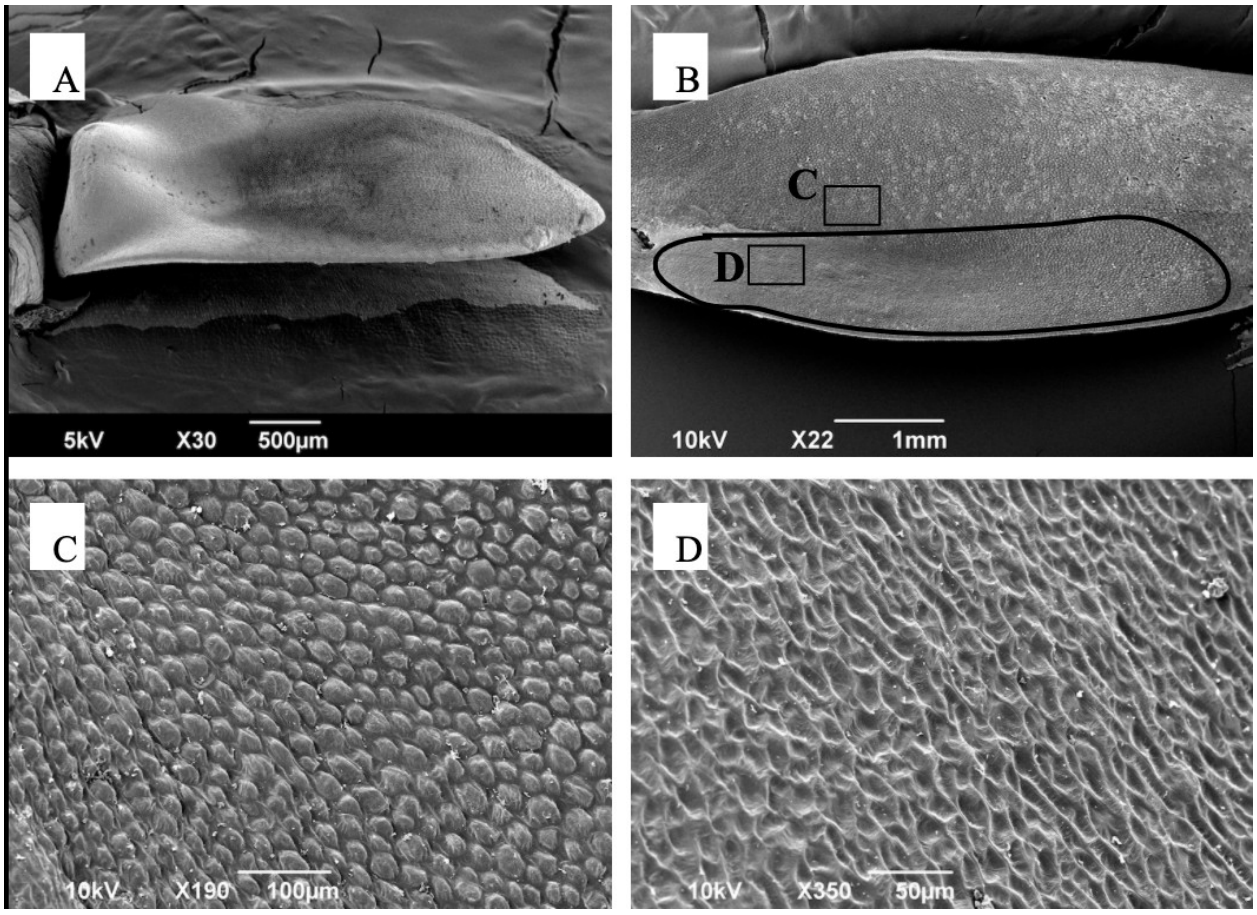

Figure 36.
Electron micrographs of Pleurothallis sandemanii. A. whole lip oriented horizontally tilted to expose vertical topography. B. low magnification image of a P. sandemanii petal with two cell morphologies enclosed in boxes. Presumed secreting region enclosed in black oval. C. higher magnification of non-secreting region of petal. D. higher magnification image of secreting region of petal.

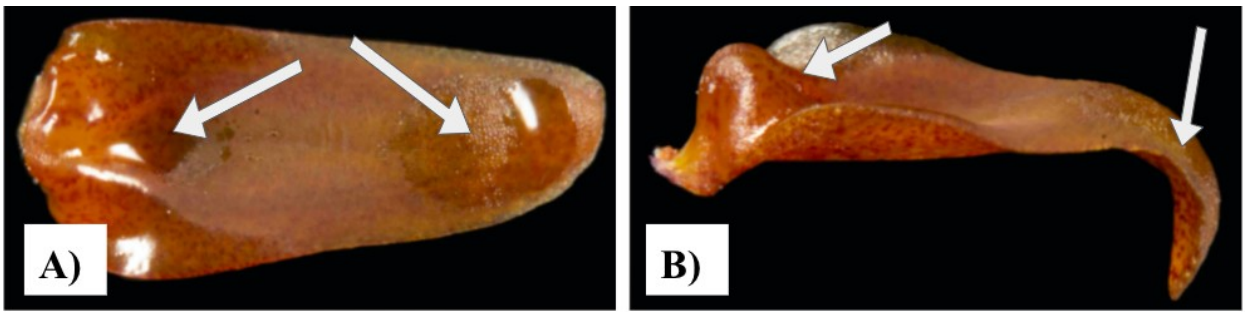

Figure 37. Photographs of fresh isolated Pleurothallis sandemanii labellum taken from above A) and the side B). Arrows identifying pools of nectar-like liquid on the hypochile and epichile. Photos taken by Dr. Mark Wilson.

The lip of P. sandemanii has a relatively uniform surface with only one cell type that is assumed to be the nectar secreting source (Fig. 36A). The petals of P. sandemanii have been observed to have nectar on the lower portion (Figure 36B block circle) but it was of unknown origin. Higher magnification images show two distinct cell morphologies on the petal separated into secreting and non-secreting areas. The presumably non-secreting cells (Fig. 36C) appear normal in their preservation but the presumably secreting cells appear sunken in (Fig. 36D).

Subgenus Pleurothallis subgroup "Mesoamerican"

Pleurothallis sanchoi

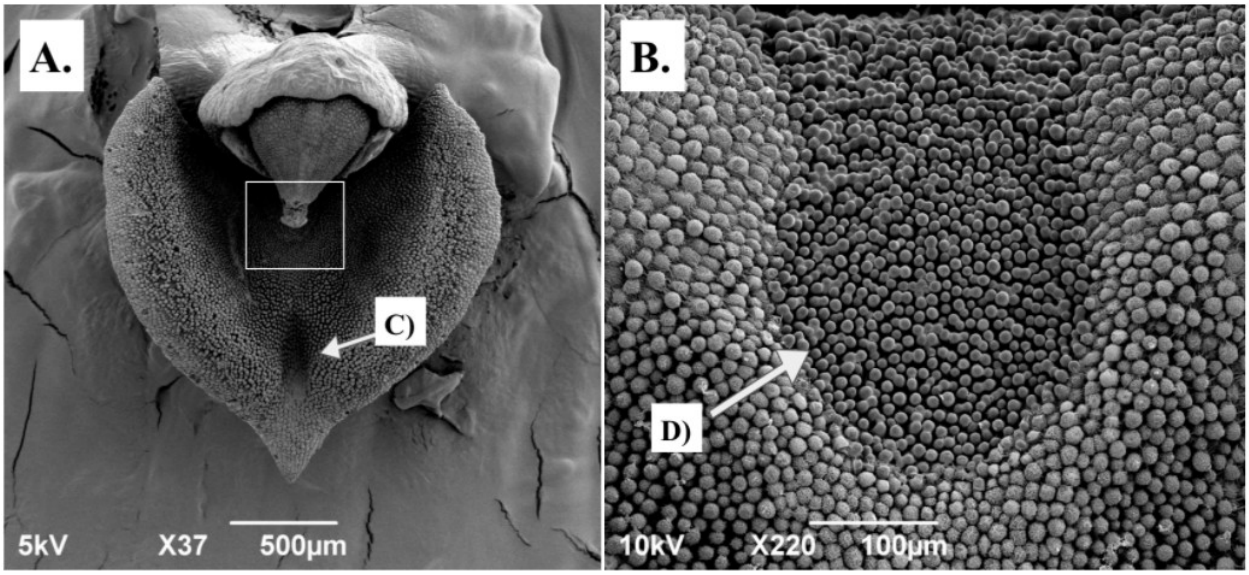

Figure 38.
Electron micrographs of Pleurothallis sanchoi. A. low magnification image of the whole lip and column showing the glenion enclosed in a box and an apical depression C). B. higher magnification of the glenion area D) after the column was removed.

The lip of P. sanchoi was mounted while attached to the synsepal with the column intact and photographed, after which the column was removed to expose the glenion area at the base of the lip (Fig. 38). P. sanchoi is mainly made up of one cell type that covers most of the lip, these cells are round, relatively large, and covered in a cuticle layer. There is an apical depression C) that does not appear to be deep, but also does not resemble the nectar funneling sulcus of similar rewarding species. The glenion D) of P. sanchoi is round and made up of typical glenion cells that are small and smooth in appearance.

Phylogenetic Results

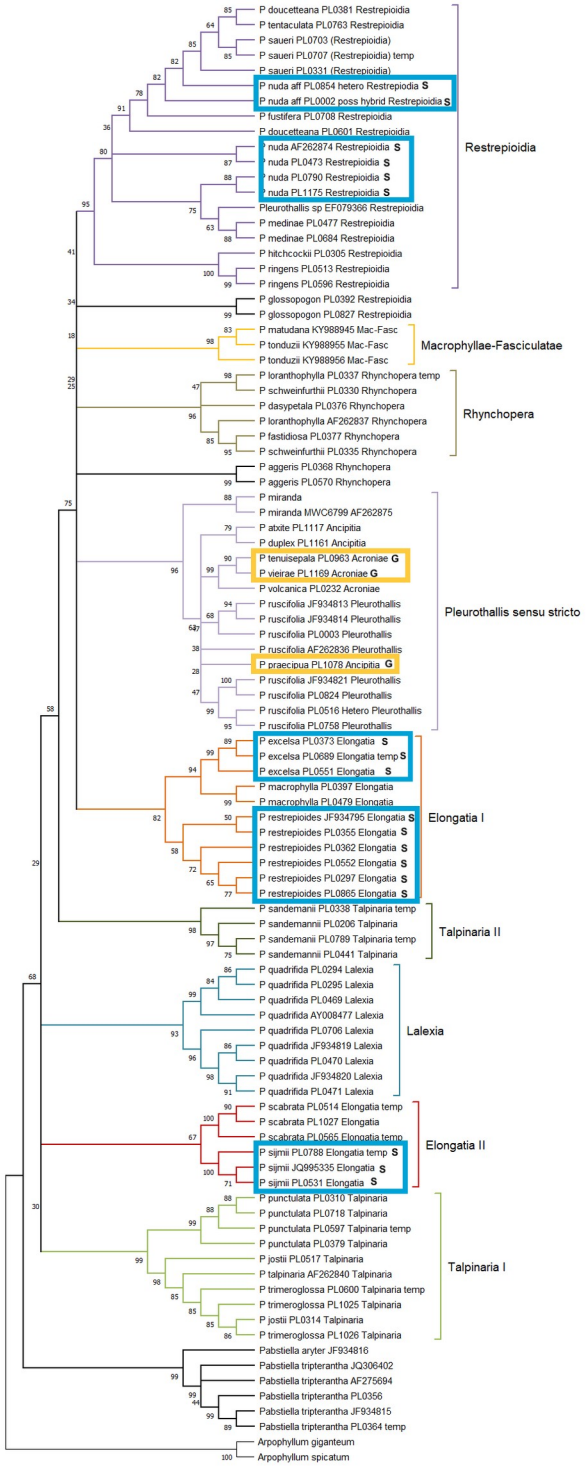

Figure 39. MP ITS phylogeny of Genus Pleurothallis
Maximum parsimony ITS phylogeny with 1000 bootstrap replications, the outgroup is Pabstiella. This ITS phylogeny includes a limited number of species in the Pleurothallis genus representing the seven major subgenera. Secretory tissue of unique morphology (marked with "S" and a blue box) occurs in Restrepioides and Elongatia. Species with distinct glenions (marked "G" with yellow boxes) occur in Pleurothallis sensu stricto and Macrophyllae-Fasciculatae.

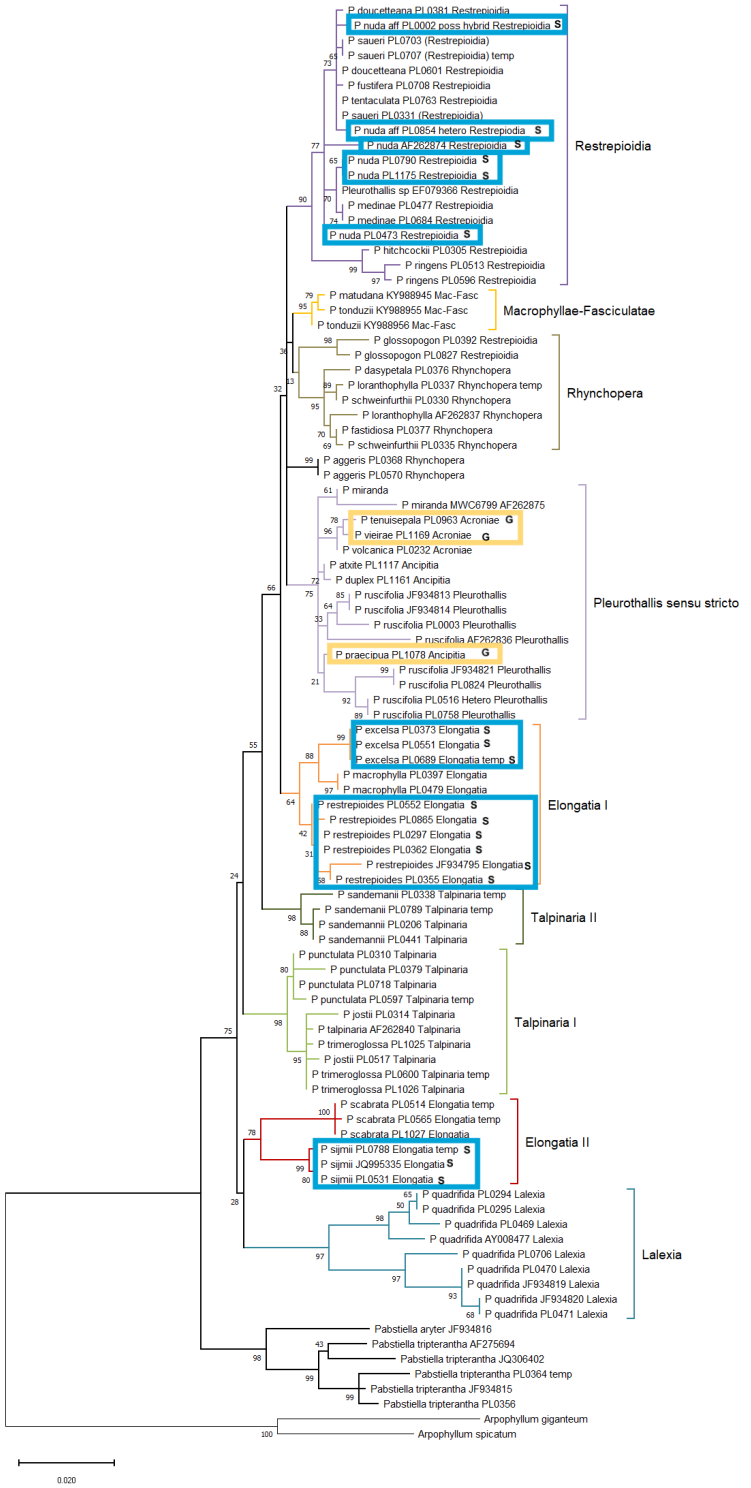

Figure 40. ML ITS phylogeny of Genus Pleurothallis
Maximum likelihood branch length ITS phylogeny with 1000 bootstrap replications, the outgroup is Pabstiella. This phylogeny contains a sample of species from Pleurothallis in the seven major subgenera. Secretory tissue of unique morphology (marked with "S" and a blue box) occurs in Restrepioides and Elongatia. Species with distinct glenions (marked "G" with yellow boxes) occur in Pleurothallis sensu stricto and Macrophyllae-Fasciculatae.

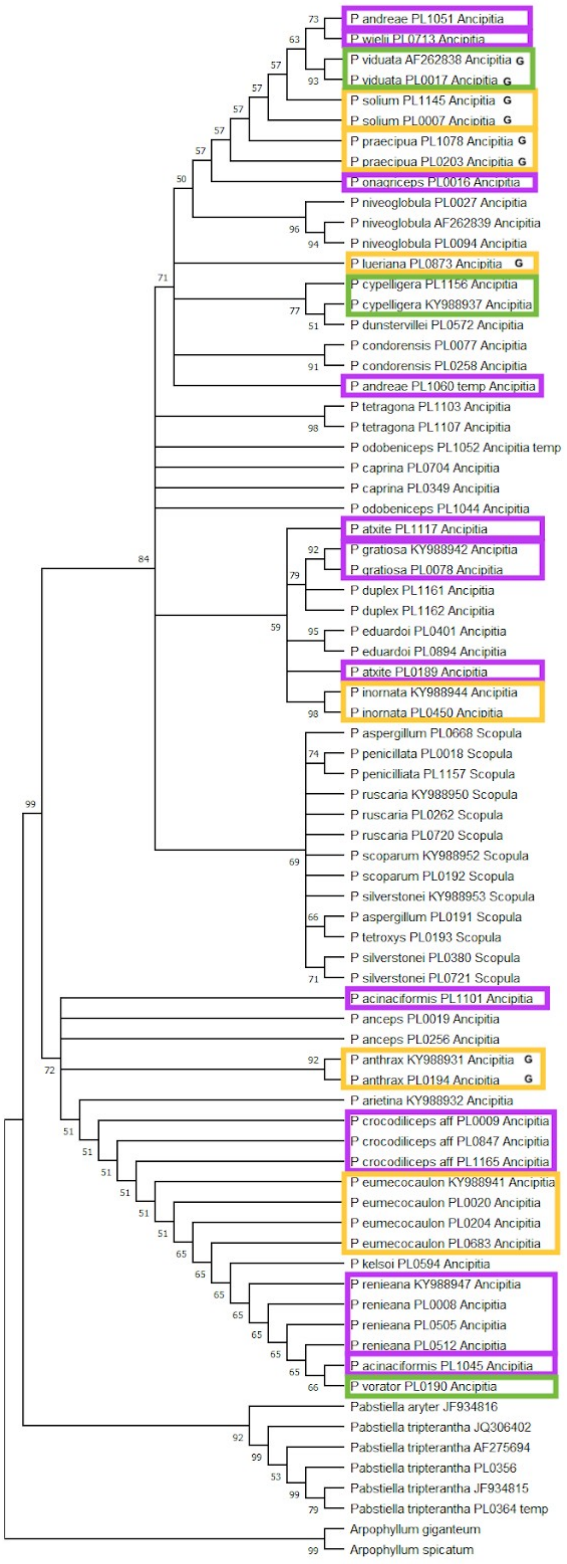

Figure 41. MP ITS Phylogeny of Pleurothallis Subgenus Ancipitia
Maximum parsimony ITS phylogeny of the Ancipitia subsection of Pleurothallis with 1000 bootstrap support nodes below 50% support collapsed. Deceptive species marked with purple boxes, rewarding species marked with yellow boxes, and inconclusive species marked in green. Rewarding species with glenions marked “G”.

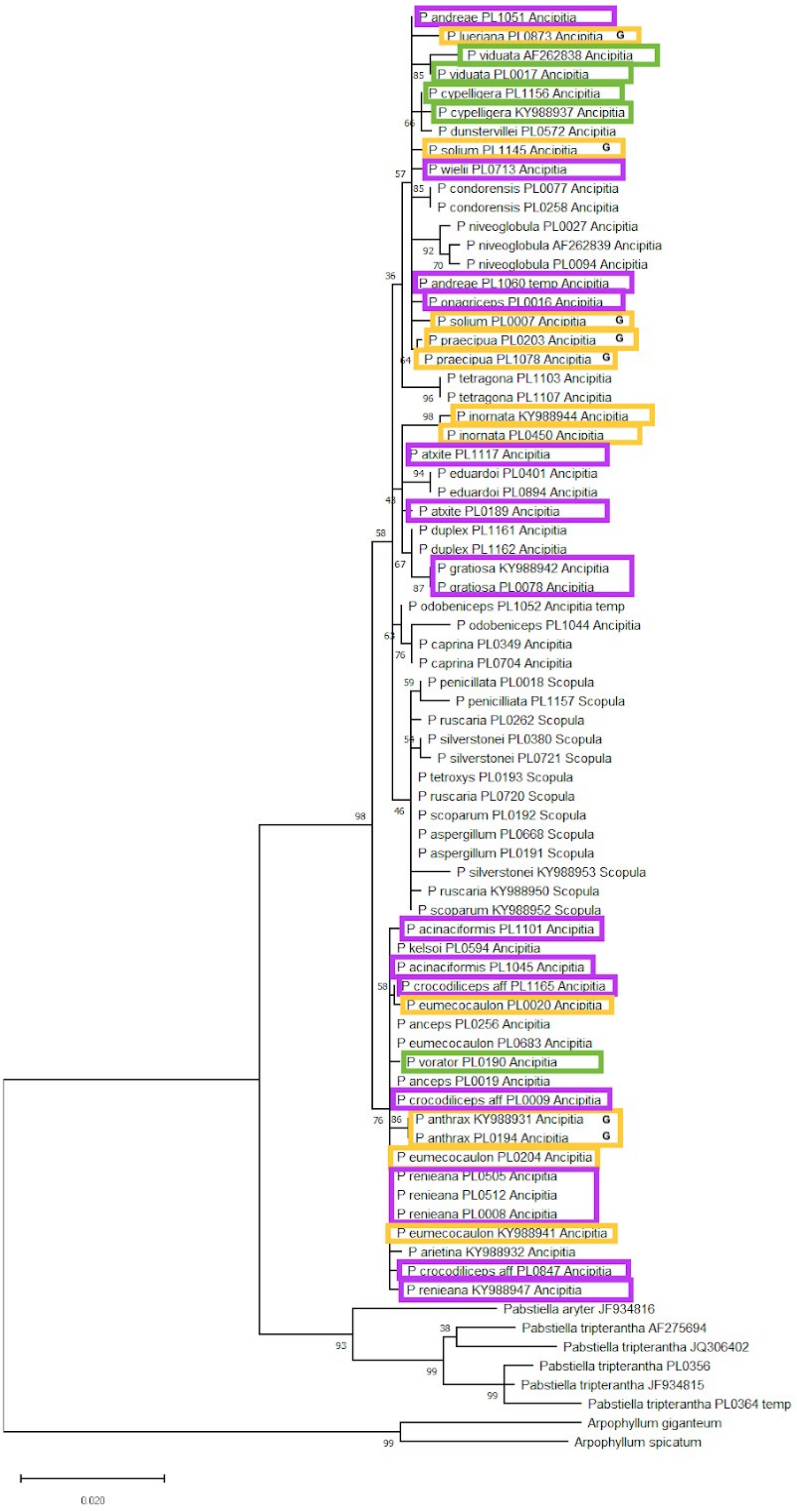

Figure 42. ML ITS Phylogeny of Pleurothallis Subgenus Ancipitia
Maximum likelihood branch length ITS phylogeny of the subsection Ancipitia with 1000 bootstrap support. Deceptive species marked with purple boxes, rewarding species marked with yellow boxes, and inconclusive species marked in green. Rewarding species with glenions marked “G”.

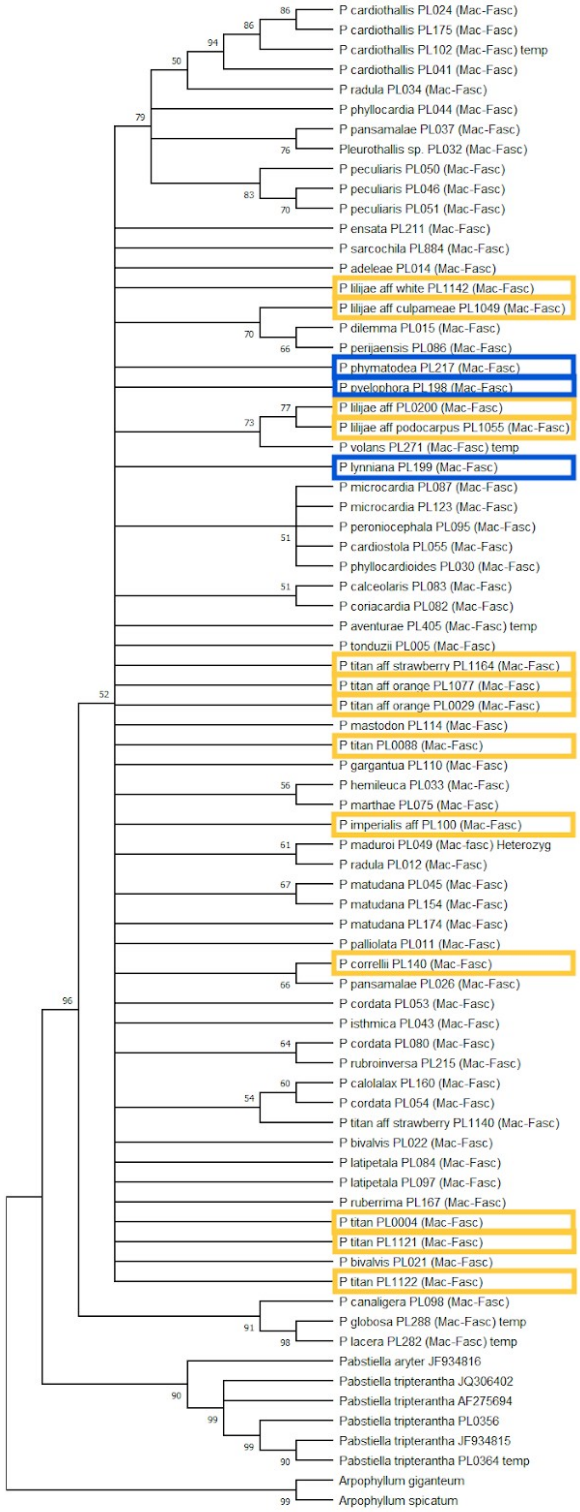

Figure 43. MP ITS Phylogeny of Pleurothallis Subsection Macrophyllae-Fasciculatae
Maximum parsimony ITS phylogeny of subsection Macrophyllae-Fasciculatae with 1000 bootstrap support nodes collapsed below 50%. Gleniform species marked with yellow boxes and non-resupinate species with a distinct pollination strategy marked with dark blue boxes.

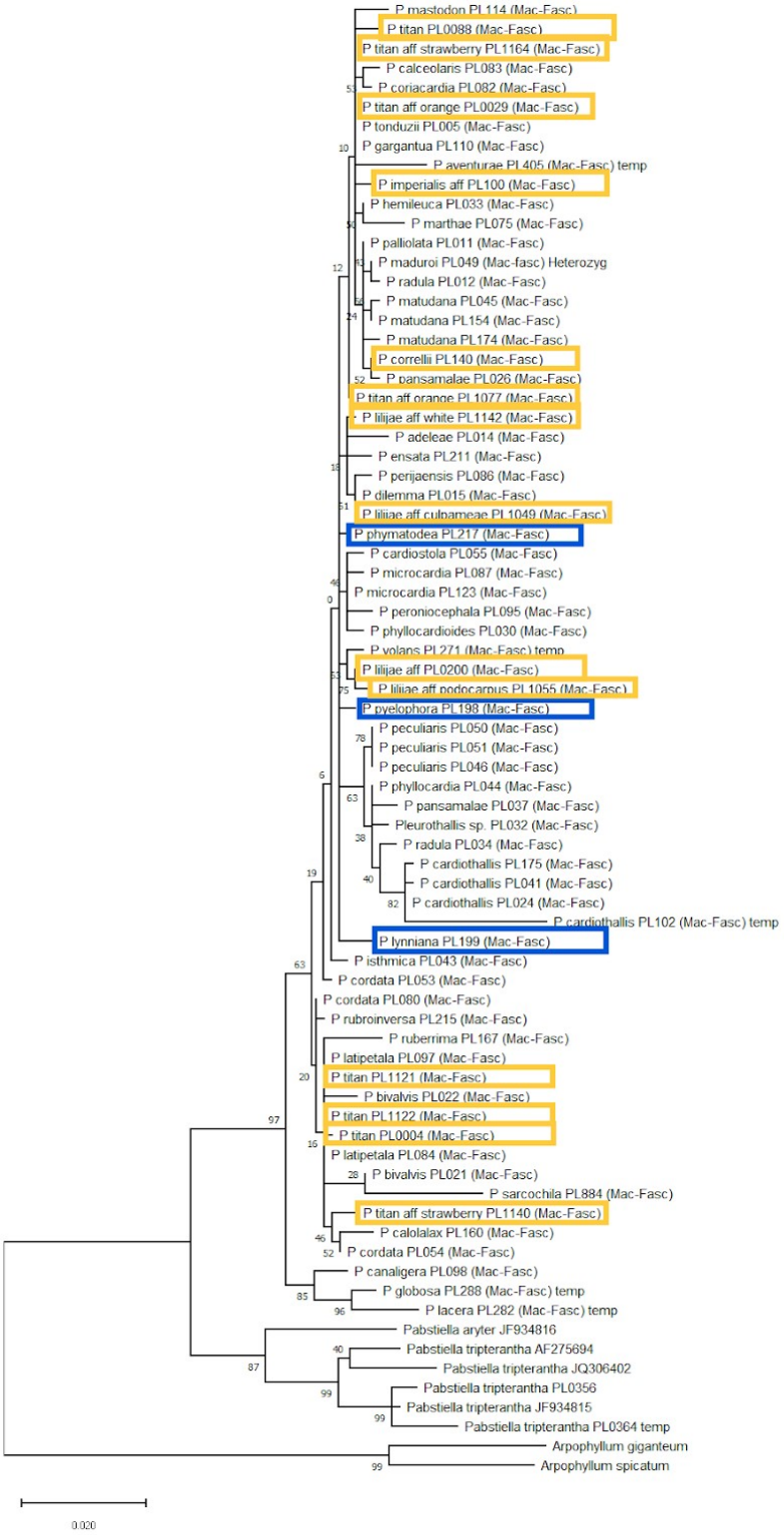

Figure 44. ML ITS Phylogeny of Pleurothallis Subsection Macrophyllae-Fasciculatae
Maximum likelihood branch length ITS phylogeny of subsection Macrophyllae-Fasciculatae with 1000 bootstrap support. Gleniform species marked with yellow boxes and non-resupinate species with a distinct pollination strategy species marked with dark blue boxes.

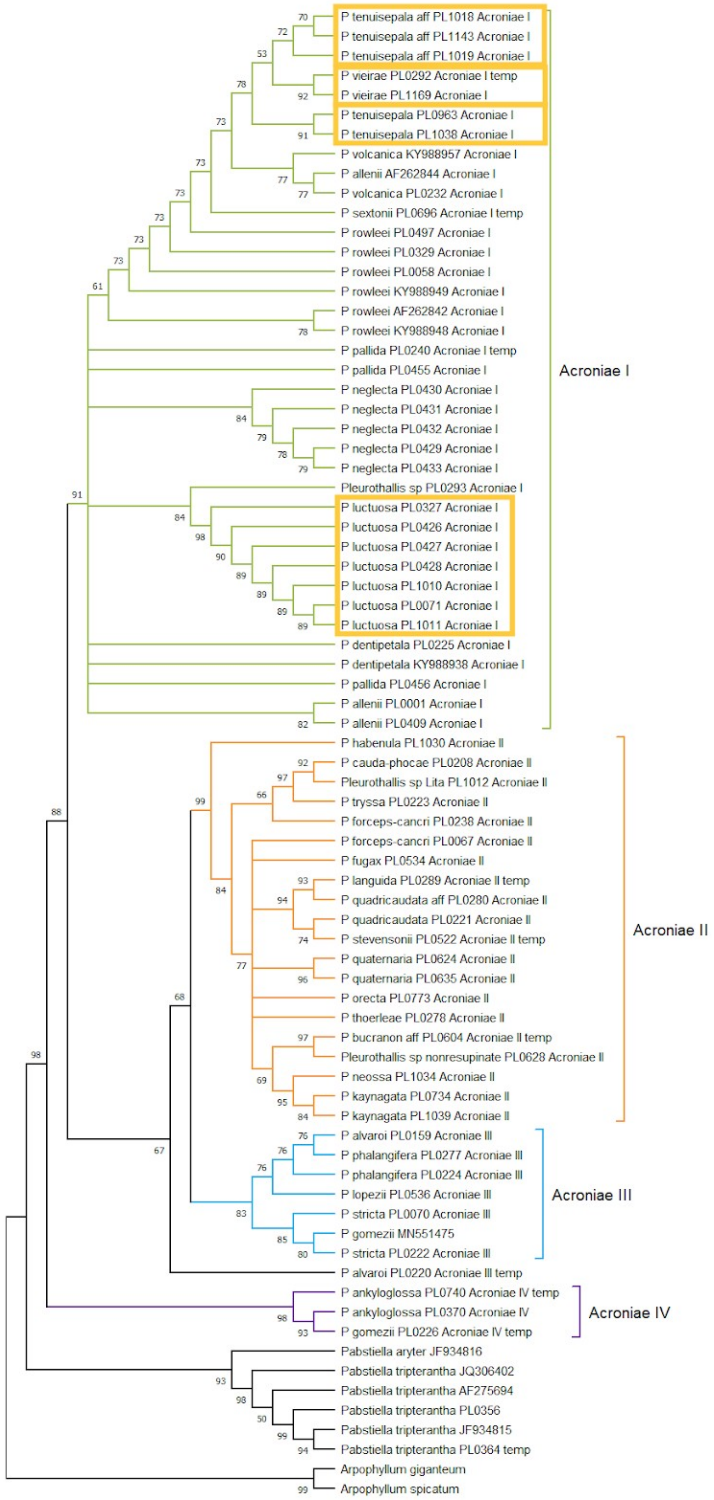

Figure 45. MP ITS Phylogeny of Pleurothallis Subsection Acroniae
Maximum parsimony ITS phylogeny of subsection Acroniae with 1000 bootstrap support, nodes collapsed below 50%. Subsection Acroniae is split into four distinct monophyletic clades, Acroniae I (light green), Acroniae II (orange), Acroniae III (light blue), and Acroniae IV (purple). Gleniform species marked by yellow boxes.

Figure 46. ML ITS Phylogeny of Pleurothallis Subsection Acroniae
Maximum likelihood branch length ITS phylogeny of subsection Acroniae with 1000 bootstrap support. In this phylogeny subsection Acroniae is split into three clades, Acroniae I (light green), Acroniae II (orange), and Acroniae IV (purple). Acroniae III is not as well supported in this phylogeny and has species in two groups. Gleniform species marked by yellow boxes.

Genus-level phylogeny

Very few new ITS sequences were added to the MP and ML Pleurothallis genus phylogenies (Fig. 39 and 40). The new sequences for P. aff. nuda group together, suggesting it is an undescribed species belonging in the subgenus Restrepioidia. The glenion was mapped onto these phylogenies to illustrate the recurring presence of the structure in the subgenus Pleurothallis sensu stricto. Although the Macrophyllae-Fasciculatae species represented on these phylogenies (P. matudana and P. tonduzii) were not examined by SEM in this study, the glenion is also highly represented in this subgenus. Unique labellar secretory tissue was mapped onto these phylogenies to illustrate the presence of this structurally similar tissue type in species from Restrepioidia and Elongatia. This could be an example of convergent evolution towards a successful pollination strategy of rewarding species.

These phylogenies suggest the recognized subgenus Elongatia could be separated into two subgenera, labelled Elongatia I and Elongatia II (Fig. 39 and 40). These two clades have moderate support with 82% and 67% bootstrap support in the MP phylogeny (Fig. 39) and the 64% and 78% bootstrap support in the ML phylogeny (Fig. 40). The subgenus Talpinaria also appears polyphyletic in these phylogenies and could be split into two subgenera, labelled Talpinaria I and Talpinaria II (Fig. 39 and 40). These clades are well supported with 99% and 98% bootstrap support in the MP phylogeny (Fig. 39), and 98% and 98% support in the ML phylogeny (Fig. 40). However, the only species included in what could be considered Talpinaria II is P. sandemanii, so perhaps this species should just be removed from subgenus Talpinaria.

Ancipitia phylogeny

Several ITS sequences were added to the MP and ML phylogenies for the subsection Ancipitia (Fig. 41 and 42). Both rewarding and deceptive species are numerous in this subsection, and occur in several clades. Additional SEM work to continue mapping rewarding and deceitful species in this subsection is necessary to propose a historical pollination strategy. For Pleurothallis, the ancestral pollination syndrome is hypothesized to be rewarding because the majority of species in the genus have rewarding characteristics. However, it is possible there is a clade within a subsection like Ancipitia where the common ancestor was deceitful and reward pollination has evolved secondarily. The glenion presents in several species in the subsection, but can neither be classified as ancestral or acquired in any individual clades without further morphological data.

Macrophyllae-Fasciculatae Phylogeny

The phylogenetic trees for subsection Macrophyllae-Fasciculatae show one large polytomy with insufficient differentiation between the species to form any distinct clades (Fig. 43 and 44). This is likely representative of the rapid diversification possible in orchids over evolutionarily short timescales. The glenion is a common feature in this subgenus, occurring in some form or another in almost all of the P. aff. lilijae species analyzed, the only exceptions being P. aff. lilijae “Riksen” and P. pyelophora both of which have a deep cavity that prevented visualization of the lip base. It is possible that reward pollination, and gleniform pollination specifically, is ancestral to this subgenus. Crateriform species are also mapped onto these phylogenies and do not form a monophyletic clade.

Acroniae Phylogeny

MP and ML phylogenetic trees of the subsection Acroniae show a few well supported clades (Fig. 45 and 46). In the MP phylogenetic tree, four clades, Acroniae I-IV, all have high bootstrap support, 91, 99, 83, and 98 respectively. In the ML tree, only Acroniae I, II, and IV are well supported, with bootstrap values of 91, 97, and 95 respectively. In this tree Acroniae III is not supported as a group and appears as two separate groups in a polytomy. In this study, ITS sequences were added only for species in Acroniae I (P. tenuisepala, P. aff. tenuisepala, and P. vieirae). Species with a glenion were identified only in Acroniae I, but they are very likely to occur in the other clades as well.

LC-MS

Using LC-MS data we were able to identify and quantify the carbohydrates fructose, glucose, and sucrose on several flower samples (Table 2). When whole flower and lip samples produced high amounts of sugar molecules (total >100 ppm) this was considered high support for that species being classified as rewarding. Medium sugar production of 20-100 ppm total carbohydrates, was also considered support for that species being rewarding.

Some species that morphologically appear to employ the sexual deception pollination strategy and group with other sexually deceptive orchids in the volatile analysis (P. acinaciformis and P. gratiosa), still showed carbohydrates in the LC-MS data. The values for the deceptive species were P. acinaciformis 2.16 ppm total carbohydrates and P. gratiosa 6.11 ppm total carbohydrates. These values are relatively low carbohydrates readings and could represent sugars that leaked from the phloem of the broken flower stalk. For this reason, all LC-MS data taken on torn floral tissue might be expected to show some low level of carbohydrates. However, at this preliminary stage of data analysis it is difficult to create a cutoff value that indicates a species is producing extra carbohydrates to act as a reward, and impossible to use this data to say definitively that a species is not rewarding. There are several reasons why a rewarding species would not register high levels of carbohydrates. For example, various floral organs may or may not produce a nectar solution including the petals, sepals, and lip. There may be significant natural variation in the amount of nectar produced by each flower even on the same inflorescence. There may also be a very specific window of nectar production after anthesis that can only be identified by noting the opening of the flower bud and sampling flowers at daily intervals afterwards. In the current data set some of the repeated samples from the same species show very different amounts of carbohydrates. Due to the potential for variation between flowers, any sample with an increased amount of carbohydrates will be considered evidence of a rewarding species.

Although the amount of carbohydrates considered evidence for rewarding varied significantly (20.72-463 ppm), this will not be used to classify species as more or less rewarding. The method of data collection included different combinations of floral parts in some samples, and multiple whole flowers or lips in some samples, making an absolute comparison of the total sugar content between species impossible. Therefore, claims will only be made to the degree that LC-MS data supports or does not currently support the hypothesis that a given species is producing a reward.

Currently the data provide evidence that P. aff. titan “strawberry lip”, P. aff. lilijae “culpameae”, P. aff. lilijae “white”, P. aff. lilijae “Podocarpus”, P. scurrula, P. pyelophora, P. sanchoi, P. nuda, P. sijmii, P. vieirae, and P. sandemanii are all rewarding species. Further LC-MS analyses will likely identify additional rewarding species.

Table 2. LC-MS data for Pleurothallis specimens processed in the Colorado College Chemistry and Biochemistry department by Murphy Brasuel ar
collected for each sample for each sugar type (first half of table) was corrected to a standard volume of 1 mL (second half of table). Resulting total s
coded; lowest <10 ppm (orange), low 10-20 ppm (yellow), medium 20-100 ppm (green), and high >100 ppm (blue).

Species	Sample #	Washate Vol (mL)	PPM Fruct	PPM Gluc	PPM Sucr			Other Disaccharides (not sucrose)	PPM All	Correction factor =(X mL/1 mL)	Corrected PPM Fruct	Corrected PPM Gluc	Corrected PPM Sucr	
BATCH #1														
P. sijmii (lip)	#1	1.00	25.00	3.00	6.00			2.00	36.00	1.00	25.00	3.00	6.00	
P. sandemanii type 1 (lip+petals)	#2	1.00	22.00	2.00	4.00			1.00	29.00	1.00	22.00	2.00	4.00	
P. sijmii (lip)	#3	1.00	23.00	3.00	11.00			2.00	39.00	1.00	23.00	3.00	11.00	
P. sandemanii type 1 (4 petals)	#4	1.00	27.00	2.00	2.00			1.00	32.00	1.00	27.00	2.00	2.00	
P. sandemanii type 1 (2 lips)	#5	1.00	21.00	3.00	4.00			1.00	29.00	1.00	21.00	3.00	4.00	
P. nuda (1 flower)	#6	1.00	165.00	13.00	27.00			0.00	205.00	1.00	165.00	13.00	27.00	
P. nuda (1 flower)	#7	1.00	163.00	15.00	24.00			0.00	202.00	1.00	163.00	15.00	24.00	
P. pyelophora (1 flower)	#8	1.00	309.00	82.00	71.00			1.00	463.00	1.00	309.00	82.00	71.00	
P. aff. titan "strawberry lip" (lip)	#9	1.00	3.00	1.00	1.00			0.00	5.00	1.00	3.00	1.00	1.00	
P. correllii (10 flowers)	#10	1.00	9.00	1.00	2.00			1.00	13.00	1.00	9.00	1.00	2.00	
P. nuda (lip)	#11	1.00	17.00	3.00	0.00			0.00	20.00	1.00	17.00	3.00	0.00	
P. scurrula (lip)	#12	1.00	39.00	10.00	7.00			0.00	56.00	1.00	39.00	10.00	7.00	
P. aff. lilijae white" (1 flower)	#13	1.00	49.00	10.00	10.00			0.00	69.00	1.00	49.00	10.00	10.00	
P. pyeophora (1 flower)	#14	1.00	25.00	12.00	22.00			1.00	60.00	1.00	25.00	12.00	22.00	
P. aff. lilijae "culpameae" (1 flower)	#15	1.00	29.00	6.00	3.00			0.00	38.00	1.00	29.00	6.00	3.00	
P. praecipua (1 flower)	#16	1.00	1.00	0.00	2.00			0.00	3.00	1.00	1.00	0.00	2.00	
P. sijmii (lip)	#17	1.00	18.00	3.00	2.00			3.00	26.00	1.00	18.00	3.00	2.00	
P. sijmii (2 lips)	#18	1.00	92.00	11.00	11.00			3.00	117.00	1.00	92.00	11.00	11.00	
BATCH #2	Sample #	Washate Vol	PPM Fruct	PPM Gluc	PPM Sucr	PPM Lact	PPM Malt	PPM Unknown (est)	PPM All	Correction factor	Corrected PPM Fruct	Corrected PPM Gluc	Corrected PPM Sucr	Corrected PPM Lact
P. correllii (5 lips)	#19	0.50	7.77	0.59	3.78	0.00	0.98	0.25	13.37	0.50	3.89	0.30	1.89	0.00
P. praecipua (4 lips)	#20	1.00	1.01	0.30	7.30	0.00	0.00	0.00	8.61	1.00	1.01	0.30	7.30	0.00
P. pyelophora (2 flowers)	#21	1.00	26.99	28.07	110.74	0.00	5.23	0.00	171.03	1.00	26.99	28.07	110.74	0.00
P. vieirae (1 flower)	#22	1.00	21.14	3.02	17.46	0.40	0.40	0.00	42.42	1.00	21.14	3.02	17.46	0.40
P. sanchoi (2 flowers)	#23	1.00	5.62	4.11	10.99	0.00	0.00	0.00	20.72	1.00	5.62	4.11	10.99	0.00
P. gratiosa (3 flowers)	#24	1.00	0.28	0.40	5.17	0.13	0.13	0.00	6.11	1.00	0.28	0.40	5.17	0.13
P. sandemanii type 1 (1 flower)	#25	1.00	10.41	1.24	1.71	0.00	0.12	0.00	13.48	1.00	10.41	1.24	1.71	0.00
P. cypelligera (1 flower)	#26	0.25	0.24	0.25	10.32	0.00	0.00	0.00	10.81	0.25	0.06	0.06	2.58	0.00
P. fustifera (1 flower)	#27	1.00	5.65	0.96	0.85	0.00	0.00	0.00	7.46	1.00	5.65	0.96	0.85	0.00
P. praecipua (lip?)	#28	0.08	present not	present not	2.33	0.00	0.00	0.00	2.33	0.08			0.19	0.00
P. scurrula (lip)	#29	0.08	83.37	69.80	5.98	0.00	0.18	0.00	159.33	0.08	6.67	5.58	0.48	0.00
P. aff. scrabrilinguis (lip)	#30	0.08	19.10	13.77	10.29	0.00	1.05	0.00	44.21	0.08	1.53	1.10	0.82	0.00
P. sp. "garnet lip" (1 lip)	#31	0.20	2.64	1.70	0.51	0.00	0.00	0.00	4.85	0.20	0.53	0.34	0.10	0.00
P. octavioi (lip)	#32	0.20	8.44	4.11	1.63	0.00	0.09	0.00	14.27	0.20	1.69	0.82	0.33	0.00
P. cypelligera (1 flower)	#33	0.20	0.40	0.13	11.75	0.00	0.00	0.00	12.28	0.20	0.08	0.03	2.35	0.00
P. schweinfurthii (1 flower)	#34	0.40	5.93	3.23	1.23	0.00	0.00	0.00	10.39	0.40	2.37	1.29	0.49	0.00
P. schweinfurthii (1 flower)	#35	0.40	2.91	1.87	0.87	0.00	0.00	0.00	5.65	0.40	1.16	0.75	0.35	0.00
P. acinaciformis (1 flower)	#36	0.40	2.77	1.72	0.90	0.00	0.00	0.00	5.39	0.40	1.11	0.69	0.36	0.00
P. nuda (lip)	#37	0.40	1.48	0.49	0.54	0.00	0.00	0.00	2.51	0.40	0.59	0.20	0.22	0.00
P. nuda (lip)	#38	1.00	2.77	0.68	0.27	0.00	0.00	0.00	3.72	1.00	2.77	0.68	0.27	0.00
P. aff. scabrilinguis (1 flower)	#39	0.40	3.72	3.13	4.02	0.59	0.00	0.00	11.46	0.40	1.49	1.25	1.61	0.24
P. aff. acestrophylla (3 flowers)	#40	0.40	3.24	1.72	30.33	0.14	0.00	0.00	35.43	0.40	1.30	0.69	12.13	0.06
P. aff. lilijae "podocarpus" (1 flower)	#41	0.40	148.01	21.15	24.20	0.00	1.39	0.00	194.75	0.40	59.20	8.46	9.68	0.00
P. sp. "garnet lip" (1 flower)	#42	0.30	14.52	3.83	2.56	0.00	0.00	0.00	20.91	0.30	4.36	1.15	0.77	0.00
P. ruberrima (lip+synsepal)	#43	0.40	9.83	4.73	0.50	0.07	0.00	0.00	15.13	0.40	3.93	1.89	0.20	0.03
P. microcardia (1 flower)	#44	0.50	7.69	22.02	7.66	1.51	0.00	0.00	38.88	0.50	3.85	11.01	3.83	0.76
P. aff. titan "strawberry lip" (lip)	#45	0.75	22.43	3.99	1.40	0.21	0.00	0.00	28.03	0.75	16.82	2.99	1.05	0.16
P. vieirae (2 lips)	#46	0.05	45.14	7.46	92.70	0.00	6.66	0.00	151.96	0.05	2.26	0.37	4.64	0.00
P. aff. nuda (lip)	#47	1.00	1.44	0.59	1.94	0.07	0.00	0.00	4.04	1.00	1.44	0.59	1.94	0.07
	#48		12.91	0.75	9.59	0.20	0.20	0.00	23.65		0.00	0.00	0.00	0.00
	#49		30.27	6.93	2.42	0.00	0.00	0.00	39.62		0.00	0.00	0.00	0.00
	#50		0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00

Discussion

Rewarding Species: Rugose Tissue and the Glenion

Five species were observed with SEM to have morphologically similar secretory tissue (Fig. 47). We refer to the tissue type as "rugose" because low magnification images of the tissue on P. aff. restrepioides, on which the tissue was first visualized, make it look like ripples on sand or wrinkles. The tissue is hypothesized to be the location of nectar secretion on each of the species because it occurs either in a channel down the center of the lip (P. nuda, P. aff. nuda, P. restrepioides, and P. aff. restrepioides), or covers almost all of the lip surface between the lateral lobes (P. sijmii). This would suggest that the tissue has a role in leading the pollinator towards the base of the lip to increase contact with the pollinarium. LC-MS data supports the hypothesis that P. nuda and P. sijmii are both rewarding species. LC-MS data does not currently support the hypothesis that P. aff. nuda is rewarding, but it was only sampled once and the flower may not have been secreting at that time. Photographs of P. aff. nuda with nectar-like secretions (Fig. 29B) suggest a reward is produced, although possibly not a sugar containing one. If all of these species are nectar rewarding, that could be tied to this tissue type, and it could potentially become a new morphological indicator of nectar secreting flowers. The similarity in the appearance and texture of the tissue, represented in two different Pleurothallis subgenera strongly suggests convergent evolution towards a common pollination strategy (Fig. 39 and 40). It is likely this tissue type will be discovered to occur in other species in these subgenera, or perhaps even in other subgenera within Pleurothallis.

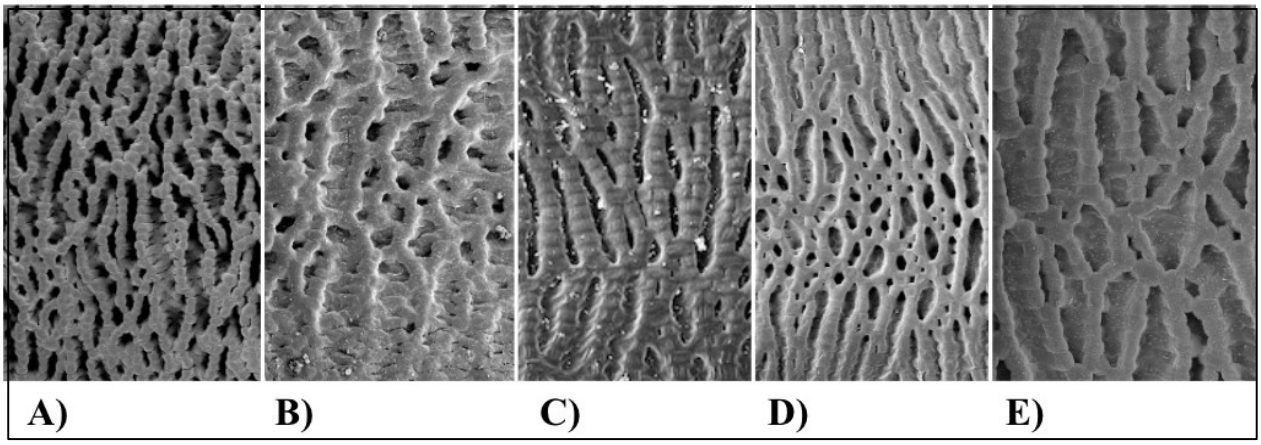

Figure 47.
Comparing secretory tissue similarities on the center of the lip for several species. A. P. nuda. B. P. aff. nuda. C. P. restrepioides. D. P. aff. restrepioides. E. P. sijmii.

Identifying the presence of a glenion with SEM was one of the predominant goals of this study. In the Pleurothallis subsections Ancipitia, Acroniae, and Macrophyllae-Fasciculatae, seventeen species were identified to have some sort of glenion tissue. Previous studies have described a typical glenion as a small round area of secretory cells at the base of the lip, made up of smaller less densely packed cells with a smooth surface texture (Zhao, 2019). In this study, a group of cells at the base of the lip that were distinguishable from the rest of the lip surface was considered a glenion. This removes the emphasis from the structural elements and redefines the glenion as a location. The proposed function of the glenion, to aid in positioning the pollinator at

the base of the lip, could be achieved by many vastly different morphological configurations and this new definition does not exclude atypical glenions from consideration.

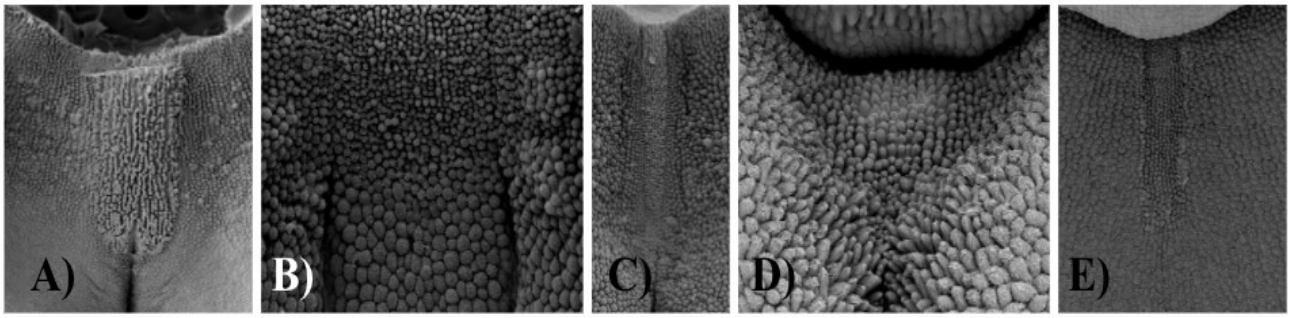

Figure 48.
Comparing the glenion structure variation in Pleurothallis subsection Ancipitia. A. P. lueriana. B. P. solium. C. P. anthrax. D. P. viduata. E. P. praecipua.

Within Ancipitia there is high variation between the glenions. Some glenions appear elongated and narrow (P. lueriana, P. anthrax, and P. praecipua), while others are very small and all but covered by the column (P. viduata). The glenion region of P. solium does not have a clearly defined shape, but does have cells with globules that may result from a dried secretion (Fig. 45). Glenions were imaged on two species in subsection Acroniae (P. vieirae and P. aff. tenuisepala), and closely resemble one another (Fig. 49). These glenions are both very circular, and contain the most typical glenion cell type. Both are nested in a shallow well with a sulcus leading towards the epichile that is hypothesized to retain any liquid secretions and draw pollinators up the lip.

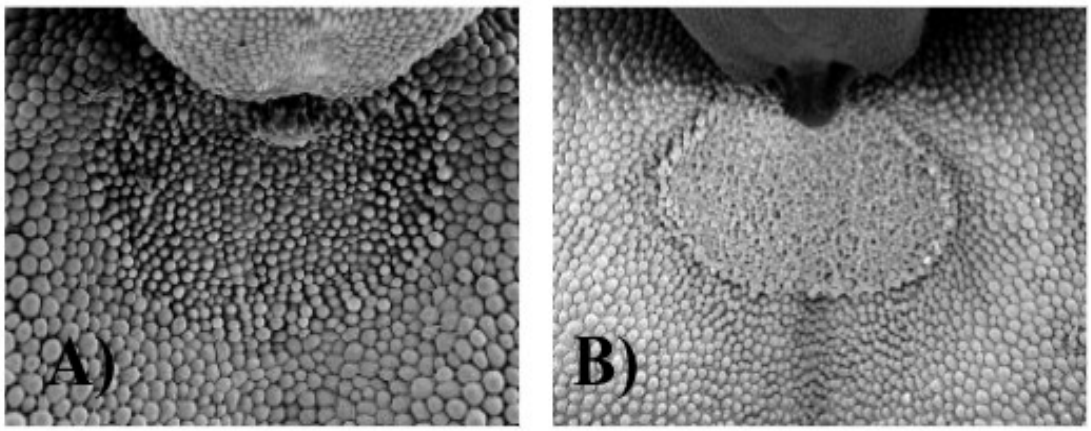

Figure 49.
Comparing the glenion A. P. aff. tenuisepala and P. vieirae, both in subsection Acroniae.

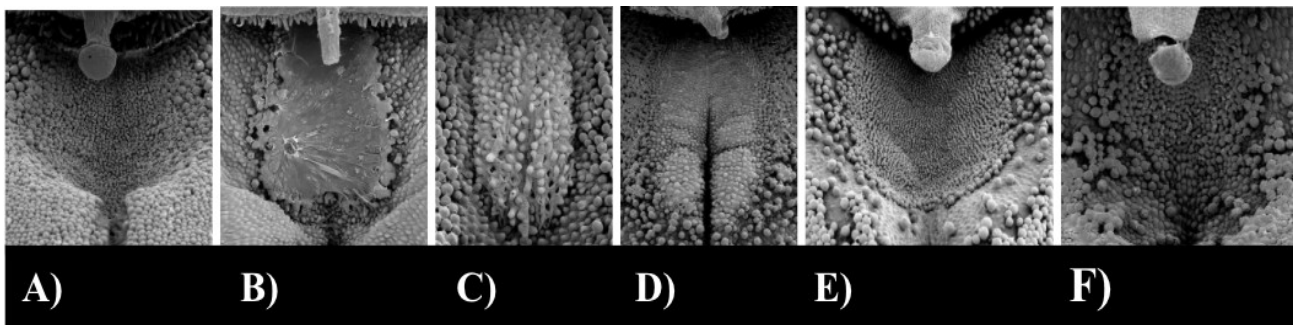

Figure 50.
Comparing the glenion structural variation in the P. aff. lilijae complex within Pleurothallis subsection Macrophyllae-Fasciculatae. A. P. aff. lilijae "Podocarpus". B. P. aff. lilijae "white". C. P. aff. lilijae KH006. D. P. aff. lilijae KH011. E. P. lanigera F. P. aff. lilijae "culpameae".

Several glenions in Macrophyllae-Fasciculatae are atypical. Even among the gleniform species in the P. aff. lilijae species complex there is a high degree of variation (Fig. 50). The glenions of P. aff. lilijae "Podocarpus" (Fig. 50A), P. aff. lilijae "white" (Fig. 50B), P. lanigera (Fig. 50E), and P. aff. lilijae "culpameae" (Fig. 50F), closely resemble one another. These glenions are all relatively large, circular, and have a similar cell type. In contrast, the glenions of P. aff. lilijae KH006 (Fig. 50C) and P. aff. lilijae KH011 (Fig. 50D) appear very different. The glenion cells of these species are much rougher and show no obvious signs of dried secretions. While the glenion of P. aff. lilijae KH006 is much smaller and more raised than that of P. aff. lilijae KH011, these species both have secretory cells in patches on either side of the glenion area resembling the glenion cells of the other four species. Additionally, the other four gleniform species compared (Fig. 51) all show evidence of dried liquid on their glenion cells.

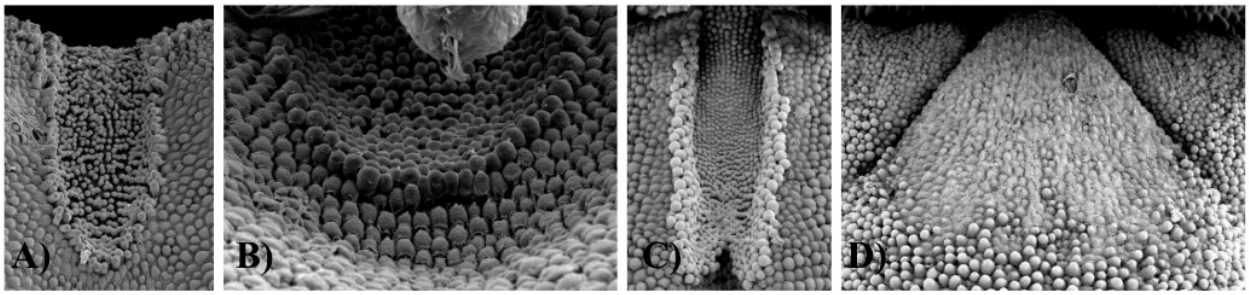

Figure 51.
Comparing the glenion structure variation in other species in the Pleurothallis subsection Macrophyllae-Fasciculatae. A. P. aff. titan "orange". B. P. aff. titan "strawberry lip". C. P. scurrula. D. P. aff. imperialis.

Crateriform Pollination

Although most species investigated in Macrophyllae-Fasciculatae had some form of a glenion structure, some had a very different overall lip morphology that can be collectively referred to as "crateriform" species. Crateriform pollination has not been extensively investigated, but is used to refer to the morphological similarities in the species P. pyelophora, P. phymatodea, and P. lynniana. These species are all non-resupinate and have a lip with the edges curved in to create a large central crater. P. aff. lilijae "Riksen" is also non-resupinate and has a small crater formed in the labellum. It is possible these species secrete a nectar reward in the crater and are pollinated

when flies, or other small insect pollinators, reach into the crater, thereby encountering the viscidium with their front legs, head, or the bottom part of its thorax. LC-MS data on P. pyelophora suggests it produces a nectar reward (Table 2), but no pollinating interactions have been observed for these species. Like gleniform pollination, this hypothesis also describes rewarding species, but is distinct in the hypothesized way these flowers physically interact with their pollinators. Crateriform species are mapped onto the Macrophyllae-Fasciculatae phylogenies in blue boxes (Fig. 40 and 41).

Labellar Crystals

Labellar crystal structures were found on two species of Pleurothallis, P. viduata in subsection Ancipitia, and P. aff. restrepioides in subgenus Elongatia (Fig. 49). While a direct size comparison cannot be made because SEM measurements were not taken, the crystals on P. aff. restrepioides are much smaller than those protruding from the cells of P. viduata. The crystals on P. aff. restrepioides appear along much of the labellar tissue, and are not confined to a distinct location (Fig. 49B), alternatively, the crystals on P. viduata appear exclusively on a limited number of cells at the very base of the lip (Fig. 52A). The function of the crystals, if any, remains unknown. Neither of these crystal types resemble the crystals of calcium oxalate hydrate observed on the perianth of species of Stelis. A proposed hypothesis for the function of extracellular crystals on Stelis is as reflective pseudonectar to attract pollinators (Chase and Peacor, 1987). However, P. aff. restrepioides produces visible nectar on the lip surface so it is unlikely the crystals function as pseudonectar. It is possible the crystals were produced from the nectar, or another labellar secretion, during the dehydrating or drying process, but this was not observed in other species.

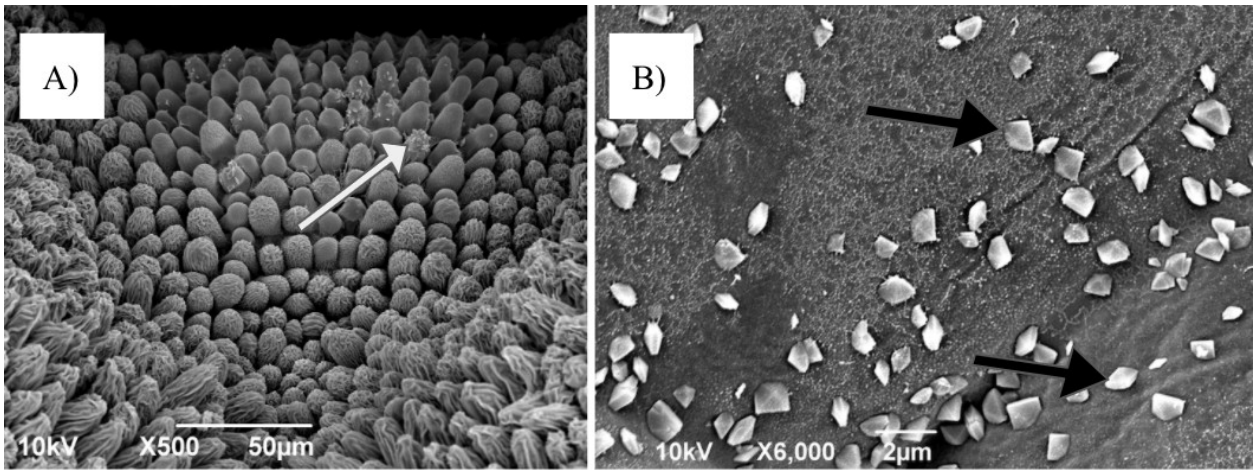

Figure 52. Comparison of crystalline protrusions from the cells at the base of the lip of P. viduata A) (white arrow) and surface crystals of P. aff. restrepioides B) (black arrows).

Possible Mixed Pollination Syndromes

The lip of P. viduata has several features suggestive of reward pollination, including a small glenion and sulcus. However, these features seem unlikely to aid significantly in a pollination strategy simply because of the small size of the lip compared to the column. The proposed “glenion” area, while appearing very similar to other glenions, may even be inaccessible to an insect pollinator due to its position tucked directly under the base of the column (Fig. 4). The lip could be a landing location for a small fly species, but the lip does not fulfill the typical role of

a rewarding species because it is not large enough to produce a large amount of nectar or function as an aggregation site. Visually, liquid droplets have been recorded on the dark purple areas of the petals, but SEM did not show any differentiation between these potentially secretory tissues and the surrounding cells. GC-MS data on this species places it among deceptive species (Hensley pers. com.); P. gratiosa and P. wielii. This means the morphological and the chemical data for this species conflict, with morphology suggesting reward and volatiles suggesting deceit. LC-MS could be used to analyze the petals and lip for potential carbohydrate secretions, but this would not be definitive evidence either way. This species may be an example of the transition between pollination strategies, with the rewarding characteristics becoming vestigial after a volatile evolution towards sexual deceit. This species could also just be an example of the many orchid species that do not exhibit one pollination strategy and employ several mechanisms of attraction.

Compared with the channel or depression of purportedly rewarding species (specifically P. praecipua), the three-pronged cavity on the epichile of the lip of P. vorator is much deeper (Fig. 8). This depression does not appear to fulfill the hypothesized role of the nectar sulcus in rewarding species and could be an indicator of the transition from a historically rewarding species to a sexually deceptive species. The volatile analysis of P. vorator shows that it is most similar to other rewarding species, but it is notable that it produced the chemical (Z)-9-tricosene, the pheromone of the female house fly (Carlson et al., 1971). The production of a well-documented dipteran pheromone by this species could be an important indicator of a shift in the pollination strategy as it could greatly affect the insects attracted to the flowers. Carbohydrate data on this flower could be an important distinguisher of pollination strategy depending on whether or not it shows significant levels of sugar molecules on the lip.

P. cypelligera morphologically seems similar to other rewarding species in Ancipitia. There is a distinct sulcus (Fig. 2) and photographs by Alfonso Doucette and Mark Wilson show liquid droplets on the surface of the lip. Droplets in photographs are suggestive of nectar secretions, but cannot be identified as such without corroborating carbohydrate data. It is always possible the droplets were not genuinely secreted by the flower and were instead created by a misting device in a greenhouse. At this point, LC-MS data for two P. cypelligera flowers do not show the presence of any rewarding carbohydrates (Table 2). This could always mean the flowers were not producing a nectar at the time of sampling, but if several more flower samples solidify this preliminary finding it could imply P. cypelligera is not actually a rewarding species. The volatiles produced by P. cypelligera place it among the other putatively rewarding species, but this could also be evidence of a nectar deceptive species. Without confirmation of rewarding sugars from LC-MS analysis, this species cannot be classified as rewarding. It is important to note that P. praecipua, while categorized as rewarding because of its identifiable glenion, also did not show any rewarding level of lip surface carbohydrates with three separate samples processed. Food-deceptive orchids often have a faint or a highly variable scent, to prevent strong pollinator associations and avoidance (Jersáková et al., 2009). Using this information to analyze the abundance of volatile molecules recorded in these samples, as well as processing more samples from different plants, could be used to investigate the possibility of food-deceptive species in Pleurothallis.

Table 3. Provided to organize the results of this study, and to show where the data can be expanded. Each column details one technique used to identify pollination strategy and shows the conclusions for each species in each technique.

Species	Subgenus	Morphology Hypothesis (evidence source)	Volatiles Hypothesis (source)	Carbohydrate Hypothesis (source)
P. tetragona	Ancipitia	No Data	No Data	No Data
P. odobeniceps	Ancipitia	No Data	Rewarding (Hesley, 2020)	No Data
P. duplex	Ancipitia	No Data	No Data	No Data
P. solium	Ancipitia	Rewarding (This study)	Rewarding (Hesley, 2020)	No Data
P. praecipua	Ancipitia	Rewarding (This study)	Rewarding (Hesley, 2020)	Rewarding not supported
P. cypelligera	Ancipitia	Inconclusive (Possibly Rewarding)	Rewarding (Hesley, 2020)	Rewarding not supported
P. inornata	Ancipitia	Rewarding (Photographs)	Rewarding (Hesley, 2020)	No Data
P. vorator	Ancipitia	Inconclusive (Possibly Deceptive)	Rewarding (Hesley, 2020)	No Data
P. viduata	Ancipitia	Inconclusive (traits of both)	Deceptive (Hensley, 2020)	No Data
P. atxite	Ancipitia	Deceptive (This study)	No Data	No Data
P. andreae	Ancipitia	Deceptive (Photographs)	No Data	No Data
P. penicillata	Ancipitia	No Data	No Data	No Data
P. acinaciformis	Ancipitia	No Data	Deceptive (Hensley, 2020)	Rewarding not supported
P. gratiosa	Ancipitia	Deceptive (Dupree, 2016)	Deceptive (Hensley, 2020)	Rewarding not supported
P. eumecocaulon	Ancipitia	Rewarding (Zhao, 2019)	Rewarding (Hesley, 2020)	No Data
P. aff. titan "strawberry lip"	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	Support for Reward
P. aff titan "orange"	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	No Data
P. titan	Macrophyllae-fasciculatae	Rewarding (Photographs)	No Data	No Data
P. aff. lilijae "culpameae"	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	Support for Reward
P. aff. lilijae "white"	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	Support for Reward
P. aff. lilijae "riksen"	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	No Data
P. aff. lilijae "podocarpus"	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	Support for Reward
P. lanigera	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	No Data
P. aff. lilijae sp.	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	No Data
P. aff. lilijae sp.	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	No Data
P. aff. imperialis	Macrophyllae-fasciculatae	Inconclusive (Possibly Rewarding)	No Data	No Data
P. scurrula	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	Support for Reward
P. pyelophora	Macrophyllae-fasciculatae	Rewarding (visible nectar)	No Data	Support for Reward
P. sanchoi	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	Support for Reward
P. anthrax	Macrophyllae-fasciculatae	Rewarding (This study)	No Data	No Data
P. renieana	Macrophyllae-fasciculatae	Deceptive (This study)	Deceptive (Hensley, 2020)	No Data
P. nuda	Restrepioidia	Rewarding (This study)	No Data	Support for Reward
P. aff. nuda	Restrepioidia	Rewarding (This study)	No Data	Rewarding not supported
P. restrepioides	Elongatia	Rewarding (This study)	No Data	No Data
P. aff. restrepioides	Elongatia	Rewarding (This study)	No Data	No Data
P. sijmii	Elongatia	Rewarding (This study)	No Data	Support for Reward
P. tenuisepala	Acroniae	Rewarding (Photographs)	No Data	No Data
P. aff. tenuisepala	Acroniae	Rewarding (This study)	No Data	No Data
P. vieirae	Acroniae	Rewarding (This study)	No Data	Support for Reward
P. sandemanii	Talpinaria	Rewarding (This study)	No Data	Support for Reward

Conclusions

1. The preliminary LC-MS data referenced in this study definitively identify several species of Pleurothallis that produce large amounts of sugar that can be identified as a nectar reward.

2. This study recorded a glenion structure in 18 Pleurothallis orchids and catalogs several atypical glenions. The recognition of the glenion in all shapes and forms could expand the definition of the glenion and justify electron microscopy for more species and afford a closer look at previously imaged species.

3. Preliminary data provided for new pollination strategies involving "rugose" secretory tissue and crateriform pollination. Both of these pollination strategy indicators require additional exploration but could represent convergent evolution.

4. Several species in this study could not be easily classified as either rewarding or deceptive. While additional LC-MS data and more extensive analysis of GC-MS data could be useful, there are some species that may never align perfectly with the artificial designations of rewarding and deceitful pollination strategies.

5. Additional SEM analysis should be done to investigate the hypothesis that sunken cells can be an indicator of secretory tissue (Fig. 33).

6. There is significant SEM, GC-MS, LC-MS, and phylogenetic work to be done on the Pleurothallis orchids. While this study mainly focuses on nectar rewards and sexual deceit pollination strategies, it is important to keep in mind the dozens of other pollination strategies orchids employ, and always be on the lookout for new ones.

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