PHYLOGENETICS AND SCANNING ELECTRON MICROSCOPY OF THE ORCHID GENUS *ANDINIA SENSU LATO*:

EVIDENCE FOR TAXONOMIC EXPANSION AND POLLINATION STRATEGIES

A THESIS

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By

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Abstract

Andinia Luer (Orchidaceae: Pleurothallidinae) is a small genus from the Andes mountains. Generic circumscriptions within the large subtribe Pleurothallidinae have been under debate as newer molecular evidence aims to correct polyphyletic genera that were created based on potentially homoplasious floral characteristics. Data from the nuclear internally transcribed spacer (ITS) gene region have previously indicated that *Andinia* should be expanded to include the pleurothallid genera *Neooreophilus*, *Masdevalliantha*, and *Xenosia*. In this study, phylogenetic trees were constructed from an expanded ITS data set, a new data set from the *matK* gene of the plastid genome, and composite of the two sequences. This broader phylogenetic analysis shows strong support for the monophyly of an expanded *Andinia*, reinforcing the original recommendation that Andinia be expanded. Additionally, scanning electron microscopy (SEM) was used to image morphological floral characteristics from an array of species spanning the breadth of the analyzed taxa. SEM images suggest multiple pollinator attraction strategies within *Andinia sensu lato*. Many of the floral characteristics and inferred pollinator attraction strategies are evident elsewhere in Pleurothallidinae, supporting the idea that certain floral characteristics have evolved multiple times and are therefore unreliable for organizing Pleurothallidinae.

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Chapter One

Expanding Andinia: Evidence from ITS nrDNA and *matK* cpDNA

Introduction

Pleurothallidinae Lindl. (Orchidaceae) is the largest of the ten subtribes within tribe Epidendreae Lindl., subfamily Epidendroideae Kostel. (Dressler 1981). The subtribe Pleurothallidinae contains approximately 4,100 species, but the number of genera remains a point of contention. Molecular phylogenetic evidence suggests as few as 32 (Chase *et al.* 2003), whereas an approach using morphological systematics suggests as many as 96 (Luer 2002). The largest genus in the subtribe is *Pleurothallis* R. Br., containing roughly half the species in the subtribe (Higgins 2009). Luer lamented that previous keys to the Pleurothallidinae either relied on characteristics that did a poor job of perceiving differences between species or relied on single characteristics that were difficult, if not impossible, to distinguish in all but the most well preserved herbarium specimens. Thus, Luer undertook the monumental task of revising the classification of the Pleurothallidinae according to, in order of importance, vegetative characteristics, characteristics of the inflorescence, and floral parts, weighting obvious traits over more subtle ones (Luer 1986a).

The species discussed here are all part of the subtribe Pleurothallidinae and many have a complex taxonomic history because of the difficulty inherent in using morphological systematics to classify them. Circumscribing pleurothallid genera by morphological characteristics is complicated by convergent evolution in floral traits (Borba & Semir 2001). For example, in their phylogenetic analysis of the subtribe Pleurothallidinae using morphological traits, Neyland, Urbatsch, &

Pridgeon (1995) showed that number of pollinia—an example of a morphological trait that Luer found largely useless—is an unreliable trait for determining phylogenetic relationships, as certain numbers of pollinia have evolved multiple times. Within Orchidaceae, floral homoplasy is not unique to Pleurothallidinae. Davies, Stpiczyńska, & Rawski (2014) showed that floral elaiophores (oil glands) were structurally similar in four species that had previously been assigned to the same genus, *Oncidium* Swartz, but had more recently been separated into other genera on the basis of molecular evidence. Their findings indicate the potential for floral homoplasy across multiple genera, and illustrate the risks of building phylogenies using floral traits.

The species in this study originate from three subgenera within *Pleurothallis* and from another pleurothallid genus, *Lepanthes* Swartz. *Lepanthes* is characterized by its “lepanthiform” sheaths, which are tubular and ribbed, enclosing the ramicauls in an imbricate series (i.e. overlapping, like roof tiles) (Luer 1986a). Ramicauls are the aerial, more or less erect, secondary stems in orchids, typically bearing a single leaf (Stern & Pridgeon 1984). Luer (1986a) transferred what would eventually become the first two species of *Andinia*, *Lepanthes dielsii* Mansfeld and *L. pensilis* Schlechter to the small genus *Salpistele* Dressler. Though *Salpistele* shares many similarities with *Lepanthes*, it lacks the characteristic lepanthiform sheaths enclosing its ramicauls. Like many species of *Lepanthes*, species of *Salpistele* have a bipartite lip attached to the underside of a column, which lacks a foot. The column, or gynostemium, is derived from a fusion of male and female reproductive parts into a single organ

and can have a basal extension, called the column foot, to which the lip, or labellum, is attached. The name *Salpistele* refers to a trumpet-like shape of the column apex, which terminates in a disc surrounding the apical anther and stigma, which sit in a shallow concavity (Dressler 1979).

Five years after adding *S. dielsii* (Mansfeld) Luer and *S. pensilis* (Schlechter) Luer to *Salpistele*, Luer (1991) subdivided the genus into two subgenera. Luer placed *S. dielsii* and *S. pensilis* into the subgenus *Andinia* and the other four species of the genus into subgenus *Salpistele*. Though the floral qualities of subgenus *Andinia* were similar to the rest of *Salpistele,* Luer segregated them based on their different growth habit and distribution, proposing the possibility that the shared floral structures are analogues (i.e. products of convergent evolution). While species of the subgenus *Salpistele* are found in Central America and have a caespitose habit, those of *Andinia* are distributed, as the name indicates, in the Andes mountains and have a repent growth habit (Luer 1991). Eventually, partially influenced by new information from unpublished DNA sequence data (Pridgeon & Chase 2001), Luer recognized the differences between the subgenera *Salpistele* and *Andinia* as distinct enough to elevate *Andinia* to generic status (Luer 2000). The new genus still contained only two species, *A. dielsii* (Mansfeld) Luer and *A. pensilis* (Schlechter) Luer, though this did not last long, as several more species were about to be added from elsewhere in Pleurothallidinae.

The first phylogenetic analysis of Pleurothallidinae using DNA sequence data, including both nuclear and plastid sequences (Pridgeon et al. 2001),

resulted in radically different groupings as compared to the earlier morphological analysis of Neyland, Urbatsch, & Pridgeon (1995). However, both morphological and DNA analyses showed polyphyly in *Pleurothallis,* indicating that it should likely be separated into several discrete genera in order to better reflect evolutionary history (Pridgeon et al. 2001). At the time of the first volume of *Icones Pleurothallidinarum*, Luer stated that determining phylogenetic order from morphological traits was impossible (Luer 1986a) and that the generic groupings were necessarily artificial (i.e. more for practical purposes of identification than intended to reflect evolutionary relationships). Folowing the phylogenetic study of Pridgeon et al. (2001), Luer claimed that it had overlooked the basic aim of taxonomy, ease of identification, and that *Pleurothallis* was best treated as a polyphyletic alliance of monophyletic subgenera (Luer 2002). Despite disagreements about generic circumscriptions, the studies of Pridgeon *et al.* (2001) and Luer (2002) agreed on the polyphyly of *Pleurothallis*, which allowed subgenera to be removed without making the remaining genus non-monophyletic.

Shortly following the broad phylogenetic study of Pleurothallidinae (Pridgeon *et al.* 2001), Pridgeon & Chase (2001) included an additional species, *Pleurothallis lappacea* of subgenus *Aenigma* Luer, in a larger phylogeny and found that it was sister to *Andinia pensilis* with 99% bootstrap support. This find was evidence enough for them to expand the concept of *Andinia* Luer to include *Pleurothallis* subgenus *Aenigma*, bringing the total number of species in *Andinia* to eleven. *Pleurothallis* subgenus *Aenigma* Luer is characterized by short, repent

(i.e. prostrate), ascending rhizomes (root-bearing stems that send up ramicauls at intervals) and somewhat fasciculated (i.e. bunched), abbreviated ramicauls (Luer 1986b). At the time of its description as a subgenus, *Aenigma* contained five species, four of which were placed in section *Aenigmata* and one of which was placed into a monotypic section, *Vestigipetalae*. The two sections were distinguished primarily by differences in the ovaries, *Aenigmata* with long-spiculate ovaries and *Vestigipetalae* having glabrous ovaries, as well as vestigial petals (Luer 1986b). Morphological similarities of the rhizome, ovaries, lip, and column between the genus *Andinia* and *Pleurothallis* subgenus *Aenigma* as well as overlapping distributions in the Andes, from Colombia to Bolivia, supported the expansion of *Andinia* (Pridgeon & Chase 2001).

Just after the expansion of *Andinia, A. vestigipetala* was recognized as distinct enough to pull it out of *Andinia* into the monotypic genus *Lueranthos* Szlachetko & Margonska (2001). The description of the genus included vestigial petals and a lip that is erect, enveloping the column (Szlachetko & Margonska 2001). Luer also recognized the dissimilarities between *A. vestigipetala* and the rest of the newly expanded *Andinia*. Despite only one species of *Pleurothallis* subgenus *Aenigma* being sequenced, Luer (2002) generally agreed with the proposed expansion of *Andinia*, except he continued to consider *P. vestigipetala* a part of *Pleurothallis*. Luer did not comment directly on the creation of *Lueranthos*. Another addition to the genus *Andinia* came when Luer described the species *A. hirtzii*, which Luer (2005) describes as being morphologically very similar to *A. schizopogon*.

Like the first two species placed in *Andinia* Luer, the first species of what has since become the genus *Neooreophilus* Archila also originated in the genus *Lepanthes*. When Reichenbach described *Lepanthes nummularia* (1856), he recognized that, while all other species of *Lepanthes* had longer ramicauls than rhizomes, the inverse was true in *L. nummularia*. He created two sections; placing *L. nummularia* into the monotypic *Lepanthes* section *Brachycladae*, meaning “short branches”, and the rest of the genus into *Lepanthes* section *Macrocladae*. Luer (1986a) elevated *Lepanthes* section *Brachycladae* Swartz to subgeneric status, giving it the name *Lepanthes* subgenus *Brachycladium* Luer. Luer (1994) later described ten new species and delineated two new sections of the subgenus, which contained by that time twenty-four species.

Eventually, Luer (2005) would elevate *Lepanthes* subgenus *Brachycladium* to generic status under the name *Brachycladium* (Luer) Luer, comprising by then 35 species. However, the name *Brachycladium* was already occupied by *Brachycladium* Corda (Fungi Imperfecti), rendering Luer’s combinations illegitimate and motivating Archila and Higgins (2008) to propose the name *Oreophilus* W.E. Higgins & Archila. Once again, this name was rendered invalid, as Higgins and Archila had included three species of the genus *Andinia* Luer, including the type species *A. dielsii* (Mansfeld) Luer, without explanation. Realizing the mistake, Archila (2009) proposed another name for the genus, *Neooreophilus* Archila, this time validly. Unaware of the publication of *Neooreophilus,* Luer and Thoerle (2010) published the name *Penducella* to

replace *Brachycladium* and *Oreophilus,* but it was superfluous after the validly proposed name *Neooreophilus* and therefore rendered invalid.

Phylogenetic analyses by Wilson & Jost (2009) using DNA sequences of the nuclear ITS gene region showed that the former *Lepanthes* subgenus *Brachycladium* (now the genus *Neooreophilus*) was monophyletic and was not closely related to *Lepanthes*. Instead, *Neooreophilus* was most closely related to *Andinia pensilis, A. dielsii*, and the former *Pleurothallis* subgenus *Aenigma,* now part of the genus *Andinia* (Wilson & Jost 2009). Archila and Higgins (2008) also apparently recognized this relationship, because they included *A. dielsii, A. pensilis,* and *A. hirtzii* in their proposed circumscription of *Oreophilus* (validly, *Neooreophilus*), despite the lack of explanation for doing so and invalidity of the proposed generic name. The connection between *Andinia* and *Brachycladium* (validly, *Neooreophilus*) was confirmed following sequencing of additional species in the genera in a broadened analysis (Wilson & Jost 2011).

In their broadened analysis, Wilson and Jost (2011) also included species from the pleurothallid genera *Masdevalliantha* (Luer) Szlachetko & Margonska and *Xenosia* Luer based on vegetative morphological similarities to some species in *Andinia*. Both of the genera had been created from subgenera of *Pleurothallis*. Luer & Escobar (1983) had already noted the possible relatedness of *Pleurothallis xenion* Luer & Escobar and *P. spiralis* (Ruiz & Pavon) Lindley (syn. *Stelis spiralis* Persoon) and recognized similarities in the growth habit of *P. xenion* and *P. longiserpens.* Luer (1986b) went on to create *Pleurothallis* subgenus *Xenion* for two species, *P. spiralis* and *P. xenion*. Luer described these

species as sharing a climbing growth habit and short ramicauls with other pleurothallid taxa, particularly *Pleurothallis* subgenus *Aenigma*, which Pridgeon & Chase (2001) would later add to the genus *Andinia*. The species of *Pleurothallis* subgenus *Xenion* are unique in their centrally excavated, three-lobed lip, which attaches at its base to the column foot (Luer 1986b). Luer (2004) later elevated *Pleurothallis* subgenus *Xenion* to generic status, giving it the name *Xenosia*. In his massive reorganization of *Pleurothallis,* Luer (1986b) also created the subgenus *Masdevalliantha*, the etymology of which refers to the floral similarities shared with the genus *Masdevallia* Ruiz & Pavón. Later, Szlachetko & Margonska (2001) elevated *Masdevalliantha* to generic status, encompassing only two species, *M. longiserpens* (C. Schweinf.) Szlachetko & Margonska and *M. masdevalliopsis* (Luer) Szlachetko & Margonska, the type species.

The broadened phylogenetic analyses of Wilson & Jost (2011) indicated highest bootstrap support at the node subtending the clade containing all four genera—*Andinia*, *Masdevalliantha*, *Neooreophilus*, and *Xenosia*. However, these results are based upon DNA sequence data from a single gene region referred to as ITS, the 5.8S ribosomal RNA gene and two internally transcribed spacers, ITS1 and ITS2, flanking it on either side (White 1990). The ITS region is easily aligned, has minimal length variation, and has been used to identify relationships at the species level in other plant families (Baldwin 1992) as well as in Orchidaceae (Cox *et al.* 1997). Because different genomes (e.g. nuclear and plastid) have different evolutionary histories, confidence in phylogenetic trees can be greatly improved by analyzing sequence data from more than one

(Savolainen & Chase 2003). The maturase K gene (*matK*) is found in the plastid genome and typically codes for the MatK protein responsible for splicing the *trnK* intron in which it is located (Vogel *et al.* 1997). Phylogenetic analysis using *matK* in the tribe Diurideae indicates that *matK* may be a pseudogene in Orchidaceae, evidenced by the occurrence of stop codons within the gene and indels creating reading frame shifts (Kores *et al.* 2001). Alternatively, Barthet (2006) showed that an out-of-frame *matK* start codon in some orchid species buffers against substitutions that would otherwise destroy the reading frame.

We hypothesized that the evolutionary relationships suggested by the nuclear ITS phylogeny would be corroborated by phylogenetic analysis using the plastid *matK* gene and that consequently, the circumscription of *Andinia* can be expanded to include the genera *Neooreophilus*, *Masdevalliantha,* and *Xenosia.*

Materials and Methods

Plant Material:

Sources of plant material are listed in Table 1. In this study, we included 8 of the 14 species of *Andinia,* one of the two species of *Masdevalliantha* (missing *M. masdevalliopsis*), both species of *Xenosia,* and 12 of the 45 species in *Neooreophilus*, including multiple morphotypes of *N. nummularius* (Wilson, unpublished data). Designation of *Laelia* as the outgroup taxa was based on a phylogenetic study of Laeliinae using ITS nrDNA (van den Berg *et al.* 2000). *Laelia* falls outside of the Pleurothallidinae, but is still part of the subfamily Epidendroideae, and its designation as an outgroup produces branch lengths in the Maximum Likelihood analyses that are longer than any between ingroup taxa,

but are not so long as to obscure distances between them. Authorities for plant names follow the International Plant Names Index.

Andinia (AN) project number	**Tentative Identification**	**Donor/Plant Number/Origin**	**ITS Genbank Accession Number**	***matK* Genbank Accession Number**
AN005	*Andinia dalstroemii* (Luer) Pridgeon & M.W.Chase	Ecuagenera	KP012339	pending
AN068	*Andinia dalstroemii* (Luer) Pridgeon & M.W.Chase	Ecuagenera	KP012346	
AN079	*Andinia dielsii* (Mansf.) Luer	Ecuagenera		pending
AN018	*Andinia dielsii* aff.	Karremans #AK5429; Hirtz	KC425739	
AN011	*Andinia lappacea* (Luer) Pridgeon & M.W.Chase	O'Shaughnessy #01428	KP012343	KP012516
AN015	*Andinia lappacea* (Luer) Pridgeon & M.W.Chase	Pridgeon #AP108; Ecuagenera	KC425837.1	
AN022	*Andinia lappacea* (Luer) Pridgeon & M.W.Chase	Manning #090602	pend. update	pending
AN002	*Andinia pensilis* (Schltr.) Luer	Ecuagenera	KP012336	pend. update
AN014	*Andinia pensilis* (Schltr.) Luer	Pridgeon #AP200; Ecuagenera	KP012344	KP012517
AN080	*Andinia pensilis* (Schltr.) Luer	Pridgeon #MWC8007	AF262826	AF265455.1
AN001	*Andinia pogonion* (Luer) Pridgeon & M.W.Chase	Jost #8293	KP012335	pending
AN003	*Andinia pogonion* (Luer) Pridgeon & M.W.Chase	Ecuagenera	KP012337	KP012515
AN009	*Andinia pogonion* (Luer) Pridgeon & M.W.Chase	O'Shaughnessy #02004	KP012341	
AN010	*Andinia pogonion* (Luer) Pridgeon & M.W.Chase	O'Shaughnessy #03845	KP012342	
AN004	*Andinia schizopogon* (Luer) Pridgeon & M.W.Chase	Ecuagenera	KP012338	pending
AN019	*Andinia schizopogon* (Luer) Pridgeon & M.W.Chase	Karremans #AK5783; Dubbeldam	KC425740	
AN069	*Andinia schizopogon* (Luer) Pridgeon & M.W.Chase	Ecuagenera	KP012346	pending
AN076	*Andinia schizopogon* (Luer) Pridgeon & M.W.Chase	Andy's	KP012350	pend. update
AN006	*Andinia* sp.	Ecuagenera	KP012340	pending
AN073	*Andinia trimytera* (Luer & R.Escobar) Pridgeon & M.W.Chase	Vieira	pending	pending
AN075	*Andinia vestigipetala* (Luer) Pridgeon & M.W.Chase	Thoerle	KP012349	pending
AN012	*Masdevalliantha longiserpens* (C.Schweinf.) Szlach. & Marg.	O'Shaughnessy #04515	KP012353	pend. update
AN013	*Masdevalliantha longiserpens* (C.Schweinf.) Szlach. & Marg.	O'Shaughnessy #01755; Ecuagenera	KP012354	pending
AN020	*Masdevalliantha longiserpens* (C.Schweinf.) Szlach. & Marg.	Karremans #AK5724; Dubbeldam	KC425744	
AN021	*Masdevalliantha longiserpens* (C.Schweinf.) Szlach. & Marg.	Ecuagenera	KP012356	KP012521
AN057	*N. ciliaris* (Luer & Hirtz) Archila	O'Shaughnessy #01380; Ecuagenera	KP012372	pending
AN066	*N. compositus* (Luer & R.Escobar) Archila	O'Shaughnessy #03688; Ecuagenera	KP012377	pending
AN065	*N. lynnianus* (Luer) Archila	O'Shaughnessy #02869; Ecuagenera	pending	pending
AN051	*N. nummularius* (Rchb.f.) Archila	O'Shaughnessy #00125; Ecuagenera	pending	KP012526
AN056	*N. nummularius* (Rchb.f.) Archila	O'Shaughnessy #01317; Colombia, Niessen	KP012371	
AN060	*N. nummularius* (Rchb.f.) Archila	O'Shaughnessy #02359; Ecuagenera	pending	KP012530

AN050	*N. nummularius* (Rchb.f.) Archila	O'Shaughnessy #00096; Ecuagenera	pending	KP012525
AN030	*N. nummularius* (Rchb.f.) Archila (form A)	Jost #6803; Tapichalaca	pending	
AN043	*N. nummularius* (Rchb.f.) Archila (form C)	Jost #8316	KP012365	
AN047	*N. nummularius* (Rchb.f.) Archila (form D)	Jost #8320	KP012367	
AN026	*N. nummularius* (Rchb.f.) Archila (form E)	Jost #5043; Sumaco-Galeras	pending	
AN071	*N. ortizianus* S.V.Uribe & Thoerle	Vieira	KP012378	
AN054	*N. persimilis* (Luer & Sijm) Archila	O'Shaughnessy #00982; Ecuagenera	KP012369	
AN055	*N. pilosellus* (Rchb.f.) Archila	O'Shaughnessy #01008; Ecuagenera	pend. update	pending
AN063	*N. pilosellus* (Rchb.f.) Archila	O'Shaughnessy #02624; Colombia, Niessen	pend. update	pend. update
AN033	*N. pilosellus* (Rchb.f.) Archila (spotted)	Jost #7026; Los Cedros	pend. update	
AN032	*N. pilosellus* (Rchb.f.) Archila (yellow)	Jost #7025; Los Cedros	pend. update	
AN052	*N. pilosellus* (Rchb.f.) Archila	O'Shaughnessy #00146; Colombia, J&L Orchids	KP012368	pending
AN017	*N. platysepalus* (Luer & R.Escobar) Archila	Karremans #AK4847; Leiden	JQ995331	KC425864
AN064	*N. platysepalus* (Luer & R.Escobar) Archila	O'Shaughnessy #02625; Colombia, Niessen	KP012376	pending
AN029	*N. pseudocaulescens* (L.B.Sm. & S.K. Harris) Archila	Jost #5444; Pastaza	KP012360	
AN024	*N. stalactites* (Luer & Hirtz) Archila	#6; Ecuagenera	pend. update	KP012523
AN059	*N. stalactites* (Luer & Hirtz) Archila	O'Shaughnessy #02248; Ecuagenera	KP012374	KP012529
AN072	*N. vieira-perezianus* P.Ortiz	Vieira	KP012379	
AN058	*N. werneri* (Luer) Archila	O'Shaughnessy #01492; Ecuagenera	KP012373	pend. update
AN053	*N. werneri* (Luer) Archila	O'Shaughnessy #00508; Ecuagenera		pending
AN007	*Xenosia spiralis* (Ruiz & Pav.) Luer	Ecuagenera	KP012351	pending
AN070	*Xenosia spiralis* (Ruiz & Pav.) Luer	Ecuagenera	KP012357	pending
AN008	*Xenosia xenion* (Luer & R.Escobar) Luer	Ecuagenera	KP012352	pend. update
AN016	*Xenosia xenion* (Luer & R.Escobar) Luer	Pridgeon #AP250; Ecuagenera	KP012355	
AN074	*Xenosia xenion* (Luer & R.Escobar) Luer	Almanza	KP012358	KP012522

Table 1: A list of taxa sampled for ITS nrDNA sequencing and/or *matK* cpDNA sequencing. The ANxxx number assigned to each sample was given in the order that the sample was acquired. When the sample was acquired from another collector or researcher, they are indicated as the donor, followed by the number they assigned the sample and the original source of the plant, where available. When the sample was acquired directly from a retailer, no donor name is indicated.

DNA Extraction

Genomic DNA was extracted from fresh or frozen (-20°C) leaf tissue. Leaf tissue was frozen using liquid nitrogen and ground to a fine powder using a ceramic mortar and pestle. The mortar and pestle were cleaned with bleach between uses to prevent contamination. Genomic DNA was extracted from ground leaf tissue using a DNeasy Plant Mini Kit (Qiagen). Genomic DNA concentration was estimated by running a sample on a 0.8% agarose 1X TAE gel against known quantities of λ DNA (10, 25, 50, 75, 100, and 150 ng) at 100 V for 15 min.

Sequencing matK and ITS

PCR amplification of matK

The primer pair 390F (CGATCTATTCATTCAATATTTC) and 1326R (TCTAGCACACGAAAGTCGAAGT) was used to amplify the plastid gene maturase K (*matK*) as described by Cuénoud et al. (2002). A master mix was created using 12.5 µL 2x PCR Master Mix (Promega), 1 µL 390f (25 µM), 1 µL 1326r (25 µM), and 0.5 µL molecular biology grade water per reaction, for a total of 15 µL. In a 0.2 mL PCR tube, 10 µL containing approximately 2.5 ng template DNA was added to 15 µL mastermix and mixed thoroughly by pipetting. Three to five 25 µL PCR reactions were performed for each sample. PCR amplification was performed using an iCycler thermal cycler (Bio-Rad Laboratories, Inc.) with the following program:

matK thermal cycler program:

30 cycles:

94°C 1 min

48°C 30 s

72°C 1 min

1 cycle:

72°C 7 min

1 cycle:

4°C hold

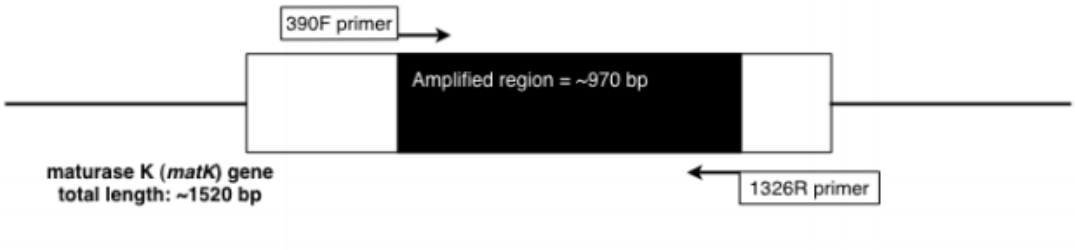

Figure 1: A schematic diagram of the maturase K (*matK*) plastid gene, showing the approximate binding sites of the forward primer (390F) and reverse primer (1326R) used in amplification. (Sheade 2012).

PCR amplification of nuclear ITS

The primer pair 17SE(ACGAATTCATGGTCCGGTGAAGTGTTCG) and 26SE(TAGAATTCCCCGGTTCGCTCGCCGTTAC) was used to amplify the nuclear internally transcribed spacer (ITS) as described by Sun *et al.* (1994). A master mix was created using 12.5 µL 2x PCR Master Mix (Promega), 1 µL 17SE (25 µM), 1 µL 26SE (25 µM), 1 µL dimethyl sulfoxide (DMSO), and 4.5 µL

molecular biology grade water per reaction, for a total of 20 µL per reaction. In a 0.2 mL PCR tube, 5 µL containing approximately 10 ng template DNA was added to 20 µL mastermix and mixed thoroughly by pipetting. Three 25 µL PCR reactions were performed for each sample. PCR amplification was performed using a thermal cycler (Bio-Rad Laboratories, Inc.) with the following program:

ITS thermal cycler program

1 cycle:

94°C 5 min

5 cycles:

94°C 1 min

60°C 1 min

72°C 3 min

30 cycles:

94°C 1 min

58°C 1 min

72°C 3 min

1 cycle:

72°C 15 min

1 cycle:

4°C hold

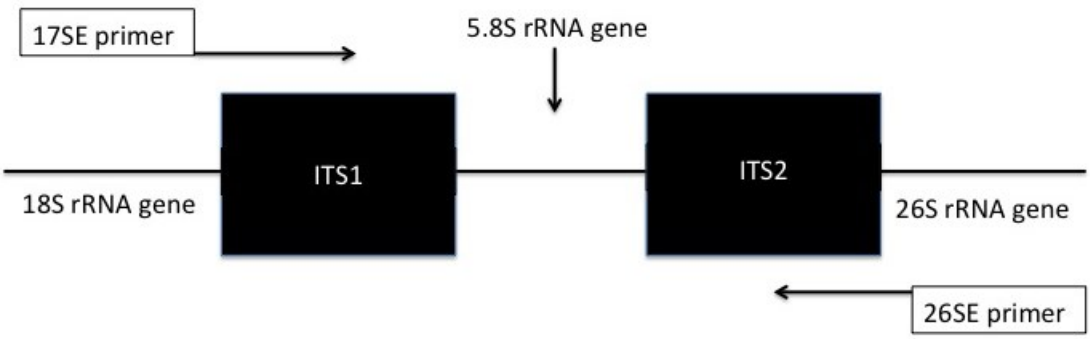

Figure 2: Schematic diagram of the nuclear 5.8S rRNA gene and the two internally transcribed spacers (ITS1 and ITS2) flanking it, collectively known as the ITS gene region, and the two primers used in its amplification: the forward primer 17SE and the reverse primer 26SE (Shum 2011).

Gel Purification of PCR products

A preparative gel was created using 50 mL 1x Tris/Acetate/EDTA (TAE) buffer, 1.5% (0.75 g) agarose, and 3 µL ethidium bromide. The three (for ITS) or five (for *matK*) PCR reactions for each sample were combined to give a total volume of 75 or 125 µL. The PCR product solution was mixed with one-sixth total volume (i.e. 15-25 µL) 6X Blue/Orange Loading Dye (Promega) and loaded into a triple-sized well. PCR products were run alongside a 100 bp ladder in order to verify the desired product (ITS = ~875 bp, *matK* = ~930 bp). Gels were run at 100 V for 90 min, or until separation between the desired product and any non-specific bands was obtained. Gels were photographed using a BioDoc-It Imaging System (UVP). Using a UV transilluminator (FisherBiotech FBTIV-614), the target band was excised from the gel using a razor blade, trimmed of excess agarose, and weighed to determine appropriate buffer volumes during gel extraction.

PCR products were extracted from excised gel cubes using a QIAquick Gel Extraction Kit (QIAGEN) according to the protocol provided. Concentration (ng/μL) and purity (A_{260}/A_{280}) of purified DNA were estimated on a NanoDrop 2000 Spectrophotometer (Thermo Scientific) or Biophotometer (Eppendorf).

Preparation of DNA for Sequencing

Purified PCR products were submitted either to GeneWiz or to University of Michigan DNA Sequencing Core (UM) for sequencing. ITS PCR products were sent with the primers 17SE, 26SE, ITS1 (TCCGTAGGTGAACCTGCGG) (White et al. 1990), and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). *matK* PCR products were sent with the primers 390F, 1326R, Nina-matK-F (GCGATTGTTTTTCCACGAAT) (Sheade 2012), and Nina-matK-R (TCCGCTGTGATAACGACAAA) (Sheade 2012).

Sequence Analysis and Phylogeny Construction

Trace files were downloaded from either GeneWiz or UM and viewed in FinchTV (Geospiza) to ensure sequence viability and to confirm that peaks were called correctly, edited for accuracy when they were not, and truncated at the appropriate sites. Trace files were then exported as FASTA files and aligned by eye using Se-Al software to create consensus sequences for each specimen. When sequences produced a poor consensus, with ambiguous nucleotides or a lack of corroboration between multiple sequences, samples were re-amplified, purified, and sent back to UM or GeneWiz for another round of sequencing. Consensus sequences were exported to MEGA 6.06 and aligned by

muscle. A combined ITS and *matK* phylogeny was constructed by concatenating the two sequences in MEGA for all samples for which both ITS and *matK* were sequenced. Phylogenies were constructed both as Maximum Parsimony (MP) trees and as Maximum Composite Likelihood (i.e. Maximum Likelihood [ML]) trees with 1000 bootstrap replicates. MP trees were obtained using the Subtree-Pruning-Regrafting algorithm (Nei & Kumar 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). ML trees were created based on the model of Tamura & Nei (1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value (Tamura *et al.* 2013). All trees were rooted with four outgroup sequences from *Laelia* spp. In addition to species from *Andinia sensu lato*, analyzed taxa included five species of *Pleurothallis* (identified as a sister genus in a 9-region tree, Whitten & Pridgeon, *pers. comm.*) to offer context within Pleurothallidinae.

Results

ITS—In this study, 9 new ITS sequences were generated and incorporated into the alignment: AN003, AN021, AN022, AN023, AN032, AN053, AN074, AN075, and AN076. The resulting aligned ITS nrDNA matrix of 67 taxa comprised 790 characters, of which 201 (25.44%) were variable and 153 (19.36%) were potentially parsimony informative. MP analysis produced a single most parsimonious tree with length 381 steps. Consistency Index (CI) =

(0.635542), Retention Index (RI) = (0.892157), Composite Index = 0.608821 (0.567003) for all sites and parsimony-informative sites (in parentheses). The ML analysis produced a tree with a highest log likelihood of -3573.3364. In the MP bootstrap consensus tree (Fig. 3), the node subtending *Andinia*, *Neooreophilus, Masdevalliantha*, and *Xenosia* received 91% bootstrap support (A). However, this clade only received moderate support (74%) in the ML tree (Fig. 4). There is moderate support in both the MP (77%) and ML (71%) trees for a clade encompassing all *Neooreophilus* spp. (B). However, support is much stronger for two smaller groups of *Neooreophilus*; *N. nummularius* and *N. stalactites* cluster together with 90% support in the MP tree and 83% in the ML tree (C) and all other *Neooreophilus* spp. included in the analysis cluster together with 94% support in the MP tree and 90% support in the ML tree (D). *Andinia* in its present sense does not form a monophyletic clade in either tree, though smaller groups within the genus are well supported. *A. trimytera* does not cluster with any other species of *Andinia* in either tree (E), except for the unidentified *Andinia* sp. (AN006), which was received from Ecuagenera as *Andinia vestigipetala*, but never flowered and does not cluster with *A. vestigipetala* in any analysis. Both MP and ML analyses show moderate support (74% MP, 77% ML) for a group (F) containing *A. dielsii* aff., *A. pensilis*, *A. lappacea*, and *A. vestigipetala.* The sample labeled "*A. dielsii* aff." (AN018) is likely *A. dielsii,* the type species of *Andinia*, but morphological similarities in floral characteristics between *A. dielsii* and *A. pensilis* made absolute confirmation impossible. A second clade (G) containing *A. pogonion, A. schizopogon,* and *A. dalstroemii* has 95% support in

the MP phylogeny and similarly strong support (94%) in the ML tree. *Xenosia* and *Masdevalliantha* cluster together (H) with moderate (64%) support in the MP tree and in the ML tree (69%), though the level of support increases to 68% and 86%, respectively, with the exclusion of *Xenosia spiralis*.

matK—All *matK* sequences with ANxxx numbers were generated in this study. The aligned *matK* cpDNA matrix of 47 taxa comprised 821 characters, of which 136 (16.57%) were variable and 84 (10.23%) were potentially parsimony informative. MP analysis resulted in 6 most-parsimonious trees of length 173 steps, CI = (0.806723), RI = (0.935754), Composite Index =0.811348 (0.754894) for all sites and for parsimony-informative sites (in parentheses). The ML analysis produced a tree with a highest log likelihood of -2335.5793. The MP (Fig. 5) and ML (Fig. 6) bootstrap consensus trees both show equally strong support (97%) at the node subtending *Andinia*, *Neooreophilus*, *Xenosia*, and *Masdevalliantha* (A). All *Neooreophilus* spp. included in the analysis cluster together into a single clade with strong (92% MP, 95% ML) support (B). Within the larger *Neooreophilus* clade, there is also a smaller moderately- to strongly-supported (86% MP, 85% ML) clade of *N. platysepalus, N. pilosellus,* and *N. compositus* (D1). However, *N. werneri, N. lynnianus*, and *N. ciliaris*, which clustered with the aforementioned group in the ITS analysis, do not group with it in the *matK* phylogeny (D2). *Andinia pensilis* clusters with *A. dielsii* (90% MP, 89% ML) (F1) and the clade encompassing *A. schizopogon, A. pogonion*, and *A. dalstroemii* (G) is strongly supported (99% MP and ML). No other species of *Andinia* cluster together into smaller clades within the large, well-supported *Andinia*-

Neooreophilus-Xenosia-Masdevalliantha clade (A), nor do any species of *Xenosia* (H1, H2) or *Masdevalliantha* (H3).

Composite analysis—A composite alignment was created from the concatenated sequences of ITS nrDNA and *matK* cpDNA of all samples for which both were sequenced. The aligned matrix of 45 taxa comprised 1609 characters, of which 327 (20.32%) were variable and 228 (14.17%) were potentially parsimony informative. The MP analysis produced 2 most parsimonious trees of length 518 steps. The Consistency Index (CI) = (0.697842), the Retention Index (RI) = (0.886179), and the Composite Index is 0.670622 (0.618413) for all sites and parsimony-informative sites (in parentheses). The ML analysis produced a tree with highest log likelihood of -5705.7064 (Fig. 8). There is 100% support at the node subtending *Andinia*, *Neooreophilus*, *Xenosia*, and *Masdevalliantha* (A) in the MP tree (Fig. 7) and 99% for the same clade in the ML tree. Of the smaller groupings, the clade of all *Neooreophilus* spp. in the analysis received 99% bootstrap support in the MP tree and 98% support in the ML tree (B). Within *Neooreophilus,* the clade containing *N. nummularius* and *N. stalactites* (C) has 93% support in the MP tree and 86% support in the ML tree. The node subtending all other *Neooreophilus* spp. (D) received 94% support in the MP tree and 95% support in the ML analysis. *Andinia* did not cluster as a monophyletic clade. *A. vestigipetala* and *A. lappacea* clustered with *A. pensilis* (F), receiving moderate support in the MP analysis (77%) and the ML analysis (78%). The clade containing *A. pogonion*, *A. schizopogon*, and *A. dalstroemii* (G) was strongly supported in both trees

(100%). *A. trimytera* did not cluster with any other species of *Andinia,* except for the unidentified sample AN006 (E). *Xenosia xenion* and *Masdevalliantha longiserpens* cluster together (H2) with moderate (69%) support in the ML tree, and weaker support (54%) in the MP tree; the level of support drops to 64% and 48%, respectively, with the inclusion of *Xenosia spiralis* (H1).

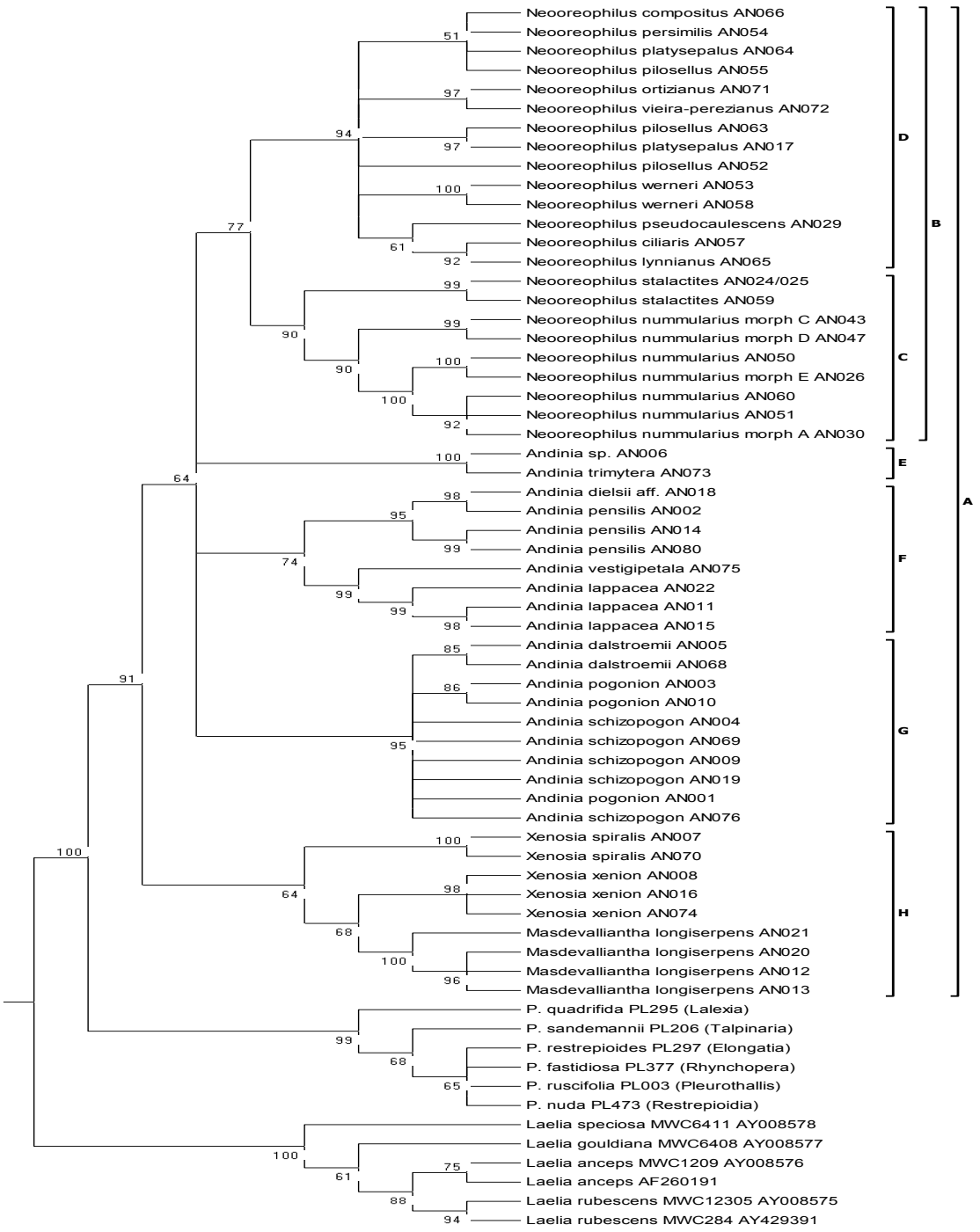

Figure 3: Bootstrap consensus phylogenetic tree created from the ITS nrDNA data set using MP analysis with 1000 bootstrap replicates. Nodes with bootstrap values under 50% are condensed. Labels were assigned to clades in accordance with the order in which they are discussed. Values at each node reflect percent bootstrap support.

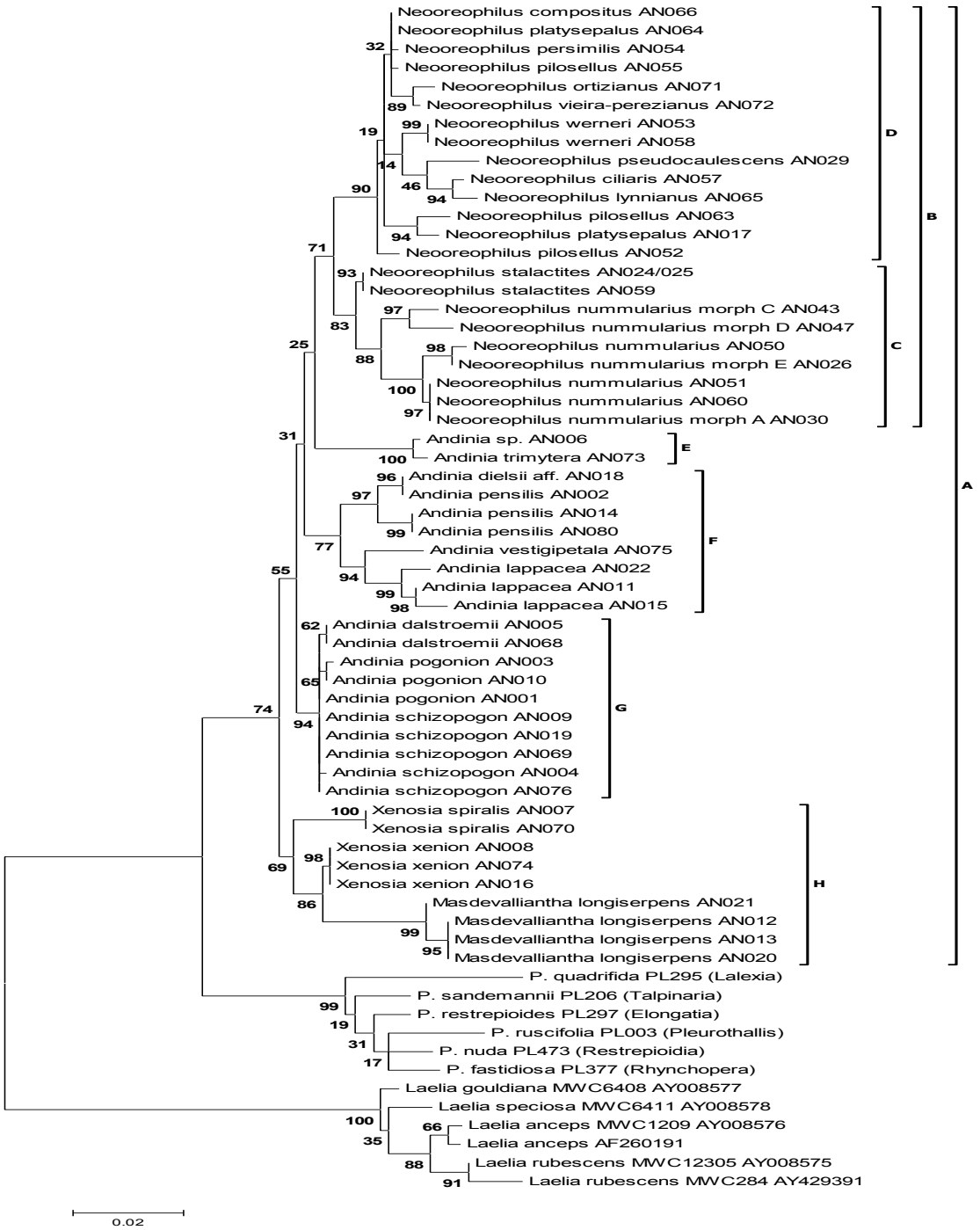

Figure 4: Phylogenetic tree created from the ITS nrDNA data set using ML analysis with 1000 bootstrap replicates. The tree displayed is the one with the highest log likelihood (-3573.3364). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Branch length values are not shown in order to improve legibility. Values at each node reflect percent bootstrap support.

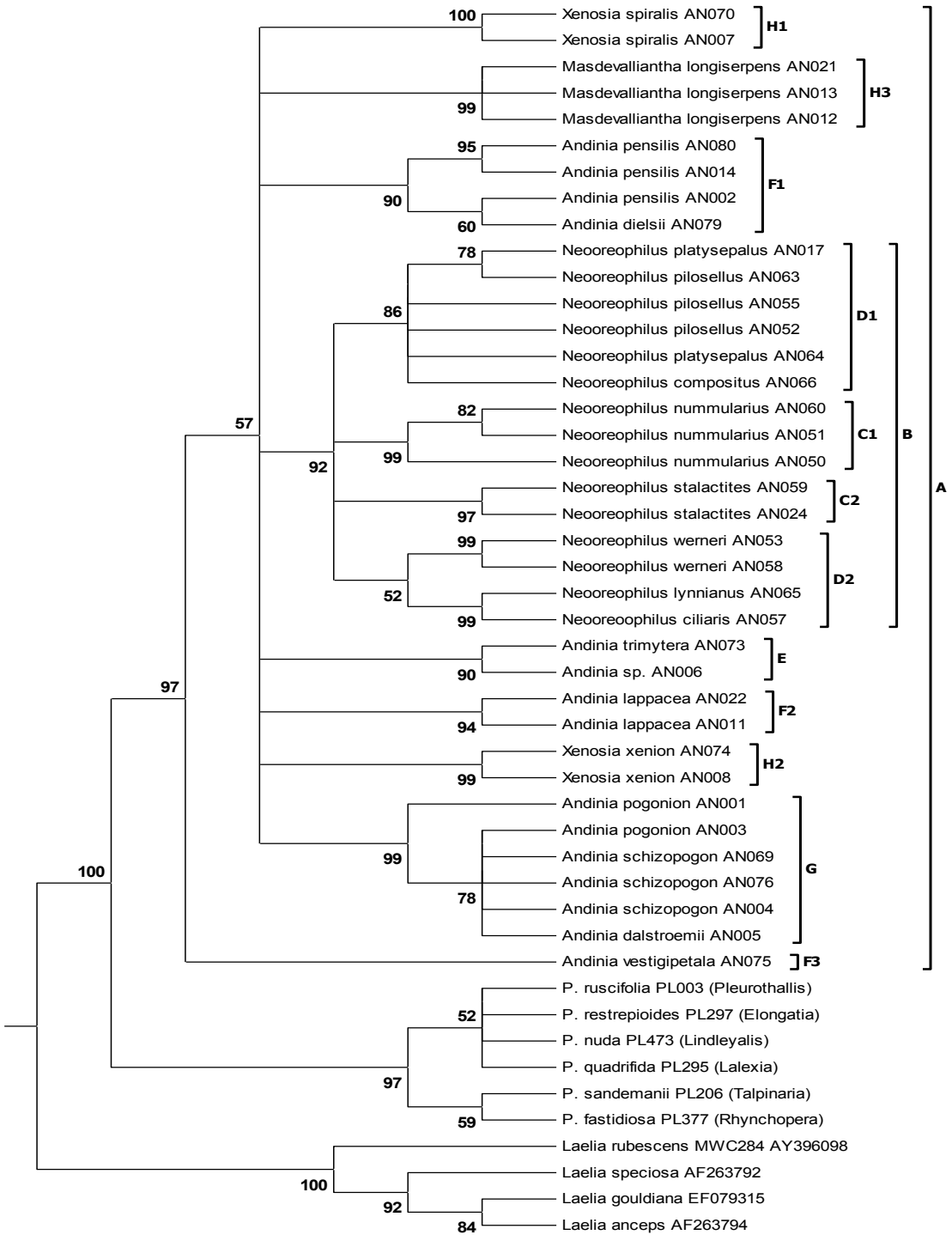

Figure 5: Bootstrap consensus phylogenetic tree created from the *matK* cpDNA data set using MP analysis with 1000 bootstrap replicates. Values at each node reflect percent bootstrap support and nodes with bootstrap values under 50% are condensed. Labels reflect clades from the ITS MP tree (Fig. 3).

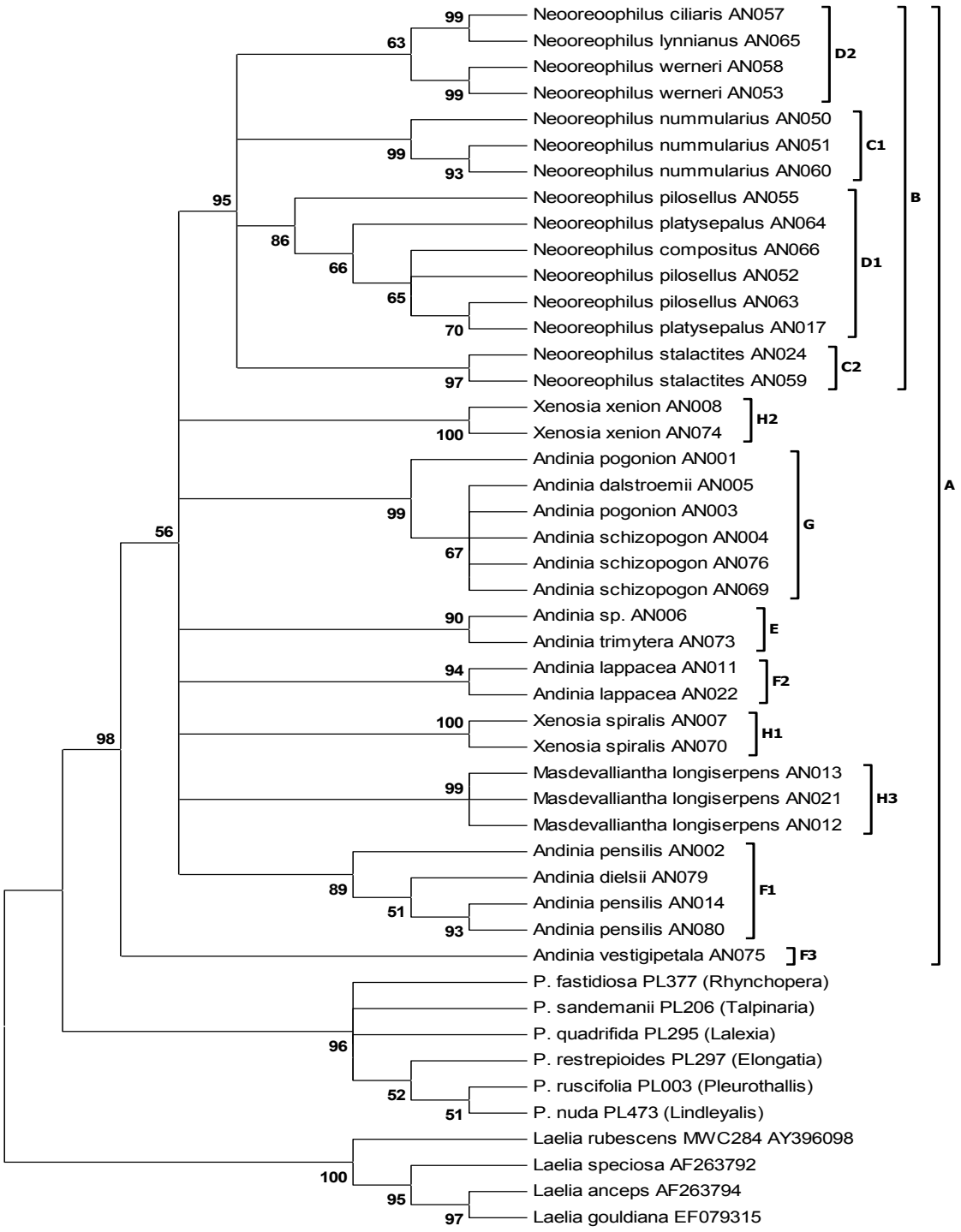

Figure 6: Phylogenetic tree created from the *matK* cpDNA data set using ML analysis with 1000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Branch length values are not shown in order to improve legibility. Values at each node represent percent bootstrap support.

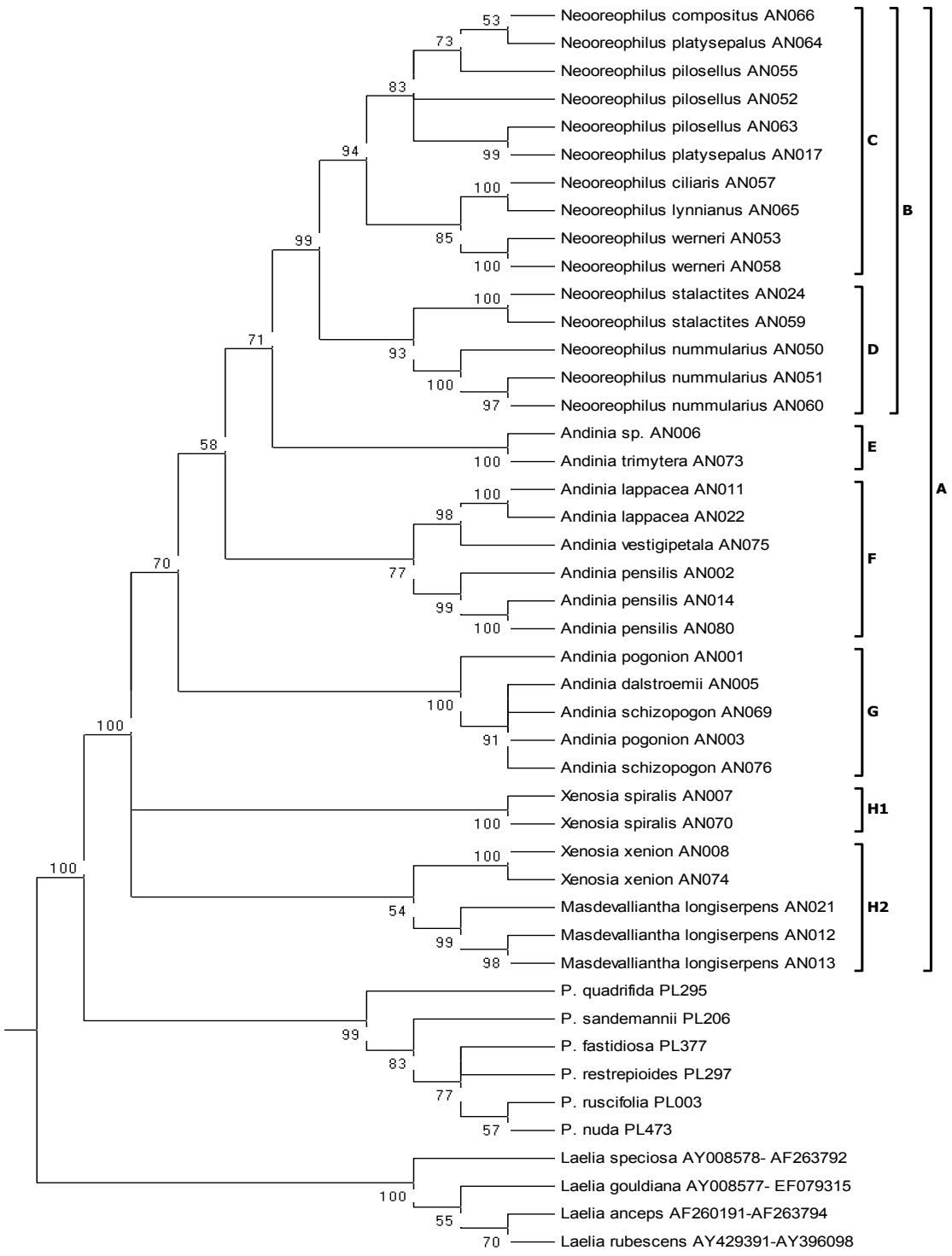

Figure 7: Bootstrap consensus phylogenetic tree created from the concatenated ITS nrDNA and *matK* cpDNA data set using MP analysis with 1000 bootstrap replicates. Values at each node reflect percent bootstrap support and nodes with bootstrap values under 50% are condensed. Clade lettering reflects clades from the ITS MP analysis.

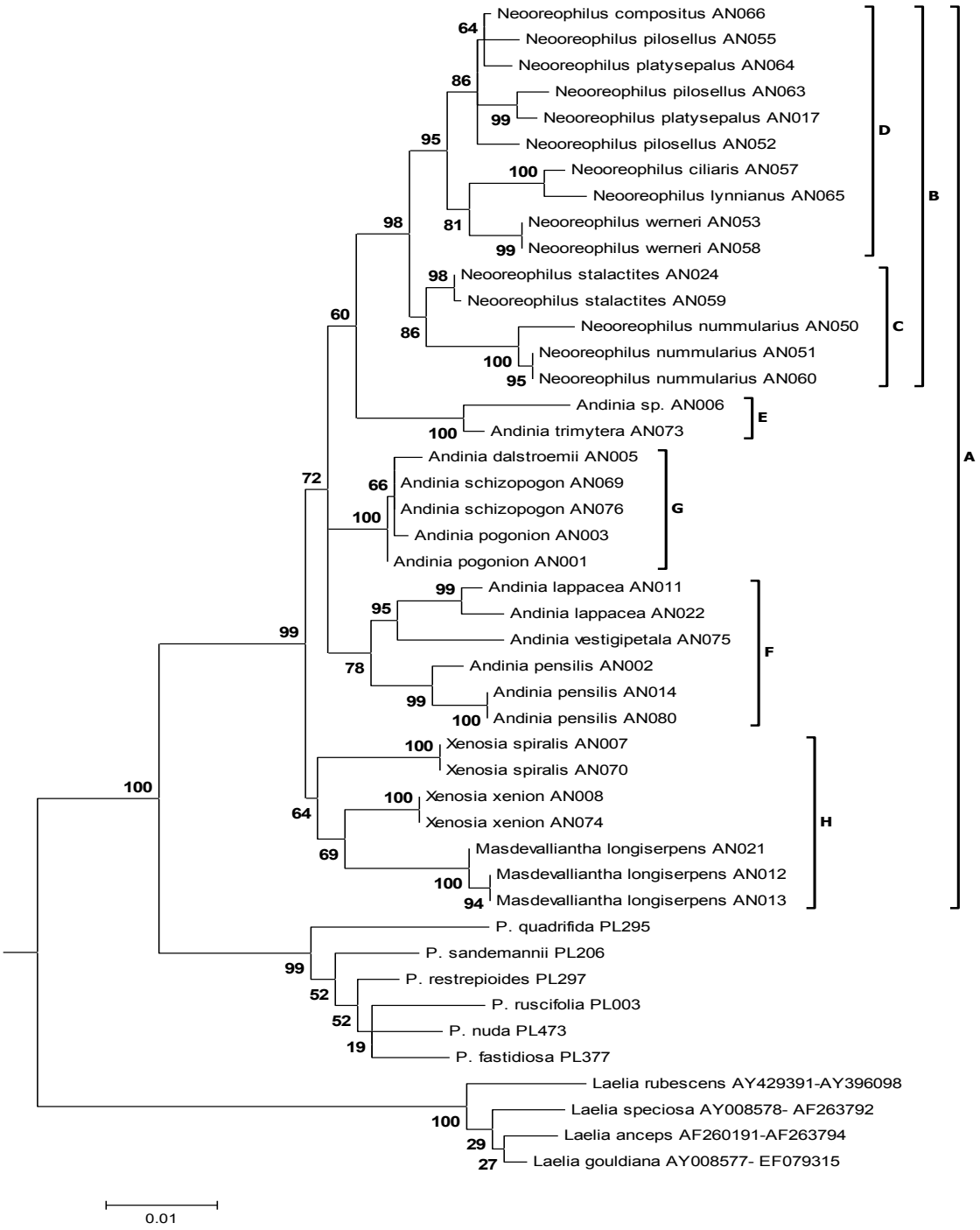

Figure 8: A phylogenetic tree with the highest log likelihood (-5705.7064) created from the concatenated ITS nrDNA and *matK* cpDNA data set using ML analysis with 1000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Branch length values are not shown in order to improve legibility. Values at each node represent percent bootstrap support.

Discussion

Analyses of the ITS, *matK*, and composite (i.e. concatenated) datasets all indicate strong support for the expansion of the genus *Andinia* to include the pleurothallid genera *Neooreophilus*, *Xenosia*, and *Masdevalliantha*. However, the two gene regions differ in their support of various infra-generic clades, perhaps reflecting the different evolutionary histories of the two gene regions. ITS nrDNA is bi-parentally inherited, whereas *matK* cpDNA is uni-parentally (maternally) inherited (Cafasso *et al.* 2005). The ITS dataset generally produced better-resolved clades than the *matK* analyses, in which there is a large polytomy. One possible explanation for the poor resolution of the *matK* phylogeny is that it is more highly conserved than the ITS gene region for the analyzed taxa. The ITS dataset had 153 parsimony informative characters of 790 total (19.4%), compared to 84 of 821 (10.2%) in *matK*. The difference in variability is likely attributable to the non-coding spacer regions, ITS1 and ITS2, in the ITS gene region, which each had approximately the same number of steps when taken individually as the entire *matK* gene in a phylogenetic analysis of the subtribe Pleurothallidinae (Pridgeon *et al.* 2001). In most plant taxa, the *matK* gene codes for the MatK protein responsible for splicing the *trnK* intron in which it is located (Vogel *et al.* 1997), though Kores *et al*. (2001) indicated that *matK* is probably a non-coding pseudogene in Orchidaceae. We first analyzed all sequences as non-coding, but when *matK* was analyzed as coding for amino acids, the resulting phylogenetic tree (not shown) showed lower support for every clade, implying that there may be indels creating reading frame shifts within the sequence.

However, this is not necessarily indicative of a pseudogene, as an additional *matK* start codon in Orchidaceae may buffer against these frame shifts (Barther 2006).

One benefit of including *matK* in the analysis is that it appears to have low rates of homoplasy in the taxa analyzed here. The Consistency Index (CI)—a measure of homoplasy—for parsimony-informative characters in the *matK* MP analysis is 0.806723, much better than the CI of 0.635542 in the ITS MP analysis (a CI of 1.0 indicates no homoplasy). The CI of the composite MP analysis is somewhere in between, as would be expected, at 0.697842. Though the level of homoplasy in *matK* seems to be lower than ITS, all of our MP analyses have strong CI values when compared to those predicted based on the number of taxa included in each analysis. Using the regression equation,

$$CI = 0.90 - 0.022(\text{number of taxa}) + 0.000213(\text{number of taxa})^2$$

(Sanderson & Donoghue 1989)

predicted CI values for our MP analyses are: 0.3822 for ITS (67 taxa), 0.3365 for *matK* (47 taxa), and 0.3413 for the composite analysis (45 taxa). For a relevant comparison, the CI for a combined matrix of ITS, *matK*, and *trnL* in a phylogenetic analysis of the subtribe Pleurothallidinae by Pridgeon *et al.* (2001) was 0.51.

The retention index (RI) (Farris 1989) is another measure of homoplasy, but measures how well synapomorphies—character changes shared by two or more groups derived in their most recent common ancestor—expected from the dataset are retained in the phylogenetic tree. All three analyses in this study had

very high retention indices (ITS = 0.892157; *matK* = 0.935754, composite = 0.886179), corroborating the low level of homoplasy inferred by the high CI scores.

The differences in variability between ITS and *matK* are likely the cause behind the incongruences in the two datasets. Gene regions with higher rates of evolution are more likely to provide better cladistic resolution, but those that evolve more slowly may better retain phylogenetic information (de Queiroz *et al.* 1995), hence the lower rate of homoplasy in our analysis of *matK*. Though both gene regions support an expanded *Andinia*, the *matK* matrices show poor resolution when it comes to infrageneric groupings. Indeed, *matK* alone has sometimes been insufficient even for resolving relationships between closely related genera (Pridgeon *et al.* 2001), let alone subgenera. Other than the expansion of *Andinia*, of the well-supported clades in the ITS analyses, *matK* only fully supports clades B and G, as well as the monotypic nature of clade E. The greater variability of ITS over *matK* means that ITS is weighted more heavily in the composite analysis, since the composite alignment was produced by concatenating the two sequences (de Queiroz *et al.* 1995). When dealing with multiple genes, there are multiple ways of dealing with the data, but concatenated sequence trees, such as the ones in this study, have been shown to more accurately reflect phylogenetic relationships than consensus gene trees, which are generated from the independent analyses of each gene region (Gadagkar *et al.* 2005).

We analyzed the concatenated sequences and both individual gene regions using two different methods of statistical analysis, MP and ML. MP analysis assumes that sequence similarities occur due to common inheritance, as opposed to homoplasy, selecting the most likely tree based on whichever requires the fewest changes to explain the data (Hall 2011). However, parsimony informative sites may be more likely to occur as a result of parallelism (i.e. convergent evolution), than due to inheritance (Felsenstein 1978). ML analyses were included in this study to mitigate the chance that the phylogenies produced—and the resulting taxonomic changes—are a result of homoplastic characters, even with the high CI values in our MP analyses. Likelihood is the hypothetical probability that an event that has already occurred will yield a specific outcome. So, the tree inferred by ML analysis shows an evolutionary history that is most likely to produce the similarities and differences in the dataset (Hall 2011).

In this study, ML analyses produced phylogenies that were highly congruent with MP phylogenies, only differing in their levels of support for certain clades. In the ITS dataset, the greatest difference between MP and ML analyses was in their support for the expansion of genus *Andinia* (clade A), though even the lower support from ML was a moderate 72%. The only other node in the condensed ITS analyses to differ by more than 5% in support between MP and ML was the grouping between *Xenosia xenion* and *Masdevalliantha longiserpens*, which was a moderate 68% in the MP analysis, but a much stronger 86% in the ML analysis. In both cases, it is clear that these species are

closely related. In the *matK* analysis, all clades with over 50% bootstrap support were composed of the same species in both MP and ML analyses, with bootstrap support differing by only 3% or less at each node in the condensed trees (Figs. 5 and 6).

Clade A— Proposed genus *Andinia*: The expansion of *Andinia* to include the genera *Neooreophilus, Masdevalliantha*, and *Xenosia* was supported in all analyses. *Andinia* Luer is the earliest name of the four genera to be included in the expanded genus and continues to reflect the distribution of the genus in the Andes mountains. The expansion will bring the total number of species in *Andinia* from 14 to 63.

Clade B— Proposed subgenus *Brachycladium*: We suggest resurrecting the subgeneric name *Brachycladium* for this clade, which contains all analyzed species of what is currently the genus *Neooreophilus*. Before its elevation to generic status, the group was *Lepanthes* subgenus *Brachycladium*, though *Brachycladium* turned out to be an invalid name at the generic level because it was already occupied by a fungal genus. While the elevation of this group to the generic level seemed warranted due to its dissimilarities from the genus *Lepanthes*, keeping it as a separate genus following our analysis would not be appropriate unless we were to separate clades E, F, G, and H out into their own genera as well, which itself is unnecessary due to the overwhelming levels of bootstrap support for clade A.

Clade C—Proposed section *Brachycladae*: In the former *Lepanthes* subgenus *Brachycladium*, *L. nummularia* and *L. stalactites* were each placed into

the monotypic sections *Brachycladae* and *Bilaminatae*, respectively. However, our results indicate that they are closely related, relative to the rest of *Neooreophilus*, and can both be included in one section, which we suggest be named *Brachycladae*, meaning “short branches”. *Brachycladae* was the earlier of the two section names and adequately reflects the characteristic of both plants having shorter ramicauls than rhizomes.

Clade D—Proposed section *Amplectentes*: This clade contains all species of the current genus *Neooreophilus* except for *N. nummularius* and *N. stalactites.* Our phylogenies have maintained the delineation between sections of the former *Lepanthes* subgenus *Brachycladium*, so we can infer that even the species of *Neooreophilus* that were not included in this study should be kept alongside the rest of the former section *Amplectentes*. We also propose that this name, *Amplectentes*, be maintained for the section in its new genus.

Clade E—Proposed subgenus Minuscula: *Andinia* as it is currently circumscribed does not form a single monophyletic clade, but instead is split into three monophyletic groupings. One of these is the monotypic clade formed in all analyses of this study by *A. trimytera*, which was formerly of *Pleurothallis* subgenus *Aenigma* before being moved to *Andinia* (Pridgeon *et al.* 2001). We suggest the subgeneric name *Minuscula* to encompass only *A. trimytera*, reflecting the small size (ca. 3 mm long x 2 mm wide) of the flowers.

Clade F— Proposed subgenus *Andinia*: This clade contains the type species of *Andinia*, *A. dielsii*, as well as the other initial species of *Andinia*, *A. pensilis*, and two species from the former *Pleurothallis* subgenus *Aenigma* (more

recently of *Andinia*), *A. vestigipetala* and *A. lappacea*. *A. vestigipetala* was previously distinguished from the rest of *Pleurothallis* subgenus *Aenigma* due to differences in floral characteristics, and was placed in the monotypic section *Vestigipetalae*, so molecular evidence for its distinction from the rest of the former *Pleurothallis* subgenus *Aenigma* is not surprising. However, the inclusion of *A. lappacea* in this group is more unexpected, as it was previously a part of the section *Aenigma* with the rest of the species found in *Pleurothallis* subgenus *Aenigma*. Although the *matK* analyses do not support the inclusion of *A. vestigipetala* and *A. lappacea* with *A. dielsii* and *A. pensilis,* they cluster together in ITS and composite analyses. Due to the inclusion of the type specimen of *Andinia*, we suggest the subgeneric name *Andinia* for this clade.

Clade G—Proposed subgenus *Aenigma*: With the exception of *Andinia lappacea* and *A. trimytera*, this clade contains all species that were once in the *Pleurothallis* subgenus *Aenigma* section *Aenigmata*, so we propose also adding all species from that section that were not included in our analysis and resurrecting the subgeneric name *Aenigma* for this group. *Pleurothallis* subgenus *Aenigma* was characterized by a shortly repent, ascending rhizome with more or less bunched, short ramicauls, which have a successively flowered inflorescence arising through an annulus (ring-shaped structure); the section was characterized by a long-spiculate ovary, semiconnate (united) lateral sepals, and a column that is shorter than the lip (Luer 1986b).

Clade H—Proposed subgenus *Masdevalliantha*: This clade is perhaps the most tenuous of those supported by our analyses, with no support in the *matK*

analyses and moderate support elsewhere. However, we are still proposing to combine what are at present the distinct genera *Xenosia* and *Masdevalliantha* into a single subgenus within *Andinia*. The alternative may be to combine *X. xenion* and *M. longiserpens*, splitting *X. spiralis* into its own subgenus. However, there is no evidence suggesting that *X. spiralis* is wholly distinct from *X. xenion* and *M. longiserpens* and this approach would ignore the moderate support for the entire group, thus poorly reflecting the evolutionary history suggested by our analyses. Luer (2004) articulated the vegetative morphological similarities between *X. xenion,* which occurs in Colombia, and *M. longiserpens*, which is found in Peru, but distinguished them on the morphology of the lip and its attachment to the column foot. Vegetatively, *X. spiralis* also shares characteristics with *M. longiserpens,* most notably the repent growth habit and the ramicauls, which in both species are described as stout, ascending-erect, and enclosed by 2-3 loose, tubular sheaths (Luer 2006).

Conclusions—The analyses here support preliminary work by Wilson & Jost (2009, 2011) exploring the relatedness between *Andinia* and other genera in the subtribe Pleurothallidinae. The *matK* cpDNA matrices strongly support the expansion of *Andinia* initially suggested by ITS nrDNA, with high similarity between results of both MP and ML analyses. The expansion of *Andinia* will add an additional 49 species of Andean orchids to the genus, two from *Xenosia,* two from *Masdevalliantha*, and the rest from *Neooreophilus*. Proposed taxonomic changes can be found in Appendix I.

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Appendix I: Proposed taxonomy

To be published shortly by Wilson, M., Karremans, A., *et al.*.

Andinia (Luer) Luer

Type: *Andinia dielsii* (Mansf.) Luer

Andinia subgenus *Andinia* Wilson & Karremans, subgen. nov.

Andinia dielsii (Mansf.) Luer

Andinia lappacea (Luer) Pridgeon & M.W.Chase

Andinia pensilis (Schltr.) Luer

Andinia vesitigipetala (Luer) Pridgeon & M.W.Chase

Andinia subgenus *Aenigma* Wilson & Karremans, subgen. nov.

Andinia schizopogon (Luer) Pridgeon & M.W.Chase

Andinia pogonion (Luer) Pridgeon & M.W.Chase

Andinia dalstroemii (Luer) Pridgeon & M.W.Chase

Andinia hirtzii Luer

Andinia hystricosa (Luer) Pridgeon & M.W.Chase

Andinia ibex (Luer) Pridgeon & M.W.Chase

Andinia panica (Luer & Dalström) Pridgeon & M.W.Chase

Andinia pentamytera (Luer) Pridgeon & M.W.Chase

Andinia subgenus *Brachycladium* Wilson & Karremans, subgen. nov.

Section *Brachycladae* Wilson & Karremans

Andinia nummularia (Rchb.f.) Wilson & Karremans, comb. nov.

Andinia stalactites (Luer & Hirtz) Wilson & Karremans, comb. nov.

Section *Amplectentes* Wilson & Karremans

Andinia ariasiana (Luer & L. Jost) Wilson & Karremans, comb. nov.

Andinia bifida (Tobar & Archila) Wilson & Karremans, comb. nov.

Andinia cardiochila (Luer & R. Escobar) Wilson & Karremans, comb. nov.

Andina caveroi (D.E. Benn. & Christenson) Wilson & Karremans, comb. nov.

Andinia chelosepala (Luer & Hirtz) Wilson & Karremans, comb. nov.

Andinia chilopsis (Luer & Hirtz) Wilson & Karremans, comb. nov.

Andinia ciliaris (Luer & Hirtz) Wilson & Karremans, comb. nov.

Andinia composita (Luer & R. Escobar) Wilson & Karremans, comb. nov.

Andinia cordilabia (Luer) Wilson & Karremans, comb. nov.

Andinia dactyla (Garay) Wilson & Karremans, comb. nov.

Andinia destituta (Luer & R. Escobar) Wilson & Karremans, comb. nov.

Andinia erepsis (Luer & Hirtz) Wilson & Karremans, comb. nov.

Andinia exigua (Luer & L. Jost) Wilson & Karremans, comb. nov.

Andinia geminipetala (Luer & J. Portilla) Wilson & Karremans, comb. nov.

Andinia hippocrepica (Luer & R. Escobar) Wilson & Karremans, comb. nov.

Andinia irrasa (Luer & R. Escobar) Wilson & Karremans, comb. nov.

Andinia lunaris (Luer) Wilson & Karremans, comb. nov.

Andinia lunatocheilla (Tobar & Archila) Wilson & Karremans, comb. nov.

Andinia lupula (Luer & Hirtz) Wilson & Karremans, comb. nov.

Andinia lynniana (Luer) Wilson & Karremans, comb. nov.

Andinia macrotica (Luer & Dalström) Wilson & Karremans, comb. nov.

Andinia micropetala (L.O. Williams) Wilson & Karremans, comb. nov.

Andinia mongeei (Tobar & Archila) Wilson & Karremans, comb. nov.

Andinia monilia (Luer & R. Escobar) Wilson & Karremans, comb. nov.

Andinia montis-rotunda (P. Ortiz) Wilson & Karremans, comb. nov.

Andinia octocornuta (Luer) Wilson & Karremans, comb. nov.

Andinia ortiziana (S.V. Uribe & Thoerle) Wilson & Karremans, comb. nov.

Andinia pendens (Garay) Wilson & Karremans, comb. nov.

Andinia persimilis (Luer & Sijm) Wilson & Karremans, comb. nov.

Andinia phalica (Toabr & Archila) Wilson & Karremans, comb. nov.

Andinia pholeter (Luer) Wilson & Karremans, comb. nov.

Andinia pilosella (Rchb.f.) Wilson & Karremans, comb. nov.

Andinia platysepala (Luer & R. Escobar) Wilson & Karremans, comb. nov.

Andinia pseudocaulescens (L.B. Sm. & S.K. Harris) Wilson & Karremans, comb. nov.

Andinia ricii (Luer & R. Vasquez) Wilson & Karremans, comb. nov.

Andinia triangularis (Luer) Wilson & Karremans, comb. nov.

Andinia tridactyla (Luer) Wilson & Karremans, comb. nov.

Andinia ursula (Luer & R. Escobar) Wilson & Karremans, comb. nov.

Andinia viebrockiana (Luer & L. Jost) Wilson & Karremans, comb. nov.

Andinia vieira-pereziana (P. Ortiz) Wilson & Karremans, comb. nov.

Andinia villosa (Løjtnant) Wilson & Karremans, comb. nov.

Andinia werneri (Luer) Wilson & Karremans, comb. nov.

Andinia subgenus *Masdevalliantha* subgen. nov.

Andinia longiserpens (C. Schweinf.) Wilson & Karremans, comb. nov.

Andinia masdevalliopsis (Luer) Wilson & Karremans, comb. nov.

Andinia spiralis (Ruiz & Pav.) Wilson & Karremans, comb. nov.

Andinia xenion (Luer & R. Escobar) Wilson & Karremans, comb. nov.

Andinia subgenus *Minuscula* subgen. nov.

Andinia trimytera (Luer & R. Escobar) Pridgeon & M.W. Chase

Chapter 2
Scanning electron microscopy (SEM) of floral morphological traits

Introduction

Floral characteristics in Orchidaceae are very diverse, corresponding with a wide array of mechanisms for attracting pollinators. This diversity has drawn the attention of scientists since Charles Darwin (1877), who was ahead of his time in recognizing the close relationship between floral characteristics and pollinator morphology. Classification of the subtribe Pleurothallidinae, a subset of which was the focus of the phylogenetic analysis in Chapter One, has been plagued by homoplasy in vegetative and floral morphological characteristics (Neyland, Urbatsch, & Pridgeon 1995). Many floral homoplasies are the result of convergent evolution in which species have adapted to attract similar types of pollinators. For example, attenuated petals with apical osmophores and actively mobile labella—both of which are likely adaptations for pollinators—occur in relatively unrelated species (Pridgeon *et al.* 2001, Luer 1986a).

Though the close association between a species' pollinators and its floral characteristics makes them unreliable for determining phylogenetic relationships, the association allows us to infer pollination strategies by studying morphological features. In a typical orchid flower, the gynostemium, commonly referred to as the column, lies at the center, a result of the fusion of stigma and stamen (Claessens & Kleynen 2013). Within the stamen lies the pollinarium, a unit containing a variable number of pollinia (pollen sacs held together by the sticky fluid elasctoviscin), which are connected by the caudicle to the viscidium, a viscid (i.e. sticky) structure. In myophilous species, the viscidium attaches to the

pollinator while it either attempts to obtain a food reward (Calderon-Saenz 2011) or to copulate with the flower (Alvarez). *Pleurothallis marthae* attracts fungus gnat pollinators by mimicking the smell of the fungal substrate necessary for the flies' larval development (i.e. that of rotting flesh), and uses nectar production as an incentive to keep the flies on the flower for a longer period of time, increasing the likelihood of pollination (Duque-Buitrago *et al.* 2014). Similarly, two species of *Stelis*, also of Pleurothallidinae, were shown to produce fragrances in conjunction with nectar-like substances as a method of attracting dipteran pollinators (Albores-Ortiz & Sosa 2006).

Attractive fragrances are produced by osmophores, which can be located on the petals, sepals, or lip (Vogel 1990). Olfactory attractants are often highly specific to a particular pollinator, and a diversity of sensory biases in pollinators may have driven the evolution of such diverse scents in orchids (Ramírez *et al.* 2011). Peakall *et al*. (2010) recognized differences in the chemical compounds responsible for pollinator attraction as the basis for high specificity of orchid-pollinator interactions in the Australian orchid genus *Chiloglottis* (subtribe Drakaeinae). Albores-Ortiz & Sosa (2006) suggested that two sympatric *Stelis* avoid hybridization, despite coinciding flowering times, by producing different scents that were chemically different. Thus, scent production can have a substantial impact on a species' evolutionary history.

Lepanthes glicensteinii offers no food reward to pollinators, but, in the first known instance of sexual deception in the Pleurothallidinae, pollinators always approached from downwind and the surface of the lip is covered in papillate cells.

These two observations led Blanco & Barboza (2005) to hypothesize that *L. glicensteinii* was releasing an olfactory cue, mimicking a female dipteran, from an osmophore on the lip. *Neooreophilus pendens,* a species that we are proposing to add to *Andinia*, has also been observed being pollinated through this sort of sexual deceit, a process termed pseudocopulation in which the male fly attempts to copulate with the orchid flower, under the impression that it is mating with a female (Alvarez). Successful removal of pollinia by fungus gnats has been observed during attempts at copulation with flowers of *Lepanthes yubarta* (Calderón-Sáenz 2012).

Though nearly one-third of species in Orchidaceae are deceit pollinated (Cozzolino & Widmer 2005), including the vast majority of Pleurothallidinae (Luer 1986a [in Pridgeon *et al.* 2001]) some species do offer a food reward to pollinators. *Pleurothallis marthae,* mentioned above, offers a nectar-like reward to increase the likelihood of pollination by incentivizing pollinators to stay longer (Duque-Buitrago *et al.* 2014). Similarly, *Specklinia* (*Pleurothallis*) *fuegi* has small osmophores on the ends of its petals, but secretes nectar “in a small pan on the labellum” (Vogel 1990).

Though little is known about the pollination mechanisms of *Andinia* and the genera we are proposing to add to the expanded genus, scanning electron microscopy (SEM) of the flowers should give us insight as to whether they use deceit or offer a reward. The “small pan” on the lip of some species (Vogel 1990) is termed the glenion in Pleurothallidinae and its presence is probably indicative of reward pollination. We expect to find a glenion in some of our specimens, as

some species not yet acquired for SEM, such as *Andinia lappacea*, have a glenion visually obvious to the naked eye. The absence of a glenion, the overall structure of the lip, and the presence of an osmophore may be taken to indicate that a species utilizes pseudocopulation as a pollination mechanism. Though osmophores are also present on some reward-pollinated flowers, and are therefore not a definitive indicator independent of other characteristics, they are critical to the efficacy of pseudocopulation. Since there is evidence that multiple species in Pleurothallidinae are pollinated by pseudocopulation, including *Neooreophilus pendens*, we expect that some of the specimens in our study will have structures suggesting pseudocopulation as their pollination mechanism.

Methods

Plant material

Sources of orchid flowers are listed in Table 2. We have made an effort to include species from each clade in the phylogenetic analysis of Chapter 1, but continued efforts are being made to have multiple representatives from each clade to identify pollinator attraction strategies at the infrageneric level, as both mechanisms may occur within a single clade.

Andinia (AN) project number	Latin Binomial	Donor/Plant Number/Origin	Proposed subgenus	Imaging Technique
AN021	*Masdevalliantha longiserpens* (C.Schweinf.) Szlach. & Marg.	Ecuagenera	*Masdevalliantha*	High vacuum
AN075	*Andinia vestigipetala* (Luer) Pridgeon & M.W.Chase	Thoerle	*Andinia*	Low vacuum
AN076	*Andinia schizopogon* (Luer) Pridgeon & M.W.Chase	Andy's	*Aenigma*	High vacuum
AN082	*N. nummularius* (Rchb.f.) Archila	Driessen	*Brachycladium*	Low vacuum
AN083	*N. nummularius* (Rchb.f.) Archila	Doucette	*Brachycladium*	High vacuum
AN084	*Andinia pensilis* (Schltr.) Luer	Doucette	*Andinia*	High vacuum

Table 2: A list of plant material imaged using scanning electron microscopy. The "Proposed subgenus" column refers to results of the phylogenetic study in Chapter 1.

Scanning Electron Microscopy (SEM)

Low Vacuum samples

Specimens were preserved in KEW mix, a mixture of 5% formaldehyde solution (37.6% formaldehyde), 53% methanol, 5% glycerol, and 37% deionized water. Aluminum mounts, i.e. stubs, (Electron Microscopy Sciences, Cat. #75450) were drilled with a small hole in the center and sprayed with carbon. After being transferred into a series of deionized water rinses, specimens were placed on the stubs, the holes in which were filled with deionized water to mitigate drying out of the specimens during imaging. Specimens were imaged using Jeol JSM-6390LV scanning electron microscope under a low vacuum with an accelerating voltage of 10-20 kV.

High Vacuum samples

Specimens were preserved in 70% ethanol for transportation. Upon receipt, they were fixed in KEW mix, and then dehydrated in successively higher concentrations of ethanol for 15 min each (80%, 95%, 100%, 100%) before being placed in fresh 100% ethanol. Specimens were then removed from the ethanol and placed in the critical point dryer (EMS 850). The system was first cooled to 5°C, and then the chamber was filled with liquid CO_2. While maintaining the system temperature at 5°C, the liquid CO_2 in the chamber was purged and replaced two times. Finally, the system was heated to 35°C and pressure was allowed to build until the liquid CO_2 reached its critical point and vaporized. Once dry, specimens were placed on a stub with a carbon conductive tab. Edges of the specimen were carefully painted with liquid carbon in order to improve conductivity from the specimen to the stub. After the carbon was allowed to dry, mounted specimens were subjected to three rounds of sputter coating with gold. Specimens were imaged using a Jeol JSM-6390LV scanning electron microscope with an accelerating voltage of 10-15 kV.

Results

Masdevalliantha longiserpens

High vacuum scanning electron microscopy (SEM) of *M. longiserpens* (Fig. 1) revealed a concave glenion in the center of the labellum, or lip. The column is set back behind the lip, with the anther cap still in place over the pollinarium. The viscidium is visible, as the end of the pollinarium sticking out

from under the anther cap, appearing rounded in a half-dome shape. Beneath the anther cap and pollinarium, some of the stigmatic surface is visible.

Andinia schizopogon

Under a high vacuum, SEM imaging on *A. schizopogon* (Fig. 2) shows that the pollinia have been displaced from our specimen. As a result, the entire rostellum is visible, having not yet dried up to reveal the stigmatic surface. There appears to be some sort of globular substance on the end of the rostellum. Beneath the rostellum, the stigmatic surface is visible. A large, trilobed lip sticks out at the base of the column. The glenion lies at the center of the lip, surrounded by more or less uniform protrusions that appear to be multicellular.

Andinia pensilis

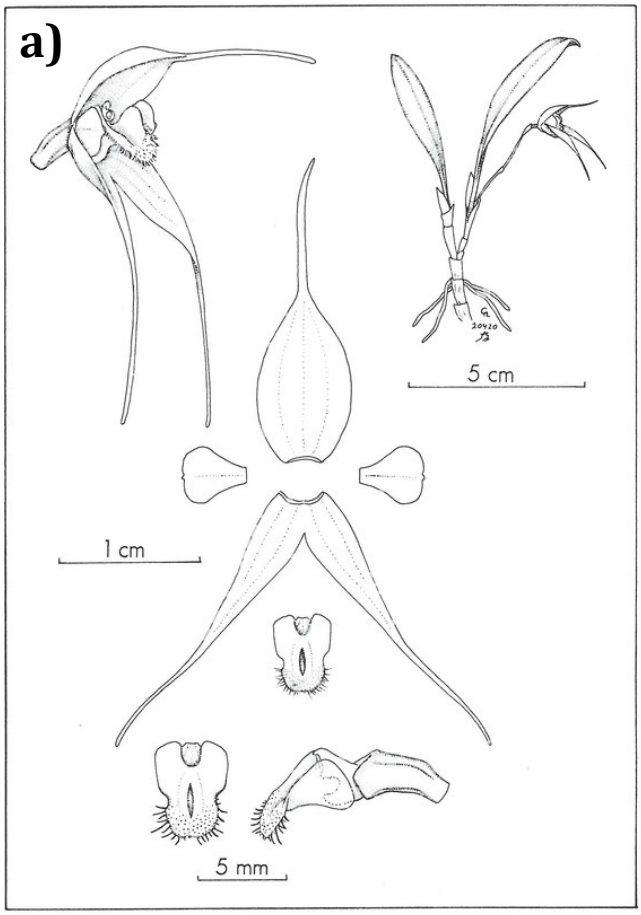
High vacuum SEM of *A. pensilis* (Fig. 3) shows that once again the pollinarium has been displaced in this specimen, leaving a rostellum that has yet to dry upward. The stigmatic surface beneath the rostellum is not as readily visible with the rostellum still in place as it was in *A. schizopogon*. The top of the column surrounding the rostellum is covered in papillate cells. The lip wraps loosely around the entire column, sticking out in the front. On the lip, just beneath the column, is a callus that protrudes from what would otherwise be the most deeply recessed part of the lip. The lip has no apparent glenion.

Neooreophillus nummularius

SEM of *N. nummularius* (Fig. 4) under a high vacuum shows that the surface of entire flower, more or less, is covered in papillate cells. The two lateral petals do no stick out far from the base of the column. On top of the column, the anther cap has remained in place on this specimen. Just beneath the anther cap, two pollinia are visible, though they do not come together into a single pollinarium. In front of the column, at the very base, a very small lip is apparent. The lip is somewhat rounded on the end that abuts the column, dips down slightly, then has a more slender end projecting upwards.

Andinia vestigipetala

SEM of the column of *A. vestigipetala* (Fig. 5) under a low vacuum resulted in poorer resolution, but nonetheless revealed that in our specimen, the pollinia had been displaced and the rostellum had dried upward, revealing the stigmatic surface. The lip sticks out slightly from the end of the column, with a fringed edge and no apparent glenion.



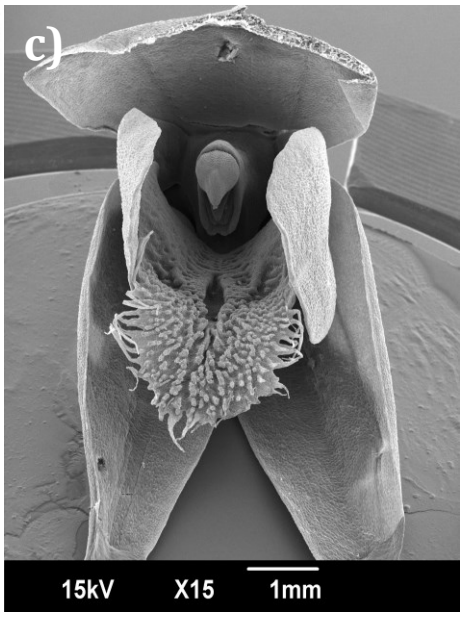
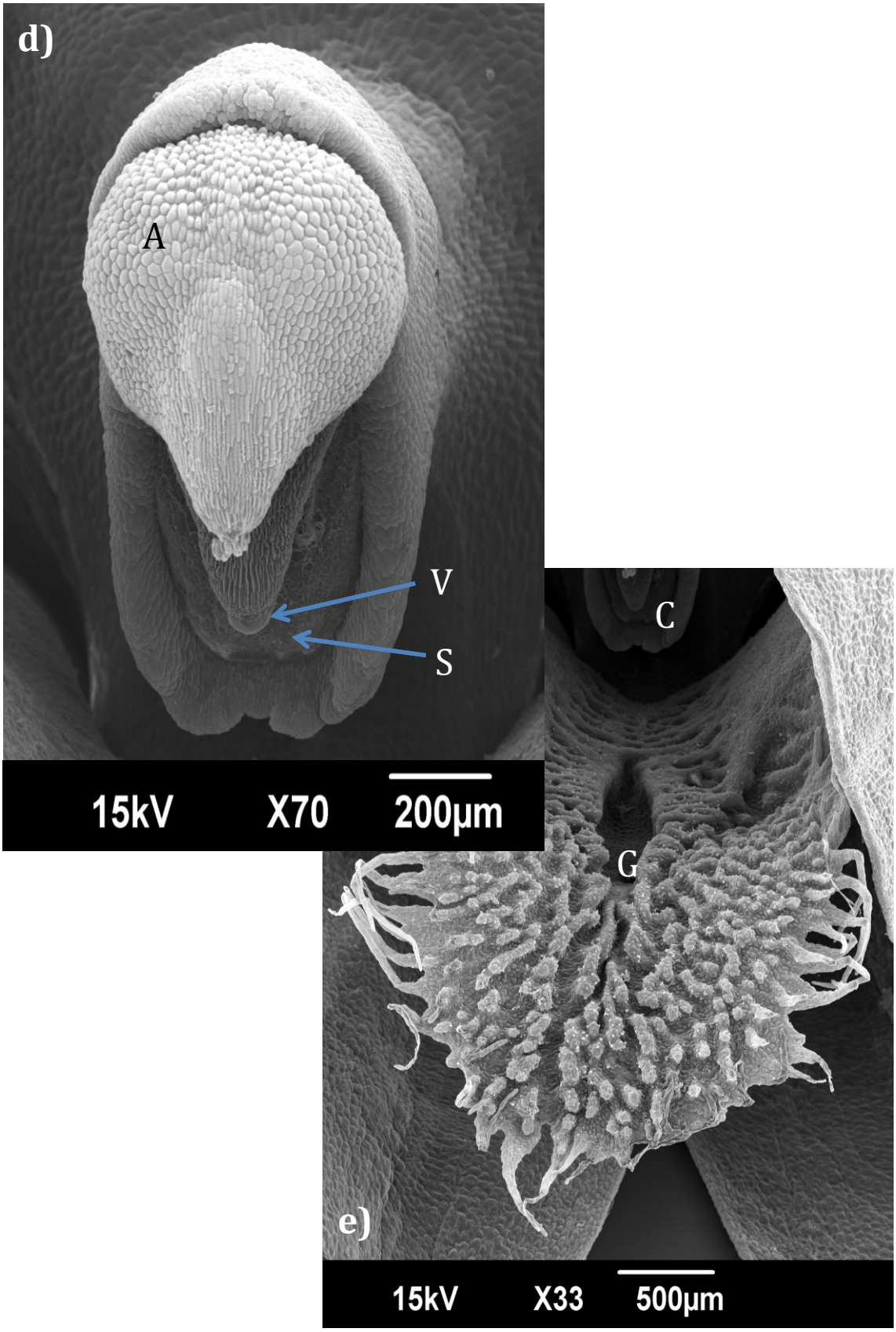


Figure 1 ***Masdevalliantha longiserpsens*** **(AN021): a)** Illustration of the vegetative and morphological characteristics of the species (Luer 2006); **b)** Photo of a flower of another specimen, with the red labellum (L) in the center particularly apparent (S. Manning); **c)** SEM micrograph of the entire flower; **d)** SEM micrograph of the column, including the anther cap (A), viscidium (V) and stigmatic surface (SS); **e)** SEM micrograph of the lip, with the glenion (G) in the center and the column (C) visible in the background.


d)
A
V
S
15kV X70 200µm
C
G
e)
15kV X33 500µm

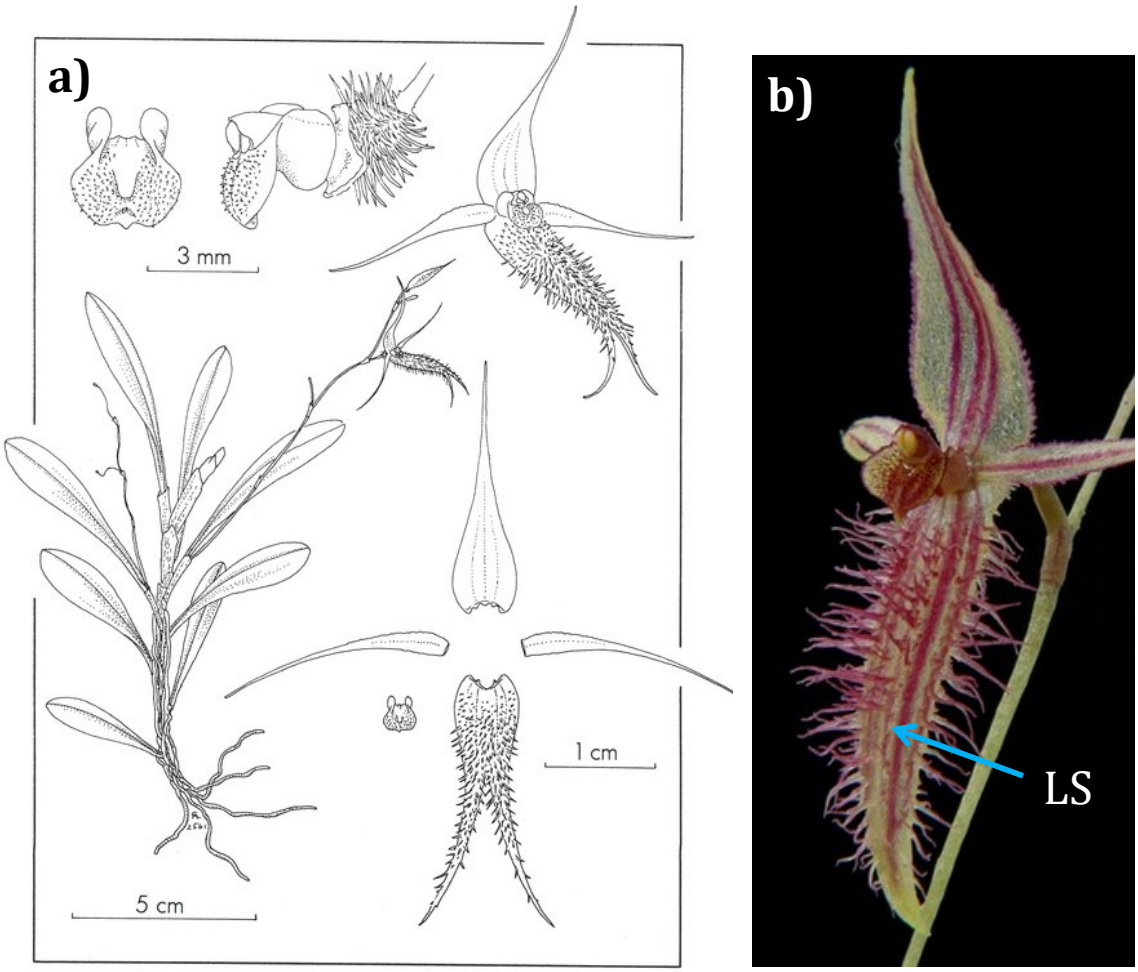
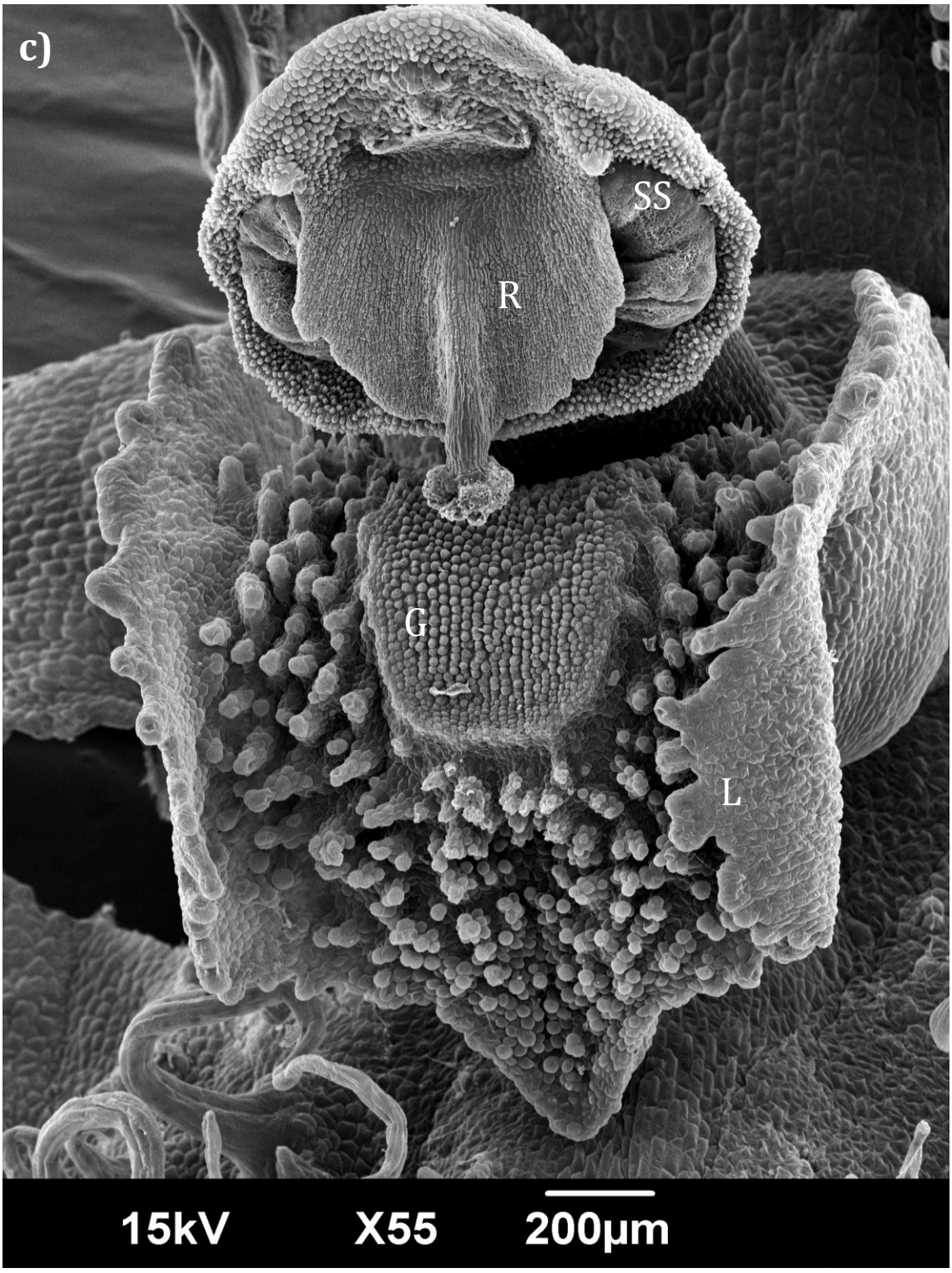
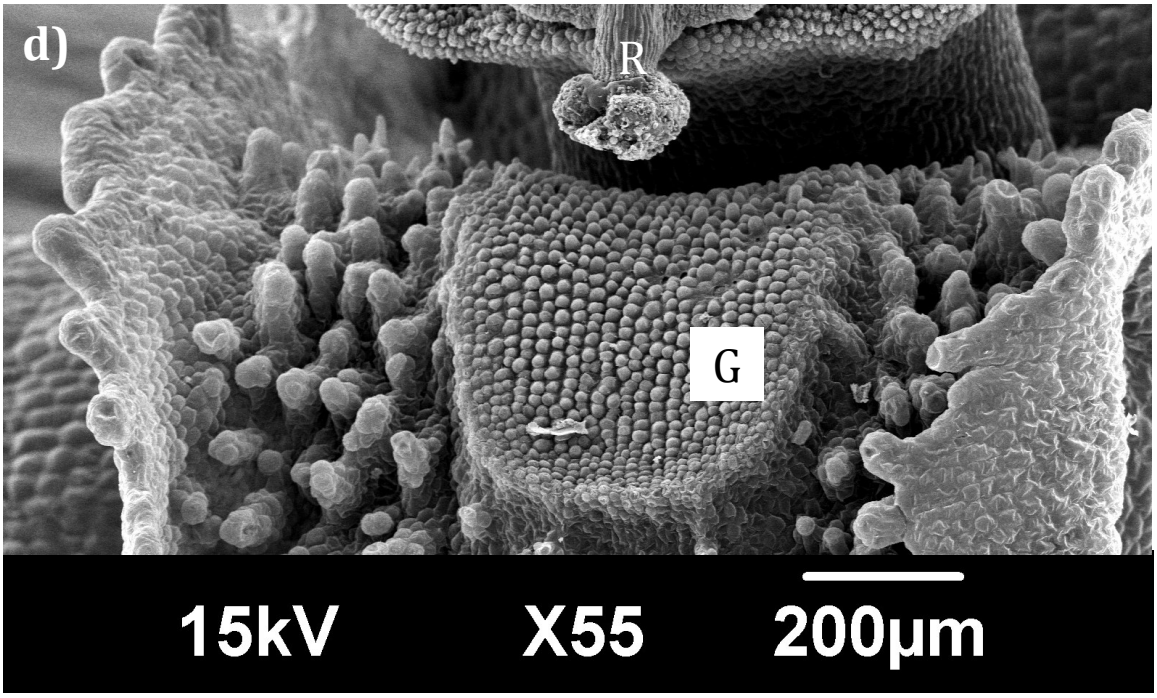
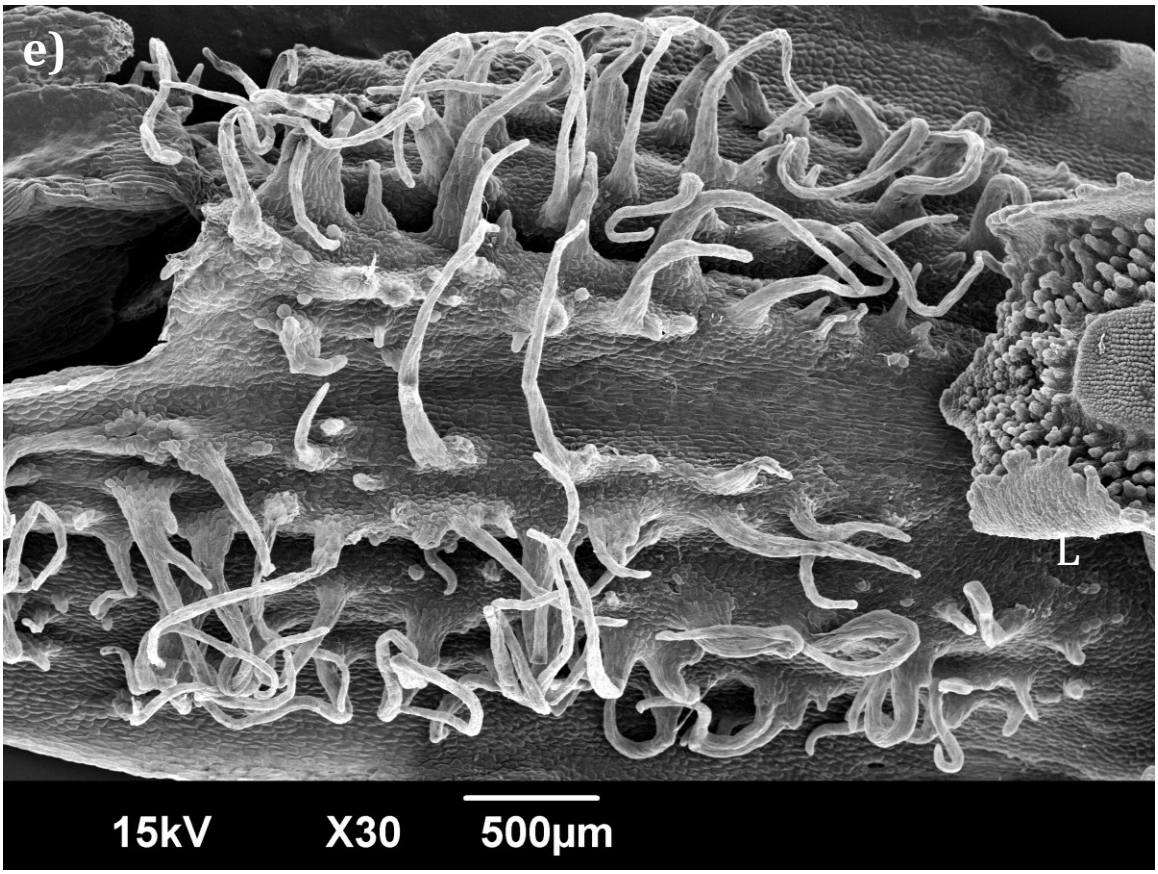


Figure 2 ***Andinia schizopogon*** **(AN076): a)** Illustration of vegetative and floral characteristics (Luer 2006); **b)** Flower of another specimen, with the long-spiculate-pubescent lateral sepals (LS) readily apparent (W. Driessen); SEM micrograph of entire flower; **c)** The column, from which the pollinia have been removed to reveal the rostellum (R)—covering the stigmatic surface (SS)—and the lip (L) with a glenion (G) in the center; **d)** A closer look at the glenion (G) with the end of the rostellum (R) dangling above; **e)** The lateral sepals, with the lip (L) to the right.


c)
SS
R
G
L
15kV
X55
200µm


d)
R
G
15kV
X55
200µm


e)
L
15kV
X30
500µm

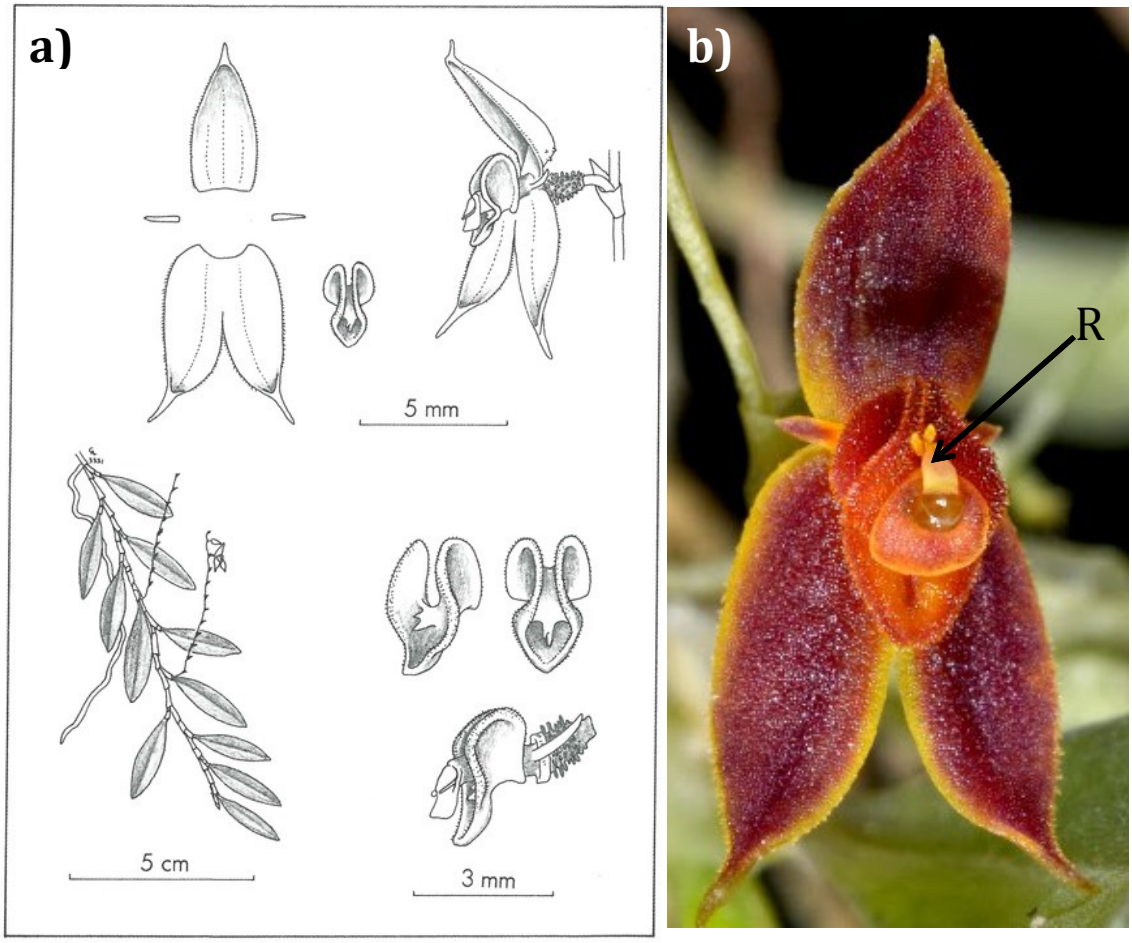
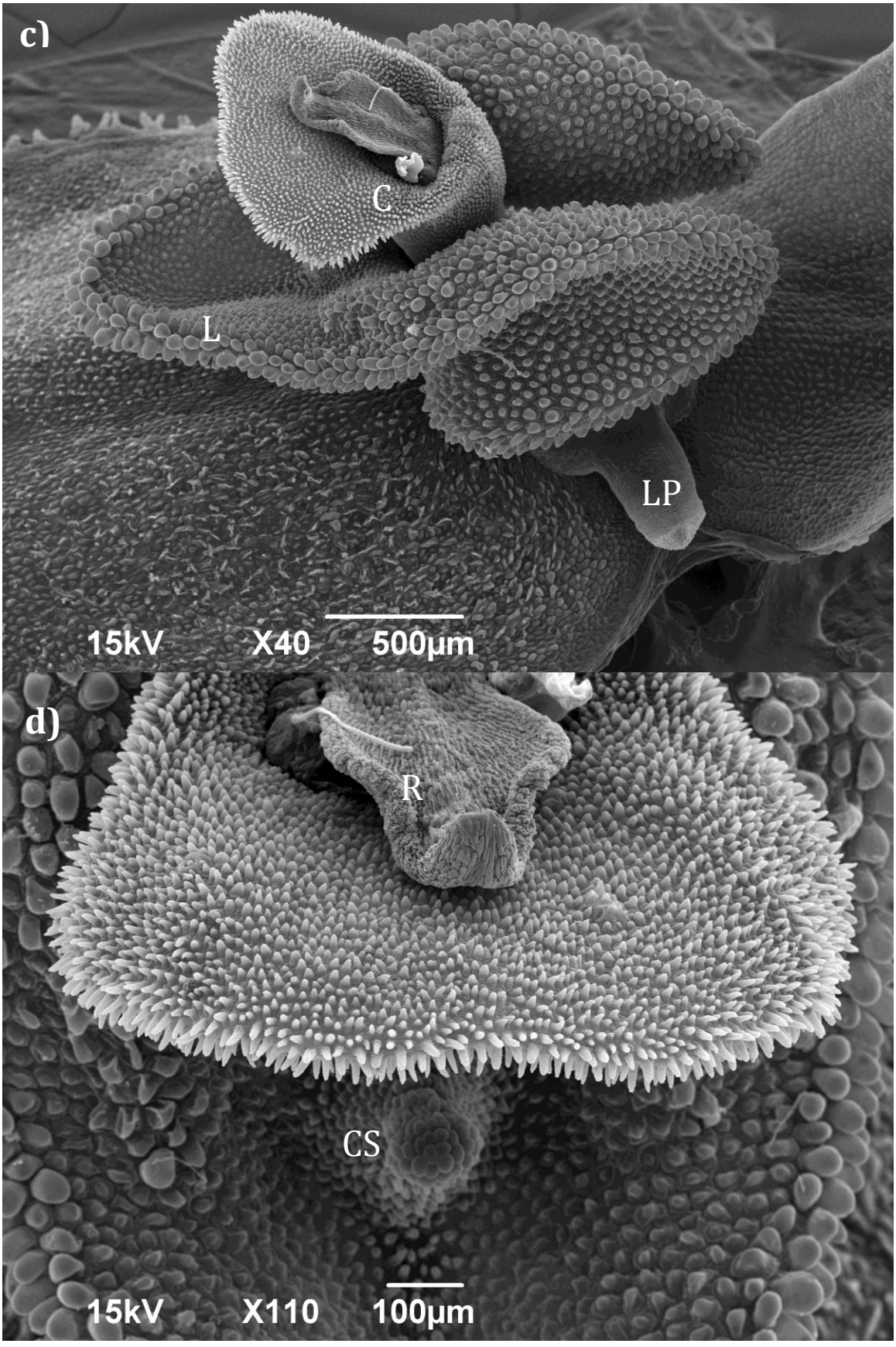
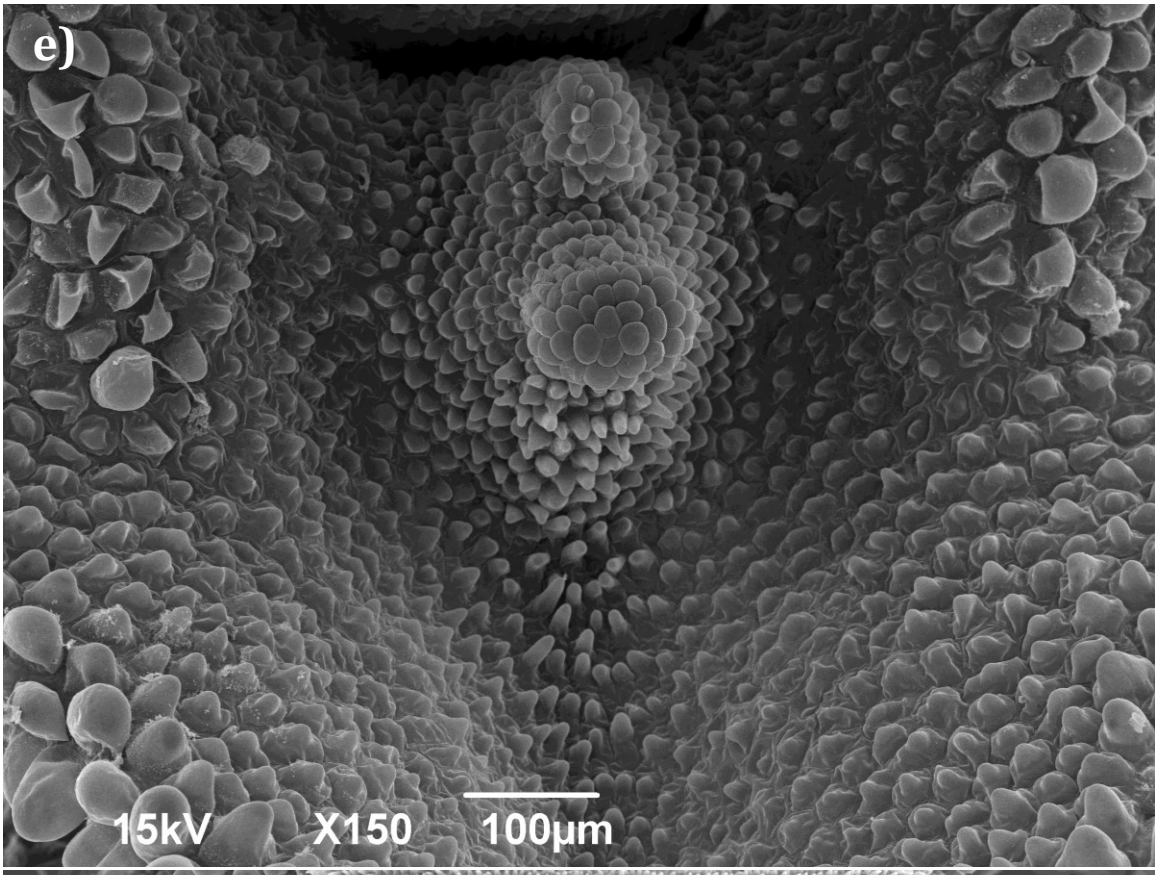
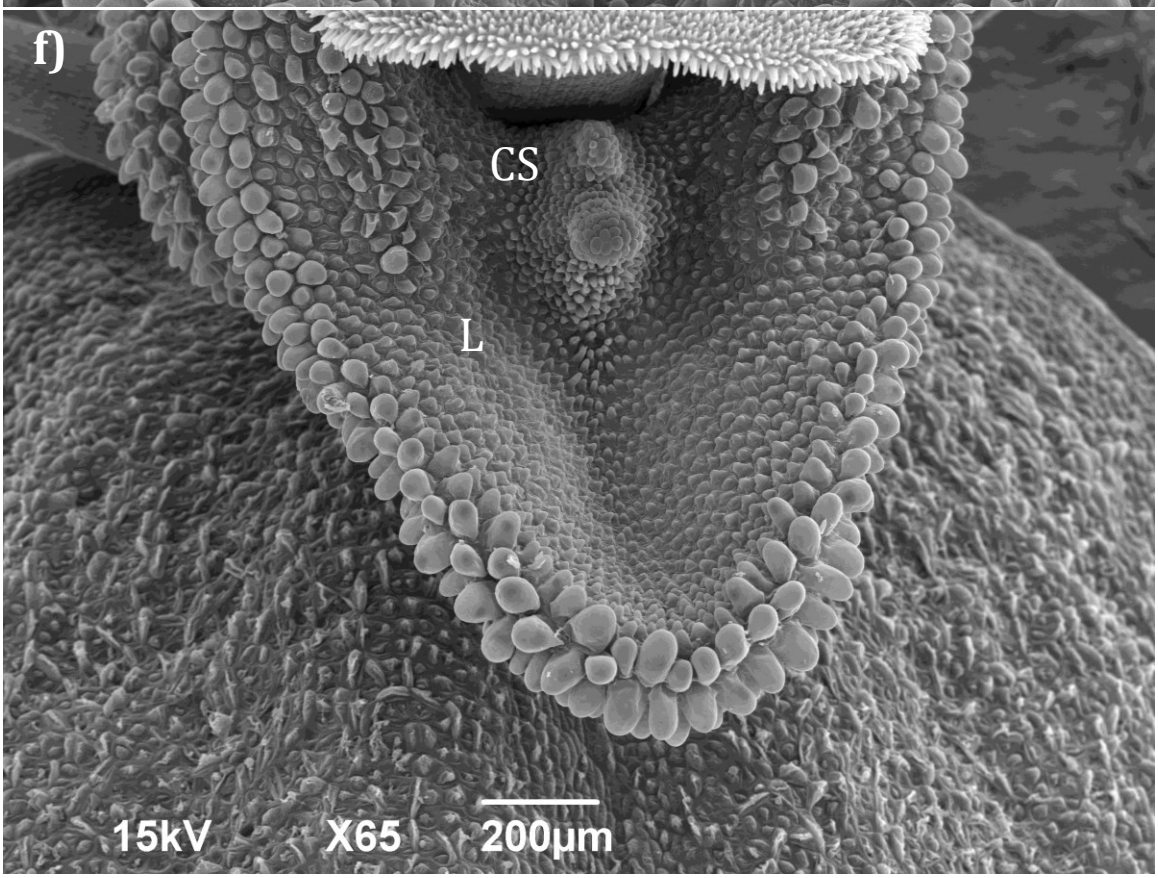


Figure 3 ***Andinia pensilis*** **(AN084): a)** Illustration of vegetative and morphological features (Luer 1991); **b)** Photograph of a flower in which the pollinia have been displaced and the rostellum (R) has dried upward to reveal the stigmatic surface (Parsons); **c)** SEM micrograph of the column (C), labellum (i.e. lip) (L), and a lateral petal (LP); **d)** SEM micrograph showing the rostellum (R) lying flat, and a callus (CS) on the lip just beneath the column; **e)** SEM micrograph sowing the callus in finer detail; **f)** SEM micrograph of the entire bottom part of the lip (L) and the callus (CS) in context.


c)
C
L
LP
15kV X40 500µm
d)
R
CS
15kV X110 100µm


e)
15kV
X150
100µm


f)
CS
L
15kV
X65
200µm

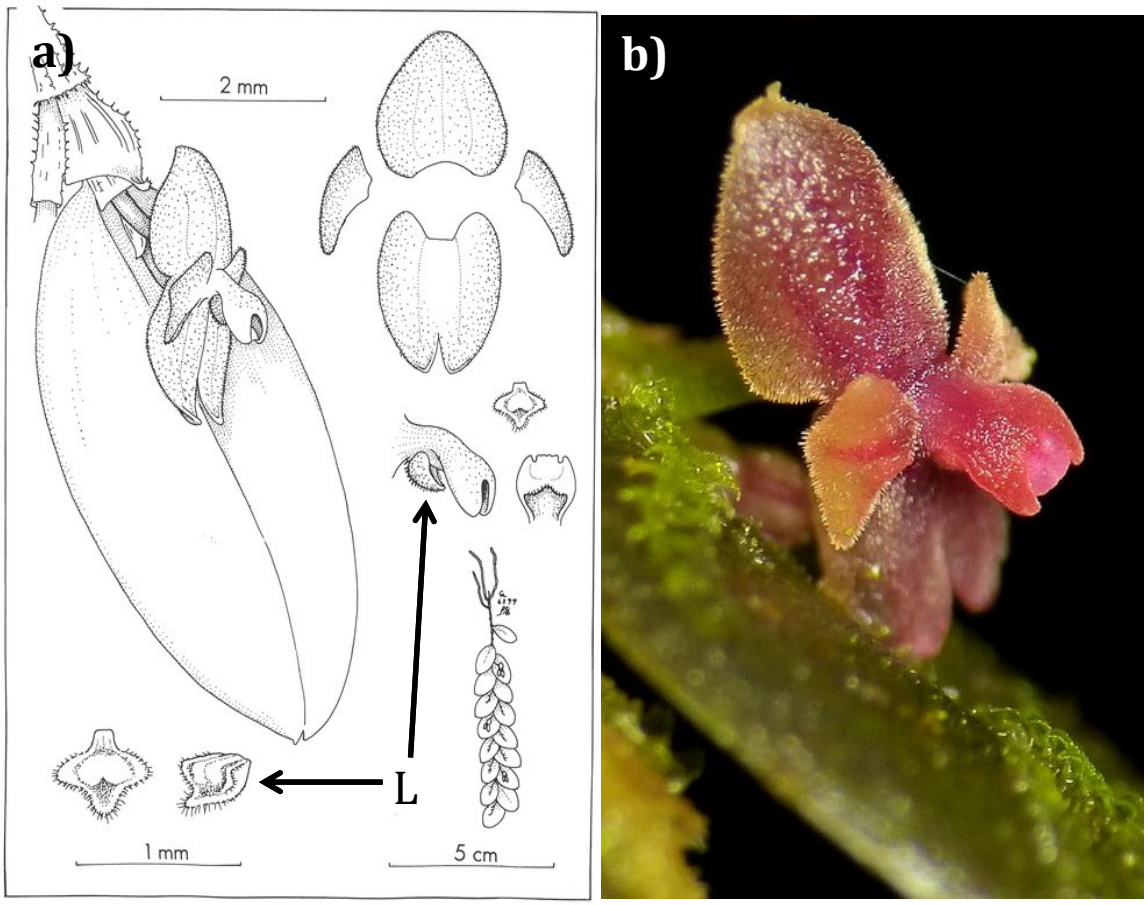
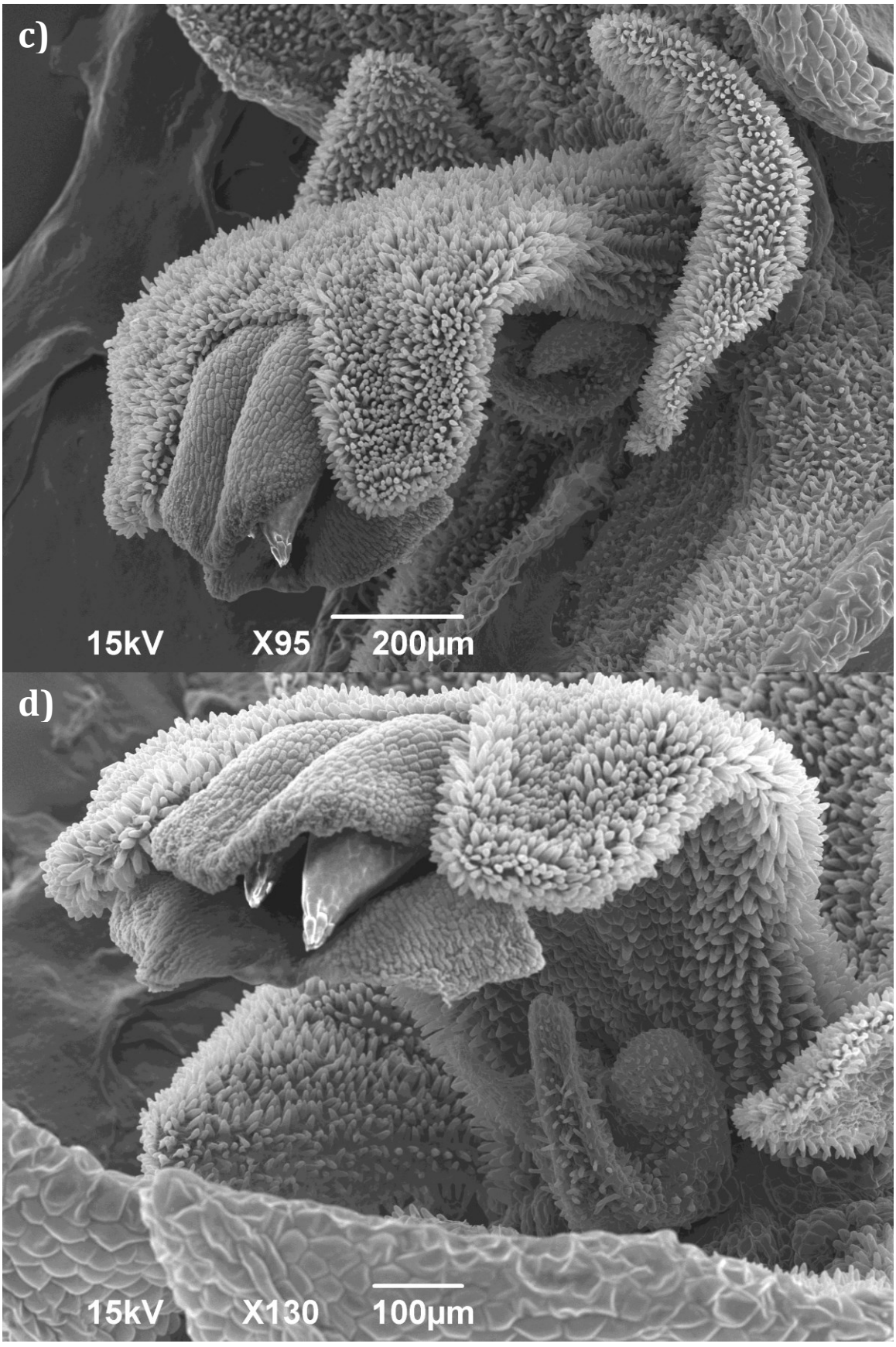
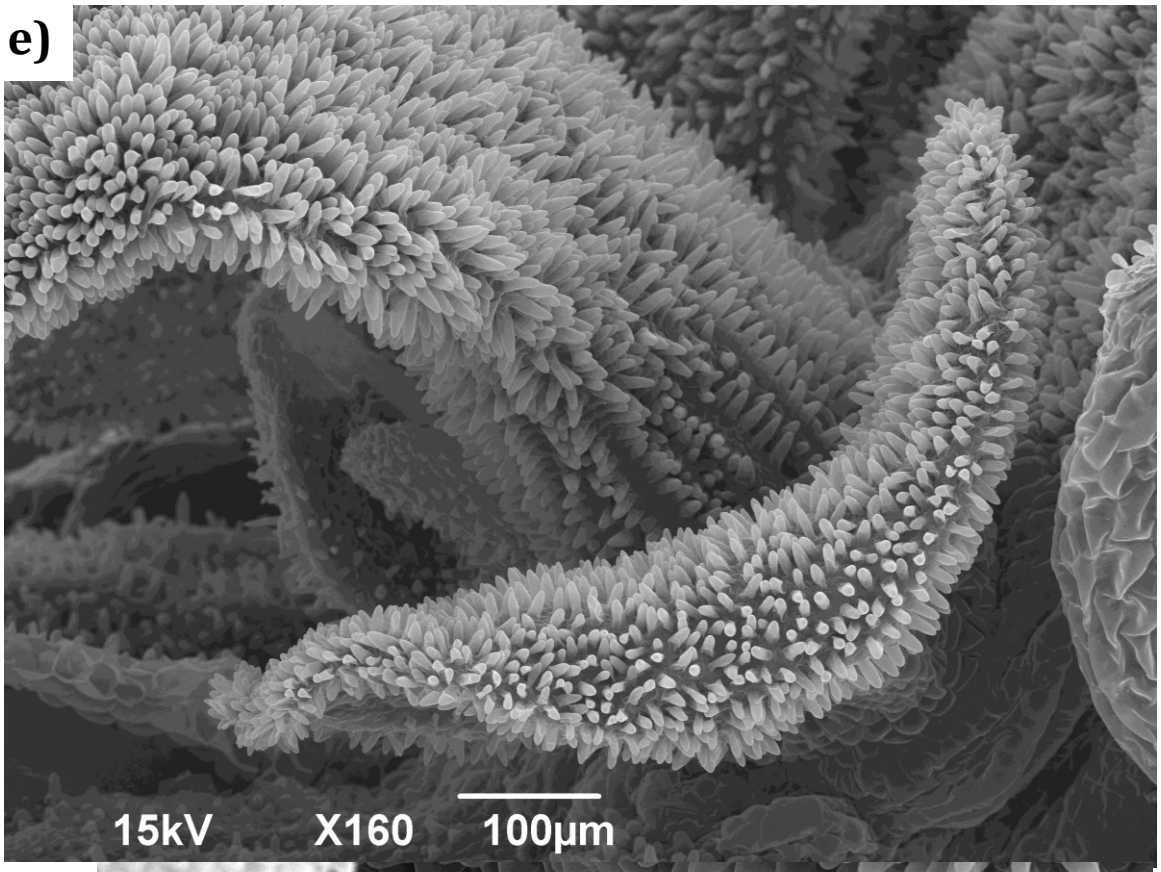
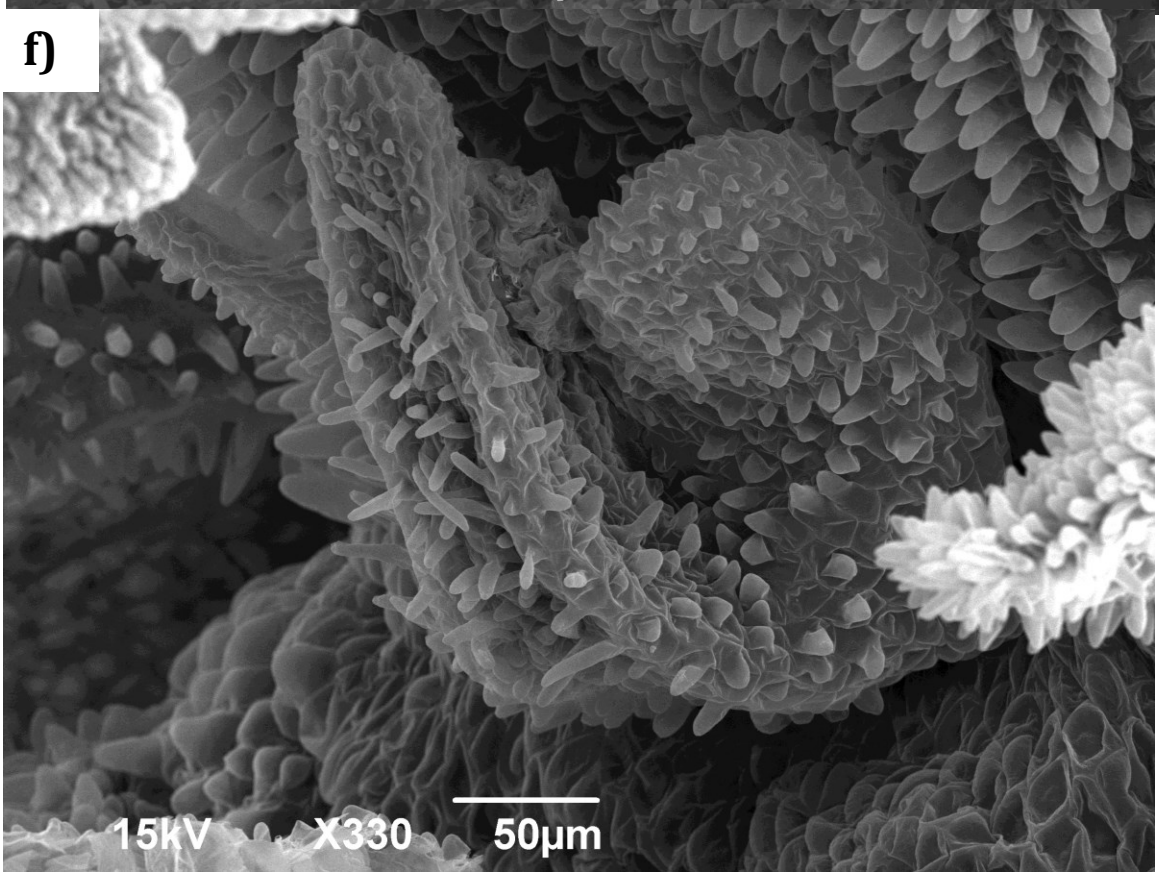
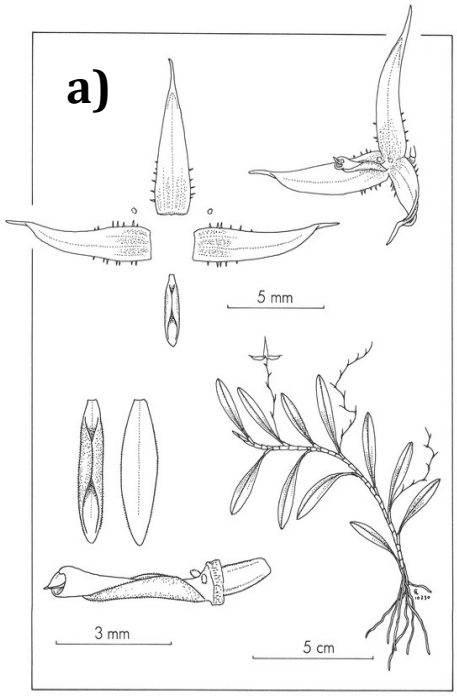


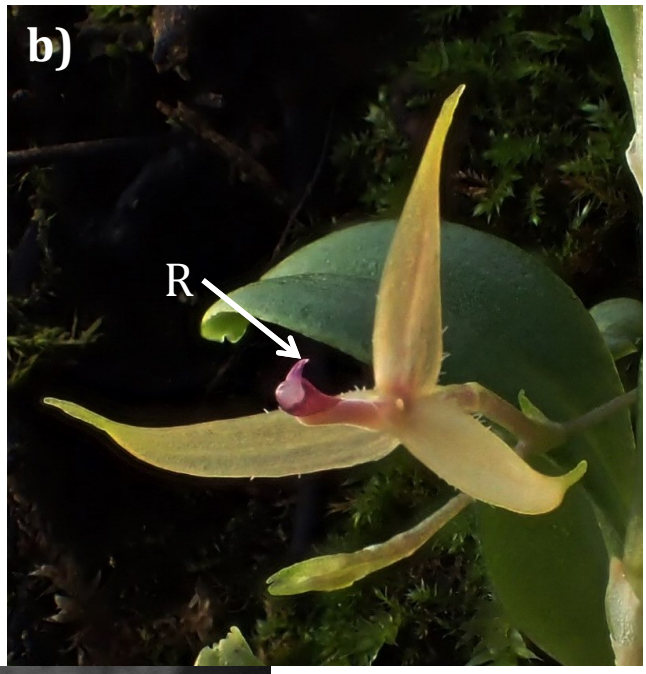
Figure 4 ***Neooreophilus nummularius*** **(AN083)**: **a)** An illustration of key vegetative and floral characteristics, notably the lip (L) (Luer 1994); **b)** Photograph of a flower *in situ* (A. Kay); **c)** SEM micrograph of the column (C) in the context of the lateral petals (LP). The anther cap (A), is still in place; **d)** SEM micrograph of the column (C) from another angle, clearly showing the labellum (L) underneath and the pollinia (P) under the anther cap (A); **e)** SEM micrograph focusing on the papillate cells of the lateral petals (LP) and column (C); **f)** SEM micrograph showing greater detail of the lip (L).


c)
15kV
X95
200µm
d)
15kV
X130
100µm


e)
15kV
X160
100µm


f)
15kV
X330
50µm





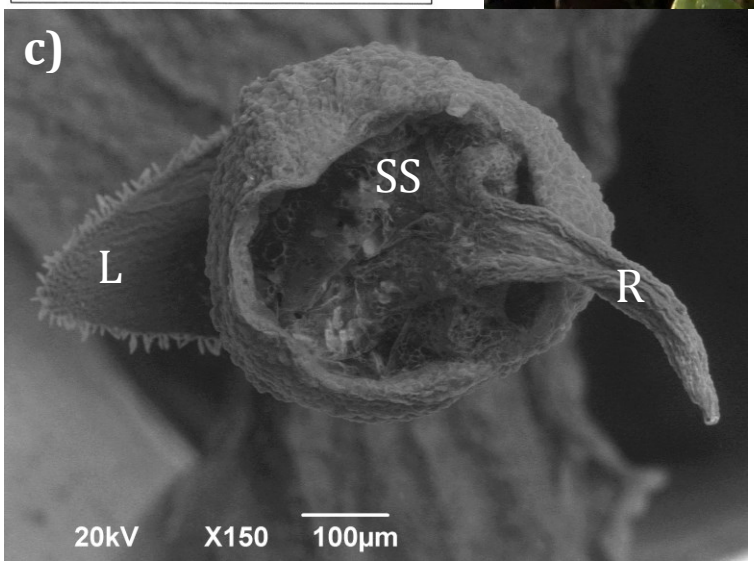


Figure 5 ***Andinia vestigipetala*** **(AN075):**

a) Illustration of floral and vegetative characteristics (Luer 1994);

b) Photo of a flower (L. Thoerle) with the pollinia displaced and the rostellum (R) dried upwards to reveal the stigmatic surface;

c) SEM micrograph of the column taken under a low vacuum. The pollinia are displaced and the rostellum (R) is dried upwards to reveal the stigmatic surface (SS), as in the photograph above; the lip (L) sticks out slightly from the column.

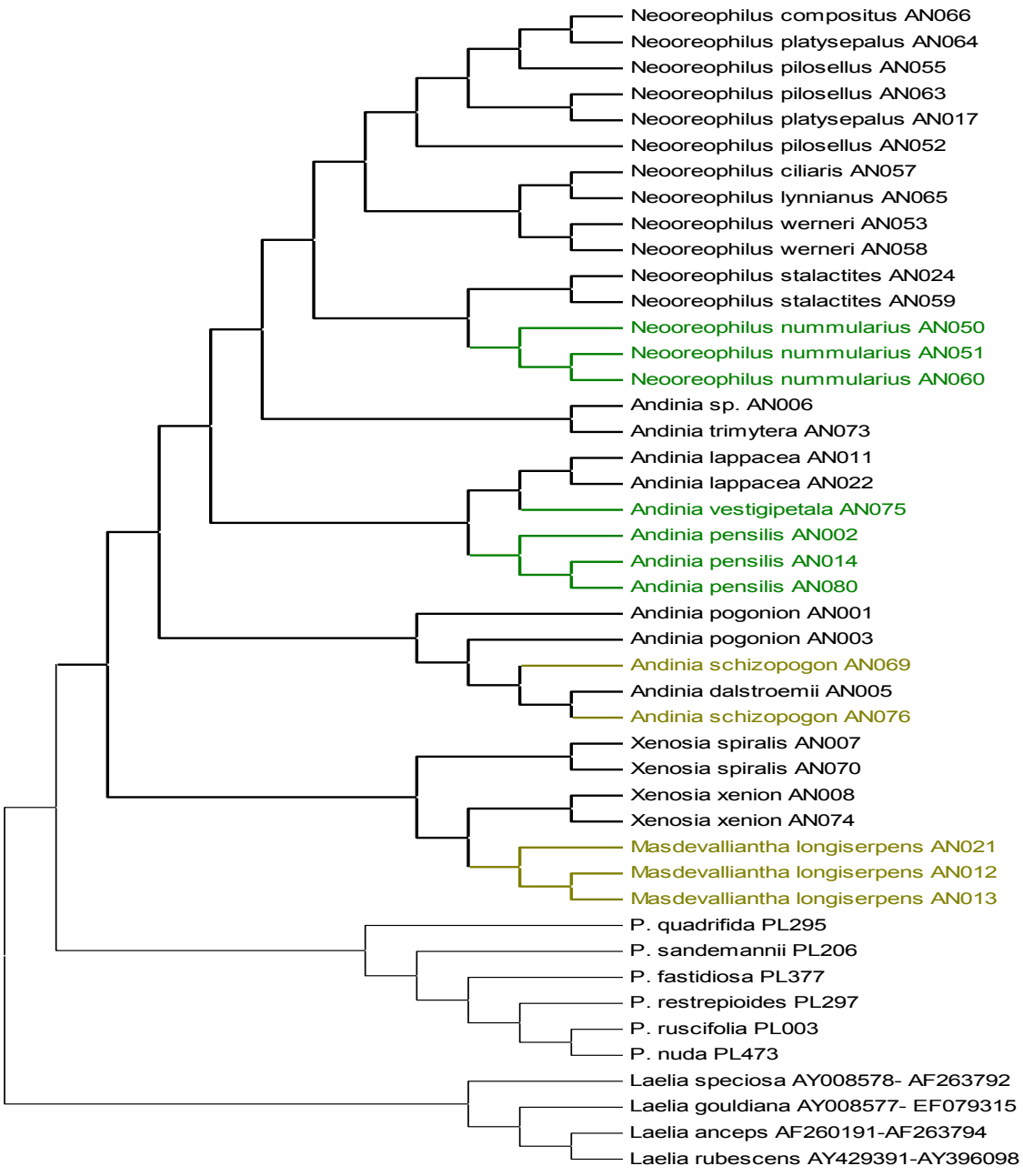


Figure 6: The pollination mechanisms suggested by SEM imaging were overlaid with the results of our phylogenetic analysis (Maximum likelihood analysis using composite ITS-*matK* sequence data). Green indicates possible deceit pollination by pseudocopulation, yellow indicates probable reward pollination, and black was used for those species not yet imaged with SEM.

Discussion

SEM imaging suggests that there are at least two possible mechanisms of pollinator attraction within the new, expanded sense of *Andinia*. The conclusions drawn from the SEM micrographs in this study are not definitive, but deductions were made according to similarities between structures observed on the flowers studied and the flowers of orchids that have been proven to utilize various attraction methods. The presence of a glenion on the flowers of *A. schizopogon* and *M. longiserpens* suggests that they both offer a nectar-like reward for pollinators. The glenion is located just beneath and in front of the viscidium of the *M. longiserpens* flower and just beneath the end of the rostellum, on which the viscidium appears to have rested before the pollinarium was removed, on the *A. schizopogon* flower, suggesting that in both species the glenion is used to attract pollinators to the vicinity of the viscidium.

A glenion is absent from the flowers of *A. vestigipetala*, *A. pensilis*, and *N. nummularius*, and they have features suggestive of pseudocopulation. The flower of *N. nummularius* is covered almost entirely in papillate cells that may comprise an osmophore (Pridgeon & Stern 1983); these cells are also visible on the lip of the *A. pensilis* flower. While osmophores are not exclusive to flowers pollinated by deceit (Duque-Buitrago *et al.* 2014), the use of deceptive scents is critical in attracting pollinators when no reward is offered (Borba & Semir 2001). The papillate cells may also mimic the body surface of a female fly in addition to releasing scents (Blanco & Barboza 2005). Another indicator of pseudocopulation is the protruberant callus found on the lip of *A. pensilis*, which

we suspect may interact with the abdomen of the pollinator as it attempts to copulate with the flower, assuming that it grabs on to the flower on its dorsal side, as in several species of *Lepanthes*, rather than backing in, as in *L. excelsa* (Blanco & Barboza 2005). The shape and small size of the lip of *N. nummularius*, as well as its location just at the base of the column, suggest that the entire lip may function similarly to the callus on the *A. pensilis* flower.

So far, only the column of *A. vestigipetala* has been imaged, and no papillate cells indicative of an osmophore were observed, though this does not necessarily rule out their presence on the sepals. Though the lip is apparently separate from the column in our SEM image, this may be an artifact of desiccation while under the low vacuum in the microscope chamber, since it appears to completely adhere to the column in photos of live flowers (e.g. Fig. 5b). Whether or not the lip is completely adhered to the column, there is no glenion present, nor is the minute surface sufficient to serve as a landing platform from which a pollinator would be able to feed on a reward. Similarly, there is no callus on the lip, nor does the shape of the lip lend itself to interaction with the pollinator abdomen, so the actual mechanism through which pseudocopulation would occur in *A. vestigipetala* remains somewhat enigmatic. However, nectar, if it was produced on the sepals, would likely be too far removed from the end of the column to encourage removal, and later deposition, of pollen, so *A. vestigipetala* is unlikely to attract pollinators using a reward.

When placed in the context of our phylogenetic analysis (Fig. 6), SEM imaging suggests that the various mechanisms for attracting pollinators may have evolved multiple times within the new circumscription of the genus. Reward-based pollinator attraction may be a plesiomorphic trait, since it is the ancestral trait in Pleurothallidinae (Kores *et al.* 2001) and seen in both *M. longiserpens* and *A. schizopogon*, whereas deceit pollination seems to be derived later and possibly multiple times. Deceit pollination would appear to be apomorphic based on the results of SEM imaging thus far, but the apparent presence of a glenion visible on the live flower of *A. lappacea* casts uncertainty over this conclusion. If further investigation confirms that *A. lappacea* possesses a glenion, then two possibilities exist. The first possibility is that the presence of a glenion is indeed plesiomorphic and that deceit pollination arose at least two separate times, in the clade containing *A. pensilis* and *A. vestigipetala* and in the proposed subgenus *Brachycladium*, in which *N. nummularius* is

Figure 7: Photograph of *Andinia lappacea* flower (F. Debruille). The dark area on the lip, beneath the column, appears to be a glenion (G).

located. The second scenario would be that the presence of a glenion in *A. lappacea* is homoplasious with the glenions of *A. schizopogon* and *M. longiserpens*, having arisen a second time after the evolution of deceit pollination in the subgenera *Neooreophilus, Andinia,* and *Minuscula*.

In any case, it is apparent that imaging a single flower per clade is insufficient for determining the comprehensive evolutionary history of pollinator attraction in the expanded genus *Andinia*. Floral characteristics, such as the presence/absence of a glenion and of probable osmophores, vary within this genus, but are shared with species in other genera (Blanco & Barboza 2005, Duque-Buitrago *et al.* 2014), supporting the notion that floral homoplasy is rampant in Pleurothallidinae and therefore unreliable for determining evolutionary relationships. It remains to be seen whether floral characteristics will be useful for categorizing new species within *Andinia*. Further studies of the genus will attempt to image as many different species as possible in order to elucidate whether the subgeneric delineations determined by our phylogenetic analysis are consistent with floral traits or if these floral characteristics exhibit a high degree of homoplasy even at the infrageneric level.

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