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Evolutionary diversification and historical biogeography of orchidaceae in Central America with emphasis on Costa Rica and Panama

Bogarin Chaves, D.G.

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EVOLUTIONARY DIVERSIFICATION AND HISTORICAL BIOGEOGRAPHY OF ORCHIDACEAE IN CENTRAL AMERICA

with emphasis on Costa Rica and Panama

Diego G. Bogarín Chaves

2019

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with emphasis on Costa Rica and Panama

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This thesis is dedicated to my parents Gerardo and Inés;
my brother, Sergio; my sister, Marielos;
and my wife Maricruz

Table of Contents

General Introduction

Chapter 1

Introduction 11
Aims of the thesis 21
Outline of the thesis 21

Taxonomy and systematics of *Lepanthes* and allies

Chapter 2

Phylogenetic comparative methods improve the selection of characters for generic delimitations in a hypediverse Neotropical orchid lineage 25

Chapter 3

Genus-level taxonomical changes in the Lepanthes affinity 49

Chapter 4

Anchored Hybrid Enrichment generated nuclear, plastid and mitochondrial markers resolve the Lepanthes horrida (Orchidaceae: Pleurothallidinae) species complex . 59

Chapter 5

Two new Lepanthes (Orchidaceae: Pleurothallidinae) from Panama 91

Pollination biology

Chapter 6

Pollination of Trichosalpinx (Orchidaceae: Pleurothallidinae) by biting midges (Diptera: Ceratopogonidae) 103

Chapter 7

Floral anatomy and evolution of pollination syndromes in Lepanthes and close relatives 139

Evolutionary diversification and biogeography of *Lepanthes* and allies

Chapter 8

Recent origin and rapid speciation of Neotropical orchids in the world's richest plant biodiversity hotspot 161

Chapter 9

Speciation and biogeography of the hyperdiverse genus Lepanthes (Orchidaceae: Pleurothallidinae) 177

General discussion and conclusions

Chapter 10

General discussion and conclusions 193

Summaries

Summary 199
Samenvatting 202
Resumen 205

References and acknowledgments

References 211
Acknowledgments 234

Curriculum vitae

Curriculum Vitae 235
List of publications 236
Abstracts 241

General Introduction

Chapter 1

Introduction

1.1 Evolutionary diversification and historical biogeography of Orchidaceae in Costa Rica and Panama

The floristic richness of the Neotropics has a complex origin. The most diverse plant family in the American continent is made up of the Orchidaceae with more than 13,000 species (Ulloa et al., 2017) and the most diverse genera of angiosperms are *Piper* L. (1,804) and *Peperomia* Ruiz & Pav. (1,133) (Piperaceae), *Miconia* Ruiz & Pav. (1,110) (Melastomataceae) and the orchid genera *Epidendrum* L. (1,459 species), *Lepanthes* Sw. (1,125) and *Stelis* Sw. (1,128). In contrast, most genera of American angiosperms (5,975) contain less than 100 species (Ulloa et al., 2017). Orchidaceae is also the most diverse plant family in Central America, concentrating 13% of the species and the number of species is triple that of other well-represented angiosperm families. Although we are still far from knowing the exact number of orchid species extant in both countries nowadays, at present Costa Rica (1,620 spp.) and Panama (1,372 spp.) together contain more than 2,000 species of orchids; representing about 8.0% of all orchid species on just about 1% of the Earth's land surface. In this region, Cymbidiae, Pleurothallidinae and Laeliinae are the most diverse groups and contain the largest genera: *Maxillaria* Ruiz & Pav. *s.l., Lepanthes*, *Oncidium* Sw*., Pleurothallis* R.Br., *Stelis* and *Epidendrum* showing the same global pattern observed in the Neotropics.

Historically, the isthmus of Costa Rica and Panama has been a source of fascination for its strategic position linking North America to South America. The geological events that led to the closure of the isthmus that started with the formation of a volcanic arc, dating from the Cretaceous to Eocene, 67 to 39 million years ago (Ma) (Montes et al., 2015), have been studied extensively but are still controversial. There is no consensus about when the isthmus closed the Central American Seaway (CAS) separating the Pacific from the Atlantic Ocean and favoring the Great American Biotic Interchange (GABI). Traditionally, it was assumed that this closure was established between 3.5-5.0 Ma, but other studies that include new information suggest a closure much earlier, between 13-15 Ma in the middle Miocene (Bacon et al., 2015; Montes et al., 2015). Despite this controversy, it is clear that with the initial emergence of a volcanic arc in the Cretaceous, orchids had millions of years to colonize some of these oceanic islands by wind dispersal of seeds and evolve there. According to a phylogenomic analysis and net diversification regimes across lineages using BAMM analysis, Givnish *et al*., (2015) proposed that Orchidaceae arose around 112 Ma in the Cretaceous, long before the formation of the arc and subsequent closure of the Isthmus of Panama. However, the most diverse Neotropical subtribes Laeliinae, Oncidi-

inae, Maxillariinae and Pleurothallidinae probably diversified between 10-25 Ma after the last acceleration of net diversification rate that occurred about 25 Ma, overlapping with the possible closure of the Isthmus proposed recently (Bacon et al., 2015; Givnish et al., 2015; Montes et al., 2015). Indeed the flora of the Isthmus is dominated mainly by species of Cymbidiae, Laeliinae and Pleurothallidinae that diversified in the past 20 Ma. Consequently, we can assess some of the factors that shaped this extraordinary diversity in the isthmus by analyzing the current floristic composition of selected orchid groups with phylogenetics, floral trait evolution, pollination evidence and biogeographical analyses (Fig. 1.1).

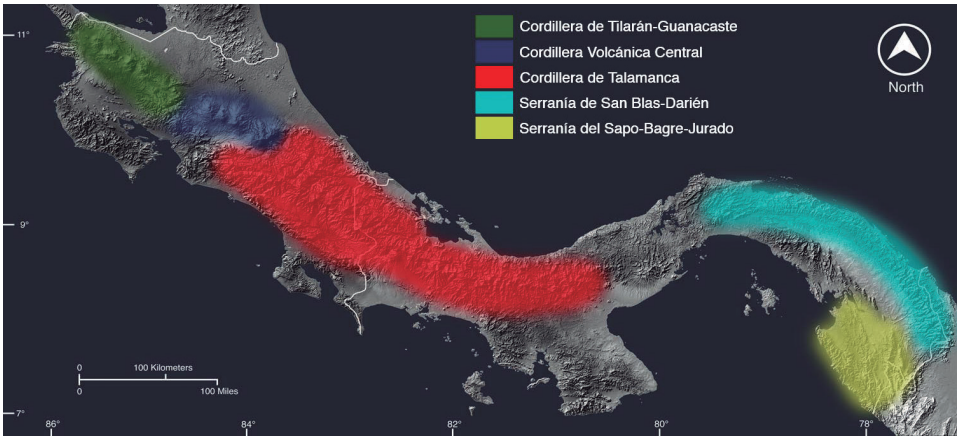

Figure 1.1. Geography of southern Central America (Costa Rica and Panama) showing the main ranges: Talamanca crossing both countries and San Blas-Darien on the southeast of Panama towards Colombia.

1.2 Orchid diversity in the hotspot of Costa Rica and Panama

Updated floristic inventories of Costa Rica and Panama (both countries treated as a biogeographic unit) resulted in the detection of 2012 species of orchids of which 934 are shared (Bogarín et al., 2014b). From these figures, 784 (39%) species are endemic to the Isthmus (Table 1.1). A strategy to analyze the current evolutionary and floristic relationships of the Orchidaceae of the Isthmus is to study the most diverse groups in the region. An analysis of the various genera in both Costa Rica and Panama shows that *Epidendrum* L., *Lepanthes* Sw. and *Stelis* s.s. Sw. contain the highest number of species and the highest percentages of endemism (Tables 1.2–1.3). These genera are also monophyletic (Pridgeon et al., 2001) and therefore there is no bias due to the use of different nomenclatural circumscriptions that might cause variations in the number of species assigned to a genus. One factor that can affect the interpretation of evolutionary and biogeographic data is the intensity of the alpha-taxonomic work. The most diverse genus in the Isthmus is *Epidendrum,* the taxonomy of which has been developed in detail and consistently by Hágsater and colleagues. *Lepanthes* is a diverse genus, and despite extensive work by (Luer, 2003a) it is expected that there are still many undiscovered species new to science, especially in the relatively unexplored areas of the Cordillera de Talamanca and Panama. If this expectation is correct, *Lepanthes* may exceed *Epidendrum* in number of species recorded in the Isthmus (Pupulin and Bogarín, 2014).

Table 1. 1. Number of species, endemics and genera in Costa Rica and Panama

	Costa Rica	Panama	Total
Species	1574	1372	2012
Endemics	485	299	784
Genera	199	187	211

Moreover, the taxonomy of *Stelis* s.s. is the least developed, and conclusions based on these data are likely biased (Luer, 2003b). Botanical exploration and alpha-taxonomy are therefore tasks that must be promoted with impetus in the region. Some other diverse groups in the Isthmus are *Camaridium* Lindl., *Dichaea* Lindl., *Oncidium*, *Pleurothallis*, *Scaphyglottis* Poepp. & Endl., *Sobralia* Ruiz & Pav., *Specklinia* Lindl. and *Telipogon* Kunth. (Fig. 1.2) These groups also maintain a tendency to hold many endemic species. The taxonomic work in these genera has also revealed new species and expanded knowledge on their geographic distributions, encouraging more potential case studies to understand the evolution and diversification of Orchidaceae in the Isthmus (Bogarín et al., 2014a; Dressler and Pupulin, 2015; Pupulin et al., 2012).

Table 1.2. The most diverse genera in Costa Rica and Panama

Costa Rica		Panama	
Genus	**Number of species**	**Genus**	**Number of species**
Epidendrum	207	*Epidendrum*	221
Stelis	88	*Lepanthes*	151
Lepanthes	66	*Stelis*	103
Pleurothallis	54	*Camaridium*	48
Camaridium	48	*Pleurothallis*	48
Scaphyglottis	39	*Specklinia*	44
Sobralia	39	*Scaphyglottis*	38
Specklinia	34	*Sobralia*	38
Oncidium	32	*Telipogon*	37
Dichaea	26	*Masdevallia*	34

1.3 Biogeography and endemism of orchids in Costa Rica and Panama

About 40% of the orchid species are endemic to the Isthmus. The highest percentages of endemism recorded could be related to geological events of its volcanic arc, vicariance and *in situ* speciation produced by the lifting of the Cordillera de Talamanca. For example, allopatric speciation in *Lycaste bruncana* Bogarín and *L. tricolor* Rchb.f. (Fig. 1.3), among other examples found in *Brassia* R.Br., *Epidendrum*, *Kefersteinia* Rchb.f., *Oncidium*, *Pleurothallis* and *Stelis,* indicate an important role of the altitudinal division in vicarance speciation induced by the Talamanca and its climate barrier effect blocking the Caribbean trade winds (Bogarín, 2007; Pupulin, 2001; Pupulin and Bogarín, 2009). The highest percentages of endemism are found in the most diverse genera. For instance, 90% of the species of *Lepanthes* are endemic and about 50% of the species of *Stelis* and *Epidendrum* (Table 1.4) occur nowhere else. The study of the factors favoring this high

Table 1.3. Genera with most endemic species in Costa Rica and Panama.

Costa Rica		Panama	
Genus	**Endemic species**	**Genus**	**Endemic species**
Lepanthes	102	*Epidendrum*	53
Epidendrum	80	*Pleurothallis*	23
Stelis	37	*Stelis*	23
Telipogon	31	*Lepanthes*	21
Pleurothallis	15	*Telipogon*	17
Sobralia	15	*Sobralia*	16
Camaridium	14	*Masdevallia*	9
Specklinia	14	*Camaridium*	8
Masdevallia	13	*Specklinia*	7

Table 1.4. The most diverse genera and the % of endemis in the Isthmus of Panama

Genus	Species in the Isthmus	% endemic species
Lepanthes	155	90.12
Epidendrum	133	46.18
Stelis	60	43.80

endemism in *Lepanthes* is key to understanding its diversification and will be further discussed in the upcoming chapters of this PhD thesis. Other genera also deserve more attention because, although not as diverse, they show high rates of endemism; one of these is *Telipogon*, in which more than 70% of the species are endemic. Current floristic relationships with other groups of orchids of the Andes is evident. For example, *Telipogon* is a diverse genus in the highlands of the Isthmus, and its northern distribution is limited. Other genera of South American affinities are *Brachionidium* Lindl., *Fernandezia* Lindl. and *Pterichis* Lindl. that almost reach their northern-most distribution in the Cordillera de Talamanca. About 10 genera are present in Panama but not in Costa Rica. These genera have a strong South American affinity: *Discyphus* Schltr., *Eloyella* P.Ortiz, *Koellensteinia* Rchb.f., *Neomoorea* Rolfe, *Rudolfiella* Hoehne and *Selenipedium* Rchb.f. They range from Central Panama to the southeast of Darien and towards Colombia, indicating a common geological history of this area but different from western Panama and southeast Costa Rica. The geological formation of foothills of Maje, Darien and San Blas in Panama and western Colombia is reflected in the species composition data. Geographical distributions of *Dinema* Lindl., *Euryblema* Dressler, *Helleriella* A.D. Hawkes and *Horichia* Jenny suggests that these genera might be present in Costa Rica (Bogarín et al., 2014b). On the other hand, 18 genera present in Costa Rica are still not recorded from Panama. Some of them show a northern distribution such as *Arpophyllum* La Llave & Lex. and *Restrepiella* Garay & Dunst. However, *Epistephium* Kunth, *Funkiella* Schltr., *Lankesterella* Ames, *Trevoria* F.Lehm., *Tropidia* Lindl. and *Warmingia* Rchb.f., all with representatives in South America, might be distributed in Panama after all. The bias resulting from less floristic and alpha-taxonomic work in Panama should be reduced in the upcoming years (Bogarín et al., 2013).

1.4 Evolutionary diversification and orchid floristic composition

There are many factors that can enhance orchid species diversifications such as orogeny, past climatic fluctuations, interactions with other organisms such as mycorrhiza, pollinators, seed dispersers or key innovations such as colonization (extrinsic) or trait evolution (intrinsic). The main aim of my PhD thesis consisted of studying the factors that led to the formation of the current species composition of Orchidaceae in the Isthmus. Based on our taxonomic experience we have selected *Lepanthes* and closely related genera as a model group to study the extraordinary species richness and evolution in Costa Rica and Panama and its relationship with the Andean flora. We intend in the future to extend this model to other diverse groups such as *Stelis*. *Epidendrum* is

Figure 1.2. Some representatives of the major groups of Orchidaceae present in Lower Central America. From left to right: *Camaridium campanulatum*, *Epidendrum nocturnum*, *Epidendrum* (*Oerstedella*) *wallisii*, *Lepanthes matamorosii*, *L. bradei*, *Pleurothallis anthurioides*, *Scaphyglottis pulchella*, *Stelis transversalis*, *Telipogon panamensis*. Photographs by Diego Bogarín.

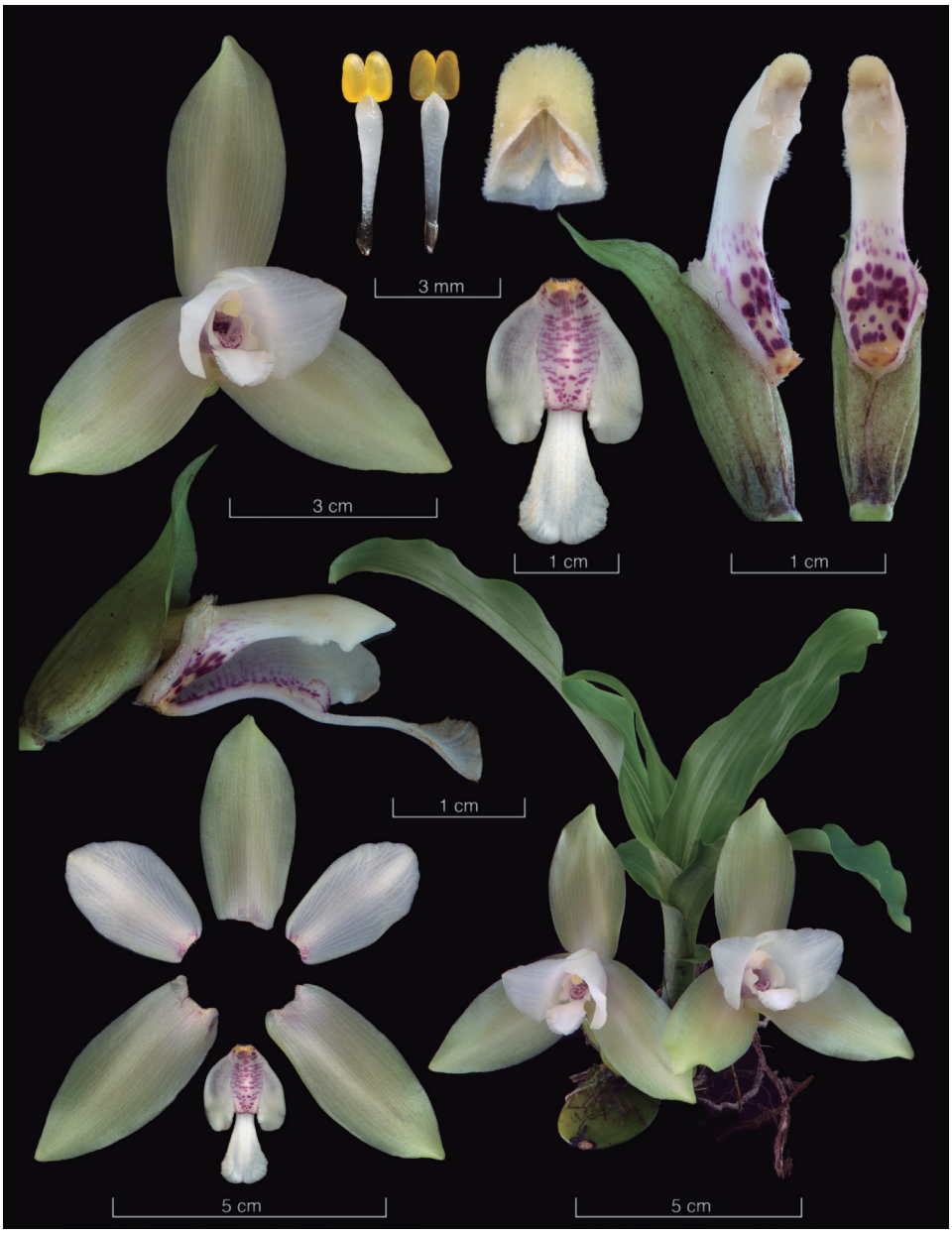

Figure 1.3. Lankester Composite Digital Plate of *Lycaste bruncana,* a species from Costa Rica and Panama restricted to the Pacific watershed of Cordillera de Talamanca.
Photograph by Diego Bogarín.

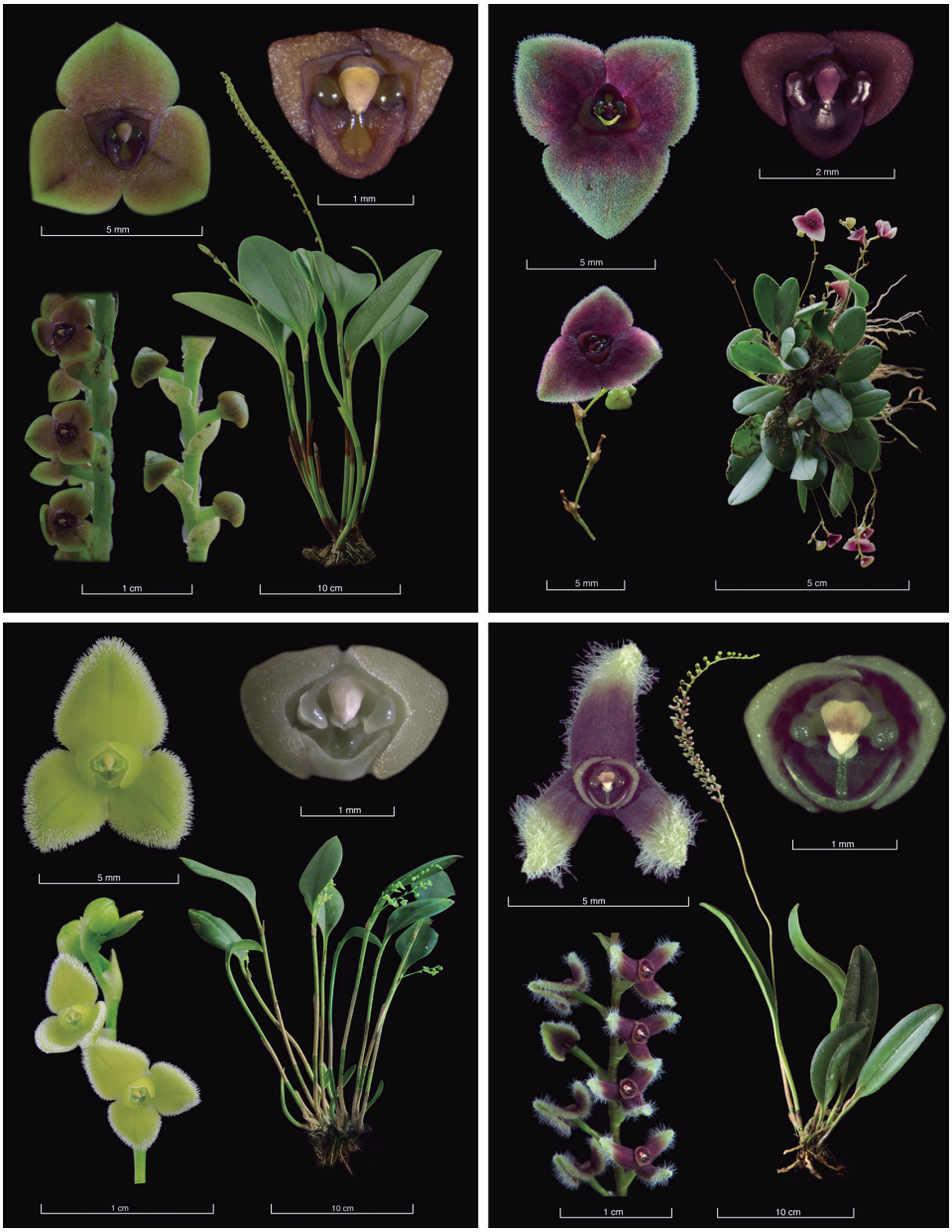

Figure 1.4. Lankester Composite Dissection Plates (LCDP) of some representative species of the highly diverse genus *Stelis* from Costa Rica and Panama. Species are currently under taxonomic review. Photographs by Diego Bogarín.

another interesting group, and it is being evaluated by Hágsater and co-workers so there will be information available in the future. Although *Stelis* s.s. remains an excellent group as a candidate to study their high evolutionary diversification, the limited taxonomic expertise and little ecological information available so far prevented us to address this group (Fig. 1.4). However, some clues about its pollination mechanism (hitherto little-known) indicate that it may be pollinated by gall midges of Cecidomyiidae under conditions that we are still exploring.

1.5 The orchid genus *Lepanthes*

Lepanthes is one of the major genera in the Pleurothallidinae. With over 1,000 spp., the genus ranges from southern Mexico and the Antilles to Peru and Bolivia, with few species in the Guianas and Brazil. Plants grow mostly from 1,500 to 3,000 m elevation in humid, often shady places. Highest diversity is found in the Andean region of Colombia and Ecuador with more than 300 species in each country (Luer, 1996b; Luer and Thoerle, 2012) (Fig. 1.6-1.7). *Lepanthes* is represented in Costa Rica and Panama with about 150 spp. Only two species are shared with Colombia and Ecuador. This may reflect the floristic influence of the Andean region in Costa Rica and Panama at the genus level but not the species level. Species are usually restricted to specific ranges or mountains, and endemism is high. Plants are recognized by the monophyllous ramicauls, enclosed by a series of lepanthiform sheaths and congested, distichous inflorescences. Floral morphology distinguishes *Lepanthes* from other genera with lepanthiform sheaths (*Draconanthes* (Luer) Luer, *Trichosalpinx* Luer and *Lepanthopsis* (Cogn.) Hoehne among others). Flowers are characterized by the ovate to elliptic sepals and the transversely bilobed petals. Lip morphology is complex (Fig. 1.6); the lip is usually bilaminate with the two blades supported by connectives that often lift the blades above the column. The central part of the lip is made up by the body, which is attached to the column. The appendix is developed from the sinuous between the connectives and varies morphologically among the species in different combinations of lobes, hairs, projections, trichomes and membranes. *Lepanthes* taxonomy has been studied by Luer and Thoerle (2012) and particularly in Costa Rica and Panama by Luer and Dressler (1986), Luer (2003a), Pupulin *et al.* (2009), Pupulin and Bogarín (2014). Givnish *et al*., (2015) pointed out that the role of limited dispersal of seeds and ineffective pollinators, limited gene flow, population bottlenecks and genetic drift deserve to be further studied, and that *Lepanthes* would be one of the best study cases for that so we focused on its systematics in Chapters 2 and 3. Although *Lepanthes* is considered a monophyletic group, it has been poorly sampled phylogenetically (Pridgeon et al., 2001). Phylogenetic analyses of the Pleurothallidinae showed that *Andinia* (Luer) Luer (including *Neooreophilus* Archila) is not closely related to *Lepanthes,* and

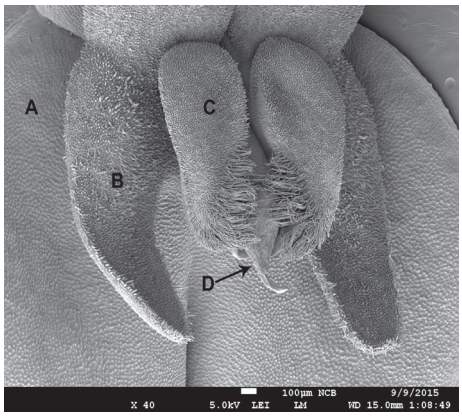

Figure 1.6. Scanning electron microscopy (SEM) of a flower of *Lepanthes horichii* showing the complex morphology in detail. **A.** Sepal. **B.** Petal (lower lobe). **C.** Lip (lobe). **D.** Column showing the apical anther. Photographs by Diego Bogarín

Figure 1.7. Some species of *Lepanthes* from Costa Rica and Panama. Species are mostly endemic and show a wide range of morphological variation around the same scheme. Note the coloration of the flowers, which might be involved in attraction of pollinators. Photographs by Diego Bogarín.

flower similarities are homplastic (Wilson et al., 2017) (Fig. 1.8). *Neooreophilus* species have a similar flower morphology as *Lepanthes,* and there is some evidence of its pollination by pseudocopulation (S. Vieira-Uribe, pers. comm. 2015). *Neooreophilus* is absent in Mesoamerica, and it might be a younger group when compared to *Lepanthes,* which is widespread in the Neotropics. Phylogenetics of these two groups could help to shed light on this hypothesis. Furthermore, the floral morphology of *Lepanthes* varies astonishingly around the same scheme in all the >1,000 species known. The flowers are developed above or beneath the leaves or sometimes in inflorescences surpassing the leaves and the petals and lip tend to be reduced or almost absent in some species. The most common colors of flowers are yellow, red, orange, purple (rarely green) or a combination of these. The appendix of the lip plays an important role in pollination of *Lepanthes* flowers. Blanco and Barboza (2005) described the first case of pseudocopulation in the

Figure 1.8. Floral convergence among the species of *Neooreophilus*. **A-B.** and *Lepanthes* **C.** Photos: A-B. by Sebastián Vieira-Uribe. C. by Diego Bogarín.

genus in which male fungus gnats of *Bradysia floribunda* (Diptera: Sciaridae) visit flowers of *L. glicensteinii* Luer, apparently attracted by sexual pheromones. The male adheres to the flower appendix during copulation. In this attempt the insect removes the pollinarium with the abdomen. Calderón-Sáenz (2012) observed the same phenomenon in *L. yubarta* E.Calderon, which is visited by another species of *Bradysia* in Valle del Cauca, Colombia. Sciaridae flies, commonly known as dark-winged fungus gnats, are a diverse group of flies with more than 8,000 species worldwide. Eggs are deposited between the lamina of sporocarps of fungi, and the larvae feed on sporocarps and other decaying organic matter such as rotten trunks or plant roots or leaves. Some species are pests of important economic crops such as mushrooms. Blanco and Barboza (2005) and Calderón-Sáenz (2012) clearly described the pollination of *Lepanthes* but left many evolutionary questions un answered. We are studying more cases of pollination in other *Lepanthes* species where morphological evidence indicates that other parts of the body of fungal gnats are being used such that the pollinia are not always attached to the abdomen. Probably, flowers produce pheromone-like compounds to attract pollinators and we have some preliminary evidence that flowers indeed use this strategy to attract males.

The anatomy of the flower was studied in order to find possible secretory structures involved in pollinator attraction (Fig. 1.7). Sciarids are attracted by yellow colors. Special traps were designed to catch flies in greenhouses made up by yellow cardboard and petroleum jelly. Although this method proved to be less effective in studying *Lepanthes* pollination (Godden, 2002), the approach works well in large populations of plants to increase the probabilities of catching gnats carrying pollinia. Sciarid flies have short life cycles (Wilkinson and Daugherty, 1970). Adults usually live less than 7 days, and they are considered poor flyers. Thus the chance to deceive inexperienced males may be high. Sciaridae is a highly diverse group but poorly known. The behavior and natural history of Sciaridae are key to understanding the evolution of *Lepanthes*. Why is *Lepanthes* more diverse than closely related genera such as *Anathallis* Barb. Rodr., *Draconanthes, Lankesteriana* Karremans, *Lepanthopsis, Trichosalpinx* and *Zootrophion* Luer? A hypothesis is that pseudocopulation triggered the high speciation levels in *Lepanthes*. To study the evolutionary diversification of *Lepanthes* and the possible triggers of speciation, it was necessary to extend the molecular phylogenetic sampling of the "*Lepanthes* clade" as described by Pridgeon *et al*., (2001) in order to find answers to the evolutionary success of *Lepanthes* as compared to its sister genera (see **Chapters 2-5**). However, the pollination mechanisms

that operate in the sister genera are also important for comparisons with *Lepanthes*. Observations on the pollination of *Trichosalpinx* revealed a frequent visitation by biting midges of the Ceratopogonidae family (see **Chapters 6-7**) . Finally, we also used biogeographical areas within the Neotropics in order to draw accurate conclusions about endemism and species distribution in biogeographical analyses (**Chapters 8-9**).

Aims of the thesis

In this thesis, I targeted the orchid genus *Lepanthes*, one of the six genera of angiosperms that surpasses 1,000 species in the Neotropics, as a study model to investigate the evolutionary processes that promoted species diversifications. To investigate some of the possible factors that shaped the diversification in *Lepanthes* and related genera we improved the taxonomy of the group by providing a solid phylogenetic framework combined with ancestral state reconstructions, assessing inter-specific relationships in species complexes with hundreds of molecular markers, and describing new species, **(Chapters 2-5)**, disclosed a new pollination system, identified morphological characters associated with similar pollination mechanisms **(Chapters 6-7)** and discussed the impact of biogeographical events and orogeny (formation of the Andes and Central America) on the extant species richness and biodiversity of *Lepanthes* **(Chapter 8-9)**. This thesis provides new insights in the complex evolution of one of the most species-rich angiosperm lineages in the Neotropics.

Outline of the thesis

Lepanthes contains more than 1,130 species and new species are constantly being discovered in the Neotropics. An approximate number of the actual species diversity is not yet known and this number tends to increase partially due to the extreme diversity of the genus but also because several regions of the Neotropics continue to be explored and the boost of alpha-taxonomic studies (Luer and Thoerle, 2012; Pupulin et al., 2018; Pupulin and Bogarín, 2019). In addition, the phylogenetic relationships of the *Lepanthes* and allied genera were problematic at the start of my PhD project, not because of the lack of sufficient DNA markers but because of insufficient taxonomic sampling and the widespread convergences in reproductive characters. Therefore, in **Chapter 2** (Bogarín et al. in review) we presented the integral discussion on the phylogenetics of the *Lepanthes* clade integrating phylogenetics and morphological evolution of character states. Consequently, in **Chapter 3** we proposed a new classification of the *Lepanthes* clade based on a more extensive taxonomic sampling and the information obtained in Chapter 2 (Bogarín et al., 2018). Similar to the poor understanding of inter-generic relationships, some inter-specific relationships are difficult to understand because of the high morphological similarity, especially in floral traits. In addition, these species complexes are challenging to resolve using standard DNA barcoding markers such as nrITS or *mat*K. Therefore, in **Chapter 4** we assessed the performance of hundreds of innovative molecular markers derived from an anchored hybrid enrichment approach (AHE) to resolve phylogenetic relationships and improve species recognition in the *Lepanthes horrida* species group (Bogarín et al., 2018). Further, some areas of the Neotropics are rich in *Lepanthes* species but much floristic work still needs to be done. This

is for instance the case for Panama, where an underestimation of species is well known but an increase of taxonomic studies is revealing new species or new records from neighboring regions (Bogarín et al., 2013). In this way, in **Chapter 5** we revealed two new species of *Lepanthes* detected during fieldwork (Bogarín et al., 2017). In addition to the systematics and the evolution of morphological traits, pollination studies are key in understanding homoplastic characters in closely related genera and the role of pollinators as drivers of species diversity. However, this is largely unknown because knowledge of pollination systems in the group is still scarce and only the pollination system of *Lepanthes* is known. Therefore, in **Chapter 6** we addressed the pollination of *Lepanthes*' closely related genus *Trichosalpinx* through study of floral anatomy, pollinator behaviour and floral traits shared with other angiosperms to elucidate its pollination mechanism (Bogarín et al., 2018). The similar floral morphology and homoplastic characters described in **Chapter 5** among *Trichosalpinx* and the closely related genera *Anathallis* and *Lankesteriana* suggest that they are pollinated by a similar system as shown in **Chapter 6**. Hence, in **Chapter 7** we assessed the micromorphological and histochemical features of floral organs to test a hypothesis on floral convergence in this clade (Bogarín et al., 2018). And finally, to understand the role of abiotic factors such as the impact of the Andean mountains in the diversification of *Lepanthes* in **Chapter 8-9** we inferred the biogeographical history and diversification dynamics of the two largest Neotropical orchid groups (Cymbidieae and Pleurothallidinae), using two unparalleled, densely sampled phylogenies coupled with geological and biological datasets (Pérez-Escobar et al., 2017a). In **Chapter 10**, I discuss further steps needed to compliment the findings presented in my PhD thesis to fully understand and better protect orchid species radiations in the Neotropics.

Taxonomy and systematics of *Lepanthes* and allies

Chapter 2

Phylogenetic comparative methods improve the selection of characters for generic delimitations in a hypediverse Neotropical orchid lineage

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Scientific Reports. In review

Abstract. Taxonomic delimitations are challenging because of the convergent and variable nature of phenotypic traits. This is particularly evident in species-rich lineages, where the ancestral and derived states and their gains and losses are difficult to assess. However, phylogenetic comparative methods help to evaluate the parallel evolution of a given morphological character, thus enabling the discovery of traits useful for classifications. In this study, we investigate the evolution of selected traits to test for their suitability for generic delimitations in the Neotropical species-richest orchid lineage *Lepanthes*. We evaluated every generic name proposed in the *Lepanthes* clade producing densely sampled phylogenies with Maximum Parsimony, Maximum Likelihood, and Bayesian approaches. In addition, we assessed with Ancestral State Reconstructions 18 phenotypic characters that have been traditionally used to diagnose the genera. Our results support the recognition of 14 monophyletic genera and provide solid morphological delimitations. We identified 16 plesiomorphies, 12 homoplastic characters, and 7 synapomorphies, the latter of which are reproductive features mostly related to the pollination by pseudocopulation and possibly correlated with rapid diversifications within *Lepanthes*. Furthermore, the ancestral states of some reproductive characters suggest that these traits are associated with similar pollination mechanisms promoting homoplasy. Our methodological approach enables the discovery of useful traits for generic delimitations in the *Lepanthes* clade. This offers various other testable hypotheses for future research on Pleurothallidinae orchids because phenotypic variation of some of the characters evaluated here also occur in other diverse genera.

2.1 Introduction

Taxonomic delimitation is essential to understand, document, and quantify earth's biodiversity. This is particularly true for species, which are regarded as the fundamental units of biological systems. Species delimitations and their numerous corresponding concepts are still hotly debated, yet relatively little has been discussed regarding supra-specific taxon delimitations (Barkman and Simpson, 2001; De Queiroz, 2007, 2005). Among such higher taxonomic ranges, the genera are important because they inform about discernable trait patterns shared among species groupings (Humphreys and Linder, 2009), and are widely used as biodiversity indicators of biogeographical areas (Gentry, 1986), and even biomes (Ulloa et al., 2017). Generic delimitations are based on several criteria that are often informed by morphological traits, the principle of monophyly, statistical node supports in phylogenies, and even lineage size (i.e. species number). Among these, morphology is perhaps the most common invoked criterion to segregate or subsume species aggregates (Humphreys and Linder, 2009), yet morphological characters are often variable and converge across the angiosperm tree of life (Stull et al., 2018), thus rendering the selection of suitable morphological characters for generic delimitations quite difficult.

The orchid family includes about 25,000 species and *ca.* 750 genera. Its generic classification system is quite dynamic, with hundreds of genera having been subsumed and segregated during the last decade (Chase et al., 2015). Among recalcitrant lineages with complicated generic delimitations are the Pleurothallidinae, the species-richest subtribe in the Neotropics (5,200 species; (Karremans, 2016; Luer, 2007; Pridgeon et al., 2001)). The high species diversity derived from recent and rapid diversifications and the exceptionally wide spectrum of morphological features have made the classification of this group challenging (Pérez-Escobar et al., 2017a). Previous cladistic and contemporary systematic studies were largely based on morphology (Luer, 1986a; Neyland et al., 1995). Using these studies as a framework, Pridgeon et al. (2001) proposed the first molecular phylogenetic classification of the subtribe by sequencing nuclear and plastid regions of 185 selected taxa (3.5% of the species of the Pleurothallidinae). This study laid the foundation for the classification system followed in *Genera Orchidacearum* (Pridgeon et al., 2005) which divided the subtribe in nine main clades. In the past 10 years, several phylogenetic studies, aimed to increase taxon sampling or add more markers to the previous phylogenetic reconstructions, supported or redefine most of the taxonomic and generic concepts proposed by Pridgeon et al. (2001) and Luer (2006). These phylogenetic re-evaluations covered almost all clades across the subtribe (Abele, 2007; Chiron et al., 2012; Karremans et al., 2013; Karremans et al., 2016; Karremans et al., 2016).

One of the few remaining puzzling groups with phylogenetic relationships poorly understood in the Pleurothallidinae is the *Lepanthes* clade (Bogarín et al., 2018c; Karremans, 2016; Luer, 1986b; Pridgeon et al., 2001) (. 2.1). In its current circumscription, it comprises the genera *Anathallis* Barb.Rodr. (116 spp.), *Draconanthes* (Luer) Luer (2), *Epibator* Luer (3), *Frondaria* Luer (1), *Lankesteriana* Karremans (21), *Lepanthes* Sw. (>1200), *Lepanthopsis* (Cogn.) Ames (44), *Trichosalpinx* Luer (24) and *Zootrophion* Luer (26). Moreover, four generic concepts needed to attain monophyly, were recently erected by Bogarín (Bogarín et al., 2018c): *Gravendeelia* Bogarín & Karremans (1), *Pendusalpinx* Karremans & Mel.Fernández (7), *Stellamaris* Mel.Fernández & Bogarín (1), and *Opilionanthe* Karremans & Bogarín (1) as well as the reinstatement of

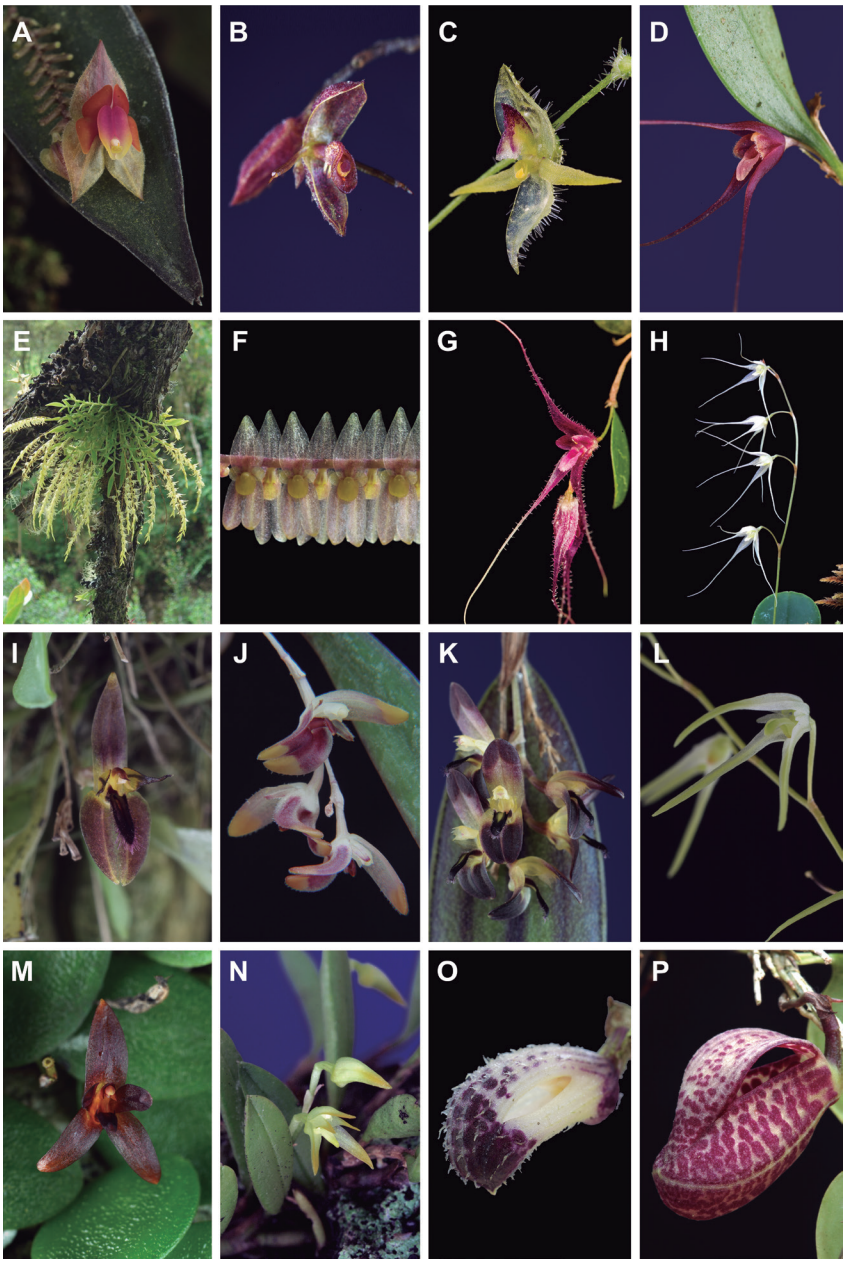

Figure 2.1. Flower morphology of the representatives of the *Lepanthes* clade: **A.** *Lepanthes*. **B.** *Draconanthes*. **C.** *Pseudolepanthes*. **D.** *Stellamaris*. **E.** *Frondaria*. **F.** *Lepanthopsis*. **G.** *Gravendeelia*. **H.** *Opilionanthe*. **I.** *Lankesteriana*. **J.** *Pendusalpinx*. **K.** *Trichosalpinx*. **L.** *Tubella*. **M.** *Anathallis*. **N.** *Anathallis*. **O.** *Zootrophion*. **P.** *Zootrophion* (*Epibator*). Photographs A-B, D, F, I, K-O by D.Bogarín, C,G by S. Vieira-Uribe, E by J. Portilla (Ecuagenera), H,J,P by W. Driessen.

Pseudolepanthes (Luer) Archila (10) and *Tubella* (Luer) Archila (79). The species-richest genus is *Lepanthes*, which comprises more than 77% of the species of the clade, whereas the remaining genera represent less than 8% of the species diversity each.

The *Lepanthes* clade is widely distributed in the Neotropics ranging from Mexico and Florida to southern Brazil and Argentina, including Central America and the Antilles. The species are characterized by infundibular sheaths, also called "lepanthiform sheaths" along the ramicauls of unknown functionality (Luer, 1996b; Pridgeon et al., 2001). These sheaths are unornamented and imbricating in *Anathallis*, *Lankesteriana* and *Zootrophion*, foliaceous with expanded leaf sheaths in *Frondaria* and sclerotic with ornamentations (spiculate or muriculate) along the ramicauls in the remaining genera (Figs. 2.1-2.2). Regardless of the relative uniformity in plant vegetative characters, flower morphology is highly dissimilar among genera and no single diagnostic floral character distinguishing the group has been recognized. Floral trait variation is most evident in the flower shape (spread, flattened or cupped sepals and petals), color (red, yellow, white, green, purple or maculated), anthesis timing in the inflorescence (simultaneous or successive), shape of sepals, petals and lip (elongated, flattened, ciliated, bilobed), anther position (apical or ventral), pollinaria-associated structures (with or without viscidium), and presence/absence of a synsepal and column foot (Luer, 1986a, 1986b; Pridgeon, 2005) (Fig. 2.1).

Previous multi-locus phylogenies strongly supported the monophyly of the *Lepanthes* clade (Chase et al., 2015; Pridgeon et al., 2005), yet the number of genera to be recognized and their phylogenetic relationships are still unclear. This is likely due to the widespread convergences in reproductive characters in the lineage and the insufficient phylogenetic taxon sampling. Earlier phylogenetic studies in the Pleurothallidinae did not investigated morphological evolutionary patterns, homoplasy and contrasting differences in reproductive traits by combining ancestral state reconstructions (ASR) and a solid phylogenetic framework (Karremans, 2016; Pridgeon et al., 2001). This is essential to test hypotheses of morphological evolution and to disentangle recalcitrant generic delimitations due to phenotypic similarities. More importantly, theory predicts that synapomorphies or homoplastic characters are attributed to shifts or convergences due to dipteran pollination, but this remains yet to be tested due to the scarce pollination observations across the subtribe. The role of pollinator interactions in the evolution of the *Lepanthes* clade is currently unknown because only two pollination systems have been reported so far for *Lepanthes* and *Trichosalpinx* (Blanco and Barboza, 2005; Bogarín et al., 2018a).

Here, we explore the utility of molecular trees and phylogenetic comparative methods to discover suitable morphological characters for generic delimitations. To achieve this, we evaluate the relationships among members of the *Lepanthes* clade by assessing morphological characters within a phylogenetic framework. We performed ASRs on 18 floral morphological characters using a well resolved phylogenetic inference from nuclear nrITS and plastid *matK* markers of 122 species covering all recognized genera within the clade (Bogarín et al., 2018c). We want to answer the following questions: (1) which monophyletic genera can be recognized based on a phylogenetic framework? (2) what are the phylogenetically informative characters of each clade based on ASRs? (3) how did such diagnostic morphological characters evolve in the clade? We also provide a detailed generic circumscription of *Lepanthes*.

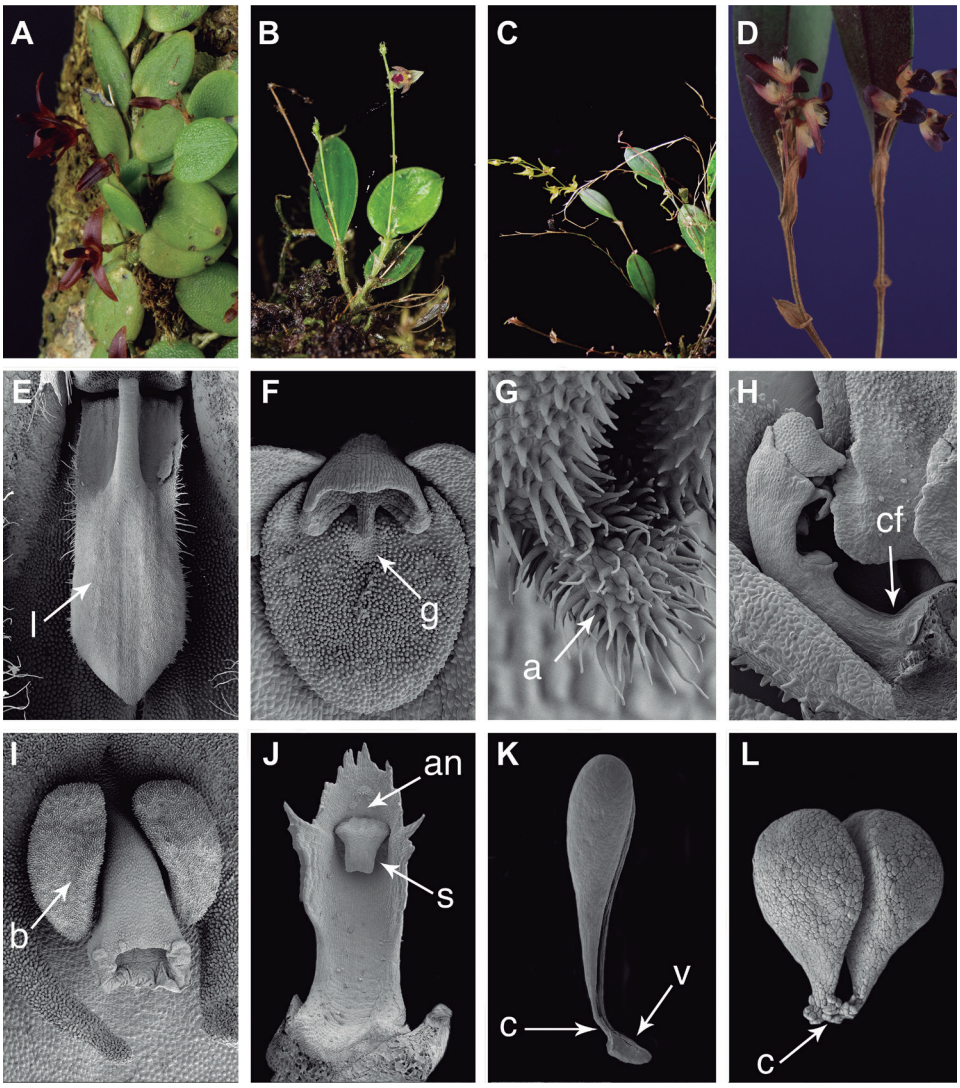

Figure 2.2. Vegetative and flower morphology of the characters evaluated: **A.** repent habit in *Anathallis*. **B.** caespitose habit with longer inflorescences than leaf in *Pseudolepanthes*. **C.** prolific ramicauls in *Tubella*. **D.** ornamented lepanthiform bracts in *Trichosalpin*x. **E.** laminar, mobile lip (i) of *Trichosalpinx*. **F.** bilobed stigma and glenion (g) in *Lepanthopsis*. **G.** Appendix (a) at the lip base of *Lepanthes*. **H.** Column foot (cf) and ventral anther in *Gravendeelia*. **I.** Bilobed lip (b) and apical anther in *Lepanthes*. **J.** Ventral anther (an) and stigma (s) in *Anathallis*. **K.** Pollinarium with viscidium (v) and caudicles (c) in *Lepanthes.* **L.** Pollinarium with caudicles (c) in *Trichosalpinx*. Photographs A-L by D.Bogarín, b. by S. Vieira-Uribe.

2.2 Materials and Methods

2.2.1 Taxon sampling

We sampled 148 accessions of 120 species from every generic name erected in the group. We included *Anathallis* (6 spp.), *Draconanthes* (1 sp.), *Frondaria* (1 sp.), *Gravendeelia* (1 sp.), *Lankesteriana* (5 spp.), *Lepanthes* (61 spp.), *Lepanthopsis* (6 spp.), *Opilionanthe* (1 sp.), *Pendusalpinx* (8 spp.), *Pseudolepanthes* (2 sp.), *Stellamaris* (1 sp.), *Trichosalpinx* (8 spp.), *Tubella* (14 spp.) and *Zootrophion* (6 spp.). Members of the *Trichosalpinx* subgenus *Xenia* Luer (five spp.) were not sampled due to unavailability of material. Voucher information, NCBI GenBank accessions, and references for each DNA sequence are listed in Appendix S1 (). A total of 88 sequences were newly generated (49 from nrITS and 39 from *mat*K) and complimented with sequences from previous studies (Karremans, 2014; Pérez-Escobar et al., 2017a; Pridgeon et al., 2001). *Acianthera cogniauxiana* (Schltr.) Pridgeon & M.W. Chase and *Acianthera fenestrata* (Barb.Rodr.) Pridgeon & M.W.Chase were chosen as outgroups based on Pridgeon et al., (2001).

2.2.2 Phenotypic character selection

We scored 18 macro-morphological characters (Table 2.1) which are considered taxonomically informative or ecologically important that have been used to characterize some of the genera. Data were obtained by direct observations from herbarium material (CR, AMES, JBL, K, L, PMA, UCH, W herbaria) and living material collected in the field or cultivated at Lankester Botanical Garden, the Hortus botanicus Leiden or private orchid collections. Observations were complimented with morphological data compiled from monographs on the Pleurothallidinae (Luer, 1986a; b, 1991, 1996a, 1997a, 2004, 2006; Pridgeon, 2005; Luer and Thoerle, 2012) and with digital documentation (photographs and drawings) from JBL databases. We generated additional macro-morphological data with a Scanning Electron Microscope (SEM) using fixed flowers dehydrated in a series of ethanol solutions (70%–96%–≥99.9%) and acetone ≥99.8%. Critical-point drying was performed in an Automated Critical Point Dryer Leica EM CPD300 (Leica Microsystems, Wetzlar, Germany) following the manufacturer's procedures. Samples were sputter-coated with 20 nm of Pt/Pd in a Quorum Q150TS sputter-coater and observed with a JEOL JSM-7600F (Tokyo, Japan) field emission scanning electron microscope, at an accelerating voltage of 10 kV. For macro-photography we used a Nikon® D7100 (Tokyo, Japan) digital camera and a PB-6 Nikon bellows. We edited the images in Adobe Photoshop® CC (Adobe Systems Inc., California, U.S.A).

2.2.3 DNA extraction

We extracted total genomic DNA from about 50-100 mg of silica gel dried leaf/flower tissue. Each sample was placed in 2 ml Eppendorf® tube with three glass beads (7 mm) and sterile sand. The tubes were frozen in liquid nitrogen for about 1-2 minutes and powdered in a Retsch MM 300 shaker for 3 minutes. We followed the 2× CTAB (Hexadecyltrimethylammonium bromide) protocol for isolating DNA (Doyle and Doyle, 1987). DNA was quiantified with a Qubit 3.0 Fluorometer (TermoFischer Scientific®).

Table 2.1. Characters and scoring of the 18 morphological traits assessed with ancestral character estimations and the main references illustrating or discussing these characters.

Characters	States	References
Habit	(0) caespitose; (1) repent	(Luer, 1986a; Pridgeon, 1982; Stern et al., 1985)
Ramicauls	(0) non-prolific; (1) prolific	(Luer, 1986a; Pridgeon, 1982; Stern et al., 1985)
Ramicauls' bracts	(0) unornamented; (1) ornamented; (2) foliaceous	(Luer, 1991, 1990)
Inflorescence	(0) simultaneously flowering; (1) successively flowering	(Luer, 1986a, 1983)
Inflorescence length	(0) shorter than leaves; (1) longer than leaves	(Luer, 1986a, 1983)
Flowers	(0) fully opening; (1) bud-like	(Luer, 1982)
Dorsal sepal concavity	(0) concave; (1) flattened	(Luer, 1996b; Luer, 2006)
Synsepal	(0) absent; (1) present	(Luer, 1986a; Luer, 1996b; Luer, 1997)
Sepal shape	(0) oblong-acute; (1) ovate-acuminate (2) ovate-acute	(Luer, 1986a; Luer, 1996b; Luer, 2006)
Petals shape	(0) dissimilar; (1) subsimilar	(Luer, 1997; Luer, 2006, 1986a)
Lip shape	(0) laminar; (1) bilobed	(Luer, 1996b; Luer, 2006)
Lip mobility	(0) mobile; (1) sessile	(Bogarín et al., 2018a; Luer, 2006)
Glenion of the lip	(0) absent; (1) present	(Luer, 1991)
Appendix of the lip	(0) absent; (1) present	(Luer, 1996b)
Column foot	(0) absent; (1) present	(Benzing and Pridgeon, 1983; Luer, 1986a)
Stigma shape	(0) entire; (1) bilobed	(Luer, 1991, 1990)
Anther position	(0) ventral; (1) dorsal	(Luer, 1996b)
Pollinaria-associated structures	(0) with caudicles; (1) with caudicles+viscidium	(Karremans et al., 2013; Stenzel, 2000)

2.2.4 Amplification, sequencing and alignment

The polymerase chain reaction (PCR) mixture, the primers for the nrITS (17SE and 26SE) and plastid *mat*K (2.1aF and 5R) regions and amplification profiles followed Karremans (Karremans et al., 2016). Sanger sequencing of both regions was conducted by BaseClear (https://www.baseclear.com) on an ABI 3730xl genetic analyzer (Applied Biosystems, Foster City, California, U.S.A). Sequences were deposited in NCBI GenBank. We used Geneious® R9 (Biomatters Ltd., Auckland, New Zealand (Kearse et al., 2012)) for the editing of chromatograms and pairwise alignment. Sequences were aligned in the online MAFFT platform (Multiple Alignment using Fast Fourier Transform, http://mafft.cbrc.jp/alignment/server/) using default settings. We adjusted and trimmed the resulting alignment manually. The concatenated dataset (nrITS +*mat*K) was built with Sequence Matrix v100.0 (Vaidya et al., 2011). When sequences were not available, they were analyzed as missing data.

2.2.5 Phylogenetic analyses

We analyzed the individual and concatenated datasets of nrITS and *mat*K with Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) analyses. The model of evolution and the parameters were calculated using the Akaike Information Criterion (AIC) in jModelTest2 v2.1.7 (Darriba et al., 2012). All analyses were run in the CIPRES Science Gateway V. 3.1 (http://www.phylo.org/sub_sections/portal/) (Miller et al., 2010). To evaluate the incongruence between plastid and nuclear datasets we followed the pipeline implemented by Pérez-Escobar et al. (2017a) using the Procrustean Approach to Cophylogeny (PACo) application (Balbuena et al., 2013) in R (http://data- dryad.org/review?doi=doi:10.5061/dryad.q6s1f). This procedure identifies potential conflicting outliers contributing to incongruent phylogenies. The *mat*K sequences from the retrieved conflicting terminals were removed and replaced by missing data because inferences derived from plastid markers are usually more in conflict with morphological observations as compared with inferences derived from nuclear markers (Pérez-Escobar et al., 2016a). A new concatenated matrix was re-aligned using the cleaned *mat*K dataset and then analyzed with BI, ML, and MP approaches. These analyses were contrasted with the original inferences from concatenated datasets.

We performed the Bayesian inference analyses with MrBayes v.3.2.6 on XSEDE (Huelsenbeck and Ronquist, 2001) with the following parameters: number of generations Ngen=50×10^6 for the combined and individual datasets, number of runs (nruns=2), number of chains to run (nchains=4), temperature parameter (temp=2) and sampling frequency of 1,000 yielding 50,001 trees per run. The log files from MrBayes were inspected in Tracer v.1.6 to check the convergence of independent runs (i.e. with estimated sample size (ESS) > 200). The initial 25% of trees were discarded as burn-in and the resulting trees were used to obtain a 50% majority-rule consensus tree. Maximum likelihood analyses were performed with RAxML-HPC2 on XSEDE (8.2.10) (Stamatakis et al., 2008) choosing the GTRGAMMA model for bootstrapping and 1,000 bootstrap iterations. Parsimony analyses were performed with PAUPRat: Parsimony ratchet searches using PAUP* (Nixon, 1999; Sikes and Lewis, 2001; Swofford, 2002) with 1,000 ratchet repetitions, seed value=0,20% percent of characters to perturb (pct=20), original weights 1 for all characters (wtmode=uniform) and a tree bisection-reconnection branch swapping algorithm (swap=TBR). The 50% majority rule consensus trees for ML and MP were obtained with PAUP v4.0a152. and observed in FigTree v.1.3.1. The statistical support of the clades was evaluated with the values of posterior probability (PP) for BI reconstruction, bootstrap for ML (MLB) and parsimony bootstrap for MP (MPB). The support values (PP) were added to the branches on the Bayesian 50% majority-rule consensus tree with additional support values shown for ML and MP when the same topology was retrieved. We considered clades with MPB ≥ 70%, MLBS ≥ 70% and PP ≥ 0.95% as well supported. To investigate phylogenetic relationships among genera, we also conducted a network analysis with 3,000 tree replicates of the BI inference of the combined dataset in Splits Tree4 v.4.11.3 (Huson and Bryant, 2006) with a 0.20 cutoff value. Resulting trees were manipulated with R programming language (R Core Team, 2017) under R Studio (Gandrud, 2013) using the packages APE, ggtree and phytools (Paradis et al., 2004; Revell, 2012; Yu et al., 2017). Final trees were edited in Adobe® Illustrator CC (Adobe Systems Inc., California, U.S.A).

To obtain ultrametric trees for the character evolution assessments we estimated the divergence times in BEAST v.1.8.2 using the CIPRES Science Gateway (Miller et al., 2010). The clock-likeness of the data was tested by observing the coefficient of variation (CV) of relaxed clock models. Speciation tree model selection was achieved by executing the Bayes factor test on Yule Process (Y), Birth Death-Process (BD) and Birth-Death-Incomplete Sampling (BDIS) models under strict and uncorrelated lognormal molecular clock models. For each model, we assigned a normal prior distribution of 16.45 (±2.5 standard deviations) Ma to the root node of the *Lepanthes* clade and 12.93 (±2.5 standard deviations) Ma to the node of *Zootrophion* with the remainder of the members of the *Lepanthes* clade using the values calculated from the fossil-calibrated chronogram of the Pleurothallidinae by Pérez-Escobar et al. (2017a). We performed two MCMC with 50×10^6 generations and sampling every 1,000 generations with a Marginal likelihood estimation (MLE) of 50 path steps, 10×10^5 length of chains and log likelihood for every 1000 generations. We inspected the convergence of independent runs size in Tracer v.1.6 as explained above. To compare the divergence time estimates among the speciation models (Y, BD and BDIS) we used Bayes factors calculated with marginal likelihood using stepping stone sampling derived from the MLE path sampling.

2.2.6 Ancestral State Reconstruction (ASRs)

Ancestral state reconstructions were assessed with ML, stochastic character mapping (SCM), and BI using phylograms and ultrametric trees. For the ML approach we explored the following models: equal rates (ER), symmetrical (SYM) and all rates different (ARD). We relied on the re-rooting method of Yang et al. (1995) and the function ACE implemented in the R-package phytools. The best-fitting model was selected by comparing the log-likelihoods among these models using likelihood ratio tests. Scaled likelihoods at the root and nodes were plotted in the time-calibrated consensus phylogenetic tree. For the stochastic mapping analyses based on joint sampling we performed 100 replicates on 100 randomly selected trees (10,000 mapped trees) from the best fitting time-calibrated BEAST analysis. The trees were randomly selected using the R function *samples.trees* (http://coleoguy.blogspot.de/ 2012/09/randomly-sampling-trees.html). Results of transitions and the proportion of time spent in each state were calculated and summarized in phytools with the functions *make.simmap* and *describe.simmap* (Bollback, 2006; Revell, 2012). These analysis were performed following the scripts by Portik and Blackburn (2016). ML and BI inferenced were executed in the program BayesTraits V3 (Pagel, 1999, 1994; Pagel and Meade, 2006). To account for phylogenetic uncertainty, ancestral character estimates were calculated using a randomly sampled set of 1000 trees from the post burnin sample of the 50,000 ultrametric trees obtained from the best fitting time-calibrated BEAST analysis as described above. We used the option *AddNode* for reconstruction of internal nodes of interest comprising every generic group of the *Lepanthes* clade and the root node. For the ML approach, we used the method *Multistate* with 10 ML attempts per tree and 20,000 evaluations in order to preliminary assess prior distributions. For the BI, we chose the method *Multistate* and MCMC parameters of 30,010,000 iterations, sample period of 1,000, burnin of 10,000, auto tune rate deviation and stepping stones 100 10,000. We used the method Reversible-Jump MCMC with hyper-prior exponential to assess the best fitting models in proportion to their posterior probabil-

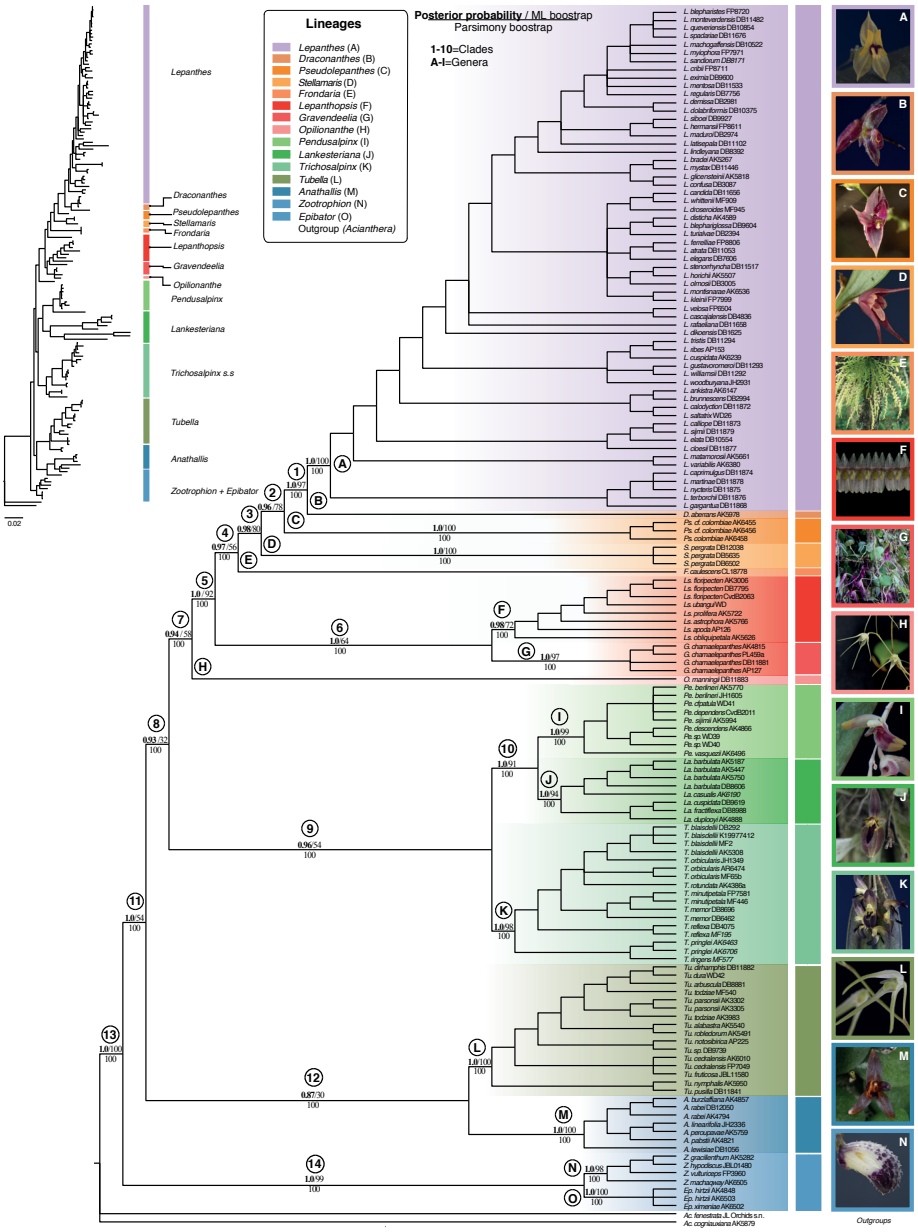

Figure 2.3. The 14 genera recognized in the *Lepanthes* clade in the 50% majority-rule consensus tree based on BI analysis of concatenated dataset. Plotted branch values for MPB, MLBS and PP are given for each well-supported clade of interest. Letters represent genera and numbers clades grouping the genera. Photographs A,B, D, F-G, J-N by D.Bogarín, C by S. Vieira-Uribe, E by J. Portilla (Ecuagenera), H-I by W. Driessen.

ities according to the MCMC approach. We chose the hyper-prior approach as recommended by Meade and Pagel (2016) in order to reduce the arbitrariness when choosing priors. Therefore, we selected the option reversible jump hyper-prior exponential with prior distribution set according to the transition ranges obtained from a preliminary ML analysis. The input files for BayesTraits V3 were partially constructed with Wrappers to Automate the Reconstruction of Ancestral Character States (WARACS) (Gruenstaeudl, 2016). The BayesTraits outputs files were analyzed in R with the BayesTraits wrapper (btw) by Randi H Griffin (http://rgriff23.github.io/projects/btw.html) and other functions from btrtools and BTprocessR (https://github.com/hferg). The MCMC stationarity of parameters (ESS values >200) and convergence of chains were checked in Tracer v1.6.0 and plotted in R with the packages coda (Plummer et al., 2006) and the function *mcmcPlots* of BTprocessR. We reconstructed the ancestral states for all nodes of the tree and plotted the mean probabilities retrieved at each node with phytools.

2.3 Results

Matrix statistics of the 148 accessions from the 120 species (including two outgroup accessions) and parsimony information for nrITS, matK and concatenated datasets are summarized in Appendix S2.

2.3.1 Gene trees

The inferences of the BI, ML and MP from the nrITS dataset yielded similar topologies and high support for the 14 genera recognized as members of the *Lepanthes* clade but with some differences in the topology among the relationships of those clades (Appendices S3,S4). Some differences were observed in the placement of *Anathallis*, *Lankesteriana*, *Pendusalpinx*, *Trichosalpinx* and *Tubella* and in the position of *L. obliquipetala*, which was placed outside the clade *Lepanthopsis+Gravendeelia*. The relationships among *Lepanthes*, *Draconanthes*, *Pseudolepanthes*, *Stellamaris* were consistent. In contrast, the inferences from the *matK* dataset showed several polytomies and low support values for most of the clades Appendices S4,S5).

2.3.2 Incongruence between nuclear and plastid datasets

A total of 24 terminals were detected as incongruent with ML and 34 with BI. Of those, 20 terminals were retrieved as incongruent by both inferences (Appendix S1; S6). The topology of the BI, MP and ML trees inferred from the concatenated datasets excluding/including the plastid conflicting sequences recognized essentially the same generic clades but showed some differences in the topology and support values in their intergeneric relationships (Appendices S1, S6, S7).

2.3.3 Concatenated approach (nrITS + matK)

Consistent with the inferences based on nrITS, the BI, ML and MP analyses from the concatenated dataset converged in the same generic groupings with high support values for all the genera

of the *Lepanthes* clade (Fig. 2.2 and Appendix S1). The support values slightly increased after removing the potential outliers from the plastid dataset. In contrast, despite the consistent topologies and high support obtained for all genera, the relationships among them differed using the original datasets (as well as the nrITS dataset alone). However, these relationships were higher supported in the analyses after removing the detected potential outliers from the *mat*K dataset and the phylogenetic relationships obtained were topologically most similar among BI, ML and MP (Fig. 2.3, Appendices S6,S7). In addition, we show the support values of the inferences with/without PACo in Appendix S6. Consistent with the high support values obtained with BI inference, the inferred network did not show phylogenetic uncertainty in the clades of the 14 genera of the *Lepanthes* clade.

2.3.4 Phylogenetic relationships and generic clades

We obtained strong support for recognizing 14 genera within the *Lepanthes* clade (Figs. 2.3-2.4). *Lepanthes* (Clade A) was supported as monophyletic in all the analyses (MPB=100, MLBS=100 and PP=1.0) and sister to *Draconanthes* (Clade B). The clustering of *Lepanthes+Draconanthes* was well supported in all the analysis (MPB=100%, MLBS=100% and PP=1.0). The accessions of *Pseudolepanthes* (Clade C) grouped together with high support (MPB=100%, MLBS=100% and PP=1.0) and this genus was sister to *Lepanthes+Draconanthes* (Clade 1). The accessions of *Stellamaris pergrata* (Ames) Mel.Fernández & Bogarín (Clade D) were well supported and the group was sister to *Lepanthes+Draconanthes+Pseudolepanthes* (Clade 2) (MLBS=80% and PP=0.98). When phylogenetic incongruence was not considered, these two genera clustered in a clade with strong support in the MP tree (MPB=100). The genus *Frondaria* (Clade E) was found to be related to *Lepanthes*, *Draconanthes*, *Pseudolepanthes*, *Stellamaris* (Clade 3), well supported (MPB=100 and PP=0.97) but lacking support in the ML analysis (MLBS=56%). Clade 4 made up by Clade 3+*Frondaria* and comprised the species more related to the core of *Lepanthes* whereas *Lepanthopsis* (Clade F) and *Gravendeelia* (Clade G) both clustered in Clade 6 as its sister group (Clade 5). Most of the nodes of these clades were well supported (MPB>100%, MLBS>72% and PP>0.98) with the only exception being Clade 6 with low support for ML but well supported by MPB>100% and PP>0.98. The genus *Opilionanthe* was sister to Clade 5 + Clade 6 with high support for MPB=100%, moderately supported by BI (PP=0.94) and low support for ML (MLBS=58%). Topologically, *Opilionanthe* always clustered apart from the other generic clades discussed here. Related to the groups of Clade 7 (members of the core of *Lepanthes* and *Lepanthopsis*) was a group consisting of species related to *Trichosalpinx* s.s. (Clade K), *Pendusalpinx* (Clade J) and *Lankesteriana* (Clade I) all highly supported as genera (MPB=100%, MLBS≥94% and PP=1.0). This topology was retrieved with high to moderate support (MPB=100%, MLBS≥54% and PP≥0.96) after removing incongruences. *Tubella* (Clade L) and *Anathallis* (Clade M) were highly supported as genera (MPB=100%, MLBS=100% and PP=1.0). The internal relationships of Clade 12 received low support with ML (MPB≤30%) and BI (PP≤0.87) but high support by MP (MPB=100%). Clade 14 comprising *Zootrophion* (Clade N) and *Epibator* (Clade O),was well supported in all the analyses (MPB=100%, MLBS≥98% and PP=1.0). The most constant well supported topologies among all the analyses were the clustering of *Zootrophion* (MPB=100%, MLBS≥99%, PP=1.0), *Lankesteriana* and *Pendusalpinx*

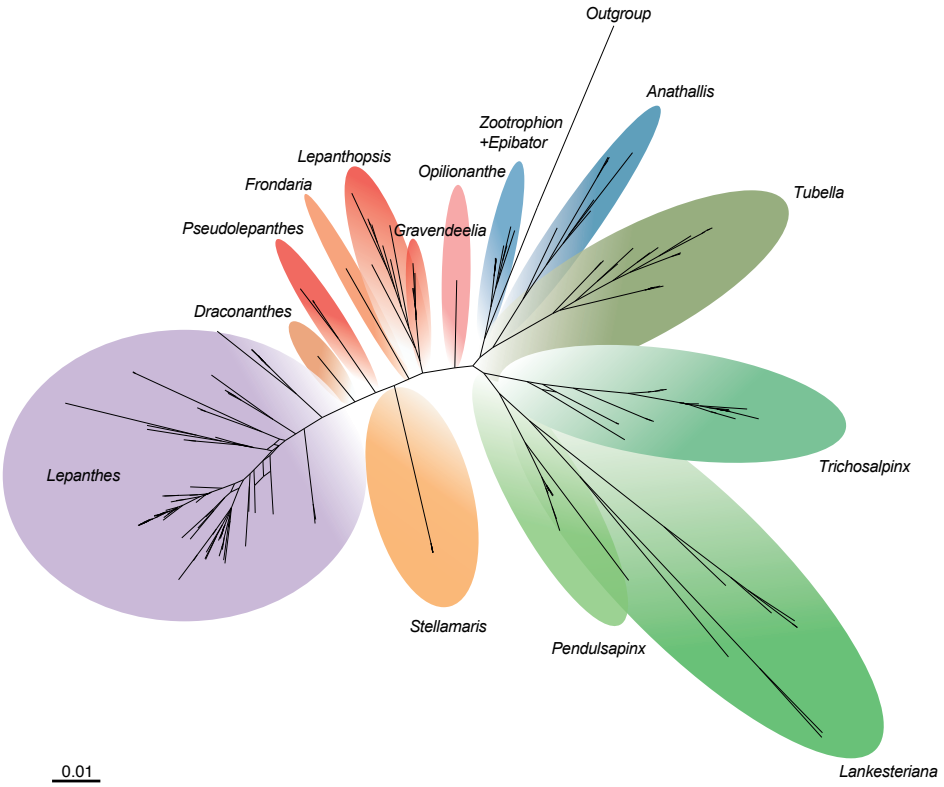

Figure 2.4. Split network showing the 14 genera of the *Lepanthes* clade inferred from 3,000 tree replicates of the BI inference. The network shows well supported groups without uncertainty in the relationships.

(MPB=100%, MLBS≥91%, PP=1.0), *Lepanthes+Draconanthes* (MPB=100%, MLBS≥88%, PP=1.0) and the clustering of the genera related to the core of *Lepanthes* (Clade 4) with *Lepanthopsis+Gravendeelia* (Clade 6) (MPB=100%, MLBS≥88% and PP=1.0).

2.3.5 Character evolution

The ASR were based on the one-rate model ER which was consistently better than the SYM and ARD models (Appendices S8,S10). These estimations were obtained using phylograms from MrBayes and ultrametric trees from BEAST calculated under the BD as the best model speciation model according to Bayes Factors test (Appendix S11). Estimations based on the Reversible-Jump MCMC model yielded similar results compared to the rates obtained with SCM (Appendices S12, S14). For the MCMC approach with BayesTraits V3 the best results were obtained with the hyperprior adjusted to the previously obtained ML transition rates (from 0 to 0.03). The ACE, SIMMAP and re-rooting methods yielded identical scaled-likelihoods at the root state and the estimations with MCMC revealed essentially the same results obtained with

ACE and SIMMAP with ambiguous estimations for the characters of inflorescence length and synsepal (Table 2.2). Characters states of the common ancestor suggest that plesiomorphic features are a caespitose habit with non-prolific, unornamented ramicauls, simultaneously flowering inflorescences, fully opening flowers with concave, ovate-acute dorsal sepals, dissimilar petals, the presence of a column foot, a laminar, mobile lip without glenion and a ventral anther with entire stigma (Table 2.3).

The most common character state transitions are: a caespitose to repent/pendent habit, ornamented to unornamented bracts, non-prolific to prolific ramicauls, simultaneously flowering to successively flowering inflorescences, shortening of inflorescences, fully opening flowers to bud-like flowers, ovate-acute to ovate-acuminate/oblong-acute sepals, concave to flattened dorsal sepals, dissimilar to subsimilar petals, loss of a column foot and synsepal, movable to sessile lip, entire to bilobed stigma, ventral to dorsal anther and pollinarium with naked caudicles to caudicles with a viscidium (Figs. 2.5-2.6). Probabilities favoring reversal transitions from prolific to non-prolific ramicauls, foliaceous to ornamented/unornamented bracts, repent to caespitose habit, bud-like to opening flowers, subsimilar to dissimilar petals, oblong-acute to ovate-acuminate/ovate-acute sepals, presence of a glenion to absence, sessile to mobile lip, absence of a column foot to presence, dorsal to apical anther, bilobed to entire stigma and pollinarium with caudicles and a viscidium to lack of a viscidium, were found to be unlikely. Lip shape from laminar to bilobed and vice-versa showed a similar probability (Figs. 2.5-2.6). Twelve homoplastic characters and seven synapomorphic characters were detected (Table 2.3). The combination of a sessile lip, absence of a column foot, dorsal anther and pollinarium with caudicles and viscidium are features only observed in *Lepanthes*, *Draconanthes*, *Pseudolepanthes* and *Lepanthopsis*, whereas mobile lips, a column foot, ventral anther and pollinarium with caudicles are observed in all other genera investigated.

2.4 Discussion

2.4.1 Phylogenetics of the *Lepanthes* clade

In this section, we discuss the nomenclatural changes needed to redefine the *Lepanthes* clade as proposed by Bogarín (Bogarín et al., 2018) as well as the relationships among these genera based on the phylogenetic insights and morphological evolution of key characters as presented in this study. Basically, the *Lepanthes* clade comprises four main clades: *Zootrophion*, *Anathallis*, *Trichosalpinx* and *Lepanthes*. *Zootrophion* is, with the inclusion of *Epibator*, confirmed as one of the early diverging clades. The next early diverging clade is *Anathallis*, which is here related to *Tubella* (see further discussion on *Trichosalpinx* s.l). *Anathallis* was initially re-established for the species of *Pleurothallis* subgenus *Acuminatia* sect. *Alata* and *Pleurothallis* subgenus *Specklinia* sect. *Muscosae* (Luer, 1999). *Anathallis* is confirmed monophyletic with the inclusion of *Panmorphia* Luer, and the exclusion of members of *Pleurothallis* subgenus *Acuminatia* sect. *Acuminatae*, that belong to *Stelis* s.l. (Karremans et al., 2013). In addition, Karremans (2014) established the genus *Lankesteriana* because its members were closely related to *Pendusalpinx* rather than to *Anathalllis* s.s. as suggested by Pridgeon et al. (2001). *Trichosalpinx* as previously

circumscribed (Luer, 1997; Pridgeon et al., 2005) is confirmed as polyphyletic, and therefore recircumscribed. The species belonging to *Pendusalpinx* and *Tubella* are confirmed to be unrelated to *Trichosalpinx* and therefore excluded, while the genera *Gravendeelia*, *Opilionanthe*, *Pseudolepanthes* and *Stellamaris* are placed for the first time in a phylogenetic framework and recognized as distinct (Bogarín et al., 2018). The polyphyly of *Trichosalpinx* was suggested in previous studies but the former genera were not evaluated or the sampling was too incomplete to allow a redefinition of these groups (Karremans, 2016). The relationships recovered here also suggest that only *Pendusalpinx* and *Lankesteriana* are closely related to *Trichosalpinx* s.s. As suggested by Pridgeon et al. (2001), members of *Tubella* are isolated from *Trichosalpinx* and *Pendusalpinx* (*P. berlineri*) but the relationships of this genus were not clearly established due to low support (Pérez-Escobar et al., 2017a) recovered *Tubella* as sister to *Lankesteriana*, *Pendusalpinx* and *Trichosalpinx* s.s. However, here with the inclusion of members of the clade not previously evaluated (*Gravendeelia, Opilionanthe, Pseudolepanthes* and *Stellamaris*), this relationship changed and *Tubella* is now recovered as sister to *Anathallis*.

The most recently diverging clade of the *Lepanthes* clade consists of *Lepanthes* and its allies: the genera *Draconanthes, Gravendeelia*, *Lepanthopsis, Opilionanthe*, *Pseudolepanthes* and *Stellamaris*. With the exception of *Draconanthes* and *Lepanthopsis*, these genera were formerly all treated under *Trichosalpinx* s.l. However, we confirm here that they are closely related to *Lepanthes* and *Lepanthopsis* rather than to *Trichosalpinx* s.s. In addition, *Lepanthopsis* is found monophyletic with the inclusion of *Expedicula*. In the next sections we discuss the morphological characters supporting the new classification of the *Lepanthes* clade proposed here.

2.4.2 Morphological evolution

Our character reconstructions improved the understanding of the evolution of phenotypic traits used to classify the genera of the *Lepanthes* clade. We identified homoplastic characters, that are not suitable for generic circumscriptions, as well as synapomorphies that are useful to base classifications on (Table 2.3). Plant habit (caespitose or repent) evolved several times with a higher transition frequency from caespitose to repent. This was found for other groups within the Pleurothallidinae as well, possibly as an adaptation to different environments. Prolific ramicauls evolved from nonprolific ones independently in four clades. The lack of ornamentation of the ramicauls confused taxonomists as the close relationship of *Zootrophion*, *Anathallis* and *Lankesteriana* with *Lepanthes*, *Lepanthopsis* and *Trichosalpinx* s.l. was not recognized previously. In addition, a combination of plesiomorphic and homoplastic characters in *Trichosalpinx* s.l., such as the ornamentation of the ramicauls, concave dorsal sepals, ovate-acuminate, caudate petals, mobile, laminar lips with a column foot and ventral anthers caused misclassifications of the now separated genera *Gravendeelia, Pendusalpinx, Opilionanthe* and *Stellamaris*. Assessment of other potential diagnostic traits was needed for these genera in order to avoid a classification based on homoplastic characters. For example, the synapomorphic sub-similar petals in *Opilionanthe* are a diagnostic feature of the genus, showing a very low probability of transition back to the ancestral state, dissimilar petals.

Inflorescence type and length are also variable characters in the Pleurothallidinae (Luer, 1986a). Although groups show trends towards the presence of one of the states only, there are

Table 2.2. Marginal probability of the root state as estimated with ACE, SCM and BI.

Characters	ML(ACE)			SCM (SIMMAP)			BI (RevJump)		
	0	**1**	**2**	**0**	**1**	**2**	**0**	**1**	**2**
Habit: (0) caespitose; (1) repent	0.99	0.01	-	0.99	0.01	-	0.99	0.01	-
Ramicaul growth: (0) non-prolific; (1) prolific	1	0	-	1	0	-	1	0	-
Bracts of ramicauls: (0) unornamented; (1) ornamented; (2) foliaceous	0.78	0.21	0	0.82	0.18	0	0.73	0.19	0.08
Inflorescence: (0) simultaneously flowering; (1) successive	0.95	0.05	-	0.97	0.03	-	0.98	0.02	-
Inflorescence length: (0) shorter; (1) longer (than leaves)	0.43	0.57	-	0.46	0.54	-	0.07	0.93	-
Flower appearance: (0) fully opening; (1) bud-like	1	0	-	1	0	-	1	0	-
Dorsal sepal concavity: (0) concave; (1) flattened	1	0	-	1	0	-	1	0	-
Synsepal: (0) absent; (1) present	0.07	0.93	-	0.06	0.94	-	0.47	0.53	-
Sepals shape: (0) oblong-acute; (1) ovate-acuminate (2) ovate-acute	0.01	0.01	0.98	0.01	0.01	0.98	0.29	0.08	0.63
Petals: (0) dissimilar; (1) subsimilar	1	0	-	1	0	-	1	0	-
Lip shape: (0) laminar; (1) bilobed	1	0	-	1	0	-	1	0	-
Lip mobility: (0) mobile; (1) sessile	1	0	-	1	0	-	1	0	-
Glenion: (0) absent; (1) present	1	0	-	1	0	-	1	0.06	-
Appendix: (0) absent; (1) present	1	0	-	1	0	-	1	0	-
Column foot: (0) absent; (1) present	0	1	-	0	1	-	0	1	-
Stigma: (0) entire; (1) bilobed	1	0	-	1	0	-	1	0	-
Anther: (0) ventral; (1) dorsal	1	0	-	1	0	-	1	0	-
Pollinarium: (0) caudicles; (1) caudicles+viscidium	1	0	-	1	0	-	1	0	-

always exceptions to the rule. For example, all the species of *Lepanthes* studied here have inflorescences shorter than the leaves but some species (not studied here) have inflorescences longer than the leaf. The opposite is observed in *Trichosalpinx* (Luer, 1997). The ancestral traits recovered for the anther position, presence of a column foot, pollinarium type, and lip mobility suggest that these are associated with the pollination mechanism. In general, flowers with a column foot, movable lips, a dorsal anther and a pollinarium without viscidium are pollinated by insects that enter the flower using the laminar lip. When trying to move in reverse to depart from the flower, the dorsal part of the insect scrapes the dorsal anther off the column in the area of the caudicles and removes the pollinarium (Bogarín et al., 2018a; Borba et al., 2002; Karremans et al., 2015b; Pansarin et al., 2016). This mechanism predominates in *Zootrophion*, *Tubella*, *Anathallis*, *Trichosalpinx*, *Lankesteriana*, *Pendusalpinx*, *Opilionanthe*, *Gravendeelia*, *Frondaria* and

Table 2.3. Cladistic classification of the 18 morphological characters assessed. Plesiomorphic characters detected with marginal probability at the root state (Table 2) *=ambiguous character at the root state. Synapomorphic and homoplastic characters based on SCM calculations.

Characters	Plesiomorphy	Synapomorphy	Homoplasy
Habit	caespitose	-	repent
Ramicauls	non-prolific	-	prolific
Ramicauls' bracts	unornamented	foliaceous (*Frondaria*)	ornamented
Inflorescence	simultaneous	-	successively flowering
Inflorescence length	*	-	shorter/longer than leaves
Flower appearance	fully opening	bud-like (*Zootrophion*)	-
Dorsal sepal concavity	concave	-	flattened
Synsepal	*	-	absent/present
Sepal shape	ovate-acute	-	oblong-acute/ovate-acuminate
Petals shape	dissimilar	subsimilar (*Opilionanthe*)	-
Lip shape	laminar	bilobed (*Lepanthes*)	-
Lip mobility	mobile	-	sessile
Glenion of the lip	absent	present (*Lepanthopsis*)	-
Appendix of the lip	absent	present (*Lepanthes*)	-
Column foot	present	-	absent
Stigma shape	entire	bilobed (*Lepanthopsis*)	-
Anther position	ventral	-	dorsal
Pollinarium	with caudicles	-	caudicles+viscidium

Stellamaris. The recent discovery of biting midges of the genus *Forcipomyia* (Ceratopogonidae) as pollinators of two species of *Trichosalpinx* highlights the importance of the mobile, papillose, ciliate lip for the pollination of this group (Bogarín et al., 2018a). Additional micromorphological observations of the flowers of these three genera, such as the papillose surface of the lip with striated cuticles and secretions of proteins as possible rewards support a hypothesis of floral convergence (Bogarín et al., 2018a). The flowers of some species of *Anathallis, Tubella* and *Opilionanthe* are similar to other pleurothallids, such as the white flowered *Specklinia calyptrostele,* which is visited by biting midges of the genus *Atrichopogon* (Ceratopogonidae) (Karremans et al., 2016), suggesting that floral similarities are prone to homoplasy due to the adaptations to similar pollination mechanisms.

The predominance of an ancestral morphology adapted to pollination by biting midges makes these characters unsuitable for generic classification. The combination of a sessile lip, absence of a column foot, dorsal anther and pollinarium with caudicles and viscidium is only observed in *Lepanthes*, *Draconanthes*, *Pseudolepanthes* and *Lepanthopsis*, whereas mobile lips, a column foot, ventral anther and pollinarium with caudicles are observed in all other genera (Luer, 1996b; Luer, 1997). Synapomorphic characters of *Lepanthes,* such as an appendix, in combination with a viscidium and a sessile lip are key features for a pollination system by sexual deception (Blanco and Barboza, 2005). Even though pollination observations are documented only for a handful of species of this genus, the floral synapomorphies indicate that a pseudocopulation strategy is

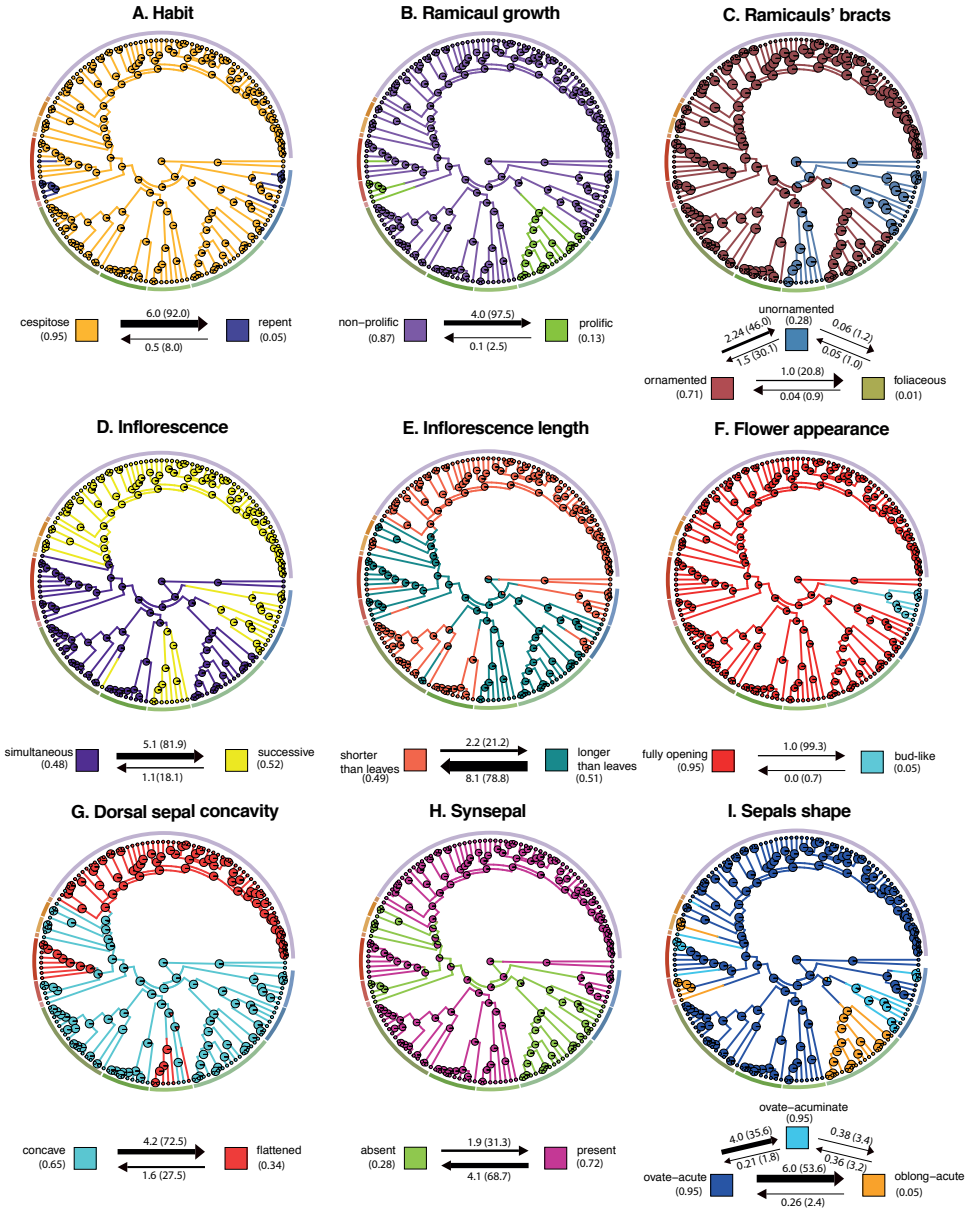

Figure 2.5. Ancestral state reconstructions of selected morphological characters from stochastic mapping analyses based on joint sampling (10,000 mapped trees). Arrows represent transitions between states and numbers represent the estimated number of evolutionary changes with proportion in parenthesis and the time spent in each state. Posterior probabilities (pie charts) are mapped in a random stochastic character map. External subdivided ring represents the 14 recognized genera. **A.** Habit. **B.** Ramicauls. **C.** Bracts of ramicauls. **D.** Inflorescence. **E.** Inflorescence length. **F.** Flowers. **G.** Dorsal sepal concavity. **H.** Synsepal. **I.** Sepal shape.

likely to be predominant in the group. *Lepanthes*-like flowers are also found in species of the former *Lepanthes* subgenus *Brachycladium* Luer, today known to belong to the unrelated genus *Andinia* (Wilson et al., 2017). The floral convergence is probably a result of pollinator selective pressure as suggested by Wilson et al. (2017) on the basis of pollination observations by Álvarez (2011). In *Lepanthopsis*, autapomorphic characters such as a glenion and bilobed stigma suggest an adaptation to different, yet unknown pollinators as compared to *Lepanthes* and *Trichosalpinx* (Blanco and Barboza, 2005; Bogarín et al., 2018a). *Lepanthopsis* and *Gravendeelia* are grouped in the same clade and the need for recognition of *Gravendeelia* is supported by autapomorphic characters of *Lepanthopsis* such as the presence of a glenion and bilobed stigma. As transitions of these characters to the ancestral state are unlikely, it seems that floral evolution in *Lepanthopsis* and *Gravendeelia* took a different path. Floral morphology of *Lepanthopsis* resembles that of *Platystele* Schltr. and the autapomorphic characters such as the presence of a glenion and bilobed stigma suggest an adaptation to different, yet unknown pollinators. In contrast, *Gravendeelia* has a floral morphology oriented towards a pollination system that likely involves the forward and reverse behavior of insects entering and leaving flowers as in *Trichosalpinx* s.s (Bogarín et al., 2018a, Chapter 6).

Ambiguous results obtained for inflorescence length and the formation of a synsepal at the root state, as well as the higher frequency of transitions between different states indicates that these traits evolved independently in several groups within the Pleurothallidinae (Pridgeon, 2005). The synsepal is made up of fused lateral sepals and this condition can be either absent or intermediate, varying between partial to complete fusion. A possible correlation between sexual mimicry and successive flowering in *Lepanthes* suggests that all flowers opening at the same time might not be an optimal strategy to fool male fungus gnats (Sciaridae), because the presence of several female-mimicking flowers together may accelerate alerting males from being tricked (Anderson and Johnson, 2006; Anderson et al., 2017). In contrast, the meagre rewards for female biting midges in *Trichosalpinx* flowers suggest that several flowers opening at the same time might be more advantageous for attracting pollinators (Bogarín et al., 2018a).

2.4.3 Circumscription of the genera in the *Lepanthes* clade

Lepanthes: it has been consistently supported as a monophyletic group by previous studies (Bogarín et al., 2018c; Pérez-Escobar et al., 2017a; Pridgeon et al., 2001). Species of the genus are known for their caespitose habit with lepanthiform sheaths of the ramicaul. Amongst its close relatives, the transversely bilobed petals, the bilobed lip with a basal appendix, the elongated column with apical anther, and the pollinarium with a viscidium are diagnostic for the genus. Several earlier proposed subgeneric divisions of *Lepanthes* (Luer, 1996a) were not supported by our molecular phylogenetic analyses and will require re-evaluation when a broader sampling becomes available.

Draconanthes: based on the former *Lepanthes* subgenus *Draconanthes* (Luer, 1996a), is currently made up of two species known only from high elevations in the Andes. It forms a clade that is sister to *Lepanthes* in the strict sense. *Draconanthes* and *Lepanthes* are morphologically similar but the former may be distinguished by the rigid sepals, linear elongated, unlobed petals and a fleshy lip with a rather rudimentary appendix-like structure in contrast with the elaborate appendixes of *Lepanthes*.

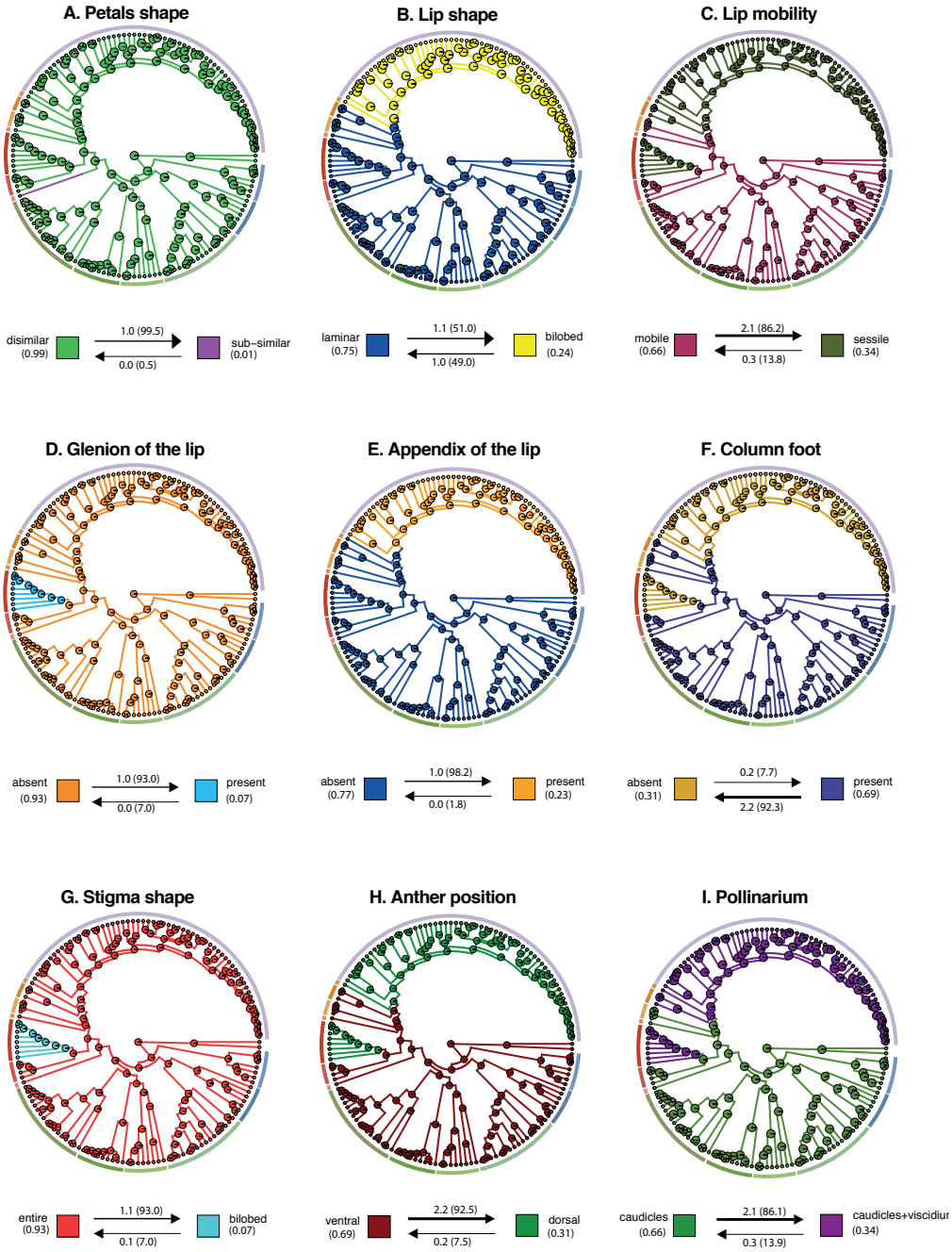

Figure 2.6. Ancestral state reconstructions of selected morphological characters: **A.** Petals shape. **B.** Lip shape. **C.** Lip mobility. **D.** Glenion of the lip. **E.** Appendix of the lip. **F.** Column foot. **G.** Stigma shape. **H.** Anther position. **I.** Pollinarium.

Pseudolepanthes: the group is sister to a clade that includes *Lepanthes* and *Draconanthes*, rather than being related to *Trichosalpinx* as previously assumed. *Pseudolepanthes* resembles species of the latter genus in plant architecture, however, its species are immediately set aside by the spreading, linear to narrowly ovate petals, and the laminar, appendix-free lip with a prominent warty callus, which suggest a different pollination strategy as compared to pseudocopulation recorded in *Lepanthes* (Luer, 1997).

Stellamaris: currently includes a single species, *Stellamaris pergrata*, previously believed to belong to *Trichosalpinx.* It is sister to a clade including *Lepanthes, Draconanthes* and *Pseudolepanthes* instead. With the latter it shares the caespitose, non-prolific habit, but it can be distinguished by a very short, few-flowered inflorescence, long-caudate sepals, a callose lip, an elongate column with an incumbent anther and a prominent column foot, and pollinia lacking a viscidium (Bogarín et al., 2018c; Luer, 1997).

Frondaria: it can be distinguished by the synapomorphic conspicuous foliaceous sheaths along the stems. Contrary to the terminal leaf, the smaller leafy bracts do not have an abscission layer which is consistent with them being overgrown, green bracts rather than true leaves. *Frondaria* produces elongate inflorescences with simultaneously opening, white flowers with spreading, acuminate sepals that are virtually indistinguishable from those of the unrelated genera *Anathallis* and *Tubella*.

Lepanthopsis: it forms a clade, together with *Gravendeelia*, that is sister to *Lepanthes*, *Draconanthes*, *Pseudolepanthes*, *Stellamaris* and *Frondaria*. Species of the genus are recognized by the inflorescences with simultaneously opening, flattened flowers, provided with a fleshy, simple lip with a glenion at the base and a short column with a bilobed stigma (Luer, 1991). A few exceptions to this scheme are found in *Lepanthopsis* subgen. *Microlepanthes* Luer (Luer, 1991).

Gravendeelia: it is a monotypic genus sister to *Lepanthopsis*. *Gravendeelia chamaelepanthes* (Rchb.f.) Bogarín & Karremans, the only species currently recognized in the genus, undoubtedly represents a species complex in need of further revision. It is morphologically different from *Lepanthopsis* by the long-prolific, pendent habit, the few-flowered inflorescences with tubular flowers, with elongated sepals, an elongate lip without a glenion and the elongate column with a distinct foot and unlobed stigma (Bogarín et al., 2018c; Luer, 1997). Both plants and flowers of *Gravendeelia* are so different from *Lepanthopsis* that their close phylogenetic clade is one of the most unexpected results of this study. The flowers resemble those of the unrelated genera *Anathallis*, *Stellamaris* and *Tubella*.

Opilionanthe: it was formerly placed in *Trichosalpinx* it is sister to a clade that includes *Lepanthes*, *Draconanthes*, *Pseudolepanthes*, *Stellamaris, Frondaria, Lepanthopsis and Gravendeelia*. The lepanthiform bracts, caespitose habit and more or less tubular white flowers are reminiscent of *Tubella*, thus the isolated phylogenetic placement of this species was unexpected. However, *O. manningii* (Luer) Karremans & Bogarín is immediately distinguished from species belonging to other genera by the sub-orbicular leaves and the long-caudate petals, which are subsimilar to the sepals (Bogarín et al., 2018c).

Lankesteriana, Pendusalpinx and Trichosalpinx: the three genera are florally similar as they share purplish flowers with a mobile, ciliate lip, attached to a column foot, and an ventral anther and stigma (Bogarín et al., 2018c; Karremans, 2014; Luer, 1997). The vegetative morphology, however, is quite distinct. Species of *Lankesteriana* can be easily distinguished from *Trichosalpinx* and *Pendusalpinx* by the extremely small habit with ramicauls that lack ornamented lepanthiform bracts that are shorter than the leaves and the successively flowering inflorescences (Karremans, 2014). *Trichosalpinx* and *Pendusalpinx* are vegetatively similar to each other, with a large habit with long ramicauls and simultaneously flowered inflorescences. *Pendusalpinx* differs in having a pendent habit with large, whitish lepanthiform bracts and glaucous leaves (Bogarín et al., 2018c). Based on vegetative morphology alone it is rather unexpected that *Lankesteriana* and *Pendusalpinx* are sister to each other. However, these findings are congruent with those of previous studies (Chiron et al., 2012; Karremans, 2014). On the other hand, contrary to what was found by these authors, *Lankesteriana* and *Pendusalpinx* are here found to be sister to the genus *Trichosalpinx* as previously supported by Pérez-Escobar et al. (2017a). Due to the contradictory inferences, the relatively long branches of the *Lankesteriana* accessions, and the highly diverging morphologies, we remain cautious as to the true phylogenetic relationships between these three genera. It is possible that the similar floral morphology was caused by convergent evolution due to a similar pollination strategy rather than a shared evolutionary history (Bogarín et al., 2018c).

Anathallis and Tubella: they are related but with moderate to low support in the BI and ML analyses, therefore, their more detailed relationship remains unresolved. Both taxa received high support as separate genera though. *Anathallis* is distinguished by the non-lepanthiform sheaths, non-proliferating ramicauls, and the free, star-shaped perianth (Karremans, 2014; Luer, 2006). Some species have purple flowers with mobile lips whereas others share similar micromorphological characters with *Lankesteriana*, *Pendusalpinx* and *Trichosalpinx* s.s. such as the striated cuticles and secretion of proteins (Bogarín et al., 2018a). Members of *Pleurothallis* subgenus *Acuminatia* sect. *Acuminatia* are phylogenetically related to *Stelis* s.l. and should therefore not be considered as part of *Anathallis* (Karremans et al., 2013). Allied to *Lepanthes*, *Lepanthopsis*, *Trichosalpinx* and their allies (named here Clade 8) are members of *Tubella*, a group traditionally recognized as a subgenus of *Trichosalpinx* (Luer, 1997; Luer, 1983). It comprises mostly slender plants, with proliferating ramicauls, simultaneously flowering inflorescences with whitish flowers and elongated sepals.

Zootrophion: it was recovered sister to all other members of the *Lepanthes* clade. It can be distinguished by the partial opening of the flowers due to the apical fusion on the sepals. As a consequence, the flowers have a single opening on each side, giving them a unique appearance. This feature, present in all species of *Zootrophion*, is not present in the other members of the *Lepanthes* clade; however, it is present in other unrelated genera of the Pleurothallidinae. The synsepal is thick and verrucose, the lip is minute. The bracts are large, unornamented and loose.

2.5 Conclusions

Generic delimitations based on morphological characters are daunting because of overwhelming homoplasy of the characters traditionally used for circumscriptions. The *Lepanthes* clade challenged systematists and taxonomists for centuries due to the floral homoplasy untangled here which is possibly resulting from similar pollination systems. We provide evidence for recognizing 14 well supported genera as members of the clade based on a combination of molecular phylogenetics and a solid morphological assessment identifying both synapomorphies and homoplastic characters. Future research should focus on members of *Trichosalpinx* subgenus *Xenia* which are extremely rare but need to be phylogenetically evaluated in order to obtain a complete evolutionary scenario for the *Lepanthes* clade. Based on morphology, we suspect that some members might be related to *Lepanthopsis* and allies but this hypothesis needs further evaluation. In addition, it is desirable to increase sampling in other groups such as *Lepanthopsis* (mainly the Antillean species) and *Tubella* because of floral similarities. Our phylogenetic framework and methodological approach enables the discovery of useful traits for generic classifications, and paves the way for more comprehensive assessments on generic delimitations of similar recalcitrant lineages based on DNA sequences and morphological characters to further improve the systematics of the subtribe.

Chapter 3

Genus-level taxonomical changes in the *Lepanthes* affinity (Orchidaceae: Pleurothallidinae)

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Phytotaxa 340, 128–136. 2018.

Abstract. We propose a new classification of the *Lepanthes* affinity based on phylogenetic re-evaluation of the Pleurothallidinae. Fourteen genera are recognized as belonging to the affinity. They are found highly supported in a DNA-based phylogenetic inference of combined plastid (*mat*K) and nuclear (nrITS) datasets. The necessary changes, including four novel generic concepts, needed to reorganize the *Lepanthes* affinity, are proposed here to insure monophyly. The integral discussion on the phylogenetics and biogeography of the group, together with morphological characterization of each clade is presented in Chapter 2.

3.1 Introduction

With about 5,200 known species, Pleurothallidinae is currently the most species-rich subtribe in the Neotropics, and one of the richest in the Orchidaceae. After the first phylogenetic study of the subtribe by (Pridgeon et al., 2001), and the subsequent proposal to recircumscribe most of its genera (Pridgeon and Chase, 2001), numerous studies aimed to refine or redefine generic concepts in the different clades of Pleurothallidinae have been published. Among the nine greater clades within the subtribe, the *Lepanthes* Sw. affinity (Karremans, 2016) is one of the most species-rich, encompassing more than 1,400 species. The currently recognized genera that are members of this clade are *Anathallis* Barbosa Rodrigues [116], *Draconanthes* (Luer) Luer [2], *Frondaria* Luer [1], *Lankesteriana* Karremans [21], *Lepanthes* [1122], *Lepanthopsis* (Cogn.) Ames [45], *Trichosalpinx* Luer [124] and *Zootrophion* Luer [26] (Chase et al., 2015; Karremans, 2016). The polyphyletic nature of some of these genera, especially *Anathallis* and *Trichosalpinx*, was suggested by several independent DNA-based phylogenetic analyses and supported by morphological observations (Chiron et al., 2012; Karremans, 2014; Luer, 1997; Luer, 2006; Pérez-Escobar et al., 2017a; Pridgeon and Chase, 2001; Rykaczewski et al., 2017). Nevertheless, no integrate, corrective, classification system was proposed, most likely due to the difficulty of adequately inferring relatedness on the basis of morphology on its own, the availability of DNA data from far too few members of the affinity and the difficulties in sampling poorly known species of restricted distribution. We propose a new classification of the *Lepanthes* affinity based on our previous studies (Karremans, 2016, 2014; Pérez-Escobar et al., 2017a) and a phylogenetic re-evaluation of the Pleurothallidinae from a broad set of species belonging to the majority of the genera and subgenera proposed within the group (Bogarín et al. in review). To avoid dealing with nomenclatural issues in the cited study, the necessary changes needed to reorganize the *Lepanthes* affinity are proposed here to assure that its genera are monophyletic and reflect the nature of its relationships (Fig. 3.1). Within the *Lepanthes* affinity fourteen genera can be recognized with high support in maximum likelihood (ML) and Bayesian inference (BI) analyses (Fig. 3.1). This study supports the more generally accepted genera such as *Anathallis*, *Draconanthes*, *Frondaria*, *Lankesteriana*, *Lepanthes*, *Lepanthopsis*, *Trichosalpinx* and *Zootrophion*. Also, highly supported as distinct clades are the less widely accepted genera *Pseudolepanthes* (Luer) Archila and *Tubella* (Luer) Archila. In addition to these, four novel generic concepts are required. They are *Gravendeelia*, *Opilionanthe*, *Pendusalpinx* and *Stellamaris*. In order to attain monophyly, and insure the least nomenclatural instability within this affinity, the following changes were proposed (Bogarin et al., 2017c):

3.2 Taxonomical treatment

3.2.1 ***Anathallis*** Barb.Rodr., Gen. Sp. Orchid. 1: 23. 1877.

Type: *Anathallis fasciculata* Barb.Rodr., Gen. Sp. Orchid.1: 23–24. 1877.

Comments: *Anathallis* species are easily recognized by the non-lepanthiform sheaths of the ramicaul, and the starshaped flower, with free perianth parts. The linear to lanceolate, acute to acuminate petals are similar to the sepals in size and shape. The sensitive lip is perpendicularly

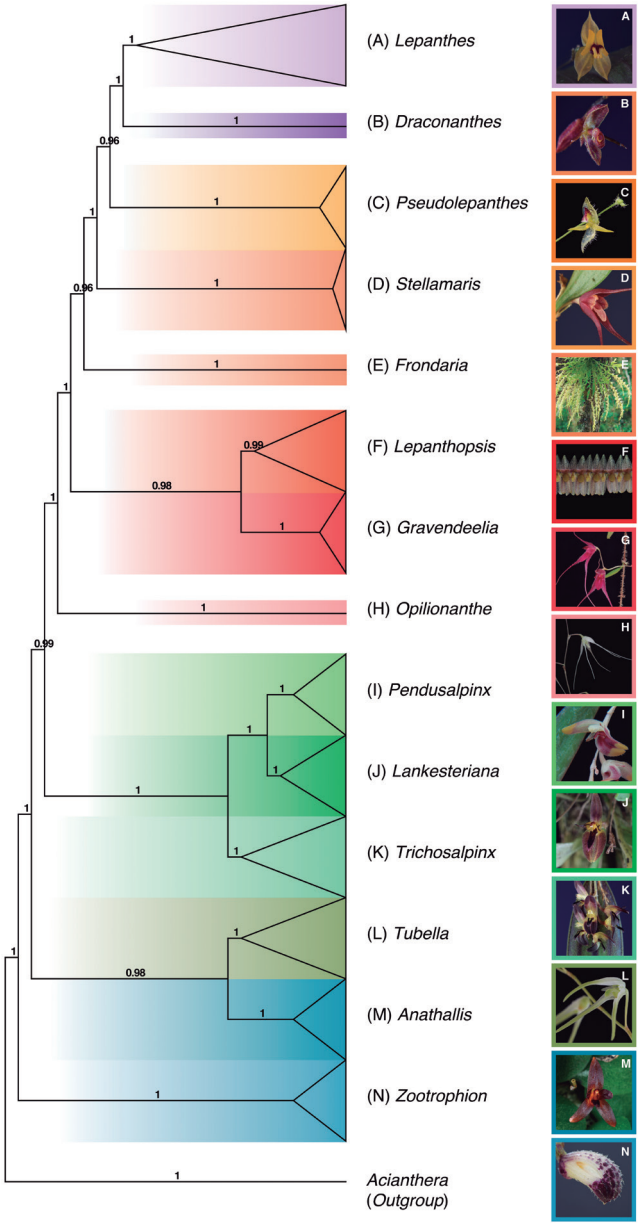

Figure 3.1. Phylogenetic analysis based on Bayesian inference of the *Lepanthes* affinity (nrITS and plastid matK sequences) from more than one-hundred different species. Terminals ending in triangles represent genera with multiple species and single terminals are monospecific genera (*Draconanthes*, *Frondaria*, *Gravendeelia*, *Opilionanthe* and *Stellamaris*). Photographs: A, B, D, F, J–K, L–N by D. Bogarín; C by S. Vieira-Uribe; E by Ecuagenera; G, H, I by W. Driessen.

hinged to the column foot, and its general shape is linear-ligulate but frequently it has small lobes at the base and/or middle. The column is sharply winged and prominently fimbriate. The pollinaria come in pairs and have reduced flat caudicles. There are currently 118 accepted species of *Anathallis*, including the one added hereafter. They are distributed from western Mexico through Central America, the Antilles and down to Argentina. They are most diverse in Brazil at low to mid elevations. *Anathallis*, as defined by Karremans (2014), is highly supported in our analyses and is modified only by the inclusion of the following species:

Anathallis convallium (Kraenzl.) Karremans & Mel.Fernández, Phytotaxa 340(2): 130. 2018. Basionym: *Pleurothallis convallium* Kränzlin, Ark. Bot.16(8): 12. 1921.

This name has been placed under *A. linearifolia* (Cogn.) Pridgeon & Chase, a species from which it differs significantly.

3.2.2 *Gravendeelia* Bogarín & Karremans, Phytotaxa 340(2): 130. 2018.

Type: *Pleurothallis chamaelepanthes* Rchb.f., Bonplandia 3: 240. 1855.
Diagnosis: *Gravendeelia* is most closely related to *Lepanthopsis*. It can be easily distinguished from that genus by the long-prolific, pendent habit (vs. caespitose, rarely prolific, erect), the few-flowered inflorescence (vs. generally multi-flowered), the cupped flower with extremely long sepals (flowers flat, sepals and petals similar), the elongate lip with two central keels (vs. lip compact, with a basal glenion), the elongate column with a distinct foot (vs. column short, stout, footless), the incumbent anther and ventral, entire stigma (vs. apical anther and bilobed stigma). Morphologically, *Gravendeelia* is reminiscent of *Tubella*, however it can be distinguished by the pendulous plants, the hirsute ovary (vs. glabrous), the hirsute sepals (vs. glabrous), and the short column foot (vs. prominent).
Comments: The only species currently known to belong to this genus is relatively common in Colombia and Ecuador, and is likely to represent a species complex in need of revision (the name bears two heterotypic synonyms at this time). The recognition of the novel genus *Gravendeelia* is highly supported in our analyses, the accessions of its only species formed a highly supported clade (Fig. 3.1) (PP=1.0), sister to *Lepanthopsis* (Fig. 3.1; P.P.: 0.98), and not closely related to any of the other species previously placed in *Trichosalpinx*. Treating *Gravendeelia* as part of a broadly defined *Lepanthopsis* is undesirable as it would result in an undiagnosable genus, whilst when kept separate they are easily recognizable.
Eponymy: The name honors orchid evolutionary biologist Dr. Barbara Gravendeel, Leiden University and Naturalis Biodiversity Center, The Netherlands, who has continuously supported these phylogenetic studies in the Pleurothallidinae.

Gravendeelia chamaelepanthes (Rchb.f.) Bogarín & Karremans, Phytotaxa 340(2): 130. 2018. Bas. *Pleurothallis chamaelepanthes* Rchb.f., Bonplandia 3: 240. 1855.

3.2.3 *Stellamaris* Mel.Fernández & Bogarín, Phytotaxa 340(2): 131. 2018.

Type: *Pleurothallis pergrata* Ames, Schedul. Orch. 4: 24–25. 1923.
Diagnosis: *Stellamaris* is phylogenetically allied to *Draconanthes*, *Lepanthes* and *Pseudolep-*

anthes. From *Pseudolepanthes* it can be easily distinguished by the very short, few-flowered inflorescence (vs. elongate, multiflowered inflorescence), the long-caudate sepals (vs. shortly acuminate, similar to the petals), the ecallose lip (vs. lip with a prominent verrucose callus), the elongate column, with a prominent column foot (vs. column short, reflexed, footless), and the pollinia with a pair of flattened caudicles, lacking a viscidium (vs. pollinia with obsolete caudicles, with viscidium). From *Lepanthes*, *Stellamaris* can be recognized by the laminated petals (vs. transversally bilobed), the un-lobed lip (vs. lip bilobed, with a basal appendix), the incumbent anther and ventral stigma (vs. anther and stigma apical), and the pollinia without visicidum (vs. pollinia with a viscidium). From *Draconanthes*, *Stellamaris* can be distinguished by the very short, few-flowered inflorescence (vs. elongate, multi-flowered inflorescence), the laminate, un-lobed, elongate lip (vs. bilobed, with a rudimentary appendix, embracing the column). *Stellamaris* is florally most similar to the unrelated genus *Tubella*, however, it can be immediately set aside by the non-prolific habit, the hirsute lepanthiform sheaths, the inflorescence shorter than the leaf bearing one or two flowers, and an extremely reduced pedicel.
Comments: The only species currently known to belong to this genus is variable across its distribution, from Costa Rica to Colombia, and is likely to represent more than a single species. The recognition of the novel genus *Stellamaris* is highly supported in our analyses, the accessions of its only species formed a highly supported clade (Fig. 3.1) (PP=1.0), sister to a clade including *Lepanthes*, *Draconanthes* and *Pseudolepanthes* (Fig. 3.1) (PP=1.0), which are all morphologically distinct. Even though *Stellamaris*, *Gravendeelia* and *Tubella* show superficially similar flowers, they are not closely related phylogenetically.
Etymology: Derived from the Latin *Stellamaris* "starfish", in allusion to the red or crimson starfish-like flowers with long-tailed sepals.

Stellamaris pergrata (Ames) Mel.Fernández & Bogarín, Phytotaxa 340(2): 131. 2018. Bas. *Pleurothallis pergrata* Ames, Schedul. Orch. 4: 24–25. 1923.

3.2.4 *Opilionanthe* Karremans & Bogarín, Phytotaxa 340(2): 131. 2018.

Type: *Trichosalpinx manningii* Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 88: 113, f. 28. 2002.
Diagnosis: *Opilionanthe* has apparently no close relatives, it is phylogenetically sister to a clade which includes *Lepanthes*, *Lepanthopsis* and all of their allies. The cupped flower with long-caudate sepals is somewhat reminiscent of species of *Gravendeelia*, *Stellamaris* and *Tubella*, however, it can be immediately distinguished from those by the long-caudate petals which are similar to the sepals (vs. acute to obtuse, conspicuously shorter than the sepals). From the first two genera it may also be distinguished by the long, multi-flowered inflorescence (vs. short, few-flowered). In species of *Anathallis*, the sepals and petals are frequently similar to each other, however, *Opilionanthe* can be distinguished from species of that genus by the lepanthiform-bracts and prolific habit.
Comments: The recognition of *Opilionanthe* is highly supported in our analyses, the accessions of its only species formed a highly supported clade (Fig. 3.1) (PP=1.0), sister to a clade that includes *Draconanthes*, *Frondaria*, *Gravendeelia*, *Lepanthes*, *Lepanthopsis*, *Pseudolepanthes* and *Stellamaris* (Fig. 3.1) (PP=1.0). The single species known to belong this genus is endemic to Peru.

Etymology: From Opiliones, an order of arachnids known as harvestmen, harvesters or daddy longlegs, and the Greek anthos, "flower", in allusion to the long, slender acuminate petals and sepals reminiscent to the long-legged opiliones, distinctive of this genus among its relatives.

Opilionanthe manningii (Luer) Karremans & Bogarín, Phytotaxa 340(2): 131. 2018. Basionym: *Trichosalpinx manningii* Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 88: 113, f. 28. 2002.

3.2.5 *Pendusalpinx* Karremans & Mel.Fernández, Phytotaxa 340(2): 131–132. 2018.

Type: *Pleurothallis berlineri* Luer, Selbyana 3(1–2): 60. 1976. Synonym: *Trichosalpinx berlineri* (Luer) Luer, Phytologia 54(5): 394. 1983.
Diagnosis: *Pendusalpinx* is sister to genus *Lankesteriana*, but can be immediately distinguished by the large, up to 30 cm tall, pendulous plants (vs. short, less than 3 cm tall, erect), with ramicauls longer than or similar to the leaf (vs. much shorter than the leaf), covered by large, lepanthiform bracts (bract inconspicuous, not lepanthiform), the glaucous leaves twisted at the base (vs. green, straight), the pendent inflorescence, shorter than the leaf, with several flowers open at once (vs. erect to arching, longer than the leaf, with one flower open at a time), the petals are triangular to elliptic (vs. generally lanceolate), and the lip flat (vs. with a deep mid-line depression). Species of *Pendusalpinx* are superficially more similar to *Trichosalpinx*, but can be distinguished by the pendulous plants, the ramicauls covered by conspicuous, whitish bracts (vs. smaller, brown bracts), the glaucous leaves, pendent, basally twisted (vs. green, erect, straight) leaves, and a pair of broad angled wings above the middle of the column (vs. without broad angled wings above the middle).
Comments: The genus includes six species that are distributed from Colombia and Venezuela to Bolivia and Peru. They are not present in Central America, the Antilles and Brazil. The recognition of the novel genus *Pendusalpinx* is highly supported in our analyses, the accessions of several of its species consistently formed a highly supported clade (Fig. 3.1) (PP=1.0), sister to *Lankesteriana* (Fig. 3.1) (PP=1.0), as was previously found by Karremans (2014) and Pérez-Escobar *et al.* (2017). The two genera are highly supported, genetically well separated and morphologically distinct in virtually every aspect. *Pendusalpinx* species share several features with *Trichosalpinx*, nevertheless, they are consistently found sister to *Lankesteriana* instead. The two genera are here highly supported as sisters of *Trichosalpinx* in the strict sense, nevertheless, such a relationship has not been found in previous DNA based studies, and in the interest of stability and definability they are recognized as distinct.
Etymology: Derived from the Latin *pendulous* "pendent" and *salpinx* "funnel-shaped" (taken from *Trichosalpinx*); a pendent *Trichosalpinx*.

Pendusalpinx berlineri (Luer) Karremans & Mel.Fernández, Phytotaxa 340(2): 132. 2018. Basionym: *Pleurothallis berlineri* Luer, Phytologia 54(5): 394. 1983.

Pendusalpinx dependens (Luer) Karremans & Mel.Fernández, Phytotaxa 340(2): 132. 2018. Basionym: *Pleurothallis dependens* Luer, Selbyana 3(1–2): 94, f. 150. 1976.

Pendusalpinx echinata (Luer & Hirtz) Karremans & Mel.Fernández, Phytotaxa 340(2): 132. 2018. Basionym: *Trichosalpinx echinata* Luer & Hirtz in Luer, Selbyana 30: 24, f. 47. 2009.

Pendusalpinx glabra (D.E.Bennett & Christenson) Karremans & Mel.Fernández, Phytotaxa 340(2): 132. 2018. Basionym: *Trichosalpinx glabra* Bennett & Christenson, Brittonia 46(3): 256, 258–259, f. 18. 1994.

Pendusalpinx patula (Luer) Karremans & Mel.Fernández, Phytotaxa 340(2): 132. 2018. Basionym: *Trichosalpinx patula* Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 65: 82. 1998.

Pendusalpinx sijmii (Luer) Karremans & Mel.Fernández, Phytotaxa 340(2): 132. 2018. Basionym: *Trichosalpinx sijmii* Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 8: 113–114, f. 29. 2002.

Pendusalpinx vasquezii (Luer) Karremans & Mel.Fernández, Phytotaxa 340(2): 132. 2018. Basionym: *Trichosalpinx vasquezii* Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 64: 35, f. 24. 1997.

3.2.6 ***Pseudolepanthes*** (Luer) Archila, Revista Guatemal. 3(1): 76. 2000.

Basionym: *Trichosalpinx* subgen. *Pseudolepanthes* (Luer) Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 64: 5. 1997.
Type: *Trichosalpinx pseudolepanthes* Luer & Escobar, Orquideología 16(2): 183. 1984.
Comments: This genus has not received wide recognition as distinct among authors (Pridgeon, 2005). However, our initial phylogenetic sampling supports this group as sister to *Draconanthes* and *Lepanthes* and not particularly closely related to *Trichosalpinx* (Luer, 1997). From those genera, it is distinguished by the presence of a large, verrucose callus on the disc of the lip. It is distinguished from *Lepanthes* by the absence of a basal appendix and the unlobed petals (vs. transversally bilobed), the lip is not bilobed with the lobes embracing the column as in *Draconanthes* and most of the *Lepanthes* species. From *Trichosalpinx* it differs in the progressively elongated, successively flowered inflorescences longer than the leaves (vs. several flowered inflorescences, shorter or as long as the leaves) and the short, footless column (vs. elongated, footed).
Etymology: Derived from the Latin pseudo "false" and *Lepanthes*, a "false *Lepanthes*" referring to the morphological similarities with the genus *Lepanthes*.

3.2.7 ***Tubella*** (Luer) Archila, Revista Guatemal. 3(1): 46. 2000.

Basionym: *Trichosalpinx* subgen. *Tubella* Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 15: 66. 1986. Type: *Pleurothallis acremona* Luer, Selbyana 5(2): 157. 1979. Synonym.: *Trichosalpinx* subgen. *Tubella* sect. *Tubellae* Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 15: 68. 1986.
Type: *Pleurothallis acremona* Luer, Selbyana 5(2): 157. 1979. Synonym.: *Pleurothallis* sect. *Acuminatae* subsect. *Lepanthiformes* Lindley, Fol. Orchid. Pleurothallis 32. 1859, *nom. illeg.* Type. *Pleurothallis arbuscula* Lindley, Edwards's Bot. Reg. 28: Misc. 72–73. 1842.
Comments: Species of *Tubella* have a slender habit, commonly with proliferating ramicauls covered by lepanthiform sheaths, the inflorescence is longer than the leaf, the ovary is glabrous, the sepals membranaceous, glabrous, shortly acuminate, concave, the petals much shorter, entire, elliptic, the lip simple, commonly three-lobed, the base unguiculate, lacking lobules, the column elongated, apically winged, with a prominent column foot (Fernández, 2014). Species of *Tubella* are phylogenetically related to *Anathallis* from which they are separated by the slender habit, proliferating ramicauls with lepanthiform sheaths (vs. creeping or caespitose without proliferat-

ing ramicauls, and lacking the lepanthiform sheaths), and inflorescences longer than the leaves bearing several flowers (vs. inflorescences frequently shorter than the leaf and few-flowered). The flowers of *Tubella* are superficially similar to *Gravendeelia*, *Stellamaris* and *Opilionanthe* in the cupped flower with long caudate sepals and elongate column, however, they are not related phylogenetically. *Tubella* is redefined from its previous circumscription by the exclusion of the species belonging to *Gravendeelia*, *Opilionanthe*, and *Stellamaris*, which are not closely related, and by the inclusion of the following six species:

Tubella adnata (I.Jiménez) Mel.Fernández & Bogarín, Phytotaxa 340(2): 133. 2018. Basionym: *Trichosalpinx adnata* Jiménez, Lankesteriana 15(3): 194. 2015.

Tubella carmeniae (Luer) Mel.Fernández & Bogarín, Phytotaxa 340(2): 133. 2018. Basionym: *Trichosalpinx carmeniae* Luer, Harvard Pap. Bot. 17: 366, f. 42. 2012.

Tubella gabi-villegasiae (I.Jiménez) Mel.Fernández & Bogarín, Phytotaxa 340(2): 133. 2018. Basionym: *Trichosalpinx gabi-villegasiae* I.Jiménez, Lankesteriana 15(3): 196. 2015.

Tubella giovi-mendietae (I.Jiménez) Mel.Fernández & Bogarín, Phytotaxa 340(2): 133. Basionym: *Trichosalpinx giovi-mendietae* I.Jiménez, Lankesteriana 15(3): 199. 2015.

Tubella reticulata (Thoerle & C.Soto) Mel.Fernández & Bogarín, Phytotaxa 340(2): ***133.*** Basionym: *Trichosalpinx reticulata* Thoerle & Soto, Lankesteriana 15(1): 95–96, f. 1A–F, 2. 2015.

Tubella werneri (Luer) Mel.Fernández & Bogarín, Phytotaxa 340(2): 133. Basionym: *Trichosalpinx werneri* Luer, Monogr. Syst. Bot. Missouri Bot. Gard. 88: 114, f. 30. 2002.

3.3 Additional Nomenclatural Changes

Platystele kayi (Thoerle & Cornejo) Bogarín & Karremans, Phytotaxa 340(2): 133. 2018. Basionym: *Lepanthopsis kayi* Thoerle & Cornejo Harvard Pap. Bot. 21: 247. 2016.

Comments: This recently described species was placed by the authors in *Lepanthopsis*. However, the ramicaul much shorter than the long petiolate leaf (vs. ramicaul normally longer than the non-petiolate leaf), bearing tubular sheaths (vs. sheaths lepanthiform), the lip that exceeds the length of sepals (vs. lip shorter than sepals), and the obsolete, truncate rostellum (vs. rostellum conspicuously triangular) are indicative of *Platystele* Schltr., not *Lepanthopsis*.

3.4 Conclusions

The polyphyletic nature of the genera *Anathallis* and *Trichosalpinx* has been previously recognized by several authors (Chiron et al., 2012; Karremans, 2014; Pridgeon et al., 2001). Nevertheless, no alternative classification proposal was published for the species belonging to the genera for lack of a clear overview of the relationships amongst their members, and other close relatives (Karremans, 2016; Pridgeon, 2005). Ongoing phylogenetic studies, including a broad set of species from this group, demonstrate the need of an integrate reclassification of the *Lepanthes* affinity (Bogarín et al., in review). Species previously assigned to genus *Trichosalpinx* (in the sense of

Luer (1997) and Pridgeon, (2005)) were found to belong to six unrelated clades, diversely allied to several other traditionally recognized genera. Species belonging to *Gravendeelia*, an ally of *Lepanthopsis*, *Tubella*, an ally of *Anathallis*, and *Stellamaris*, allied to *Lepanthes*, are not particularly closely related but have superficially similar flowers. In Pleurothallidinae, and orchids in general, similarity in floral morphology as a response to pollinator pressure is a well-known trend (Papadopulos et al., 2013b), and it is not farfetched to suspect that such is the case here as well. Each of these clades is recognized as a distinct genus, rather than including them in broader circumscriptions of their respective sister genera. The plant and floral morphology of the species belonging to these clades are so different from that of their respective sister genera, and so similar amongst each other, that it would leave the resulting broader genera completely undiagnosable. Species of *Lankesteriana*, previously believed to be related to some *Anathallis*, are confirmed instead sister to *Pendusalpinx* with high support, and both in turn sister of *Trichosalpinx* in the strict sense. However apparently closely related, these three clades are recognized as distinct genera here. Species of *Lankesteriana* have accumulated many genetic and morphological differences, as evidenced by their unusually long branch lengths. Their plant morphology is distinct from that of *Pendusalpinx* and *Trichosalpinx* in almost every aspect (Karremans, 2014). Joining these two genera would result in a morphologically undiagnosable genus, and would suppress the diverging evolutionary path of these groups. *Gravendeelia* and *Stellamaris* are here proposed as monotypic genera, which is unfavored by some authors. Nevertheless, both are typified by a common, broadly distributed species, which is highly variable along its distribution. It is likely that these in fact represent species complexes rather than a single species, thus being currently monotypic is not a strong argument for their inclusion in broader concepts of their sister genera, especially when morphological discrepancies are evident. *Opilionanthe*, also monotypic, is sister to several well recognized genera, and clearly represents a unique lineage within the group.

Chapter 4

Anchored Hybrid Enrichment generated nuclear, plastid and mitochondrial markers resolve the *Lepanthes horrida* (Orchidaceae: Pleurothallidinae) species complex

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Abstract. Phylogenetic relationships in species complexes and lineages derived from rapid diversifications are often challenging to resolve using morphology or standard DNA barcoding markers. The hyper-diverse genus *Lepanthes* from Neotropical cloud forest includes over 1200 species and many recent, explosive diversifications that have resulted in poorly supported nodes and morphological convergence across clades. Here, we assess the performance of 446 nuclear-plastid-mitochondrial markers derived from an anchored hybrid enrichment approach (AHE) coupled with coalescence and species network-based inferences to resolve phylogenetic relationships and improve species recognition in the *Lepanthes horrida* species group. In addition to using orchid-specific probes to increase enrichment efficiency, we improved gene tree resolution by extending standard angiosperm targets into adjacent exons. We found high topological discordance among individual gene trees, suggesting that hybridization/polyploidy may have promoted speciation in the lineage via formation of new hybrid taxa. In addition, we identified ten loci with the highest phylogenetic informativeness values from these genomes. Most previous phylogenetic sampling in the Pleurothallidinae relies on two regions (ITS and matK), therefore, the evaluation of other markers such as those shown here may be useful in future phylogenetic studies in the orchid family. Coalescent-based species tree estimation methods resolved the phylogenetic relationships of the *L. horrida* species group. The resolution of the phylogenetic estimations was improved with the inclusion of extended anchor targets. This approach produced longer loci with higher discriminative power. These analyses also disclosed two undescribed species, *L. amicitiae* and *L. genetoapophantica*, formally described here, which are also supported by morphology. Our study demonstrates the utility of combined genomic evidence to disentangle phylogenetic relationships at very shallow levels of the tree of life, and in clades showing convergent trait evolution. With a fully resolved phylogeny, is it possible to disentangle traits evolving in parallel or convergently across these orchid lineages such as flower color and size from diagnostic traits such as the shape and orientation of the lobes of the petals and lip.

4.1 Introduction

Identification and inventory of plant species' diversity remains an arduous task in tropical countries. Species identification in many plant groups is still largely based on phenotypic differences and proportionally very little of this has been supported from molecular evidence (Fujita et al., 2012; Granados Mendoza et al., 2013; Yang and Rannala, 2010). Although morphological differences are sometimes sufficient to separate species, precise circumscriptions of closely related species in species complexes with poor morphological differentiation or recently diversified lineages often require the support of additional sources of evidence, including molecular data. Molecular species delimitations are also important in recognizing potential unknown cryptic species or to assess taxon descriptions previously proposed on the basis of morphology only (Fujita et al., 2012; Lahaye et al., 2008).

Orchidaceae are a prime example of a highly diverse plant family with recent explosive diversifications leading to a server's worth of backlogged species to identify in the tropics (Givnish et al., 2015). In this species rich family, molecular information has been largely used to assess generic and supra-generic relationships but not as commonly for assessing species delimitations (Karremans et al., 2015a; Lahaye et al., 2008; Ramos-Castro et al., 2012). This is particularly true for hyperdiverse, young tropical lineages derived from rapid diversifications, for which standard DNA molecular markers are insufficient to resolve phylogenetic relationships at very shallow levels (Pérez-Escobar et al., 2016b). Molecular-based approaches of species delimitations could lead to false inferences when single or few-locus datasets are analyzed because of topological discordances among gene trees and species trees (Edwards, 2009). The main sources of phylogenetic incongruence in single locus datasets and resulting gene trees are caused by systematic or stochastic errors and by biological evolutionary processes such as hybridization, introgression, gene duplication, deep coalescence and branch length heterogeneity (Chan et al., 2017; Maddison, 1997; Mallo and Posada, 2016; Pérez-Escobar et al., 2016a).

To cope with phylogenetic incongruence and resolution, recent studies have focused on the assessment of large multi-locus datasets derived from multiple genomic compartments (i.e. nuclear, plastid and/ or mitochondrial) to achieve species delimitation (Brandley et al., 2015; Granados Mendoza et al., 2013; Hamilton et al., 2016; Peloso et al., 2016; Ruane et al., 2015). Xi, Liu, and Davis (2015) found that genes with low phylogenetic resolution produce unreliable gene trees affecting species tree estimations based on gene tree coalescent methods, a problem that can be solved by sampling more genes. Thereby, inferences based on large multi-locus datasets gathered from Next Generation Sequencing (NGS) or High-throughput-sequencing (HTS) techniques theoretically help improving the accuracy of species-tree based delimitations because of the higher amount of genomic data analyzed and the higher levels of sequence divergence obtained with respect to traditional approaches employing only a few markers (Jeffroy et al., 2006; Wagner et al., 2013). One of the NGS protocols for high-throughput phylogenomics which allows the capture of hundreds of orthologous markers is Anchored Hybrid Enrichment (AHE) (Buddenhagen et al., 2016; Fragoso-Martínez et al., 2016; Lemmon et al., 2012; Wanke et al., 2017). Multi-locus datasets derived from AHE of plants usually contain fragments from plastid, mitochondrial and nuclear genomes. These genomes might have linked but different evolutionary histories because their different modes of inheritance, hence the resulting phylogenetic re-

lationships might be incongruent (Jeffroy et al., 2006; Pérez-Escobar et al., 2016a). Increasing the number of analyzed genes does not guarantee *per se* the inference of an accurate species tree because the detection of topological discordance in species/ gene trees is pervasive in multi-locus based inferences (Jeffroy et al., 2006). However, these incongruences are informative because they provide clues on relevant biological phenomena for speciation such as hybridization and polyploidization (Maddison, 1997; Soltis and Soltis, 2016).

In addition to inconsistencies among multiple genome regions inherent to evolutionary processes, different species tree estimation methods might produce discordant results as well. Methods using concatenation of multi-locus datasets assume that the loci analyzed evolved in a similar way and thus phylogenies or species delimitations are inferred from standard concatenation of hundreds of anonymous markers (Lemmon and Lemmon, 2012; Mirarab and Warnow, 2015; Xi et al., 2015). However, the results produced by concatenation approaches can differ from coalescent species tree reconstructions because multi-species coalescent models recognize that gene trees exhibit different evolutionary histories and thus reduce the influence of incomplete lineage sorting (ILS) and gene duplication. Recently, Sukumaran and Knowles (2017) argued that multi-species coalescent models delimit genetic structure without making any statistical distinction between structure due to population-level processes or due to speciation. Therefore, it is possible that population structure might be misidentified as a putative species boundary. In conclusion, the authors suggested that hypotheses based on multispecies coalescent models require validation with other evidence such as morphological or ecological information (Pyron et al., 2016).

The performance of AHE multi-locus datasets in plants has been assessed in several groups, including Arecaceae, Fabaceae, Lamiaceae, Oxalidaceae, Pinaceae, Proteaceae, Sarraceniaceae and Zingiberales (Fragoso-Martínez et al., 2016; Heyduk et al., 2016; Mitchell et al., 2017; Wanke et al., 2017). However, no studies assessing species delimitations based on concatenation or coalescent species tree estimations from AHE datasets in the Orchidaceae have been published. In this study, we focus on a species complex of the highly diverse Neotropical orchid genus *Lepanthes* Sw. (Pleurothallidinae), which contains more than 1,200 species. Recent studies on the evolutionary diversification of Neotropical orchids revealed that *Lepanthes* is a relatively young group which diverged 5–10 Ma and that shows the highest net diversification rates across the Pleurothallidinae (Pérez-Escobar et al., 2017a). In *Lepanthes*, the percentage of endemism is high, especially in relatively young mountain ranges such as the Cordillera de Talamanca in southern Central America and other Andean regions.

The *Lepanthes horrida* group consists of five taxa endemic to Costa Rica and Panama along the Cordillera Volcánica Central and Cordillera de Talamanca: *L. chameleon* Ames, *L. horrida* Rchb.f., *L. maxonii* Schltr., *L. nymphalis* Luer, and *L. wendlandii* Rchb.f. Although previously known specimens belonging to the *Lepanthes horrida* group could be easily separated morphologically, a clear distinction between recent collections from populations in Cordillera de Talamanca could not be made morphologically nor in combination with conventional phylogenetic analysis of the traditional markers used in Pleurothallidinae, namely nrITS and *mat*K.

To investigate the utility of AHE in resolving species complexes in lineages with rapid diversifications we inferred the phylogenetic relationships of the species in the *Lepanthes horrida* group. We obtained 446 target orthologous loci, generated with AHE by specific probes designed

for Pleurothallidinae orchids across the recently published *Phalaenopsis equestris* (Schauer) Rchb.f. (Cai et al., 2014) and *Dendrobium catenatum* Lindl. (Zhang et al., 2016) genomes. The performance of loci recovered was evaluated with both concatenation and coalescent-based methods (including and excluding missing sequences) and with analyses of phylogenetic informativeness (Townsend, 2007). The loci were identified and classified in three separate groups: plastid, mitochondrial and nuclear; each dataset was evaluated for incongruences inherent to multi-locus based inferences (Jeffroy et al., 2006). This study assesses the evolutionary relationships of the species of the *Lepanthes horrida* group by answering the following questions: (i) can the application of NGS with concatenated and multi-species coalescent models disclose species relationships in recently diverged clades which were unsolved with Sanger sequencing generated nuclear (ITS) and plastid markers (*mat*K)? (ii) are the hypotheses of supermatrix and species coalescent delimitations consistent with morphological evidence? (iii) are there phylogenetic incongruences among inferences based on plastid, mitochondrial and nuclear multi-locus datasets and what are the possible sources of this discordance?.

4.2 Materials and Methods

4.2.1 Taxon sampling

Living plant specimens were collected in the field and cultivated at JBL between 2013 and 2017. We sampled the five taxa belonging to the *L. horrida* group including individuals that do not correspond morphologically to any of the known species. As outgroup, we selected *Gravendeelia chamaelepanthes* (Rchb.f.) Bogarín & Karremans, a closely related species to the genus *Lepanthes* according to the latest phylogenetic studies in the group (Bogarín et al., 2018c) in addition to *Lepanthes elata* Rchb.f., *Lepanthes gargantua* Rchb.f. and *Lepanthopsis prolifera* Garay (Table 1). Vouchers were preserved as herbarium/spirit specimens for future reference at CR, JBL and L.

4.2.2 DNA extraction

Total genomic DNA was extracted from about 100 mg of silica gel dried leaf/flower tissue. Each dried sample was frozen in liquid nitrogen and powdered in a Retsch MM 300 shaker for 5 min. We followed the 2×CTAB (Hexadecyltrimethylammonium bromide) protocol for isolating DNA (Doyle and Doyle, 1987). Resulting total DNA was treated with Ribonuclease A (RNase A, Qiagen) and quantified with a Qubit 3.0 Fluorometer (TermoFischer Scientific®) to ensure 2.0 µg of DNA per sample in 130 µl of buffer. All DNA samples (4 µL of sample DNA and 2 µL of 6× loading dye) were checked on a 2% agarose gel in 1× TAE (Tris-Acetate-EDTA) buffer running for 90 min at 120 V with a size ladder of 100 bp–1000 bp fragments.

4.2.3 Anchored phylogenomics locus selection and probe design

We aimed to collect data for the Angiosperm AHE target loci (Buddenhagen et al., 2016; Léveillé-Bourret et al., 2018; Wanke et al., 2017). Refinement of the target regions and corresponding probes for data collection in orchids was conducted at the Center for Anchored Phylogenomics (www.anchoredphylogeny.com). To improve enrichment efficiency in Orchidace-

ae, we leveraged the published genomes of two orchid species, *P. equestris* (Cai et al., 2015; NCBI Bioproject PRJNA192198), and *D. catenatum* (Zhang et al., 2016; NCBI Bioproject PRJNA192198). Following the approach of (Ruane et al., 2015), we obtained AHE target locus sequences for these two species using three reference sequences from the Angiosperm AHE V1 kit: *Lactuca sativa* L., *Arabidopsis thaliana* (L.) Heynh., and *Oryza sativa* L. In addition to using orchid-specific probes, we extended the standard angiosperm targets into adjacent exons to obtain longer loci. Candidate regions identified using spaced kmers (17 of 20 matches) were verified as a good match if at least 55 of 100 consecutive bases matched between the orchid sequence and one or more of the references. For each locus, a 4,000 bp region centered on the best-matching region was isolated for each species. For each locus, alignments containing the isolated *P. equestris* and *D. catenatum* sequences in addition to the three corresponding reference sequences were then estimated using MAFFT v.7 (Katoh and Standley, 2013). The alignments were inspected in Geneious (R9; Biomatters Ltd., Kearse et al., 2012) and the largest well-aligned region containing the AHE V1 probe region was identified. This procedure produced target loci substantially larger than that found in the V1 design, such that neighboring loci sometimes overlapped. When this occurred, the smaller target locus was removed as a target region. Finally, sequences were profiled following (Hamilton et al., 2016) and repetitive regions were masked. Final alignments used for probe design represented 451 target loci (448 of which contained both species). The loci averaged 887 bp in length (90% were between 261 bp and 1,973 bp) and had pairwise identity values averaging 77.7% (90% of loci had values between 65.5% and 87.5%). Probes of length 120 bp were tiled uniformly at 10x density across the two orchid sequences in each alignment, producing 53,881 probes in total.

4.2.4 Sample processing

Data were collected by the Center for Anchored Phylogenomics. Following DNA extraction, a Covaris E220 Focused ultrasonicator with Covaris microTUBES was used to fragment genomic DNA to a distribution of 300–800 bp. Libraries were prepared and indexed (8 bp) on a Beckman-Coulter Biomek FXp liquid-handling robot that implemented a modified version of Meyer and Kircher (2010) protocol. All libraries were then pooled at equal quantities, and enrichments were performed using an Agilent SureSelect XT probe kit containing the probes described above. Enriched library pools were pooled and sequencing on one half of an Illumina HiSeq2500 lane (23.9 Gb of raw data). Sequencing was performed in the Translational Science Laboratory in the College of Medicine at Florida State University.

4.2.5 Raw data processing

Raw sequence reads were processed using Illumina's CASAVA pipeline (v1.8) and low-quality reads were quality filtered using the high chastity setting. Quality filtered reads were then demultiplexed using 8bp indexes, which differed by at least 2 bases. Reads with corresponding indexes not matching one of the 16 expected indexes were discarded. Read accuracy and length were enhanced through pairedread merging, which was performed following the approach of Rokyta et al. (2012). Reads were assembled using a quasi de novo approach described by Prum et al. (2015) and Hamilton et al. (2016). Reads were mapped to probe region sequences from the orchid design

Table 4.1. Voucher specimens and species analyzed.

Sample code	Species	Voucher	Country
DB01	*Lepanthes nymphalis* Luer	DB11781 (JBL)	Costa Rica
DB02	*Lepanthes elata* Rchb.f.	DB11778 (JBL)	Costa Rica
DB03	*Lepanthes genetoapophantica* Bogarín & Gravend.	DB9745 (JBL)	Costa Rica
DB04	*Lepanthes wendlandii* Rchb.f.	DB11827 (JBL)	Costa Rica
DB05	*Lepanthes amicitiae* Bogarín & Pupulin	DB5911 (JBL)	Panama
DB06	*Lepanthes elata* Rchb.f.	AK6632 (JBL)	Costa Rica
DB07	*Lepanthes genetoapophantica* Bogarín & Gravend.	DB8682 (JBL)	Costa Rica
DB08	*Lepanthes chameleon* Ames	DB8371 (JBL)	Costa Rica
DB09	*Lepanthes wendlandii* Rchb.f.	DB11885 (JBL)	Costa Rica
DB10	*Lepanthes maxonii* Schltr.	DB5914 (JBL)	Panama
DB11	*Lepanthes amicitiae* Bogarín & Pupulin	AK6144 (JBL)	Costa Rica
DB12	*Lepanthes horrida* Rchb.f.	DB11459 (JBL)	Costa Rica
DB13	*Lepanthes wendlandii* Rchb.f.	DB11946 (JBL)	Costa Rica
DB14	*Gravendeelia chamaelepanthes* (Rchb.f.) Bogarín & Karremans	DB11881 (L)	Colombia
DB19	*Lepanthopsis prolifera* Garay	DB12048 (L)	Colombia
DB32	*Lepanthes gargantua* Rchb.f.	DB11868 (L)	Ecuador

described above. The assembly approach involves applying divergent references to initiate the assembly of each locus in the conserved probe region, and then assembling subsequent reads to the initially-mapped reads in a reference-based assembly style. To prevent low-level contamination from being included in downstream analyses, consensus sequences derived from fewer than 742 reads were removed from further analysis. Orthology across consensus sequences was established using pairwise distances following Hamilton et al. (2016). Haplotypes were phased assuming diploidy following (Pyron et al., 2016) and the haplotypes were aligned for each locus using MAFFT v.7 (Katoh and Standley, 2013). Lastly alignments were trimmed using the methods of Hamilton et al. (2016), but requiring 50% of bases in a site to be identical to identify a site as conserved, a minimum of 14 conserved sites in a 20 bp stretch for the stretch to be retained, and allowing only 18% missing data at each site for a site to be retained (see Hamilton et al. (2016) for details). Following the automated alignment trimming/masking procedure, alignments were manually inspected in Geneious R9 (Biomatters Ltd., Kearse et al., 2012) to verify the absence of misaligned regions and obvious paralogs (one locus was removed due to the presence of these issues).

4.2.6 Loci identification and datasets

The 446 loci retrieved were identified and classified in plastid, mitochondrial and nuclear genome datasets by conducting automatic BLAST searches in NCBI GenBank (https://blast.ncbi.nlm.

Table 4.2. Datasets analyzed in this study. All tree inferences contained 32 terminals (16 species with two haplotypes). Datasets derived from the three separate genomes did not have any missing sequences.

Inference	Dataset	Loci	Description
ML-423/ASTRAL-423	Complete matrix	423	All loci with/without missing sequences
ML-305/ ASTRAL-305	Reduced Matrix	305	All loci without missing sequences
ML-n/ ASTRAL-n	Nuclear	254	All loci from the nuclear genome
ML-m/ ASTRAL-m	Mitochondrial	14	All loci from the mitochondrial genome
ML-p/ ASTRAL-p	Plastid	37	All loci from the plastid genome

nih.gov) with an in-house designed script (https://github.com/dickgroenenberg/ Bogarin_Anchored_Phylogenomics) and subsequent further characterization in the TAIR database (https://www.arabidopsis.org). The BLAST hits mostly matched with annotated genes identified in the sequenced genomes of the orchid species *D. catenatum* (Zhang et al., 2016) and *P. equestris* (Cai et al., 2014) and other monocots with fully sequenced genomes such as oil palm, maize and rice. We performed inferences based on five datasets of trimmed loci matrices: (1) using the 305 loci alignments with 100% coverage (without missing sequences), (2) using 423 loci alignments with ~72% coverage (118 with missing sequences for one-two accessions), this because excluding/ including loci with missing sequences from some individuals can affect the outcome derived from both types of datasets (Huang and Lacey Knowles 2016; Mitchell et al. 2017), (3) with 254 loci derived from nuclear, (4) 14 from mitochondrial and (5) 37 from plastid datasets (Table 2). After the initial filtering by number of reads mapped to each locus and checking for orthologs, 446 loci were retained; however, once all data was aligned, if a locus had no sequence data for 3 or more of the 16 taxa in the alignment (x > 18.75%), it was not included in the analyses (a total of 23 alignments, see Results).

4.2.7 Supermatrix (concatenation)

We inferred a maximum likelihood (ML) species tree with the supermatrix approach using the ML-305 and ML-423 datasets. Statistical support was calculated with bootstrap support (BS) and the analysis was performed in RAxML-HPC2 on XSEDE v. 8.2.11 under the GTRGAMMA model for bootstrapping phase and 1000 bootstrap iterations in CIPRES Science Gateway V. 3.3 (Miller et al., 2015; Stamatakis, 2014). We applied the same ML analysis to the concatenated mitochondrial (ML-m), nuclear (ML-n) and plastid (ML-p) datasets.

4.2.8 Gene tree estimation

We generated unrooted ML gene trees for each locus with 100 bootstrap replicates using rapid bootstrapping with RAxML v. 8.2.11 under the GTRGAMMA model (command raxmlHPC-PTHREADS -f a -x 12,345 -p 12,345 -# 100 -k -m GTRGAMMA) as inputs for calculating species-tree estimations and further analysis on concordance and conflict among gene and species trees. The model of evolution for each loci was calculated using the Akaike Information Criterion (AIC) in jModelTest2 v2.1.7 (Darriba et al., 2012).

4.2.9 Super tree estimation

We used the estimated ML gene trees with collapse nodes with >33% bootstrap support in the R package phyloch to infer species trees with four programs developed under the coalescent model: ASTRAL-II v. 5.5.7 (Mirarab and Warnow, 2015; Sayyari and Mirarab, 2016), which is a disagreement reduction method (ASTRAL) and multi-locus bootstrapping (ASTRAL-mlbs) with support calculated with local posterior probability (LPP) and bootstrap support (BS) respectively, MP-EST v1.6 (Liu et al., 2010), NJst (Liu and Yu, 2011) and STAR (L. Liu et al., 2009) which are single evolutionary process methods considering ILS (Liu et al., 2015; Mallo and Posada, 2016; Shaw et al., 2013). NJst and STAR were run with R programming language (R Core Team, 2017) under R Studio (R Studio Team, 2016) using the package phybase (Liu and Yu, 2010) and the STRAW webserver (http://bioinformatics.publichealth.uga.edu/SpeciesTreeAnalysis/index.php). Gene trees were rooted online in the STRAW webserver. We inferred a species network with Phylonet v. 3.6.1 (https://bioinfocs.rice.edu/phylonet) using ML and estimated branch lengths of the gene trees for the inference. The network analysis was visualized in Dendroscope v.3 (Huson and Scornavacca, 2012). Phylonet is a multiple evolutionary processes method that reconstructs phylogenetic networks of reticulate evolutionary events (considering ILS and hybridization) (Than et al., 2008; Yu et al., 2014). We also inferred a species network with SplitsTree4 v 4.13.1 (Huson and Bryant, 2006) using the 423 loci dataset and excluding outgroup species with the following settings: Jukes-Cantor for "Characters" and NeighborNet method for "Distances". We tested these methods to evaluate possible discrepancies in the topology of each resulting species tree and the supermatrix approach (Simmons and Gatesy, 2015). We also inferred ASTRAL species trees from mitochondrial (ASTRAL-m), nuclear (ASTRAL-n) and plastid (ASTRAL-p) datasets and for each multi-locus alignment of 305 (ASTRAL-305) and 423 loci (ASTRAL-423). Final trees were manipulated in R using the packages APE, ggtree, phangorn and phytools (Paradis et al., 2004; Revell, 2012; Schliep, 2011; Yu et al., 2017) and later edited in Adobe Illustrator CS6 (Adobe Systems Inc., California, USA).

4.2.10 Gene and species tree concordance/discordance

We evaluated the topological concordance among gene trees, supermatrix approach, species trees and the analysis from the different genomic datasets with the R package TreeSpace v. 1.10.19 (Jombart et al., 2017). We identified clusters of similar trees with Metric Multidimensional Scaling (MDS) based on Robinson-Foulds (RF) symmetric difference (Robinson and Foulds, 1981) (unrooted, topological) and Ward clustering method and topological concordance among gene trees with RF and Kendall-Colijn metric vector (Kendall and Colijn, 2016) in order to test possible differences in unrooted and rooted based tests, respectively. We evaluated the level of concordance among gene trees (without missing data and collapsed nodes with < 33% support) against the mapping reference ASTRAL-305 species tree, ASTRAL-m, ASTRAL-n and ASTRAL-p and the ML supermatrix tree, ML-m, ML-n and ML-p with the program PhyParts (https://bitbucket.org/blackrim/phyparts) (Smith et al., 2015). Trees were previously rooted in R with the package APE and *G. chamaelepanthes* as outgroup. The output obtained with Phyparts was visualized by plotting pie charts on the ASTRAL species tree and ML concatenated tree with the script PhyPartsPieCharts (https://github.com/mossmatters/MJPythonNotebooks) using the ETE3 Python toolkit (Huerta-Cepas et al., 2016).

4.2.11 Phylogenetic informativeness

We evaluated the performance of the AHE datasets by measuring the net phylogenetic informativeness (PI) through an arbitrary time scale (tips assigned to time 0 and root to time 1) as described by Townsend (2007). This method has been used to calculate the power of each locus in resolving a node over time in AHE datasets (Fragoso-Martínez et al., 2016; Pyron et al., 2014; Wanke et al., 2017). To estimate the PI, we converted the rooted consensus ML trees (ML-305 and ML-423) to ultrametric trees with PATHd8, a program for phylogenetic dating without a molecular clock (https://www2.math.su.se/PATHd8/) (Britton et al., 2007; Schoch et al., 2009). The PATHd8 method calculates ultrametric trees with branch lengths proportional to the number of substitutions and these substitutions rates are smoothed locally (Britton *et al*., 2007). The partitioned concatenated matrices were built in SequenceMatrix v100.0 from the trimmed loci alignments (Vaidya et al., 2011). These input files were uploaded in the web application PhyDesign (López-Giráldez and Townsend, 2011; http://phydesign. townsend.yale.edu/) to estimate phylogenetic informativeness profiles with the HyPhy substitution rates algorithm for DNA sequences (Kosakovsky Pond et al., 2005). For the identification of sites with unusually high substitution rates that could cause phylogenetic noise, we followed the R script and filtering method described by Fragoso-Martínez et al. (2016). Sites with rate values higher than five were removed manually from the alignments using Geneious R9 (Biomatters Ltd., Kearse et al., 2012) and these corrected matrices were uploaded again to PhyDesign as described above.

4.3 Results and discussion

4.3.1 New phylogenetic markers generated

A total of 446 innovative loci generated from 16 plant samples and two haplotypes (diploidy as default assumption) for each locus were identified (supplementary material: Table 4.3; https://www.sciencedirect.com/science/article/pii/S1055790318301623#s0310). Of these, 367 (82.10%) loci were nuclear, 19 (4.47%) mitochondrial and 58 (13.42%) plastid derived. Only two fragments could not be assigned to any genome (scored as N/A in Table 4.3); 54 (12.11%) could not be linked to any protein (product scored as uncharacterized in Table 3). To the best of our knowledge, no studies using AHE have characterized and annotated loci recovered under this approach. The average locus length was 1074 bp, the shortest was 114 bp and the longest was 4644 bp. The number of loci recovered is similar to that obtained in *Salvia* (Lamiaceae) (448) and higher than other monocots (i.e. Arecaceae: 133, and Zingiberales: 308) (Fragoso-Martínez et al., 2016). The average locus length is higher than previous AHE datasets in *Aristolochia* (670–687 bp), *Protea* (551 bp) and *Salvia* (704 bp) (Heyduk et al., 2016; Mitchell et al., 2017; Wanke et al., 2017). The optimization of orchid probes to increase enrichment efficiency and the extension of the standard angiosperm targets into adjacent exons successfully improved the discriminative power in terms of orchid gene tree resolution. When working on shallow scales, there is the potential for extending anchor regions to produce longer loci that increases the chances of producing well-resolved gene trees. This approach has been successfully tested in salamanders and is currently under development in other non-model organisms (McCartney-Melstad et al., 2016). From the 446 loci alignments, two concatenated matrices were produced. One matrix

comprised of 31,905 bp and contained 305 loci with complete representation of taxa. The other matrix comprised of 444,631bp and contained 423 loci, including loci with missing sequences for one-two accessions (taxa). The remaining 23 loci alignments were not included in either matrix due to missing three or more accessions. We obtained 305 gene trees based on alignments without missing sequences and 118 gene trees from alignments with missing sequences. From the 305 gene trees, a total of 254 gene trees were derived from nuclear, 14 from mitochondrial and 37 from plastid datasets. The models of evolution calculated from each locus belonged to the General Time Reversible (GTR) family (supplementary material: Table 3).

4.3.2 Super tree estimation and supermatrix (concatenation)

The topology of the ASTRAL and ASTRAL-mlbs based on 305 loci was identicaL. Most of the nodes showed high support values of bootstrap and LPP. A total of 7,166,891 induced quartet trees were retrieved in the ASTRAL-305 species tree accounting for 65.34% of all quartet trees found in the species tree and for the ASTRAL-423, 63.18% of the quartet trees and 8,775,615 induced quartet trees. The MP-EST, NJst and STAR showed identical topologies compared to the ASTRAL analyses (Figs. 4.1 and 4.2A, B). The species tree analyses were congruent and recognized three main clades with high support (ASTRAL: LBS = 100, LPP ≥ 0.91): (1) *L. wendlandii*, (2) *L. horrida* and (3) *L. maxonii*. The *L. wendlandii* clade (1) clustered with the grouping of *L. horrida* (2) + *L. maxonii* (3) clades. Within the *L. horrida* clade (2), *L. horrida* clustered with *L. chameleon* and two accessions of the here described species *L. genetoapophantica* (DB8682 and DB9745). The two samples of *L. genetoapophantica* did not cluster together showing paraphyly; *L. genetoapophantica* (DB8682) was linked to *L. chameleon* and the other sample of *L. genetoapophantica* (DB9745) was linked in a more internal node to the two previous accessions (but with low support, ASTRAL: LPP < 0.56 and BS < 81). ASTRAL-305, MP-EST, NJst and STAR clustered all haplotype sets (1 and 2) from each sample (Fig. 4.2B–D). However, entire missing sequences in individual gene matrices caused discrepancies in the topology of gene trees, lower support (ASTRAL: LPP < 0.41 and BS < 15) and resulting species tree inferences in ASTRAL-423 and ML423 supermatrix approaches (BS < 84) (Fig. 4.2B–D). These incongruences were restricted to the *L. horrida* clade and the main discrepancy was the splitting of the two haplotypes and resulting paraphyly of *L. horrida*. All the inferences with missing and non-missing sequences recognized similar topologies for the *L. wendlandii* and *L. maxonii* clades (Fig. 4.2). The *L. maxonii* clade (3) contained *L. nymphalis* as a species related to a group made up of *L. maxonii* and two accessions of the undescribed species *L. amicitiae* (AK6144 and DB5911). Low LPP values are related to incongruences among gene trees (see further discussion Section 3.4.).

The ML supermatrix approaches retrieved essentially the same clades as the species tree analyses with the highest bootstrap support (BS) of 100% for most of the nodes (Fig. 4.2C and D). High support in inferences derived from NGS datasets are related to the markedly increasing supermatrix size (Wagner et al., 2013). Composition of the *L. wendlandii* clade (1) and *L. maxonii* clade (3) was similar in the topology of the ASTRAL (with 305 and 423 loci), ASTRAL-mlbs, MP-EST, NJst and STAR analyses, however, the most problematic clade for these analyses was again the *L. horrida* clade (2) because of the unexpected separation of the two haplotypes of *L. horrida* (DB11459). This separation was also observed in the ASTRAL-423 analyses (Fig.

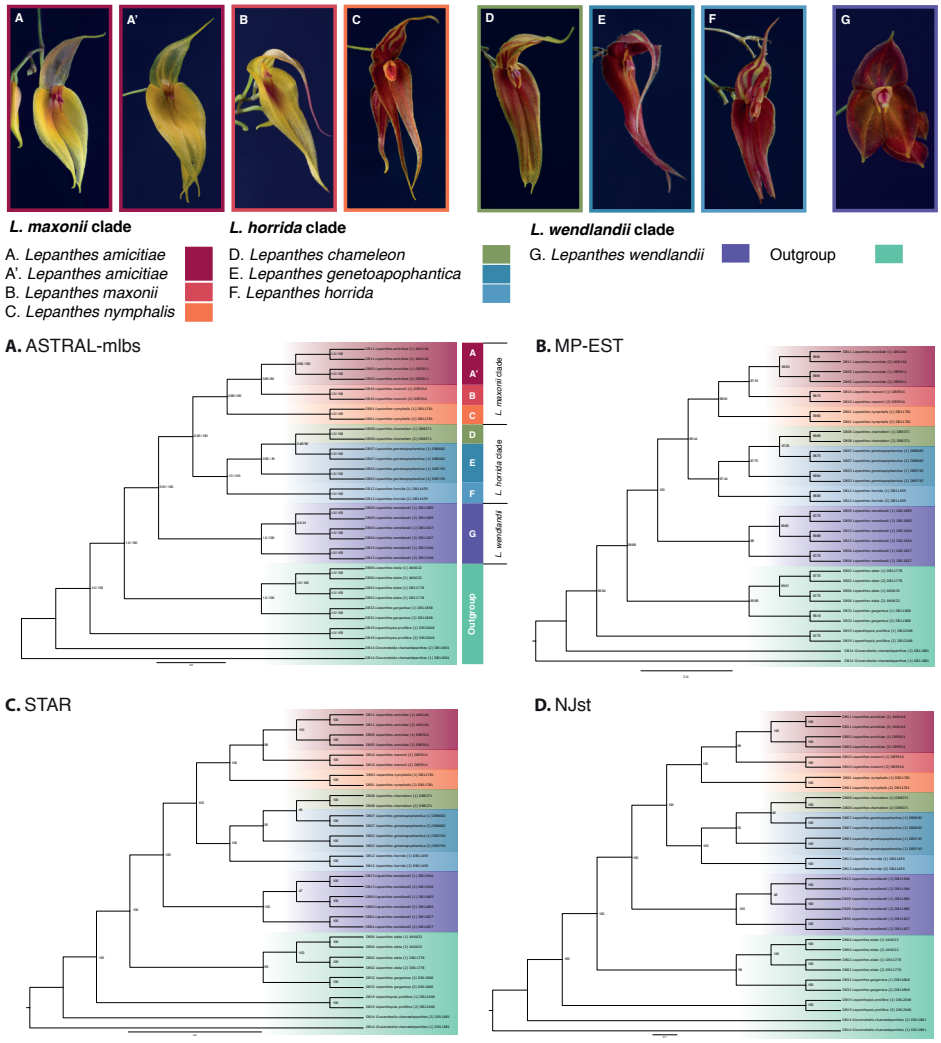

Figure 4.1. Flower morphology of the species of the *Lepanthes horrida* group and inferred species tree topologies from A. ASTRAL-mlbs, B. MP-EST, C. NJst and D. STAR. Local posterior probability/bootstrap support is shown for the nodes of ASTRAL-mlbs. These analyses support the clustering of three main clades: *L. maxonii* (**A.** A', *L. amicitiae*+ **B.** *L. maxonii*+ **C.** *L. nymphalis*) and *L. horrida* (**D.** *L. genetoapophantica*+ **E.** *L. chameleon*+ **F.** *L. horrida*) both sister to *L. wendlandii* (G)**.**

4.2B). Both ML supermatrix approaches including/excluding missing data did not group both haplotypes of *L. horrida* together (Fig. 4.2C-D). In the ML-305 supermatrix, the two samples of *L. genetoapophantica* were clustered but with low support (BS < 51%) and haplotype 1 of *L. horrida* was placed as sister to a clade made up of *L. genetoapophantica*, *L. chameleon* and haplotype 2 of *L. horrida* (Fig. 4.2C). In addition, lower bootstrap support values were observed for the internal nodes of the *L. maxonii* clade, in particular the node linking *L. nymphalis* with

L. maxonii + *L. amicitiae* (LBS = 60%). The support for this node was higher in the ML supermatrix including missing data (LBS = 97%). Also, in the *L. wendlandii* clade, one node linking two samples of *L. wendlandii* showed low bootstrap support (LBS = 61%) (Fig. 4.2C). In the ML-423 supermatrix haplotype 2 of *L. horrida* was grouped with *L. chameleon* (DB8371), BS = 84% and haplotype 1 with *L. genetoapophantica* (DB8682), BS = 93% (Fig. 4.2D). These were the only two nodes with bootstrap values less than 100% in the ML-423 analyses. In addition, the two samples of *L. genetoapophantica* were separated: *L. genetoapophantica* (DB9745) clustered with *L. chameleon* and to haplotype 2 of *L. horrida* whereas the other sample of *L. genetoapophantica* (DB8682) clustered with haplotype 1 of *L. horrida*. Similar to the species tree, the PhyloNet approach grouped together the haplotypes of *L. horrida* (DB11459), however, the two samples of *L. genetoapophantica* were not grouped together: *L. genetoapophantica* (DB9745) clustered with *L. chameleon* (DB8371) and *L. genetoapophantica* (DB8682) with *L. horrida* (DB11459) (Fig. 4.3A). This topology was similar to the tree inferred with ASTRAL-305, MP-EST, NJst and STAR. Similar as in the species tree analyses, the two samples of *L. amicitiae* were grouped together and *L. maxonii* ended up as closely related to this species. One sample of *L. wendlandii* (DB11827) did not cluster with the other two samples of *L. wendlandii* (DB11885 and DB11946). The network derived from SplitsTree did not cluster the two samples of *L. genetoapophantica* together and separated both haplotypes of *L. horrida* (DB11459) (Fig. 4.3B).

4.3.3 Species recognition

Species delimitations based on coalescent methods were consistent with the morphology of the species and agreed with previous species circumscriptions. The results also supported the recognition of two undescribed species and resolved the species relationships that were not previously disclosed using only nrITS and matK. Species tree estimations showed a strong tendency to recognize *L. wendlandii* as sister to *L. maxonii* + *L. horrida*. *Lepanthes wendlandii* is the most divergent species of the group. Unlike *L. maxonii* + *L. horrida*, plants of *L. wendlandii* are all characterized by short-pubescent, blackish ramicauls often longer than 20 cm, reddish flowers with the sepals widely ovate, obtuse and short-caudate and a cylindrical column (Luer, 2003a). Individuals of *L. wendlandii* show little morphological variation. Although in some analyses, one sample of *L. wendlandii* (DB11827) was positioned apart from the other two samples, the morphological evidence presented above suggests that the three samples belong to the same species. As suggested by by Pyron et al. (2016) assessments of species delimitations with computational genetic models should include traditional morphological data for species recognition so we refrain from recognizing additional taxa in this complex. The sister group of *L. wendlandii* comprises all the species related to the *L. maxonii* and *L. horrida* clades. It is characterized by plants with hirsute ramicauls, elongated, acuminate sepals and a column conspicuously flattened at the apex (Luer, 2003a). Within this group, the topology of the *L. maxonii* clade was constant in all the analyses. The clade clustered *L. nymphalis*, an endemic species to the Cordillera Central of Costa Rica with reddish flowers and distinctive for its long ciliated lip blades with two yellow-flowered species, *L. amicitiae* and *L. maxonii*, both endemic to the Cordillera de Talamanca between Costa Rica and Panama. Morphological differences suggest that individuals with yellow flowers correspond to two different species. One distinguished by the rounded shape of the upper lobe of

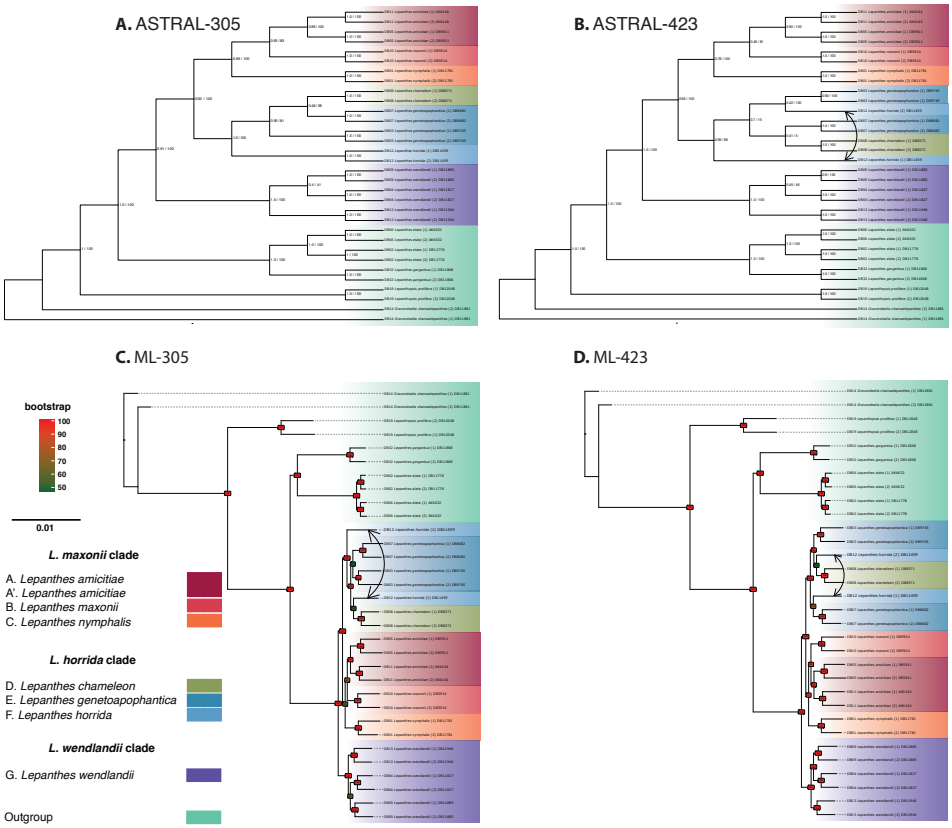

Figure 4.2. ASTRAL and Maximum likelihood (ML) inferences of concatenated datasets: **A.** ASTRAL of 305 loci without missing sequences. **B.** ASTRAL of 423 loci. **C.** ML-p based on 305 loci. **D.** ML-m based on 423 loci. Arrows in C and D show the splitting of the two haplotypes of *L. horrida* (DB11459), that do not cluster together. Note the lower BS support.

the petals (*L. amicitiae*) and other by the elongated lobes of the petals (*L. maxonii*). This hypothesis was supported consistently by ML and species tree analyses. Recognition of the *L. horrida* clade was constant in the grouping of *L. horrida*, *L. chameleon* and the undescribed species *L. genetoapophantica* (DB8682 and DB9745). Morphological evidence supports the recognition of these three species. However, unlike the *L. wendlandii* and *L. maxonii* clades, the topology of the *L. horrida* clade showed discrepancies in the positioning of *L. genetoapophantica* and both haplotypes of *L. horrida* and consequently low support BS in the ML analyses. Theoretically, in absence of ILS and gene flow, mutation is the only possible source of allelic variation. Therefore, haplotypes retrieved from the same sample are expected to be monophyletic. When haplotypes are not monophyletic in concatenated analyses (because concatenation does not take into account other gene evolutionary processes), it is possible that other sources of allelic variation operate such as gene flow or ILS (Pyron et al., 2016).

Hybrid origin may be one of the possible explanations to the nonmonophyly of species. *Lepanthes genetoapophantica* is morphologically similar to *L. horrida*, but it is distinguished by the smaller, divergent blades of the lip in contrast to the larger and elongated blades of *L. horrida*. The paraphyly of the two samples of *L. genetoapophantica* in the ASTRAL-305, MP-EST, NJst, STAR and Phylonet analyses supports a hybrid affinity or shared genetic diversity due to ILS or ancestral polymorphisms. This possible hybrid affinity is likely ancestral and not due to actual spontaneous hybridization because populations of *L. horrida*, endemic to the Cordillera Central of Costa Rica, are geographically isolated from the Cordillera de Talamanca where *L. chameleon* and *L. genetoapophantica* are endemic. In addition, there is no evidence of morphological variation between the characters that distinguish *L. horrida* and *L. genetoapophantica* that could suggest spontaneous hybridization. Thus, ancient hybridization could be a hypothesis for the discordant grouping of these species and might have contributed to speciation through the formation of new hybrid taxa (Abbott et al., 2013). Artificial hybridization in *Lepanthes* is possible but few natural hybrids have been documented probably because of the highly specialized pollination system (sexual mimicry).

An alternative hypothesis explaining the non-monophyly of *L. horrida* is polyploidy. Wanke et al. (2016) found that "assumed" diploidy in phased AHE alignments in *Aristolochia* yielded non-monophyletic allelic groupings from the same sample, however, upon assuming tetraploidy, these allelic groupings could be forced into monophyly in their analyses, suggesting that polyploidization could have occurred during the evolution of these species. Allopolyploidy occurs at high frequency in plants and can create postzygotic reproductive barriers in speciation events mediated by hybridization. Allopolyploids are common in the Orchidaceae and are expected to occur in the Pleurothallidinae as well because of the high intercompatibility among species and the many artificial hybrids created for commercial purposes. In Pleurothallidinae, polyploidy has been recorded in the genera *Octomeria* and *Scaphosepalum* (de Oliveira et al., 2015) but no data are yet available for *Lepanthes*. However, in absence of experimental evidence (i.e. data on genome size, chromosome counts, the relative success of artificial hybrid crossings, selfing and outcrossing), we refrain from favoring any of the current hypotheses that may explain the sources of reticulation in the *L. horrida* complex.

4.3.4 Concordance among gene trees/ASTRAL and ML trees

Even though the backbone nodes showed LBS = 100 in the supermatrix approach, the Phyparts analysis on the ASTRAL species tree and the ML supermatrix tree from the 305 gene trees showed a similar high degree of gene tree conflict (Fig. 4.4). In the ASTRAL-305 and ML-305 supermatrix tree (Fig. 4.4A and B), the only well supported clade in the gene trees was the *L. wendlandii* clade (1), supported by 257 (~84%) of the 305 loci tree topologies. The remaining 16% of the gene trees supported alternative topologies (Fig. 4.4A). In contrast, a very low number of gene trees (3.6%) supported the separation of the *L. horrida* (2) and *L. maxonii* (3) clades. This node showed dominance for other conflicting topologies (indicated in red in the pie charts of Fig. 4.4). The same pattern was shown in the nodes linking the species within the two groups. Low gene tree support and dominance of other conflicting bipartitions was also observed in the *L. maxonii* clade (3). *Lepanthes nymphalis* as sister to *L. maxonii* and *L. amicitiae* was support-

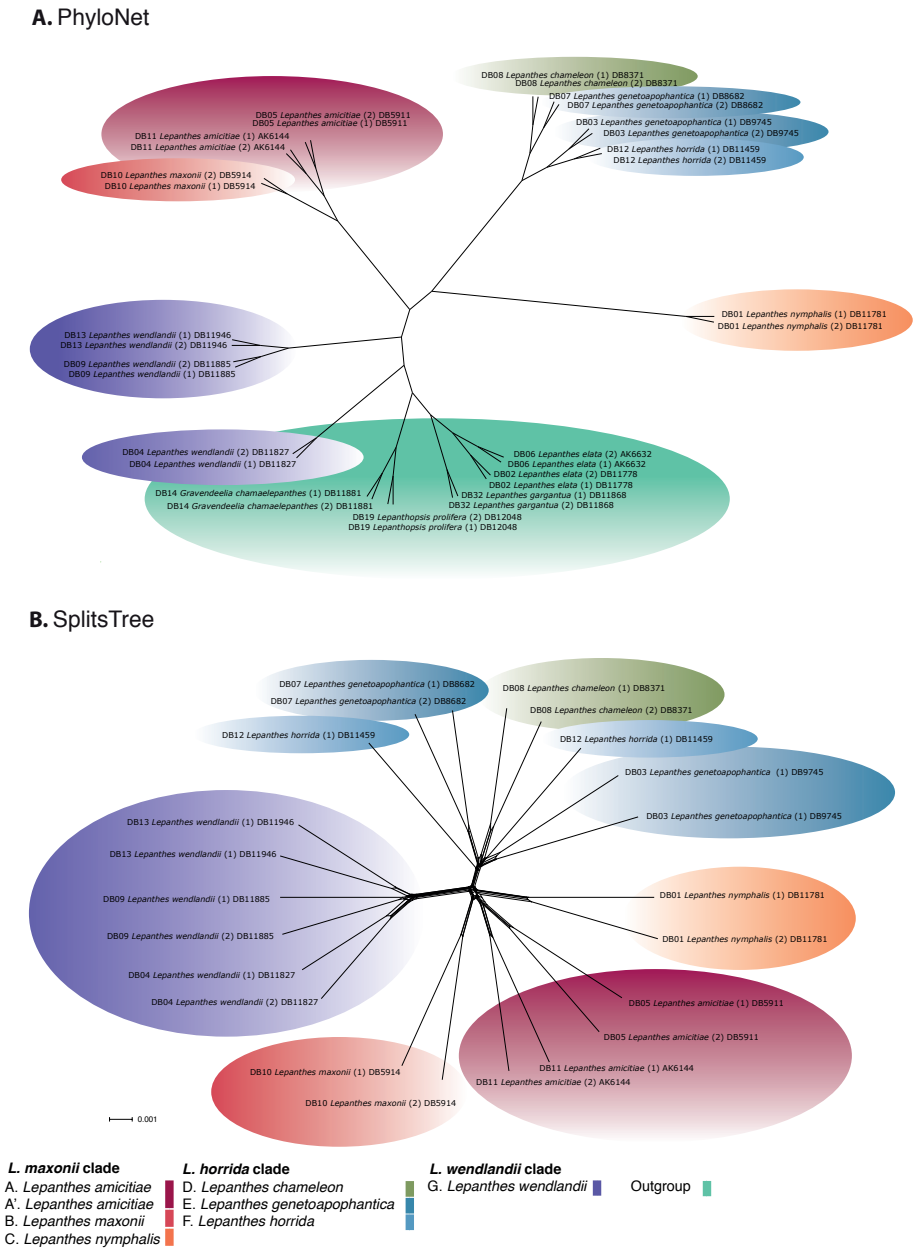

Figure 4.3. Inferred species network analyses of **A.** PhyloNet approach showing a similar clustering of species compared to the species tree analyses but grouping the two samples of *L. genetoapophantica* separately: one grouped with *L. horrida* and the other with *L. chameleon*. The affinity of *L. maxonii* and the two samples of *L. amicitiae* is also evident **B.** SplitsTree network showing the non-monophyly of *L. horrida*, the separate clustering of *L. genetoapophantica* and the networking on the three clades.

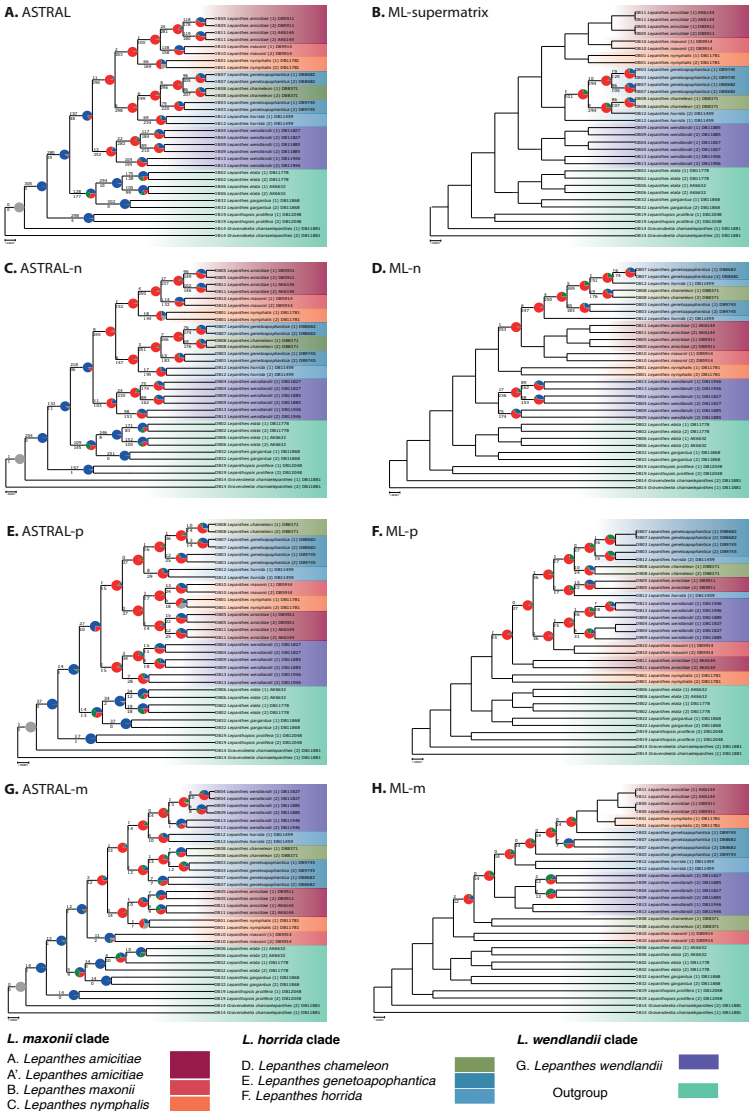

Figure 4.4. ASTRAL and ML species trees inferred from 305 ML gene trees and genomic analyses: **A.** ASTRAL-mlbs. **B.** ML-concatenated. **C.** ASTRAL-nuclear. **D.** ML-nuclear (based on 288 nuclear loci) **E.** ASTRAL-plastid. **F.** ML-plastid (based on 40 plastid loci). **G.** ASTRAL- mitochondrial. **H.** ML-mitochondrial (based on 18 mitochondrial loci). Numbers on branches represent the gene trees supporting each node (top) and the number of gene trees in conflict with the shown topology of the species tree (bottom). Pie charts show the proportion of gene trees concordant with the shown topology (blue), conflict with the shown topology that support the main alternative for that clade (green), other dominant alternative (conflicting) supported bipartitions (red) and unsupported nodes due to conflicting bipartitions with less than 70% bootstrap support (gray). Pie charts are shown only where the topology differs in the ML with respect to the ASTRAL analyses.

ed only by two (~1%) gene trees, *L. maxonii* as sister to *L. amicitiae* by only five (1.6%), and only 24 (7.8%) gene trees supported the grouping of the two samples of *L. amicitiae* in a single clade. The nodes of the *L. maxonii* and *L. horrida* clades mostly showed alternative (conflicting) bipartitions (indicated in red in the pie charts of Fig. 4.4). Increasing support for the shown topologies was observed in the tip nodes grouping all haplotypes together (indicated in blue in the pie charts of Fig. 4.4), however, conflicting bipartitions were still observed (dominance of red and to a lesser extent green in the pie charts in Fig. 4.4). The topology of *L. horrida* clade (2) was the main difference between the ASTRAL and ML supermatrix approach (Fig. 4.4A and B). In the ASTRAL analyses, only six (1.9%) gene trees supported the relationship between *L. horrida* and *L. chameleon* + *L. genetoapophantica* and six (1.9%) the clustering of *L. genetoapophantica* (DB9745) and *L. chameleon* + *L. genetoapophantica* (DB8682). The grouping of the latter species was supported by eight (2.6%) gene tree topologies (Fig. 4.4A). In the ML supermatrix, the separate clustering of haplotype 1 of *L. horrida* was supported by six (1.9%) gene trees and the clustering of haplotype 2 with *L. chameleon* was supported by nine (~3%) gene trees (Fig. 4.4B). In addition, the nodes showed higher dominance of other main topologies (indicated in green in the pie charts in Fig. 4.4) as compared to ASTRAL.

Similar to other studies on animals and plants in which the performance of multi-locus datasets was evaluated, we found that the analyses of multiple gene copies do not necessarily result in concordance or high support of the topologies obtained with coalescent-based methods of species tree estimations and individual gene trees (Jeffroy et al., 2006; Sun et al., 2015; Tang et al., 2015). On the contrary, individual gene trees with divergent topologies are common in many groups, suggesting that hybridization, horizontal gene transfer, gene duplication and ILS are pervasive phenomena and could be important causes of these topological discordances (Jeffroy et al., 2006; Mallo and Posada, 2016; Sun et al., 2015; Yu et al., 2013).

Incomplete lineage sorting might cause discordances in groups of closely related species with rapid diversifications in part because the alleles within a population do not have enough time to coalesce (Degnan and Rosenberg, 2009; Tsutsumi et al., 2016). Recent studies in the evolution of the Pleurothallidinae revealed that *Lepanthes*, with an estimation of over 1200 species, radiated in the last 2.5 million years (Pérez-Escobar et al., 2017a). In addition, Tremblay and Ackerman (2001) found that genetic drift is important in population differentiation due to small population size and restricted gene flow common in *Lepanthes*. Therefore, ILS could be a plausible explanation for the incongruences observed as well, next to hybridization and polyploidy.

Individual gene tree clustering based on RF and Kendall-Colijn distance showed a wide array of topologies and consequently little resolution in the topologies as compared to species tree and concatenated methods (Fig. 4.5A and B). The species tree analyses clusters produced in group six showed similar topologies according to RF based clustering (Fig. 4.5C and D). Only 17 gene trees (16 nuclear and one plastid) clustered in the same group of the supermatrix while the remaining 288 gene trees (94.4%) and the species tree analyses clustered separately, thus showing other topologies (Fig. 4.5C). The topologies of both ML concatenated supermatrix approaches were slightly divergent with respect to ASTRAL, MP-EST, NJst and STAR species trees (Figs. 4.1 and 4.5D). The most notable topological difference among them was the separation of the haplotypes of *Lepanthes horrida* (DB11459) and the two samples of *L. genetoapophantica* as discussed above.

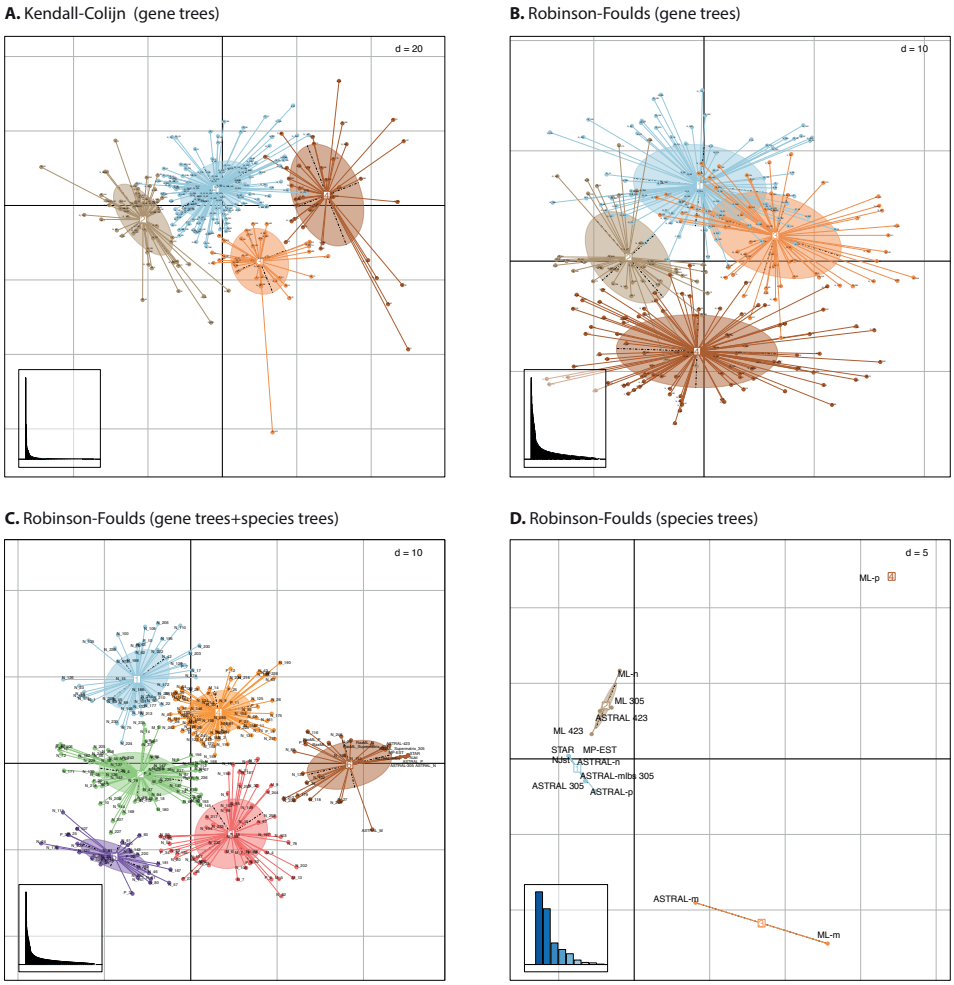

Figure 4.5. Gene trees and species trees cluster analysis of Metric Multidimensional Scaling (MDS) **A.** Clustering of the 305 gene trees (rooted) based on the Kendall-Colijn metric vector and four clusters. M=mitochondrial, N=nuclear, P=plastid. **B.** Clustering of the 305 gene trees (unrooted) based on Robinson-Foulds (RF) symmetric difference. **C.** Clustering of the 305 gene trees, species (ASTRAL, MP-EST, NJst, STAR) trees and ML concatenated datasets based on RF. Cluster 6 contains the species trees and ML trees, (d) MDS of species trees and ML concatenated datasets based on RF.

In addition to ILS and deeper speciation, incongruence could also be the result of estimation errors in gene trees derived from alignments containing missing sequences, long-branch attraction or phylogenetic noise (Mallo and Posada, 2016; Mirarab and Warnow, 2015). Excluding loci alignments with missing sequences indeed produced different topologies. The topology of the ASTRAL-423 (inferred from alignments with missing sequences in less than two samples) was similar to the topology of the ML supermatrix approaches and ML-n (Figs. 4.2 and 4.4D–5) mostly because it recovered a non-monophyletic *L. genetoapophantica* and *L. horrida* (Figs. 4.1

and 2A). In contrast, ASTRAL-305 grouped these haplotypes together similar to the other species tree analyses (Figs. 4.1 and 5D), possibly because according to Huang and Lacey Knowles (2016), information is lost because of reduction of matrix size and biased representation of mutations when missing data are excluded.

4.3.5 Nuclear and organellar datasets

As observed in the species tree and supermatrix approaches, a low number of gene tree topologies supported the topologies of the species trees based on the nuclear/organellar datasets (Fig. 4.4C–H). The gene trees from each genomic dataset did not form specific conglomerates because the topologies were mixed without showing any pattern that could be linked to a unique genomic origin (Fig. 4.5A–C). Plastid and nuclear derived gene trees were observed across the six clusters and the mitochondrial derived gene trees were only absent in cluster six where the species trees and ML analyses were placed (Fig. 4.5C). The topology of ASTRAL-n was similar to ASTRAL-305 and to the other species tree analyses (Fig. 4.5D) but a low number of gene trees supported this topology (Fig. 4.4A, C and 4.5C, D). In contrast, ML-305 differed from ML-n in the separation of the two samples of *L. genetoapophantica* in ML-n, which were grouped together in ML-305 but with low support and a low number of gene tree topologies supporting (BS < 54% in ML-305 and BS < 62% in ML-n) the internal branches of the *L. horrida* cade (Figs. 4.2C and 4.6A). ASTRAL-n and ML-n differed in the placement of both haplotypes of *L. horrida* because ASTRAL-n clustered them together contrary to ML-n. However, both agreed on the topology of the *L. maxonii* clade.

The analyses based on the mitochondrial datasets ASTRAL-m and ML-m (Figs. 4.4G–H and 4.5D) and the plastid ML-p dataset (Figs. 4.4F and 4.5D) were the most divergent with respect to the ML-supermatrix, ML-n, ASTRAL-n, ASTRAL-p and all the species tree analyses (Fig. 4.5D). These trees showed very low bootstrap support for most of the internal branches of the three main clades (BS < 70%) (Fig. 4.6C and D). All ML analyses failed in clustering the two haplotypes of *L. horrida* (DB11459) together. Although the ML-m analyses grouped both haplotypes of *L. horrida* together, BS was low and this analysis also failed in clustering the two haplotypes of *L. genetoapophantica* (DB9745) together (Fig. 4.6C). The ASTRAL-m analyses showed the most divergent species tree topology (Figs. 4.5C, D and 4.6C) as compared to ASTRAL-n and ASTRAL-p (Fig. 4.5C and D), which were more similar to the ASTRAL, MP-EST, NJst and STAR species trees (Fig. 4.1). However, this topology was supported by a very low number of gene trees (< 3 gene trees, 16.6%). In addition, the topology of the ML-m analyses was divergent as well, the main differences being the placement of *L. maxonii* and *L. chameleon* as sister to the rest of the species of the group and the separation of the haplotypes of one sample of *L. genetoapophantica* (DB9745). These topologies were poorly supported by individual gene trees (Fig. 4.4H). In contrast, the ASTRAL-p topology was more similar to the species tree analyses. It recognized the same clades with the same topology for the *L. wendlandii* and *L. horrida* clades, but a different topology for the *L. maxonii* clade (3), because the two *L. amicitiae* samples clustered together and they were grouped with a clade formed by *L. nymphalis* and *L. maxonii*. However, the alternative topology of the ASTRAL-p analyses was supported by a low number of gene trees (< 3 gene trees, 7.5%) (Fig. 4.4E). The paraphyly of *L. horrida* was the main difference between the ASTRAL-p and ML-p analyses.

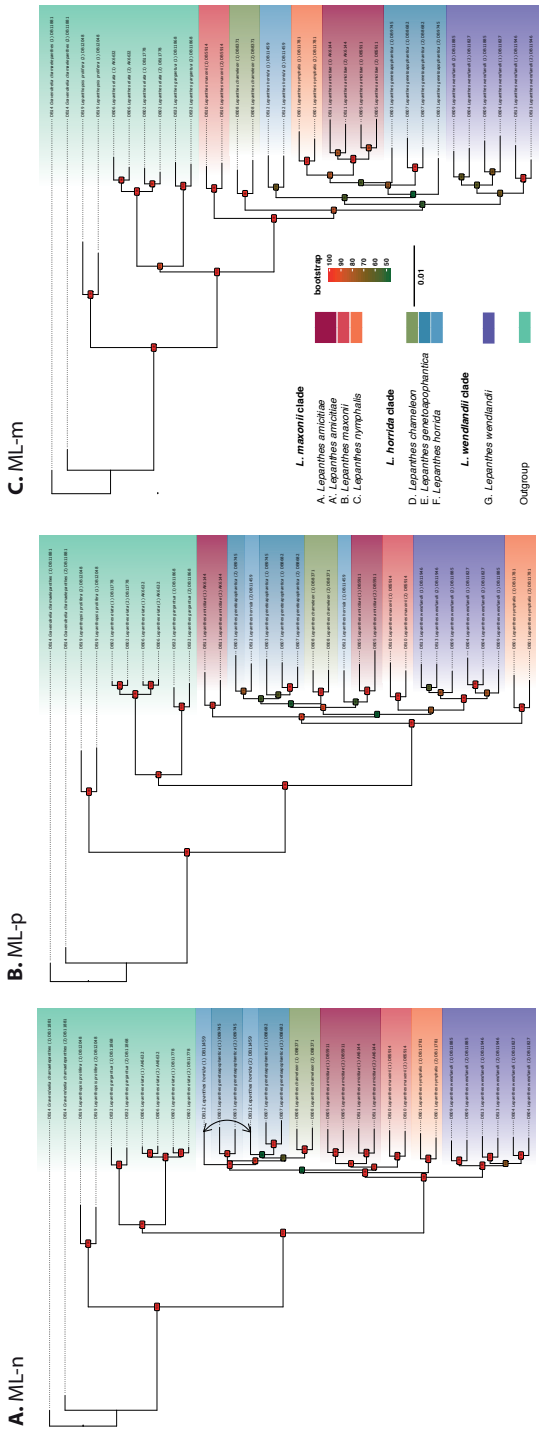

Figure 4.6. Maximum likelihood (ML) inferences of concatenated datasets showing bootstrap support for each node. **A.** ML-n based on 288 nuclear loci. **B.** ML-p based on 40 plastid loci,. **C.** ML-m based on 18 mitochondrial loci.

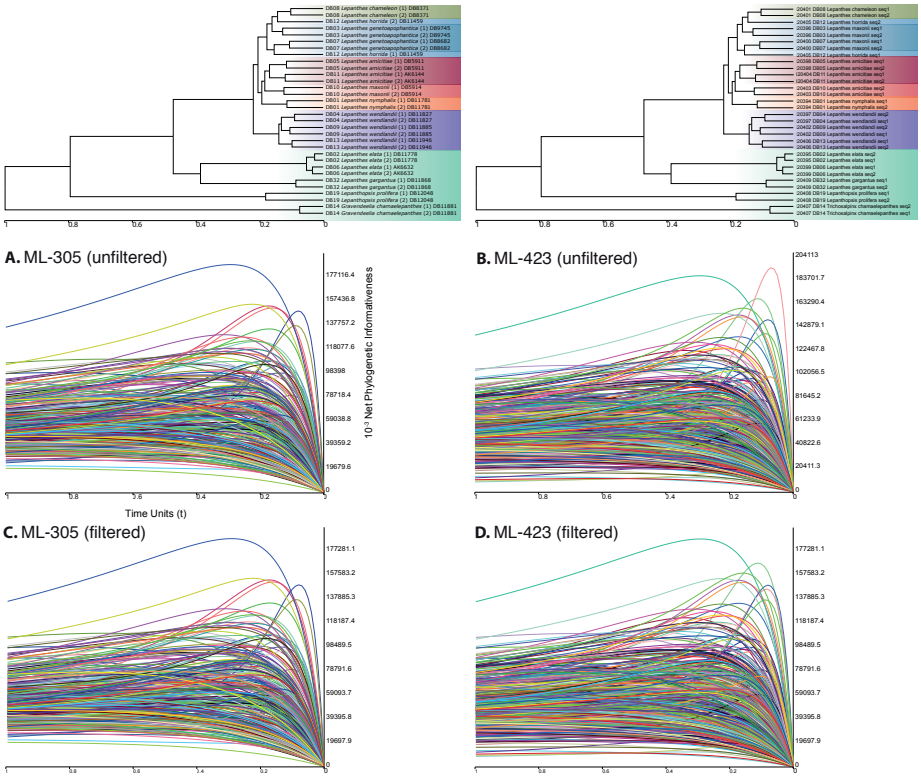

Figure 4.7. Net phylogenetic informativeness profiles per each locus of the ML-305 and ML-426 datasets. Ultrametric trees were obtained with PATHd8 using a relative time scale (0 to 1): **A.** ML-305. **B.**ML-423, **C.** Filtered ML-305. **D.** Filtered ML-423. Curves are smoother in unfiltered and filtered datasets. The analyses recovered the same topology and the BS values slightly increased in the filtered datasets.

The greater number of nuclear genes obtained likely produced a stronger influence in the topology of the concatenated species tree analyses that was almost identical to the inferences based on nuclear genes. Incongruences between nuclear and plastid datasets might also suggest hybridization. Studies in Rosidae showed conflicts in the topology derived from plastid, nuclear and mitochondrial datasets likely produced by ILS and ancient hybridization (Sun et al., 2015). The high incongruence observed in our results among individual nuclear loci (if not related to estimation/stochastic errors) suggests that conflict among nuclear and organellar datasets is due to biological evolutionary events such as ILS and/or ancient hybridization. Mitochondrial derived trees showed disparate topologies that disagree with the morphology of the species. Because our results were based on a few mitochondrial gene trees only, these could be misleading due to undersampling (Parks et al., 2017).

4.3.6 Phylogenetic informativeness

Species net phylogenetic informativeness plots showed slightly increasing, stable curves over time in most of the loci from both ML approaches (Fig. 4.7A and B). A total of 75% of the

loci reached a maximum net PI between 78.03 and 44.33 at a reference time (t) between 0.30 and 0.81. The obtained unfiltered datasets showed plots with smooth curves lacking "phantom" spikes and the filtering method (with rate values > 5) detected only six loci with high substitution rates (Fig. 4.7C and D). In addition, filtered and unfiltered datasets recovered identical topologies and almost the same BS values (but slightly higher in the filtered datasets). These findings are similar to those found in AHE datasets from Aristolochiaceae and Lamiaceae (Fragoso-Martínez et al., 2016; Wanke et al., 2017).

The ten individual loci with the highest PI values for each analysis are shown in Table 4. They were derived from all three separate genomes (despite the dominance of nuclear genes in our datasets), thus highlighting the importance of organellar loci in phylogenetic analyses. The fragment with the highest net PI was the inhibitor of Bruton tyrosine kinase like (IBTK) gene, of which 25,98bp were analyzed. Phylogenetic analyses of *Lepanthes*, (Pleurothallidinae in general), have been based mostly on nrITS and *mat*K only, so the recovery of the new loci published here would be useful for future molecular systematic studies in this group (Pridgeon et al., 2001).

4.4 Conclusions

Anchored hybrid enrichment coupled with coalescence-based methods is a powerful tool to solve complicated phylogenetic relationships in linages derived from recent, rapid diversifications. Despite the high discordance in the topology of the gene trees reconstructed, combined ASTRAL, MP-EST, NJst and STAR analyses could resolve the phylogenetic relationships of the *L. horrida* species group. These analyses also disclosed two undescribed species, *L. amicitiae* and *L. genetoapophantica*. These results could not have been obtained by morphology and standard nrITS and matK analyses. Phenomena such as ILS, hybridization and polyploidy may be common in groups recently diverged such as Pleurothallidinae causing discordance among datasets. The data presented here showed high incongruences in the topologies among individual loci that were probably produced by different biological phenomena. Due to the various sources of incongruence, species delimitations based on multi-locus datasets should be interpreted in conjunction with traditional morphological observations. Only with a large number of innovative phylogenetic markers generated from three different genomes, the phylogeny could be fully resolved and this enabled us to separate traits evolving in parallel or convergently across these orchid lineages, such as flower color and size, from real diagnostic traits such as the shape and orientation of the lobes of the petals and lip.

4.5 Taxonomic treatment

Key to the species of the Lepanthes horrida group

1. Sheaths of the ramicaul shortly pubescent; synsepal broadly ovate-orbicular, the free apices obtuse — ***L. wendlandii***

1”. Sheaths of the ramicaul densely ciliate-hirsute; synsepal narrowly lanceolate, the free apices linear-acuminate — 2

2. Margins of the sepals ciliate-dentate — 3

3. Synsepal glabrous at the base; lateral lobes of the lip long ciliate-hispid along the margins — ***L. nymphalis***

3”. Synsepal hirsute at the base; lateral lobes of the lip glabrous — ***L. chameleon***

2”. Margins of the sepals glabrous — 4

4. Lateral sepals fused almost to the apex, yellow, striped with red; lateral lobes of the lip rose-purple, large, covering the column almost to the apex — ***L. horrida***

4”.Lateral sepals fused just to the middle of their length or less, solid red or yellow, sometimes with a basal reddish blotch, but never striped; the lateral lobes of the lip yellow to orange, small, only covering the basal part of the column — 5

5.Flowers red; upper lobes of the petals long, narrowly linear, acute; lower lobe of the petals as long as the upper lobe; blades of the lip diverging at apex — ***L. genetoapophantica***

5”.Flowers yellow; upper lobes of the petals short, elliptic to rounded, sub truncate; lower lobe of the petals three times longer as the upper lobe; blades of the lip parallel at apex — 6

6. Upper lobes of the petals oblong-elliptic, acute, subtruncate; lower lobe of petals subequal to the upper lobe (elliptic); appendix shorter than the conectives of the lip — ***L. maxonii***

6”. Upper lobes of the petals rounded, lower lobe of the petals three times longer as the upper lobe (filiform); appendix longer or as long as the connectives of the lip — ***L. amicitiae***

Table 4.4. The 10 loci with the best performance (highest PI values) in both ML-305 and ML-423 analyses.

Dataset	Loci ID	Product	Maximum PI value	Max. PI value at time (t)	Length (bp)	Genome
RaxML 423						
	T272_L184	inhibitor of Bruton tyrosine kinase (IBTK)	65.38	0.99	2598	Nuclear
	T272_L45	histidine biosynthesis bifunctional protein hisIE (HISN2)	53.76	0.85e	1595	Plastid
	T272_L277	tRNA:m(4)X modification enzyme TRM13 (TRMT13)	48.78	0.44	1197	Nuclear
	T272_L317	transcription factor bHLH140 (BHLH140)	47.44	0.61	1592	Nuclear
	T272_L217	uracil-DNA glycosylase (UNG)	47.31	0.56	695	Mitochondrial
	T272_L8	Ribosomal-protein-alanine-acetyltransferase (Rps13)	45.20	0.75	873	Nuclear
	T272_L162	chromatid cohesion protein (DCC1)	45.11	0.95	1783	Nuclear
	T272_L55	cytochrome c biogenesis protein CCS1 (Ccs1)	45.00	0.67	1950	Plastid
	T272_L248	Uncharacterized	44.88	0.79	1272	uncharacterized
	T272_L420	yrdC domain-containing protein (YRDC)	43.35	0.73	1296	Mitochondrial
RaxML 305						
	T272_L184	inhibitor of Bruton tyrosine kinase (IBTK)	67.58	0.99	2598	Nuclear
	T272_L45	histidine biosynthesis bifunctional protein hisIE (HISN2)	57.82	0.77	1595	Plastid
	T272_L217	uracil-DNA glycosylase (UNG)	50.20	0.56	695	Mitochondrial
	T272_L55	cytochrome c biogenesis protein CCS1 (Ccs1)	50.14	0.59	1950	Plastid
	T272_L162	chromatid cohesion protein (DCC1)	47.98	0.88	1783	Nuclear
	T272_L248	uncharacterized	47.58	0.74	1272	uncharacterized
	T272_L420	yrdC domain-containing protein (YRDC)	46.10	0.68	1296	Mitochondrial
	T272_L286	PCNA domain-containing protein (PCNA)	45.17	0.77	879	Nuclear
	T272_L365	calcium sensing receptor (CAS)	44.83	0.94	1674	Plastid
	T272_L352	glycosylphosphatidylinositol anchor attachment 1 protein (GPAA1)	44.04	0.94	1607	Nuclear

4.5.1 *Lepanthes amicitiae* Bogarín & Pupulin, Molec. Phylogen. Evol., 129: 40–43. 2018., Figs. 4.8F and 9A.

Type: Costa Rica-Panama. Puntarenas-Bocas del Toro: Coto Brus-Valle del Risco, línea fronteriza sobre la divisoria de aguas ingresando por el camino de la Finca Sandí-Hartmann "El Capricho", 8°57'12.34"N 82°43'32.69"W, 2154 m, bosque pluvial montano bajo, 11 diciembre 2013, *A. P. Karremans 6144, D. Bogarín, M. Fernández & L. Sandoval* (holotype: JBL).
Diagnosis: This species is similar to *Lepanthes maxonii* Schltr. but it differs in the rounded upper lobe of the petals (vs. oblong-elliptic) and linear-acuminate lower lobe (vs. elliptic), subfalcate, convergent lobes of the lip (vs. straight. divergent) and hirsute appendix (vs. pubescent).
Description: Epiphytic, densely caespitose, erect herb, up to 30 cm tall. Roots slender, filiform, glabrous, to 1 mm in diameter. Ramicauls ascending to erect, 10–25 cm long, enclosed by 5–22 lepanthiform sheaths, long-ciliate along the thickened ribs, dilated at apex into a horizontal, ovate, acute ostia with densely ciliate margins. Leaf erect, subcoriaceous, elliptic, acute, emarginated with a short apiculous at apex, 3.0–3.8 cm long, 1.8–2.3 cm wide, the base cuneate into a petiole ca. 2 mm long. Inflorescence a dense, distichous, successively many-flowered (up to 20 or more flowers) raceme to 8 cm long, borne by a filiform peduncle 4.0–4.8 cm long. Floral bract broadly ovate-triangular, cucullate, obtuse, sparsely glandular, ca. 2 mm long. Pedicel terete, glabrous, 6–8 mm long. Ovary terete-subclavate, the intralocular ridges along the veins thickened into low, rounded crests, ca. 4.0 mm long. Flowers relatively large for the genus, the dorsal sepal widespread, yellow-hyaline, the veins solid yellow; the laterals sepals yellow, often with a large rose-red blotch at the base; the petals yellow to orange, sometimes purple along the mid vein and flushed purple along the lower lobe; the lip orange to orange-pink; the column rose-purple. Dorsal sepal ovate, slightly concave, acute, long acuminate, glabrous, 6.0–6.5 × 2.0–2.3 mm, connate to the lateral sepals for about 2 mm. Lateral sepals partially fused at the base into a bifid, lanceolate synsepal, 7.0–7.4 × 2.8–3.0 mm, connate for about 5 mm, the free apices narrowly acute-subacuminate. Petals transversely bilobed, linear-subfalcate, 0.5–0.7 × 3.5–3.7 mm; the upper lobe shorter and broader than the lower lobe (ca. 1mm long), rounded,the lower lobe linear-acuminate, subsigmoid. Lip 3-lobed, the lateral blades elliptic, rounded, subfalcate, 0.6–0.7 mm long, held parallel to the column, the connectives cuneate to subrectangular, connate to the column near the middle, the appendix hirsute, slender, ligulate. Column hemiterete, flattened at apex into elliptic, rounded wings, 1.2 mm long; the anther dorsal, the stigma ventral Pollinia 2, narrowly obpyriform-complanate, on an elliptic, orange brown viscidium.
Distribution and ecology: Endemic to the Cordillera de Talamanca between south-eastern Costa Rica and western Panama in montane cloud forests at 2100-2500 m.
Etymology: From the Latin *amicitia*, friendship, in allusion to La Amistad (The Friendship) International Park, a protected area which spans over southeastern Costa Rica and western Panama, where the type specimen was collected, and alluding to the friendship among the researchers of the University of Costa Rica and the University of Chiriquí, who are linked by a long-term, common floristic project.
Discussion: This species is mostly closely related to *L. maxonii*, another species with similar yellow sepals and a red blotch at the base of the synsepal. Rudolf Schlechter described *L. maxonii* from Cerro de Horqueta, Chiriquí, Panama from a collection by R.W. Maxon in 1911. The type

specimen was destroyed in the herbarium B during the Second World War, however, the drawing based on the holotype specimen shows the oblong-elliptic upper lobe of the petals, which differs from the rounded, suborbicular lobe of *L. amicitiae*.
Additional specimens examined: Costa Rica-Panama. Puntarenas-Bocas del Toro: Coto Brus-Valle del Risco, línea fronteriza sobre la divisoria de aguas ingresando por el camino de la Finca Sandí-Hartmann "El Capricho", 8°57'12.34"N 82°43'32.69"W, 2154 m, bosque pluvial montano bajo, 11 diciembre 2013, fl. 8 Jan. 2014, *D. Bogarín et al. 10751* (JBL). PANAMA. Chiriquí: Bugaba, Las Mirandas, Las Nubes, ca. 3 km al noroeste de Cerro Punta, Parque Internacional La Amistad, 2500 m, colectada por E. Olmos, setiembre 2008, floreció en cultivo en Finca Drácula, Chiriquí, Guadalupe, Panamá, 10 diciembre 2008, *D. Bogarín 5911* (JBL).

4.5.2 *Lepanthes chameleon* Ames, Schedul. Orchid. 4: 28. 1923. Figs. 4.8D and 4.10C.

Type: Costa Rica: near Cartago, *C. H. Lankester s.n*. (holotype: AMES; detail of type: AMES).
Distribution and ecology: Endemic to the Cordillera de Talamanca, Costa Rica in montane cloud forests at 2200-2700 m.
Etymology: From the Latin *chameleon*, "a ground lion", a group of lizards called *chameleon*s (Chamaeleonidae) which are able to change their skin coloration under certain circumstances. Oakes Ames noted that the purple color of young flowers of *L. chameleon* fades away in mature flowers, hence the comparison.
Discussion: This species is closely related to *L. genetoapophantica* and *L. horrida*, but it is easily distinguished by the ciliate-dentate sepals and the hirsute synsepal (mostly at the base) contrasting with the entire, glabrous sepals of *L. genetoapophantica* and *L. horrida*.

4.5.3 *Lepanthes genetoapophantica* Bogarín & Gravend., Molec. Phylogen. Evol. 129: 44. 2018., Figs. 4.8C and 4.10D.

Type: Costa Rica. Puntarenas: Coto Brus, Sabalito, Zona Protectora Las Tablas, 15 km al noreste de Lucha, Sitio Tablas, Finca Sandí-Hartmann "El Capricho", camino a El Surá, 8°57'0.63"N 82°44'59.72"W, 2017 m, bosque pluvial montano bajo, 10 diciembre 2013, *D. Bogarín 10644, A. Karremans, M. Fernández & L. Sandoval* (holotype: JBL).
Diagnosis: This species is similar to *Lepanthes horrida* but it differs in the linear-subfalcate petals, the yellowish, diverging, sub-trapezoid lobes of the lip, the lower apices of the lip not reaching the anther cap, the appendix extending beyond the lower apex of the lip and the truncate apex of the column.
Description: Epiphytic, densely caespitose, erect herb, up to 25 cm tall. Roots slender, filiform, glabrous. Ramicaus ascending to erect, stout, 8–13 cm long, enclosed by 4–12 lepanthiform sheaths, long papillous-ciliate along the thickened ribs, dilated at apex into an oblique, ovate, acuminate ostia with densely ciliate margins. Leaf erect, coriaceous, ovate to elliptic, acute, emarginated with a short apiculous at apex, 3.5–5.5 cm long, 1.5–2.5 cm wide, the base cuneate into a distinct petiole 2–3 mm long. Inflorescence a dense, distichous, successively many-flowered (up to 40or more flowers) raceme to 12 cm long, borne by a filiform peduncle 2–3 cm long. Floral bract broadly ovate-triangular, cucullate, acute, glabrous, ca. 2 mm long. Pedicel terete,

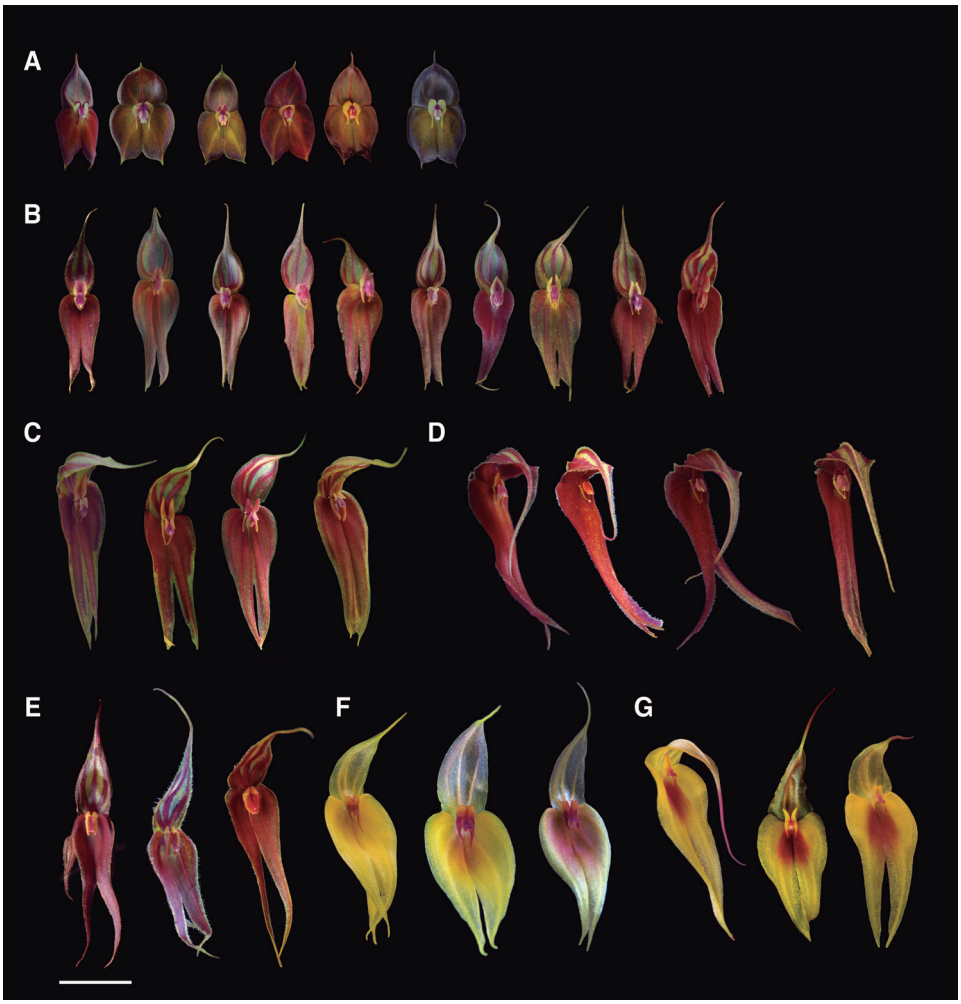

Figure 4.8. Flower morphology and variation among individuals of **A.** *Lepanthes wendlandii*, **B.** *L. horrida*, **C.** *L. genetoapophantica*. **D.** *L. chameleon*. **E.** *L. nymphalis*. **F.** *L. amicitiae*. **G.** *L. maxonii*.

glabrous, 5 mm long. Ovary terete-subclavate, the intralocular ridges along the veins provided with low, semi hyaline-cartilaginous crests, 3.5–4.0 mm long. Flowers relatively large for the genus, the dorsal sepal widespread, yellow, suffused with purple along the veins and the margins; the laterals red, edged in yellow on the external margin toward the apex; the petals yellow, suffused with orange-red at the base; the lip yellow; the column rose-purple. Dorsal sepal elliptic, concave, acute, long acuminate, glabrous, 1.2–1.4 × 0.4–0.6 cm, connate to the lateral sepals for about 1 mm. Lateral sepals fused at the base into a bifid, lanceolate synsepal, 1.2–15 × 0.5–6 cm, connate for about 8 mm, the free apices narrowly acute. Petals transversely bilobed, linear-subfalcate, 0.8–1.0 × 3.7–4.5 mm, the outer margin between the lobes provided with a minute, rounded apiculum; the upper lobe shorter and broader than the lower lobe, linear, rounded, the

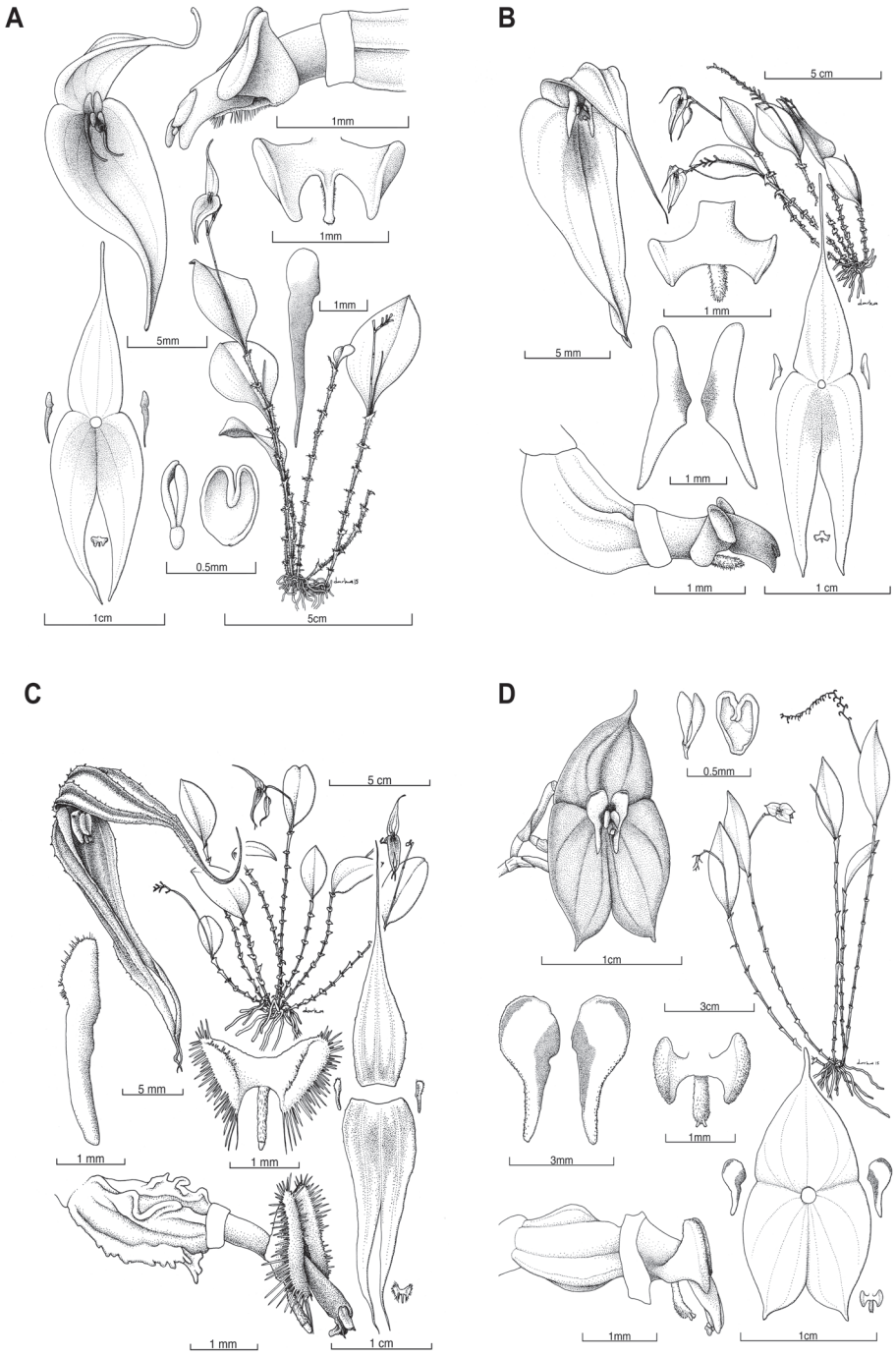

Figure 4.9. Composite-line drawings of **A.** *L. amicitiae* (Bogarín 10751). **B.** *L. maxonii* (Bogarín 5914). **C.** *L. nymphalis* (Bogarín 8307). **D.** *L. wendlandii* (Pupulin 6711).

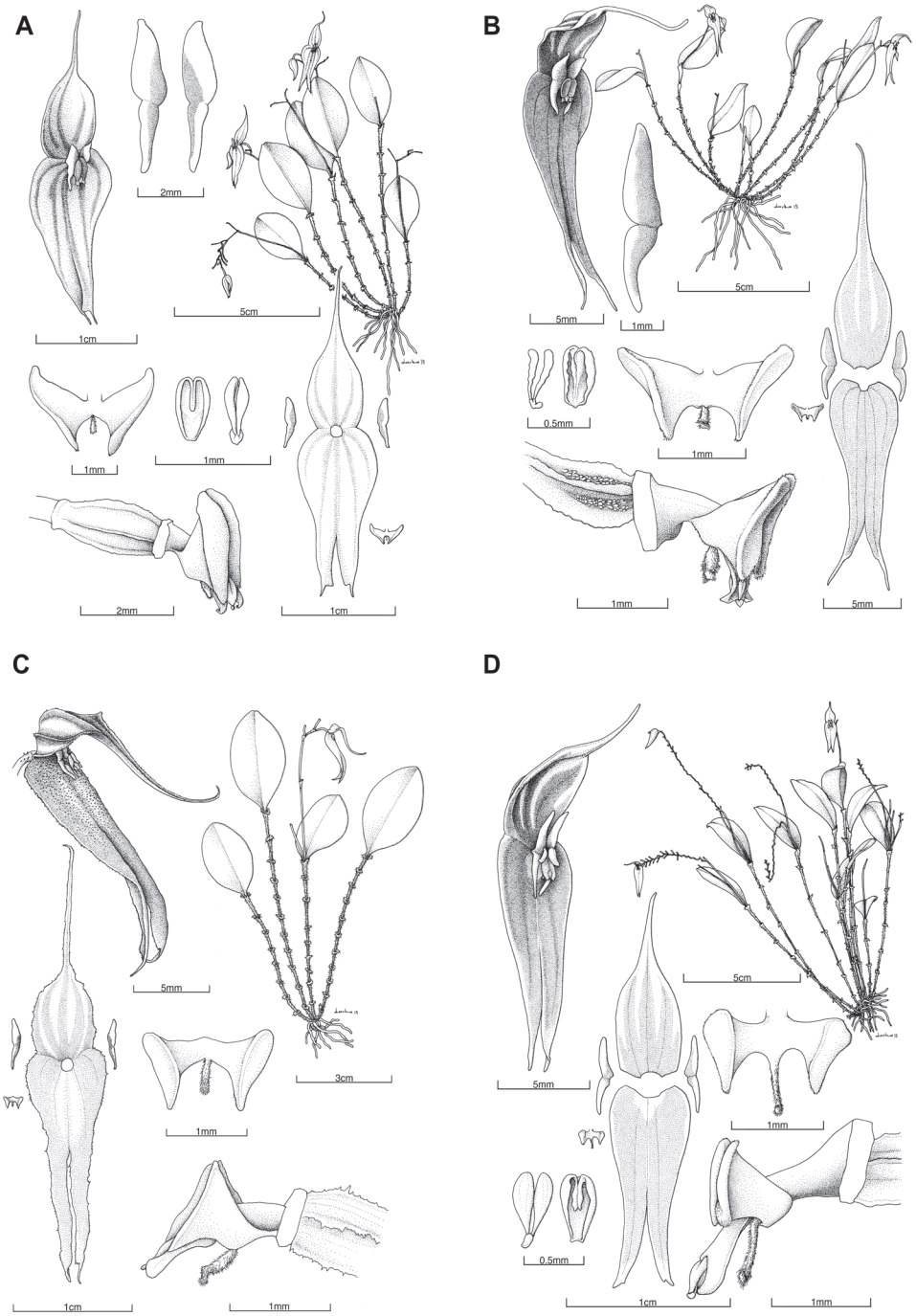

Figure 4.10. Composite-line drawings of **A.** *L. horrida* (Bogarín 272). **B.** *L. horrida* (Bogarín 11489). **C.** *L. chameleon* (Pupulin 4277). **D.** *L. genetoapophantica* (Bogarín 10644).

lower lobe subfalcate, acuminate, rounded at the apex. Lip 3-lobed, the lateral blades subtrapezoid, rounded, ca. 1 mm long, the apices diverging when erect, the connectives cuneate, connate to the column near the middle, the appendix pubescent, slender, ligulate, with an apical subquadrate gland. Column hemiterete, flat, dilated at apex into elliptic, acute wings, ca. 2 mm long; the anther dorsal, the stigma ventral Pollinia 2, narrowly obpyriform, sub attenuate at the base, on a elliptic, orange brown viscidium.

Distribution and ecology: Endemic to the Cordillera de Talamanca between south-eastern Costa Rica and western Panama in montane cloud forests at 2183-2624 m.

Etymology: Named after the Greek words γένεσις (genesis), origin, generation, and ἀποφαίνω (apophaino), to make visible, in reference to the genetic work, carried out with the aid of next generation sequencing techniques, that revealed the hidden identity of this species among its relatives.

Discussion: This species is closely related to *L. chameleon* and *L. horrida*, all with similar red flowers. It was confused by us with *L. maxonii* however, after studying the type material we realized that it corresponds to the yellow-flowered species most closely allied to *L. amicitiae*. Therefore, we proposed it here as a new species. From similar *L. horrida* it differs in the linear-subfalcate petals (vs. ovate, erect), the yellowish, diverging, sub-trapezoid lobes of the lip (vs. pink, parallel, ovate-elliptic), the lower apices of the lip not reaching the anther cap (vs. reaching the anther cap), the appendix extending beyond the lower apex of the lip (vs. shorter, not extending) and the truncate apex of the column (vs. cleft). From *L. chameleon* it differs in the glabrous, entire sepals (vs. hirsute, denticulate).

Additional specimens examined: Costa Rica. Limón: Talamanca: Bratsi, Parque Internacional La Amistad, Valle del Silencio, camio del refugio hacia el jardín (Turbera), orillas del Río Terbi, bosque pluvial montano, 2471 m, 9°07’05.12”N 82°57’40.95”W, 15.08.2012, *D. Bogarín et al. 9817* (JBL); same collection data, *D. Bogarín et al. 9842* (photo-JBL); Bratsi, Parque Internacional La Amistad, Valle del Silencio, camino del refugio hacia el jardín (Turbera) antes de cruzar el Río Terbi, bosque pluvial montano, 2411 m, 9°07’45.53”N 82°57’31.23”W, 18.9.2014, *A. Karremans et al. 6395* (JBL). Puntarenas: Buenos Aires, Buenos Aires, Olán, de la falda noreste del Cerro Tinuk hacia la falda sureste de Cerros Utyum, 9°17’33.9” N 83°09’47.5” W, 2587 m, bosque pluvial montano bajo, epífitas en bosque primario, 26 julio 2012, *D. Bogarín et al. 9745* (JBL); Buenos Aires, Buenos Aires, Olán, de la falda noreste del Cerro Tinuk hacia la falda sureste de Cerros Utyum, 9°17’37.1” N 83°09’40.1” W, 2624 m, bosque pluvial montano bajo, epífitas en bosque primario, 26 julio 2012, *D. Bogarín et al. 9752* (JBL); Puntarenas-Chiriquí: Coto Brus-Renacimiento, línea fronteriza hacia el Cerro Pando, después del mojón N.338, 8°55’11.22”N 82°43’18.18”W, 2446 m, bosque muy húmedo montano bajo, epífitas en bosque primario, “*in sylvis virginis versus montium Pando in itinere ad summum Costa Rica austro-orientalis in finibus utrimque Costa Rica et Panama*”, 19 abril 2011, *D. Bogarín et al. 8682* (photo). PANAMA. Chiriquí: Bugaba, Cerro Punta, Parque Internacional La Amistad, sendero Las Nubes, Mirador La Nevera, 8°54’00.3”N 82°37’12.8”W, 2436 m, bosque pluvial montano bajo, epífitas en *Podocarpus* sp., J & L. Harrison, Z. Samudio & Z. Serracín, 25 febrero 2014, florecieron en cultivo, 3 marzo 2014, *D. Bogarín 10974* (UNACHI). Renacimiento, Santa Clara, Cotito, camino a la divisoria de la sierra, 8°53’57.7”N 82°42’07.8”W, 2183 m, bosque pluvial montano bajo, epífitas en bosque secundario, 6 marzo 2014, *D. Bogarín et al. 10986* (UCH);

Guadalupe, camino de Finca Drácula al Parque Internacional La Amistad, 1200 m, epífitas en bosque secundario a orillas del camino, en cultivo Finca Drácula, 12 diciembre 2006, *D. Bogarín 2966 & R.L. Dressler* (JBL); Bugaba, Cerro Punta, Guadalupe, 2000 m, planta colectadas por Erick Olmos & A. Maduro, sin más datos de recolecta, en cultivo en Finca Drácula, 19 diciembre 2008, *D. Bogarín 5961* (JBL).

4.5.4 *Lepanthes horrida* Rchb.f., Beitr. Orchid.-K. C. Amer. 91. 1866. Figs. 4.8B and 4.10A-B.

Type: [Alajuela-Heredia]: Desengaño in Costa Rica, 9 May 1857, *H. Wendland s.n.* (holotype: W; illustration of type: AMES).
Distribution and ecology: Found in Costa Rica in secondary forests at elevations of 1500 to 2500 m. along the Cordillera Volcánica Central and Cordillera de Tilarán.
Etymology: Even though the Latin word *horridus* commonly refers to dreadful, horrible, or inspiring fear. However, the adjective bears the alternative meaning of bristly, referring to the stiff trichomes covering the ramicauls of this species.
Discussion: This species is closely related to *L. genetoapophantica* but it differs in the ovate, erect petals (vs. linear-subfalcate), pink, parallel, ovate-elliptic lobes of the lip (vs. diverging, sub-trapezoid) and the shorter appendix, not extending beyond the lower apex of the lip (vs. extending far beyond the lobes of the lip).

4.5.5 *Lepanthes maxonii* Schltr., Repert. Spec. Nov. Regni Veg. 12: 204. 1913. Figs. 4.8G and 9B.

Type: Panama. An Gaumstämmen in feuchten Wäldern zwischen Alto de Las Palmas und dem Gipfel des Cerro de Horqueta (Chiriqui), 2100-2268 m, blühend im Mar 1911, *W.R. Maxon 5494* (holotype: B, destroyed; isotypes: NY, US; illustrations of type: AMES-100633, AMES-100634).
Distribution and ecology: Endemic to Panama around Cerro Horqueta, Chiriquí in the Cordillera de Talamanca at 2100-2268 m in cloud forests.
Eponymy: Named after William Ralph Maxon (1877–1948), American botanist, who worked for the United States National Museum, a part of the Smithsonian Institution.
Discussion: This species is closely related and morphologically similar to *L. amicitiae*, both having similar yellow flowers. However, *L. maxonii* differs in the oblong-elliptic upper lobe of the petals (vs. rounded, suborbicular in *L. amicitiae*), the lower lobe of the petals being subequal to the upper lobe (vs. filiform) and the appendix shorter than the connectives of the lip (vs. longer or as long as the connectives).

4.5.5 *Lepanthes nymphalis* Luer, Phytologia 54: 357. 1983. Figs. 4.8E and 4.9C.

Type: Costa Rica. Heredia: epiphytic in cloud forest, Alto Gallito, alt. 2000 m, beyond the pass north of El Castillo, 21 June 1981, *C.A. Luer & J. Luer 6356* (holotype: SEL; isotype: CR).
Distribution and ecology: Endemic to the Cerro Delicias and Alto Gallito in the Cordillera Volcánica Central, Heredia, Costa Rica at around 2000 m. It grows epiphytically in cloud forests.

Etymology: From the Latin *nymphalis*, "of a nymph, a mythological woodland deity," referring to the dark, mossy, wooded habitat of the species.
Discussion: This is a very distinctive species related to *L. amicitiae* and *L. maxonii*, from which it differs in the red flowers and the long ciliate-hispid blades of the lip.

4.5.6 ***Lepanthes wendlandii*** Rchb.f., Beitr. Orchid.-K. C. Amer.: 91. 1866. Figs. 4.8A and 4.9D.

Type: Vulkan de Barba in Costa Rica, 11 Jul 1857, *H. Wendland s.n.* (holotype: W; illustration of type: AMES).
Distribution and ecology: This species is found in oak cloud forest along the Cordillera Volcánica Central and the Cordillera de Talamanca in Costa Rica and Panama at 2200-2800 m.
Eponymy: Named after the German botanist, collector and gardener Hermann Wendland (1825–1923), from the Herrenhauser Gardens in Hannover, Germany.
Discussion: This is the most divergent species of the group characterized by the glabrous sheaths of the ramicaul and the broadly ovate-orbicular synsepal with free obtuse apices (not elongated as in the other related species).

Appendix A. Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.07.014.

Chapter 5

Two new *Lepanthes* (Orchidaceae: Pleurothallidinae) from Panama

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Nordic Journal of Botany 36(1–2): 1-8. 2017.

Abstract. Panama is still far from completing its orchid flora inventories, where large genera such as *Lepanthes* (Pleurothallidinae) reveal novelties regularly. Here, we describe and illustrate two new species of *Lepanthes*. *Lepanthes aures-ursinae* is similar to *L. micellilabia* but differs by the orbicular-ovate, obtuse, convex leaves, larger sepals 2.7–3.0 × 2.2–2.3 mm the yellow petals, shorter column to 1 mm long and Y-shaped bi-laminate lip with the blades embracing the column and the body elongated towards the base of the column forming a cylindric structure. *Lepanthes vertebrata* is most similar to *L. demissa*, from which it can be distinguished by the inflorescences bearing 20–73 pedicels in well-developed inflorescences, the vinaceous flowers, the larger lateral sepals to 6.0 × 2.3 mm, wider petals to 4.5 mm with the lower lobe longer than the blades of the lip, narrowly oblong upper lobe of petals, oblong lower lobe, shorter lip ca. 1 mm long with ovate pink blades and ventral stigma. Data on distribution, habitat and ecology, etymology and phenology are provided for each species.

5.1 Introduction

Comprising about 1,000 species, *Lepanthes* Sw. is one of the largest genera of the Pleurothallidinae (Luer and Thoerle, 2012). The genus ranges from southern Mexico and the Antilles to Peru and Bolivia, with a few species in the Guianas and Brazil. The highest diversity is found in the Andean region of Colombia and Ecuador with more than 300 species in each country (Luer, 1996c; Luer and Thoerle, 2012). In Mesoamerica, most of the species are concentrated in the southeast, in the highlands of Costa Rica and Panama (Ossenbach et al., 2007). Plants of *Lepanthes* are mostly found in montane and premontane rain forests at elevations of 1000–2500 m a.s.l. in the Cordillera de Talamanca, which extends from central Costa Rica towards western Panama. However, a number of species are recorded in humid mid-elevation to coastal lowlands (below 1,200 m a.s.l.) such as the slopes of Cerro Azul (571 m a.s.l.), Campana (1,030 m a.s.l.), Gaital (1,185 m a.s.l.), and Jefe (1007 m a.s.l.) in central Panama, or the Caribbean plains of the Bocas del Toro province (Luer and Dressler, 1986). Few records are known from the provinces of Colón, Darién and Los Santos where intensive fieldwork is needed (Bogarín et al., 2013). The level of endemism in *Lepanthes* is high. The species often have narrow distributions, and they are usually found in certain ridges or mountains with similar geological or climatic characteristics (Luer and Thoerle, 2012). For instance, Costa Rica and Panama share 41 species (66% of the species known to Panama), most of which are endemic to the Cordillera de Talamanca (Bogarín et al., 2014b). *Lepanthes* has been little studied in Panama. Extensive collections led by Henri Pittier and William R. Maxon during 1911 in the region of Cerro de La Horqueta, in Boquete, Chiriquí, led to the description of the first two known species for the country: *Lepanthes* eciliata Schltr. and *Lepanthes maxonii* Schltr. (Schlechter, 1913). In 1915, Charles H. Powell continued the botanical exploration, mostly based in the Canal Zone and Chiriquí highlands. He collected another species named *L. chiriquensis* Schltr. (Schlechter, 1922). In the Orchidaceae chapter for the flora of Panama, (Williams, 1946) provided the first treatment of the genus for Panama. Based upon collections by Mary E. Spence Davidson and Paul H. Allen, L.O. Williams included five species, in addition to the three species described by (Schlechter, 1922). There were no further additions until 1984, when (Luer, 1984; Luer, 1996a, 1997) and (Luer and Dressler, 1986) published several species new to Panama. (Luer, 2002) described 26 species and added 10 new records for the country (mostly species already described from Costa Rica). Later, he reduced five species to synonymy (Luer, 2003a). Only one species has been described after Luer‘s contributions (Pupulin et al., 2009). Our latest account of the genus in Panama revealed 66 species, 21 of which are endemics (Bogarín et al., 2014b). As part of the botanical activities for the project aimed to complete the inventory of the Orchidaceae of Panama carried out by Jardín Botánico Lankester of the Universidad de Costa Rica and Herbario UCH of the Universidad Autónoma de Chiriquí, we are currently revising the genus. Herein, we describe and illustrate two new species.

5.2 Materials and Methods

This study was conducted at Herbario UCH of the Universidad Autónoma de Chiriquí, Panamá and Finca Drácula, Guadalupe, Chiriquí. Sketches and images were prepared from living specimens with a Leica® MZ 9.5 stereomicroscope with drawing tube, Nikon® D7100 digital

camera with a AF-S VR Micro-NIKKOR 105mm f/2.8G IF-ED lens and Epson Perfection Photo Scanner V600. Composite plates were diagrammed in Adobe Photoshop CS6. Ink drawings were prepared on smooth Fabriano paper of 240 g m with a Rotring Rapidograph 0.1 mm using black capillary cartridges and traced in Artograph LightPad A920. Herbarium specimens were deposited at UCH and PMA. Phenological data were recorded in the field and from cultivated specimens. The map and georeferences for specimens were obtained by using Google Earth and data from JBL, MO, PMA and UCH herbaria.

5.3 Taxonomic treatment

5.2.1 ***Lepanthes aures-ursinae*** Bogarín and Serracín, Nordic J. Bot., 36 (1–2(e01292)): 2–58, f. 1A–F, 2A. 2017. (Fig. 5.1, 5.2A, 5.3).

Diagnosis: A Lepanthi micellilabia Luer and Escobar foliis orbicularibus obtusis convexis, sepalis latioribus, petalis flavis, laminis labelli amplectentibus, connectivis basi elongatis, columna brevioribus differt.
Type: Panama, Coclé, El Valle de Antón, La Pintada, collected by E. Olmos and A. Maduro, 1,200 m a.s.l., without further locality data, cultivated in Finca Drácula, Chiriquí, Guadalupe, Panamá 2006, flowered in cultivation at Finca Drácula, Guadalupe, Cerro Punta, Chiriquí, 10 Dec 2008, *D. Bogarín 5932* (holotype: UCH).
Etymology: The epithet is derived from the Latin *auris*, 'ears' and *ursus*, 'bear', in reference to the appearance of the petals resembling a bear's ears.
Description: Plant epiphytic, caespitose, erect, to 5.2 cm tall. Roots slender, flexuous, up to 0.5 mm in diameter. Ramicaul erect, up to 1.5–3.3 cm, enclosed by 6–8, tightly fitting, glabrous, blackish lepanthiform sheaths, each 4–6 mm long; ostia slightly dilated, acute. Leaves convex, erect, coriaceous, with a prominent median vein, green, orbicular or oblong-ovate, obtuse; emarginate with a short apiculus, 1.5–1.9 × 1.4–1.6 cm; the rounded base abruptly contracted into a petiole to 1.5 mm long. Inflorescence racemose, distichous, successively flowered, remaining on abaxial surface of the leaf, shorter than the leaves, to 1.2 cm long, with peduncle 9 mm long and rachis 3 mm. Floral bracts to 1 mm long, triangular-ovate, glabrous. Pedicel to 1.3 mm long, persistent. Ovary to 0.8 mm long. Flowers with yellow sepals, the base of the lateral sepals tinged with red; petals yellow with red lip. Dorsal sepal ovate, acute, dorsally carinate, entire, slightly convex at the base, connate to the lateral sepal for about 0.7 mm, 3.0 × 2.3 mm, 3-nerved. Lateral sepals ovate, acute, flat, entire, connate at the base for about 1.2 mm, 2.7 × 2.2 mm, 2-nerved. Petals reniform-suborbicular, minutely papillose, obscurely bilobed, inconspicuous, 0.5 0.7 mm; lobes rounded, subsimilar. Lip bi-laminate, adnate to about the middle of the column, Y-shaped, 1.1 × 1.2 mm; blades transversely triangular, with rounded apices; connectives cuneate, embracing the column; body elongated towards the base of the column but not adnate to the column, forming a cylindric structure with some part of the tissue folding, up to 0.8 mm long; appendix cylindric, very small, minutely ciliate. Column cylindric, 1 mm long; anther apical; stigma subapical. Pollinia two, ovoid, joined at the base by an obovate viscidium. Anther cap cucullate.
Phenology: The species has been recorded in flower throughout the year but mostly from December to June.

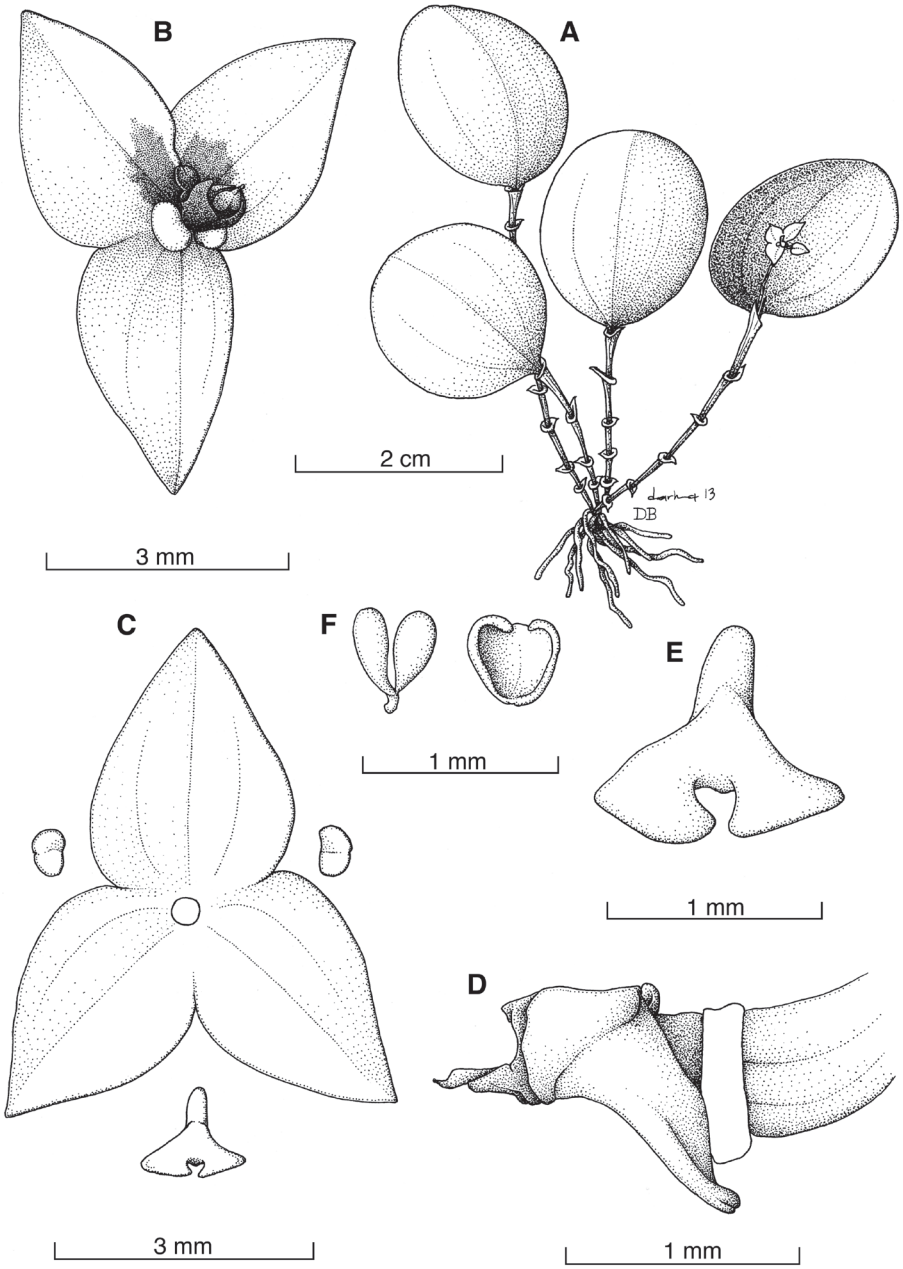

Figure 5.1. *Lepanthes aures-ursinae*, **A.** habit. **B.** flower. **C.** dissected perianth. **D.** ovary, column and lip (lateral view). **E.** lip (spread showing the cylindric structure made up by the elongation of the body). **F.** pollinarium and anther cap. Drawn by D. Bogarín and D. Solano from the holotype.

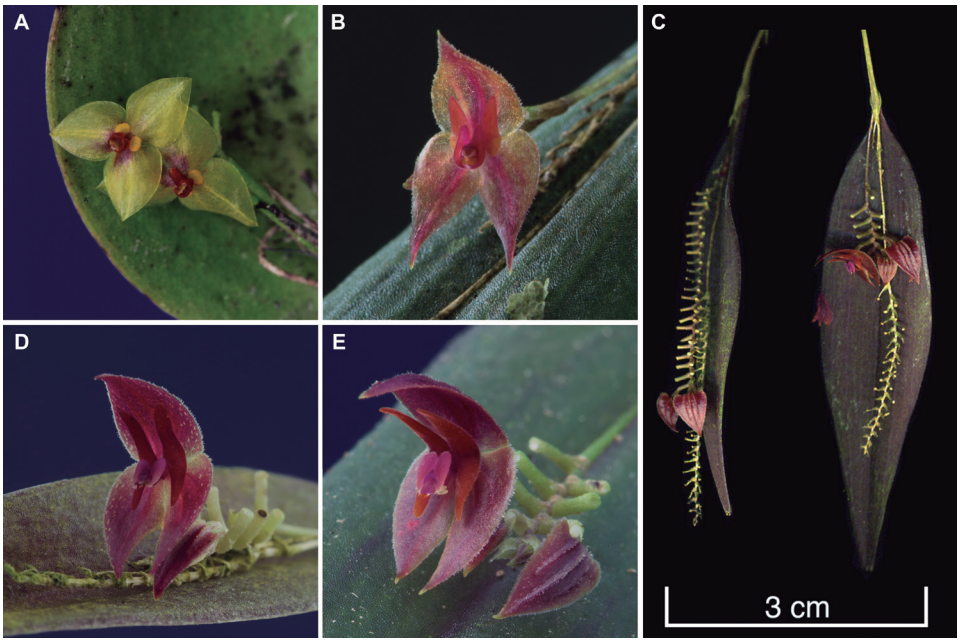

Figure 5.2. *Lepanthes aures-ursinae,* **A.** flower (Bogarín 5932). *Lepanthes demissa*, **B.** flower (Bogarín 10973). *Lepanthes vertebrata* **C.** detail of the inflorescence showing the elongate rachis of the inflorescence with several persistent pedicels (Bogarín 2975). **D.** flower (Bogarín 2975), (E) flower (Serracín 1006). Photos by D. Bogarín.

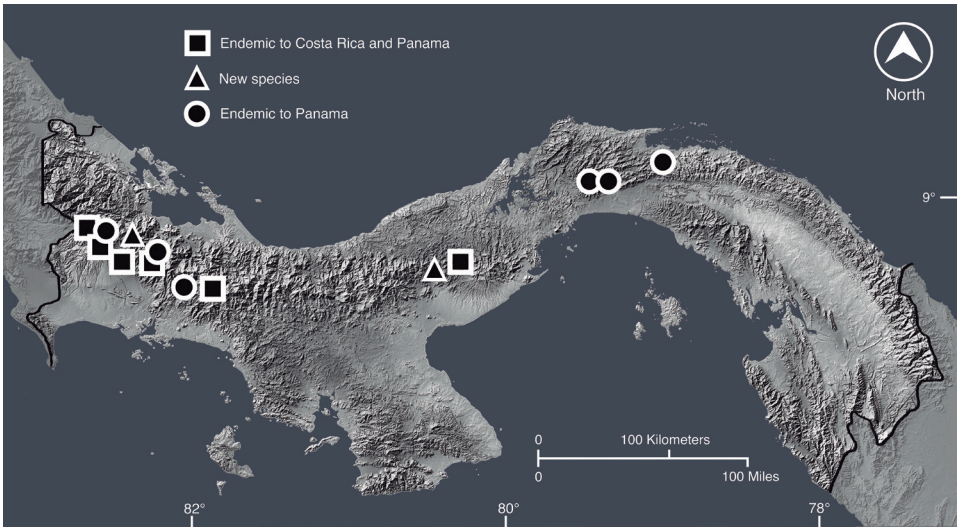

Figure 5.3. Distribution map of collecting sites of *Lepanthes* in Panama showing the endemic species (circles), endemics to Costa Rica and Panama (squares) and the new species here described (triangles).

Habitat, ecology and distribution: *Lepanthes aures-ursinae* is only known from Panama where it is an epiphyte in primary and secondary forest in Valle de Antón, Coclé, at around 1,200 m a.s.l.
Similar species: *Lepanthes aures-ursinae* is similar to *L. micellilabia*, but differs by the orbicular-ovate, obtuse, convex leaves (rather than ovate, flat, acute), the larger lateral sepals 2.7 × 2.2 mm (rather than 2.50 × 1.25 mm), the yellow petals (rather than red) and the shorter column to 1 mm long (rather than 1.5 mm long). However, the most important feature distinguishing *L. aures-ursinae* is the Y-shaped bilaminate lip with the blades embracing the column and the body that is elongated towards the base of the column forming a cylindric structure (rather than reduced lip at the base of the naked column). No other species of *Lepanthes* of Panama has this feature. Flowers of *L. aures-ursinae* have minute petals compared to the expanded triangular sepals. This characteristic is also present in several other species of *Lepanthes*, and may have evolved several times in different unrelated groups within the genus (Pupulin et al., 2010). *Lepanthes equus-frisiae* Pupulin and H. Medina, *Lepanthes isosceles* Luer and R. Escobar, *L. micellilabia*, *L. pelorostele* Luer and Hirtz, *L. rigidigitata* Luer and Hirtz and *L. vestigialis* Bogarín and Pupulin are among the species characterized by the presence of extremely reduced, simple petals and lip. The differences among these species are summarized in Table 5.1. The plant habit of *L. aures-ursinae* is similar to the species of the *Lepanthes disticha* Garay & R.E. Schult. complex, characterized by the erect ramicauls with blackish, amplectent lepanthiform sheaths with narrow ostia and coriaceous leaves. In the new species, the leaves are orbicular-ovate and convex, resembling the habit of *L. dotae* Endres ex Luer and *L. whittenii* Pupulin and Bogarín. However, the new species can be distinguished from them mainly by the pro portionally very small, reniform-orbicular petals and the Y-shaped lip.

5.2.2 *Lepanthes vertebrata* Bogarín, Mel.Fernández and Serracín, Nordic J. Bot. 36(1–2(e01292)): 518–7, f. 2C–E, 4A–E. 2017. (Fig. 5.2C, 5.2D, 5.2E, 5.4).

Diagnosis: A Lepanthi demissa Luer inflorescentia longiore, floribus vinaceis, sepalis latioribus, lobulis petalorum subequalibus lobo infero falcato et laminis labelli ovatis roseis differt.
Type: Panama, Chiriquí, Boquete, without further locality data, collected by Erick Olmos and A. Maduro, 30 Oct 2000, flowered in cultivation at Finca Drácula, Guadalupe, Cerro Punta, Chiriquí, 12 Dec 2006, *D. Bogarín 2975* (holotype: UCH, isotype: PMA).
Etymology: From the Latin *vertebratus*, 'vertebrate, jointed' in allusion to the elongate rachis of the inflorescence with persistent pedicels forming a vertebral spine-like or fish backbone-like structure.
Description: Plants epiphytic, caespitose, pendent, to 21.5 cm tall. Roots slender, flexuous, up to 0.5 mm in diameter. Ramicaul pendent or suberect, straight, up to 2.5–15.5 cm, enclosed by 5–15-ciliate, lepanthiform sheaths, 1.0–1.8 cm long; ostia markedly dilated, acuminate. Leaves pendent, subcoriaceous, with a prominent mid vein, purplish-green, elliptic to narrowly elliptic, acute to acuminate, emarginate with a short apiculus, 5.3–8.6 × 1.3–2.2 cm, with cuneate base narrowing into a petiole ca 1 mm long. Inflorescence racemose, distichous, successively flowered, born above the leaf, in some specimens becoming longer than the leaf as it elongates and produces new flowers, to 4.0–5.5 cm or longer, forming a conspicuous, congested chain of pedicels with age (20–73 pedicels on each rachis); peduncle 1.5–2.0 cm long; rachis 2.8–4.0 cm. Flo-

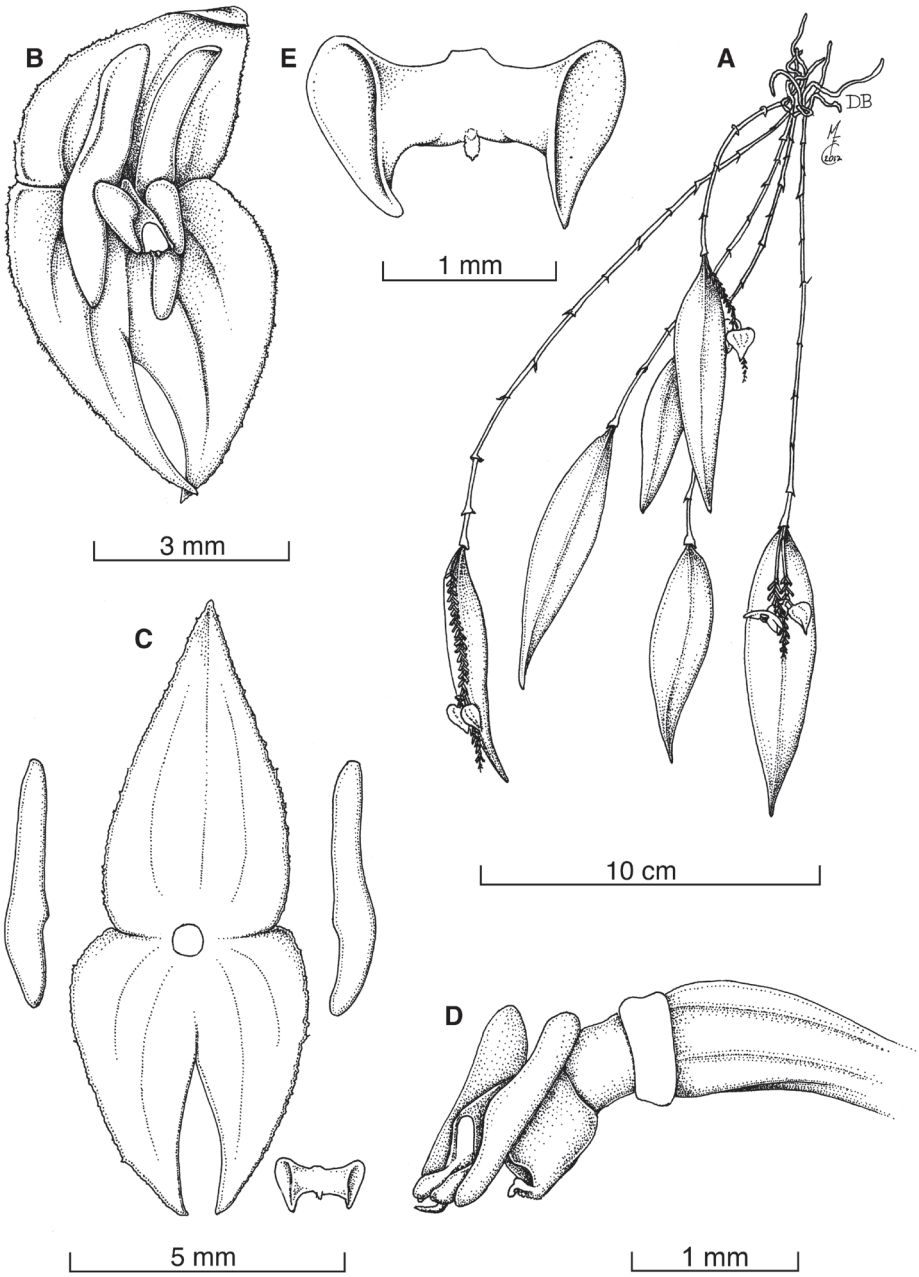

Figure 5.4. *Lepanthes vertebrata* **A.** habit. **B.** flower. **C.** dissected perianth. **D.** ovary, column and lip (lateral view). **E.** lip (front view, spread). Drawing by D. Bogarín and M. Fernández from the holotype.

Table 5.1. Morphological differences among *L. aures-ursinae* and similar species.

Character	*L. aures-ursinae*	*L. equus-frisiae*	*L. micellilabia*	*L. vestigialis*
Habit	erect	suberect-prostrate	erect	suberect-prostrate
Plant size (cm)	5.5	to 3.5	8	2.5
Flower color	yellow with red lip	reddish-brow with pink	yellow suffused with red	yellowish-pink
Dorsal sepal (mm)	3.0 × 2.3	5.0 × 1.5	2.5 × 1.5	4.0 × 1.5
Lateral sepals (mm)	2.7 × 2.2	4.5 × 1.2	2.50 × 1.25	4.0 × 1.5
Petals (mm)	reniform-suborbicular, 0.5 × 0.7 mm	reniform-suborbicular, 0.3 × 0.4 mm	oblong, 0.25 × 0.5	0.5 × 0.5
Lip (mm)	Y-shaped, 1.1	subspherical, 0.3	cordate, 0.25	triangular, 0.5
Stigma	subapical	apical	apical	subapical

ral bracts triangular-ovate, glabrous, 0.5 mm long. Pedicel to 2.5 mm long, persistent. Ovary up to 1.4 mm long, cylindrical, carinate. Flowers with red-vinaceous sepals, externally suffused with dark vinaceous color along veins, submarginal area pale yellow; petals and lip pink vinaceous. Dorsal sepal ovate, acute, concave, dorsally carinate, ciliate, connate to the lateral sepal for about 1 mm, 6.0 × 3.4 mm, 3-veined. Lateral sepals ovate, acute, subfalcate, flat, ciliate, connate at the base for about 1 mm, 6.0 × 2.3 mm, 2-veined. Petals microscopically pubescent, ciliate, transversely bi-lobed, 0.8 × 4.5 mm; upper lobes narrowly oblong, rounded at apex; lower lobes oblong, subfalcate, with rounded apex, smaller than the upper lobes. Lip bi-laminate, adnate to the column, ca 1 × 2 mm; blades ovate, microscopically pubescent, with rounded, falcate, ciliate apices; connectives cuneate, up to 1 mm long; body oblong, connate to about the middle of the column; appendix external, oblong, hirsute. Column cylindrical, ca 1.5 mm long; anther apical; stigma ventral Pollinia two, ovoid. Anther cap cucullate.

Phenology: Plants have been recorded in flower throughout the year but mostly from December to March.

Habitat, ecology and distribution: *Lepanthes vertebrata* is only known from western Panama where it is an epiphyte in primary and secondary forest on the Pacific watershed of Cordillera de Talamanca, north of Boquete, Chiriquí between 1600–2000 m a.s.l. A population was found on twigs of a Melastomataceae tree.

Similar species: *Lepanthes vertebrata* is morphologically similar to *L. demissa* Luer (Fig. 5.2B). Both species have hanging narrowly elliptic-ovate, dark-green leaves. However, *Lepanthes vertebrata* is distinguished by the inflorescences bearing 20–73 pedicels in well-developed inflorescences (vs less than 10 persistent pedicels), vinaceous flowers (vs reddish-orange), larger sepals to 6.0 × 2.3 mm (vs 5.0 × 1.8 mm), wider petals 4.5 mm (vs 1.8 mm), narrowly oblong upper lobe of petals (vs oblong-ovate), oblong lower lobe which is longer than the blades of the lip (vs ovate, shorter), shorter lip ca 1 mm long with ovate pink blades (vs 1.4 mm long with narrowly oblong, orange blades) and ventral stigma (vs apical). *Lepanthes vertebrata* is recorded between 1600–2000 m a.s.l. in premontane forests whereas *L. demissa* is found in cloud forest mostly on *Podocarpus* sp. and *Quercus* spp. above 2300 m a.s.l. Another similar species is *L.*

Table 5.2. Morphological differences among *L. vertebrata* and similar species.

Character	*L. vertebrata*	*L. demissa*	*L. machogaffensis*
Habit	pendent	pendent	suberect
Plant size (cm)	to 21.5	to 15	to 12
Rachis	2.8–4.0 cm, longer than the peduncle	2.5 cm, shorter or equal to the peduncle	1.5 cm, shorter than the peduncle
Pedicels in mature inflorescences	>20	< 10	< 10
Flower color	red-vinaceous	reddish-orange	purple-red
Dorsal sepal	6.0 × 3.4 mm	5 × 2.6 mm	7.0 × 2.5 mm
Lateral sepals	6.0 × 2.3 mm, acute	5.0 × 1.8 mm, acute to acuminate	7.0 × 1.9 mm, acuminate-attenuate
Petals	0.8 × 4.5 mm	0.5 × 1.8 mm	0.7 × 2.5 mm
Petal (upper lobe)	narrowly oblong	oblong-ovate	subrectangular
Petal (lower lobe)	oblong	ovate	narrowly ovate-subfalcate
Lip blades	ovate	narrowly oblong	ovate
Lip length	ca. 1.0 mm	1.4 mm	1.0 mm
Stigma	ventral	apical	apical

machogaffensis from Costa Rica (Pupulin et al., 2009). However, this species differs mainly by the shorter plants < 12 cm, mature inflorescences with <10 pedicels, rachis 1.5 cm, shorter than the peduncle, acuminate-attenuate sepals and apical stigma. The differences among these species are summarized in Table 5.2.

Additional specimens examined (paratypes): Panama. Chiriquí-Bocas del Toro, Cerro Pata de Macho, north of Boquete, 2000 m a.s.l., collected Mar 2005, cultivated by Steve and Marjorie Sarner, no. 624, Boquete, Panamá, 19 Dec 2008, *D. Bogarín 5995* (UCH-spirit); Panama, Chiriquí, Boquete, Los Naranjos, Bajo Mono, Sendero Culebra, orillas del Río Caldera, 8°50’55.0” N, 82 °29’36.9” W, 1904 m a.s.l., epífita a orillas del camino, 14 Jul 2014, *D. Bogarín 11151, J. Harrison, L. Harrison, Z. Samudio and Z. Serracín* (UCH); Panama, Chiriquí: Boquete, Los Naranjos, Bajo Mono, Sendero Culebra, orillas del Río Caldera, 8°50’34.1” N, 82°28’53.0”W, 1673 m a.s.l., epífitas a orillas del camino, 18 Jun 2015, *Z. Serracín 1006, D. Bogarín, Z. Samudio and C. Rodríguez* (UCH) (Fig. 5.2E).

Pollination biology

Chapter 6

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

Diego Bogarín, Melania Fernández, Art Borkent, Anton Heemskerk, Franco Pupulin, Erik Smets and Barbara Gravendeel

Botanical Journal of the Linnean Society 186: 510–543. 2018.

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

6.1 Introduction

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

Pleurothallidinae are the largest Neotropical orchid subtribe comprising > 5100 species, of which most are probably pollinated by Diptera (Pridgeon et al., 2005). Various pollination strategies are known in detail for some species of *Acianthera* Scheidw. (Phoridae), *Dracula* Luer (Drosophilidae), *Lepanthes* Sw. (Sciaridae), *Octomeria* R.Br. (Sciaridae), *Pleurothallis* R.Br (Mycetophilidae) and *Specklinia* Lindl. (Drosophilidae), all belonging to phylogenetically unrelated groups in the subtribe and pollinated by unrelated Diptera (Barbosa et al., 2009; Blanco and Barboza, 2005; Duque-Buitrago et al., 2014; Duque, 1993; Endara et al., 2010; Karremans et al., 2015b; Pansarin et al., 2016; Policha et al., 2016). One of the most interesting pollination systems of these genera evolved in *Lepanthes*, in which species are pollinated by sexual deception through genitalic pseudocopulation with male fungus gnats of the genus *Bradysia* (Sciaridae), probably attracted by a pheromone-mimicking strategy (Blanco and Barboza, 2005). *Lepanthes* is the most species-rich genus of the subtribe (> 1200 spp.), with *Masdevallia* Ruiz & Pav., *Pleurothallis* and *Stelis* Sw. accounting for 60% of all species of Pleurothallidinae. *Lepanthes* forms a monophyletic group with the much less diverse *Anathallis* Barb.Rodr., *Draconanthes* Luer, *Frondaria* Luer, *Lankesteriana* Karremans, *Lepanthopsis* (Cogn.) Ames, *Trichosalpinx* Luer s.l. and *Zootrophion* Luer (Chiron et al., 2012; Karremans, 2014; Pridgeon et al., 2001). These genera display extraordinary divergent floral morphologies suggesting adaptation to different pollinators. In addition, this clade underwent rapid speciation in the highlands of the Andes and Central America and exhibits the highest rates of species diversification in Pleurothallidinae (Givnish et al., 2016; Pérez-Escobar et al., 2017a). However, the role of pollinators as drivers of species diversity in the group is largely unknown. Apart from pollination of three *Lepanthes* spp., nothing is known about the pollination of the sister groups (Blanco and Barboza, 2005) (Fig. 6.1). To better understand the role of such biotic factors in the evolution of *Lepanthes* and close relatives, we investigated the pollination system of two *Trichosalpinx* spp. *Trichosalpinx* comprises c. 110 species, ranging from Mexico and Central America to the Andean regions of Peru and Bolivia, Venezuela, French Guiana, southern Brazil and the Antilles (Luer, 1983). The genus

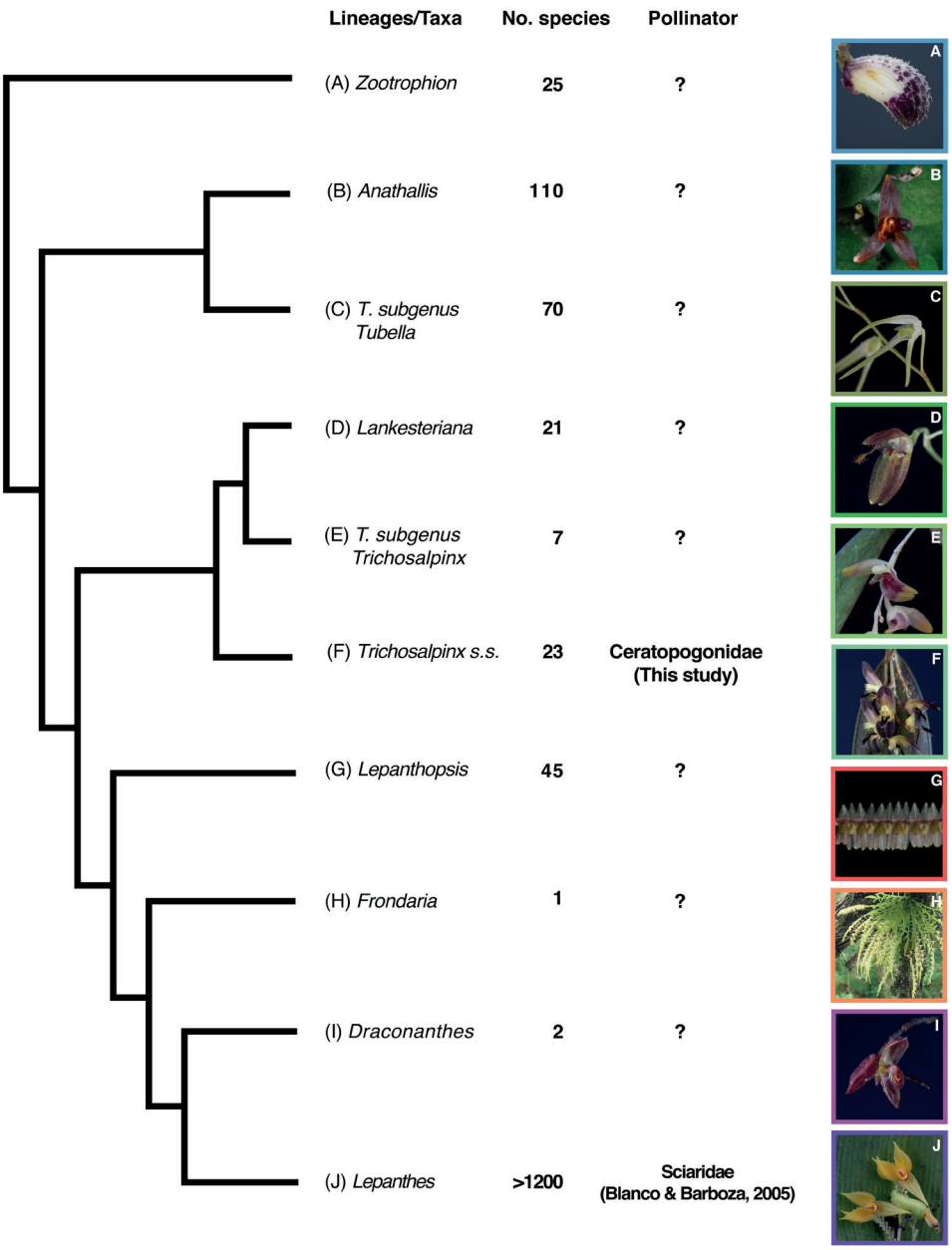

Figure 6.1. Phylogenetic summary of the *Lepanthes* clade showing the main lineages/taxa, the number of species in each taxa and pollinator information. *Lepanthes* accounts for 85% of the species of the clade. Only two cases of pollination have been documented for the entire clade. Phylogenetic tree based on our unpublished data using combined ITS and *matK* with Bayesian inference.

is polyphyletic according to initial phylogenetic evidence (Chiron et al., 2012; Karremans, 2014; Pridgeon et al., 2001). Thus, in this study we focused on three species of *Trichosalpinx* subgenus *Trichosalpinx* or *Trichosalpinx* s.s. (herein referred to as *Trichosalpinx*) that belong to one of the subclades closely allied to *Lepanthes* (Fig. 6.1). *Trichosalpinx* spp. have non-prolific ramicauls, racemose inflorescences, which are usually shorter than the leaves, and produce purple, pinkish or reddish-vinaceous flowers that open simultaneously (Fernández and Bogarín, 2011; Luer, 1997). One of the most visible features of *Trichosalpinx* flowers regarding pollination is the dark purple, ciliated lip, which is movable under the weight/momentum of the pollinators and which vibrates with the air due to the union of the lip base with the column foot through a flexible, thin labellar ligament . Motile lips also evolved independently in the closely related *Anathallis* and *Lankesteriana* (Karremans, 2014; Luer, 2006; Pridgeon et al., 2001) and other Pleurothallidinae such as *Specklinia*, *Stelis* s.l. (*Condylago* Luer), *Masdevallia* and *Porroglossum* Schltr.(Pridgeon et al., 2005). The pantropical *Bulbophyllum* Thouars (Dendrobiinae), which is another diverse but unrelated genus thought to contain many myophilous species, also exhibits a wide variety of motile lips and appendages (Bartareau, 1994; Davies and Stpiczyńska, 2014; De Pádua Teixeira et al., 2004; Kowalkowska et al., 2014; Phillips et al., 2014; Stpiczyńska et al., 2015).

Vogel (2001) conducted extensive studies on the role of motile, vibrant structures called 'flickering bodies' that are mostly present on the sepals, petals or lip in some species of the *Bulbophyllum*, *Pleurothallis*, *Specklinia* Lindl. and *Trichosalpinx*. They are diverse in structure and comprise appendages, trichomes, cilia or vibratile hairs. These structures are associated with deceptive systems, which involve mimicry of insect prey models (Gibernau et al., 2004; Heiduk et al., 2016, 2015, 2010; Oelschlägel et al., 2015). Vogel (2001) considered flowers bearing flickering bodies as deceptive, even if they offer some nectar rewards. However, most of these pollination syndromes have not been tested experimentally. Dark purple, motile floral appendages are present in several unrelated angiosperm families (magnoliids, monocots and eudicots) such as Aristolochiaceae (*Aristolochia* L. and *Pararistolochia* Hutch. & Dalziel), Apocynaceae (*Caralluma* R.Br., *Ceropegia* L., stapeliads), Malvaceae (*Abroma* Jacq., *Herrania* Goudot and *Theobroma* L.) and Orchidaceae (*Bulbophyllum*, *Caladenia* R.Br., *Disa* P.J.Bergius, *Genoplesium* R.Br., some Pleurothallidinae, *Pterostylis* R.Br. and *Telipogon* Kunth) (Jürgens et al., 2006; Meve and Liede, 1994; Ollerton et al., 2009; Phillips et al., 2014; Vogel, 2001; Williams and Adam, 2010; Young and Severson, 1994). Pollination strategies in some of these taxa involve dipterans belonging to different groups such as Ceratopogonidae (biting midges), Chloropidae (grass flies), Drosophilidae (vinegar flies), Milichiidae (filth flies), Cecidomyididae, Sciaridae and Phoridae (gall and fungal gnats), Sarcophagidae (flesh flies) and Calliphoridae (blowflies) (Bartareau, 1994; Borba and Semir, 1998; Gamisch et al., 2014; Heiduk et al., 2015; Humeau et al., 2011; Ollerton et al., 2009; Stpiczyńska et al., 2015; Woodcock et al., 2014). In this study, we identified and documented the behaviour of pollinators of two *Trichosalpinx* spp., described the anatomy, ultrastructure and histochemistry of the flowers and compared these results with other angiosperms pollinated by similar insects to address the following questions: (1) What is the pollination mechanism of *Trichosalpinx*? (2) What is the function of the motile lip and how does the flower attract pollinators? (3) What are the anatomical features shared with other plants pollinated by similar insects? By answering these questions, we hope to improve understanding of the evolution of members of the *Lepanthes* clade.

6.2 Material and Methods

6.2.1 Study site and sample collection

We studied the pollination of *Trichosalpinx blaisdellii* (S.Watson) Luer and *Trichosalpinx reflexa* Mel.Fernández & Bogarín (Fig. 6.2) in semi-open greenhouses at Lankester Botanical Garden (JBL), University of Costa Rica, Cartago, and San Miguel de Santo Domingo, Heredia, Costa Rica. In addition, we studied a wild population of *T. reflexa* occurring along the shores of the Turrubaritos river, Turrubares, San José, Costa Rica between 2014 and 2016 (Supporting Information, Table 6.S1). *Trichosalpinx* reflexa is endemic to the lowland areas of the northern and central Pacific of Costa Rica. Plants bloom during the rainy season from August to February and form large populations mostly on 'wild cashew' or 'espavel', *Anacardium excelsum* (Bertero & Balb. ex Kunth) Skeels (Anacardiaceae), and on species of *Ficus* L. (Moraceae). *Trichosalpinx blaisdellii* is found between 0 and 1800 m and blooms between June and March (Luer, 1997). Midges were filmed, photographed and collected with a pooter between 7:00 and 17:00 and at night between 18:00 and 19:00 for a total of c. 36 h of observation. Samples were stored in absolute ethanol for DNA barcoding, and other samples were mounted on microscope slides for morphological identification following Borkent and Bissett (1990). Midge vouchers were deposited at JBL, L, Canadian National Collection (Ottawa, Canada) and Museo de Insectos of the University of Costa Rica (San José, Costa Rica). Plant vouchers were deposited at CR, JBL (spirit), L, and USJ (Supporting Information, Table 6.S1).

6.2.2 Photography, video and digital imaging

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

6.2.3 Fixation of flowers for microscopy

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

6.2.4 Insect DNA barcoding identification

We extracted total genomic DNA from midge leg tissue with the Dneasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) and obtained a 666 bp DNA barcode fragment of the cytochrome

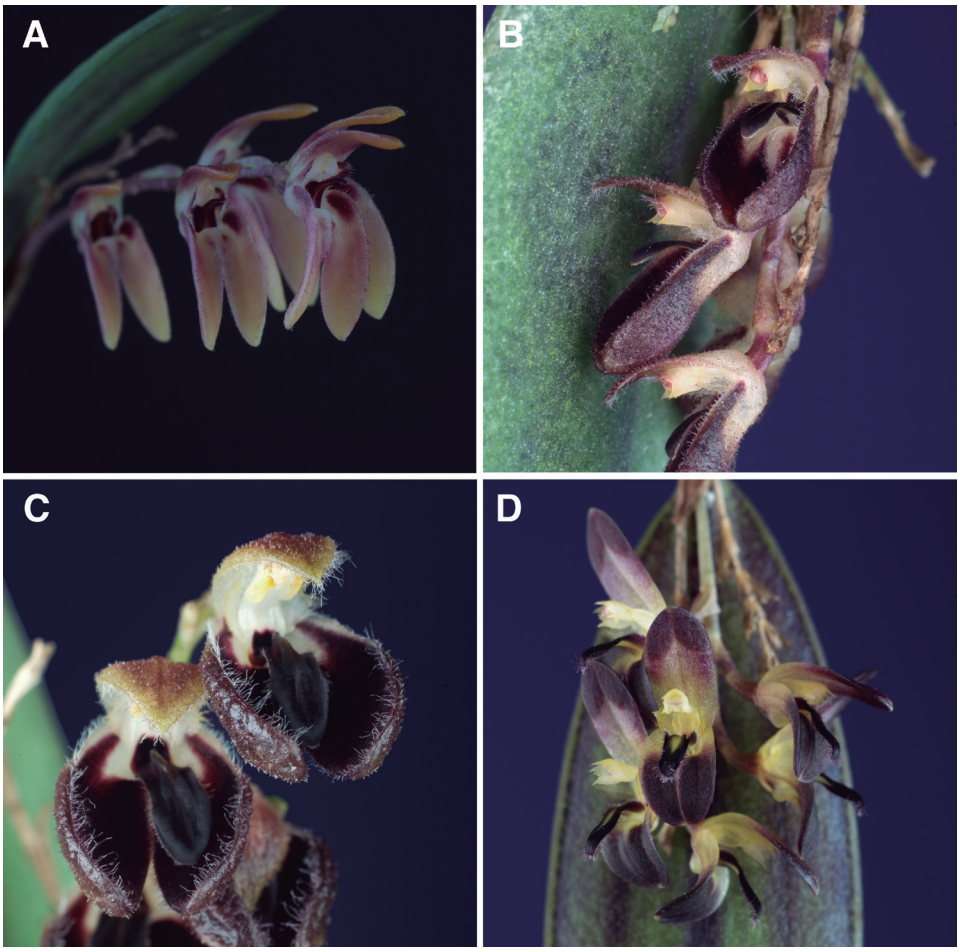

Figure 6.2. Some representatives of *Trichosalpinx* subgenus *Trichosalpinx*. **A.** *T. blaisdellii*. **B.** *T. memor*. **C.** *T. minutipetala.* **D.** *T. reflexa.* Photographs by D. Bogarín.

c oxidase subunit I (COI) gene with the primers LCO1490 5I (Folmer et al., 1994) and Lep-F1 5CO1490 5I (Hebert et al., 2004). Polymerase chain reaction (PCR) followed Karremans *et al*., (2015). Sanger sequencing was conducted by BaseClear (http://www.baseclear.com), and sequences were deposited in NCBI GenBank (Supporting Information, Table 6.S1).

6.2.5 Fragrance sampling and gas chromatography–mass spectrometry

Floral volatiles were extracted by storing at least five flowers collected at anthesis in 4-ml amber glass vials with melamine cap and PTFE liner (Supelco–Sigma– Aldrich Co.) filled with 0.5 mL of chromatography grade hexane. We removed water and particles accumulated with a custom-made column filled with silica gel. Hexane was reduced down to 100 µL by evaporation using a stream of N_2 gas and subsequently analysed by gas chromatography mass spectrometry

(GC/ MS). Analyses were carried out using a HP 6890/5973 system equipped with HP-5 fused silica capillary column (30 m × 0.25 mm × 0.25 µm). We identified the compounds using the NIST08 mass spectral database and the NIST MS Search software v2.0. Threshold detection was adjusted to exclude those peaks that were < 3% than the largest peak.

6.2.6 Histochemistry

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

6.2.7 Light microscopy

Epoxy resin: fixed samples were dehydrated for 15 min in a series of ethanol and successive 1% UAR-EMS uranyl acetate replacement. The ≥ 99.9% ethanol was later replaced with propylene oxide. The samples were infiltrated in a mixture of propylene oxide and Epon. After overnight evaporation of the remaining propylene oxide, the samples were placed in fresh Epon for 3 h, polymerized at 60 °C for 48 h and sectioned at 1.5 µm with a Reichert Jung 2040 rotary microtome. Sections were mounted on microscope slides following Hamann et al., (2011). Epon sections were observed with transmission electron microscopy (TEM) and LM. The Epon sections for LM were stained with toluidine blue O (TBO) 1% (w/v) in 1% (w/v) sodium borate and PAS as described above and mounted in Entellan. LR White: fixed samples were embedded following following Hamann et al., (2011). Each sample was polymerized at 60 °C for 48 h, sectioned (4 µm thickness), mounted and stained as described above for Epon samples. Paraffin-Paraplast: fixed samples were rinsed in water and dehydrated in a series of ethanol:xylene solutions. Then, they were stored in xylene for 8 h, infiltrated in Leica Paraplast and placed in an oven at 60 °C for 1 day. Infiltrated samples were solidified and sectioned at 4–8 µm thickness. Deparaffination

of samples was performed in a series of xylene:ethanol and later stained with TBO 1% (w/v) in 1% (w/v) sodium borate and PAS as described for Epon and LR White samples. Etzold's staining (Basic Fuchsin 10 mg, Safranin 40 mg, Astra Blue 150 mg, acetic acid 2 ml and distilled water to complete 100 mL) was performed by submerging the sections for 30 min in Etzold's, rinsed in tap water for 5 min and demineralized water for 1 min. Dehydration of samples for Etzold's, TBO and PAS was performed by a series of ethanol:xylene solutions.

6.2.8 Scanning electron microscopy

Fixed flowers were dehydrated in a series of ethanol solutions and twice in fresh ≥ 99.8% acetone. Critical point drying was performed using ≥ 99.8% acetone and liquid CO2 with an Automated Critical Point Dryer Leica EM CPD300 following the manufacturer's protocols (Leica Microsystems, Wetzlar, Germany). Samples were sputter-coated with 20 nm of Pt/Pd in a Quorum Q150TS sputter-coater and observed with a JEOL JSM-7600F field emission scanning electron microscope, at an accelerating voltage of 10 kV.

6.2.9 Transmission electron microscopy

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

6.2.10 Reproductive biology

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

6.3 Results

6.3.1 Pollination biology and insect behaviour

Female biting midges of an undescribed species belonging to *Forcipomyia* subgenus Euprojoannisia (Diptera: Ceratopogonidae) exclusively visited and pollinated the flowers of the *Trichosalpinx* spp. studied (Figs. 6.3, 6.4A, B). Formal description of the new species was not undertaken since males are unknown and these generally exhibit diagnostic features. However, we obtained a COI barcode of five specimens to aid with future identification of males (Supporting Information, Table 6.S1). We collected 21 midges (Supporting Information, Table 6.S1) that visited the flowers mostly from 7:00 to 15:00 h, but we documented nocturnal visitation once (18:00). At least ten midges visited the five flowers of a single inflorescence and one to six individuals accessed a single flower simultaneously in *T. reflexa* (Supporting Information, Video 6.S3). The latter was the most frequently visited species and four midges removed the pollinarium (Fig. 6.3; Supporting Information, Video 6.S1). One midge removed the pollinarium of *T. reflexa* from the anther and deposited it on the stigma of another flower (Fig. 6.4A, B). We also observed six

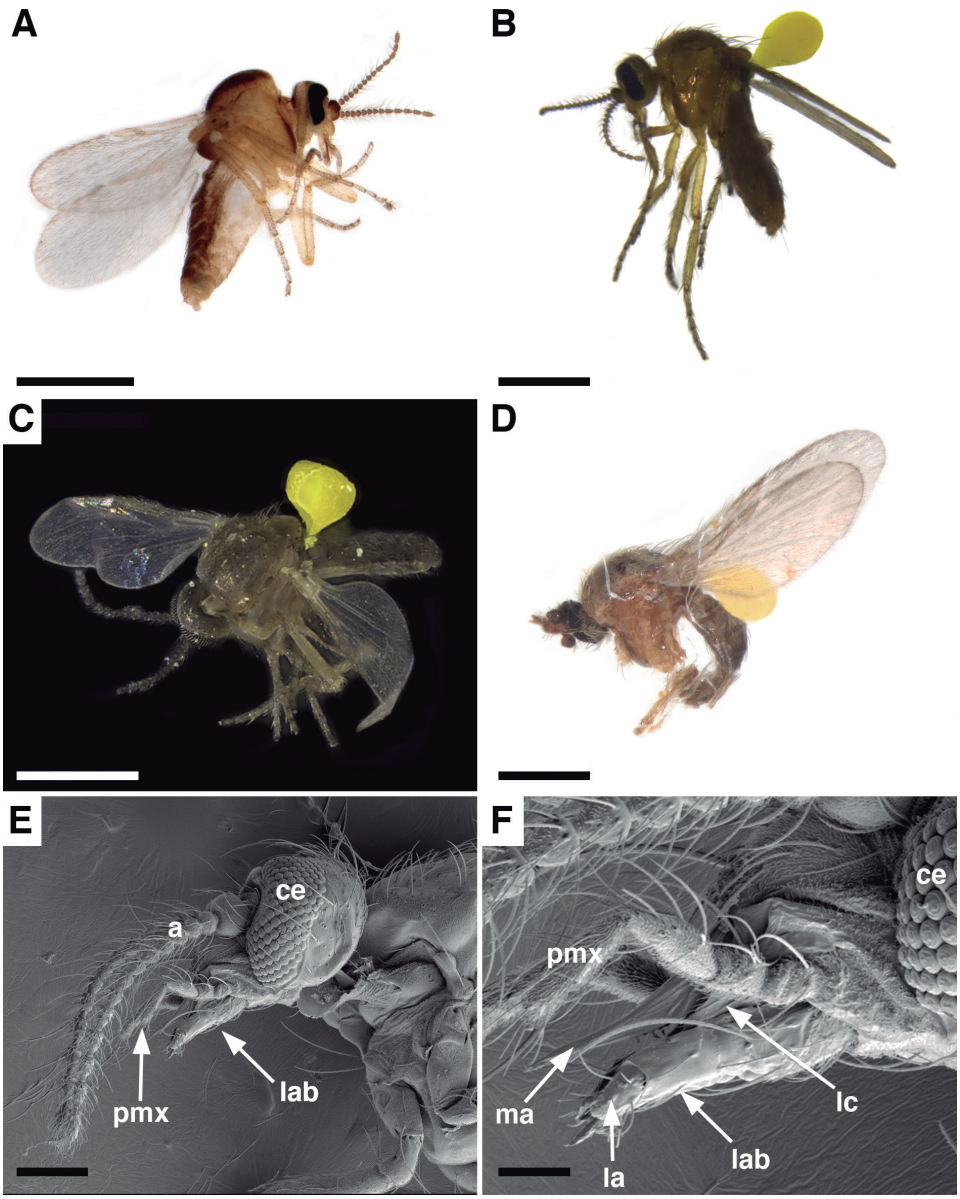

Figure 6.3. Females of *Forcipomyia* (*Euprojoannisia*) sp. collected visiting specimens of *Trichosalpinx*. **A.** female (*D. Bogarín 12058, JBL*) visiting *T. reflexa* (*D. Bogarín 7879, JBL*). **B.** *Forcipomyia* sp. (*D. Bogarín 11420, L*) carrying pollinia of *T. reflexa* (*D. Bogarín 11415, JBL*). **C.** *Forcipomyia* sp. (*D. Bogarín 12060, JBL*) carrying pollinia of *T. reflexa* (*D. Bogarín 7879, JBL*). **D.** *Forcipomyia* sp. (*D. Bogarín 11421, L*) carrying pollinia of *T. blaisdellii* (*D. Bogarín 7250, JBL*). **E.** SEM image of *Forcipomyia* sp. showing the head, compound eyes, antenna and mouth parts. **F**., detail of mouth parts showing the antenna, mandibles, lacinia and maxillary palps. Scale bars = A–D, 0.5 mm. E, 100 µm and F, 30 µm. a, antenna; ce, compound eye; la, labella; lab, labium; lc, lacinia; ma, mandibles; pmx, maxillary palp. Photographs by D. Bogarín.

midges visiting *T. blaisdellii* (pollinaria removal and pollination only once; Supporting Information, Table 6.S1 and Video 6.S5) and *Trichosalpinx minutipetala* (visitation only). We did not observe visitation, pollination and fruit production in other *Trichosalpinx* spp. cultivated in the same greenhouse. Five individuals of *T. reflexa* and two of *T. blaisdellii* developed fruits under greenhouse conditions (Fig. 6.4C, D).

The female *Forcipomyia* sp. approached the flowers in an irregular zigzag flight and landed on the lateral sepals. They immediately walked to the lip and began to inspect the papillose surface from the apex to the base. They were particularly interested in the apex of the ciliate margin of the lip and the short papillae on the surface where they sought and sucked exudates from the cuticular surface using the labella of their mouthparts. The midges did not pierce the lip with mouthparts (Supporting Information, Videos 6.S1–S4). Occasionally they also walked to the sepals and sucked substances from the surface. Sometimes, the midges walked on the purple surface of the sepals and attempted to suck substances or stopped to rest, but they paid most attention to the lip. They showed no interest in the petals (Supporting Information, Video 6.S5). When they walked to the lip from the apex, torque forces initially kept the lip horizontally. When the *Forcipomyia* sp. female approached the base near the callus, at the balance point, the weight of the midge initiated a lever movement rapidly lifting the lip c. 30–40° upwards. In this movement, the midge was slammed against the column (Supporting Information, Videos 6.S1, 6.S2). When more than one midge was at the apex of the lip, the lever mechanism did not work, apparently because the weight of several individuals did not trigger the lip movement (Supporting Information, Video 6.S3). While they were on the flower, they were occasionally observed cleaning their antennae and mouthparts with their front legs or rubbing their hind legs.

In the struggle to get free, the dorsal part of the midge scraped the apex of the column in the area of the caudicles and removed the pollinarium (or deposited the pollinarium on the stigma if it already carried one). The lip returned to the original horizontal position allowing the midge to fly to another flower or remain on the same. The lateral sepals also served as landing surface when the midge managed to get free from the column after capture. The pollinarium was attached to either the postnotum or first abdominal segment of the midge (Figs. 6.3B–D, 6.4B). The midge was more easily released if it tried to make a turn to the side after touching the caudicles. On a few occasions, the insect was not able to release the pollinarium and get free; thus, it was trapped and subsequently died in the flower (Fig. 6.4B). In addition, we did not observe any oviposition behaviour and the flowers observed in scanning electron microscropy (SEM) did not reveal any (traces of) eggs or larvae. Moreover, we did not observe any males visiting the flowers and consequently no sexual behaviour.

6.3.2 Anatomy and ultrastructure of the flowers

Floral morphology of *Trichosalpinx* was described by Luer (1997, 1983) and vegetative anatomy by Pridgeon (1982) and Pridgeon (2005). However, the ultrastructure and histochemistry of the flowers has not been previously studied. Here, we describe the ultrastructure of flowers of *T. blaisdellii* and *T. reflexa* focusing on their adaptations to pollination. Histochemical tests and results are consistent between the studied species (unless specified) and summarized in Supporting Information (Table 6.S2). The sepals have flattened epidermal cells at the base that lack

Figure 6.4. A. females of *Forcipomyia* sp. visiting *T. reflexa*. **B.** *Forcipomyia* female dead in *T. blaisdellii*. C. fruit of *T. blaisdellii*. D. fruit of *T. reflexa*. Both fruits developed under greenhouse conditions after midge visitation. Photographs by D. Bogarín.

anthocyanins, whereas at the apex, the cells are globose, with anthocyanins, thickened cell walls, lipids (OsO_4) and areas that react with VK (Figs. 6.5A–C, 6.6A–F, 6.7B). The subepidermal parenchyma lacks pigments and contains idioblasts and starch grains (PAS) (Fig. 6.7A, B). The cell wall contains acidic lipids, phospholipids and fatty acids (NBA), insoluble polysaccharides

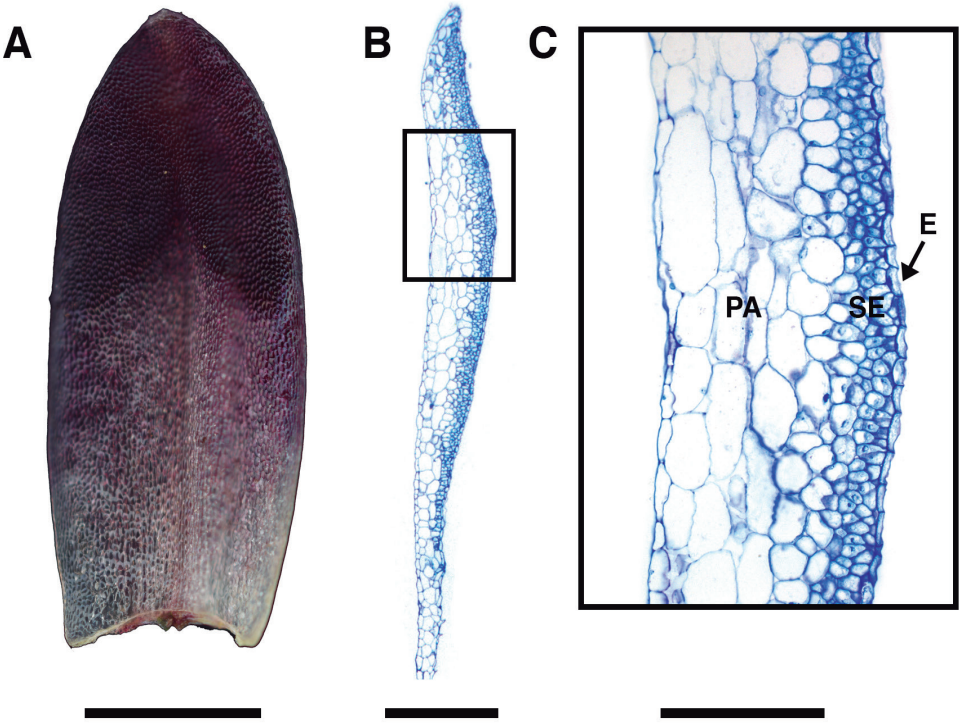

Figure 6.5. Anatomy of the dorsal sepal of *T. reflexa*. **A.** dorsal sepal of *T. reflexa* showing the colourless base and purple apex. **B.** longitudinal section of sepal showing anatomical differentiation of the cells of the epidermis and mesophyll. Stained with toluidine blue. **C.** detail of the longitudinal section of the sepal. Scale bars = 1 mm, 500 µm and 200 µm, respectively. E, epidermis; PA, ground parenchyma; SE, subepidermal layer. Photographs by D. Bogarín.

(PAS) and mucilage (RR) (Fig. 6.7A–D). Stomata are uncommon and located near the midrib or the adaxial surface close to the margin (Fig. 6.7E, F). The cuticle is conspicuous, smooth, with a lipidic layer (SIV) and various epicuticular secretions such as crystals (VK), waxes (SIV), insoluble polysaccharides (PAS) and proteins (ABB, CBB) (Fig. 6.8A–F). Proteins and carbohydrates are seen as cotton-like substances, usually mixed with crystals (Fig. 6.8E). The base of the sepals shows unicellular trichomes (Fig. 6.8F). TEM observations revealed a reticulate cuticle, dense cytoplasm, plastids with plastoglobuli, osmiophilic droplets, mitochondria, rough endoplasmic reticulum (RER) and occasionally dictyosomes. An exchange of lipidic substances (OsO_4) occurs between the cytoplasm and cell wall of sepals and lip; these compounds migrate into the cuticle and accumulate in channels under the ridges of the cuticle (Fig. 6.9A–F). The sepals and the ovary have scattered secretory trichome-like colleters, which reacted with PAS, SIV, VK and NR (Fig. 6.10A–F). In *Trichosalpinx memor*, we detected fungal hyphae associated with colleters on the sepals (PAS) (Fig. 6.10F). Moreover, flowers of this species have a more obvious anatomical differentiation in the epidermal and subepidermal layers in the synsepal as compared with *T. blaisdellii* and *T. reflexa* (Fig. 6.11A–D). Furthermore, in *T. memor* (Rchb.f.) Luer and *T. minutipetala* (Ames & C.Schweinf.) Luer, the synsepal is concave unlike in *T. blaisdellii* and *T. reflexa*,

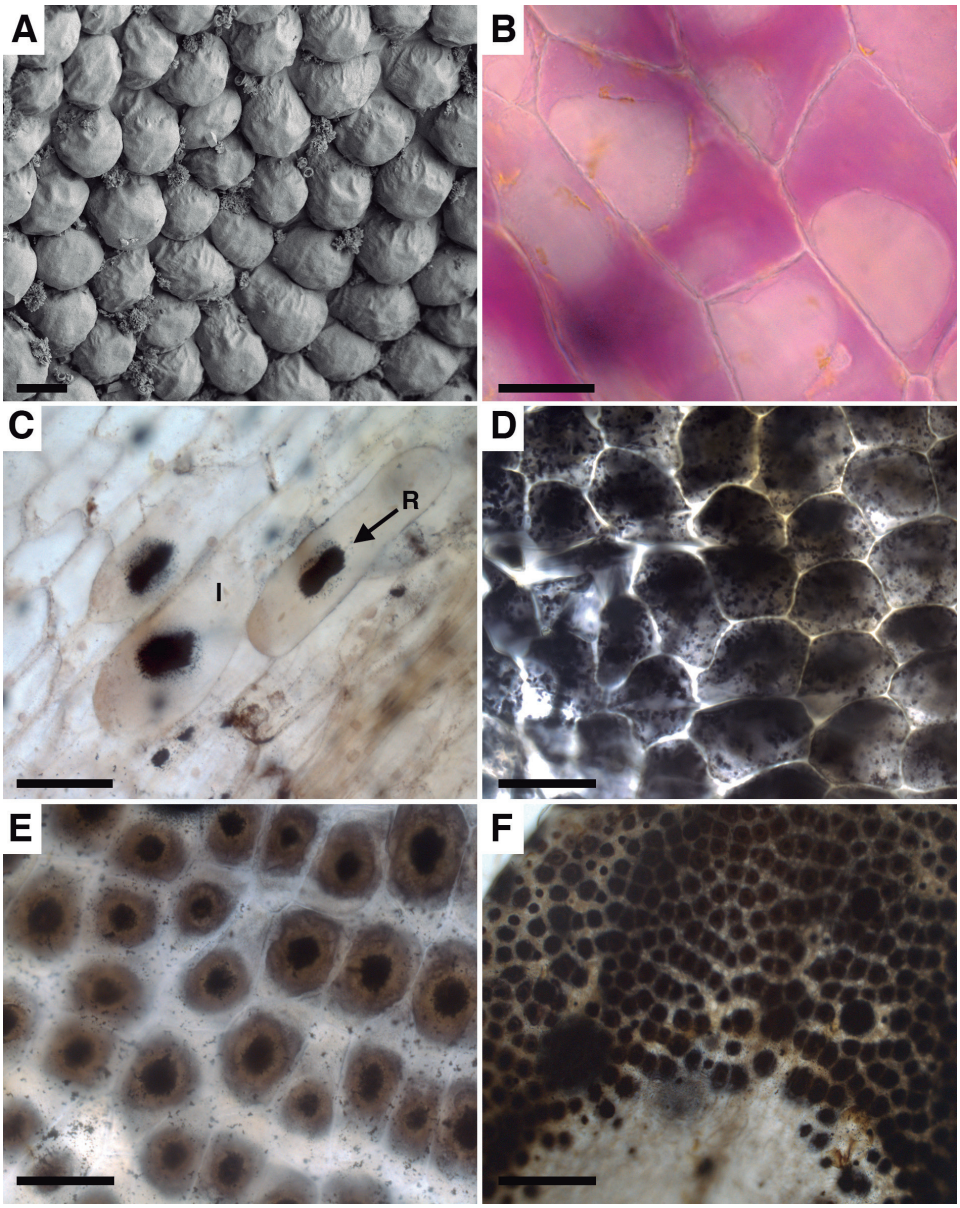

Figure 6.6. Anatomy and micromorphology of the sepals of *T. reflexa*. **A.** SEM of the surface of the apical part showing the dome-shaped cells of epidermis and epicuticular secretions. **B.** LM of unstained sepal showing pigmentation (anthocyanins). **C.** LM of raphides of parenchyma stained with VK (black). **D.** LM of cells of the epidermis stained with OsO_4 showing lipid droplets (black). **E.** LM of the epidermal cells of the dorsal sepal stained with VK (ions, phosphate, urates). **F.** LM of the apex of the dorsal sepal. Note that only the cells of the purple apical area react with VK. Scale bars = 10, 20, 50, 50, 50 and 200 µm, respectively. I, idioblast; R, raphides. Photographs by D. Bogarín and M. M. Chabert.

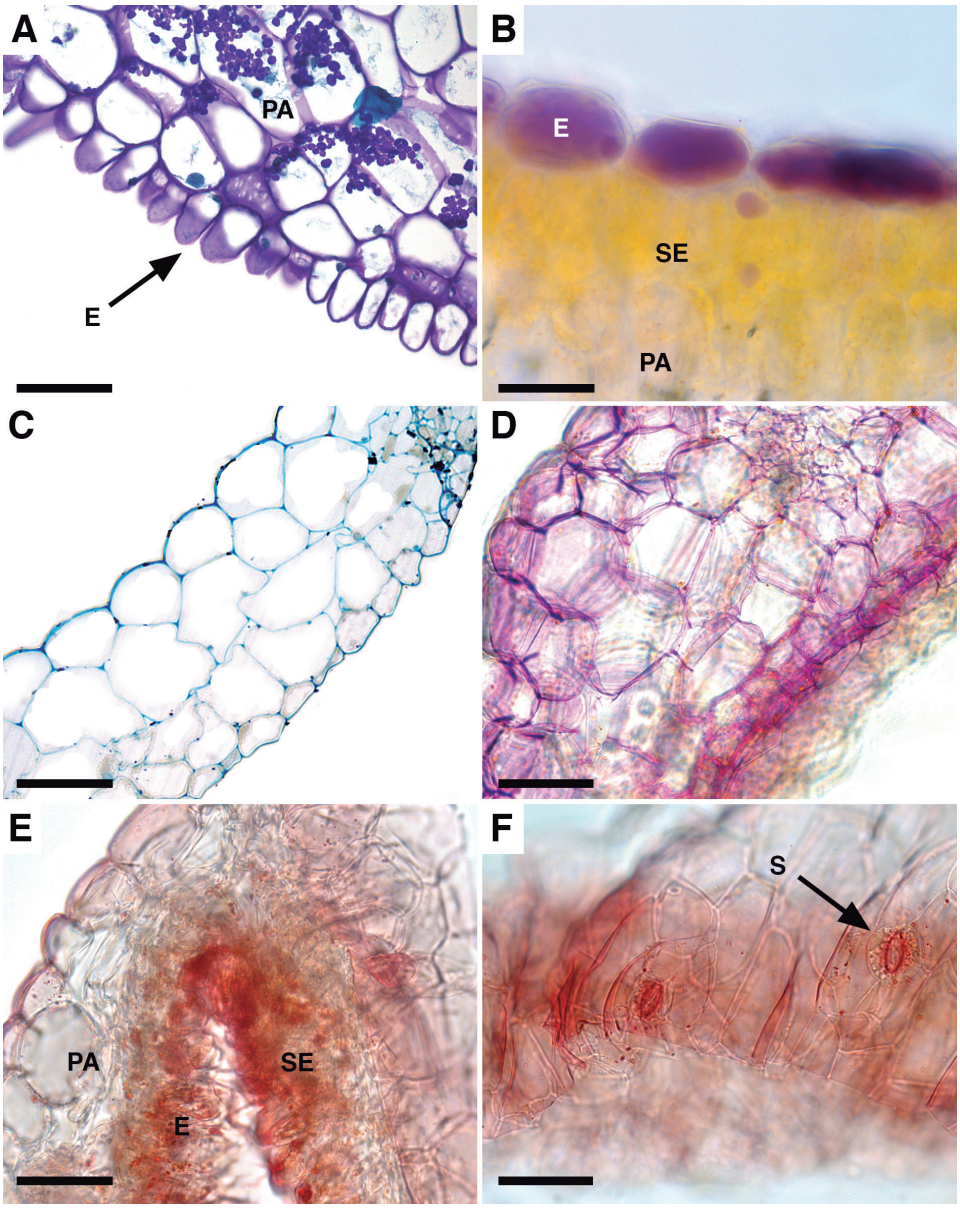

Figure 6.7. Anatomy and micromorphology of the sepals of *T. reflexa* under LM. **A.** ground parenchyma with starch grains (PAS) and ciliate epidermal cells with striate purple cell walls. **B.** hand-section of unstained sepal showing natural pig- mentation in the epidermal cells (anthocyanins) and parenchymatous cells (carotenoids or xanthophylls). **C.** transverse section stained with NB. Cell walls contain acidic lipids, phospholipids and fatty acids. **D.** transverse section stained with RR (mucilage). **E.** transverse section stained with SIV showing lipid concentration in the subepidermal cells. **F.** transverse section stained with SIV showing stomata and lipid concentration in the guard cells. Scale bars = 50, 20, 50, 50, 50 and 50 µm, respectively. E, epidermis; PA, ground parenchyma; S, stomata; SE, subepidermal layer. Photographs by D. Bogarín and M. M. Chabert.

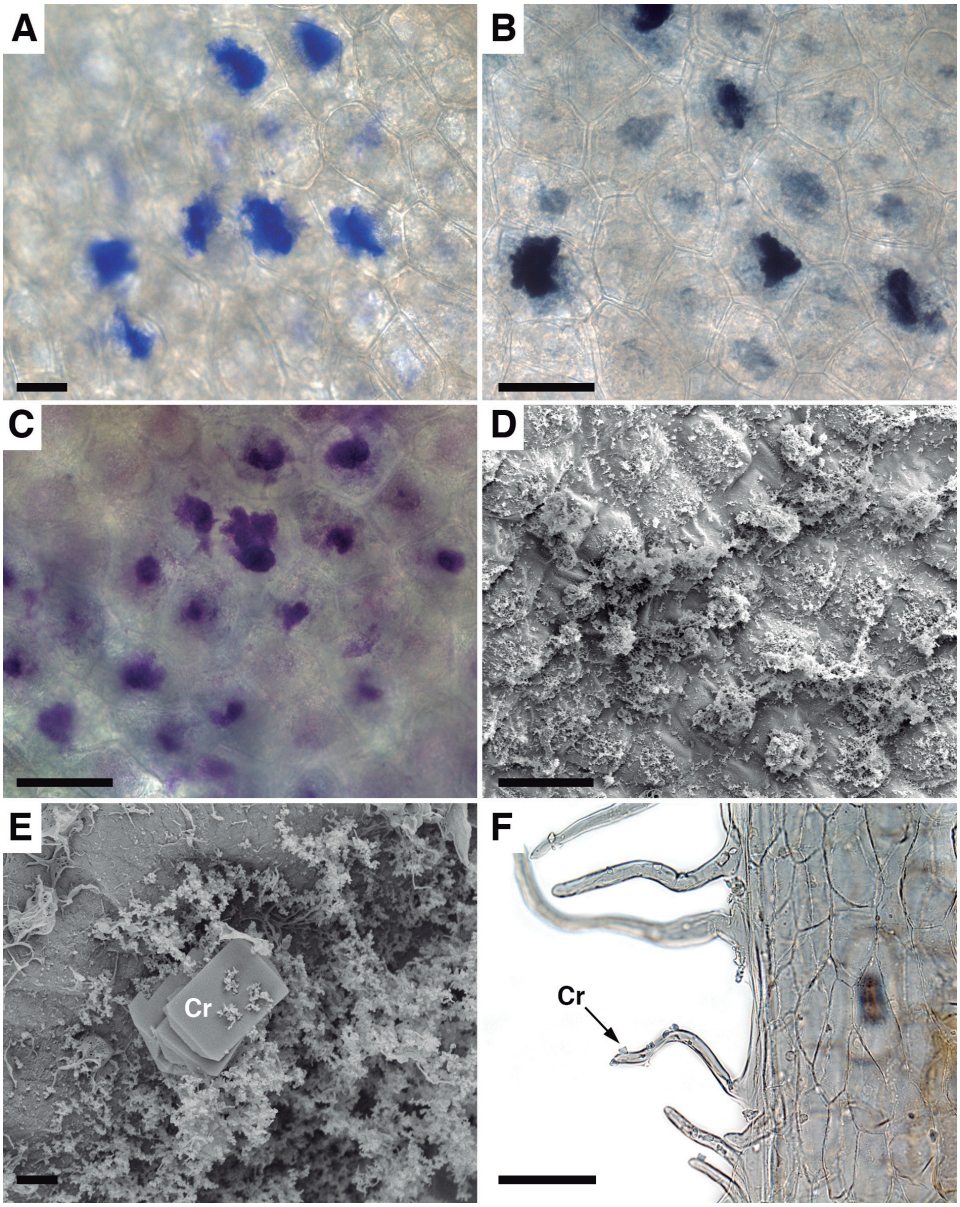

Figure 6.8. Histochemistry and micromorphology of the sepals of *T. reflexa* under LM and SEM. **A.** epicuticular proteins on surface of sepals detected with CBB (blue). **B.** epicuticular proteins on sepals detected with ABB (dark blue). **C.** epicuticular insoluble polysaccharides detected with PAS (purple). **D.** SEM of the surface of sepals where the epicuticular polysaccha- rides and proteins (seen as cotton-like substances) were detected. **E.** SEM of the surface of sepals showing crystals among the epicuticular compounds. **F.** LM of the margin of sepals of *T. memor* with calcium oxalate crystals on the surface of uni- cellular trichomes and epidermis. Scale bars = 50, 20, 50, 40, 10 and 50, respectively. Cr, crystal.
Photographs by D. Bogarín and M. M. Chabert.

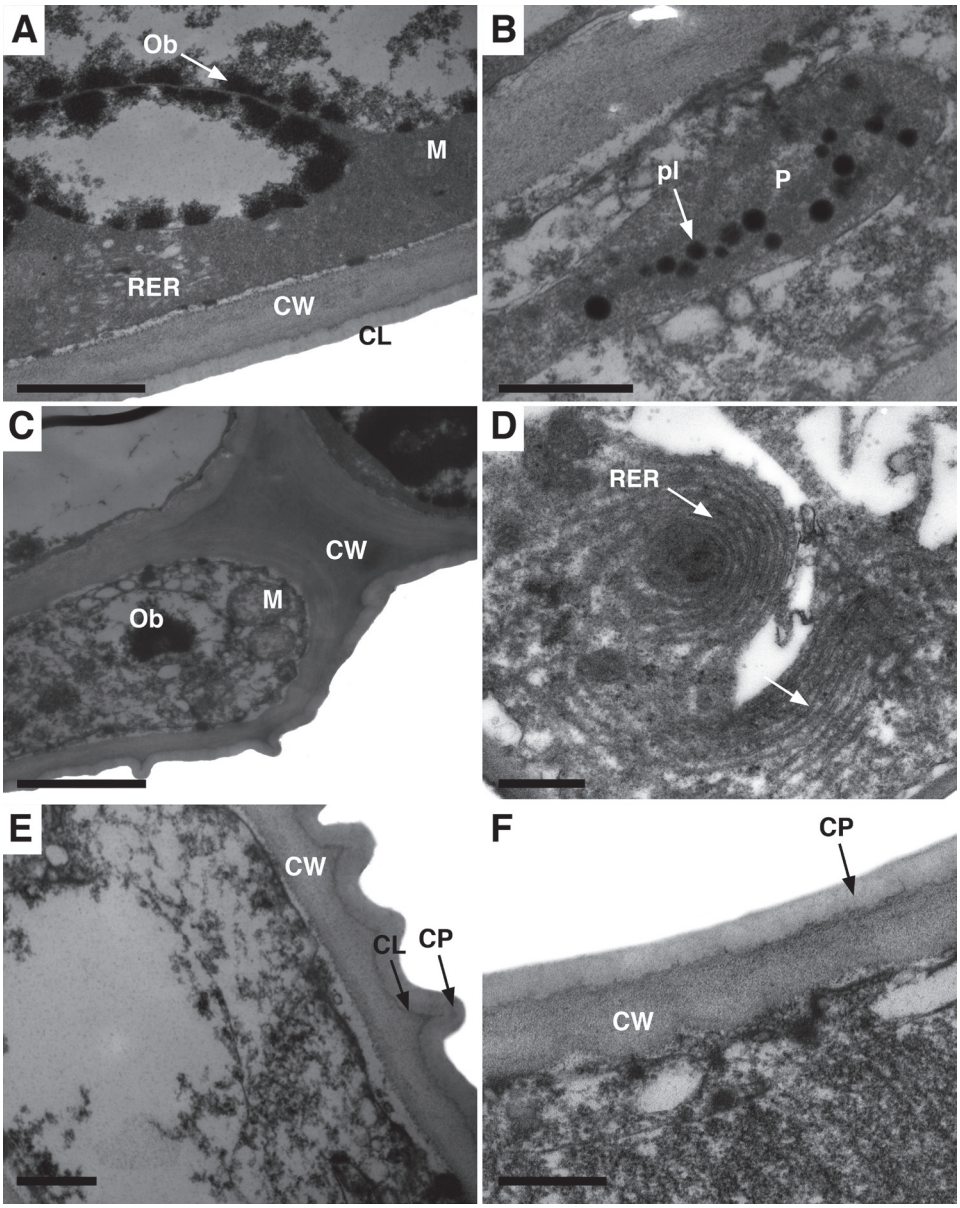

Figure 6.9. TEM images of the sepals of *T. blaisdellii*. **A.** osmiophilic bodies (black), mitochondrion and rough endoplasmic reticulum. **B.** plastid with plastoglobuli. **C.** epidermal cell with osmiophilic bodies and mitochondrion. **D.** rough endoplasmic reticulum in the epidermal cell. **E.** cell wall, cuticle layer and undulate cuticle proper of the epidermal cells. **F.** flat cuticle layer and osmiophilic bodies in the cytoplasm migrating towards the cell wall. Scale bars = 50, 20, 50, 40, 10 and 50 µm, respectively. CL, cuticle layer; CP, cuticle proper; CW, cell wall; M, mitochondrion; P, plastid; pl, plastoglobuli; RER, rough endoplasmic reticulum. Photographs by D. Bogarín and R. Langelaan.

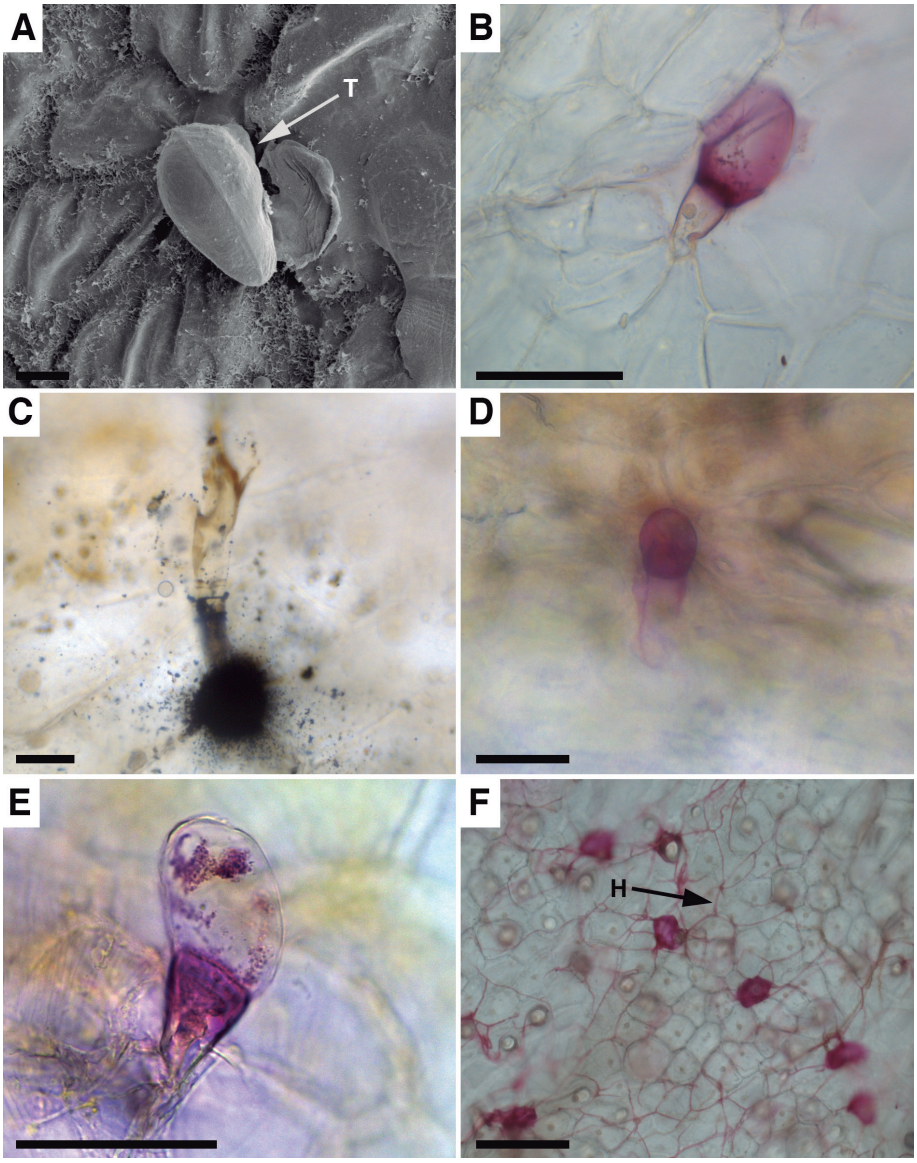

Figure 6.10. Histochemistry and micromorphology of the trichome-like colleters of sepals of *T. reflexa* and *T. memor* under LM and SEM. **A.** SEM of trichome-like colleter showing a globose apex among flat epidermal cells. **B.** LM of cylindrical base and globose apex of the trichome-like colleters showing fragrance emission and secretory activity detected with NR (red) concentrated at the apex. **C.** LM of the base of the colleter stained positively with VK (ions, phosphate, urates) (black). **D.** LM showing lipid concentration at the apex of the colleter (red). **E.** LM of the colleter showing insoluble polysaccharides at the base and some at the apex detected with PAS (pink). **F.** LM fungal hyphae network associated with colleters detected with PAS in *T. memor*. Scale bars = 50, 50, 20, 50, 50 and 100 µm, respectively. T, trichome-like colleter; H, fungal hyphae. Photographs by D. Bogarín and M. M. Chabert.

which is convex or flattened (Fig. 6.2).

The petals are usually colourless at the base and the cells show few cytoplasmic contents. Histochemical tests yielded no positive results (Figs. 6.2, 6..4, 6.12A, B). In contrast, the lip is the structure of interest for the insects, where they reside most of the time (Figs. 6.13, 6.14, 6.15). The base of the lip is attached to the base of the column by a membranous tissue that provides the necessary flexibility for slamming a midge against the column (Fig. 6.13). The lip has two auricles at each side and a raised callus in the middle (Figs. 6.14, 6.15A). The margins have unicellular elongated cells with a noticeably striated cuticle. The upper epidermis shows shorter cilia than the cells of the margin but with identical cuticle (Fig. 6.15A–E). We detected polysaccharides (PAS), lipophilic compounds (SBB) and osmiophilic bodies (OsO_4) (Fig. 6.16A, B) within the papillae, indicating a secretory function. The apices of the papillae along the entire blade reacted positively with NR (Fig. 6.16C), CBB (Fig. 6.16D–E) and ABB (Fig. 6.16F), indicating the presence of scents and proteins, respectively. Anthocyanins are restricted to the upper epidermis and chromoplasts are scattered in the epidermal and subepidermal cells (Fig. 6.17). In a few samples, we observed crystals of calcium oxalate (VK) exuded by the apices of the papillae (Fig. 6.18). SEM and TEM revealed a smooth cuticle with accumulations of compounds on the apices of papillae and exudates on the surface (and on crests of the reticulate cuticle at the base) (Figs. 6.19–21). The cell content of the epidermal layer is remarkably dense and complex in comparison with the cells of the parenchyma (Fig. 6.20B). Osmiophilic substances (revealed by OsO_4) are present between the cytoplasm, plasmalemma and the cell wall and under the ridges of the cuticle (Fig. 6.21). The cytoplasm contains a dense protein matrix (CBB), an extensive network of RER and osmiophilic bodies (Fig. 6.20C, E). In addition, we did not detect nectaries or starch grains in the lip. The arcuate, footed column has similar papillae to those observed on the sepals and lip. The apex is erose or ciliated with two small arms (Fig. 6.22A). The stigma is ventral, separated from the incumbent anther by a conspicuous membranous rostellum. The pollinarium consists of two globose pollinia with pollen grains arranged in triads or tetrads and gemmate ornamentation. At the base, the pollinarium has sticky caudicles that appear to be tetrads derived from immature pollen grains (Fig. 6.22).

6.3.3 Fragrance compounds detected by GC/MS

We detected acid chlorides, esters, fatty acids and long-chain aliphatic hydrocarbons with GC/MS. Methyl ester hexadecanoic acid and lactic acid were found in *T. blaisdellii* but not in *T. reflexa*, whereas tridecyl ester octanoic acid was found only in *T. reflexa*. Acid chlorides and aliphatic hydrocarbons were abundant in both species. Compounds detected with the NIST08 mass spectral database and the NIST MS Search software v2.0. are summarized in Supporting Information (Fig. 6.S1 and Table 6.S3).

6.3.4 Breeding system

No flowers of *T. reflexa* that were hand pollinated with pollinia from the same (n = 20) or different flowers from the same inflorescence (n = 26) developed fruits as all ovaries abscised 2 days after the flowers were pollinated. After hand pollination of flowers from different plants, although, 11 fruits developed (n = 15).

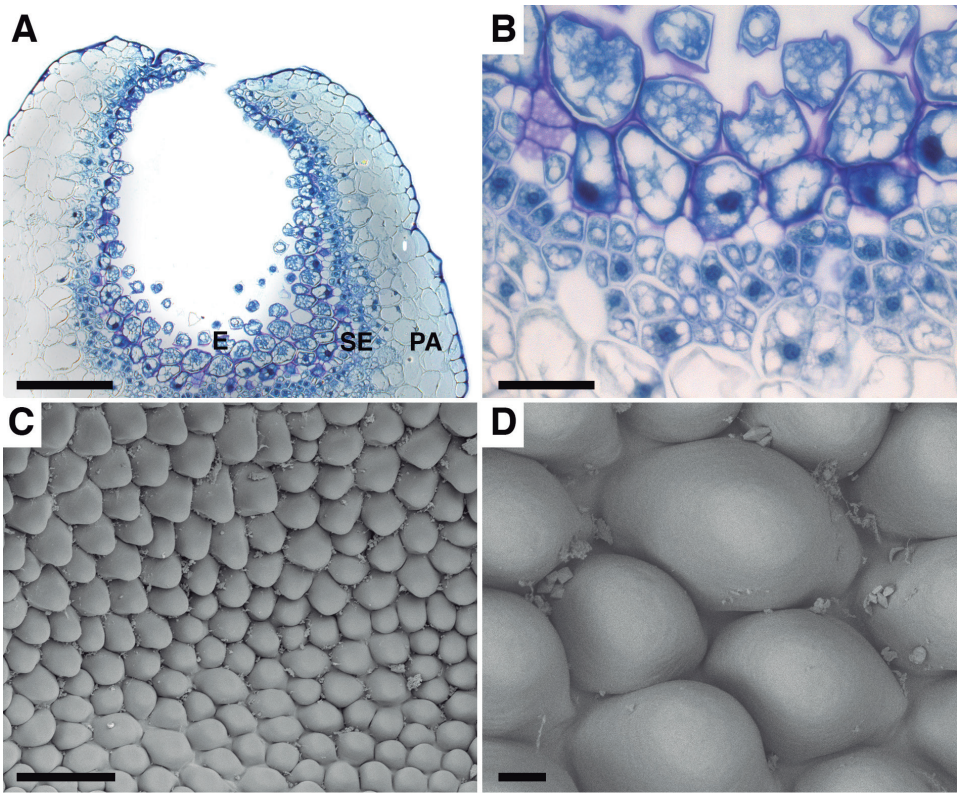

Figure 6.11. Anatomy and micromorphology of the lateral sepals of *T. memor* under LM and SEM. **A.** LM of the synsepal showing the anatomical differentiation of the cells of the epidermis and mesophyll. **B.** LM detail of the epidermal tissue. **C.** SEM of the epidermal surface showing the papillose surface and epicuticular secretions. **D.** SEM of the detail of epidermal surface. Scale bars = 200, 50, 100 and 10 µm, respectively. E, epidermis; PA, ground parenchyma; SE, subepidermal layer. Photographs by D. Bogarín.

6.4 Discussion

6.4.1 Pollination system of *Trichosalpinx*

Ceratopogonidae are a diverse group of Diptera with a worldwide distribution and 6,267 named species (Borkent, 2016). The exclusive presence of females of one *Forcipomyia* (Euprojoannisia) sp., the absence of males as pollinators and the secretion of proteins on the lip suggest that *Trichosalpinx* might stimulate protein collection behaviour of females (for egg production) through prey related colours, odours and movement of flickering bodies (Vogel, 2001). Adult females of earliest-branching lineages in Ceratopogonidae are vertebrate blood feeders and, like other biting flies, require a protein meal to produce eggs (some are autogenous or facultatively autogenous) (Borkent, 2004). Males and females also require a source of nutrition to fuel flight, and this may be in the form of sugars from nectar or honeydew. Subfamily Forcipomyiinae, also an early lineage, includes two genera, *Forcipomyia* and *Atrichopogon*. The adult females are

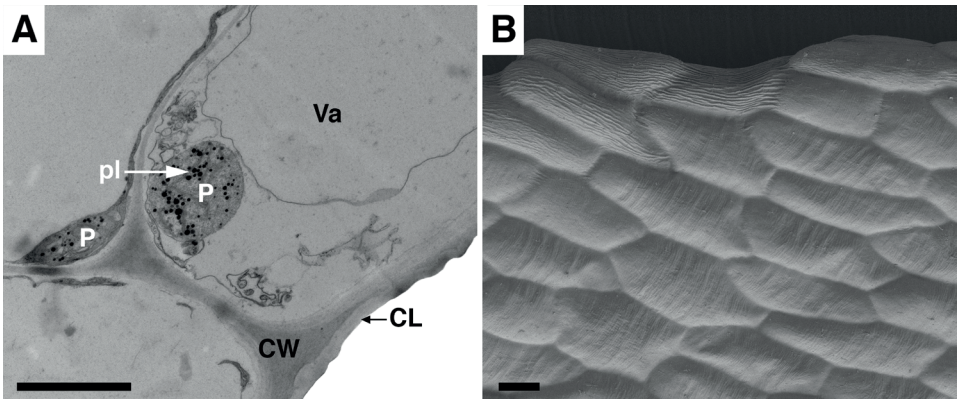

Figure 6.12. A. TEM image of the epidermal cells of petals of *T. blaisdellii* showing simple cytoplasm containing few plastids rich on plastoglobuli with a system of internal membranes sometimes elongated next to the cell wall, vacuoles, cell wall and cuticle layer. **B.** SEM image of the epidermal surface of *T. blaisdellii* showing smooth cuticles of the internal cells and the striate patter of the cells along the margin. Scale bars = 2 and 10 µm, respectively. CL, cuticle layer; CW, cell wall; P, plastid; pl, plastoglobuli; Va, vacuole. Photographs by D. Bogarín and R. Langelaan.

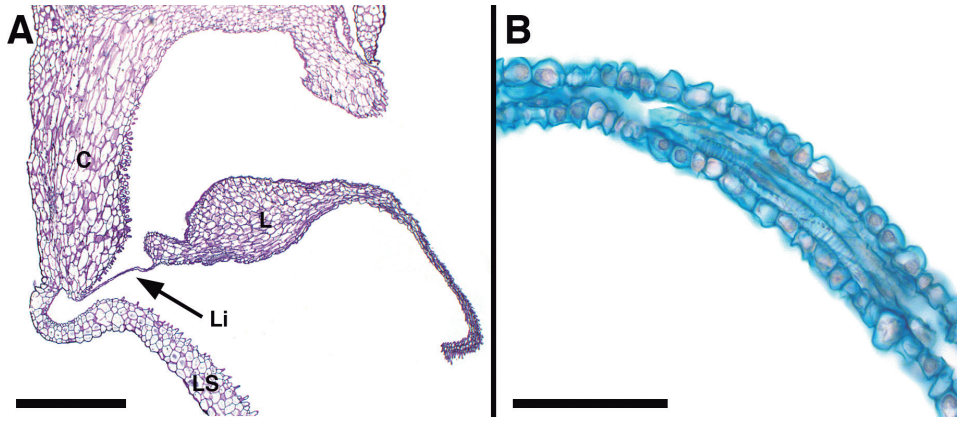

Figure 6.13. Section of a flower of *T. blaisdellii* (LM). **A.** LM transverse section showing column foot, labellar ligament with the hinged lip and part of the lateral sepal (stained with PAS). **B.** longitudinal section of the labellar ligament showing two layers of quadrate cells crossed in the middle by vascular bundles (stained with Etzold's). C, column; L, lip; Li, labellar liga- ment; LS, lateral sepal. Scale bars = 500 and 500 µm, respectively. Photographs by D. Bogarín and M. M. Chabert.

nearly all ectoparasites of other insects much larger than themselves and suck their haemolymph to obtain proteins for egg production. Species of *Forcipomyia* subgenus Lasiohelea are the only vertebrate blood feeders in the subfamily and, in the New World, are known to feed on the blood of frogs. The remaining *Forcipomyia* and all *Atrichopogon* have been recorded from a wide array of hosts, including spiders, phasmids, caterpillars, the wings of Odonata, Chrysopidae and Lepidoptera, Meloidae, Oedemeridae and adult Tipulidae and Culicidae (Borkent and Rocha-Filho,

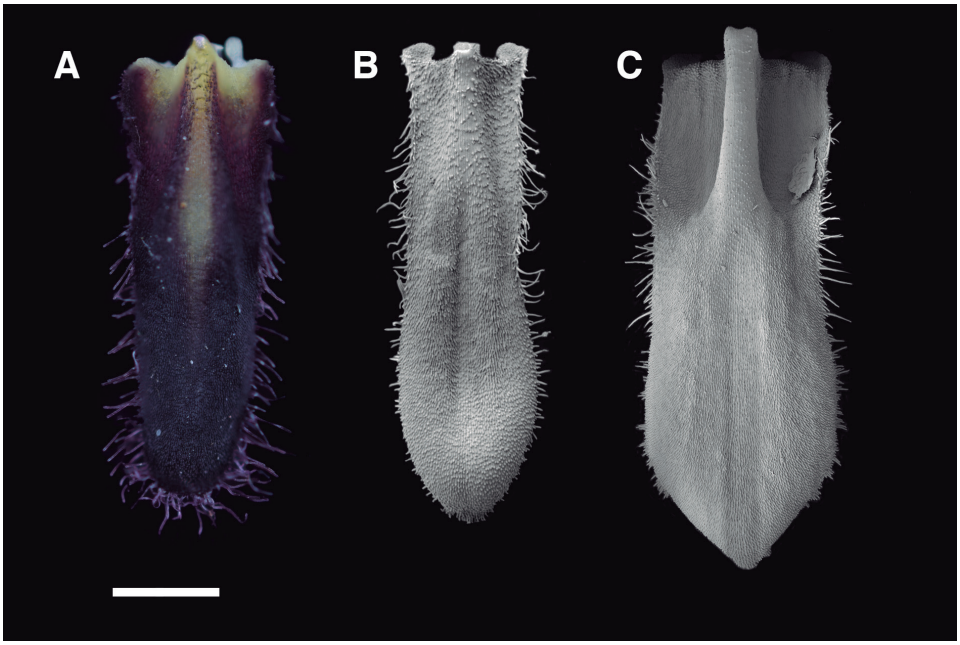

Figure 6.14. Morphology of the lip of *Trichosalpinx* subgenus *Trichosalpinx*. **A.** macrophotography of the lip of *T. reflexa* showing natural pigmentation. **B.** SEM image of *T. blaisdellii*. **C.** lip of *T. minutipetala* all showing ciliate margins, papillose surfaces, a basal raised callus and a pair of auricles at the base. Scale bar = 500 µm. Photographs by D. Bogarín.

2006; A Borkent and Spinelli, 2007; Marcus, 2016). A few records have involved the observation of *Atrichopogon* and *Forcipomyia* being kleptoparasitic on dead insects captured in spider webs (Borkent and Spinelli, 2007; Marshall et al., 2015).

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

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

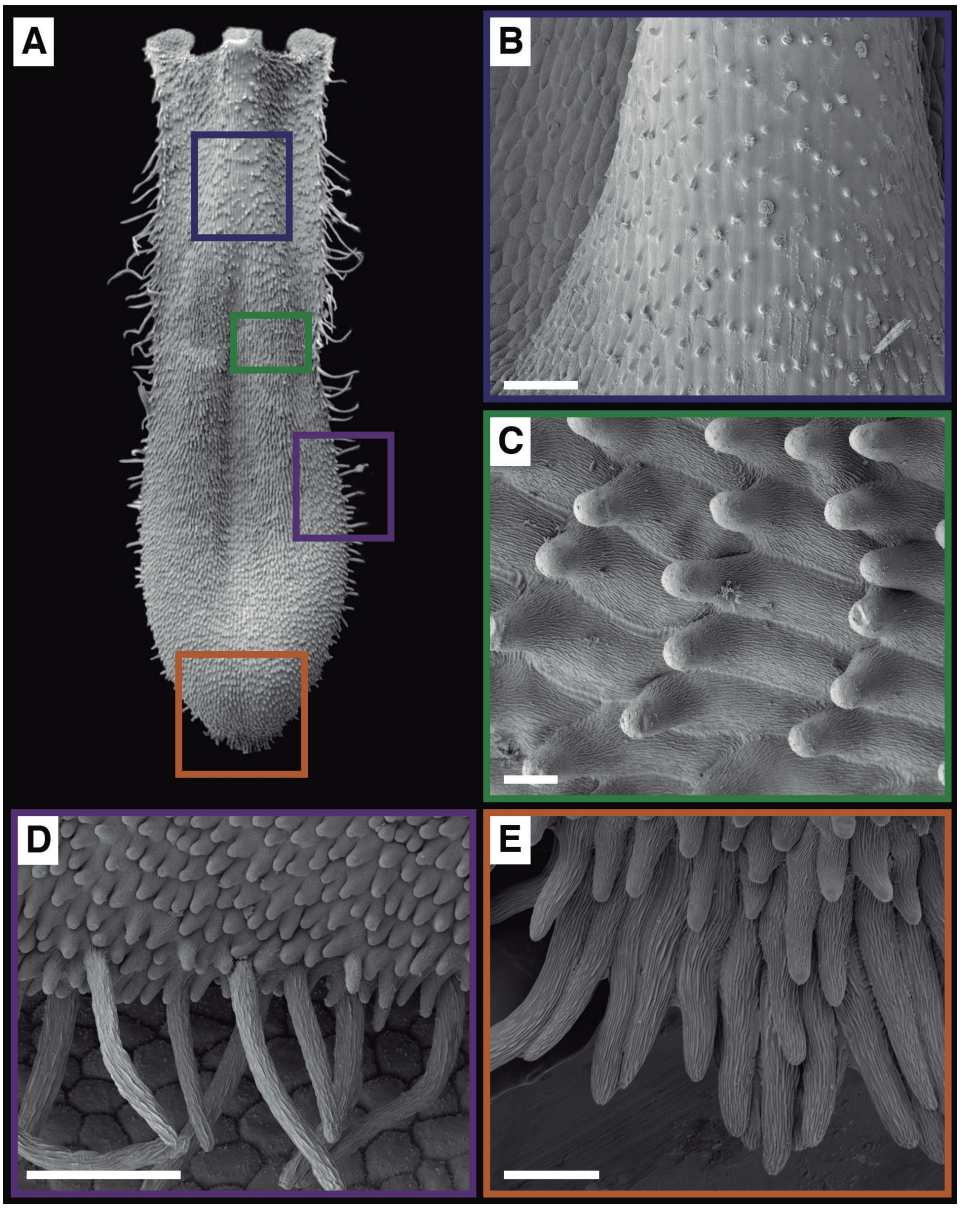

Figure 6.15. Anatomy of the lip of *T. blaisdellii* (SEM). **A.** morphology of the lip. **B.** detail of the callus surface showing scat- tered papillae and epicuticular substances. **C.** papillose surface of the lip with characteristic striated pattern of the cuticle and smooth apex of papillae. **D.** ciliate margin of the lip with elongated unicellular hairs and striate cuticle. **E.** apex of lip with elongated unicellular cells with striated pattern. Scale bars = 50, 50, 10 and 30 µm, respectively. Photographs by D. Bogarín, M. M. Chabert and F. Gardien.

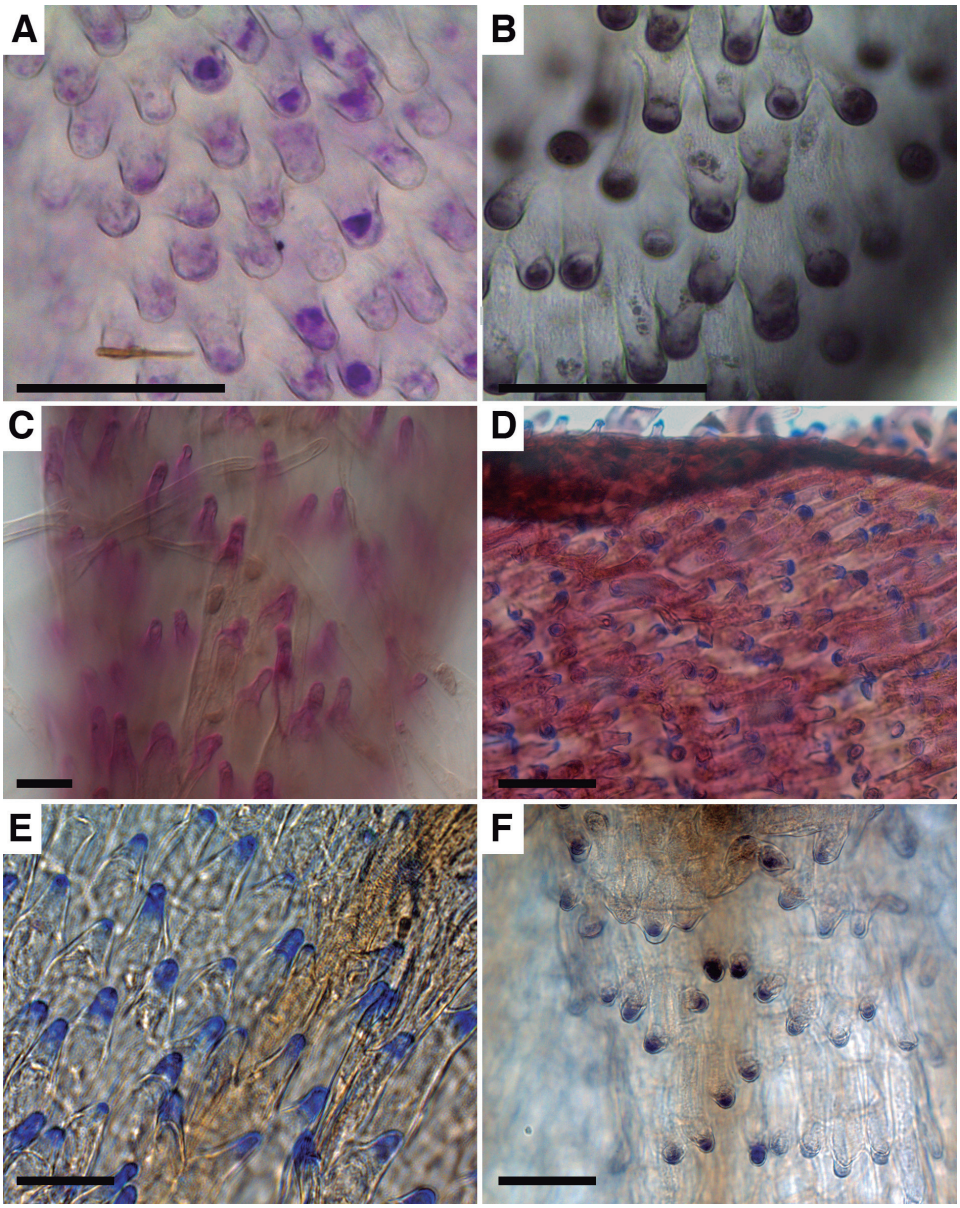

Figure 6.16. Histochemistry of the papillose surface of the lip of *T. reflexa* (LM). **A.** insoluble polysaccharides stain pink (PAS). **B.** lipids stain black (SBB). **C.** detection of scent emission with NR (red/pink). **D.** staining of fresh tissue lip with CBB yielded positive results for proteins (blue tips). E. decoloured lip also yielded positive results for proteins (CBB) blue tips. **F.** papillae of the callus showing proteins at the apices (ABB). Scale bars = 500 µm, 500 nm, 20 µm, 50 µm, 50 µm and 50 µm, respectively.

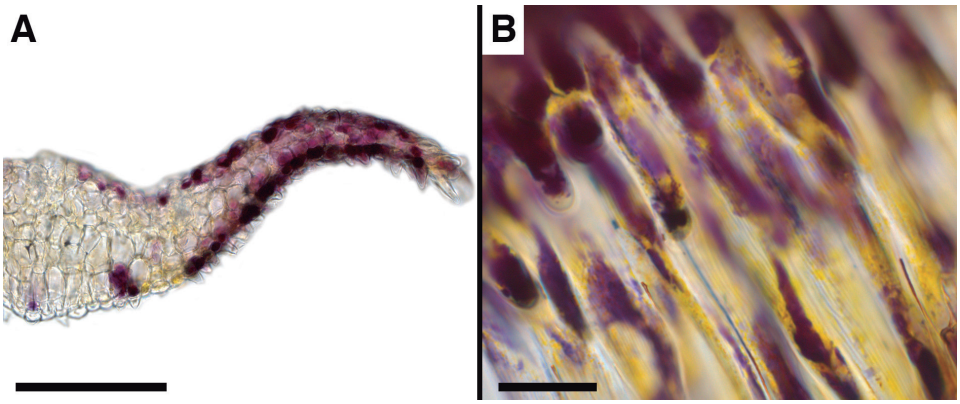

Figure 6.17. A. unstained transverse hand-section of the lip showing the purple epidermal cells containing anthocyanins, ciliate margins and the uncoloured ground parenchyma. **B.** detail of the unstained papillose surface of the lip showing the anthocyanins (purple) and carotenoids (yellow) and the striated cuticle. Scale bars = 50 and 20 µm, respectively. Photographs by D. Bogarín and M. M. Chabert.

Borba and Semir (1998), found that females of *Pholeomyia* spp. (Diptera, Milichiidae) are the sole pollinators of three *Bulbophyllum* spp. in Brazil and they theorized that females are attracted by their oviposition instinct, although, they did not observe any flies ovipositing. Davies and Stpiczyńska (2014) suggested that offering proteins as a reward by a species of *Bulbophyllum* section *Racemosae* Benth. & Hook.f. is strong evidence that the attraction is due to the stimulation of the instinct of protein collection in the females. Our observations agree with the hypothesis of Davies and Stpiczyńska (2014), and we consider that *Trichosalpinx* might represent an analogous case. We could not identify the proteins synthesized by *Trichosalpinx* or demonstrate that these offered proteins are sufficient for females in terms of egg production. The females of *Forcipomyia* (Euprojoannisia) collected have well-developed mandibles and poorly developed laciniae, as do other species in this subgenus. This strongly indicates that the flies primarily feed on invertebrates (either live or dead) to draw protein-rich haemolymph for egg development, suggesting that the orchids are not probably their primary source of proteins.

Few documented observations of invertebrate prey of *Forcipomyia* (Euprojoannisia) are known. *Forcipomyia hardyi* feeds on caterpillars of Geometridae and Sphingidae (Lepidoptera) and one species is kleptoparasitic, attracted by immobilized wrapped termites captured by spiders (Bystrak and Wirth, 1978; Marshall et al., 2015). Kleptoparasites use olfactory signals to locate their food sources including predator venoms, predator digestive secretions, odours of decaying insects, prey defense secretions released on disturbance or odours of freshly killed insects (Heiduk et al., 2016, 2015, 2010; Oelschlägel et al., 2015). Some angiosperms can mimic these fragrances luring kleptoparasites (Sivinski and Stowe, 1980). Oelschlägel et al. (2015) and Heiduk et al. (2016, 2015) introduced the concept of kleptomyiophily, which is pollination by kleptoparasitic flies (food thieves which feed on prey of other predators) attracted to flowers that mimic insect related odours such as semiochemicals to stimulate food-seeking behaviour. *Aristolochia rotunda* L., *Ceropegia dolichophylla* and *Ceropegia sandersonii* use a kleptomyiophilous strategy to fool kleptoparasitic female flies of Chloropidae and Milichiidae.

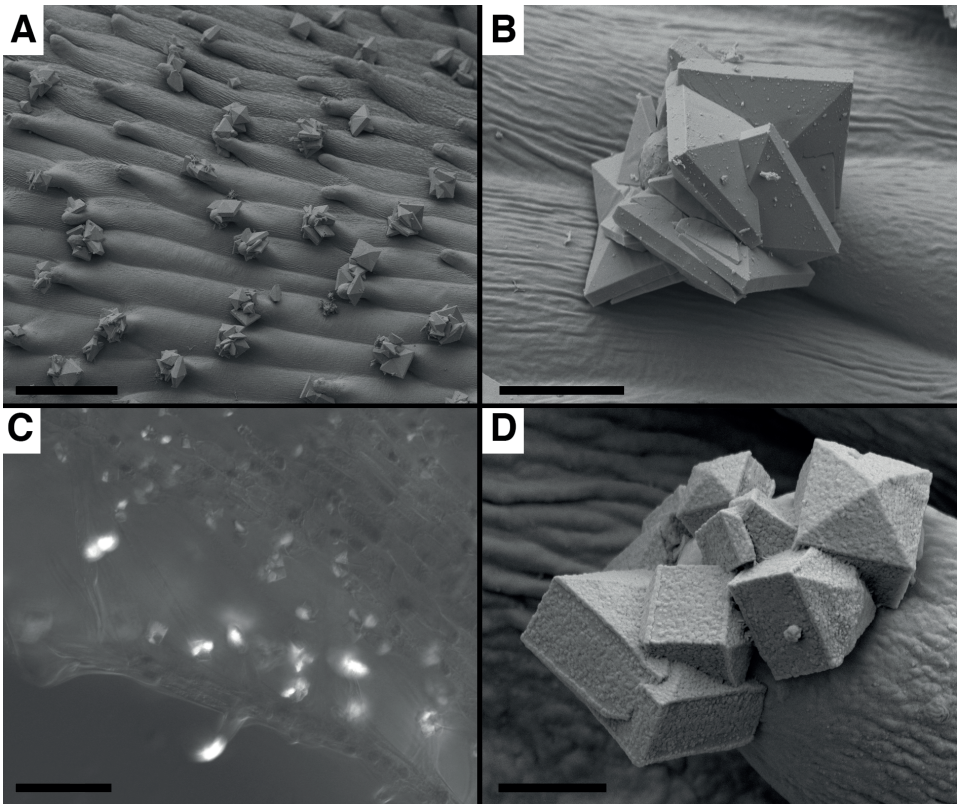

Figure 6.18. SEM and LM images of the epicuticular crystals of the lip of *T. blaisdellii*. **A.** crystals on the apices of the papillae. **B.** detail of the pyramid-shaped crystals with smooth surface. **C.** birefringence of crystals under LM DIC mode. **D.** crystals of the apex of papillae with rough surface. Scale bars = 30, 5, 50 and 2 µm, respectively. Photographs by D. Bogarín and M. M. Chabert.

According to Oelschlägel et al. (2015), Ceratopogonidae are less important pollinators of *A. rotunda*, but they may be deceived in a similar way as the chloropids and milichiids because kleptoparasitism occurs in this group. Scents of these plants simulate those emitted by trapped prey or venoms injected by predators such as spiders. The presence of kleptoparasitism in *Forcipomyia* (Euprojoannisia) raises the possibility that kleptomyiophily may have evolved in *Trichosalpinx*. The ciliated lip, which moves due to vibration or wind, might produce a visual effect similar to that of a prey trapped and immobilized in, for example, a spider web and could activate gregarious instinctual responses in predatory dipterans (Meve and Liede, 1994; Vogel, 2001) (Supporting Information, Video 6.S3). The movement of the lip may also aid in dispersing attractive floral fragrances produced in the epithelium as observed in *Bulbophyllum* (da Silva et al., 1999). The evolution of kleptomyiophily has not been fully documented in Orchidaceae. However, it probably occurs in the Australian orchid *Genoplesium littorale* D.L.Jones as suggested by (Bower et al., 2015).

An alternative hypothesis could be an initial long-distance stimulus mediated by the imitation of aliphatic hydrocarbons and esters recorded in cuticles of insects and spiders (see ‘Floral

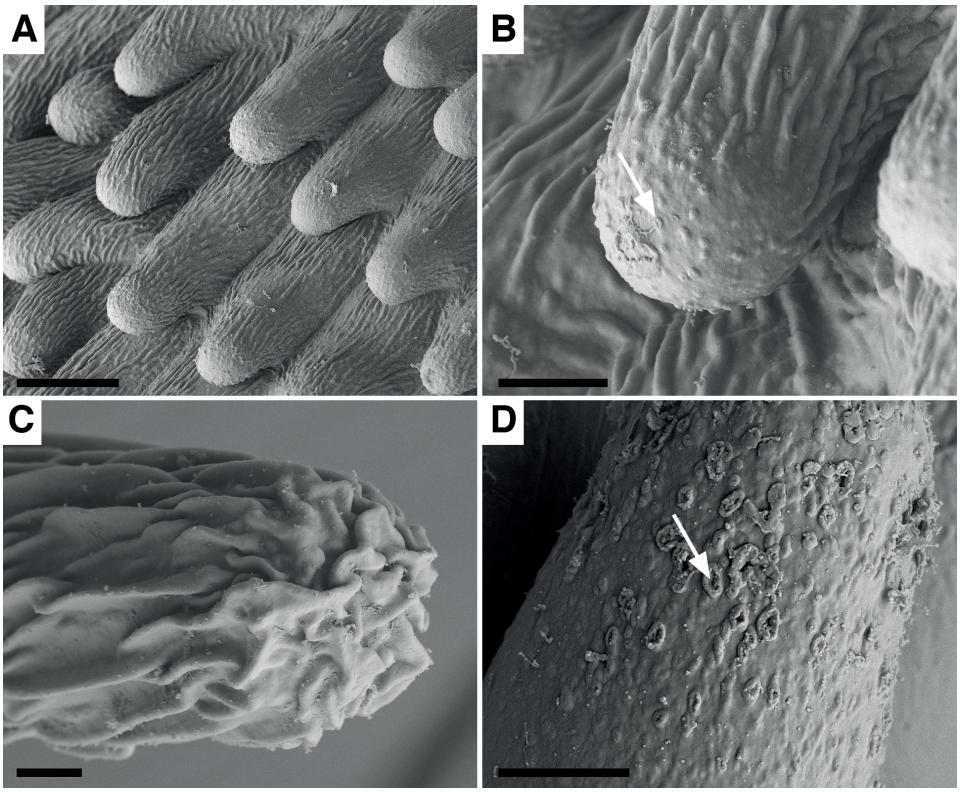

Figure 6.19. Anatomy of the papillae of the lip of *T. blaisdellii*. **A.** detail of the papillose surface with striated cuticle. **B.** apex of the papillae showing a smooth surface with pores and substances at the apex. **C.** striate cuticle of the elongated unicellular cells of the margin of the lip. **D.** magnification of the apex of the papillae showing pores and accumulation of substances (white arrow). Scale bars = 10 µm, 3 µm, 3 µm, 1 µm, 500 nm and 500 µm, respectively. Photographs by D. Bogarín and M. M. Chabert.

fragrances'). This would suggest that the flowers attract *Forcipomyia* females by mimicking an invertebrate host prey first by a long-distance fragrance and second by a short distance tactile (hairy surfaces of the lip), visual (purple colour) and mechanical (movement of the lip) cues that mimic the body surface of caterpillars or possibly spiders (Bystrak and Wirth, 1978; Marcus, 2016). However, the hypothesis of kleptomyiophily or the alternative imitation of the cuticular hydrocarbons and body of an invertebrate host should be further tested, as we have little information about the feeding habits and biology of these pollinators.

The small quantities of proteins and insoluble carbohydrates on the surface of the lip suggest that *Trichosalpinx* is primarily food deceptive. Proteins could be a strong enough signal, a tease, to lure female biting midges into the flower and guide them to the point of balance of the lip (Borba and Semir, 1998; Vogel, 2001). Generally, both sexes of *Forcipomyia* feed on floral nectar to fuel flights. However, *T. blaisdellii* and *T. reflexa* lack nectaries and the absence of males suggests that the insoluble carbohydrates do not stimulate male visitation. Similar examples were

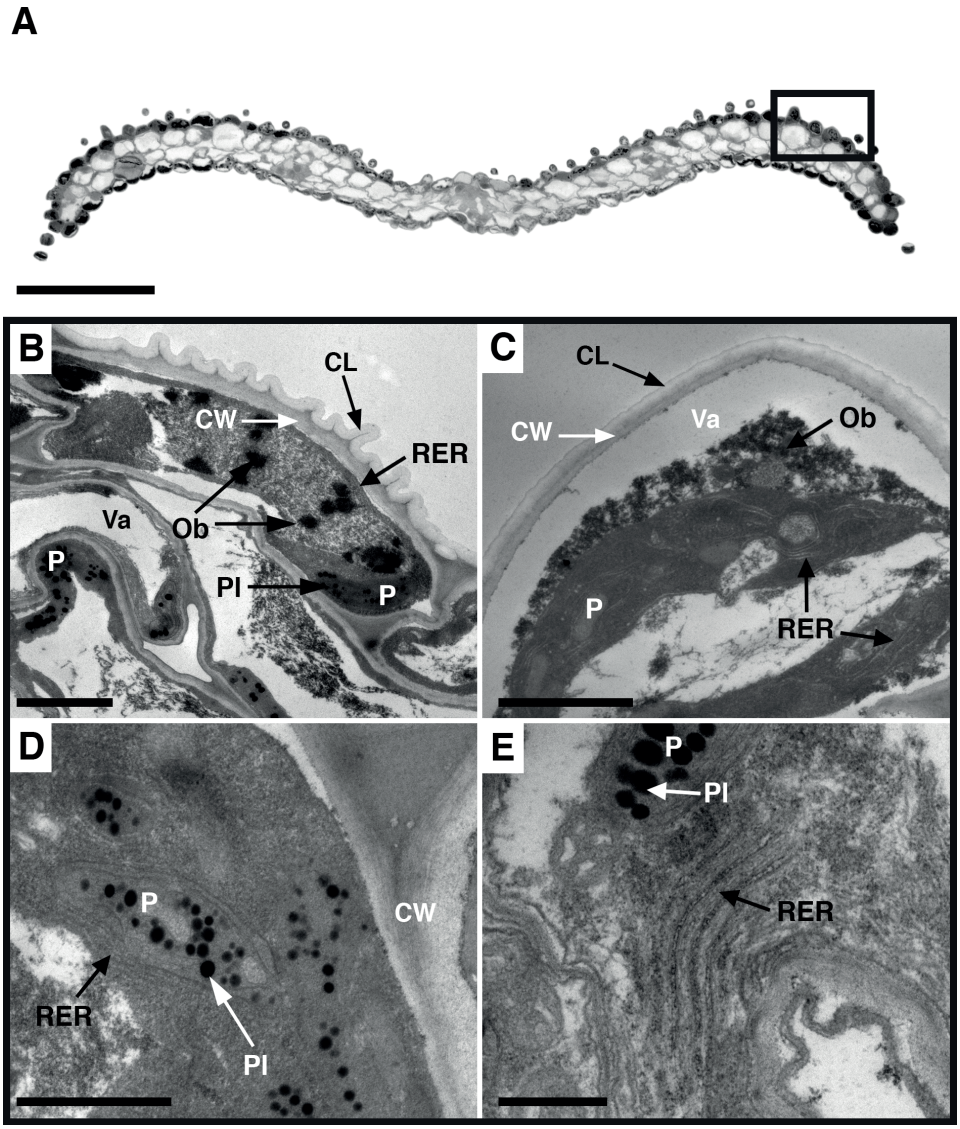

Figure 6.20. LM and TEM images of the lip of *T. reflexa*. **A.** transverse Epon section stained with OsO_4 showing osmiophilic contents restricted to the epidermal layers and the unstained ground parenchyma. B–E, TEM images of upper epidermal cells. **B.** epidermal cell showing the striate cuticle, parietal cytoplasm, endoplasmic reticulum profiles, plastids with plastoglobuli and osmiophilic bodies. Some plastids with plastoglobuli are observed in the sub-parenchyma. **C.** another epidermal cell showing dense cytoplasmic contents, with profiles of endoplasmic reticulum, vacuoles and plastids. **D.** plastids with plastoglobuli and endoplasmic reticulum. **E.** detail of rough endoplasmic reticulum with ribosomes. Scale bars = 10 µm, 2 µm, 2 µm, 1 µm and 500 nm, respectively. CL, cuticle layer; CW, cell wall; Ob, osmiophilic body; P, plastid; Pl, plastoglobuli; RER, rough endoplasmic reticulum; Va, vacuole. Photographs by D. Bogarín and R. Langelaan.

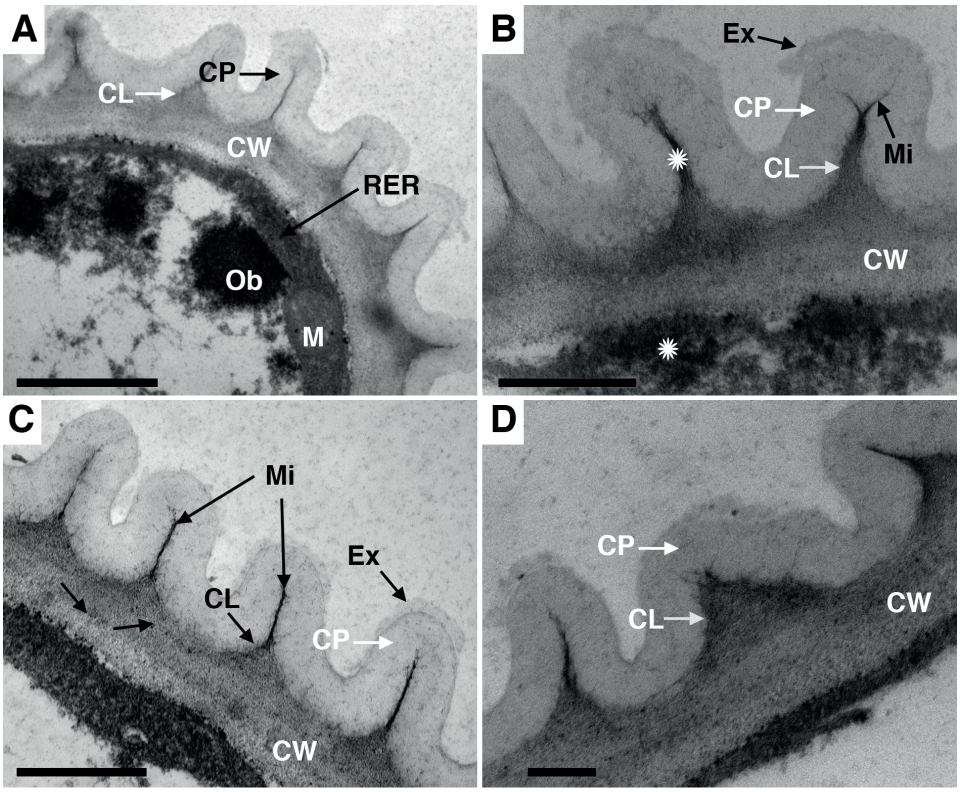

Figure 6.21. TEM images of the cuticle and cell wall of the lip of *T. reflexa* showing the migration of substances from the cytoplasm towards the cuticle. **A.** osmiophilic bodies close to the cell wall and profiles of rough endoplasmic reticulum. **B.** reticulate cuticle layer and cuticle proper with exudates penetrating the cell wall (asterisk) and bifurcated microchannels with osmiophilic substances. **C.** osmiophilic substances crossing the cell wall (black arrows) and accumulation of substances in the folds of the cuticle. C, detail of reticulate cuticle proper with osmiophilic substances accumulated along the plasma- lemma and crossing the cell wall. CL, cuticle layer; CP, cuticle proper; CW, cell wall; Ex, exudates; M, mitochondrion; Mi, microchannels; Ob, osmiophilic body; RER, rough endoplasmic reticulum. Photographs by D. Bogarín and R. Langelaan. Scale bars = 1 µm, 500 nm, 500 nm, 1 µm and 500 nm, respectively.

observed in the nectar-producing flowers of *Ceropegia* L. (Apocynaceae). Vogel (2001, 1990) considered these flowers deceptive because the small amount of nectar offered is unlikely to be the primary award for the pollinators. In addition, the wind-assisted fly pollinated *Bulbophyllum ipanemense* Hoehne (a species that needs wind movement because the flies are unable to tilt the lip with their weight) is exclusively visited by female flies that are not attracted primarily to any nectar offered and males were instead observed feeding on extrafloral nectaries of sympatric *Epidendrum secundum* Jacq. (Borba and Semir, 1998). Thus, a partial reward deception may have evolved in these orchids. In addition, it is possible that a reward is provided to aid in correctly positioning the pollinator in the flower, but not enough to encourage fidelity. The advantage of offering a minimum reward or a tease over offering no reward might be that it extends the visitation

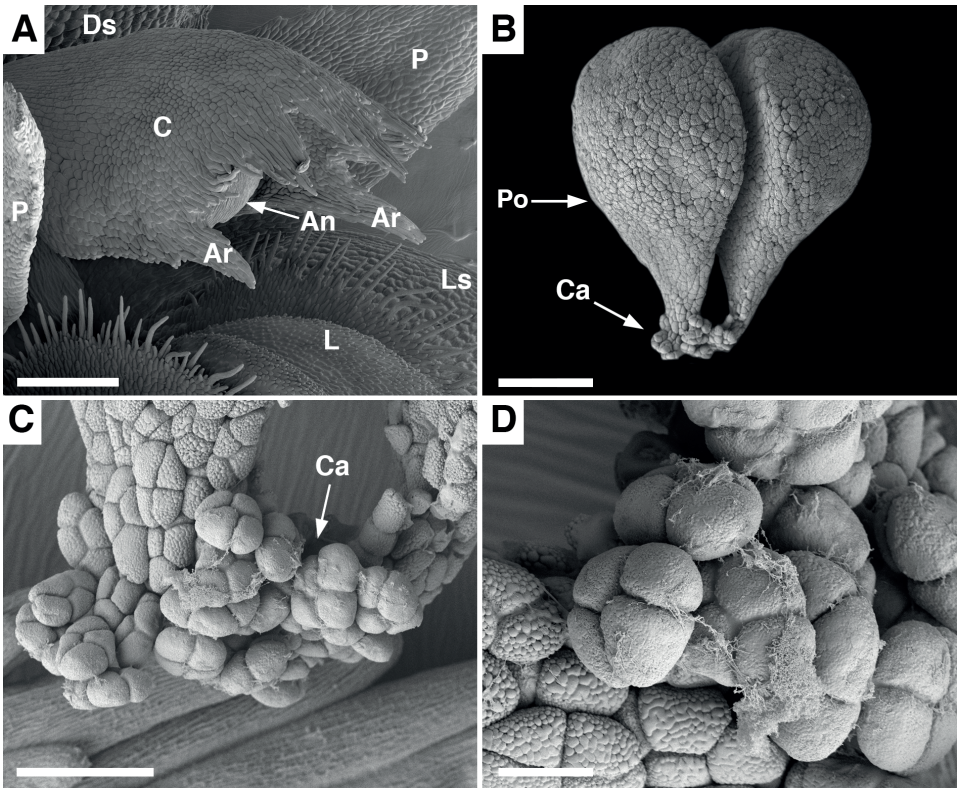

Figure 6.22. Floral morphology of *T. reflexa*: **A.** portion of the column showing the erose apex and column arms, anther cap, the papillose, ciliate lip and smooth surface of sepals and petals. **B.** pollinarium composed by two pollinia and sticky caudicles. **C.** detail of the base of the pollinia and caudicles showing gemmate ornamentation of cells of the pollinia and the smooth tetrads of the caudicle. **D.** magnification of the tetrads of the caudicle with a sticky substance. Scale bars = 300, 100, 30 and 10 µm, respectively. An, anther cap; Ar, column arms; Ca, caudicle; C, column; L, lip; P, petal; Ds, dorsal sepal; Ls, lateral sepal. Photographs by D. Bogarín and M. M. Chabert.

time of insects and that the insects are not stimulated to learn to avoid the orchid as may occur in non-rewarding species where the pollination efficiency is lower (Tremblay et al., 2005). Many flies, including Ceratopogonidae, are able to release saliva on dried sugars and suck up the resultant fluid. In *Trichosalpinx*, it takes longer for the midges to collect those meagre rewards, thus enhancing the possibility of pollination. Moreover, production of small rewards or teases might not require a lot of energy (Jersáková et al., 2006). Ackerman et al. (1994) and Salguero-Faría and Ackerman (1999) studied the advantage of offering meagre rewards in *Comparettia falcata* Poepp. & Endl. They found that hummingbirds still perceive rewards despite the low quantities and concentration. In addition, an increase in the production of rewards does not necessarily imply an increase in pollinator visitation, pollen transfer or reproductive success. In the rewardless *Anacamptis morio* (L.) R.M.Bateman, Pridgeon & M.W.Chase, the artificial addition of nectar increased not only pollinator visitation but also geitonogamous pollination (Johnson et al., 2004).

6.4.2 Plant features

The sepals and lip are the most important structures for the attraction of pollinators. Some fly-pollinated Pleurothallidinae have anatomical and ultrastructural differences between the base and the apex of sepals and show osmophoric tissue at the apex (de Melo et al., 2010; Pansarin et al., 2016; Pridgeon and Stern, 1985, 1983; Vogel, 1990). In *Trichosalpinx*, the absence of osmophoric tissue suggests that the apices of the sepals act as visual rather than olfactory stimuli (Pridgeon and Stern, 1985). The capitate trichome-like colleters of the sepals in several Pleurothallidinae secrete and synthesize fragrances (Mayer et al., 2011). In addition, some *Bulbophyllum* show glandular trichomes with similar histochemical features as *Trichosalpinx* (Nunes et al., 2015, 2014; Stpiczyńska et al., 2015). Cardoso-Gustavson et al. (2014) concluded that floral colleters in the Pleurothallidinae do not produce fragrances because secretion stopped before anthesis. However, our observations suggest that they secrete scents. Cardoso-Gustavson et al. (2014) also found fungal infections restricted to the colleters of the ovary. The fungal species producing these hyphae and their possible role in pollination are still unknown.

Crystals occur in the sepals, petals and lip of many other Pleurothallidinae (D. Bogarín, pers. observ.). However, little is known about their role in pollination. Chase and Peacor (1987) suggest that refractile properties of crystals in *Stelis* might mimic nectar droplets (pseudonectar) and are acting as visual attractants to lure pollinators. Crystals also occur in flowers of *Bulbophyllum* and other orchids (Davies and Stpiczyńska, 2014; Kowalkowska et al., 2014; Nunes et al., 2015, 2014; Prychid and Rudall, 1999; Stpiczyńska et al., 2015). *Trichosalpinx*, some fly-pollinated Pleurothallidinae and myophilous *Bulbophyllum* have unicellular papillae and secretory activity restricted to the adaxial epidermis of the lip (De Pádua Teixeira et al., 2004; Nunes et al., 2015, 2014; Stern et al., 1985). Moreover, in some *Bulbophyllum*, *Restrepia* Kunth and *Scaphosepalum* Pfitzer, the synthesis of fragrances in the papillae is associated with starchless plastids rich in plastoglobuli, ER and osmiophilic droplets as found in *Trichosalpinx* (Kowalkowska et al., 2014; Pridgeon and Stern, 1983; Stern et al., 1985). Lipophilic compounds and osmophoric tissue suggest a synthesis of fragrances on top of the papillae. Vogel (1990) documented the fragrance synthesis by the epithelium or 'emission layer' in *Ceropegia*. Similar to the lip of *Trichosalpinx* and *Bulbophyllum* (Davies and Stpiczyńska, 2014), fragrance synthesis in *Ceropegia* takes place in the glandular epithelium of the distal lobar ends of the corolla (Vogel, 1990). *Trichosalpinx* flowers lack stomata on the lip indicating that the epithelium releases fragrances by diffusion through the cuticle (De Pádua Teixeira et al., 2004; Kowalkowska et al., 2014). The osmophoric papillae of the lip and trichomes of the sepals indicate that two types of olfactory signals might be used. Likewise, two heterogeneous centers of fragrance synthesis also occur in *Bulbophyllum ornatissimum* (Rchb.f.) J.J.Sm. (Vogel, 1990). Dense cytoplasmic contents and an extensive network of RER are associated with the synthesis and secretion of proteins in the epithelium of the lip. This observation agrees with the ultrastructure and anatomy of the lip in some *Bulbophyllum*, which also produce abundant protein secretions probably as floral rewards or teases (Davies and Stpiczyńska, 2014). Similarly, *Bulbophyllum wendlandianum* (Kraenzl.) Dammer produces higher concentrations of protein in the epithelium rather than in the parenchyma as observed in *Trichosalpinx* (Kowalkowska et al., 2014). The species of *Bulbophyllum* pollinated by an insect weight mechanism also lack nectaries but synthesize lipid droplets (De Pádua Teixeira et al.,

2004). The papillae of the lip lack starch granules, suggesting that no nectar is produced there. The striated cuticle of epithelial cells of the lip is another feature shared by *Bulbophyllum* and *Trichosalpinx* (Davies and Stpiczyńska, 2014; Kowalkowska et al., 2014; Nunes et al., 2015, 2014; Stpiczyńska et al., 2015). This cuticle pattern could be linked to a mechanism of light diffraction, producing iridescence or more intense 'structural colours' and thus acting as visual cues (Antoniou Kourounioti et al., 2012; Nunes et al., 2015). It can also increase the emitting surface area of the lip or function as a tactile stimulus to insects (Vogel, 1990). The arcuate, incumbent column with a foot and the anatomy of the ligament of the lip that acts as a hinge evolved in several Pleurothallidinae and *Bulbophyllum* (Borba and Semir, 1998; Karremans et al., 2015b; Nunes et al., 2015; Vogel, 2001). The pollinarium of *Trichosalpinx*, made up of two pollinia and sticky caudicles, occur in several Pleurothallidinae related to *Trichosalpinx* (Stenzel, 2000) and in some *Bulbophyllum* (Nunes et al., 2015).

6.4.3 Pollination of angiosperms by biting midges

Biting midges of 13 genera have been recorded on the flowers of a wide array of angiosperms. Specifically, pollination by *Forcipomyia* spp. evolved independently in unrelated angiosperm families (Gibernau et al., 2004; Ollerton et al., 2009; Razzak et al., 1992). Although the mechanism of attraction used by these plants is largely unknown, the flowers adapted to the pollination of *Forcipomyia* often have dark, vinaceous, hirsute floral structures of different homology in combination with green, yellow or white structures (Figs. 6.23, 6.24).

Biting midges pollinate *Aristolochia bracteolata* Lamk (Aristolochiaceae) (Razzak et al., 1992) (Fig. 6.23A) and *Aristolochia watsonii* Wooton & Standl. (Aristolochiaceae) (Fig. 6.23B), possibly attracted by their hairy, dark purple limb and mouth of flowers (Crosswhite and Crosswhite, 1984; Woodcock et al., 2014) (Fig. 6.23B). In addition, adult female biting midges are the main pollinators of at least 19 species of *Ceropegia* (Apocynaceae) and the vine *Pararistolochia praevenosa* (F.Muell.) Mich.J.Parsons (Aristolochiaceae) (Fig. 6.23E). (Fig. 6.23C–E). These species have tubular bristly, purple corolla apices with some white, green or yellow parts (Ollerton et al., 2009; Williams and Adam, 2010). Two *Culicoides* spp. pollinate *Arum conophalloides* Kotschy ex Schott (Araceae), which has a purple spadix and spathe that produce scents that might mimic the vertebrate prey of the pollinator (Gibernau et al., 2004). The pollination of cacao, *Theobroma cacao* (Malvaceae), has been studied intensively due to its economic importance. Some authors claim that several species of flies (mostly biting midges) are probably attracted to and feed on compounds secreted by the purple trichomes of the staminodia (O'Doherty and Zoll, 2012; Winder, 1978) (Fig. 6.23F). In Orchidaceae, *Forcipomyia sauteri* pollinates the Australian *Bulbophyllum macphersonii* Rupp (Fig. 6.24B) (Bartareau, 1994). *Trichosalpinx* spp. and *B. macphersonii* may be examples of evolutionary convergence towards a common mechanism of pollination (Figs. 6.23, 6.24).

6.4.4 Floral fragrances

Some *Forcipomyia* spp. are attracted by aliphatic esters such as decyl hexanoate and hexyl hexanoate (Sugawara and Muto, 1974). However, the authors did not discuss any foraging behaviour of the flies in this experiment. The latter compound was also detected in the floral fragrance of

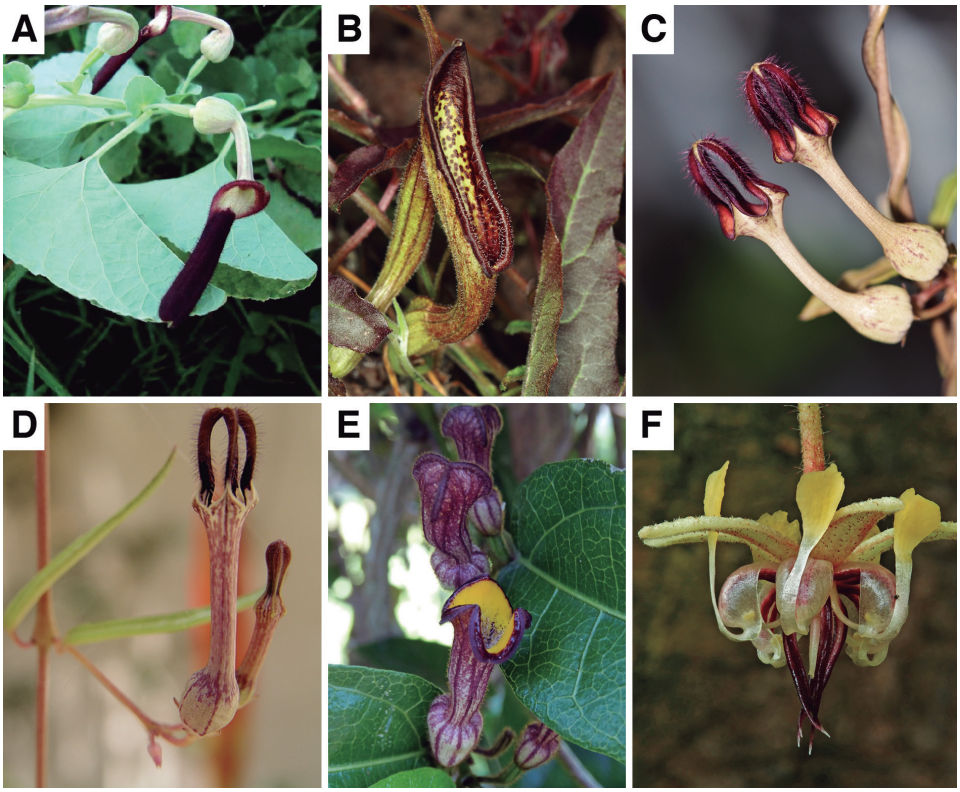

Figure 6.23. Floral morphology of some angiosperms pollinated by *Forcipomyia* spp. **A.** *Aristolochia bracteolata* from Asia showing the purple, hirsute limb. **B.** *Aristolochia watsonii* from North America with a purple-spotted, greenish limb. **C.** Asian *Ceropegia bulbosa* with the purple, hirsute apices of the corolla. **D.** *Ceropegia linearis* from Southern Africa showing the purple, hirsute apices of the corolla. **E.** *Pararistolochia praevenosa* from Australia showing the hirsute, spotted corolla with purple margin and the inner yellow surface. **F.** flower of the Neotropical *Theobroma cacao* with the purple, hairy stami- nodia. Photographs by A, ©Dr K. Sambandan. B, ©Russ Kleinman. C, ©Jason da Silva. D, ©David Midgley. E, ©Foam Bark Gully Gang. F, © H. Zell.

kleptomyiophilous *A. rotunda* (Oelschlägel et al., 2015). We detected lactic acid in *T. blaisdellii*, a compound which acts as long-distance attractant in *Forcipomyia taiwana*, a vertebrate feeder (W.-Y. Liu et al., 2009). Arsene et al. (2002) recorded *n*-alkenes such as heptacosene in the epicuticle of the cabbage white butterfly *Pieris rapae* (Lepidoptera). Other *n*-alkanes, alkenes and methylalkanes have been recorded as cuticular hydrocarbons in insects and spiders (Mant et al., 2005). We detected alkanes in both *T. blaisdellii* and *T. reflexa*, suggesting a correlation between insect epicuticles and floral fragrances of these orchids. Hexadecanoic acid (palmitic acid), octadecadienoic acid and 17-pentatriacontene, also detected in the floral fragrance of *Trichosalpinx*, are also wax components in spider webs (Prouvost et al., 1999; Trabalon and Assi-Bessekon, 2008). Heiduk et al. (2010) found mainly spiroacetals and aliphatic compounds in two *Ceropegia*

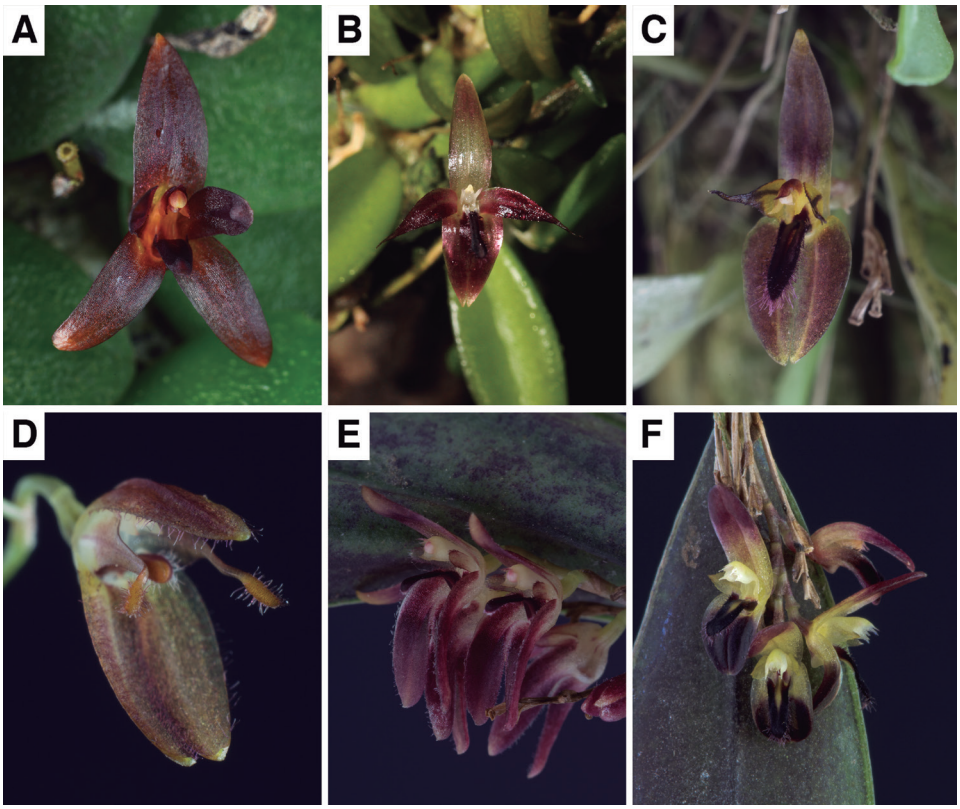

Figure 6.24. Flower morphology of some orchids pollinated by *Forcipomyia* spp. or probably adapted to a similar pollination system. **A.** Neotropical *Anathallis lewisiae* (Ames) Solano & Soto Arenas (*A. Karremans 6444*, JBL). B. *Bulbophyllum macphersonii* from Northern and North Eastern Queensland, Australia. **C.** *Lankesteriana barbulata* (Lindl.) Karremans (*D. Bogarín 12041*, JBL) from Costa Rica. **D.** *L. fractiflexa* (Ames & C.Schweinf.) Karremans (*E. Jiménez 2558*, JBL) from Costa Rica. **E.** Neotropical *T. blaisdellii* (*JBL-22846*, JBL). **F.** Costa Rican endemic *T. reflexa*. Photographs by A, C, D, E, F ©Diego Bogarín. B, ©John Varigos.

spp. They argue that these species mimic a chemical signal that attracts kleptoparasites. Indeed, the milichiid flies that pollinate both *Ceropegia* spp. are kleptoparasites that feed on haemolymph of prey in spider webs (Heiduk et al., 2016, 2015, 2010).

Heiduk et al. (2010) detected mostly aliphatic esters in the fragrance of *C. dolichophylla*, which also attract kleptoparasitic flies. *Aristolochia rotunda* uses a similar strategy, but fools kleptoparasitic females of Chloropidae that feed on true bugs (Miridae) captured by spiders. The main attractants are also aliphatic esters, aliphatic hydrocarbons and aliphatic alcohols (Oelschlägel et al., 2015). Further experimentation such as bioassays is needed to prove the role of these scents in the pollination of *Trichosalpinx* as has been performed in other deceptive systems (Oelschlägel et al., 2015; Phillips et al., 2014).

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

6.4.5 Breeding system

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

6.5 Conclusions

Trichosalpinx spp. might exclusively attract female midges by exploiting their protein collection instinct for egg production. The similar floral structures of other kleptomyophilous angiosperms compared to *Trichosalpinx* and the kleptoparasitic habits of *Forcipomyia* (Euprojoannisia) (only one report so far) suggest that kleptomyiophily may have evolved in *Trichosalpinx*. The well-developed mouthparts of the midges studied here indicate that they normally draw protein-rich haemolymph from animal hosts, so *Trichosalpinx* flowers are probably offering small quantities of proteins and sugars as meagre rewards. Although deception in pollination biology is usually equated with rewardlessness, it is possible that flowers use a deceit mimicking strategy, but they still provide rewards (Ackerman et al., 1994; Salguero-Faría and Ackerman, 1999; Vogel, 2001). In the phylogenetic context, at least two families of Diptera are involved in the pollination of species in the *Lepanthes* clade: Sciaridae males in *Lepanthes* and Ceratopogonidae females in *Trichosalpinx*. Most of the 25 *Trichosalpinx* spp. show similar floral traits and therefore we hypothesize that other *Trichosalpinx* spp. are pollinated via a similar system. The similarities among *Trichosalpinx* and the closely related *Anathallis* and *Lankesteriana* suggest that they also have similar pollination mechanisms. The pollination mechanisms of other related genera such as *Lepanthopsis*, *Tubella* and *Zootrophion* and the role of their pollinators as drivers of species diversification in the *Lepanthes* clade remain unknown. Future research should investigate the natural history of the *Forcipomyia* sp. studied here, including the discovery of the males, their feeding and breeding sites, diets and prey. Dietary analysis, bioassays and behavioural studies of both this *Forcipomyia* sp. and their insect prey and GC/MS analyses of their pheromones and

cuticular scents and the floral fragrance of other *Trichosalpinx* spp. are necessary to further test our hypotheses. Further biochemical characterization of the proteins, carbohydrates and crystals produced by the flowers and the detection of these compounds using stable isotopes to determine the extent of plant vs. animal food sources should also be conducted. Moreover, the fossil record of Ceratopogonidae is one of the best among Diptera. Research on amber fossils is key for the understanding of the evolution of pollination by biting midges and orchids (Borkent and Spinelli, 2007; Ramírez et al., 2007).

Chapter 7

Floral anatomy and evolution of pollination syndromes in *Lepanthes* and close relatives

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

7.1 Introduction

With more than 5100 species, Pleurothallidinae (Epidendreae) is the largest Neotropical orchid subtribe (Pridgeon et al., 2005). The species diversity of the group is concentrated in few genera. One of these is *Lepanthes* Sw., containing over 1200 species (about 25% of the species of Pleurothallidinae). The genus is phylogenetically most closely related to *Anathallis* Barb.Rod., *Draconanthes* (Luer) Luer, *Frondaria* Luer, *Lankesteriana* Karremans, *Lepanthopsis* (Cogn.) Ames, *Trichosalpinx* Luer and *Zootrophion* Luer, all considered members of the *Lepanthes* clade (Chase et al., 2015; Karremans, 2016) (Fig. 7.1). These genera are much less diverse than *Lepanthes* and account for only 1% of the species of the clade (Bogarín et al., 2018c). Studies on the evolution of the Pleurothallidinae showed that the most speciose lineages of the subtribe diversified recently (within the last 15 Ma) and the *Lepanthes* clade underwent the highest rate of species diversification (Pérez-Escobar et al., 2017a). However, the factors that have shaped this incredible species diversity in the Pleurothallidinae such as the role of plant-pollinator interactions are still largely unknown because the lack of knowledge on pollination strategies. Shifts in pollination strategies or adaptations to new pollinators exert evolutionary forces that enhance rapid speciation in angiosperms (Johnson, 2010; Kay and Schemske, 2008; Smith, 2010). In other plant groups, however, species radiations without changes in pollinator specialization have been documented (Ollerton et al., 2009) and other studies pointed out that certain pollination systems may increase species diversification independently of the pollination shift (Valente et al., 2012). In the *Lepanthes* clade, nothing is known about the pollination strategies of the members of the clade besides the pollination studies of a few species of *Lepanthes* and *Trichosalpinx*, and this hampers the understanding of evolutionary relationships within this group and the associated pollination shifts. *Lepanthes* flowers, which are mostly characterized by a bilaminate lip with a central appendix, exhibit a highly specialized pollination system involving sexual deception. Flowers are specifically pollinated by male fungus gnats of the genus *Bradysia* (Diptera, Sciaridae) probably attracted by a pheromone-mimicking strategy (Blanco and Barboza, 2005). On the other hand, the closely related *Trichosalpinx s.s.* (further referred to simply as *Trichosalpinx*), exhibits very different floral traits as compared to *Lepanthes* and consequently a different pollination mechanism. *Trichosalpinx* targets exclusively females of genus *Forcipomyia* (Diptera, Ceratopogonidae). Flowers attract the insects with the motile, ciliate, papillose surface of the lip blade, which secretes proteins and carbohydrates. The presence of females, the absolute absence of males and secretion of protein rewards indicate that *Trichosalpinx* imitates a model aimed at stimulating the protein collection behavior of females for egg production through a complex deceptive system likely related to kleptomyophyly (Bogarín et al., 2018a).

Some members of the clade exhibit similar floral traits, suggesting they may share similar pollination syndromes. For example, purple flowers with motile lips attached to the column foot by a thin ligament, as found in *Trichosalpinx,* are present in both *Anathallis* and *Lankesteriana*. These traits most likely evolved to accommodate pollination by biting midges since these features are also present in *Trichosalpinx* and other angiosperm pollinated by similar strategies such as *Bulbophyllum* Thouars, *Ceropegia* L. spp. (Asclepiadaceae) and *Theobroma cacao* L. (Malvaceae) (Bartareau, 1994; Bogarín et al., 2018a; O'Doherty and Zoll, 2012; Ollerton et al., 2009). However, other genera of the *Lepanthes* clade display floral traits that suggest pollination

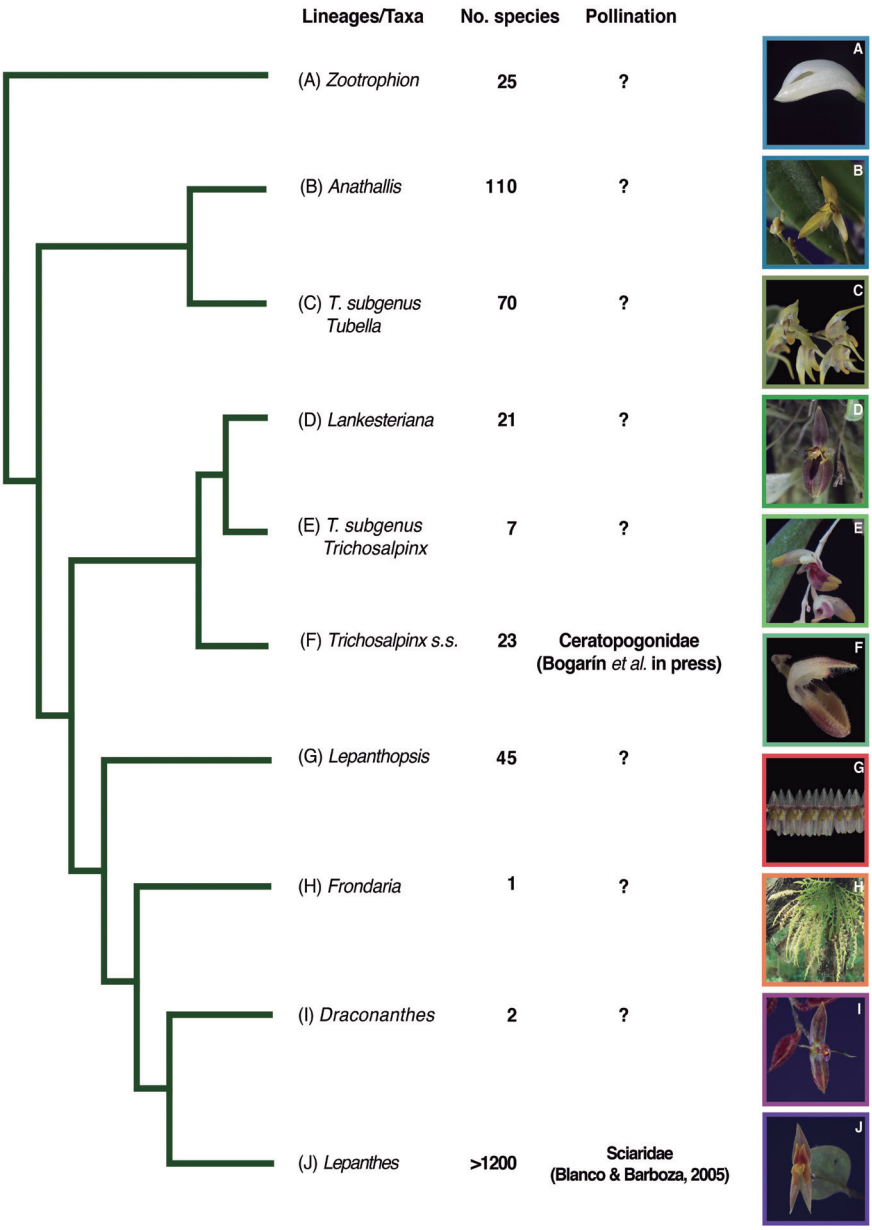

Figure 7.1. Phylogeny summary of the *Lepanthes* clade showing the main lineages/taxa, the number of species in each taxa and pollinator information based on our unpublished data using ITS and matK.

through different syndromes. For example, *Lepanthopsis* flowers exhibit reduced petals, a glenion at the base of the lip and a short column with bilobed stigma, much more similar to what is found in many *Pleurothallis* R.Br. and *Stelis* Sw. species rather than any of its closest relatives. In

Zootrophion, the flowers are very fleshy, with the sepals fused at the base and apex, and leaving only a window-like aperture at each side of the flower, features which are unique among its close relatives but found as well in a few unrelated genera, including *Acianthera* Scheidw., *Phloeophila* Hoehne & Schltr., *Specklinia* Lindl. and *Stelis*. In *Trichosalpinx* subgenus *Tubella* (=further referred to simply as *Tubella* Archila) flowers are mostly white with an entire lip blade, which contrast with the purple ciliated lips of *Trichosalpinx* (Luer, 1997; Luer, 2006, 2004).

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

7.2 Materials and Methods

7.2.1 Study site and sample collection

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

7.2.2 Digital Imaging and Microscopy

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

7.2.3 Microscopy fixation

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

7.2.4 Light Microscopy (LM) and Histochemistry

Entire fresh flowers and hand-cut sections of flowers were stained to detect lipids, polysaccharides and proteins. Flowers fixed in 70% ethanol for several days were also used for staining. Heavily pigmented tissue areas of fresh flowers were cleared for 10-60 min in 10% (v/v) commercial solution of sodium hypochlorite and rinsed in 30% ethanol for 1 hour before staining to avoid the interference of tissue coloration in staining results (Ruzin, 1999). Neutral or acidic lipids, phospholipids and fatty acids were detected with a solution of Nile Blue A 1% (NBA) (w/v, demi water) at 37ºC for 1 minute and differentiated in 1% acetic acid for 30 seconds at 37ºC and rinsed in demi water (Ruzin, 1999). Sudan IV 0.5% (SIV) (w/v, 70% ethanol) and Sudan Black B (SBB) 0.07% (w/v, ethanol 70%) were used to detect lipids (fats, oils and waxes) (Bronner, 1975; Ruzin, 1999). Insoluble polysaccharides and starch were detected with a periodic acid–Schiff reaction (PAS) by oxidizing the samples in aqueous solution of periodic acid (HIO_4) 5% (m/v) for 10 minutes, rinsing 3 times in distilled water for 2 minutes and submerging for 15 minutes in Schiff's reagent, and finally submerging in tap water at 50-60ºC for 5 minutes (Ruzin, 1999). Mucilage-secreting areas with acidic compounds, pectic acids or hexuronic acids were detected with a solution of Ruthenium Red 0.05% (RR) (w/v, tap water) for 15-20 minutes (Southworth, 1973). Proteins were detected with Aniline Blue-Black (ABB) 1% in 7% acetic acid for 10 minutes at 50-60ºC (Jensen, 1962; Fisher, 1968) and Coomassie brilliant blue R-250 (CBB) in a solution of 0.25% CBB, 50% ethanol, and 7% acetic acid for 3 minutes and rinsed in tap water (Fisher, 1968; Jensen, 1962). Areas of fragrance emission were detected by submerging the samples in a solution of Neutral Red 0.1% (NR) (w/v, tap water) for 15-20 minutes and differentiated with tap water (Ruzin, 1999).

7.2.5 Scanning Electron Microscopy (SEM)

Fixed flowers were dehydrated for 20 minutes in a series of ethanol solutions (70%–96%–≥99.9%) and twice in fresh ≥99.8% acetone. Critical-point drying using ≥99.8% acetone and liquid CO_2 as exchange fluids was performed in Automated Critical Point Dryer Leica EM CPD300 (Leica Microsystems, Wetzlar, Germany). The drying protocol included a cooling step at 15°C, 50% stirrer speed with auto version, slow CO_2 influx in the pressure chamber, with a delay of 120 seconds after influx CO_2 and before starting the exchange process, 18 exchange cycles (CO_2: 99.8% acetone) at a speed of 5, a fast heating speed and medium gas out speed. Dried samples were mounted in stubs with adhesive carbon conductive tabs and sputter-coated with 20 nm of Pt/Pd in a Quorum Q150TS sputter-coater. Resulting samples were observed with a JEOL JSM-7600F field emission scanning electron microscope, at an accelerating voltage of 10 kV.

7.3 Results

Anathallis: As in *Trichosalpinx* and *Lankesteriana*, the lip of the members of this genus is motile because it is hinged to the column foot by a thin membranous ligament. The papillose lip shows slightly striated cuticles and various secretions such as lipids (SIV), insoluble polysaccharides (PAS) but most notably proteins (ABB, CBB) on the apex of the papillae and occasionally prismatic crystals of calcium oxalate, indicating a secretory function similar to *Trichosalpinx* (Fig.

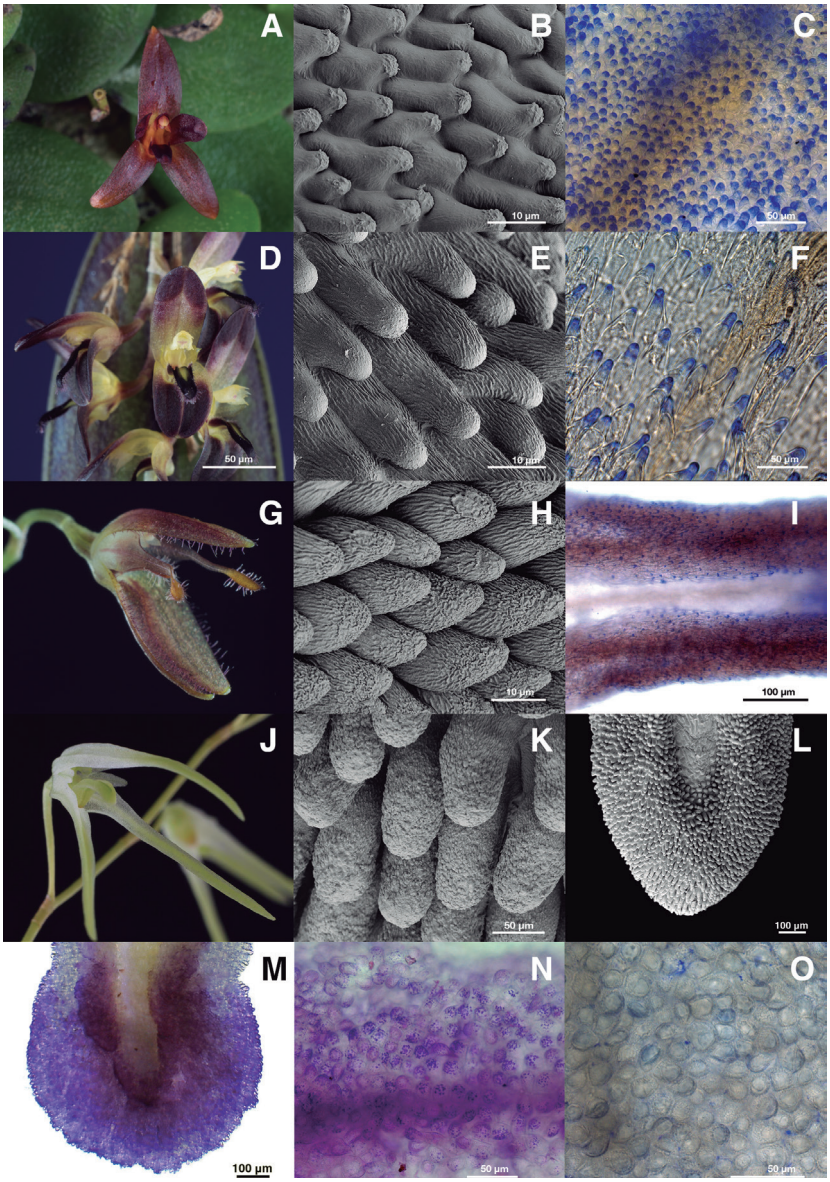

Figure 7.2. Flower morphology of some representatives of *Anathallis, Lankesteriana, Trichosalpinx* and *Tubella*. **A-C:** *Anathallis lewisiae*: **A.** Flower. **B.** Papillose surface of the lip. **C.** Detection of proteins with CBB in the epidermis of the lip. **D.** *T. reflexa.* **D-F:** *Trichosalpinx reflexa*: Flower. E. Papillose surface of the lip. **F.** Detection of proteins with CBB in the epidermis of the lip. **G-I:** *Lankesteriana fractiflexa*: **D.** Flower. **E.** Papillose surface of the lip. **F.** Detection of proteins with CBB in the epidermis of the lip. J-O: *Tubella arbuscula*: **J.** Flower. **K.** Papillose surface of the lip. **L.** Papillose apex of the lip with the median groove. **M.** Apex of the lip with positive detection of carbohydrates (PAS). **N.** Papillose side of the lip with positive detection of carbohydrates (PAS). **O.** Apex of the lip with negative detection of proteins (CBB) Photos: D. Bogarín).

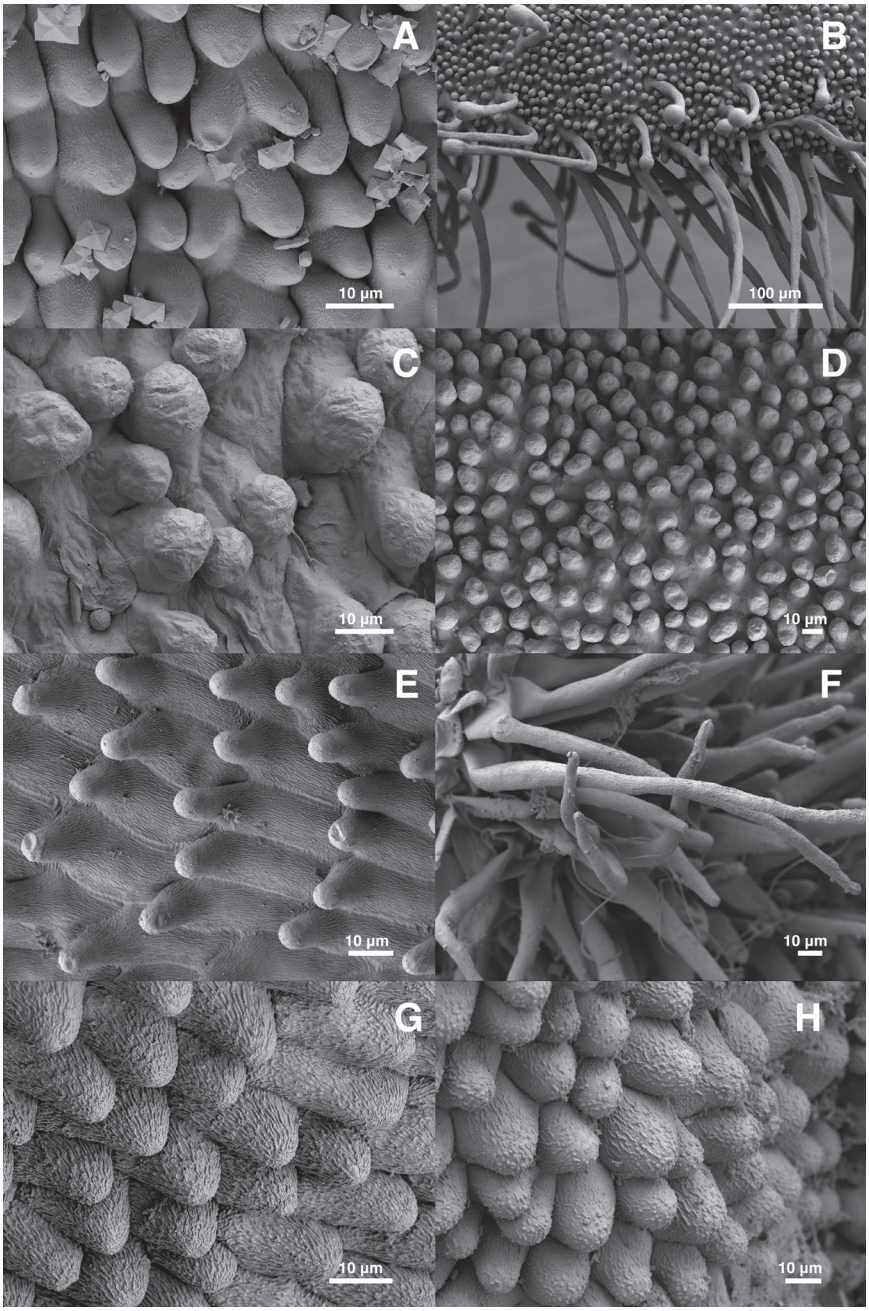

Figure 7.3. SEM micrographs of the epidermis of the lip of: **A.** *Anathallis lewisiae*. **B.** *Lankesteriana barbulata*. **C.** *G. chamaelepanthes*. **D.** *T. pergrata*. **E.** *T. reflexa*. **F.** *T. ringens*. **G.** *T.* cf. *patula*. **H.** *Tubella dura*. (Photos: D. Bogarín).

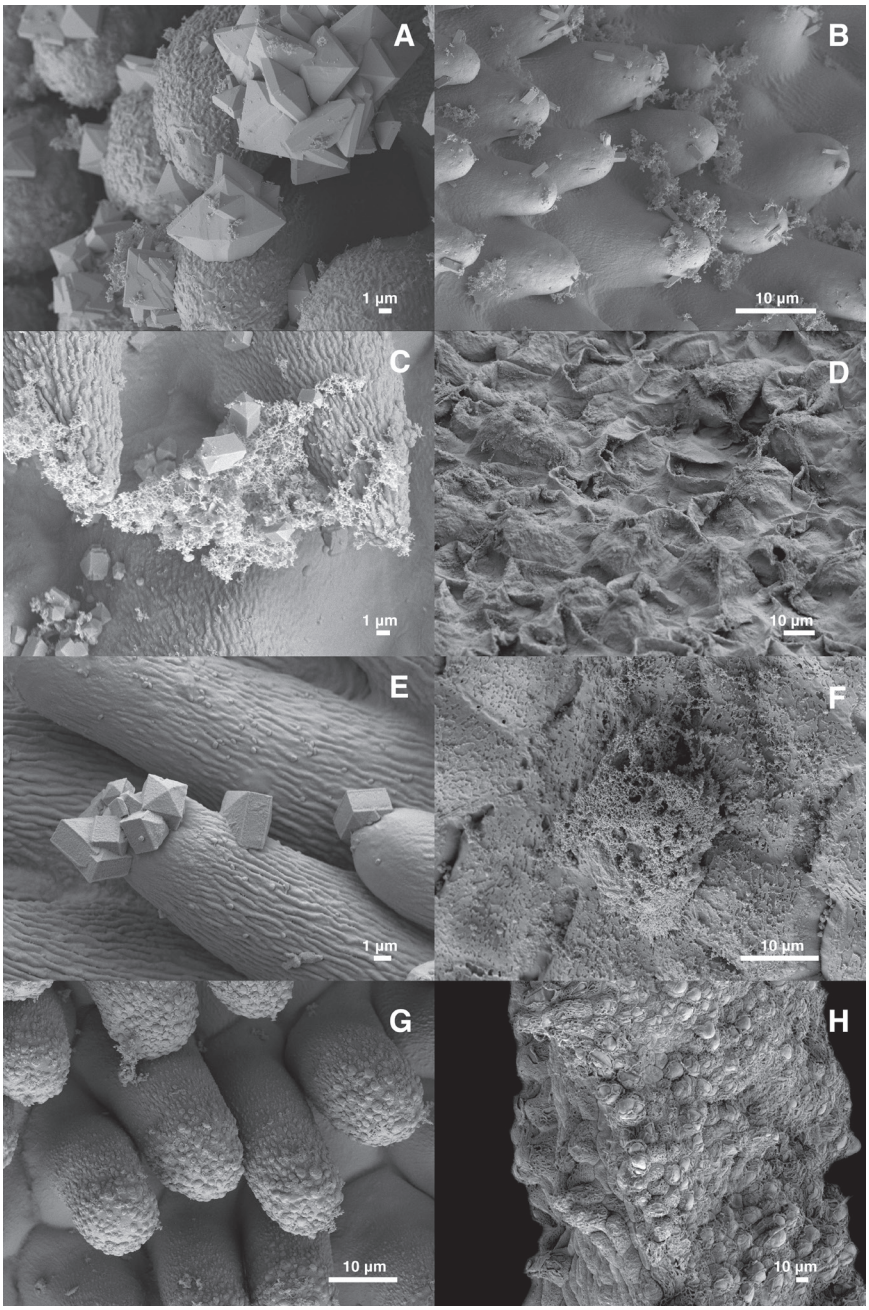

Figure 7.4. SEM micrographs of the epicuticular secretions in the epidermis of the lip of: **A.** *Anathallis funerea*. **B.** *Lankesteriana barbulata*. **C.** *Lepanthes chameleon*. **D.** *Stellamaris pergrata*. **E.** *T. reflexa*. F. *T. ringens*. **G.** *Tubella dura*. **H.** *Z. endresianum*. (Photos: D. Bogarín).

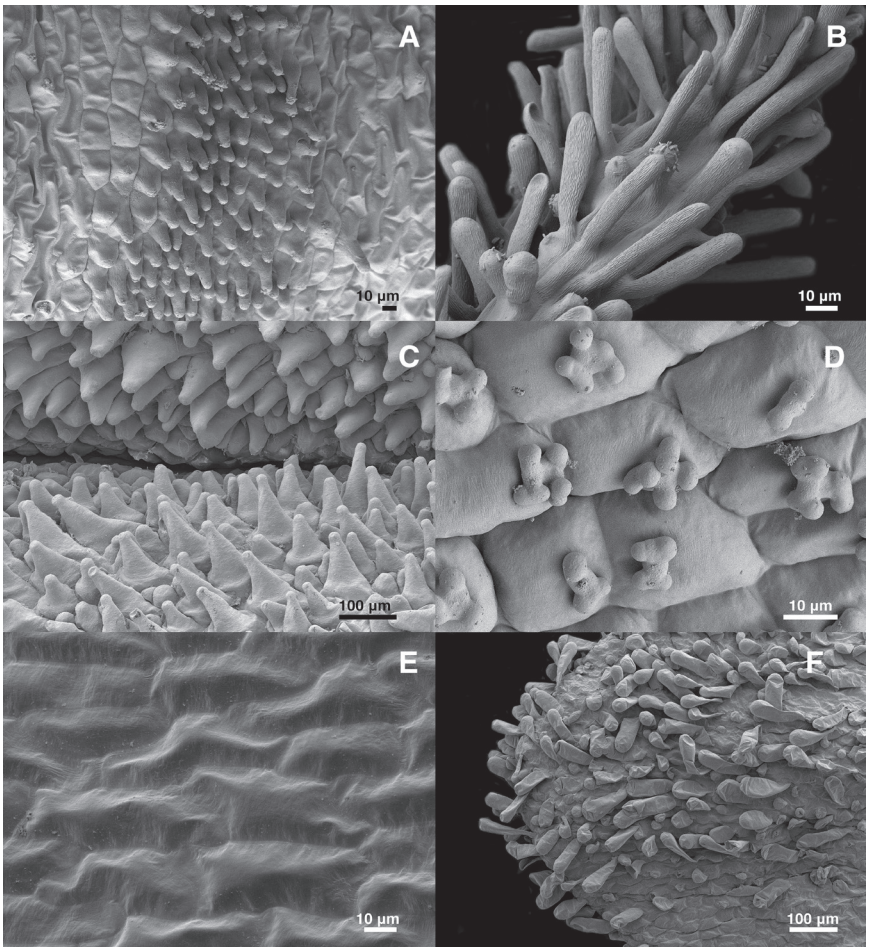

Figure 7.5. SEM micrographs of the epidermis of the petals of: **A.** *Anathallis lewisiae*. **B.** *Lankesteriana fractiflexa*. **C.** *Lepanthes chameleon*. **D.** *Lepanthopsis prolifera*. **E.** *T. reflexa*. **F.** *S. pergrata* (Photos: D. Bogarín).

7.1A-C, 7.3A, 7.4A). Differences with *Trichosalpinx* and *Lankesteriana* include the absence of elongated cells towards the margins and the raised callus at the base of the lip. In *A. lewisiae* (Ames) Solano & Soto Arenas, the petals are also papillose, with striated cuticles and secretions at the apices unlike the petals of *Trichosalpinx* (Fig. 7.5E). Flowers of some *Anathallis* species have purple colors, but other species have white or yellowish flowers.

Lankesteriana: Species belonging to the genus also show similarities with the lip of *Trichosalpinx* and *Anathallis* species, including the papillose surface of the lip with striated cuticle and secretory activity (Fig. 7.1G-I, 7.3B, 7.4B). We detected lipids (SIV), insoluble polysaccharides (PAS) and again proteins on top of the papillae (ABB, CBB) (Fig. 7.1I). Also, the lip shows elongated cells with widened apices scattered towards the lip apex unlike the elongated

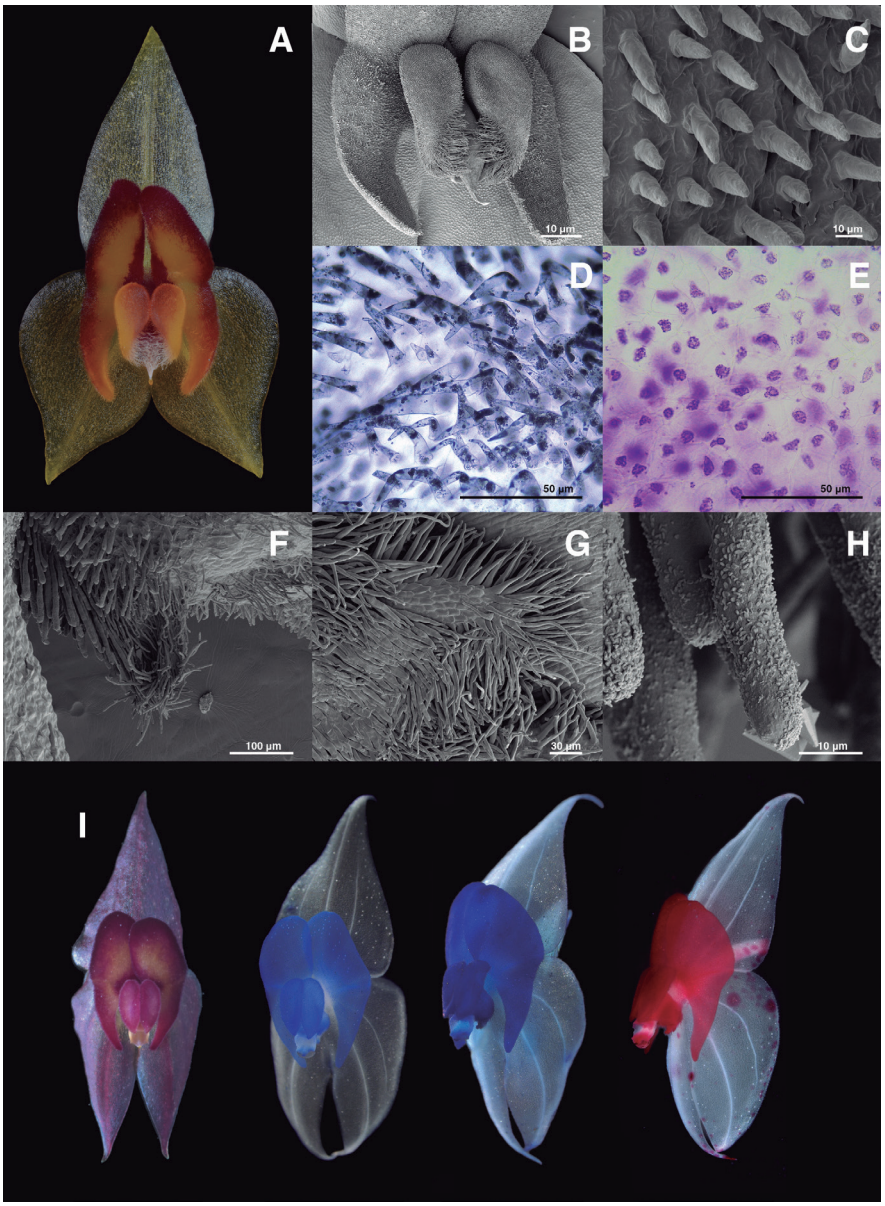

Figure 7.6. Flower anatomy and histochemistry of: A-H: *Lepanthes horichii*. **A.** Flower. **B.** SEM of the lip and petals. **C.** papillose surface of petals. **D.** LM of cells of the epidermis of the petal stained with SBB showing lipid droplets (black). **E.** LM of the epidermal cells the petal stained with PAS (carbohydrates). **F.** SEM micrographs of the hairy appendix of the lip. **G.** detail of the appendix. **H.** detail of the elongated cells (hairs) of the appendix. **I.** Histochemistry of *Lepanthes bradei* showing the positive reaction with stains, from left to right: flower with natural pigmentation, CBB, ABB, NR (Photos by D. Bogarín and M.M. Chabert).

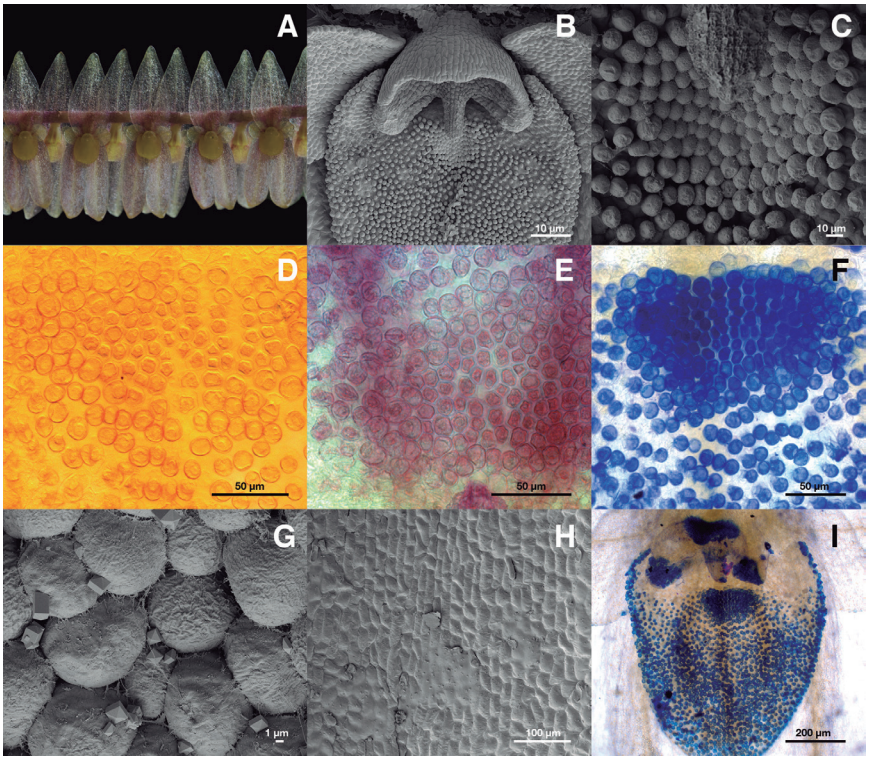

Figure 7.7. Flower anatomy and histochemistry of: **A-I:** *Lepanthopsis floripecten*, **A.** Flowers. **B.** SEM of the petals, lip and column. **C.** papillose surface of the glenion. **D.** LM of cells of the glenion stained with SIV showing lipids surrounding the cell walls of the papillae. **E.** LM of cells of the glenion stained with PAS detecting carbohydrates. **F.** LM of cells of the glenion stained with CBB detecting proteins. **G.** detail of the papillae of the glenion with some epicuticular secretions. **H.** detail of the smooth surface of the petals. **I.** LM of the papillose lip stained with CBB detecting proteins (Photos by D. Bogarín and M.M. Chabert).

cells of *Trichosalpinx* that are restricted to the margins and not widened at apex (Fig. 7.3B). The combination of brown-purple with white in the flowers of some *Lankesteriana* is similar to the flowers of *Trichosalpinx* (Fig. 7.1D, G). The lip of *Lankesteriana* is grooved unlike that of *Trichosalpinx* and *Anathallis*. Like *Anathallis*, the apices of the petals are papillose, secretory, with striated cuticles, but sometimes with elongated cells (Fig. 7.5A-B). Some *Lankesteriana* species have elongated, ciliated or papillose petals, ending in widened apices such as *L. fractiflexa* (Ames & C.Schweinf.) Karremans. Other species such as *L. barbulata* (Lindl.) Karremans have *Trichosalpinx*-like petals without cilia but with irregular margins.

Trichosalpinx: in *Trichosalpinx* species [including the relatives of *P. berlineri* (Luer) Karremans & Mel.Fernández], the base of the lip is attached to the column foot by a membranous ligament. The lip blade is papillose with elongated cells appearing towards the margins. The papillae of the lip blade exhibit a noticeable striated cuticle and towards the apices the cuticle is smooth and se-

cretory. We detected lipophilic compounds (SBB), polysaccharides (PAS) and proteins (CBB and ABB) within and on the apex of the papillae, indicating a secretory function (Fig. 7.1D-F, 7.3C, E, F, G, 7.4D, E, F). Tests with (NR) were positive in the apices of the papillae. In particular, the lip of *T. ringens* Luer has elongated hairs with some striations in the cuticle unlike the short papillae of most of *Trichosalpinx* species (Fig. 7.3F). Crystals of calcium oxalate were exuded on the apices of the papillae but were not present in all the specimens analyzed (Fig. 7.4E). The petals are oriented parallel to the column and they do not have papillose surfaces (Fig. 7.5E) and no secretory activities were detected because histochemical tests yielded no positive results. The column is arcuate with a rounded foot where the lip is attached. At the base of the column foot, there are similar papillae to those observed on the sepals and lip.

Lepanthes: flowers of *Lepanthes* are characterized in general by a bilobed lip with an appendix at the base, and an elongated column with apical anther and stigma (Fig. 7.6A, B, F-H). Our studies indicate a generalized pattern in the epidermal surface of the sepals and petals. The sepals have flattened, smooth cells and, in contrast, the petals and lip are always papillose (Fig. 7.5C, 7.6B, C). The sepals did not react to histochemical stains but the petals reacted to all the stains applied for carbohydrates (PAS, RR), lipids (SBB, SIV), proteins (ABB, CBB) and scents (NR) (Fig. 7.6D, E, I). The appendix of the lip is ciliated, hirsute or with a combination of elongated and flattened cells (Fig. 7.6F-H). Prismatic crystals and compounds on the surface of the cells were observed (Fig. 7.4C).

Lepanthopsis: it is easily recognized by the flattened flowers with reduced petals and the presence of a glenion at the base of the papillose lip, a feature not found in any of the sister genera (Fig. 7.7). In *L. floripecten*, the glenion is made up of an aggregation of papillose and secretory cells just in front of the very reduced column. Flowers of some species resemble those of *Platystele* Schltr. (Fig. 7.7B-C, 7.8A-D). In *L. astrophora*, the glenion is also papillose but sunken (Fig. 7.8B). The papillose lip reacts for proteins on top of the papillae over the lip (ABB, CBB) but most notably in the glenion (Fig. 7D). SEM images show several compounds in the surface of the glenion that also react positively for lipids (SIV) and insoluble polysaccharides (PAS) (Fig. 7.7 D, E). Petals and sepals have flattened cells and do not react for proteins and carbohydrates but probably contain epicuticular waxes (SIV). However, other species such as *L. prolifera* have epidermal cells with characteristic projections (Fig. 7.5D). We observed prismatic crystals on the surface of the cells but mostly concentrated on the glenion (Fig. 7.7G).

Tubella: Flowers of this group are generally white-greenish to yellowish (Fig. 7.1, J). The lip is papillose with striated cuticles like in *Anathallis*, *Trichosalpinx* and *Lankesteriana* but with a median groove of flattened cells and without cilia along the margins (Fig. 7.2K-M). Some areas of the lip at the sides and towards the apex contain insoluble polysaccharides (PAS) but the blade does not react for proteins (ABB, CBB) and lipids (SIV) (Fig. 7.2M-O). Some species are fragrant such as *T. arbuscula* (Lindl.) Luer. The petals are flattened, not ciliated and without papillae. The sepals are elongated and also entire. Some species classified have smooth papillose surfaces on the lip such as *G. chamaelepanthes* (Rchb.f.) Bogarín & Karremans and *S. pergrata* (Ames) Luer (Fig. 7.3C, D). Also, the apex of the petals of *S. pergrata* are papillose in contrast of those of *T. arbuscula* and *T. dura* (Lindl.) Luer (Fig. 7.5E).

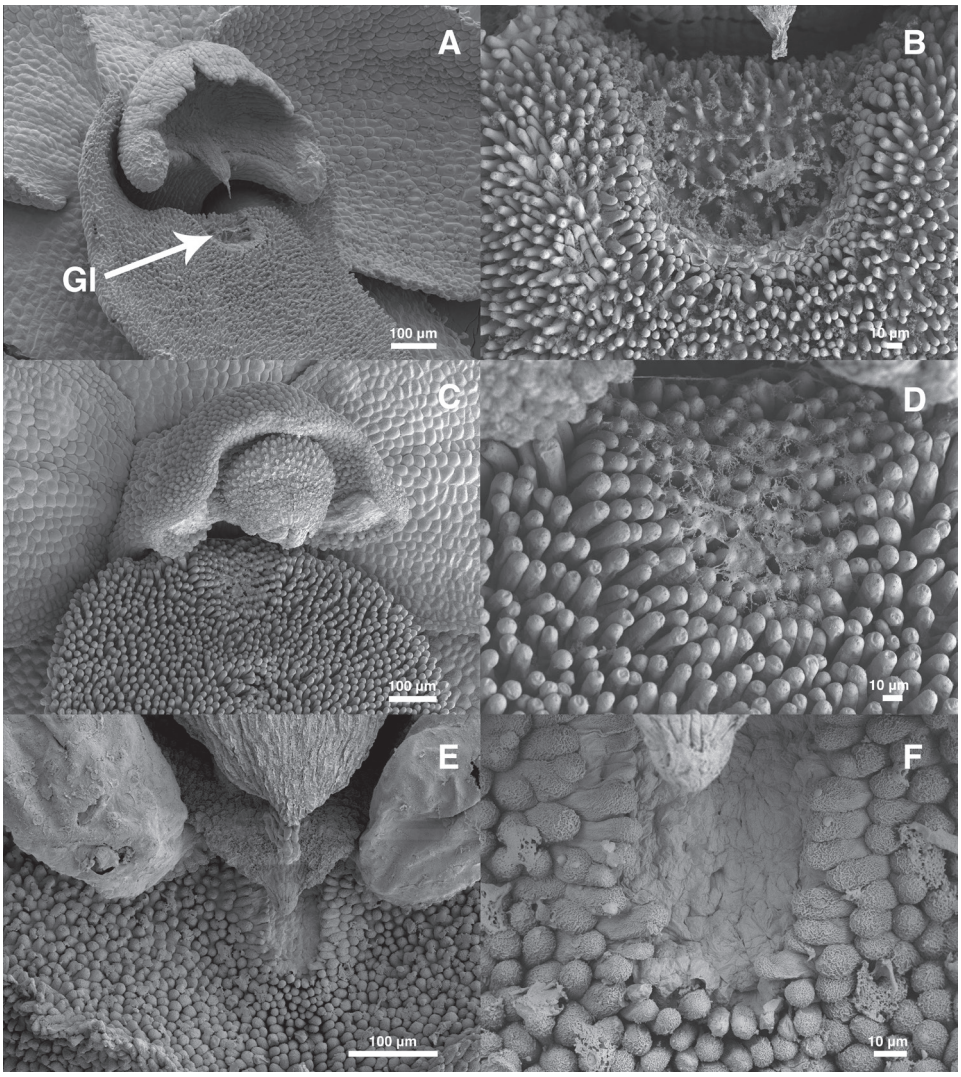

Figure 7.8. SEM micrographs of the column and glenion at the base of the lip of: **A.** *Lepanthopsis astrophora*. **B.** Detail of the glenion of *Lepanthopsis astrophora*. **C.** *Platystele sp*. D. Detail of the glenion of *Platystele sp*. **E.** *Stelis sp.* **F.** Detail of the glenion of *Stelis sp*. (Photos: D. Bogarín).

Zootrophion: Flowers of this genus are unique within the group. The basally and apically fused sepals form a closed flower with only two open sides, resembling windows. The color of the flower varies from white and yellow to pinkish and purple, sometimes with spotted sepals and petals (Fig. 7.9A). The inner surface of the synsepal is made up of papillose or rugose surfaces that react to carbohydrates (PAS) and lipids (SB). In *Z. vulturiceps* (Luer) Luer, a species with white flowers, we did not detect proteins (CBB) on the rugose surface (Fig. 7.9B-F). However,

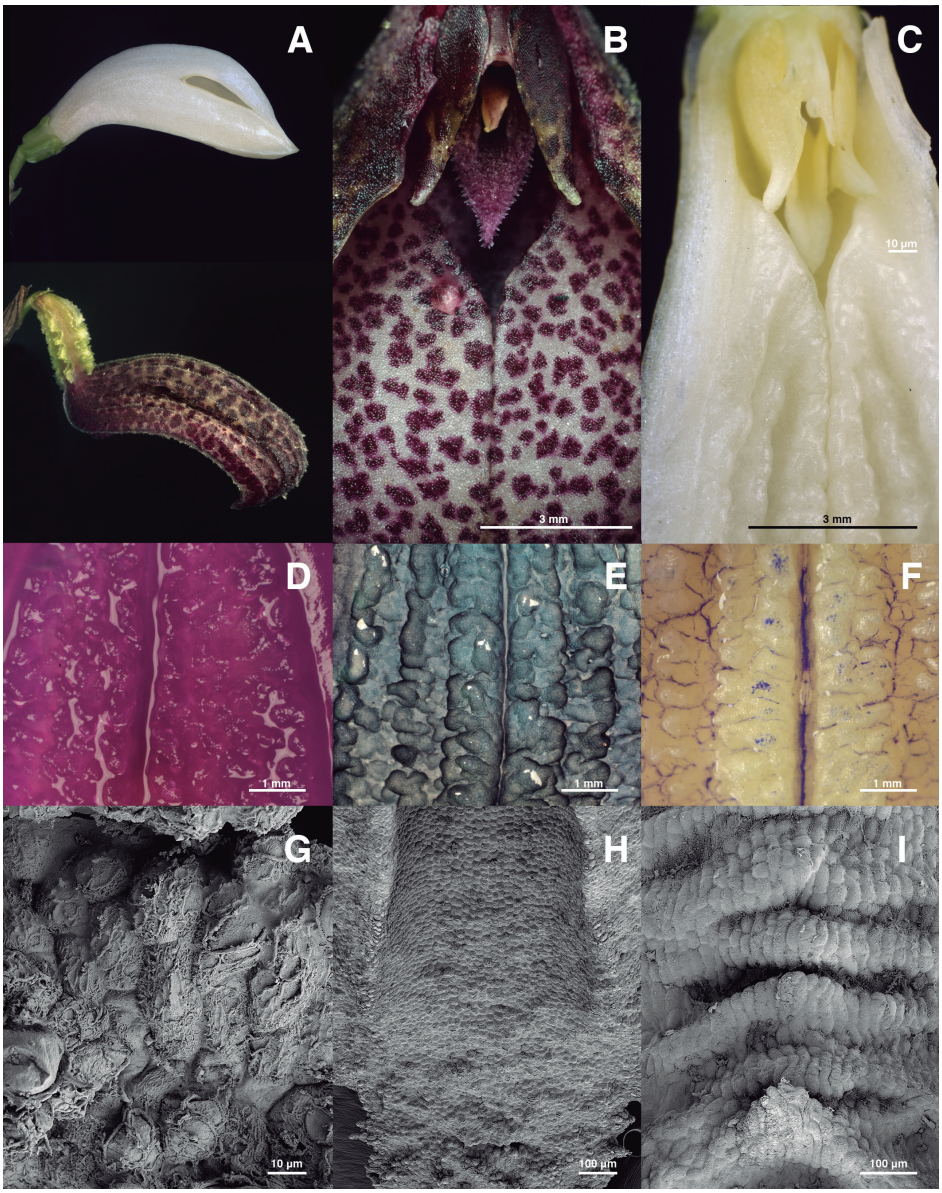

Figure 7.9. Flower anatomy and histochemistry of *Zootrophion*: **A.** Flower of *Z. vulturiceps* (upper), *Zootrophion* sp. (lower). **B.** Inner view of the flower of *Zootrophion* sp. showing the papillose surface of sepals and the shorter column, lip and petals. **C.** Inner view of the flower of *Z. vulturiceps* showing the rugose surface of sepals and the shorter column, lip and petals. **D-F:** Histochemistry of cells of the epidermis of the synsepal of *Z. vulturiceps*. **D.** staining with PAS (carbohydrates). **E.** staining with SBB showing lipids (black). **F.** staining with CBB showing proteins (blue). **G-F:** SEM micrographs of the rugose surface of the lip of *Z. endresianum*. **G.** epicuticular compounds on the epidermis. **H.** view of the papillose mid part of the lip. **I.** rugose surface of the base of the lip.

the papillose area of the synsepal of the purple spotted *Zootrophion* sp. shows a positive reaction for proteins (CBB). The dorsal sepal is smooth and does not react with the stains. The lip is very reduced and motile and it is attached to the column foot by a membranose tissue securing mobility. The surface is papillose and rugose with various evident secretions on the epidermal cells (Fig. 7.4H, 7.9G-H). The column is footed and elongated like in *Anathallis*, *Trichosalpinx* and *Lankesteriana*. The petals are parallel to the column and enclose the column and lip.

7.4 Discussion

7.4.1 Floral micromorphology and histochemistry

The epidermal secretory papillae of the lip of *Anathallis, Lankesteriana* and *Trichosalpinx* is also present in other myophilous species of *Bulbophyllum* and fly-pollinated Pleurothallidinae (De Pádua Teixeira et al., 2004; Nunes et al., 2015, 2014; Pridgeon and Stern, 1985). Secretion of proteins on the lip occurs in species of *Bulbophyllum* sect. *Racemosae* Benth. & Hook.f., possibly as floral rewards for female flies (Davies and Stpiczyńska, 2014). In addition, flowers of *B. wendlandianum* contained protein secretions in the epithelium (Kowalkowska et al., 2014), as observed previously in *Trichosalpinx* and in the species of *Anathallis* and *Lankesteriana* studied here. Positive reaction with NR and lipophilic compounds also indicate scent synthesis in the papillose epidermis. (Vogel, 1990) documented fragrance emission in the epithelium of the distal lobar ends of the corolla of *Ceropegia*, which is mostly pollinated by biting midges. These structures are purple, papillose or hairy, like the lip of some species of *Anathallis, Lankesteriana* and *Trichosalpinx*. The striated cuticle of the papillae is another feature shared by species of these genera and also with some myophilous *Bulbophyllum* species (Davies and Stpiczyńska, 2014; Kowalkowska et al., 2014; Nunes et al., 2015, 2014; Stpiczyńska et al., 2015). Striated cuticles have been associated with light diffraction producing more intense “structural colors” acting as a visual effect on pollinators (Antoniou Kourounioti et al., 2012). Nunes et al., (2015), postulated that the striated cuticular patterns in *Bulbophyllum* sect. *Napellii* Rchb.f. are related to these visual cues. Our findings on *Anathallis* and *Lankesteriana* support previous observations in *Trichosalpinx* and *Bulbophyllum* in which the striated cuticles are present only in the purple-colored areas of the lip and petals, whereas the whitish or translucent areas have flattened and smooth epidermal cells. The papillose epidermal areas of flowers might increase the area of emission of scents or “emission layer” (Vogel, 1990). In *Lepanthes*, the active parts of the flower in terms of compound synthesis are the papillose epidermis of petals and scattered colleters of sepals. The role of this tissue in the production of pheromone-like odors that attract male fungus gnats as pollinators has to be tested experimentally but our histochemical evidence indicates that the papillose petals are involved in scent synthesis. In addition, this papillose epidermis is mostly not striated and does not concentrate the secretions on the apex of the papillae as observed in *Anathallis, Lankesteriana* and *Trichosalpinx*, indicating that *Lepanthes* flowers do not produce collectable rewards. This is consistent with the hypothesis of sexual deception and behavior of pollinators in the flowers that do not search for rewards. On the other hand, papillose or verrucose areas of the synsepal of *Zootrophion* are secretory and the epicuticular compounds on the cells of the epidermis of the lip observed with SEM may indicate that *Zootrophion* flowers offer rewards.

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

The detection of proteins and carbohydrates on the apex of the papillae of the lip and particularly in the glenion of *Lepanthopsis floripecten* (Rchb.f.) Ames suggests that pollinators are guided towards this point. The glenion has been defined as a circular structure or callus at the base of the labellum, placed right in front of the reduced column, which occurs in several unrelated genera of Pleurothallidinae, namely *Brachionidium* Lindl., *Lepanthopsis*, *Platystele*, *Pleurothallis* R.Br.. *Stelis* Sw. and *Teagueia* (Luer) Luer (Pridgeon et al., 2005) (Fig. 7.8). The function of the glenion in the pollination of species of these genera is discussed in further detail by Karremans and Díaz-Morales (2018). Initial evidence indicates that this structure is an aggregation of papillose or flattened cells (sometimes sunken) of secretory activity. The anatomy of the glenion varies across these genera and more ultrastructural and histochemical comparative studies are needed to characterize the micromorphology and its role in pollination.

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

7.4.2 Pollination syndromes in the *Lepanthes* clade

The recent discovery of the pollination of *Trichosalpinx* by biting midges allows us to make inferences about the pollination systems of other members of the *Lepanthes* clade (Bogarín et al., 2018a). *Trichosalpinx*, *Lankesteriana* and the *P. berlineri* group have a close affinity according to the latest phylogenetic analyses of the Pleurothallidinae and unpublished data (Karremans, 2016, 2014; Pérez-Escobar et al., 2017a) (Fig. 7.1). Species of *Lankesteriana* and the *P. berlineri* group have a mobile, ciliate lip that is almost indistinguishable from those of *Trichosalpinx* and some *Bulbophyllum* (Bartareau, 1994; Luer, 2006). Although no data on pollination of *Lankesteriana* and *P. berlineri* group are available, our findings suggest pollination by biting midges. The papillose epidermis with a striated cuticle and secretion of proteins are consistent with the anatomical features found previously in the *Trichosalpinx* species pollinated by females of *Forcipomyia* that search for proteins (Bogarín et al., 2018a). In addition, this hypothesis is strengthened by floral traits present in other angiosperm groups pollinated by biting midges such some *Aristolochia L.* and *Pararistolochia* Hutch. & Dalziel in the Aristolochiaceae, *Caralluma* R.Br., *Ceropegia L.* in the Apocynaceae and *Abroma* Jacq., *Herrania* Goudot and *Theobroma* L. in Malvaceae (Davies

and Stpiczyńska, 2014; Kowalkowska et al., 2014; Nunes et al., 2015, 2014; Stpiczyńska et al., 2015; Vogel, 1990). In the Orchidaceae, the flowers of the distantly related Australian *Bulbophyllum macphersonii* Rupp., a species pollinated by biting midges, are very similar to those of *Trichosalpinx, Lankesteriana* and some *Bulbophyllum* spp. of sections *Hybochilus* Schltr., *Oxysepalum* Schltr. and *Polyblepharon* Schltr. (Bartareau, 1994). Common features among these species are again the ciliated, purple, mobile lip with two basal auricles, and the purple sepals and petals. *Trichosalpinx* and *B. macphersonii* are an example of evolutionary convergence towards a common mechanism of pollination, and this is likely occuring in *Lankesteriana* and the species of the *P. berlineri* group as well.

Similarity in floral traits are also present in some *Anathallis* species, such as *A. lewisiae* (Fig. 7.2A), *A. microgemma* (Schltr. ex Hoehne.) Pridgeon & M.W. Chase and *A. nanifolia* (Foldats) Luer as noted by Luer (1997). *Anathallis* appears to be related to species of *Tubella* in the phylogenetic analysis and are not embedded within the *Trichosalpinx* clade in the strict sense (Bogarín et al., 2018c) (Fig. 7.1). However, some species have purple flowers and a mobile lip (though not ciliated) hinged by a membrane at the bottom of the column foot. Pollinators of *Anathallis* are not yet known, but we hypothesize that some species showing striated papillae and secretion of proteins in the epidermis of the lip represent another case of evolutionary parallelism to attract the same type of pollinator guild as *Lankesteriana* and some *Bulbophyllum* and *Trichosalpinx* species.

Floral morphology of *Stellamaris pergrata* (Ames) Luer, *T. ringens* Luer and *T. sanctuarii* Mel. Fernández & Bogarín is different from the species of *Trichosalpinx* s.s. For example, the absence of a trembling lip with a flexible membrane and the *Acianthera*-like flowers of *T. ringens* and *T. sanctuarii* indicate that these species may be pollinated by different pollinator groups. The red flowers of *S. pergrata* with papillose apices of petals and two nectary-like structures in the column also suggest another, yet unknown, pollination mechanism (Fernández and Bogarín, 2013, 2011; Luer, 1997).

The species of *Tubella* (*T.* subgenus *Tubella*) are not strictly embedded within the *Trichosalpinx* clade (Karremans, 2016; Pridgeon et al., 2001; Rykaczewski et al., 2017). Species of *Tubella* have white or yellowish flowers and the lip is not ciliate. Besides the preference of biting midges for flowers with purple and hirsute structures, they have also been documented to visit plants like rubber, *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg. (Euphorbiaceae) and mango, *Mangifera indica* L. (Anacardiaceae), with white flowers (Borkent and Spinelli, 2007). Males and females seek nectar in these small white flowers to meet their energy needs. Art Borkent (pers. comm.) observed biting midges of the genera *Atrichopogon* and *Dasyhelea* Kieffer, a group with reduced mouthparts and without blood sucking behaviour, visiting flowers of *Epidendrum piliferum* Rchb.f. in Monteverde, Costa Rica, an orchid with white flowers and purple nectar guides on the blade of the lip. (Pedersen, 1995) recorded biting midges of the genus *Forcipomyia* as a visitor of *Dendrochilum longibracteatum* Pfitzer, an orchid species with white flowers and a brownish lip. As already noted by (Luer, 1997), white flowers with caudate petals are present in species of *Specklinia* subgen. *Hymenodanthae*, such as *S. calyptrostele* (Schltr.) Pridgeon & M.W.Chase, which resemble flowers of *Tubella*. Karremans (2016) recorded pollination of *S. calypstrotele* by a Ceratopogonidae species, possibly *Atrichopogon*. Therefore, the pollinators of *Tubella* may be biting midges as well but the operating mechanism is probably

different, similar to the anthophilous nectar-seeking flies pollinating the white flowers of *Hevea* or *M. indica*. Although more pollination observations and anatomical and histological studies are needed in this genus, the presence of carbohydrates found in the papillae of the lip instead of proteins support these hypotheses.

Of the other genera belonging to the *Lepanthes* clade, there is no information available on pollinators yet. The floral morphology of these groups is unlike those already studied suggesting that at least two additional mechanisms may be in place. *Lepanthopsis* is unique amongst its close relatives in having a papillose secretory glenion of the lip. The sepals and petals are generally flat and the sepals are caudate. The column is short, broad and footless, the anther is apical with a bilobed stigma (except for a few species). This type of column suggests that the pollinarium is positioned either on the head, antenna, or legs, but most likely not on the dorsal part of the thorax or abdomen of the pollinator. Undoubtedly, this represents another pollination mechanism yet unknown but different from the currently documented cases in *Lepanthes* and *Trichosalpinx*. Other unrelated genera like *Brachionidium*, *Platystele*, *Pleurothallis, Stelis* and *Teagueia* exhibit similar floral traits, specifically flat flowers with a short column with a bilobed stigma and glenion at the base of the lip (Luer, 1990). Some of these groups are pollinated by Mycetophilidae and Sciaridae (Duque-Buitrago et al., 2014), and these families may be involved in the pollination of *Lepanthopsis* as well, as further discussed by (Karremans and Díaz-Morales, 2018). Some species of the aberrant group *T.* subgenus *Xenia* such as *Trichosalpinx ballatrix* Luer & Escobar*, T. escobarii* Luer and *T. tenuiflora* (Schltr.) Luer are somewhat florally similar to some *Teagueia* (such as *T. barbeliana* L.Jost & A.Shepard and *T. puroana* L.Jost & A.Shepard). Unfortunately, we do not have any, anatomical, phylogenetic or pollination data available for these groups yet (Luer, 1997).

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

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

7.5 Conclusions

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

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

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

Evolutionary diversification and biogeography of *Lepanthes* and allies

Chapter 8

Recent origin and rapid speciation of Neotropical orchids in the world's richest plant biodiversity hotspot

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Abstract. The Andean mountains of South America are the most species-rich biodiversity hotspot worldwide with c. 15% of the world's plant species, in only 1% of the world's land surface. Orchids are a key element of the Andean flora, and one of the most prominent components of the Neotropical epiphyte diversity, yet very little is known about their origin and diversification. We address this knowledge gap by inferring the biogeographical history and diversification dynamics of the two largest Neotropical orchid groups (Cymbidieae and Pleurothallidinae), using two unparalleled, densely sampled orchid phylogenies (including more than 400 newly generated DNA sequences), comparative phylogenetic methods, geological and biological datasets. We find that the majority of Andean orchid lineages only originated in the last 20–15 Ma. Andean lineages are derived from lowland Amazonian ancestors, with additional contributions from Central America and the Antilles. Species diversification is correlated with Andean orogeny, and multiple migrations and recolonizations across the Andes indicate that mountains do not constrain orchid dispersal over long timescales. Our study sheds new light on the timing and geography of a major Neotropical diversification, and suggests that mountain uplift promotes species diversification across all elevational zones.

8.1 Introduction

Species richness is unevenly distributed in time (Simpson, 1953), space (Willis, 1922) and across the Tree of Life (Vargas and Zardoya, 2014). An understanding of the processes underlying current patterns in species richness and distribution therefore constitutes a major scientific challenge. The Andean mountains of South America contain c. 15% of the world's plant species, in only 1% of the world's land surface, resulting in the most species-rich biodiversity hotspot worldwide (Myers et al., 2000). A large proportion of this diversity is found in high-altitude grasslands, and is suggested to have resulted from recent rapid speciation events (Hughes and Eastwood, 2006; Hughes and Atchison, 2015). By contrast, Andean seasonally dry forests experienced much slower diversification and have older origins (Pennington et al., 2010), suggesting contrasted macroevolutionary histories within the Andean biodiversity hotspot (Pennington et al., 2010; ter Steege et al., 2013; Valencia et al., 1994).

In a seminal paper, Gentry (1982) postulated that mountain uplift was a major trigger of Andean mega-diversity, although he posited that this might have occurred indirectly via biotic interactions. A pivotal result of Gentry's floristic analyses was the discovery of two patterns of plant distribution in the Neotropics: 'Amazonian-centred' and 'Andean-centred' taxa. Amazonian-centred taxa consist mostly of canopy trees and lianas, whereas Andean-centred taxa are almost exclusively epiphytes and shrubs (Gentry, 1982). The latter occur mostly in the Northern Andes, with secondary centres in the Brazilian coastal mountains and Central America, together accounting for c. 33% of all Neotropical plants (Gentry, 1982), and thus largely contributing to the world's most species-rich biodiversity hotspot, the tropical Andes (Myers *et al.*, 2000).

Contrasting with the dominant views at the time, Gentry (1982) hypothesized that the Andean-centred flora resulted from 'recent, very dynamic speciation', a hypothesis that we test here. Gentry and Dodson (1987) further suggested that the high diversity of epiphytes in the Northern Andes and southern South America could have resulted from the finer niche partitioning in these forests, allowing for high alpha diversity, the high microsite differentiation of mountain areas, fostering high beta diversity, and explosive speciation driven by genetic founder effects because of the environmental dynamicity, implying frequent relocation. Orchids are one of the most characteristic and diverse components of the Andean flora (Gentry and Dodson, 1987; Krömer and Gradstein, 2003; Parra-Sánchez et al., 2016; Richter et al., 2009). They often make up 30–50% of the total epiphytic species number reported along the Northern Andes (Kreft et al., 2004; Küper et al., 2004), and epiphytic orchids account for 69% of all vascular epiphytes world-wide (Zotz and Winkler, 2013). Neotropical epiphytic orchids are generally characterized by narrowly restricted populations with small numbers of individuals (Crain and Tremblay, 2012; Jost, 2004; Pandey et al., 2013; Tremblay and Ackerman, 2001). Despite the ecological importance and prominence of epiphytic orchids (and of epiphyte diversity overall) in the Andean flora, their origin and diversification have not been explicitly studied because of the difficulties in generating densely sampled and strongly supported phylogenies. We address these issues by studying the evolutionary history of the two largest Neotropical orchid clades, namely Cymbidieae and Pleurothallidinae. The Cymbidieae comprise over 3700 species, 90% of which occur in the Neotropics (the remaining species occur in tropical Africa and Australasia). Cymbidieae comprise 12 subtribes, four of which are the most

speciose and include Andean-dwelling subclades (i.e. Maxillariinae, Oncidiinae, Stanhopeinae and Zygopetalinae; Pridgeon *et al*., 2009). Pleurothallidinae comprise 44 genera and 5100 exclusively Neotropical species (Karremans, 2016) distributed mostly in the highlands of the Northern Andes and Central America. Together, they are the most representative elements of the Andean orchid flora (Pérez-Escobar *et al.,* 2009; Pridgeon *et al.*, 2009; Kolanowska, 2014) and make up most of their species richness. In addition, these lineages have evolved a rich array of pollination syndromes and mating systems (including protandry, unisexuality, cleistogamy; Gerlach and Schill, 1991; Borba *et al.*, 2011; Pérez-Escobar *et al.*, 2016a) that have long fascinated botanists and naturalists (Darwin, 1877; Lindley, 1843). This is particularly true for Cymbidieae, in which up to seven pollination syndromes have been recorded (Pridgeon et al., 2009; van der Cingel, 2001), ranging from species exclusively pollinated by male euglossine bees (Ramírez et al., 2011) to those pollinated only by oil bees. Data on the pollination ecology of Pleurothallidinae are very scarce, but scattered reports across the clade suggest that they are mostly pollinated by a vast array of dipteran lineages (Blanco and Barboza, 2005; Pupulin et al., 2012). Rapid Andean orogeny could have promoted orchid species richness by creating ecological opportunities, such as increasing the landscape, mediating local climate change, creating novel habitats and forming insular environments that affected migrations and allopatric speciation through isolation (Gentry and Dodson, 1987; Hoorn et al., 2013). This effect should have been most accentuated over the last 10 Ma, during which c. 60% of the current elevation of the Andes was achieved (Gregory-Wodzicki, 2000). Diversification studies of Andean centred clades have provided evidence for rapid diversification that temporally matches the Andean surface uplift, for instance in the plant genera *Lupinus*, *Espeletia*, *Halenia* and *Heliotropium*, and in the families Campanulaceae and Annonaceae (von Hagen and Kadereit, 2003; Bell and Donoghue, 2005; Donoghue and Winkworth, 2005; Hughes and Eastwood, 2006; Pirie *et al.*, 2006; Antonelli *et al.*, 2009b; Luebert *et al.*, 2011; Drummond *et al.*, 2012; Madriñán *et al.*, 2013; Lagomarsino *et al.*, 2016; Diazgranados and Barber, 2017). Taken together, these studies suggest that rapid Andean uplift yielded new niches that fostered both adaptive and non-adaptive radiations (Nevado et al., 2016). Other Andean groups, such as hummingbirds, diversified mostly before Andean uplift (McGuire et al., 2014) or after it had attained most of its current height (Smith et al., 2014). We address the impact of the Andean uplift on the diversity and distribution of orchids by inferring the dynamics of speciation, extinction and migration, whilst simultaneously incorporating surface uplift of the two largest Andean Neotropical orchid clades Cymbidieae and Pleurothallidinae. We rely on model-based inference methods in historical biogeography, ancestral area and character estimation approaches, and a series of diversification analyses to investigate the following questions. From which geographical area(s) do Andean orchids mostly originate? Is there evidence for the Andes acting as a dispersal barrier for epiphytic lowland taxa? Did the Andean uplift enhance orchid diversification and, if so, was this effect evident on all species from the Andean region or just those from the highest elevations? Is Andean diversity derived from pre-adapted (i.e. high elevation) lineages or rather descendants of lowland migrants (either local or from other areas)? In addition, we use the limited available data to evaluate whether shifts in pollination syndromes are associated with changes in diversification rates. Our results support Gentry's prediction (Gentry, 1982) that Andean-centred groups have resulted from recent rapid speciation, suggesting that Andean

orogeny provided opportunities for rapid orchid species diversification in the world's premier plant biodiversity hotspot. Such diversity is derived from lowland lineages but, more rarely, from migrants already pre-adapted to cool environments, a more frequent situation documented from other mountain environments (Merckx et al., 2015).

8.2 Materials and Methods

8.2.1 Taxon sampling, DNA sequencing and phylogenetic analysis

To generate solid phylogenies of the tribe Cymbidieae and subtribe Pleurothallidinae, we newly generated a total of 420 sequences of the nuclear ribosomal internal transcribed spacer (ITS) and a c. 1500-bp fragment of the gene ycf 1 of underrepresented lineages of key biogeographical importance. DNA amplification, PCR product purification and sequencing were conducted as described previously in Irimia *et al.* (2014) and Pérez-Escobar *et al.* (2016a). Voucher information and GenBank accession numbers are provided in Supporting Information Tables 8.S1 and 8.S2. We merged our novel dataset with previously generated data from the studies of Blanco *et al.* (2007), Whitten *et al.* (2014), Karremans *et al.*, (2016a,b), and Ramírez *et al.* (2011), using the R-package Megaptera v.1.0 (available at https://github.com/cran/megaptera.git). We retrieved 3541 sequences of nuclear (ITS) and plastid (matK, trnL-F region, psbA, ycf1). We selected outgroup taxa representing the old and new world subtribes Polystachyinae, Aeridinae and Laeliinae. Trees were rooted on *Calypso bulbosa* (for Cymbidieae) and *Arpophyllum giganteum* (for Pleurothallidinae) following Whitten *et al.* (2014). Poorly aligned positions were excluded from the alignments using GBLOCKS v.0.9 (Talavera and Castresana, 2007). To statistically detect potential incongruences between plastid and nuclear DNA phylogenies, we used the tool Procrustes Aapproach to Cophylogeny (PACo; http://www.uv.es/cophylpaco/) (Balbuena *et al.*, 2013; Pérez-Escobar *et al.*, 2016b). Maximum likelihood (ML) tree inference was performed using RAXML-HPC v.8.0 (Stamatakis, 2014), under the GTR + G substitution model with four gamma categories (best model for both datasets as inferred via the Akaike information criterion (AIC) in jModelTest v.2.1.6; Darriba *et al.*, 2012), with 1000 bootstrap replicates and data partitioning by genome compartment. All phylogenetic and dating analyses were performed in the CIPRES Science Gateway computing facility (Miller et al., 2015).

8.2.2 Molecular clock dating

A few unambiguous orchid macrofossils are available for Orchidaceae (*Dendrobium winikaphyllum*, *Earina fouldenensis*, *Meliorchis caribea*; Ramírez *et al.*, 2007; Conran *et al.*, 2009), but these are assigned to lineages very distantly related to our groups of interest. Using distant outgroups to calibrate our Cymbidieae and Pleurothallidinae phylogenies would have created extensive sampling heterogeneities, which can result in spurious results (Drummond and Bouckaert, 2014). Thus, we had to rely on secondary calibrations. In order to obtain the best secondary calibration points possible, we first generated an Orchidaceae-wide, fossil-calibrated phylogeny, sampling 316 orchid species and four loci (nrITS, matK, rbcL and trnL-F), sampled as evenly as possible along the tree. Detailed settings and fossil calibrations used to generate an Orchidaceae-wide phylogeny are provided in the extended Methods 8.S1.

Secondary calibration points were obtained from our Orchidaceae-wide dated phylogeny, and the most recent common ancestor (MRCA) of Cymbidieae + Vandeae was dated to 34±7 Ma, 95% credible interval (CI), whereas that of Pleurothallidinae + Laeliinae was estimated to 20±7 Ma. We therefore used a normal prior (with values of mean = 34, SD = 4 for Cymbidieae; mean = 20, SD = 3 for Pleurothallidinae, to reflect the 95% CI from our fossil-calibrated tree) to calibrate our phylogenies using these secondary constraints, which were designed to reflect the uncertainty previously estimated for the root node of Cymbidieae and Pleurothallidinae.

8.2.3 Ancestral range estimation

Species ranges were coded from the literature (Pridgeon *et al.*, 2009) and from herbarium specimens through a survey of virtual collections and loans of several herbaria (AMES, COL, F, MO, SEL, US, M), as well as the Global Biodiversity Information Facility (GBIF) repository. To query the GBIF database, we relied on the function "occ" of the R-package SPOCC (Chamberlain et al., 2016). A total of 19,486 distribution records were compiled for the Cymbidieae, and 9042 records for the Pleurothallidinae. Protocols for distribution maps and species richness pattern analyses are detailed in Methods S1. Distribution maps for Cymbidieae and Pleurothallidinae (summarized in Figs. 8.S1, 8.S2) and extant distribution patterns identified for other plant lineages (e.g. Rubiaceae, Antonelli *et al.*, 2009b) allowed the identification of 10 main distribution areas (see the inset in Figs. 8.1, 8.2). Species were assigned to one of these regions: Central America (comprising southern Florida to Panama); West Indies (i.e. Caribbean Islands); Northern Andes (mountain ranges from elevations higher than 500 m in Colombia and Venezuela); Central Andes (from Peru to the Tropic of Capricorn, from elevations higher than 500 m); Amazonia (including lowlands and montane forest below 500 m in Colombia, Ecuador, Peru, Brazil, Venezuela, Guyana, Suriname and French Guiana); the Guiana Shield (including elevations higher than 500 m in north-eastern South America (Brazil, Guyana, Suriname and Venezuela)); South-eastern South America (including the Brazilian shield, but also lowlands in eastern Brazil and northern Argentina); Chocó (comprises lowlands below 500 m of the western Andes in Colombia and Ecuador); Africa; and Australasia. To infer the ancestral range of all examined lineages in Cymbidieae and Pleurothallidinae, we used the R-package BioGeoBEARS v.0.2.1 (Matzke, 2014, 2013). In addition, in order to estimate the number of migrations, dispersals, extinctions and within-area speciation events from our phylogeny, we used biogeographical stochastic mapping (BSM) (Matzke, 2014) under the best-fit model, as implemented in BioGeoBEARS (for detailed settings, see Methods 8.S1).

8.2.4 Rates of species diversification

To infer the diversification dynamics of the Cymbidieae and Pleurothallidinae, we first used a time-dependent model implemented in BAMM v.2.5.0 (Rabosky, 2014) to estimate the rates of extinction and speciation across the phylogenies. Incomplete taxon sampling was accounted for by assigning a sampling fraction of 25% of the extant orchid diversity of Cymbidieae, and 13% of Pleurothallidinae (sampling fractions of every genus sampled were incorporated according to Chase *et al.*, 2015). We performed three runs with 1 million Markov chain Monte Carlo (MCMC) generations, sampling parameters every 10,000 generations. Diversification rates and

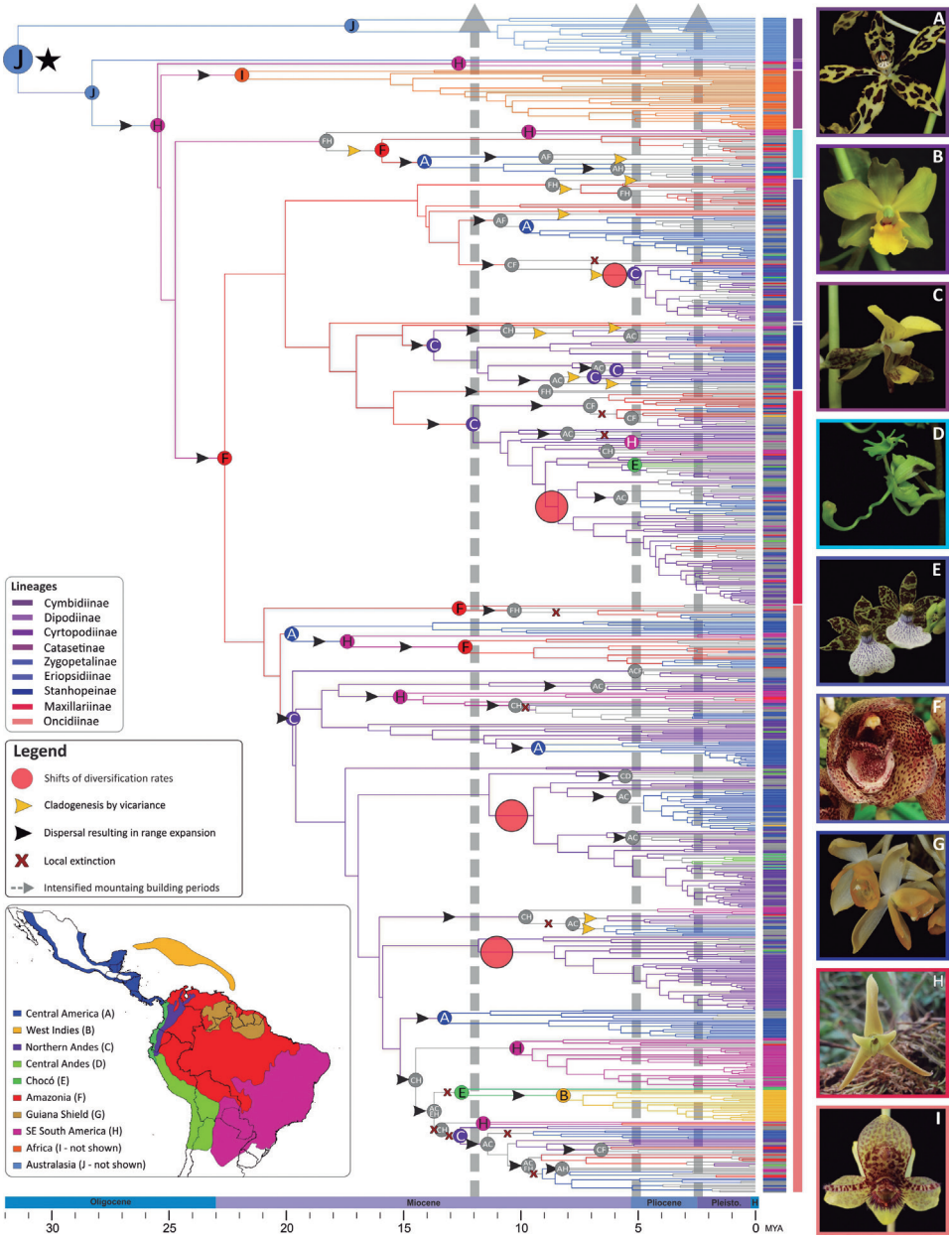

Figure 8.1. Biogeographical history of Cymbidieae orchids. Letters on the coloured circles at the nodes indicate the estimated ancestral area with the highest probability as inferred by BIOGEOBEARS. Branches are colour coded following the reconstructed area of their corresponding node, and the geographical ranges of every taxon are shown as vertical bars in front of the terminals. The black star indicates the most recent common ancestor of Cymbidieae. Grey arrows show the periods of accelerated Andean uplift (Gregory-Wodzicki, 2000).Changes on shifts of diversification rates are shown as pale red circles on the

rate shift configurations were plotted using the R-package BAMMtools (Rabosky *et al*., 2014). We checked the convergence of the runs by plotting the loglikelihood across MCMC generations sampled in the 'mcmc_out' file. To evaluate the best model generated by BAMM (compared with a null M_0 model with no diversification rate shifts), we relied on Bayes Factors calculated with the *computebayesfactor* function of BAMMtools. We examined the 95% credible set of macro-evolutionary shift configurations using the BAMMtools function *credibleShiftSet*. We sought cross validation of our BAMM results with RPANDA (Morlon et al., 2016), and details about the settings are provided in Methods S1.

8.2.5 Geographical state-dependent analyses

We used GeoSSE (Goldberg et al., 2011), an extension of the BiSSE model that allows lineages to occur simultaneously in two areas and to test whether one area has overall higher speciation rates, as implemented in the R-package Diversitree v.0.9-7 (FitzJohn, 2012). To test whether lineages restricted to the Northern Andes ('A') had higher diversification rates than lineages absent from the Northern Andes (collectively called 'B' here), we used Bayesian MCMC GeoSSE analyses of 1 million generations on the maximum clade credibility tree from BEAST (in the particular case of Cymbidieae, only Neotropical representatives were included). Implemented models in GeoSSE and settings of tailored simulations to account for Type I error biases in GeoSSE are provided in Methods S1.

8.2.6 Mapping speciation rates in the Neotropics

Based on the speciation and extinction rates inferred for orchid lineages, and their geographical occurrence, it is possible to identify important areas of diversification as plotted on a heat map (Condamine et al., 2013). For this purpose, we designed a novel method that involves retrieving speciation rates from BAMM analyses using the function *GetTipsRates* in BAMMtools v.2.1 (Rabosky et al., 2014a) and to link them to species occurrences. Rates were further associated to known distribution records of Cymbidieae and Pleurothallidinae and interpolated to a polygon representing the currently known distribution of Cymbidieae and Pleurothallidinae species, using the inverse distance weight method implemented in the software ARCMap v.9.3 (Esri). To account for geographical sampling biases, we divided the geographical range of species records into a grid of 0.5° 9 0.5° cells. We then randomly sampled occurrences arrayed on every grid cell using the R package Raster (Hijmans and Elith, 2016), so that a single occurrence per grid cell was kept.

branches. Range expansions, local extinctions and cladogenetic events via vicariance are indicated on the branches with black and yellow arrow heads and red crosses, respectively. Subtribe members of Cymbidieae are colour coded. Right panels showselected representatives of **A.** Cymbidiinae (*Grammatophyllum measuresianum*); **B.** Cyrtopodiinae (*Cyrtopodium macrobulbon*; photograph by D. Bogarín); **C.** Eulophiinae (*Eulophia streptopetala*); **D.** Catasetinae (*Cycnoches egertonianum*); **E.** Zygopetalinae (*Zygopetalum* aff. *brachypetalum*); **F.** Coeliopsidinae (*Peristeria cerina*); **G.** Stanhopeinae (*Sievenkingia* sp.); **H.** Maxillariinae (*Cryptocentrum sp*.); **I.** Oncidiinae (*Trichoceros* sp.). Photographs (except B): O. Pérez. (Inset) Coded areas for biogeographical analysis. Political divisions obtained from DIVA-GIS (http://www.diva-gis.org/gdata). Timescale shown at bottom is expressed in million years ago(Ma).

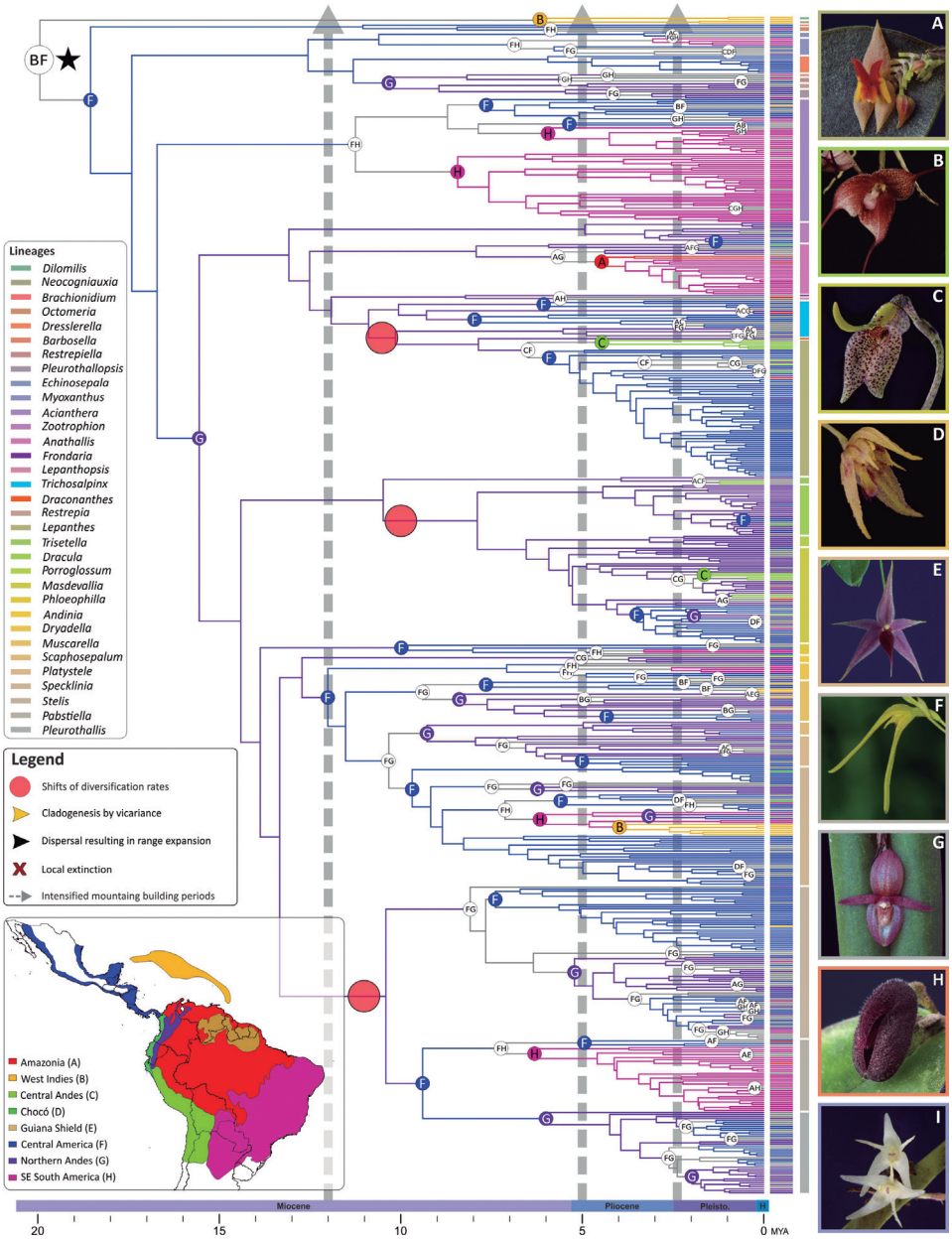

Figure 8.2. Biogeographical history of Pleurothallidinae orchids. Letters on coloured circles at the nodes indicate the estimated ancestral area with the highest probability as inferred by BIOGEOBEARS. Branches are colour coded following the reconstructed area of their corresponding node, and geographical ranges of every taxon are shown as vertical bars in front of the terminals. The black star indicates the most recent common ancestor of Pleurothallidinae. Grey arrows show the periods of accelerated Andean uplift (Gregory-Wodzicki, 2000). Changes on shifts of diversification rates are shown as pale red circles on the

8.2.7 Palaeo-elevation-dependent diversification

We tested the effect of past environmental change on the diversification of Cymbidieae and Pleurothallidinae using birth–death models that allow speciation and extinction rates to vary according to a quantitative, time-dependent, environmental variable (Condamine *et al.*, 2013), here the palaeo-elevation of the Northern Andes (Hoorn *et al.*, 2010; Lagomarsino *et al.*, 2016). The R-package PSPLINE (Ramsey and Ripley, 2010) was used to interpolate a smooth line for Andean palaeo-elevation. This smooth line was sampled during each birth–death modelling process to give the value of the palaeo-elevation variable at each time point. Speciation and extinction rates were then estimated as a function of these values along the time-calibrated phylogenies, according to the parameters of each model. The palaeo environmental dependent model is implemented in the Rpackage RPANDA v.1.1 (Morlon et al., 2016). Implemented models in RPANDA are provided in Methods S1.

8.2.8 Ancestral character state estimation

To account for potential biotic variables as drivers of Neotropical orchid diversification, such as shifts on pollination syndromes (Givnish et al., 2015), we compiled information on the pollination syndromes of Cymbidieae from the literature (Gerlach, 2011; Pansarin et al., 2009; Pridgeon et al., 2009; Ramirez et al., 2011; Singer, 2002; van der Cingel, 2001), and consulted experts on specific groups (see the Acknowledgements section). As a result of a dearth of detailed information on pollination ecology (i.e. available for c. 6% of taxa sampled only), we followed a generalist coding approach, and seven pollination syndromes, (i.e. bee, bird, butterfly, lepidopteran, fly, wasp and self-pollination) were coded. To account for missing information on pollination syndromes, we assigned equal probabilities to all character states to taxa with unknown pollination syndromes. To estimate ancestral elevation ranges in Pleurothallidinae and Cymbidieae, we obtained absolute elevation values from herbarium records for every taxon sampled in our phylogenies. We obtained a mean of five values per taxa sampled, and we coded mean elevation values as a continuous character. We followed the classification of major Andean ecoregions proposed by Rangel-Churio *et al.* (1997) and Jørgensen and León-Yánez (1999), and taxa occurring at elevations higher than 1100 m were considered to inhabit sub-Andean (montane) forests (1100–2400 m). Species occurring at elevations of < 1100 m were considered as lowland inhabitants. Detailed settings for ancestral character state of altitude and pollination syndromes are provided in Methods 8.S1.

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branches. Range expansions, local extinctions and cladogenetic events via vicariance are indicated on the branches with black and yellow arrowheads and red crosses, respectively. Generic members of Pleurothallidinae are colour coded. Right panels show selected representatives of **A.** *Lepanthes* (*Lepanthes* sp.); **B.** *Dracula* (*D. astuta*); **C.** *Masdevallia* (*M. utriculata*); **D.** *Muscarella* (*M. exesilabia*); **E.** *Platystele* (*P. porquinqua*); **F.** *Pabstiella* (*P. ephemera*); **G.** *Pleurothallis* (*P. adventurae*); **H.** *Dresslerella* (*D. pilosissima*); **I.** *Myoxanthus* (*M. colothrix*). Photographs: A. Karremans, D. Bogarín and O. Pérez. (Inset) Coded areas for biogeographical analysis. Political divisions obtained from DIVA-GIS (http://www.diva-gis.org/gdata). Timescale shown at bottom is expressed in million years ago, Ma.

8.3 Results

8.3.1 Phylogenetics, age and biogeography of Andean orchids

Analyses of phylogenetic incongruence detection identified 259 and 125 potential conflicting tips in Cymbidieae and Pleurothallidinae, respectively (Figs. 8.S3, 8.S4), all of which clustered in weakly to moderately supported clades (< 75% bootstrap support, BS) or in clades with extremely long branches. These analyses indicated the absence of supported phylogenetic incongruence (Mason-Gamer and Kellog, 1996; Pérez-Escobar *et al.*, 2016b). In the absence of supported phylogenetic conflicts, nuclear and plastid partitions of Cymbidieae and Pleurothallidinae were concatenated. For the Cymbidieae, our molecular dataset consisted of 6.6 kb DNA (five markers) for 816 species, and yielded the first strongly supported phylogeny of the tribe (Fig. 8.S5). The Pleurothallidinae dataset was composed of 2.4 kb DNA (two markers) and 684 terminals, including, in total, 420 newly generated sequences (Fig. 8.S6). Both orchid phylogenies are strongly supported at most important nodes, with 618 nodes (76%) with BS > 75% for the Cymbidieae, and 321 nodes (47%) with BS > 75% for the Pleurothallidinae (Figs. 8.S5, 8.S6).

Ages obtained on our wide orchid-dated phylogeny were very similar to those of other recent orchid dating studies (Chomicki *et al.*, 2015; Givnish *et al.*, 2015). A chronogram for the orchid family showing absolute ages and 95% CIs for every node is provided in Fig. 8.S7. The absolute ages obtained for Cymbidieae and Pleurothallidinae chronograms are also in agreement with previously published dated phylogenies (e.g. Ramírez *et al.*, 2011; Chomicki *et al.*, 2015; Givnish *et al.*, 2016). Divergence time estimates and 95% CIs inferred for all nodes of Cymbidieae and Pleurothallidinae chronograms are shown in Figs. 8.S8 and 8.S9.

Our dating and biogeographical analyses identified the Dispersal– Extinction–Cladogenesis model with founder speciation event (DEC + J) as the best fitting model for both Cymbidieae and Pleurothallidinae (Table s S3, S4). Under this model, an Australasian origin of the Cymbidieae around the Eocene–Oligocene boundary (34 8Ma) was inferred (Figs. 8.1, 8.S8, 8.S10). We inferred a late Oligocene dispersal from Australasia to South America following the estimation of southern South America as the ancestral area of *Cyrtopodium* and the rest of the Cymbidieae (Figs. 8.1, 8.S10). Such dispersal corresponds to the final break-up of Gondwana (split between Antarctica and South America at Drake Passage). From the late Oligocene to the early Miocene, our analyses indicate dispersal from east to west in the Neotropics. The Northern Andean region was reached four times from Amazonia by MRCAs nested in Oncidiinae c. 19± 5Ma, Maxillariinae c. 11±5Ma, Stanhopeinae c. 13± 4Ma and Zygopetalinae c. 5±2Ma.

Ancestral state estimations of mean altitude further show that the MRCA of Cymbidieae was probably adapted to lowland environments (ancestral elevation value of c. 900 m; Figs. 8.S11, 8.S12). Three of the MRCAs of Amazonian migrants that reached the Andes (i.e. nested in Maxillariinae, Stanhopeinae and Zygopetalinae) were not pre-adapted to montane habitats (mean elevation values of c. 1050, 900 and 1000 m, respectively (< 1000–1100 to 2400 m; Cuatrecasas, 1958; Rangel-Churio *et al.*, 1997; Figs. 8.S11, S12). The MRCA of Oncidiinae that reached the Northern Andes, by contrast, was probably adapted to montane habitats (c. 1200 m). Strikingly, Oncidiinae and Maxillarinae are the species-richest lineages in Cymbidieae (1584 and 819 species, respectively; (Chase et al., 2015)), and are derived from both lowland Amazonian and

montane pre-adapted migrants. Stanhopeinae subsequently dispersed to several other Neotropical regions, particularly Central America (Figs. 8.1, 8.S10).

Different from the Cymbidieae, we infer an origin of Pleurothallidinae in Central America or the West Indies in the early Miocene, followed by a migration to the Northern Andes c. 16.5 Ma (Figs. 8.2, 8.S9, 8.S13), before the main uplift periods, but within a timeframe in which the Northern Andes had already achieved peak mean elevations of c. 1500 m. However, the majority of early divergent Pleurothallidinae and their sister groups are from the Antilles, and thus the inference of Central America as the ancestral area of Pleurothallidinae most probably reflects our inability to sample extensively the early diverging Antillean lineages. As inferred by ancestral state estimations, the MRCA of Pleurothallidinae was probably adapted to montane habitats (mean elevation of c. 1200 m), and all Pleurothallidinae migrants to the Northern Andes were probably adapted to montane–cloud forest environments (mean elevation of c. 1200–1300 m; Figs. 8.S14, 8.S15). BSM indicates that *in situ* speciation was the dominant biogeographical process in both clades, whereas processes of range expansion (dispersal and vicariance) and range contraction (subset speciation) were scarcer and relatively evenly distributed across lineages (Figs. 8.1, 8.2, 8.S16, 8.S17).

8.3.2 Diversification of Andean orchids

The diversification analyses performed with BAMM strongly rejected a constant-rate model (Bayes factor = 151.3, Table 8.S5) and, instead, identified four rate shifts during the evolutionary history of Cymbidieae (Figs. 8.3b, 8.S18, 8.S19). The best model configuration identified four shifts in speciation rate in the most speciose Cymbidieae lineages: one in Maxillariinae, one in Zygopetalinae and two in Oncidiinae.We further identified three rate shifts in the Pleurothallidinae (Table 8.S6): at the MRCA of *Lepanthes* + *Lepanthopsis*, MRCA of *Dracula* + *Porroglossum* + *Masdevallia*, and MRCA of *Stelis* + *Pabstiella* + *Pleurothallis* (Figs. 8.4b, 8.S20, 8.S21). All shifts in diversification rates in Cymbidieae and Pleurothallidinae were further confirmed using the RPANDA method (Figs. 8.S22, 8.S23; Tables 8.S7, 8.S8).

The diversification rate shifts are all located at clades that already inhabited the Northern Andes, and temporally match with periods of accelerated Andean uplift in this region (Cymbidieae, Fig. 8.1; Pleurothallidinae Fig. 8.2). To further explore this apparent correlation with either accelerated Andean uplift or presence in the Northern Andes and fast diversification, we used a trait dependent approach (GeoSSE) that estimates region-dependent speciation rates. Here, a model with free rates fitted best our Cymbidieae and Pleurothallidinae datasets (Table 8.S9), indicating significant differences in speciation (sA sB) and diversification (dA dB) rates highly if not maximally supported (0.99 and 1 Bayesian posterior probabilities, respectively). GeoSSE analyses further indicated that speciation rates in Northern Andes are consistently higher than in any other biogeographical region (Figs. 8.3c, 8.4c) in both Cymbidieae and Pleurothallidinae datasets. We evaluated and confirmed the robustness of these results through extensive data simulations (Fig. 8.S24). Here, the null distribution of GeoSSE ΔAIC values obtained from analyses with reshuffled area states was centred towards values of −20 000 and far away from the ΔAIC values obtained under analyses with real area states. We developed a novel method to generate a ‘speciation rate map’ using inferred speciation rates for each orchid lineage and georeferenced

species occurrences (see the Materials and Methods section). Our speciation rate maps are in agreement with GeoSSE results, and we confirmed that speciation rates in the Northern Andes were significantly higher than those in any other region (Figs. 8.3c, 8.4c). This is in agreement with a recent study with more limited taxon sampling for the two clades focused on here (Givnish et al., 2015). The speciation rate map (see the Materials and Methods section) further demonstrates that fastest speciation took place in the Northern Andes region, and reveals secondary speciation hotspots in the Central Andes, the Guiana Shield and Central America (Figs. 8.3d, 8.4d). These secondary hotspots are occupied by species derived from the four highly diversifying Northern Andean Cymbidieae clades (Fig. 8.S25), suggesting that the Andes acted as a major source of new lineages to the rest of the continent, thus greatly increasing Neotropical orchid diversity. This is particularly true for the Pleurothallidinae, where we identified multiple migrations from the Northern Andes of montane-adapted lineages to Central America (Figs. 8.2, 8.S26). We also found a strong geographical correlation between current species richness and diversification (Figs. 8.3d, 8.4d, 8.S27, 8.S28), suggesting that recent *in situ* speciation was the main process for species accumulation in the Neotropics.

Although these results suggest an impact of the Andean uplift on species diversification, they do not explicitly account for biotic interactions, landscape and climatic changes through time. We therefore assessed the fit of a model that explicitly integrates palaeo-elevation in diversification rate analyses (see the Materials and Methods section). In three of the four Cymbidieae clades in which BAMM inferred a speciation rate shift, the palaeo-elevation-dependent model inferred a continuous speciation increase from 10 to 6 Ma as a result of a positive correlation between speciation and palaeo-elevation (Fig. 8.3e,f; Table S10). By contrast, no positive correlation with palaeo-elevation and diversification could be detected for Pleurothallidinae (Table 8.S11). Moreover, our ancestral character estimation of pollination syndromes in Cymbidieae suggests that the MRCA of Cymbidieae was bee pollinated (Fig. 8.S29). Nine shifts of syndromes were identified along the evolutionary history of Cymbidieae, always derived from bee pollination. No reversals from other syndromes towards bee pollination were recovered (Fig. 8.S29).

8.4 Discussion

8.4.1 Andean orchids are derived from lowland Amazonian, montane Central American and local sub-Andean migrants

Our ancestral area estimations show that Andean orchid flora is derived primarily from Amazonian lowland taxa (i.e. MRCAs of Andean clades of Maxillariinae, Stanhopeinae and Zygopetalinae, from which most of the species-richest lineages in Cymbidieae originated), but also from cool pre-adapted lineages (MRCAs of both Andean Oncidiinae and most extant Andean-centred pleurothallid taxa). Previous research has revealed that mountain flora origin is strongly influenced by the immigration of cool preadapted lineages (Hughes and Eastwood, 2006; Merckx *et al.*, 2015; Uribe-Convers and Tank, 2015), and that contributions from lowland-adapted lineages is rather rare. In Borneo, a large portion of the mountain endemics of Mount Kinabalu arose from pre-adapted lineages from other cool areas (Merckx et al., 2015), but *Dendrochilum* orchid montane endemics arose from low-elevation local ancestors (Barkman and Simpson,

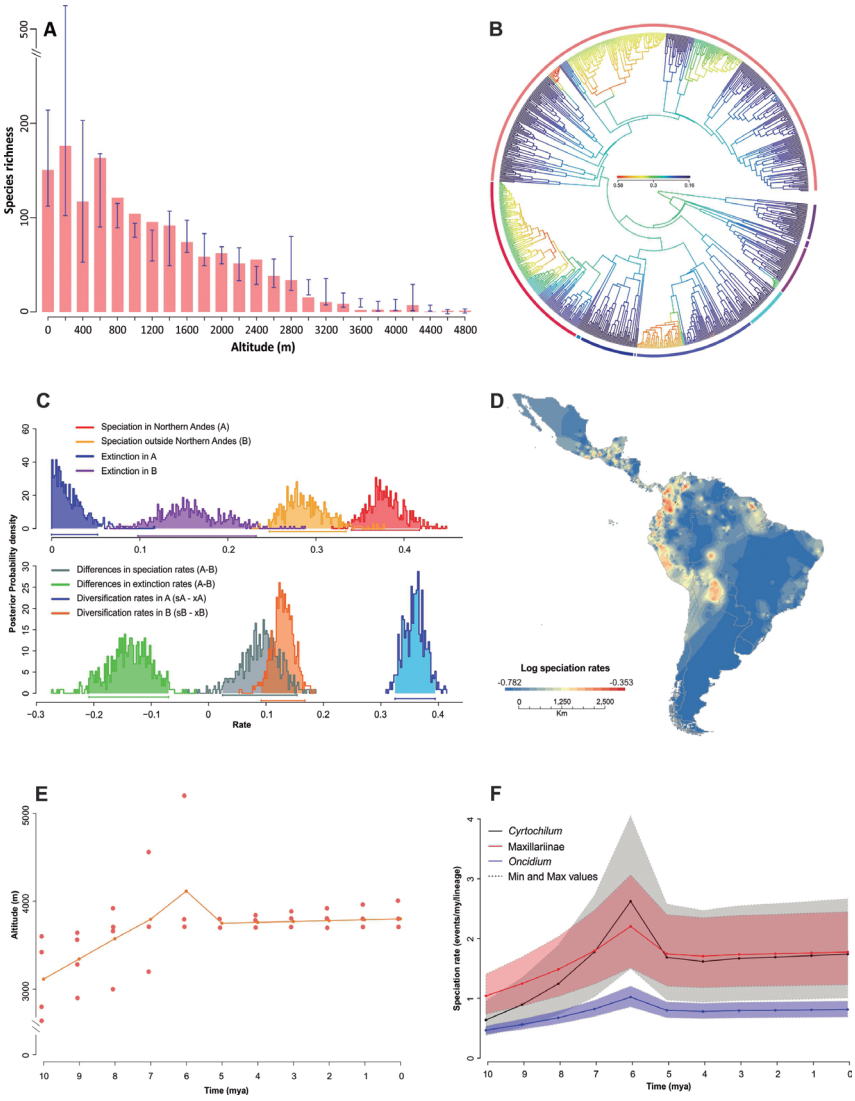

Figure 8.3. Diversification of the Cymbidieae. **A.** Richness vs elevation plot for 55%(> 20 000 herbarium records) of the c. 4,000 Cymbidieae species. Blue error bars indicate maximum and minimum species richness values. **B.** Speciation rate plot (phylorate) showing the best configuration shift identified by BAMM. Colour intensity across branches is proportional to changes in diversification rates. (c) Density probability plots of speciation, extinction and net diversification rates per area identified by GEOSSE. Area 'A' refers to species restricted to the Northern Andes; area 'B' refers to species occurring in all areas except the Northern Andes. **D.** Speciation ratemap estimated fromBAMM (see the Materials and Methods section). **E.** Average palaeo-elevation of the Central and Northern Andes. **F.** Palaeo-elevation-dependent models applied to the four clades detected by BAMM to have significantly higher diversification rates than others. Lineages in (B) are colour coded in the same way as shown in Fig. 8.1. Timescale in panels (E) and (F) is expressed in million years ago (Ma).

2001). Similarly, epiphytic, tuberous Rubiaceae (Hydnophytinae) endemics from New Guinea montane habitats originated from local lowland migrants (Chomicki and Renner, 2017). Our study points to the key role of Amazonia for the origin of Andean orchid diversity, and also reveals an ancient biological connectivity between Amazonia and the Northern Andes.

8.4.2 The Andes did not constrain orchid dispersal

The recurrent migration back and forth through the Andes, even during the period of highest palaeo-elevation, is also a central result from our study. The colonization of the Northern Andes by some clades of Cymbidieae matches in time with accelerated surface uplift (Figs. 8.1, 8.S10), and reflects the Miocene biotic connectivity between the Andes and Amazonia previously suggested for plants (Antonelli et al., 2009a), Poison dart frogs (Santos et al., 2009), and birds (Brumfield and Edwards, 2007), among others. This suggests that shifts across elevational zones were not rare, contrary to recent results in Mount Kinabalu in Borneo (Merckx et al., 2015).

Surprisingly, dispersal events across the Andes did not decrease during accelerated Andean uplift (Figs. 8.1, 8.2, 8.S10, 8.S13), suggesting that the uplift of the Andes did not act as a major dispersal barrier for Cymbidieae and Pleurothallidinae orchids, contrary to findings in other plant groups (e.g. Annonaceae, (Pirie et al., 2006); Rubiaceae, (Antonelli et al., 2009b); or Fabaceae, (Pennington et al., 2010). This result probably relates to the biology of orchids, which produce large amounts of dust-like, wind-dispersed seeds, allowing for occasional long-distance dispersal (Antonelli et al., 2009a; Arditti and Ghani, 2000; Barthlott et al., 2014; Givnish et al., 2016; Pérez-Escobar et al., 2017), enabling occasional crossing of the Andes, and perhaps more frequently migration to different elevation zones. Taken together, these findings suggest that the Andes constitutes a semipermeable barrier to biotic dispersal, and that orchids may be more geographically constrained by intrinsic factors, such as fungal symbionts and pollinator mutualists, which differ among elevational zones (Arroyo et al., 1985, 1982; Lugo et al., 2008) than by distance. The dependence of immigrant orchids on particular fungal or pollinator mutualists, matched to the available pool of mutualists, may greatly determine the success of their establishment in a new area. Our findings of widespread within-region speciation as the main biogeographical process (Figs. 8.1, 8.2, 8.S16, 8.S17), coupled with the apparent widespread permeability of the Andean mountains to lowland migrants, raise the question of the speciation mechanisms underlying these fast speciation rates. We speculate that the habitat heterogeneity, with many adjacent but distinct niches, could have favoured isolation, perhaps via peripatric or parapatric speciation. Be as it may, our work paves the way for microevolutionary studies of orchid speciation in the Andes.

8.4.3 Accelerated orchid diversification across elevational zones

Gentry's hypothesis of rapid speciation (Gentry, 1982) in the Andes was mainly based on the observation of floristic groups (e.g. 'Andean-centred taxa') with very speciose genera from the lowlands to mid-elevations in the (mostly Northern) Andes. This matches well the total altitudinal distribution of our respective study groups, with a richness vs elevation plot for > 55% of the 3,700 Cymbidieae species based on over 20,000 records (Figs. 8.3a, 8.S1), which reveals that

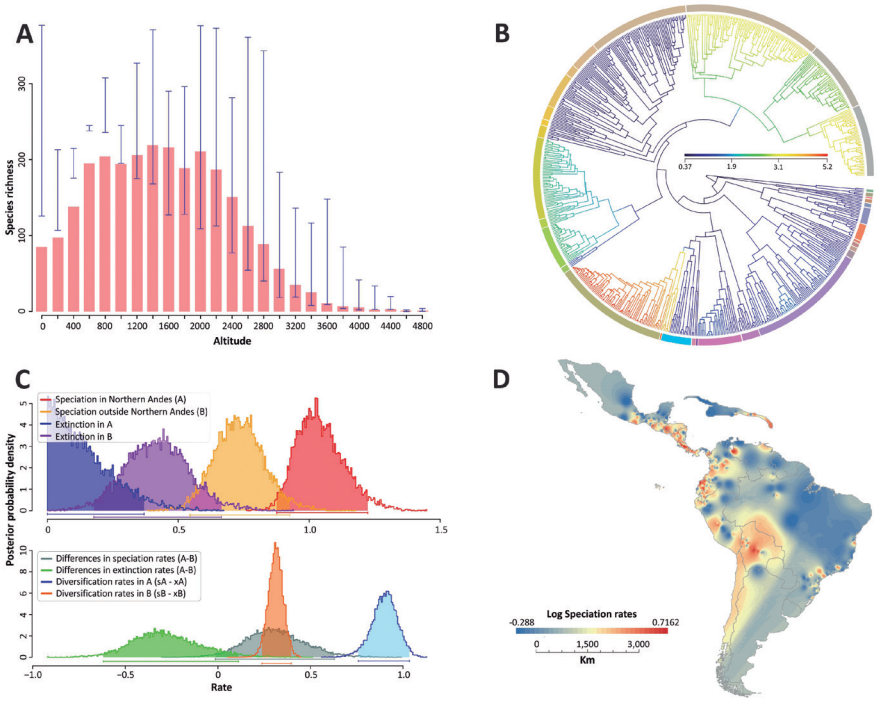

Figure 8.4. Diversification of the Pleurothallidinae. **A.** Richness vs elevation plot for 50% (> 9000 herbarium records) of the c. 5000 Pleurothallidinae species. Blue error bars indicate maximum and minimum species richness values. **B.** Speciation rate plot (phylorate) showing the best configuration shift identified by BAMM. Colour intensity across branches is proportional to changes in diversification rates. **C.** Density probability plots of speciation, extinction and net diversification rates per area identified by GEOSSE. Area 'A' refers to species restricted to the Northern Andes; area 'B' refers to species occurring in all areas except the Northern Andes. **D.** Speciation rate map estimated from BAMM (see the Materials and Methods section). Lineages in (B) are colour coded in the same way as shown in Fig. 8.2.

Cymbidieae diversity peaks at low elevations (< 1,100 m), whereas Pleurothallidinae diversity (c. 10,000 records; Fig. 8.S2) peaks at c. 1,500 m (Fig. 8.4a).

The diversification rate shifts are all located within clades that already inhabited the Northern Andes, and temporally match with periods of accelerated Andean uplift in this region (Gregory-Wodzicki, 2000; Hoorn et al., 2010) (Figs. 8.1, 8.2). The late middle Miocene and early Pliocene are the periods with the fastest documented rates of Andean uplift in the Northern Andes (i.e. Venezuelan Andes and Northern Andes of Colombia; Hoorn *et al.*, 1995; Bermúdez *et al.*, 2015). In all three Cymbidieae clades, speciation rates peaked at 6 Ma, a time at which the Northern Andes reached c. 4,000 m, their maximum mean palaeo-elevation (Bermúdez et al., 2015). Contrary to Cymbidieae, we found no correlation between Andean uplift and Pleurothallidinae diversification (Table 8.S11). We hypothesize that this is a result of the rapid diversification of migrating cool preadapted Pleurothallidinae lineages from Central America into already formed

montane environments (Hoorn et al., 2010). Similar diversification patterns have been reported for *Lupinus*, *Bartsia*, Adoxaceae, Valerianaceae and, more recently, Ericaceae (Donoghue and Sanderson, 2015; Schwery et al., 2015; Uribe-Convers and Tank, 2015).

Gentry proposed that the main mechanism underlying rapid speciation in the Andes was the evolution of novel plant–insect interactions (Gentry, 1982). The Cymbidieae are particularly known among biologists and ecologists because of the rich array of pollination syndromes and sexual systems they have evolved (e.g. sexual and food deceit, food and fragrance reward, dichogamy and environmental sex determination; Gerlach and Schill, 1991; Singer, 2002; Pansarin *et al.*, 2009; Gerlach and Pérez-Escobar, 2014). Our analyses suggest that pollinator syndrome shifts do not match with diversification rate shifts, although our data do not take into account pollinator shifts within given pollinator groups. This is particularly true for the bee pollination syndrome, which is widespread in the tribe and probably overarches several transitions from different types of bees (e.g. oil to euglossine bees as observed in Catasetinae). More field observations of pollinations are therefore needed to evaluate the relative role of pollinator shifts in contributing to Neotropical orchid diversification.

8.5 Conclusions

Based on two extensively sampled orchid phylogenies, combined with statistically robust diversification models, our results reveal that Andean orchid diversification has closely tracked the Andean orogeny. Together with studies in other mega-diverse regions (Bruyn et al., 2014; Verboom et al., 2009), our results show that rapid recent speciation has moulded this area of exceptional species richness. In addition, our results highlight the crucial role of Amazonian lowlands, as well as the Antillean and Central American regions, as biotic sources for Andean biodiversity, providing cool pre-adapted lineages that dispersed into the Andes and further diversified *in situ*.

Contrary to general expectation, the rise of the Andes had little effect on restricting orchid biotic dispersal across the Neotropics. This suggests that mountains are semi-permeable barriers to lowland organisms, whose dispersal ability is more probably related to intrinsic traits (e.g. seed size, dispersal mechanism, mutualisms). Although both abiotic and biotic processes are clearly responsible for the exceptional species richness of the world's premier biodiversity hotspot (Antonelli and Sanmartín, 2011; Hughes *et al.*, 2013; Eiserhardt *et al.*, 2017), our results suggest that geological processes played a central and direct role in the diversification process. Finally, as the highest species richness in Cymbidieae is concentrated in the lowlands and the Pleurothallidinae peak is at mid-elevation, our study shows that Andean uplift dramatically affected the evolutionary assembly of both lowland and mid-elevation Andean forests, as originally hypothesized by Gentry (1982).

Supporting information online: https://nph.onlinelibrary.wiley.com/doi/full/10.1111/nph.1462

Chapter 9

Speciation and biogeography of the hyperdiverse genus *Lepanthes* (Orchidaceae: Pleurothallidinae)

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To be submitted

Abstract. *Lepanthes* is one of the six most species-rich angiosperm genera in the Neotropics accounting for more than 1,130 species. The influence of extrinsic factors on the diversification of the genus was assessed in a broad-scale analysis in Chapter 8. Here, we used *Lepanthes* as a model to understand patterns of diversification in the Neotropics driven by intrinsic factors. We inferred the biogeographical history of the genus based on a time-calibrated chronogram obtained by a molecular phylogenetic analysis with a species sampling increased up to 25%. Our results show that *Lepanthes* likely originated in the Central Andes (CA) and diversified between 7-8 Ma. The genus reached Southern Central America (SCA) from the Andean region twice, with one recolonization to the Northern Andes (NA) from SCA. The extant lineages from Northern Central America (NCA) and the West Indies (WI) are likely derived from SCA ancestors. Cladogenesis by within-area speciation was the most common biogeographical event and the most frequent dispersal routes were SCA with NCA and NA and NA with CA. *Lepanthes* showed the highest rates of species diversification in the Pleurothallidinae and two of its most recent clades from SCA experienced shifts in species diversification with an acceleration around 2.5 Ma. This acceleration did not strictly correlate with mountain orogeny as found in Chapter 8 for the Epidendroideae as a whole. However, paleoclimatic evidence indicates that cooling periods started before 2.7 Ma and this partially correlates with *Lepanthes* diversifications in SCA. Although species sampling from SCA was intensive, the sampling of Andean, NCA and WI lineages was still low and should be increased. Likewise, more accurate species distribution data and alpha-taxonomical expertise are needed to obtain more insight in the most important intrinsic factors driving speciation and biogeography of *Lepanthes*.

9.1 Introduction

Lepanthes Sw. is one of six most species-rich angiosperm genera in the Neotropics accounting for more than 1130 species (Bogarín et al., 2018c). *Lepanthes* and closest allies are widely distributed from Mexico and Florida to southern Brazil and Argentina, including Central America and the Antilles (Luer and Thoerle, 2012). Most of the species are concentrated in Costa Rica-Panama (160 spp.), Colombia- Ecuador (>300 spp. each) and Peru-Bolivia (>100 spp.). Multiple hypotheses exist about the factors that drove this extraordinary diversity. These include intrinsic characters such as trait evolution (Chapter 2), pollinator specialization (Chapter 6, Blanco and Barboza, 2005) and extrinsic traits such as colonization, orogeny or climatic fluctuations (Chapter 8, Givnish et al., 2015, 2016; Pérez-Escobar, Chomicki, et al., 2017). The influence of extrinsic factors on the diversification of the most speciose Neotropical orchid lineages (Pleurothallidinae and Cymbidiae) was assessed in Chapter 8 (Pérez-Escobar et al., 2017a). These authors discovered that rapid recent speciation predominates in the most speciose lineages such as *Lepanthes* and that the rise of mountain ranges had little effect on constraining orchid dispersal. This suggests that mountains are semi-permeable barriers and dispersal restriction is more related to intrinsic traits. In addition, these authors found that Central America has been an important biotic source for Andean biodiversity, providing cool pre-adapted lineages that dispersed into the Andes and further diversified. In Chapter 8 we addressed those biogeographical hypotheses on a broad scale by taking the entire Pleurothallidinae as a model. As a continuation of their study, we used *Lepanthes* as a model to further understand its patterns of diversification in the Neotropics. To achieve this, we inferred the biogeographical history of *Lepanthes* by increasing taxon sampling, including both key representatives of main clades and main biogeographical areas such as Northern Central America and the West Indies, and producing a time-calibrated chronogram based on nuclear nrITS and plastid *matK* markers covering about 25% of all species of *Lepanthes* and close allies (Bogarín et al., 2018c). This chapter discusses the ancestral range of *Lepanthes*, the most likely colonization routes across the Neotropics, the most common biogeographical models and its diversification rates through time. The combined results show that *Lepanthes* underwent rapid diversification and dispersed across the Neotropics during a series of climatological changes and *in situ* speciation events.

9.2 Materials and Methods

9.2.1 Taxon sampling

We sequenced the nuclear ribosomal internal transcribed spacer (nrITS) and the plastid maturase K (*mat*K) of 351 accessions of *Lepanthes*. In addition, we included previously generated sequences of the 13 genera related to *Lepanthes* from Chapters 2-3 and 8. About 20% of the species of *Lepanthes* were sampled across the Neotropics with emphasis on Southern Central America. Voucher information, NCBI GenBank accessions, and references for each DNA sequence are listed in Table 9.1. *Acianthera butcheri* (L.O.Williams) Pridgeon & M.W.Chase and *Acianthera fenestrata* (Barb.Rodr.) Pridgeon & M.W.Chase were chosen as outgroups based on Pridgeon et al., (2001).

9.2.2 DNA extraction, amplification, sequencing and alignment

We obtained total genomic DNA from about 50-100 mg of silica gel dried leaf/flower tissue powdered in a Retsch MM 300 shaker. We followed the 2× CTAB (Hexadecyltrimethylammonium bromide) protocol for isolating DNA (Doyle and Doyle, 1987). The polymerase chain reaction (PCR) mixture, the primers for the nrITS (17SE and 26SE) and plastid *mat*K (2.1aF and 5R) regions and amplification profiles are described in Chapters 2 and 8. Sanger sequencing of both regions was conducted by BaseClear (https://www.baseclear.com) on an ABI 3730xl genetic analyzer (Applied Biosystems, Foster City, California, U.S.A). Sequences were deposited in NCBI GenBank (Table 9.1). We used Geneious® R9 (Biomatters Ltd., Auckland, New Zealand (Kearse et al., 2012)) for the editing of chromatograms and pairwise alignment. Sequences were aligned in the online MAFFT platform (Multiple Alignment using Fast Fourier Transform, http://mafft.cbrc.jp/alignment/server/) using default settings. We adjusted and trimmed the resulting alignment manually.

9.2.3 Phylogenetic analyses and divergence time estimation

We obtained gene trees for each individual nrITS and *mat*K dataset with maximum likelihood (ML) in RAxML-HPC2 on XSEDE (8.2.10) (Stamatakis et al., 2008) choosing the GTRGAMMA model for bootstrapping and 1,000 bootstrap iterations. For each dataset, the model of evolution was calculated with the Akaike Information Criterion (AIC) in jModelTest2 v2.1.7 (Darriba et al., 2012). To evaluate the incongruence between nrITS (nuclear) and *mat*K (plastid) datasets we followed the pipeline implemented by Pérez-Escobar, Balbuena, and Gottschling (2016) using the Procrustean Approach to Cophylogeny (PACo) application (Balbuena et al., 2013) in R (http://data- dryad.org/review?doi=doi:10.5061/dryad.q6s1f). The conflicting terminals were excluded from the *mat*K dataset and replaced by missing data. The resulting *mat*K dataset was further concatenated with the nrITS dataset in Sequence Matrix v100.0 (Vaidya et al., 2011). This concatenated dataset was used to estimate the divergence times in BEAST v.1.8.2. In addition, the statistical support of the clades was evaluated with the values of posterior probability (PP) for the Bayesian Inference reconstruction. We performed two MCMC with 60×10^6 generations and sampling every 1,000 generations with a Marginal likelihood estimation (MLE) of 50 path steps, 10×10^5 length of chains and log likelihood for every 1,000 generations. The clock-likeness of the data was tested with the coefficient of variation (CV) of relaxed clock models. Speciation tree model selection was achieved by executing the Bayes factor test using the MLE from the stepping stone sampling on Yule Process (Y), Birth Death-Process (BD) and Birth-Death-Incomplete Sampling (BDIS) models under strict and uncorrelated lognormal molecular clock models. For each model, we assigned a normal prior distribution of 16.45 Ma and 2.5 SD (standard deviations) to the root node, 12.93 Ma and 2.5 SD to the node of the MRCA of the *Lepanthes* clade and 12.93 Ma and 2 SD to the MRCA of *Zootrophion* and the remainder of the members of the *Lepanthes* clade. These secondary calibrations were calculated from the values obtained from the time-calibrated chronogram of the Pleurothallidinae by Pérez-Escobar et al., (2017b) (Chapter 8). We inspected the convergence of independent runs size and the MCMC stationarity of parameters (ESS values >200) in Tracer v.1.6. A maximum clade credibility (MCC) tree was obtained with a 10% of burnin using TreeAnnotator v.1.8.2. All phylogenetic analyses and dating

analyses were run in the CIPRES Science Gateway V. 3.1 (http://www.phylo.org/sub_sections/portal/) (Miller et al., 2010). Resulting trees and the 95% highest posterior density (HPD) estimations were viewed in FigTree v1.4.3 (Rambaut, 2006) and manipulated with R programming language (R Core Team, 2017) under R Studio (Gandrud, 2013) using the packages APE, ggtree and phytools (Paradis et al., 2004; Revell, 2012; Yu et al., 2017). Final trees were edited in Adobe® Illustrator CC (Adobe Systems Inc., California, U.S.A).

9.2.4 Ancestral range estimation (ARE)

For the range estimations we obtained geographical records from herbaria (AMES, CR, JBL, K, L, SEL, US, W), online databases such as TROPICOS (http://www.tropicos.org), WCSP (https://wcsp.science.kew.org/), Epidendra (www.epidendra.org) and the Global Biodiversity Information Facility GBIF (https://www.gbif.org). This information was used to encode the accessions of the concatenated dataset in eight main distribution areas according to the current distribution of the genus: Northern Central America (NCA) (comprising southern Mexico to Nicaragua); Southern Central America (SCA) (comprising Costa Rica and Panama) in addition to West Indies (WI); Northern Andes (NA); Central Andes (CA); Amazonia and Guiana Shield (A); South-eastern South America (SSA) and Chocó (Ch) as defined in Chapter 8. This range matrix and the MCC tree obtained from the BEAST dating analysis were used to infer the ancestral range of *Lepanthes* and allied genera with the R-package BioGeoBEARS v.0.2.1. BioGeoBEARS calculates probabilistic inferences of ancestral geographic ranges on a phylogeny and allows model fit selection with statistical tests (Matzke, 20018). Therefore, we evaluated six models: (1) dispersal–extinction–cladogenesis, DEC (implemented in LAGRANGE, Ree and Smith, 2008), (2) DEC+J, allowing founder-event speciation, (3) DIVALIKE, a ML version of dispersal-vicariance analysis (DIVA) (Ronquist, 1997), and (4) DIVALIKE+J, allowing founder-event speciation, (5) BAYAREALIKE, the ML version of Bayesian inference of historical biogeography (BAYAREA) (Landis et al., 2013) and (6) BAYAREALIKE+J, allowing founder-event speciation. We selected the best model-fit with the weighted AIC and likelihood ratio test (LRT) scores calculated in BioGeoBEARS (Matzke, 2014, 2013). In addition, to infer biogeographical events such as migrations, dispersals, extinctions and within-area speciations we implemented the biogeographical stochastic mapping (BSM) approach generating 50 stochastic maps in BioGeoBEARS (Matzke, 2014, 2013).

9.2.5 Rates of species diversification

To infer the diversification dynamics (extinction and speciation rates) we used a time-dependent model implemented in the C++ program BAMM v.2.5.0 (Bayesian Analysis of Macroevolutionary Mixtures) (Rabosky et al., 2014a). In this analysis, we assigned a sampling fraction of 25% of the extant diversity of the *Lepanthes* clade. We performed four runs with 5×10^6 Markov chain Monte Carlo (MCMC) generations, sampling parameters every 1,000 generations. We checked the convergence of the runs by plotting the log-likelihood across MCMC generations. The diversification rates and rate shifts calculated with BAMM were analyzed and plotted with the R-package BAMMtools v2.1.6 (Rabosky et al., 2014b). In addition, we selected the best

model with a Bayes Factors test and examined the 95% credible set of macroevolutionary shift configurations with the functions *ComputeBayesFactor* and *CredibleShiftSet* of BAMMtools. Recently, reliability BAMM has been questioned due to theoretical issues of the likelihood function and the incoherent compound Poisson process prior model (Moore et al., 2016). However, Rabosky et al., (2017) demonstrated that inferences about diversification rates have been accurate with the BAMM software and that diversification rates can be inferred using several methods such as BAMM.

9.3 Results and discussion

9.3.1 Phylogenetic relationships of *Lepanthes* and allied genera

A total of 132 terminals out of 300 of the *mat*K dataset were detected as incongruent. The best speciation tree model obtained with the Bayes Factors test was the Yule Process, therefore the MCC tree obtained from this analysis was used to infer the divergence dates, ancestral range estimation and rates of species diversification. The support values slightly increased after removing the potential outliers from the plastid dataset and the main clades received strong support in the BEAST analyses. The monophylly of *Anathallis*, *Draconanthes, Lepanthes, Pendusalpinx, Stellamaris, Trichosalpinx s.s*, *Tubella* and *Zootrophion* (all with PP=1.0), *Lepanthopsis* (PP=0.99) and *Lankesteriana* (PP=0.96) was highly supported. Although *Gravendeelia*, *Frondaria* and *Opilionanthe* were recognized as distinct lineages their phylogenetic relationships remain unresolved with the molecular markers currently analyzed. The intergeneric relationships are congruent with previous phylogenies of the group though (Bogarín et al., 2018c) (see Chapters 4 and 5). After increasing species sampling, *Lepanthes* was again recovered as a monophyletic group (PP=1.0).

9.3.2 Divergence times and historical biogeography

The DEC+J model was significantly better than the DEC model according to the LRT ($p < .00001$) and received the highest likelihood score (AIC=935.4) among the six models tested (Table 9.2). The most recent common ancestor (MRCA) of *Lepanthes* and allied genera was estimated to have evolved around 13.14 Ma (95% HPD: st.dev. 10.42-15.87 Ma) during the Miocene. Most of the extant taxa diverged in the Miocene-Pliocene with MRCAs estimated for *Zootrophion* to have evolved around 3.91 Ma (st.dev. 2.07-6.58 Ma), *Anathallis* 6.87 Ma (st.dev. 4.57-9.56 Ma), *Tubella* 6.55 Ma (st.dev. 4.2-9.35 Ma), *Trichosalpinx* 7.15 Ma (st.dev. 4.48-10.25 Ma), *Lankesteriana* 6.55 Ma (st.dev. 5.62-10.83 Ma), *Lepanthopsis* 4.64 Ma (st.dev. 2.73-6.90 Ma), *Stellamaris* 9.49 (st.dev. 7.23-12.03 Ma) and *Pseudolepanthes* 8.51 Ma (st.dev. 6.43-10.08 Ma). Unfortunately, most of the ancestral ranges of these groups remained unresolved (Fig. 9.1). The ancestral ranges of *Trichosalpinx* and *Tubella* were estimated as Southern Central America, however, more sampling from other geographical regions is needed to confirm this inference because our sampling lacked key representatives from other regions (See Chapter 5). In addition, *Draconanthes* and *Lepanthes* diverged at around 8 Ma (st.dev. 6.0-10.12 Ma) in the Andean region (marginal probabilities for each range: E=0.48; F=0.12; EF=0.31, see Fig.

Table 9.2. Comparison of the six biogeographical models implemented in BioGeoBEARS fitted on the *Lepanthes* dataset with the likelihood ratio (Chi^2) and AIC test evaluating null models against the alternative model with the founder speciation event "J" parameter. Best fitting model indicated in **boldface**.

Alt.[1] model	Null model	LnL Alt	LnL null	Dstatis-tic	pval	AIC Alt.	AIC Null	AICwt[2] Alt.	AICwt Null	AICwt ratio Alt.	AICwt ratio Null
DEC+J	DEC	-464.7	-488.9	48.29	3.70E-12	935.4	981.7	1	8.90E-11	1.13E+10	8.90E-11
DIVALIKE+J	DIVALIKE	-488.1	-503.6	30.86	2.80E-08	982.2	1011	1	5.40E-07	1844349	5.40E-07
BAYAREALIKE+J	BAYAREALIKE	-483.9	-574.2	180.7	3.50E-41	973.8	1152	1	1.60E-39	6.26E+38	1.60E-39

Table 9.3. Results from 50 BSM under the DEC+J model in BioGeoBEARS. Mean of all biogeographical events across the 50 BMSs among areas (coded by letters) for the *Lepanthes* and allied genera.

		To							
		C	**E**	**A**	**B**	**G**	**H**	**D**	**F**
From	**C**	0.00	7.3 (1.45)	9.58 (1.14)	1.42 (0.57)	1.68 (0.74)	1.54 (0.81)	1.16 (0.55)	2.64 (1.19)
	E	9.62 (1.92)	0.00	0.32 (0.55)	0.84 (0.65)	0.2 (0.4)	0.28 (0.57)	3.84 (0.58)	14.42 (2.34)
	A	1.04 (0.95)	0.22 (0.42)	0.00	0.12 (0.33)	0.3 (0.51)	0.22 (0.42)	0.12 (0.33)	0.24 (0.48)
	B	0.86 (0.81)	0.14 (0.35)	0.08 (0.27)	0.00	0.1 (0.3)	0.1 (0.3)	0 (0)	0.24 (0.43)
	G	0.12 (0.33)	0.1 (0.3)	0.2 (0.4)	0.04 (0.2)	0.00	1.22 (1)	0.2 (0.4)	0.56 (0.58)
	H	0.2 (0.57)	1.24 (0.52)	0.2 (0.4)	0.04 (0.2)	1.22 (0.86)	0.00	0.14 (0.35)	3.34 (0.66)
	D	0.12 (0.44)	0.4 (0.73)	0.08 (0.27)	0 ()	0.1 (0.3)	0.1 (0.3)	0.00	0.1 (0.3)
	F	1.88 (1.22)	7.3 (2.3)	0.44 (0.64)	0.08 (0.27)	0.7 (0.61)	0.88 (0.9)	0.16 (0.37)	0.00

9.1 for the coding of areas) and *Lepanthes* originated around the end of the Miocene, around 7.2 Ma (st.dev. 5.4-9.2 Ma) in the Andes with a slightly higher probability for the central Andes (E=0.22; F=0.39; EF=0.28) (Fig. 9.1). From this *Lepanthes* ancestor, one lineage evolved in the Central Andes during the Pliocene (F=0.91), comprising the extant species related to *L. tigrina* Luer & Thoerle*, L. terborchii* Luer & Sijm*, L. nycteris* Luer & R.Vázquez*, L. caprimulgus* Luer (*L. tigrina* group) (PP=1.0). Its sister lineage, containing the remaining species of *Lepanthes,* diverged earlier during the Miocene and likely in the Andes (E=0.22; F=0.31; EF=0.22). From this ancestor, another eminently North-Central Andean clade was derived in the end of the Miocene (E=0.20; F=0.33; EF=0.47) made up of species related to *L. juninensis* Schltr. (*L. juninensis* group) in addition to the first ancestor of a likely Southern Central American origin (E=0.09; C=0.55; CE=0.35), which diverged earlier at around 6 Ma during the Miocene-Pliocene boundary. This ancestor yielded the first lineages that originated in Southern Central America, represented today by several clades endemic to Costa Rica and Panama (C=1.0) such as *L. horrida* Rchb.f., *L. jimenezii* Schltr. and *L. minutilabia* Ames & C.Schweinf. This clade is sister to another clade also derived from a Southern Central American or Andean ancestor (E=0.09; C=0.49; CE=0.41). Shortly after this splitting, several Northern Andean ancestors evolved between 6-5 Ma represented in a serially branching pattern along the MCC tree (E=>0.74; CE=<0.24), which gave rise to South American *Lepanthes* groups such as *L. calodyction* Hook., *L. felis* Luer & R. Escobar and *L. hexapus* Luer & R. Escobar and a few extant Central American and Chocoan lineages. This suggest a possible recolonization of the Andes from Central America. Around 5 Ma, another Southern Central American ancestor descended from these linages that evolved again from a Northern Andean ancestor and diversified in the extant taxa found in Costa Rica and Panama (C=0.9). This event represents a second colonization from an Andean ancestor in Southern Central America. In addition, the lineages from Northern Central America and the West Indies were derived from this second Southern Central American colonization in the Pliocene-Pleistocene suggesting that the isthmus of Panama served as a land bridge for lineages derived from Andean ancestors. Therefore, a colonization of the West Indies and Northern Central America from the Guyanas and Amazonia is not supported. The most frequent dispersal routes recorded were among Southern Central America with Northern Central America, Southern Central America with the Northern Andes and vice versa and the Northern Andes with the Central Andes (Fig. 9.1, Table 9.3). As found in Chapter 8, the MRCA of Pleurothallidinae was likely adapted to montane habitats, and migrants to the Northern Andes were probably adapted to montane–cloud forest environments. Therefore, the rise of the Talamanca range in Southern Central America likely benefited the colonization of preadapted montane–cloud forest lineages that eventually also colonized Northern Central America and the highlands of the West Indies (Pérez-Escobar et al., 2017a). Some lineages also colonized lowland areas during Pleistocene climatic fluctuations. In addition, a few extant lowland Chocoan linages descended from both Andean and South Central American ancestors. The remaining species from Costa Rica and Panama diverged very recently (about 2.5 Ma) from Andean ancestors in the Pliocene-Pleistocene. The age estimations calculated here are similar to those obtained in Chapter 8 and other chronograms of the Orchidaceae but with narrower 95% HPD intervals (Chomicki et al., 2015; Givnish et al., 2016; Pérez-Escobar et al., 2017a).

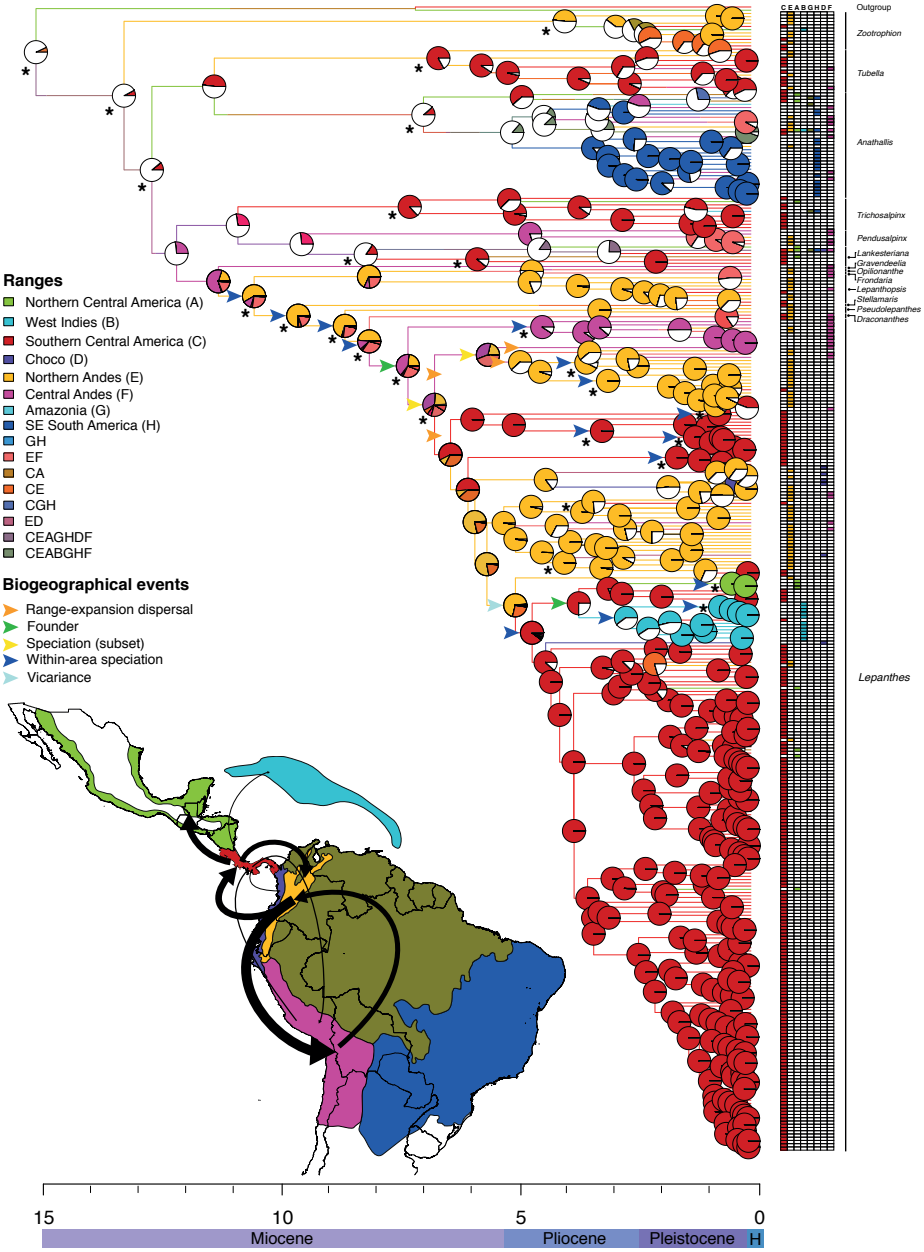

Figure 9.1. Time-calibrated phylogenetic tree of *Lepanthes* and estimated ancestral ranges with BIOGEOBEARS under the DEC+J model. Pie charts at the nodes indicate the relative probability of each estimated ancestral area and colored branches are from one of the BSM. Colored biogeographical ranges are represented with boxes and corresponding name of the range and letter (code) assigned. The combinations of letters refer to ancestral areas made up of more than one biogeographical area. Ranges of every taxon are shown as a

Table 9.4. Summary of the biogeographical stochastic mapping with the DEC+J model showing event counts across 50 BSMs, mean of events and standard deviations.

Events	Mode	Type	Number of events	Mean (SD)	%
Cladogenetic	Within-area speciation	Speciation (*in-situ*)	13751	275.02 (3.51)	68.19
		Speciation (subset)	1670	33.40 (4.57)	8.28
	Vicariance	Vicariance	755	15.10 (3.67)	3.74
	Dispersal	Founder events	1424	28.48 (4.13)	7.06
Anagenetic	Dispersal	Range-expansion dispersal (“d” parameter)	2565	51.30 (4.45)	12.72
		Range-switching dispersal (“a” parameter)	0	0	0
		Range-contraction dispersal (“e” parameter)	0	0	0
Total				403.3 (4.45)	100.00

9.3.3 Estimation of biogeographical events

Results of the BSM approach indicate that most biogeographical events correspond to cladogenesis by within-area speciation (68.20%) with minor contributions from speciation (subset), vicariance, founder events and anagenetic events (3-12% of the total estimates) (Fig. 9.3, Table 9.4). Among them, vicariance was the least favoured event whereas speciation (subset), founder events and range expansion showed similar contributions. The BSM approach suggested that *Lepanthes* likely originated from a founder event from the Northern Andes to Central Andes and subsequent speciation (subset) and range-expansion dispersal. Once the areas were colonized, within-area speciation was the most important event. The large contribution of within-area speciation is probably related to the high levels of endemism in *Lepanthes* (Luer and Thoerle, 2012), the large delimitation size of some of the Andean regions, which are rich in species (e.g. Central and Northern Andes) or the sampling bias to Southern Central American species. The rarity of vicariance events suggest that orchid dispersal is not constrained by the raise of Neotropical mountain ranges as hypothesized in Chapter 8 (Pérez-Escobar, Chomicki, et al., 2017b; Pérez-Escobar, Gottschling, et al., 2017). *Lepanthes* likely reached Southern Central America from the Andes at least twice, the first time via speciation (subset) event at about 6.5 Ma and the second time by vicariance at around 5 Ma. This suggests a biotic connectivity between the

heatmap after the terminals. Asterisks (*) indicate node supports of PP > 0.95. Some biogeographical events (range-expansion dispersal, founder, speciation (subset), within-area speciation and vicariance) are shown on the nodes and branches with colored arrowheads. (Inset map) Coded areas for biogeographical analysis corresponding to the colored ranges (boxes) and most frequent dispersal routes represented by arrows proportional to the frequency of events. Political divisions obtained from DIVA-GIS (http://www.diva-gis.org/gdata). Timescale shown at bottom is expressed in million years ago (Ma).

Andes and SCA as also found for other extant angiosperm species of the paramo and montane areas of Andean origin (e.g. *Puya*, Bromeliaceae). Although within-area speciation was the main biogeographical event, founder and vicariance were important events for colonizing new areas in *Lepanthes*. Particularly, founder events were important in the colonization of Northern Central America and the West Indies from Southern Central America.

9.3.4 Diversification of *Lepanthes* and allies

The BAMM analyses moderately rejected a null model with zero shifts (Bayes factor = 12.75) and identified three rate shifts across the *Lepanthes* clade (Table 9.5). The best model configuration identified three rate shifts, all in the genus *Lepanthes* (Fig. 9.2). The first rate shift, detected in the MRCA of *Lepanthes* around 7 Ma, was also described in Chapter 8. In comparison, the speciation rates of all closely related genera only slightly decreased over time and no shifts in diversification rates were detected, therefore, these groups (e.g. *Anathallis*, *Trichosalpinx*, *Zootrophion*) have not become as diverse as *Lepanthes* (Bogarín et al., 2018c). By increasing the species sampling for *Lepanthes* in the current study, we detected two additional rate shifts corresponding to the *L. disticha* and *L. blepharistes* groups, which occurred almost at the same time at around 2.5 Ma, towards the end of the Pliocene. These two groups are endemic to Costa Rica and Panama and taxonomically complex. Most of the new species of *Lepanthes* described recently belong to these groups (Bogarín et al., 2016; Pupulin and Bogarín, 2012). The high morphological similarity and low sequence variation in ITS and *mat*K markers observed suggests a possible correlation with fast and recent within-area speciation as inferred here (Bogarín et al., 2018d).

Overall, the rates through time in *Lepanthes* showed an acceleration around 7 Ma and at 2 Ma but these accelerations did not strictly correlate with mountain orogeny (Fig. 9.2). Similar results were observed in the Pleurothallidinae because no correlation was found among palaeo-elevation, mountain uplift and diversifications. The absence of a correlation with orogeny can be

Table 9.5. Summary of the biogeographical stochastic mapping with the DEC+J model showing event counts across 50 BSMs, mean of events and standard deviations.

		Denominator models								
	shifts	0	1	2	3	4	5	6	7	8
Numerator of models	0	1.00	0.31	0.08	0.08	0.09	0.14	0.21	0.13	0.19
	1	3.23	1.00	0.27	0.25	0.28	0.44	0.69	0.40	0.61
	2	12.13	3.75	1.00	0.95	1.04	1.65	2.60	1.52	2.27
	3	12.75	3.95	1.05	1.00	1.09	1.74	2.73	1.59	2.39
	4	11.67	3.61	0.96	0.92	1.00	1.59	2.50	1.46	2.19
	5	7.33	2.27	0.60	0.58	0.63	1.00	1.57	0.92	1.38
	6	4.67	1.45	0.38	0.37	0.40	0.64	1.00	0.58	0.88
	7	8.00	2.48	0.66	0.63	0.69	1.09	1.71	1.00	1.50
	8	5.33	1.65	0.44	0.42	0.46	0.73	1.14	0.67	1.00

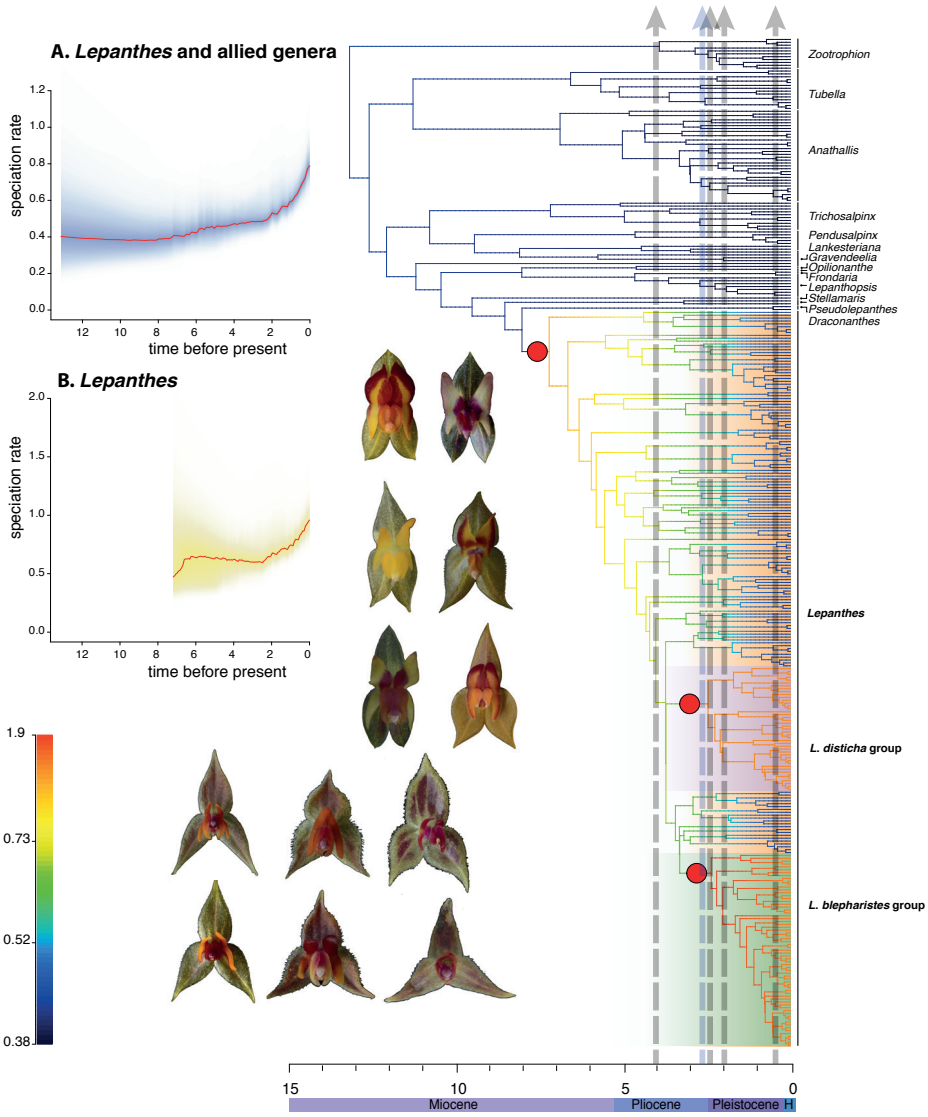

Figure 9.2. Time-calibrated phylogenetic tree of *Lepanthes* and allied genera with the best shift configuration obtained with BAMM analysis and colored according to speciation rate. Three rate shifts were detected, one at the MRCA of the genus, and two at the internal nodes corresponding to *L. disticha* and *L. blepharistes* groups (red circles). Rate-through-time analyses of speciation rates (density shading area indicates 95% Bayesian credible region of the distribution of rates) of **(A)** *Lepanthes* and allied genera and **(A)** the genus *Lepanthes* showing an acceleration of speciation rate starting at around 2.5 Ma. Grey arrows show the age of mountain ranges in SCA and the blue arrow the start of a climatic cooling period at about 2.7 Ma (Molnar, 2008). Timescale shown at the bottom is expressed in million years ago (Ma).

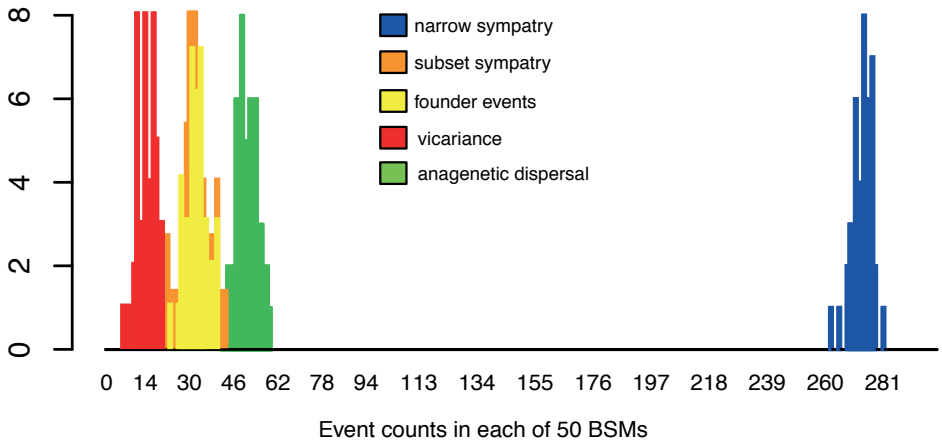

Figure 9.3. Histograms of the counts of different biogeographical events estimated in each of the 50 BSMs.

the result of the rapid diversification of migrating cool preadapted Pleurothallidinae lineages into already formed montane environments as hypothesized in Chapter 8 (Hoorn et al., 2010; Kirby, 2016, 2007). The mountain ranges of Costa Rica and Panama are among the youngest in the world and emerged as a volcanic island-arc in the Central American Seaway (CAS) around 25 Ma during the Cenozoic. Denyer and Alvarado (2007) calculated the origin of the Costa Rican Guanacaste and Central Volcanic Ranges as less than 0.5 Ma, Fila Costeña as less than 2 Ma and Cordillera de Tilarán as 5 Ma. The Cordillera de Talamanca (containing the highest peaks up to 3820 m.a.s.l.) uplifted to its present elevation during the Pleistocene and Holocene and its age was calculated as around 10-4 Ma. Historically, the closing of the CAS was assumed to start about 12 Ma and to be completed about 4.2 to 3 Ma (Denyer and Alvarado, 2007; Kirby, 2011, 2007). However, relatively new evidence suggests that the CAS closed about 15 to 13 Ma (Hoorn and Flantua, 2015; Montes et al., 2015). In both scenario's, epiphytic orchids were likely able to colonize the CAS volcanic island-arc by their wind-dispersed seeds. Furthermore, paleoclimatic fluctuations are also critical in the establishment of *Lepanthes* species (and likely also the associated pollinators and host trees) because they need humid, cold conditions to survive. Paleoclimatic evidence indicates that climatic cooling periods started before 2.7 Ma in the late Pliocene and early Pleistocene and this partially correlates with *Lepanthes* diversifications in SCA (Molnar, 2008).

Other biological factors responsible for accelerating orchid species diversifications are animal-plant interactions (Givnish et al., 2015). *Lepanthes* employs a sexual deception strategy of pollination but linking pollinator interactions to species diversifications for the entire genus is still impossible because of the scarcity of pollinator observations (only published for three out of >1,200 species) (Blanco and Vieira, 2011; Blanco and Barboza, 2005; Calderón-Sáenz, 2012). In addition, Tremblay and Ackerman (2001) found that genetic drift due to small effective population size and restricted gene flow may play a role in species diversification in *Lepanthes* because the interaction between drift and selection enhance population differentiation. Moreover,

rarity of pollinators or their inability to disperse over long distances might contribute to gene flow restriction among populations (Blanco and Barboza, 2005). Furthermore, other biotic factors such as mycorrhizal associations and availability of endophytic fungi could be limiting factors for seedling establishment in *Lepanthes*. The possible contributions of all these biotic factors to species diversification in *Lepanthes* needs further investigation.

9.4 Conclusions

Lepanthes likely originated in the Central Andes and diversified between 7-8 Ma in the Miocene. The genus reached Southern Central America from the Andean region twice, with one recolonization to the Northern Andes from Southern Central America. The extant lineages from Northern Central America and the West Indies were likely derived from Southern Central American MRCAs and not from Guyana or Amazonia. The most common biogeographical event was cladogenesis by within-area speciation and the most frequent dispersal routes recorded were Southern Central America with Northern Central America and Northern Andes, and Northern Andes with Central Andes. The genus showed the highest rates of species diversification in the Pleurothallidinae and its most recent Southern Central American clades experienced shifts in species diversification with accelerations around 2.5 Ma. These clades show a high morphological similarity and low variation in the standard DNA markers nrITs and *mat*K. Groups derived from recent, rapid diversifications should therefore be analyzed with innovative genomic techniques such as next generation sequencing in order to obtain fully resolved phylogenies (Bogarín et al., 2018d) (Chapter 8). Additionally, their molecular clock age estimates produced only 95% HPD intervals and more accurate estimations are needed. Recently, Sanmartín and Ree (2018) stated that the DEC+J model is a poor model of founder-event speciation, and that statistical comparisons with DEC are inappropriate. Therefore, comparisons with other biogeographical models should be performed to further investigate the consistency of all results obtained. Although our sampling from Southern Central America was the most intensive to date, the sampling of species from Andean, Northern Central America and West Indies lineages is still low and should be further increased. In addition, more accurate species distributions and the discovery of species belonging to new potential lineages will be key to a full understanding of the drivers of speciation and biogeography of *Lepanthes*.

General discussion and conclusions

Chapter 10

General discussion and conclusions

In this chapter, I discuss further steps needed to compliment the findings of this thesis and the work that must be continued in a general perspective to understand orchid species radiations in the Neotropics from three main topics: (i) taxonomy, systematics and evolution of floral traits, (ii) pollination biology and animal interactions and (iii) biogeography and evolutionary history.

10.1 Taxonomy, systematics and evolution of floral traits

The current angiosperm diversification in the Neotropics needs to be approached from an integrative perspective involving different sources of information. One of the most basic sources is alpha-taxonomy, primarily based on detailed morphological documentation of plants coupled with accurate locality data. This stage is the starting point to design and infer solid, densely sampled phylogenies which are appropriate to test further evolutionary hypotheses on species diversifications. However, an accurate phylogenetic sampling of *Lepanthes* is still a challenge because the number of species is overwhelming and new species continue to be discovered throughout the Neotropical region, thus producing a subestimation of the sampled lineages within the phylogenies. Therefore, alpha-taxonomic studies and botanical exploration should be made a priority to improve phylogenetic sampling as well as knowledge on species distributions. As we have shown in chapter 2 and 3, integrative taxonomy is also key in addressing species complexes difficult to separate morphologically. In this thesis, I described four new species of *Lepanthes*, two recognized primarily on morphology and two supported by morphology and a well-resolved phylogeny based on hundreds of innovative molecular markers. This was just a small contribution to the current taxonomic impediment, and I hope the discovery of additional new species will be sped up by exciting new methodologies such as for instance orchid pictures posted on social media or websites like iNaturalist (https://www.inaturalist.org) by orchid enthusiasts, naturalists or tourists (Kusuma-Wati et al., 2018).

Another challenge in understanding hyperdiverse orchid lineages such as *Lepanthes* is the explosive diversifications that resulted in poorly supported nodes and morphological convergence across clades. In these cases, inferences based on few molecular markers such as the traditional nrITS and matK regions are insufficient to provide clear species relationships in complexes of recent and rapid diversifications. Here, I demonstrated that high-throughput sequencing techniques such as anchored hybrid enrichment coupled with coalescence-based methods is a powerful tool to solve complicated phylogenetic relationships in linages derived from recent, rapid diversifications. Furthermore, phylogenomic datasets provide additional information on biological phenomena such as incomplete lineage sorting, hybridization or polyploidy that might cause discordance among individual gene trees. I found that only with a large number of innovative phylogenetic markers generated from three different genomes, the phylogeny of the *L. horrida* complex could be fully resolved and this enabled us to separate traits evolving in parallel or convergently across

these orchid lineages, such as flower color and size, from evolutionary informative diagnostic traits such as the shape and orientation of the lobes of the petals and lip. In conclusion, I recommend that more phylogenomic datasets should be generated for resolving more challenging groups within the genus *Lepanthes* and the Pleurothallidinae in general. Likewise, targeting the most informative phylogenetic markers obtained with phylogenomic datasets would be an adequate strategy to increase the sampling in hyperdiverse groups because analyzing large datasets of hundreds of species and markers might be computationally arduous. Most previous phylogenetic sampling in the Pleurothallidinae subtribe relied on two regions (nrITS and matK), therefore, the development of innovative markers such as those generated during this PhD thesis will be very useful for future phylogenetic studies of the orchid family. The advent of exciting new genomic tools such as target capture-based methods and transcriptomics in combination with custom made bioinformatics pipelines (Gravendeel et al., 2018) will definitely speed up this process.So far we conclude that alpha-taxonomy and the use of new techniques such as high-throughput sequencing are useful tools to clarify inter-specific relationships. However, another of the challenges of this thesis was to clarify the puzzling intergeneric relationships of the *Lepanthes* clade. Again, insufficient taxonomic sampling of clades precluded previous attempts to clarify generic relationships. However, the lack of a congruent system in assessing suitable morphological traits is still confusing the generic delimitations in the Pleurothallidinae. Generic delimitations solely based on morphological characters are daunting because of overwhelming homoplasy of the characters traditionally used for circumscriptions. I provided evidence for recognizing 14 well supported genera as members of the clade based on a combination of molecular phylogenetics and a solid morphological assessment identifying both synapomorphies and homoplastic characters. Future research should focus on sampling additional members of *Trichosalpinx* subgenus *Xenia*, which are extremely rare but need to be phylogenetically evaluated in order to obtain a complete evolutionary scenario for the *Lepanthes* clade. Based on morphology, we suspect that some members might be related to *Lepanthopsis* and allies but this hypothesis needs further evaluation. In addition, it is desirable to increase sampling in other groups such as *Lepanthopsis* (mainly the Antillean species) and *Tubella* because of floral similarities. Our phylogenetic framework and methodological approach enabled the discovery of useful traits for generic classifications, and paves the way for more comprehensive assessments on generic delimitations of similar recalcitrant lineages based on DNA sequences and morphological characters to further improve the systematics of the Pleurothallidinae. In conclusion, having a well resolved phylogeny and a fine delimitation of the clades is the starting point to explain the morphological evolution and the role of other biotic and abiotic factors in the diversification of the *Lepanthes* clade. This group challenged systematists and taxonomists for centuries due to the floral homoplasy untangled here, which is possibly resulting from similar pollination systems.

10.2 Pollination biology and animal interactions

The role of pollinators as drivers of species richness and morphological diversity is largely unknown because knowledge of pollination systems in Pleurothallidinae is still scarce. Pleurothallidinae are a megadiverse Neotropical orchid subtribe comprising > 5200 species, most of which are probably pollinated by Diptera. The *Lepanthes* clade accounts for about 25% of

the species of the subtribe but only one pollination system was described so far for the genus *Lepanthes*, with documented observations on just three species. I disclosed the pollination system of *Trichosalpinx*, which uses a completely different strategy compared to the *Lepanthes* pseudocopulatory system by male fungus gnats. The exclusive presence of female biting midges searching for proteins on the lip surface of *Trichosalpinx* and their well-developed mandibles and poorly developed laciniae indicate that they mainly feed on invertebrate hosts from which they draw haemolymph. Therefore, *Trichosalpinx* spp. might exclusively attract female midges by exploiting their protein collection instinct for egg production. The similar floral structures of other kleptomyophilous angiosperms compared to *Trichosalpinx* and the kleptoparasitic habits of *Forcipomyia* (Euprojoannisia) suggest that kleptomyiophily may have evolved in *Trichosalpinx*. This hypothesis derived from our study should be further tested by investigating the natural history of the *Forcipomyia* sp., discovery of the males, their feeding and breeding sites, diets and prey. Dietary analysis, bioassays and behavioural studies of both *Forcipomyia* sp. and their insect prey and GC/MS analyses of their pheromones and cuticular scents and the floral fragrance of other *Trichosalpinx* spp. are additional evidence needed. Most of the species of *Trichosalpinx* show similar floral traits, therefore, more observations in other species are necessary to confirm the pollination strategy revealed here. Likewise, the similarities among *Trichosalpinx* and the closely related *Anathallis* and *Lankesteriana* suggest that they also have similar pollination mechanisms. In addition to macromorphological similarities of the flowers of *Lankesteriana* and *Trichosalpinx* and some *Anathallis*, the species of these genera share micromorphological and histological characters that support a hypothesis of pollination by biting midges and thus parallelism. One of the most important shared characters is the secretion of proteins in the papillae of the lip and the striated cuticle of their epidermis. Species of *Trichosalpinx* employ this strategy to attract females of *Forcipomyia* for pollination and this might occur in *Lankesteriana* and some *Anathallis* as well. In the phylogenetic context, at least two families of Diptera are involved in the pollination of species in the *Lepanthes* clade: Sciaridae males in *Lepanthes* and Ceratopogonidae females in *Trichosalpinx*. However, the pollination mechanisms of the remaining 12 genera of the *Lepanthes* clade should also be investigated in order to obtain a complete picture of the evolution of pollination syndromes and floral traits related to them. These systems should be disclosed not only by describing the pollination system with innovative tools such as camera traps and Automatic Image Detection by machine learning but also by linking behavior and natural history of the pollinators to the strategy of attraction by the flowers. Consequently, other members of the group are likely pollinated by Diptera and in other groups such as *Tubella*, *Lepanthopsis* and *Zootrophion* the pollination systems are probably different from those already known. Apart from the pollination system, in *Anathallis*, *Frondaria*, *Lankesteriana*, *Tubella, Trichosalpinx s.l.* and *Zootrophion*, the pollinarium is deposited on the thorax of the pollinator since the columns are long and arcuate with an incumbent anther and a pollinarium with sticky caudicles. In contrast, in *Lepanthopsis* the pollinarium is likely not deposited on the thorax of the pollinator since the column is short and bilobed and the flower therefore does not allow for an entrance and exit as described for the genera mentioned above. Therefore, *Lepanthopsis* might employ a similar pollination strategy as *Platystele, Stelis* or *Pleurothallis*. Despite the availability of a described pollination system for three species of *Lepanthes*, more observations on other species are needed in order to fully understand the role of pollinators on the diversification and evolution of floral traits. During my

PhD project, I obtained preliminary data on pollination of *Lepanthes jugum* Luer and these data suggest that this species uses the pollinator's body in a different way as *L. glicensteinii* and *L. yubarta* to adhere its pollinarium to. In addition, the hypothesis of imitation of insect pheromones by *Lepanthes* flowers to attract male fungus gnats needs to be further tested. Finally, X-ray micro-compuated tomatography of orchid pollinators preserved in amber fossils might improve our knowledge on the timing of the evolution of this type of deceptive pollination as the fossil record of Ceratopogonidae is one of the best conserved among Diptera (Borkent and Spinelli, 2007).

10.3 Biogeography and evolutionary history

Based on two extensively sampled orchid phylogenies, combined with statistically robust diversification models, our results reveal that orchid diversification has closely tracked the Andean-Central American orogeny. Together with studies in other mega-diverse regions (Bruyn et al., 2014; Verboom et al., 2009), our results show that rapid recent speciation has moulded this area of exceptional species richness. In addition, our results highlight the crucial role of Amazonian lowlands, as well as the Antillean and Central American regions, as biotic sources for Andean, Northern Central America and Antillean biodiversity, providing cool pre-adapted lineages that dispersed into the Andes and further diversified *in situ*. The rise of the Andes had little effect on restricting orchid biotic dispersal across the Neotropics, suggesting that mountains are semi-permeable barriers to lowland organisms, whose dispersal ability is more probably related to intrinsic traits (e.g. seed size, dispersal mechanism, mutualisms). Although both abiotic and biotic processes are clearly responsible for the exceptional species richness of the world's premier biodiversity hotspot (Antonelli and Sanmartín, 2011; Hughes *et al.*, 2013; Eiserhardt *et al.*, 2017), our results suggest that geological processes played a central and direct role in the diversification process. Finally, as the highest species richness in Cymbidieae is concentrated in the lowlands and the Pleurothallidinae peak is at mid-elevation, our study shows that Andean uplift dramatically affected the evolutionary assembly of both lowland and mid-elevation Andean forests, as originally hypothesized by (Gentry, 1982). The genus *Lepanthes* likely originated during the Miocene in the Central Andes and reached Southern Central America from the Andean region twice. The extant lineages from Northern Central America and the West Indies were likely derived from Southern Central American MRCAs and not from Guyana or Amazonia suggesting that the isthmus of Panama served as a land bridge for lineages derived from Andean ancestors.

Future taxonomical and biogeographical research should focus on obtaining more accurate species distributions. For example, knowledge on orchid distributions and flowering periods can be improved by extracting image metadata from pictures (GPS location coordinates, elevation and time stamps) using command-line applications like Exiftool (https://www.sno.phy.queensu.ca/~phil/exiftool/). In addition, increasing taxon sampling and include multi-locus approaches to further test the influence of geographical barriers on current diversity patterns. Furthermore, other Neotropical areas such as northern Central America and the Antilles require more biogeographical research to complement our findings. The role of extant important biomes (most notably montane forests) in the diversification of the most diverse orchid groups within the Pleurothallidinae will then likely become much clearer. Such a result will have main conservation applications because a major group of orchids and associated pollinators and hosts are vulnerable to global warming, especially in cold, high-elevation areas, where they are most diverse.

Summaries

Summary

Historically, the isthmus of Costa Rica and Panama has been a source of fascination for its strategic position linking North America to South America. The isthmus is one of the world's most biodiverse regions where Orchidaceae is the most species-rich plant group. The area harbors more than 2,010 orchid species; representing about 8% of all species in the family on just about 1% of the Earth's land surface. Three genera of orchids are among the six most speciose angiosperm groups surpassing 1,000 species: *Epidendrum* L. (1,459 species), *Lepanthes* Sw. (1,125) and *Stelis* Sw. (1,128). The origin of this extraordinary orchid diversity has been attributed to the epiphytic habitat, CAM photosynthesis, pollination mechanisms, orogenic processes, past climatic fluctuations, or key innovations such as colonizations (extrinsic) or trait evolution (intrinsic). However, the influence of these factors in the diversification of the most speciose Neotropical orchid lineages has not been evaluated due to the insufficient knowledge on these challenging complex groups. In this thesis, I targeted the hyperdiverse orchid genus *Lepanthes*, as a study model to investigate the evolutionary processes that promoted species diversifications. To test hypotheses about the main drivers behind the evolution of these miniature orchids, we improved the taxonomy of *Lepanthes* and allies by combining morphological characters with solid, densely sampled phylogenies using phylogenetic comparative methods, described a new pollination system in the group and identified morphological characters associated with similar pollination mechanisms combining field observations, microscopy and histochemistry and discussed the impact of orogenic processes (formation of the Andes and Central America) on the actual species richness of *Lepanthes*. This thesis provides new insights in the taxonomy and systematics, pollination systems, biogeography and evolutionary history of *Lepanthes* and allies to understand the complex evolution of one of the most species-rich angiosperm lineages in the Neotropics. *Lepanthes* contains more than 1,128 species and new species are constantly being discovered. I described two new species from Panama based on morphological observations, named *Lepanthes aures-ursinae* and *Lepanthes vertebrata*. Some species are easily diagnosable based on morphological characters, however, others belonging to species complexes are difficult to separate because of the morphological similarity, especially in floral traits. In addition, lineages derived from rapid diversifications are often challenging to resolve using morphology or few standard DNA barcoding markers. Therefore, I used the anchored hybrid enrichment approach (AHE) to obtain 446 markers from the three plant genomes in order to disclose species relationships in the Costa Rican-Panamanian endemic *Lepanthes horrida* species group. I obtained a fully resolved phylogeny inferred with coalescent-based species tree estimations and disclosed two undescribed species, named *L. amicitiae* and *L. genetoapophantica*. In addition, I found high topological discordance among individual gene trees, suggesting that hybridization/polyploidy may have promoted speciation in the lineage via formation of new hybrid taxa. Similar to the poor understanding of inter-specific relationships, the inter-generic relationships in the *Lepanthes* clade have been unclear because of insufficient phylogenetic sampling and because of the convergent and variable nature of its phenotypic traits. To clarify these relationships, I used phylogenetic comparative methods to test the suitability of selected traits for generic delimitations in the *Lepanthes* clade, evaluating every generic name proposed in the group. Based on these findings, I proposed a new classification recognizing fourteen genera, including four novel generic concepts, and discussed the changes needed to reorganize the *Lepanthes* clade. From the

18 morphological traits evaluated, I identified 16 plesiomorphies, 12 homoplastic characters, and 7 synapomorphies, the latter of which are reproductive features, mostly related to pollination by pseudocopulation and possibly correlated with rapid diversifications within *Lepanthes*. Furthermore, the ancestral states of some reproductive characters suggest that these traits are associated with similar pollination mechanisms promoting homoplasy. The role of pollinators as drivers of species diversity in the *Lepanthes* clade is largely unknown because knowledge of pollination systems is scarce. The only known pollination system in the group is a pseudocopulatory strategy of *Lepanthes* involving male fungus gnats (Diptera, Sciaridae). I disclosed the pollination mechanism of the *Lepanthes*' closely related *Trichosalpinx* through the study of pollinator behavior and floral anatomy. I found that two *Trichosalpinx* spp. are pollinated exclusively by female biting midges of the genus *Forcipomyia* (Diptera, Ceratopogonidae). I detected secretion of carbohydrates and proteins on the lip with microscopy and histochemical techniques. These secretions might stimulate the protein collection instinct of female biting midges. These biting midges show well-developed mandibles and poorly developed laciniae, indicating that they mainly feed on invertebrate hosts from which they draw haemolymph. *Trichosalpinx* flowers offer small quantities of proteins and carbohydrates that may act as flavor teases as part of a complex deceptive system. Some other angiosperms such as *Bulbophyllum* (Orchidaceae), *Ceropegia* spp. (Asclepiadaceae) and *Theobroma cacao* (Malvaceae) that are also pollinated by biting midges possess similar dark purple flowers with ciliate ornamentation and use myophily, sapromyophily or kleptomyiophily as strategies to exploit different families of Diptera as pollinators. One *Forcipomyia* sp. (Euprojoannisia) is kleptoparasitic, suggesting that kleptomyiophily may have evolved in *Trichosalpinx*. The similar floral morphology among members of *Trichosalpinx* and some species of the closely related genera *Anathallis* and *Lankesteriana* suggests that they are all pollinated by biting midges. To further test this hypothesis, I studied the micromorphology and histochemistry of the flowers of *Trichosalpinx*, *Anathallis,* and *Lankesteriana* and found similar floral secretions such as carbohydrates and proteins on the lip and petals supporting a hypothesis of floral parallelism driven by pollinators.

To understand the role of abiotic factors in the diversification of *Lepanthes* such as the impact of the Andean orogeny and the influence of neighboring regions such as the Amazon, Central America and the Antilles in extant species composition, we inferred the biogeographical history and dynamics of speciation, extinction and migration of the two largest Neotropical orchid groups Cymbidieae and Pleurothallidinae, using two unparalleled, densely sampled orchid phylogenies. We found that the majority of these orchid lineages only originated in the last 20–15 Ma. Andean lineages are derived from lowland Amazonian ancestors, with additional contributions from Central America and the Antilles. Species diversification is correlated with Andean orogeny, and multiple migrations and recolonizations across the Andes indicate that mountains do not constrain orchid dispersal over long timescales. This suggests that mountain uplift promoted species diversification across all elevational zones. Derived from this study, we also found three rate shifts in the Pleurothallidinae, with the highest diversification rates in *Lepanthes*. To further investigate these diversification rates and also the biogeographical history of *Lepanthes*, I increased the species sampling to 25%. I found that *Lepanthes* likely originated in the Central Andes (CA) and diversified between 7-8 Ma during the Miocene. The genus reached Southern Central America (SCA) from the Andean region twice and the extant lineages from Northern

Central America (NCA) and the West Indies (WI) are likely derived from SCA ancestors, suggesting that the isthmus of Panama served as a land bridge for lineages derived from Andean ancestors. As found previously for the Pleurothallidinae, cladogenesis by within-area speciation was the most common biogeographical model for *Lepanthes*. The most frequent dispersal routes were SCA with NCA and NA and NA with CA. Two of the most recent clades of *Lepanthes* containing species from SCA experienced shifts in species diversification with an acceleration around 2.5 Ma. This acceleration did not strictly correlate with mountain orogeny Paleoclimatic evidence indicates that cooling periods started before 2.7 Ma and this partially correlates with species diversifications of *Lepanthes* in SCA. Botanical explorations, basic morphological documentation, and alpha-taxonomic work are the starting points to infer solid, densely sampled phylogenies in order to test evolutionary hypotheses on species diversifications in hyperdiverse orchid lineages.

In addition, new techniques such as high-throughput sequencing coupled with coalescence-based methods are a powerful tool to solve complicated phylogenetic relationships in lineages derived from recent, rapid diversifications. Selection of the most informative phylogenetic markers detected in phylogenomic datasets would be an adequate strategy to further increase the sampling because analyzing large datasets of hundreds of species and markers might be computationally arduous. Furthermore, phylogenomic datasets provide additional information on biological phenomena such as incomplete lineage sorting, hybridization or polyploidy that might cause discordance among individual gene trees.

Problematic inter-generic delimitations can be improved by assessing suitable morphological traits with phylogenetic comparative methods to detect synapomorphies and homoplastic characters. I am confident that this strategy will further improve the systematics of the Pleurothallidinae as a whole. This subtribe challenged systematists and taxonomists for centuries due to the floral homoplasy untangled here, which is possibly resulting from similar pollination systems. However, despite the new discoveries made on the pollination of *Lepanthes* and *Trichosalpinx* during my Ph.D project, many more observations on other species and genera are needed in order to fully understand the influence of pollinators on the diversification and evolution of floral traits. These systems should be disclosed not only by describing the pollination system but also linking behavior and natural history of pollinators to the strategy of attraction by the flowers. Based on two extensively sampled orchid phylogenies, combined with statistically robust diversification models, our results reveal that Andean orchid diversification has closely tracked the Andean-Central American orogeny. Further, the rise of some Neotropical mountains had little effect on restricting orchid biotic dispersal suggesting that they are semi-permeable barriers to lowland organisms, whose dispersal ability is more probably related to intrinsic traits (e.g. seed size, dispersal mechanism, mutualisms). Finally, *Lepanthes* showed the highest speciation rates across the Pleurothallidinae. The genus is estimated to have diversified recently, between 5-10 Ma. Future research should focus on increasing species sampling and adding new multiple markers to resolve recalcitrant nodes in its phylogeny. Likewise, the role of extant important biomes (i.e montane forests) in the diversification of this most diverse orchid groups within the Pleurothallidinae should be further studied to make sure that not only the orchid species but also the associated pollinators and hosts survive ongoing global warming at the cold, high- elevation areas where they are most diverse.

Samenvatting

De landengte, waar Costa Rica en Panama deel van uitmaken, is altijd een bron van fascinatie geweest voor natuurwetenschappers vanwege de strategische positie tussen Noord- en Zuid-Amerika. Het is één van ‘s werelds meest biodiverse regio’s en orchideeën zijn er de meest soortenrijke plantengroep. In het gebied komen meer dan 2.010 wilde orchideeënsoorten voor, dat is ongeveer 8% van alle soorten in de familie, op ongeveer 1% van het totale oppervlak van de aarde. Drie genera van orchideeën behoren tot de zes meest soortenrijke angiosperm-groepen met elk meer dan 1,000 soorten: *Epidendrum* L. (1,459 soorten), *Lepanthes* Sw. (1,125 soorten) en *Stelis* Sw. (1,128 soorten). Het grote aantal soorten orchideeën wordt toegeschreven aan een combinatie van epifytische leefwijzes, CAM-fotosynthese, bestuivingsmechanismen, orogene processen, historische klimaatfluctuaties en belangrijke andere innovaties zoals kolonisaties en kenmerkevolutie. De individuele invloed van al deze factoren op de soortvorming van orchideeën is nog onvoldoende bekend. In dit proefschrift heb ik het soortenrijke orchideeën genus *Lepanthes* als onderzoeksmodel gebruikt om de evolutionaire processen te onderzoeken die leiden tot het ontstaan van nieuwe soorten. Om een aantal van bovengenoemde hypothesen over soortvorming bij deze miniatuurorchideeën te testen, hebben we eerst de taxonomie van *Lepanthes* en nauwe verwanten aangepast aan de laatste wetenschappelijke inzichten. We hebben daarvoor morfologische kenmerken gecombineerd met fylogenieën, gereconstrueerd op basis van moleculaire markers, een nieuw bestuivingssysteem beschreven, en morfologische kenmerken kunnen associeren met verschillende bestuivingsmechanismen. Tot slot hebben we de mogelijke invloed van orogene processen, zoals de vorming van de Andes in Midden-Amerika bestudeerd op de huidige soortenrijkdom van *Lepanthes*. Mijn promotie-onderzoek heeft nieuwe inzichten opgeleverd in de taxonomie en systematiek, bestuivingsbiologie, biogeografie en evolutionaire geschiedenis van *Lepanthes* en nauwe verwanten, waardoor we de complexe evolutie van één van de meest soortenrijke angiospermen uit de Neotropen nu beter begrijpen. *Lepanthes* bevat momenteel 1128 soorten en nieuwe soorten worden nog voortdurend ontdekt. Ik beschreef twee nieuwe soorten uit Panama op basis van morfologische kenmerken: *Lepanthes aures-ursinae* en *Lepanthes vertebrata*. Sommige soorten zijn morfologisch gemakkelijk van elkaar te onderscheiden, maar voor soortscomplexen is dat veel moeilijker vanwege de morfologische sterk op elkaar lijkende bloemen. Daarnaast zijn fylogenieën van soorten, ontstaan uit snelle diversificaties, vaak niet betrouwbaar te reconstrueren met alleen morfologische kenmerken of slechts enkele DNA-fragmenten. Ik heb daarom gebruik gemaakt van een innovatieve methode, *Anchored Hybrid Enrichment* (AHE), om 446 nieuwe DNA-fragmenten mee te identificeren voor fylogeniereconstructie. Deze innovatieve markers waren afkomsting uit het nucleaire genoom en de genomen van de chloroplasten en mitochondria van een selectie van soorten uit de *Lepanthes horrida* groep uit Costa Rica en Panama. Met deze innovatieve markers kon een volledig opgeloste en betrouwbare fylogenie worden gereconstrueerd van de *L. horrida* groep. Op basis van de topologie van deze fylogenie heb ik nog twee nieuwe soorten beschreven: *L. amicitiae* en *L. genetoapophantica*. Omdat de verwantschappen tussen de taxa volgens de individuele markers vaak niet met elkaar overeenkomen, hebben hybridisatie en polyploïdie waarschijnlijk een rol gespeeld bij de soortvorming van de *L. horrida* groep. Handmatig gemaakte kruisingen zullen hier in de toekomst meer duidelijkheid over geven. Naast het ophelderen van de verwantschap-

pen tussen soorten in de *L. horrida* groep zijn ook de verwantschappen tussen verschillende genera in de *Lepanthes* groep door ons onderzocht. Dat was nodig vanwege de vele bloemmorfologische convergenties. Om hier meer duidelijkheid in te krijgen zijn vergelijkende fylogenetische methodes toegepast op een selectie aan diagnostische kenmerken, die van oudsher gebruikt worden om verschillende genera in de *Lepanthes* clade van elkaar te onderscheiden. Op basis van de resultaten van dit onderzoek heb ik een nieuwe systematische indeling voorgesteld, waarin 14 genera worden onderscheiden, waarvan vier nieuw voor de wetenschap. Van de 18 onderzochtte morfologische kenmerken bleken er 16 plesiomorf (basaal), 12 homoplastisch (niet evolutionair informatief) en 7 synapomorf (evolutionair informatief). De laatste categorie bestond uit reproductieve kenmerken, betrokken bij bestuiving door pseudocopulatie (zgn. sexuele mimicrie). De basale toestand van deze kenmerken bleek geassocieerd te zijn met steeds weer dezelfde bestuivingsvormen, die mogelijk tot de evolutie van convergente bloemvormen hebben geleid. De rol van bestuivers als drijvende krachten achter de huidige soortenrijkdom van *Lepanthes* bleek helaas niet goed te onderzoeken omdat er nog nauwelijks bestuivers van deze orchideeën bekend zijn. De enige gedocumenteerde bestuivers van *Lepanthes* soorten betreffen allemaal mannelijke rouwmuggen (Diptera, Sciaridae, *Bradysia*). In dit proefschrift beschrijf ik de bestuiving van het aan *Lepanthes* verwante genus *Trichosalpinx*. Verschillende soorten *Trichosalpinx* blijken bestoven te worden door vrouwelijke knutten (Diptera, Ceratopogonidae, *Forcipomyia*). Met behulp van microscopie en histochemische kleuringen heb ik ontdekt dat de lip van de bloemen van *Trichosalpinx* koolhydraten en eiwitten afscheidt. Deze afscheidingen stimuleren het eiwitverzamelingsinstinct van vrouwelijke knutten. De knutten hebben kaken met slechts rudimentaire lobjes erop, wat suggereert dat ze zich vooral voeden met hemolymfe, dat ze opzuigen uit ongewervelde prooien. Met de afgescheiden eiwitten en koolhydraten bootsen de *Trichosalpinx* bloemen ongewervelde prooien na (mimicrie). Andere angiospermen, zoals *Bulbophyllum* (Orchidaceae), *Ceropegia* spp. (Asclepiadaceae) en *Theobroma cacao* (Malvaceae), die ook donkerpaarse bloemen met trilhaartjes hebben, worden net als *Trichosalpinx* door knutten bestoven. Deze onverwante plantengenera gebruiken een vergelijkbare mimicriestrategie om bestuivers aan te trekken. Het betreft in dit geval het nabootsen van myofilie (vliegen die aangetrokken worden door nectar en pollen) , sapromyofilie (vliegen die aangetrokken worden door kadavers of mest) of kleptomyiofilie (vliegen die aangetrokken worden door hemolymfe, dat uit verse prooien druppelt, die gevangen zijn door bijvoorbeeld spinnen of bidsprinkhanen). Omdat kleptoparasitisme bekend is van *Forcipomyia* is het goed mogelijk dat kleptomyiofilie op *Trichosalpinx* bloemen van toepassing is. Soorten uit de nauw verwante genera *Anathallis* en *Lankesteriana* hebben een vergelijkbare bloemmorfologie als *Trichosalpinx*. Met behulp van microscopie en histochemische kleuringen werden ook in de lip van deze bloemen koolhydraten en eiwitten ontdekt. Onze hypothese is dat meerdere malen een vergelijkbare bloemvorm, -kleur en -chemie ontstond tijdens de evolutie van deze orchideeën, als aanpassing aan bestuiving door kleptoparasitische bestuivers. Meer waarnemingen van bestuivers zijn nodig om deze hypothese verder te onderbouwen of verwerpen. Tijdens dit promotie-onderzoek is niet alleen de invloed van biotische maar ook die van abiotische factoren op de soortvorming van orchideeën onderzocht. De focus lag daarbij op de vorming van de Andes en de Amazone in centraal Amerika en de Antillen en de invloed daarvan op ontstaan, extinctie en migratie van soorten uit twee Neotropische orchideeëngroepen, de Cymbidiae en Pleurothallidinae. Het merendeel van deze soorten ontstond 20-15 miljoen jaar

geleden. Soorten uit de Andes blijken af te stammen van voorouders uit het laagland van het Amazonegebied, Centraal Amerika en de Antillen. Soortvorming bleek in de tijd sterk gecorreleerd te zijn met de vorming van de Andes. Meerdere migraties en herkolonisaties van soorten aan verschillende kanten van de Andes laten echter zien dat deze bergketen vervolgens geen barriere vormde voor de verdere verspreiding van soorten. Het lijkt erop dat de vorming van de Andes op verschillende hoogtes tot het ontstaan van nieuwe soorten heeft geleid, dus zowel in laaglandbos als bergbos en alpiene zones. In de Pleurothallidinae hebben we drie verschillende snelheden van soortvorming gevonden en de *Lepanthes* clade bleek het snelst te divergeren en pas 10-5 miljoen jaar oud te zijn. Om meer inzicht te krijgen in de diversificatie en biogeografie van *Lepanthes* is het aantal onderzochte soorten tot 25% verhoogd. Toen bleek dat het genus waarschijnlijk 8-7 miljoen jaar geleden in de Centrale Andes ontstaan is.*Lepanthes* soorten hebben Zuidelijk Centraal Amerika vervolgens tweemaal bereikt vanuit de Andes. The huidige soorten uit Noordelijk Centraal Amerika en West-Indië zijn waarschijnlijk ontstaan uit voorouders uit Zuidelijk Centraal Amerika. De landengte, waar Costa Rica en Panama deel van uitmaken, heeft hier waarschijnlijk een belangrijke rol bij gespeeld. Net als voor het gehele tribus van de Pleurothallidinae blijkt ook voor *Lepanthes* lokale soortvorming het meest toepasselijke biogeografische model. De meest voorkomende dispersies lijken te hebben plaatsgevonden van Zuidelijk Centraal Amerika naar Noordelijk Centraal Amerika en de Noordelijke Andes en van de Noordelijke Andes naar de Centrale Andes. Twee van de meest recente soortengroepen van *Lepanthes* zijn pas 2.5 miljoen jaar geleden ontstaan. Deze soortvorming lijkt niet gelijk op te gaan met het ontstaan van plaatselijke gebergteketens maar wel met een daling van de temperatuur in Zuid Centraal Amerika ca. 2.7 miljoen jaar geleden. De meeste *Lepanthes* soorten zijn tegenwoordig te vinden in hoog gelegen mistbossen. Dit kwetsbare bioom wordt steeds meer in haar voortbestaan bedreigd vanwege de toenemende opwarming van de aarde. Het is dus zaak dat deze mistbossen zoveel mogelijk de status van beschermd natuurgebied krijgen om niet alleen de orchideeën, die daar groeien\ voor uitsterven te behoeden maar ook de bomen, bestuivers en andere organismen waar zij mee samen leven.

Resumen

Históricamente, el istmo de Costa Rica y Panamá ha sido una fuente de fascinación por su posición estratégica que une América del Norte con América del Sur. El istmo es una de las regiones con mayor biodiversidad del mundo donde Orchidaceae es el grupo de plantas más rico en especies. El área alberga más de 2,010 especies de orquídeas; representando aproximadamente el 8% de todas las especies de la familia en aproximadamente el 1% de la superficie de la Tierra. Tres géneros de orquídeas se encuentran entre los seis grupos de angiospermas más diversos que superan las 1.000 especies: *Epidendrum* L. (1,459 especies), *Lepanthes* Sw. (1,125) y *Stelis* Sw. (1,128). El origen de esta extraordinaria diversidad de orquídeas se ha atribuido al hábitat epífito, la fotosíntesis de CAM, los mecanismos de polinización, los procesos orogénicos, las fluctuaciones climáticas pasadas o las innovaciones clave, como las colonizaciones (extrínsecas) o la evolución de rasgos (intrínsecas). Sin embargo, la influencia de estos factores en la diversificación de los linajes de orquídeas neotropicales más especiosos no se ha evaluado debido al conocimiento insuficiente sobre estos desafiantes y complejos grupos. En esta tesis, me enfoqué en el género de orquídeas hiperdiversas *Lepanthes*, como modelo de estudio para investigar los procesos evolutivos que promovieron la diversificación de especies. Para probar las hipótesis sobre los principales impulsores de la evolución de estas orquídeas miniatura, mejoramos la taxonomía de *Lepanthes* y aliados mediante la combinación de caracteres morfológicos con filogenias sólidas, densamente muestreadas en conjunto con métodos comparativos filogenéticos, describimos un nuevo sistema de polinización en el grupo e identificamos caracteres morfológicos asociados con mecanismos de polinización similares combinando observaciones de campo, microscopía e histoquímica y discutimos el impacto de los procesos orogénicos (formación de los Andes y América Central) en la riqueza real de especies de *Lepanthes*. Esta tesis proporciona nuevos conocimientos en taxonomía y sistemática, sistemas de polinización, biogeografía e historia evolutiva de *Lepanthes* y aliados para entender la compleja evolución de uno de los linajes de angiospermas más ricos en especies en el Neotrópico. *Lepanthes* contiene más de 1,128 especies y constantemente están descubriendo nuevas especies. Describí dos nuevas especies de Panamá basadas en observaciones morfológicas, llamadas *Lepanthes aures-ursinae* y *Lepanthes vertebrata*. Algunas especies son fácilmente diagnosticables en función de los caracteres morfológicos, sin embargo, otras que pertenecen a complejos de especies que son difíciles de separar debido a la similitud morfológica, especialmente en los rasgos florales. Además, los linajes derivados de las diversificaciones rápidas a menudo son difíciles de resolver utilizando la morfología o unos pocos marcadores estándar de códigos de barras de ADN. Por lo tanto, utilicé el enfoque de enriquecimiento híbrido anclado (AHE por sus siglas en inglés) para obtener 446 marcadores de los tres genomas de las plantas con el fin de revelar las relaciones de las especies en el grupo de *Lepanthes horrida*, endémico de Costa Rica y Panamá. Obtuve una filogenia completamente resuelta inferida con estimaciones de árboles de especies basadas en métodos de coalescencia y revelé dos especies no descritas, llamadas *L. amicitiae* y *L. genetoapophantica*. Además, encontré una alta discordancia topológica entre los árboles de genes individuales, lo que sugiere que la hibridación o poliploidía puede haber promovido la especiación en el linaje a través de la formación de nuevos taxones híbridos. Al igual que la poca comprensión de las relaciones inter-específicas, las relaciones inter-genéricas en el clado de *Lepanthes* no han sido claras

debido al insuficiente muestreo filogenético y a la naturaleza convergente y variable de sus rasgos fenotípicos. Para aclarar estas relaciones, utilicé métodos comparativos filogenéticos para probar la idoneidad de rasgos seleccionados para delimitaciones genéricas en el clado de *Lepanthes*, evaluando cada nombre genérico propuesto en el grupo. Basándome en estos hallazgos, propuse una nueva clasificación que reconocía catorce géneros, incluidos cuatro conceptos genéricos novedosos, y discutí los cambios necesarios para reorganizar el clado de *Lepanthes*. De los 18 rasgos morfológicos evaluados, identifiqué 16 plesiomorfías, 12 caracteres homoplásticos y 7 sinapomorfías, las últimas de las cuales son características reproductivas, en su mayoría relacionadas con la polinización por pseudocopulación y posiblemente correlacionadas con diversificaciones rápidas dentro de *Lepanthes*. Además, los estados ancestrales de algunos caracteres reproductivos sugieren que estos rasgos están asociados con mecanismos de polinización similares que promueven la homoplasia. El papel de los polinizadores como impulsores de la diversidad de especies en el clado de *Lepanthes* es en gran parte desconocido porque el conocimiento de los sistemas de polinización es escaso. El único sistema de polinización conocido en el grupo es la estrategia pseudocopulatoria de *Lepanthes* que involucra mosquitos machos de hongos (Diptera, Sciaridae). Por lo tanto, divulgé el mecanismo de polinización del *Trichosalpinx* estrechamente relacionado de *Lepanthes* a través del estudio del comportamiento de los polinizadores y la anatomía floral. Encontré que dos especies de *Trichosalpinx* son polinizadas exclusivamente por mosquitos ceratopogónidos hembra del género *Forcipomyia* (Diptera, Ceratopogonidae). Detecté la secreción de carbohidratos y proteínas en el labelo de la flor técnicas de microscopía e histoquímica. Estas secreciones pueden estimular el instinto de recolección de proteínas de las moscas de la hembra. Estos mosquitos muestran mandíbulas bien desarrolladas y lacinias poco desarrolladas, lo que indica que se alimentan principalmente dela hemolinfa de invertebrados hospederos. Las flores de *Trichosalpinx* ofrecen pequeñas cantidades de proteínas y carbohidratos que pueden actuar como saborizantes dentro de un complejo sistema engañoso. Algunas otras angiospermas como *Bulbophyllum* (Orchidaceae), *Ceropegia* spp. (Asclepiadaceae) y *Theobroma cacao* (Malvaceae) que también son polinizadas por mosquitos ceratopogónidos, poseen flores similares a *Trichosalpinx* de color púrpura oscuro, ciliadas y emplean miofilia, sapromiofilia o cleptomiofilia como estrategias para explotar a diferentes familias de Dipteros como polinizadores. Una *Forcipomyia* sp. (Euprojoannisia) es cleptoparásita lo que sugiere que la cleptomiofilia pudo haber evolucionado en *Trichosalpinx*. La morfología floral similar entre los miembros de *Trichosalpinx* y algunas especies de los géneros estrechamente relacionados *Anathallis* y *Lankesteriana* sugiere que todos están polinizados por mosquitos ceratopogónidos. Para probar más a fondo esta hipótesis, estudié la micromorfología e histoquímica de las flores de *Trichosalpinx*, *Anathallis* y *Lankesteriana* y encontré secreciones florales similares como carbohidratos y proteínas en el labelo y pétalos que apoyan una hipótesis de paralelismo floral impulsado por polinizadores similares. Por otro lado, para entender el papel de los factores abióticos en la diversificación de *Lepanthes*, como el impacto de la orogenia de los Andes y la influencia de regiones vecinas como la Amazonía, América Central y las Antillas en la composición de especies existentes, inferimos la historia biogeográfica y la dinámica de la especiación, extinción y migración de los dos grupos de orquídeas neotropicales más grandes, Cymbidieae y Pleurothallidinae, utilizando dos filogenias de orquídeas densamente muestreadas. Encontramos que la mayoría de estos linajes de orquídeas solo se originaron en los últimos 20-15 Ma. Los linajes

andinos se derivan de los ancestros amazónicos de las tierras bajas, con contribuciones adicionales de América Central y las Antillas. La diversificación de especies se correlaciona con la orogenia andina, y las migraciones y recolonizaciones múltiples a través de los Andes indican que las montañas no restringen la dispersión de las orquídeas en escalas de tiempo largas. Esto sugiere que la elevación de las montañas promovió la diversificación de las especies en todas las zonas elevadas. Derivado de este estudio, también encontramos tres cambios de tasas de diversificación en Pleurothallidinae y a su vez con las tasas de diversificación más altas en *Lepanthes*. Para investigar más a fondo estas tasas de diversificación y también la historia biogeográfica de *Lepanthes*, aumenté el muestreo de especies al 25%. Encontré que *Lepanthes* probablemente se originó en los Andes Centrales (CA) y se diversificó entre 7-8 Ma durante el Mioceno. El género llegó al sur de América Central (SCA) desde la región andina dos veces y los linajes existentes del norte de América Central (NCA) y las Antillas (WI) probablemente se derivan de ancestros de SCA, lo que sugiere que el istmo de Panamá sirvió como un puente terrestre para linajes derivados de ancestros andinos. Como se descubrió anteriormente para Pleurothallidinae, la cladogénesis por especiación dentro del área fue el modelo biogeográfico más común para *Lepanthes*. Las rutas de dispersión más frecuentes fueron SCA con NCA y NA y NA con CA. Dos de los clados más recientes de *Lepanthes* que contienen especies de SCA experimentaron cambios en la diversificación de especies con una aceleración de alrededor de 2.5 Ma. Esta aceleración no se correlacionó estrictamente con la orogenia. La evidencia paleoclimática indica que los períodos de enfriamiento comenzaron antes de 2.7 Ma y esto se correlaciona parcialmente con las diversificaciones de especies de *Lepanthes* en SCA. Las exploraciones botánicas, la documentación morfológica básica y el trabajo alfa-taxonómico son los puntos de partida para inferir filogenias sólidas, densamente muestreadas útiles para probar hipótesis evolutivas sobre diversificaciones de especies en linajes de orquídeas hiperdiversas. Además, las nuevas técnicas, como la secuenciación de nueva generación junto con los métodos basados en la coalescencia, son una herramienta poderosa para resolver relaciones filogenéticas complicadas en linajes derivados de diversificaciones rápidas y recientes. La selección de los marcadores moleculares más informativos detectados en los conjuntos de datos filogenómicos sería una estrategia adecuada para aumentar aún más el muestreo debido a que el análisis de grandes conjuntos de datos de cientos de especies y marcadores puede ser computacionalmente arduo. Además, los conjuntos de datos filogenómicos proporcionan información adicional sobre fenómenos biológicos, como la clasificación de linajes incompletos, la hibridación o la poliploidía que podrían causar discordancia entre los árboles de genes individuales. Las delimitaciones intergénicas problemáticas pueden mejorarse mediante la evaluación de rasgos morfológicos adecuados con métodos comparativos filogenéticos para detectar sinapomorfías y caracteres homoplásticos. Estoy seguro de que esta estrategia mejorará aún más la sistemática de la Pleurothallidinae en su conjunto. Esta subtribu desafió a los botánicos sistemáticos y taxónomos durante siglos debido a la homoplasia floral que aquí evaluamos y que posiblemente es el resultado de sistemas de polinización similares. Sin embargo, a pesar de los nuevos descubrimientos realizados sobre la polinización de *Lepanthes* y *Trichosalpinx* durante mi proyecto de doctorado, se necesitan muchas más observaciones sobre otras especies y géneros para comprender completamente la influencia de los polinizadores en la diversificación y evolución de los rasgos florales. Estos sistemas deben ser divulgados no solo describiendo el sistema de polinización sino también relacionando el comportamiento y la histo-

ria natural de los polinizadores con la estrategia de atracción floral. Basados en dos filogenias de orquídeas ampliamente muestreadas, combinadas con modelos de diversificación estadísticamente robustos, nuestros resultados revelan que la diversificación de orquídeas andinas ha seguido de cerca la orogenia andino-centroamericana. Además, el aumento de algunas montañas neotropicales tuvo poco efecto en la restricción de la dispersión biótica de las orquídeas, lo que sugiere que son barreras semipermeables para los organismos de las tierras bajas, cuya capacidad de dispersión está más probablemente relacionada con rasgos intrínsecos (por ejemplo, tamaño de la semilla, mecanismo de dispersión o mutualismos). Finalmente, *Lepanthes* mostró las tasas más altas de especiación en las Pleurothallidinae. Se estima que el género se ha diversificado recientemente, entre 5-10 Ma. Las investigaciones futuras deberían centrarse en aumentar el muestreo de especies y agregar nuevos marcadores múltiples para resolver los nodos recalcitrantes en las filogenias. Del mismo modo, el papel de los biomas existentes e importantes en la diversificación de los grupos de orquídeas más diversos dentro de Pleurothallidinae (es decir, los bosques montanos) debe estudiarse más a fondo para garantizar que no solo las especies de orquídeas sino también los polinizadores y hospederos asociados sobrevivan el calentamiento global en curso en las zonas frías y elevadas donde éstos grupos son más diversos.

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Curriculum Vitae

Diego G. Bogarín Chaves was born on October 15th, 1982, in San José, Costa Rica, and grew up in Heredia Province. During his childhood at age 10, he developed a strong interest in orchids. Each family trip was a means to explore the rich Costa Rican ecosystems in search of different orchids species. At 12, he followed a free course "Introduction to Orchidology" at the National University of Costa Rica. After completing his secondary studies at Liceo Samuel Sáenz Flores in Heredia, his growing interest in orchids led him to study biology at the University of Costa Rica, where he obtained his B.Sc. degree in 2006. In 2001 during his studies, he became involved at the Lankester Botanical Garden (JBL), first, as a volunteer and, later, as an assistant of research in the systematic orchid projects led by his mentor and great friend, Franco Pupulin. With Franco, he witnessed the beginnings of the transformation of the JBL into an active orchid research center in the Neotropics. With the main goal of documenting the vast orchid flora of Costa Rica, Diego learned from his mentor the capacity of studying orchid species scientifically. This is why he contributed to several floristic and monographic research projects, describing more than 80 species of Neotropical orchids to date in approximately 90 scientific articles. During this time, Diego also supported new students, conducted extensive fieldwork, and was in charge of the orchid collections until the establishment of the JBL herbarium.

Since August 2005, the University of Costa Rica hired Diego as a researcher based at Lankester Botanical Garden. He first worked on the project "Conservation and Monitoring of Mesoamerican Orchids" in collaboration with the Royal Botanic Gardens, Kew, UK, where he participated in the development of DNA barcodes for Costa Rican orchids at the Jodrell Laboratory and digitized part of the orchid herbarium types at Kew. Later, in 2010, he completed his M.Sc. degree at the UCR under the supervision of Dr. Robert L. Dressler on systematics of the orchid genus *Campylocentrum*. In 2012, Diego met his co-promotor, Dr. Barbara Gravendeel, who visited JBL as part of the research activities of Adam Karremans' Ph.D. project. At that time, Dr. Gravendeel encouraged him to apply for a doctorate under her supervision to study the evolution of the species-rich genus *Lepanthes*. After obtaining a scholarship from the University of Costa Rica, Diego traveled to the Netherlands in September 2015 to begin his Ph.D. project at Naturalis Biodiversity Center and Leiden University under the supervision of Dr. Gravendeel and Dr. Erik Smets. After obtaining his doctorate, Diego will continue to work as a researcher at JBL and take charge of the chair of orchidology at the School of Biology of UCR. His main future research projects will be focused on the biotic and abiotic factors that shaped the rich orchid flora of southern Central America by applying bioinformatics, genomics, next-generation sequencing, taxonomy, and systematics. Diego is also interested in orchid in-situ conservation and the development of a research center at the herbarium UCH, at the Autonomous University of Chiriquí, Panama, where he is a Research Associate.

List of publications

Bogarín, D., E. Kaes, M. Díaz-Morales. 2019. *Lepanthes elusiva* – a New Species of *Lepanthes* (Orchidaceae: Pleurothallidinae) from Tapantí Area in Cartago, Costa Rica. Die Orchidee 5(3): 19-28.

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