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

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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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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

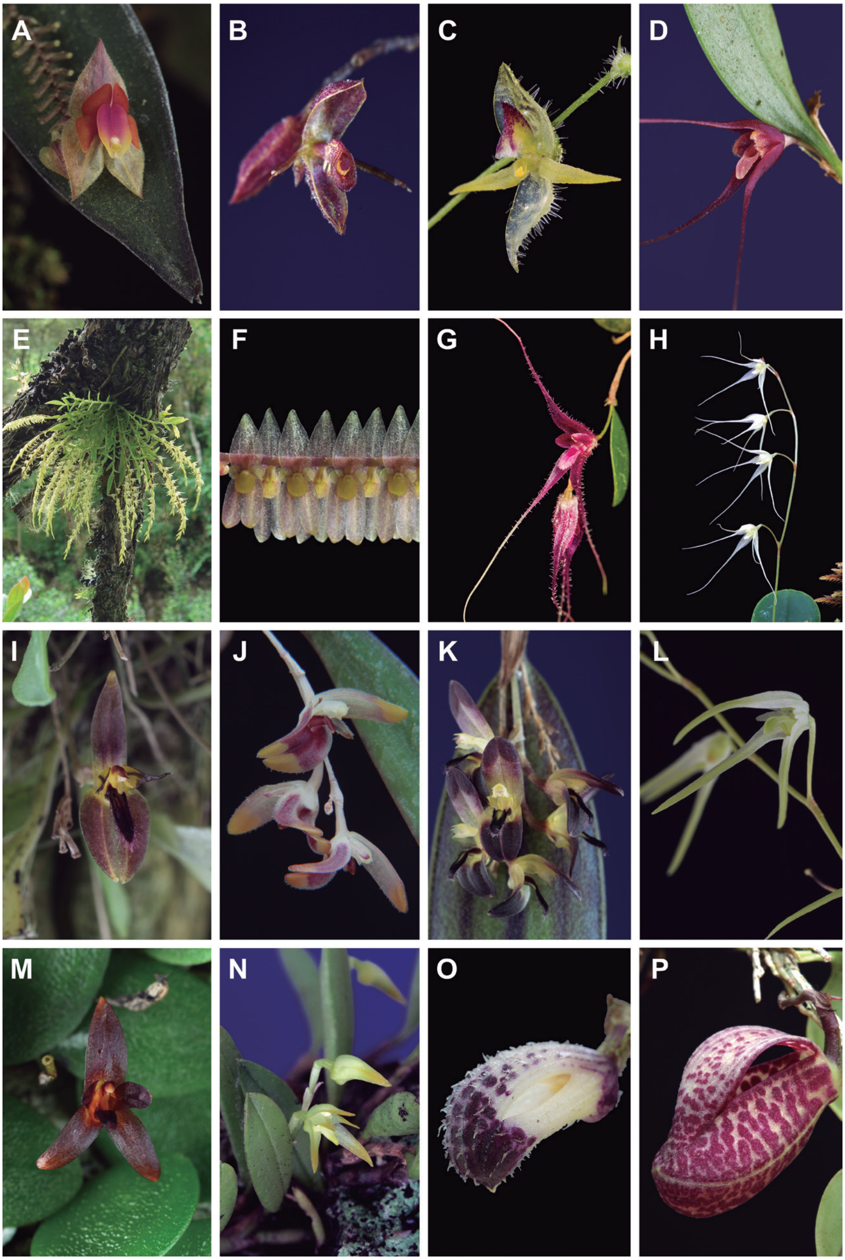

Figure 2.1. Flower morphology of the representatives of the *Lepanthes* clade: **A.** *Lepanthes*. **B.** *Draconanthes*. **C.** *Pseudolepanthes*. **D.** *Stellamaris*. **E.** *Frondaria*. **F.** *Lepanthopsis*. **G.** *Gravendeelia*. **H.** *Opilionanthe*. **I.** *Lankesteriana***. J.** *Pendusalpinx*. **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*.

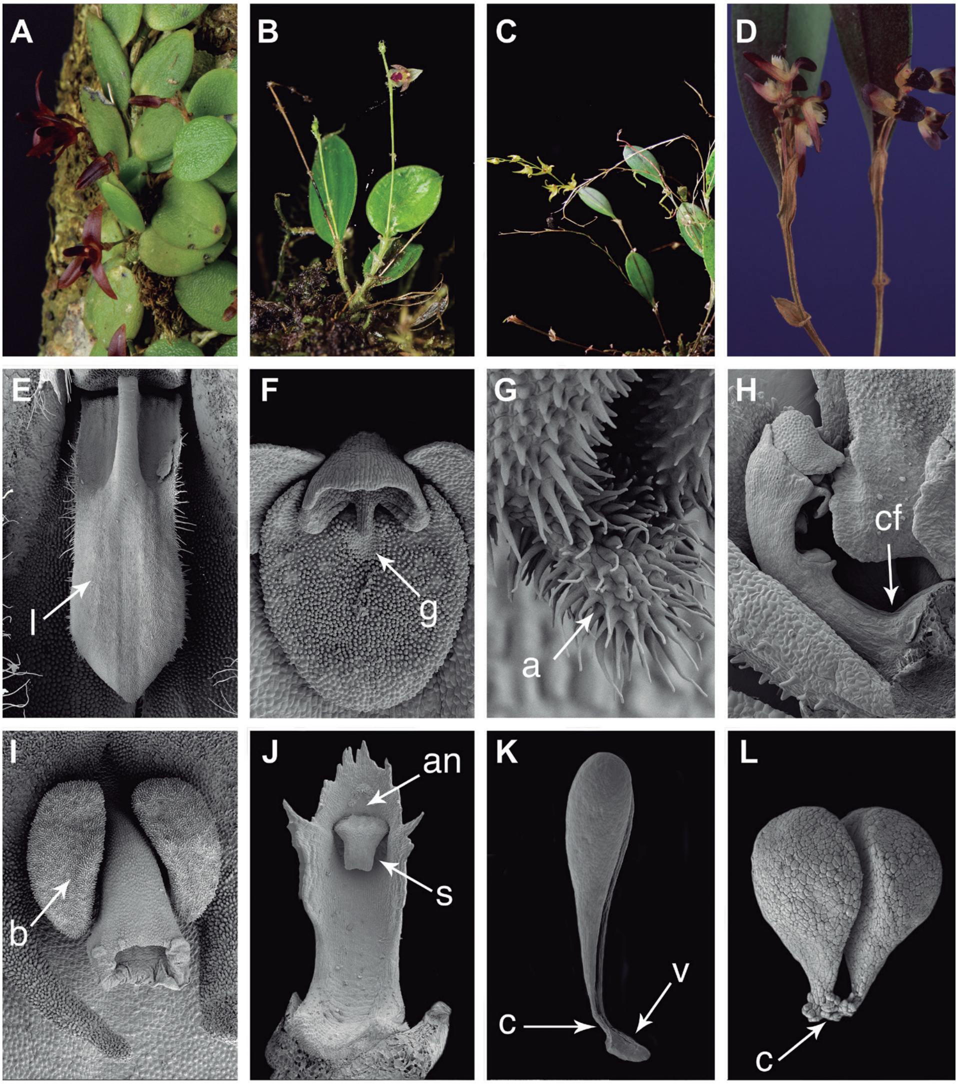

Figure 2.2. Vegetative and flower morphology of the characters evaluated: **A.** repent habit in *Anathallis*. **B.** caespitose habit with longer inflorescences than leaf in *Pseudolepanthes*. **C.** prolific ramicauls in *Tubella*. **D.** ornamented lepanthiform bracts in *Trichosalpin*x. **E.** laminar, 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-

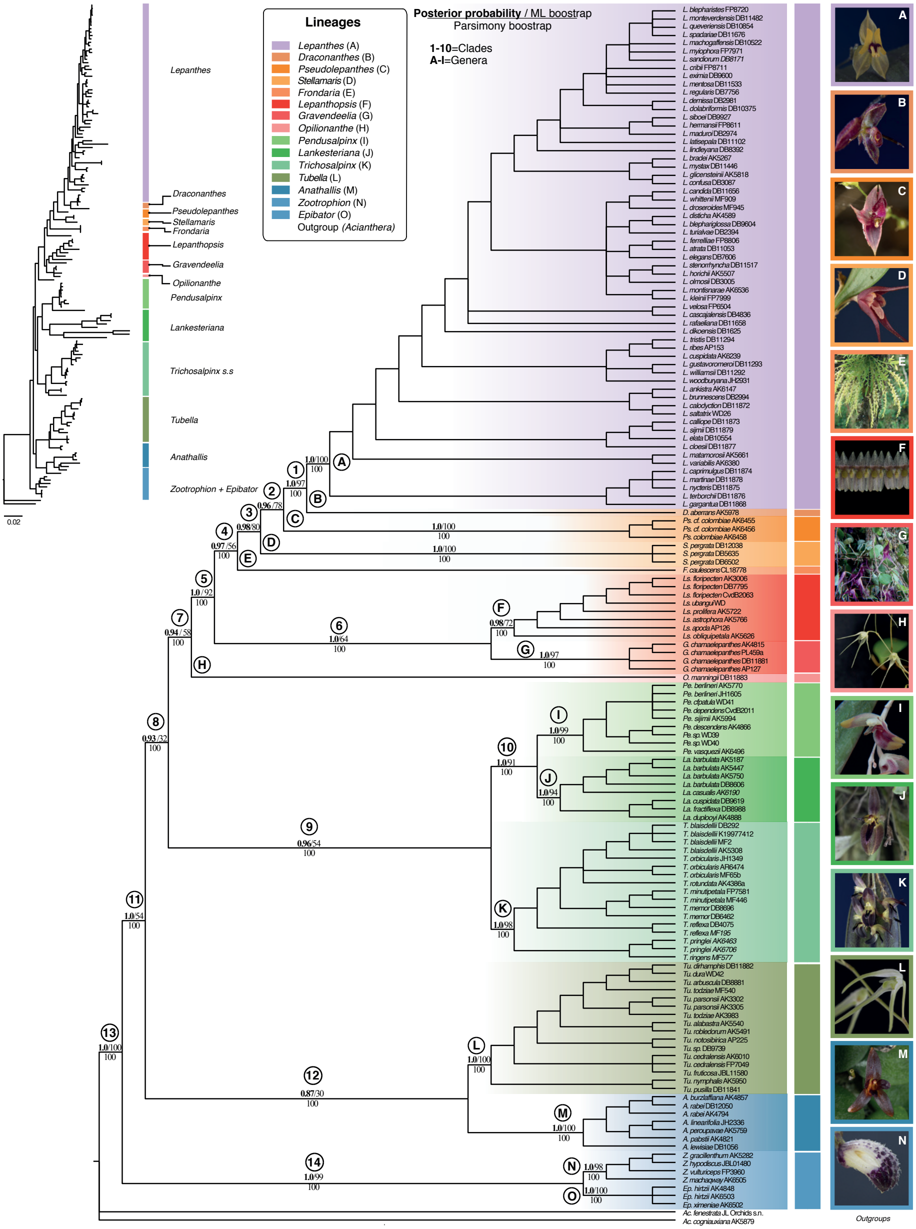

Figure 2.3. The 14 genera recognized in the *Lepanthes* clade in the 50% majority-rule consensus tree based on BI analysis of concatenated dataset. Plotted branch values for MPB, MLBS and PP are given for each well-supported clade of interest. Letters represent genera and numbers clades grouping the 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*

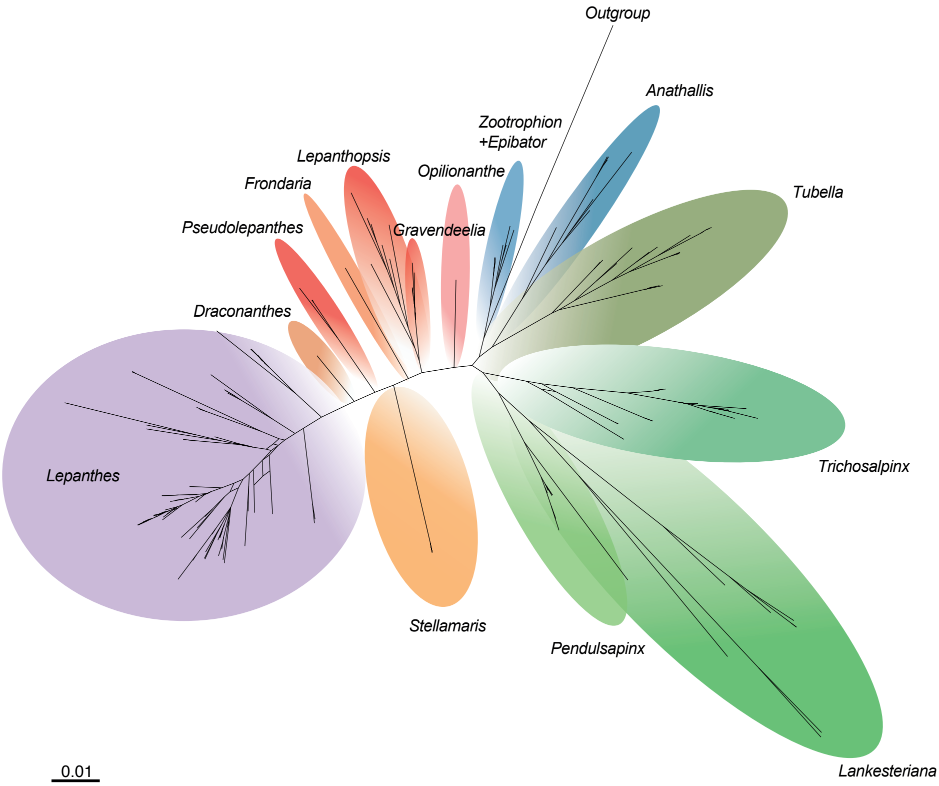

Figure 2.4. Split network showing the 14 genera of the *Lepanthes* clade inferred from 3,000 tree replicates of the BI inference. The network shows well supported groups without uncertainty in the relationships.

(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

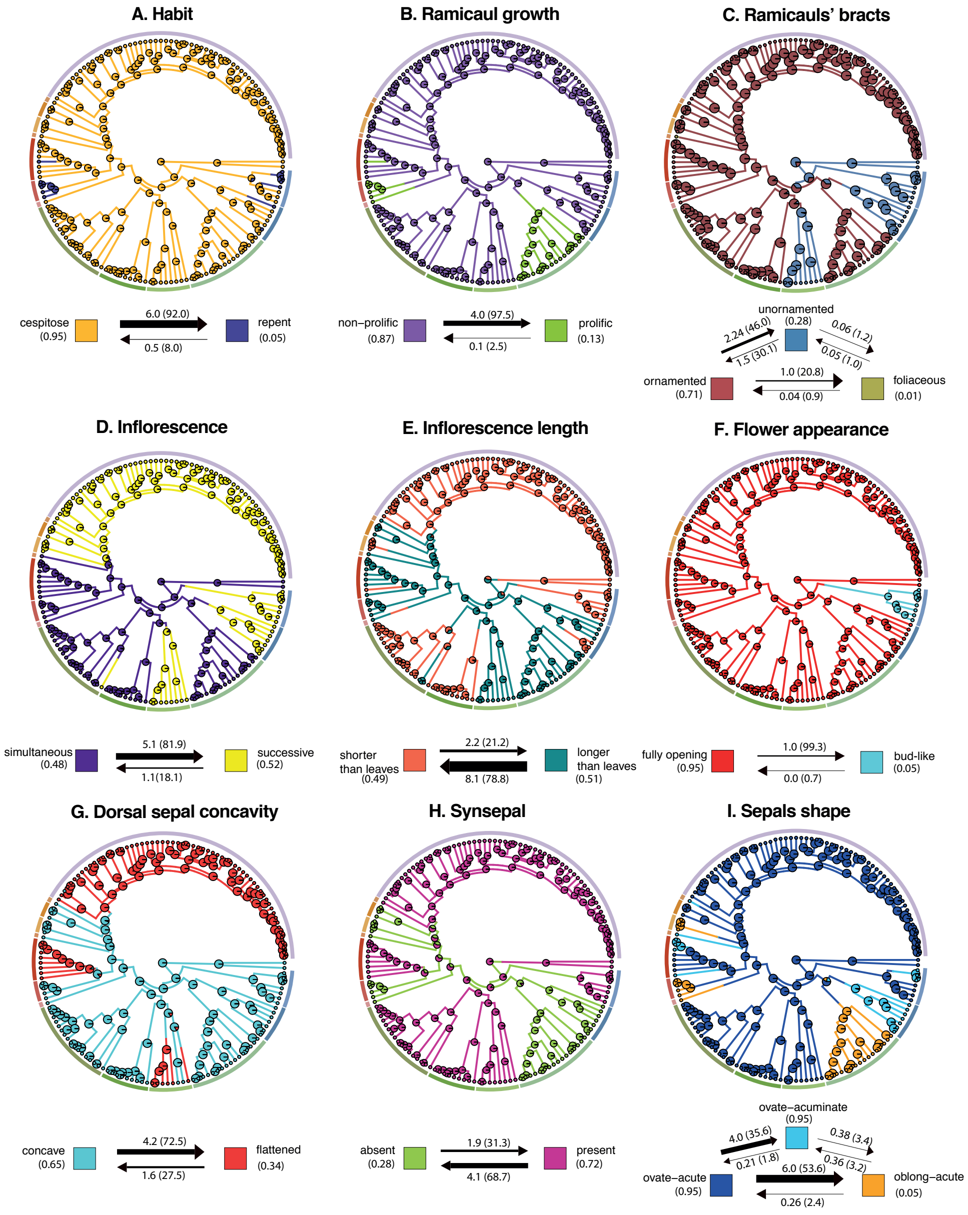

Figure 2.5. Ancestral state reconstructions of selected morphological characters from stochastic mapping analyses based on joint sampling (10,000 mapped trees). Arrows represent transitions between states and numbers represent the estimated number of evolutionary changes with proportion in parenthesis 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*.

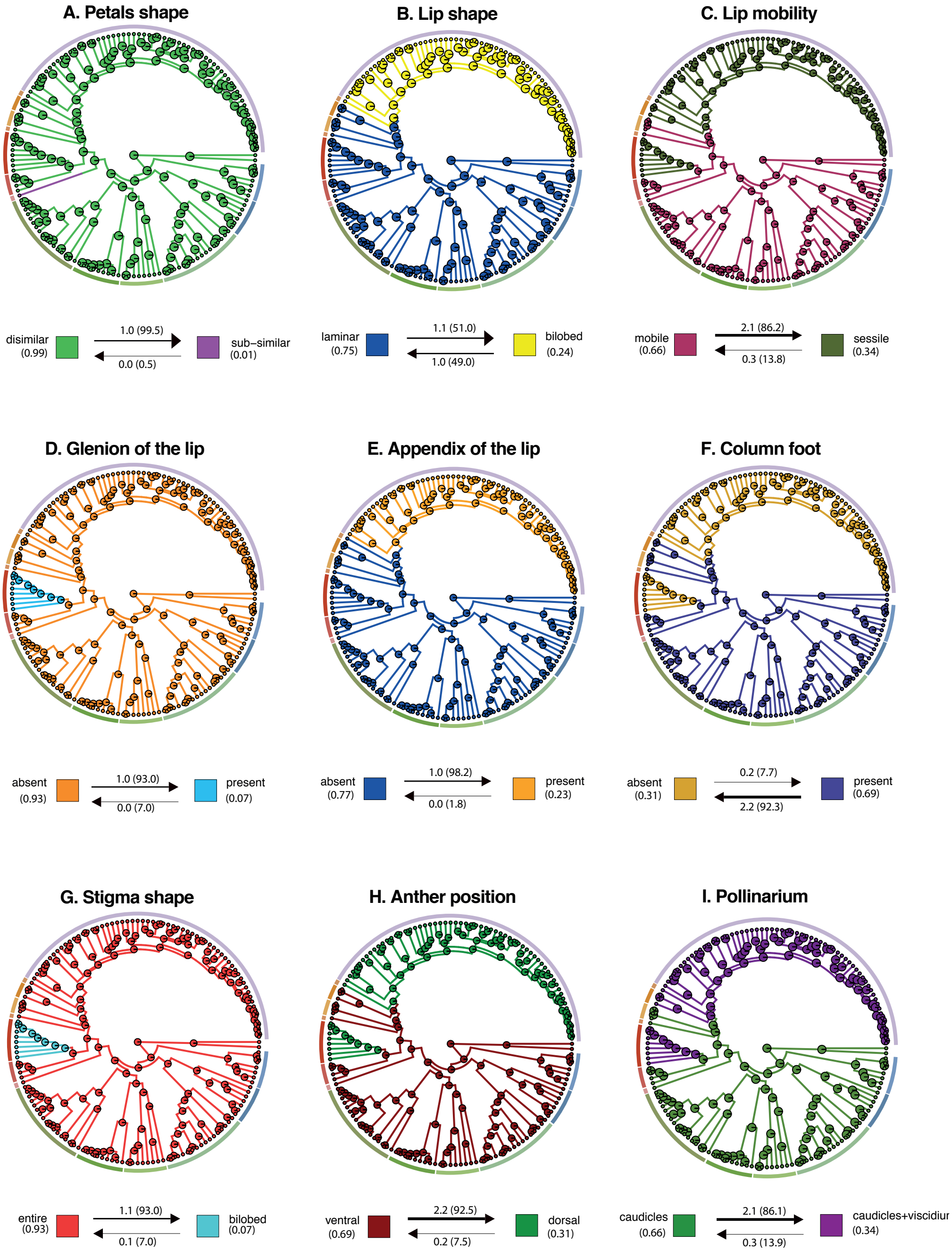

Figure 2.6. Ancestral state reconstructions of selected morphological characters: **A.** Petals shape. **B.** Lip shape. **C.** Lip mobility. **D.** Glenion of the lip. **E.** Appendix of the lip. **F.** Column foot. **G.** Stigma shape. **H.** Anther position. **I.** Pollinarium.

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.